

The effect of acute or/and chronic stress on the MnSOD protein expression in rat prefrontal cortex and hippocampus

Dragana Filipović, Jelena Zlatković and Snežana B. Pajović

Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences “Vinča”, P.O.Box 522-090, 11001 Belgrade, Serbia

Abstract. Manganese superoxide dismutase (MnSOD) is the major antioxidant in mitochondria that protect brain from neuroendocrine stress. Although MnSOD is localized in the mitochondria, the immediate subcellular distribution of MnSOD protein level in the prefrontal cortex and hippocampus of Wistar male rats exposed to acute stressors immobilization or cold, chronic stress isolation or their combinations (acute/chronic) have not been studied. Western immunoblotting revealed that acute immobilization stress resulted in an increase in mitochondrial MnSOD protein level, whereas chronic isolation compromises MnSOD protein level. Chronically stressed animals exposed to novel acute stressors showed a significant decrease in mitochondrial MnSOD protein level and reciprocal increase in this protein in the cytosolic fraction. At the same time, a significant increase in serum corticosterone level was observed after acute stressors, whereas chronic isolation led to negligible changes and caused a reduced responsiveness to a novel acute stressors. Presence of cytochrome c in mitochondrial and cytosolic fraction of both brain structures was also confirmed. Results suggest that chronic stress isolation results in mitochondrial dysfunction and MnSOD release into the cytosol.

Key words: MnSOD — Prefrontal cortex — Corticosterone — Hippocampus — Neuroendocrine stress

Introduction

Exposure to neuroendocrine stress (NES) leads to the increased release of glucocorticoids (GCs) by activation of hypothalamo-pituitary adrenal (HPA) axis. The effects of GCs in any *acute* stressful situation can be classified as *protective* for the *organism* (preserving homeostasis) against the negative sequelae of stress, while prolonged periods of exposure to elevated levels of GCs (such as occurs during chronic stress) may have deleterious biological effects, in which oxidative stress plays a major etiopathological role (Sapolsky 1992; Pardon 2007). Neurodegeneration mediated by GCs under stress conditions has been linked to an increase in generation of reactive oxygen species (ROS), which can directly damage lipids, nucleic acids and proteins (Blumberg 2004; Evans et al. 2004) resulting in mitochondrial dysfunction, cytochrome c release in the cytosol (Fujimura

et al. 1999; Chong et al. 2005) and biochemical cascade that could lead to the apoptosis (Le Bras et al. 2005). The extent of NES-triggered effects and the resulting vulnerability of cells are related to the NES duration and intensity.

One of the primary intracellular sites for *in vivo* ROS production is the mitochondria (Turrens 1997). Manganese superoxide dismutase (MnSOD) is antioxidant enzyme present in the inner membrane and matrix of mitochondria (Slot et al. 1986; Okado-Matsumoto and Fridovic 2001). It is encoded in the nuclear chromatin, synthesized as a precursor in the cytoplasm, and imported posttranslationally into the mitochondrial matrix in its mature form subsequent to the removal of its 23-amino acid NH₂-terminal leader sequence by specific proteases (Wispe et al. 1989). In the presence MnSOD, superoxide radical (O₂⁻) can be converted to the hydrogen peroxide (H₂O₂) which can then diffuse out of mitochondria in the cytoplasm. In the presence of high iron concentrations, H₂O₂ can form the highly reactive hydroxyl radical (HO) *via* the Fenton reaction. O₂⁻ can also react with nitric oxide to form the highly reactive peroxyxynitrite anion (ONOO⁻), as it has been demonstrated in brain after stress (Olivenza

Correspondence to: Dragana Filipović, Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences “Vinča”, P.O.Box 522–090, 11001 Belgrade, Serbia
E-mail: dragana@vinca.rs

et al. 2000), causing oxidative/nitrosative damage (Liu et al. 1996; Madrigal et al. 2001).

Chronic exposure to mitochondrial ROS leads to inactivation of key mitochondrial enzymes and accumulation of mitochondrial DNA mutation (Wallace 2005). It has been shown that MnSOD knockout mice display degenerative changes in large areas of the central nervous system, especially in the basal ganglia (Lebovitz et al. 1996). Overexpression of MnSOD results in neuroprotection by reducing cellular apoptosis and decreasing brain ischemic damage (Keller et al. 1998). Recent evidence has shown that chronic isolation stress increases the activity of MnSOD, whereas it is not affected by acute stressors in the brain (Pajović et al. 2006). GCs have been shown to decrease the activity of antioxidative enzymes in the hippocampus and brain cortex, both basally and in the presence of an oxidative stressor (McIntosh et al. 1998a,b). Moreover, dexamethasone can reduce basal or cytokine-stimulated MnSOD mRNA expression, suggesting that MnSOD is under glucocorticoid regulation (Valentine and Nick 1994; Antras-Ferry et al. 1997). Consequently, a decrease in the antioxidant capacity of the brain may be responsible for the stress-related oxidative damage. A possible mechanism by which stress-hormones may contribute to oxidative damage may be due to compromised brain antioxidant defense system (Michel et al. 2007; Sarandol et al. 2007).

Taking into account the above studies, we investigated the hypothesis that 2 h of acute stress immobilization or cold, 21 day of chronic stress isolation or their combinations (homotypic chronic stress followed by as heterotypic acute stress) alter MnSOD subcellular protein level in the rat prefrontal cortex as a center of cognitive brain and hippocampus which comprises the numerous centers of emotional brain, as essential components of neural circuitry mediating stress (Jacobson and Sapolsky 1991; Mizoguchi et al. 2003). We also measured serum corticosterone (CORT) level in control and all stressed rat groups, in order to examine the biological impact of the stressor. The chronic isolation stress was chosen as the model because it is a continuous stressor which contains both psychological and physiological components (Popović et al. 2000; Chourbaji et al. 2005), and may enhance anxiety in rat (Maisonnette et al. 1993; Haller and Halasz 1999) even after 7 days of isolation (Niesink and Van Ree 1982). The immediate aim of the study was to define the kind of stress (acute, chronic or combined) which led to the most pronounced changes in serum CORT levels and MnSOD subcellular protein level.

Materials and Methods

Animal treatments

Adult Wistar male rats (2–3 months old, weighing 330–400 g) were housed in groups of four individuals per cage

in a temperature $20 \pm 2^\circ\text{C}$, humidity $55 \pm 10\%$ and offered water and food *ad libitum*. The light was kept on between 7:00 a.m. and 7:00 p.m. The animal experiments were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute of Nuclear Sciences “Vinca”, which follows the guidelines of the registered “Serbian Society for the Use of Animals in Research and Education”. For experimental purposes, the animals were randomly divided into four groups: in group I four animals were kept per cage representing the unstressed animals (control, $n = 6-8$); group II was exposed to 2 h of immobilization (IM) or cold (C; 4°C) representing acute stressors (each one, $n = 6-8$); group III was exposed to chronic isolation by individual housing for 21 day (IS; $n = 6-8$), according to the model of Garzon and del Rio (1981), where the animals had relatively normal auditory and olfactory experiences but could not at any time see, touch or be touched by other colony animals; group IV was exposed to chronic IS followed by only once for 2 h of acute IM or C (4°C) stress, representing combined stressors (IS+IM, IS+C, each one, $n = 6-8$). Experiments of acute stressors were performed between 8:00 a.m. and 10:00 a.m., to avoid CORT circadian rhythm. Rats were exposed to IM action by introducing them in the prone position with all four limbs fixed to the board with adhesive tape. The head was also fixed with a metal loop over the neck area, with a consequent limitation of head motion (Kvetnansky and Mikulaj 1970). The animals exposed to C stress were initially kept at ambient temperature ($20 \pm 2^\circ\text{C}$) and then carefully transferred into a refrigerator room at 4°C . Following stress procedure, the whole brain was immediately removed and, the prefrontal cortex and hippocampus were dissected on ice. Tissue were frozen in liquid nitrogen and kept at -70°C .

Serum CORT assay

Immediately after stress, the animals were decapitated and the trunk blood was collected. Blood samples were centrifuged at 4°C for 20 min at $1500 \times g$, and serum was separated and kept at -70°C until CORT levels determination. Levels of serum CORT were determined using the Octeia ELISA kit (IDS, Boldon, U.K.) and the values were expressed as nanogram per milliliter. All samples were measured in duplicate in one assay. The variation between duplicate samples was less than 7%. The lower detection limit for hormone levels in this assay system is 25 ng/ml.

Mitochondria/cytosol fractionation

To prepare mitochondria and cytosol tissue protein extracts, frozen prefrontal cortex and hippocampus were weighed and homogenized in 2 vol. (w/v) of ice-cold homogenization buffer I (0.25 mol/l sucrose, 15 mmol/l TRIS-HCl (pH 7.9), 16 mmol/l KCl, 15 mmol/l NaCl, 5 mmol/l ethylenediamine-

tetraacetic acid (EDTA), 1 mmol/l ethylene glycol tetraacetic acid, 1 mmol/l dithiothreitol (DTT), 0.15 mmol/l spermine and 0.15 mmol/l spermidine supplemented with following protease inhibitors: 0.1 mmol/l phenylmethanesulphonylfluoride, 2 µg/ml leupeptin, 5 µg/ml aprotinin, 5 µg/ml antipain) by 40 strokes in the Potter-Elvehjem teflon-glass homogenizer. Samples were centrifuged at $2000 \times g$, 4°C for 10 min. The supernatant was centrifuged at $15,000 \times g$, 4°C for 20 min. The resulting mitochondrial pellet was washed by resuspension in homogenization buffer I followed by additional centrifugation at $15,000 \times g$, 4°C for 20 min and resuspended in 250 µl of lysis buffer (50 mmol/l TRIS-HCl (pH 7.4), 5% glycerol, 1 mmol/l EDTA, 5 mmol/l DTT, supplemented with mentioned protease inhibitors and 0.05% Triton X-100). The supernatant was further centrifuged at $100,000 \times g$ for 60 min to obtain the pure cytosolic fraction. Protein content in the mitochondrial and cytosolic fractions was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA; Sigma-Aldrich) as reference.

Electrophoresis and Western blot analysis

Equal amounts of protein of mitochondrial and cytosolic fractions of unstressed control and all stressed groups isolated from prefrontal cortex and hippocampus were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were blocked in a blocking buffer with 5% BSA in TRIS-buffered saline (20 mmol/l TRIS, 137 mmol/l NaCl (pH 7.6), containing 0.3% Tween 20) and incubated with a rabbit polyclonal anti-MnSOD antibody (SOD-110; Stressgen, Victoria, BC, Canada). Mouse anti-cytochrome c antibody (6H2; Santa Cruz Biotechnology) was used to confirm the presence of cytochrome c in mitochondrial and cytosolic fractions of both brain structures. Western blots were performed with horseradish peroxidase (HRP)-conjugated anti-rabbit (7074; Cell Signaling Technology) or goat anti-mouse immunoglobuline (SC-2005; Santa Cruz Biotechnology) for 2 h. To confirm a consistent protein loading for each lane, membranes were stained for β -actin (primary monoclonal mouse anti- β -actin antibody, A5316 Sigma-Aldrich, followed by HRP-conjugated secondary goat anti-mouse immunoglobuline). Antigen-antibody complexes were incubated with the LumiGLO substrate (7003; Cell Signaling Technology) for 5 min and immediately exposed to X-ray film. The immunoreactive bands were quantitated by Image software. Results were expressed as MnSOD/ β -actin ratio. In the figures, the MnSOD levels in stressed animals were expressed as percentage of change in relation to those in unstressed animals taken as 100% (unstressed control). The data are expressed as means \pm S.E.M. of 6–8 animals per group.

Statistical evaluation

Data were analyzed by two-way ANOVA (the factors were acute or chronic stress, and the levels for the acute stress were none, IM and C, while for the chronic stress they were none and IS). The Tukey post-hoc test was used to evaluate the differences between the groups. Statistical significance was accepted at $p < 0.05$. All data are given means \pm S.E.M.

Results

Serum CORT level in unstressed control and stressed animals

The results on serum CORT level in unstressed control and all stressed rat groups are presented in Fig. 1. Two-way ANOVA analysis revealed a significant effect of acute ($F_{2,30} = 93.19$, $p < 0.001$), chronic ($F_{1,30} = 22.45$, $p < 0.001$) or combined stress ($F_{2,30} = 6.44$, $p < 0.01$) on serum CORT secretion. In the acutely stressed animals, IM acted as an extremely potent stressor, resulted in a 5-fold increase in serum CORT level ($p < 0.001$), while C stress led to a 2-fold increase in CORT level ($p < 0.01$), compared to the unstressed control (Fig. 1, left panel). In animals chronically exposed to IS, serum CORT level was

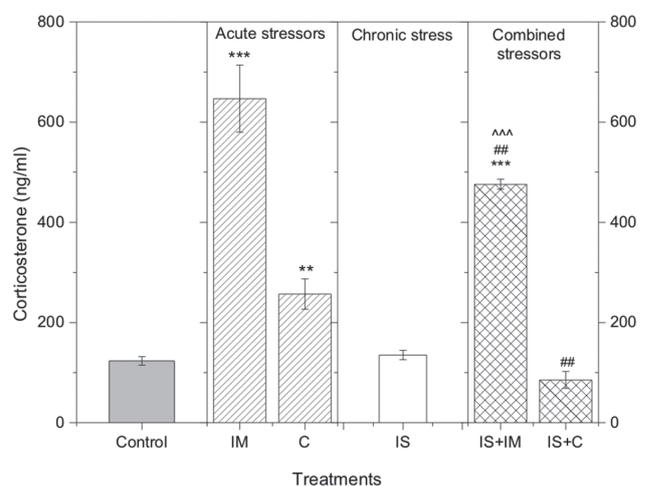


Figure 1. Changes in serum CORT levels (ng/ml) of adult Wistar male rats in unstressed control and rats exposed to acute immobilization (IM) or cold (C) stressors, chronic isolation (IS) or their combinations, as indicated. The results are expressed as mean \pm S.E.M. of 6 animals per group. Symbols indicate a significant difference between: respective stress treatment and unstressed control ** $p < 0.001$, *** $p < 0.001$; combined stressors and those respective acute stressors ## $p < 0.01$; combined stress isolation followed by immobilization (IS+IM) and chronic isolation (IS) $^{^^^}$ $p < 0.001$, by Tukey post-hoc test.

not changed ($p > 0.05$), (Fig. 1, middle panel) when compared to the unstressed control. Novel acute stressor IM showed a significant elevation of serum CORT level in the chronic IS-pretreated group and reached a 4-fold increase compared to the unstressed control ($p < 0.001$), as well as, 3-fold increase compared to the chronic IS ($p < 0.001$). On the other hand, consecutive exposure to acute C did not significantly alter serum CORT level ($p > 0.05$) compared to either unstressed control or chronic IS stress. When the results of the combined stressors were compared to those of acute stressors, it was observed a significant decrease ($p < 0.01$, Tukey post-hoc test).

MnSOD protein levels in mitochondrial or cytosolic fractions of the prefrontal cortex

An immunoreactive band of ~25 kDa corresponding to the predicted molecular mass of MnSOD protein was detected in mitochondrial and cytosolic fractions of the prefrontal cortex in unstressed control and all stressed rat groups (Fig. 2A). Two-way ANOVA analysis of mitochondrial MnSOD protein levels revealed significant effect of chronic stress ($F_{1,30} = 87.01$, $p < 0.001$), as well as, interaction effects of acute \times chronic stress ($F_{2,30} = 7.64$, $p < 0.01$). Post-hoc Tukey analysis showed a significant increase in the mitochondrial MnSOD protein level following acute IM ($p < 0.01$) (Fig. 2B, left panel), whereas acute C did not change this protein level, compared to the unstressed control. In chronically stressed animals, mitochondrial MnSOD protein level was not changed in a statistically significant manner ($p > 0.05$) compared to the unstressed control (Fig. 2B, middle panel). Additional acute stress of either IM or C showed a significant decrease in the mitochondrial MnSOD protein level in the chronic IS-pretreated group ($p < 0.01$, $p < 0.05$), relative to the unstressed control. The mitochondrial MnSOD protein levels following both combined stressors were significantly decreased from their levels after acute IM or C stressors, as indicated by Tukey post-hoc test ($p < 0.01$). For the cytosolic MnSOD protein levels in the prefrontal cortex, ANOVA analysis indicated that the main effect of chronic stress ($F_{1,30} = 27.89$, $p < 0.001$) exist in these animals. No significant differences were found in cytosolic MnSOD protein level between acute stressors and unstressed control ($p > 0.05$, Fig. 2B, left panel). A trend towards an increase in cytosolic MnSOD following chronic IS (Fig. 2B, middle panel) was found but it was not statistically significant ($p = 0.24$). Exposure to novel IM or C stress led to a significant increase in cytosolic MnSOD protein levels ($p < 0.05$) in the chronic IS-pretreated group, compared to the unstressed control (Fig. 2B, right panel). Statistically significant increase in cytosolic MnSOD protein level between combined IS+IM and acute IM stress was also observed ($p < 0.01$, Fig. 2B, right panel).

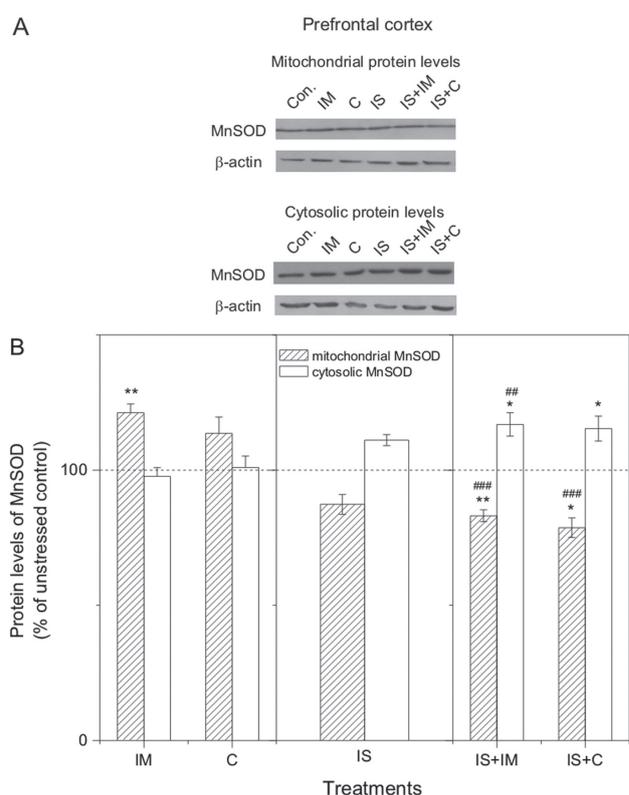


Figure 2. MnSOD protein level in mitochondrial or cytosolic fractions of the prefrontal cortex. **A.** Representative Western blots of MnSOD protein normalized to β -actin protein. **B.** Relative changes in MnSOD protein levels of rats exposed to acute immobilization (IM) or cold (C) stressors, chronic isolation (IS) or their combinations in both fractions, as indicated. The values are expressed as means \pm S.E.M. of 6–8 animals. Symbols indicate a significant difference between: the respective stress treatment and unstressed control * $p < 0.05$, ** $p < 0.01$; combined stressors and those respective acute stressors ## $p < 0.01$, ### $p < 0.001$, by Tukey post-hoc test.

MnSOD protein levels in mitochondrial or cytosolic fractions of the hippocampus

The representative hippocampal Western blots of MnSOD protein level in unstressed control and stressed Wistar male rat groups are presented in Fig. 3A. Two-way ANOVA analysis of mitochondrial MnSOD protein levels revealed significant effect of chronic stress ($F_{1,30} = 17.66$, $p < 0.001$). Post-hoc Tukey analysis showed a significantly increase in mitochondrial MnSOD protein level following acute IM stress compared to the unstressed control ($p < 0.05$) (Fig. 3B, left panel), whereas acute C did not change the level of this protein ($p > 0.05$). In chronically stressed group, mitochondrial MnSOD protein level was unchanged compared to the unstressed control level ($p > 0.05$) (Fig. 3B, middle

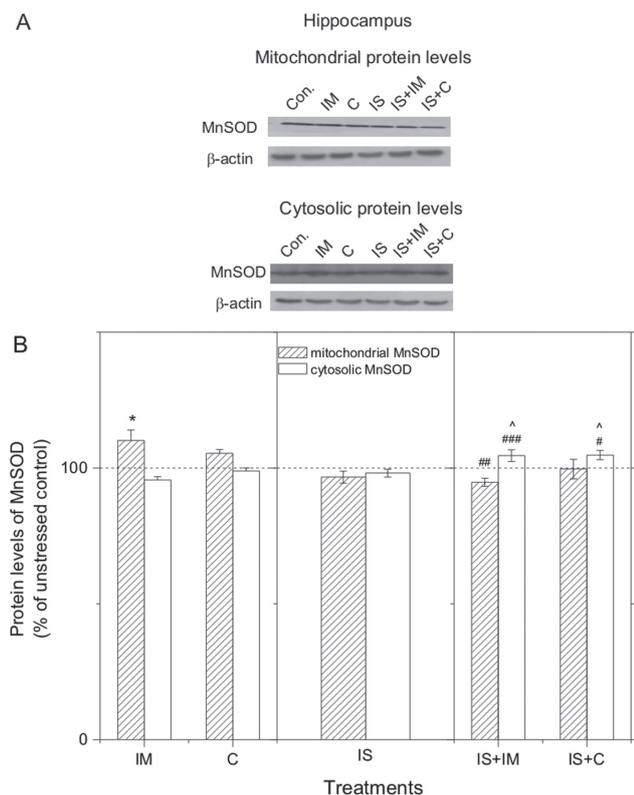


Figure 3. MnSOD protein level in mitochondrial or cytosolic fractions of the hippocampus. **A.** Representative Western blots of MnSOD protein normalized to β -actin protein. **B.** Relative changes in MnSOD protein levels of rats exposed to acute immobilization (IM) or cold (C) stressors, chronic isolation (IS) or their combinations in both fractions, as indicated. The values are expressed as means \pm S.E.M. of 6–8 animals. Symbols indicate a significant difference between: acute immobilization (IM) and unstressed control $^* p < 0.05$; combined stressors and those respective acute stressors $^{\#} p < 0.05$, $^{\#\#} p < 0.001$, $^{\#\#\#} p < 0.001$; combined stressors and chronic isolation (IS) stress $^{\wedge} p < 0.05$, by Tukey post-hoc test.

panel). In combined stress experiments, only IM led to decrease in the MnSOD protein level following IS compared to its level after acute IM stress ($^{\#\#} p < 0.01$) (Fig. 3B, right panel). Similarly to the prefrontal cortex, we found presence of hippocampal MnSOD protein in the cytosolic fraction of unstressed control and all stressed groups (Fig. 3A). Two-way ANOVA analysis revealed significant interaction effects of acute \times chronic stress ($F_{2,35} = 8.7$, $p < 0.01$). Post-hoc Tukey analysis showed a significant increase in cytosolic MnSOD level following both combined stressors (Fig. 3B, right panel) compared to either chronic IS ($^{\wedge} p < 0.05$) or those acute stressors ($^{\#\#\#} p < 0.001$, $^{\#} p < 0.05$), respectively.

Western blots demonstrating release of mitochondrial cytochrome c

To confirm the absence of mitochondrial contamination in our preparations, the same membrane from mitochondrial and cytosolic samples was reprobbed with anti-mouse cytochrome c oxidase subunit I (COX I; molecular probe 1 : 500), as the mitochondrial marker which is tightly bound to the inner mitochondrial membrane (Jin et al. 2005). The absence of COX I in the cytosolic fraction in unstressed control and all stressed groups of both brain structures confirmed that there was not contamination of mitochondria in the cytosolic fraction (Fig. 4).

As shown in Fig. 5, cytochrome c immunoreactivity was evident as a single band of molecular mass 15 kDa in the cytosolic fraction of all stressed groups of both brain structures, whereas it was barely detected in the unstressed control. The mitochondrial fractions of cytochrome c were also detected.

Discussion

This study compares the acute, chronic or combined stress models and determines the most effective stress model according to serum CORT level and subcellular distribution

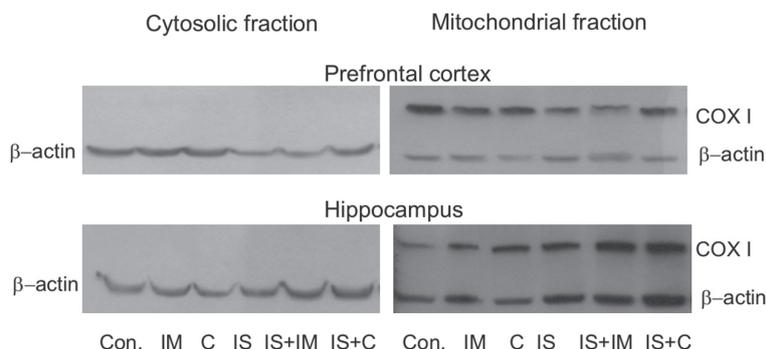


Figure 4. Cytochrome c oxidase subunit I (COX I) Western blots of mitochondrial or cytosolic fractions in unstressed control and all stressed groups from prefrontal cortex and hippocampus. β -actin was used as loading control. COX I was expressed in the mitochondrial fractions but not in the cytosolic fractions of both brain structures.

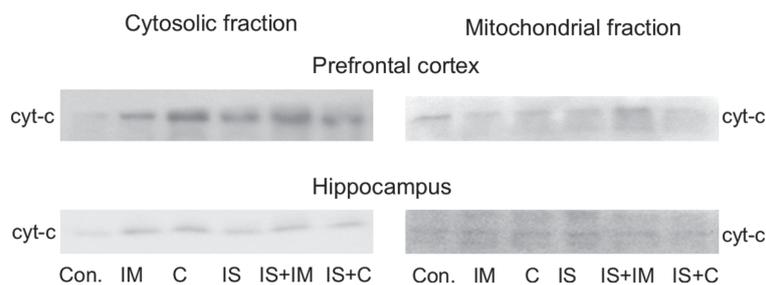


Figure 5. Cytochrome c (cyt-c) Western blots of mitochondrial and cytosolic fractions in unstressed control and all stressed groups from prefrontal cortex and hippocampus. cyt-c was detected in mitochondrial and cytosolic fractions in all stressed groups of both brain structures, whereas it was barely detected in unstressed control.

of MnSOD protein level in the prefrontal cortex and hippocampus. Western blot analysis of mitochondrial MnSOD protein level following acute IM stress showed up-regulation in both brain structures. The increased serum CORT level following both acute stressors is in accordance with previous studies (Dronjak et al. 2004; Sahin and Gümüſlü 2004) showing that serum CORT is important indicator of stress. IM as combined physical and emotional stress seems to be stronger stressor, while C is assumed to be a mild stressor, as judged by serum CORT level. Since the acute stress increases the production of ROS in the brain (McEwen 2001), increased mitochondrial MnSOD protein levels in both brain structures following acute IM are required to remove these high levels of ROS, generated under the high CORT level, and may reflect a protective response to oxidative stress, aiming to restore the cell homeostasis (Greenlund et al. 1995). Because MnSOD is localized in the inner membrane and matrix of mitochondria (Melov et al. 1999; Okado-Matsumoto and Fridovich 2001) we were surprised to find MnSOD protein level in the cytosolic fractions of unstressed control or both acute stressors of both brain structures. Absence of COX I from cytosolic fraction of unstressed control and all stressed groups showed that mitochondria fractionation procedure itself was not responsible for the release of mitochondrial MnSOD protein into the cytosolic compartment of brain (see Fig. 4). Since the MnSOD is encoded in the nuclear chromatin, synthesized as a precursor in the cytoplasm and transported to mitochondria *via* mitochondria targeting sequence, the presence MnSOD protein level in cytosolic fraction of unstressed control and following acute stressors in both brain structure, could be the results of identification of MnSOD protein that is in transit to the mitochondria after nuclear synthesis (Jin et al. 2005).

Opposite to the acute stressors, chronically stressed animals showed unchanged serum CORT level, relative to unstressed control. Furthermore, the effects of social IS on CORT level in the adult rats are not consistent among studies. Increased CORT level has been reported (Gamallo et al.

1986), whereas other groups observe no changes (Holson et al. 1991; Malkesman et al. 2006) or reduced CORT level (Sanchez et al. 1998). At the same time, chronic IS stress did not produce any significant changes in mitochondrial as well as in cytosolic MnSOD protein level of the prefrontal cortex and hippocampus, compared to the unstressed control. On the other hand, MnSOD activity data in response to the same acute or chronic stressor published by Pajović et al. (2006) were not followed by MnSOD protein expression data after either acute or chronic stressor determined in this study. The explanation for those discrepancies could be due to subcellular redistribution of the MnSOD protein in mitochondria and cytosol fraction of both brain structures examined in this study and its activity in whole extract of same brain structures performed by Pajović et al. (2006). Also, the MnSOD activity may be regulated at posttranslation level *via* phosphorylation or dephosphorylation independently of regulation of its protein synthesis (Hopper et al. 2006).

It has been shown that animals chronically exposed to a homotypic stressor display an exaggerated response of the HPA axis after exposure to heterotypic novel stressor (Marti et al. 1994; Bhatnagar and Dallman 1998). In our study, chronically isolated animals were also exposed to novel acute IM or C as heterotypic stressors. Since the chronic IS stress leads to the deregulation of glucocorticoid negative feedback mechanisms at the level of HPA axis in the prefrontal cortex and hippocampus of stressed rats (Sanchez et al. 1995; Filipović et al. 2005), novel acute stressors cannot generate appropriate answer to stimuli. This is represented by lower increase in serum CORT level than in acutely stressed animals. Our data are in agreement with the findings of Sanchez et al. (1998) who reported that 2 months of isolated rats also resulted in reduced plasma CORT concentration to 15 min of restrain stress, compared to the controls which were exposed to the acute restrain stress. Nevertheless, it is hard to say that it is a protective phenomenon, since altered activation of HPA axis is correlated with some major disorders (Tanke et al. 2008). Other possible explanation for lower

CORT levels is due to decreased secretion of corticotrophin releasing hormone that also occurs in long-term IS (Sancez et al. 1995). Moreover, novel acute IM stress produced a higher increase in serum CORT level of chronically isolated rats, comparing to the novel C stress, suggesting that response of the chronically stressed animals additionally exposed to novel acute stressors depend on the applied stress stimuli (Pacak and Palkovits 2001; Gavrilović and Dronjak 2005). At the same time, there was significant decrease in mitochondrial MnSOD protein levels in chronically stressed animals exposed to either IM or C in the prefrontal cortex, relative to the control or those levels after acute stressors, while in hippocampus it was observed only between chronic IS followed by IM and acute IM. Nevertheless, it may be claimed that our preparations reflect the average MnSOD protein changes in whole hippocampus, which could potentially mask MnSOD protein changes, and that detailed histochemical pictures would reflect protein expression more precisely. It could be speculate that chronic stress-induced changes in homeostatic mechanism could contribute to MnSOD protein inactivation by peroxynitrite (MacMillan-Crow et al. 1998; Yamakura et al. 1998; Knirsch and Clerch 2001; Filipović et al. 2007). Peroxynitrite-related MnSOD has been suggested to be related to the phosphorylation of superoxide dismutase binding proteins and to the induction of dityrosine formation and tyrosine oxidation in MnSOD. Accordingly, compromised mitochondrial MnSOD protein level could lead to the increased oxidant production within mitochondria, which could lead to nitration of other mitochondrial proteins (Madrigal et al. 2001; Cruthirds et al. 2003).

At the other side, a reciprocal increase in cytosolic MnSOD protein level between combined stressors and unstressed control in the prefrontal cortex, as well as between combined stressors and chronic or acute stressor in hippocampus, was shown. To assess whether presence of the MnSOD in the cytosol was a consequence of loss of mitochondrial membrane integrity under the combined stressors and mitochondrial MnSOD release into the cytosol, we used another mitochondrial marker, cytochrome c, to document mitochondrial damage (Jin et al. 2005). Therefore, the blots of mitochondrial/cytosolic fractions of the unstressed control and all stressed groups of prefrontal cortex and hippocampus were stripped of primary antibody and reprobred with the cytochrome c antibody. Like MnSOD, cytochrome c was observed in cytosol fraction following acute, chronic or combined stressors, whereas it was barely detected in unstressed control (Fujimura et al. 1999). It seems tempting to speculate that ROS generated under prolonged neuroendocrine stress could contribute to the release of cytochrome c to the cytosol after opening of the permeability transition pore (Yang and Cortopassi 1998; Cassarino et al. 1999; Petrosillo et al. 2001). This release may results in further ROS production by inhibition of the respiratory chain (Cai and Jones 1998). Taken

together, increased cytosolic MnSOD protein level could be derived from mitochondrial MnSOD and/or inappropriate transport of newly synthesized MnSOD into mitochondria (changes in mitochondrial targeting domain). Regardless of the mechanism, the appearance of MnSOD in the cytosolic fraction clearly indicates a loss of mitochondrial membrane integrity (Cruthirds et al. 2003; Jin et al. 2005).

The results suggest that, different stress models have different degree in influences on serum CORT and MnSOD subcellular protein level in the prefrontal cortex and hippocampus. The increased mitochondrial MnSOD protein level following acute stress might reflect a protective response to increased oxidative stress. Chronic neuroendocrine stress compromises induction of mitochondrial MnSOD protein whereas its cytosolic localization is significantly increased following combined stressors. Release mitochondrial MnSOD as well as intermembrane protein cytochrome c into the cytosol could serve as biochemical markers for mitochondrial dysfunction. Moreover, mitochondrial MnSOD protein level could serve as index for discrimination of previous chronic stress exposure. The lack of MnSOD in mitochondria may lead to further biochemical cascade causing cytochrome c release (Fujimura et al. 1999), which is known to be apoptogenic (Liu et al. 1996). Further studies are necessary to clarify whether cytochrome c plays a role in inducing the mitochondrial-dependent apoptosis cascade in acute or chronic stressed rat brain.

Acknowledgement. This work was supported by the Ministry of Sciences of the Republic of Serbia, grant No. 143044B.

References

- Antras-Ferry J., Mahéo K., Morel F., Guillouzo A., Cillard P., Cillard J. (1997): Dexamethasone differently modulates TNF-alpha and IL-1beta-induced transcription of the hepatic Mn-superoxide dismutase gene. *FEBS Lett.* **403**, 100–104
- Bhatnagar S., Dallman M. (1998): Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. *Neuroscience* **84**, 1025–1039
- Blumberg J. (2004): Use of biomarkers of oxidative stress in research studies. *J. Nutr.* **134**, 3188–3189
- Cai J., Jones D. P. (1998): Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. *J. Biol. Chem.* **273**, 11401–11404
- Cassarino D. S., Parks J. K., Parker W. D. Jr., Bennett J. P. Jr. (1999): The parkinsonian neurotoxin MPP⁺ opens the mitochondrial permeability transition pore and releases cytochrome c in isolated mitochondria via an oxidative mechanism. *Biochim. Biophys. Acta* **1453**, 49–62
- Chong Z. Z., Li F., Maiese K. (2005): Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. *Prog. Neurobiol.* **75**, 207–246

- Chourbaji S., Zacher C., Sanchis-Segura C., Spanagel R., Gass P. (2005): Social and structural housing conditions influence the development of a depressive-like phenotype in the learned helplessness paradigm in male mice. *Behav. Brain Res.* **164**, 100–106
- Cruthirds D. L., Novak L., Akhi K. M., Sanders P. W., Thompson J. A., MacMillan-Crow L. A. (2003): Mitochondrial targets of oxidative stress during renal ischemia/reperfusion. *Arch. Biochem. Biophys.* **412**, 127–133
- Dronjak S., Gavrilović Lj., Filipović D., Radojčić B. M. (2004): Immobilization and cold stress affect sympatho-adrenomedullary system and pituitary-adrenocortical axis of rats exposed to long-term isolation and crowding. *Physiol. Behav.* **81**, 409–415
- Evans M. D., Dizdaroglu M., Cooke M. S. (2004): Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res.* **567**, 1–61
- Filipović D., Gavrilović Lj., Dronjak S., Radojčić B. M. (2005): Brain glucocorticoid receptor and heat shock protein 70 levels in rats exposed to acute, chronic or combined stress. *Neuropsychobiology* **51**, 107–114
- Filipović M. R., Stanić D., Raicević S., Spasić M., Niketić V. (2007): Consequences of MnSOD interactions with nitric oxide: nitric oxide dismutation and the generation of peroxynitrite and hydrogen peroxide. *Free Radic. Res.* **41**, 62–72
- Fujimura M., Morita-Fujimura Y., Kawase M., Copin J. C., Calagui B., Epstein C. J., Chan P. H. (1999): Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome c and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. *J. Neurosci.* **19**, 3414–3422
- Gamallo A., Villanua A., Tranco G., Fraile A. (1986): Stress adaptation and adrenal activity in isolated and crowded rats. *Physiol. Behav.* **36**, 217–221
- Garzon J., del Rio J. (1981): Hyperactivity induced in rats by long-term isolation: further studies on a new animal model for the detection of antidepressants. *Eur. J. Pharmacol.* **74**, 287–294
- Gavrilović L., Dronjak S. (2005): Activation of rat pituitary-adrenocortical and sympatho-adrenomedullary system in response to different stressors. *Neuro Endocrinol. Lett.* **26**, 515–520
- Greenlund J., Deckwerth L., Johnson M. Jr. (1995): Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron* **14**, 303–315
- Haller J., Halasz J. (1999): Mild social stress abolishes the effects of isolation on anxiety and chlordiazepoxide reactivity. *Psychopharmacology (Berl.)* **144**, 311–315
- Holson R. R., Scallet A. C., Turner B. B. (1991): Isolation stress revisited: isolation-rearing effects depend on animal care methods. *Physiol. Behav.* **49**, 1107–1118
- Hopper R. K., Carroll S., Aponte A. M., Johnson D. T., French S., Shen R. F., Witzmann F. A., Harris R. A., Balaban R. S. (2006): Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium. *Biochemistry* **45**, 2524–2536
- Jacobson L., Sapolsky R. (1991): The role of the hippocampus in feedback regulation of hypothalamic-pituitary-adrenocortical axis. *Endocr. Rev.* **12**, 118–134
- Jin Z. Q., Zhou H. Z., Cecchini G., Gray M. O., Karliner J. S. (2005): MnSOD in mouse heart: acute responses to ischemic preconditioning and ischemia-reperfusion injury. *Am. J. Physiol., Heart Circ. Physiol.* **288**, 2986–2994
- Keller J. N., Kindy M. S., Holtsberg F. W., St. Clair D. K., Yen H.-C., Germeyer A., Steiner S. M., Bruce-Keller A. J., Hutchins J. B., Mattson M. P. (1998): Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J. Neurosci.* **18**, 687–697
- Knirsch L., Clerch L. B. (2001): Tyrosine phosphorylation regulates manganese superoxide dismutase (MnSOD) RNA-binding protein activity and MnSOD protein expression. *Biochemistry* **40**, 7890–7895
- Kvetnansky R., Mikulaj L. (1970): Adrenal and urinary catecholamines in rat during adaptation to repeated immobilization stress. *Endocrinology* **87**, 738–743
- Le Bras M., Clément M. V., Pervaiz S., Brenner C. (2005): Reactive oxygen species and the mitochondrial signaling pathway of cell death. *Histol. Histopathol.* **20**, 205–219
- Lebovitz R. M., Zhang H., Vogel H., Cartwright J. Jr., Dionne L., Lu N., Huang S., Matzuk M. M. (1996): Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9782–9787
- Liu J., Wang X., Shigenaga M. K., Yeo H. C., Mori A., Ames B. N. (1996): Immobilization stress causes oxidative damage to lipid, protein, and DNA in the brain of rats. *FASEB J.* **10**, 1532–1538
- Liu X., Kim C. N., Yang J., Jemmerson R., Wang X. (1996): Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147–157
- Lowry O. H., Rosebrough N. J., Farr A. J., Randall R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **123**, 265–275
- MacMillan-Crow L. A., Crow J. P., Thompson J. A. (1998): Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry* **37**, 1613–1622
- Madrigal J. L., Olivenza R., Moro M. A., Lizasoain I., Lorenzo P., Rodrigo J., Leza J. C. (2001): Glutathione depletion, lipid peroxidation and mitochondrial dysfunction are induced by chronic stress in rat brain. *Neuropsychopharmacology* **24**, 420–429
- Maisonnette S., Morato S., Brandão M. L. (1993): Role of resocialization and of 5-HT_{1A} receptor activation on the anxiogenic effects induced by isolation in the elevated plus-maze test. *Physiol. Behav.* **54**, 753–758
- Malkesman O., Maayan R., Weizman A., Weller A. (2006): Aggressive behavior and HPA axis hormones after social isolation in adult rats of two different genetic animal models for depression. *Behav. Brain Res.* **175**, 408–414
- Marti O., Gavalda A., Gomez F., Armario A. (1994): Direct evidence for chronic stress-induced facilitation of the

- adrenocorticotropin response to a novel acute stressor. *Neuroendocrinology* **60**, 1–7
- McEwen B. S. (2001): Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. *Ann. N. Y. Acad. Sci.* **933**, 265–277
- McIntosh L. J., Hong K. E., Sapolsky R. M. (1998a): Glucocorticoids may alter antioxidant enzyme capacity in the brain: baseline studies. *Brain Res.* **791**, 209–214
- McIntosh L. J., Cortopassi K. M., Sapolsky R. M. (1998b): Glucocorticoids may alter antioxidant enzyme capacity in the brain: kainic acid studies. *Brain Res.* **791**, 215–222
- Melov S., Coskun P., Patel M., Tuinstra R., Cottrell B., Jun A. S., Zastawny T. H., Dizdaroglu M., Goodman S. I., Huang T. T., Mizioro H., Epstein C. J., Wallace D. C. (1999): Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 846–851
- Michel T. M., Frangou S., Thiemeyer D., Camara S., Jecel J., Nara K., Brunklaus A., Zochling R., Riederer P. (2007): Evidence for oxidative stress in the frontal cortex in patients with recurrent depressive disorder – a postmortem study. *Psychiatry Res.* **151**, 145–150
- Mizoguchi K., Ishige A., Aburada M., Tabira T. (2003): Chronic stress attenuates glucocorticoid negative feedback: involvement of the prefrontal cortex and hippocampus. *Neuroscience* **119**, 887–897
- Niesink R. J., Van Ree J. M. (1982): Antidepressant drugs normalize the increased social behaviour of pairs of male rats induced by short term isolation. *Neuropharmacology* **21**, 1343–1348
- Okado-Matsumoto A., Fridovich I. (2001): Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J. Biol. Chem.* **276**, 38388–38393
- Olivenza R., Moro M. A., Lizasoain I., Lorenzo P., Fernández A. P., Rodrigo J., Bosca L., Leza J. C. (2000): Chronic stress induces the expression of inducible nitric oxide synthase in rat brain cortex. *J. Neurochem.* **74**, 785–791
- Pacak K., Palkovits M. (2001): Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. *Endocr. Rev.* **22**, 502–548
- Pajović S. B., Pejić S., Stojiljković V., Gavrilović Lj., Dronjak S., Kanazir D. T. (2006): Alterations in hippocampal antioxidant enzyme activities and sympatho adrenomodulatory system of rats in response to different stress models. *Physiol. Res.* **55**, 453–460
- Pardon M. C. (2007): Stress and ageing interactions: a paradox in the context of shared etiological and physiopathological processes. *Brain Res. Rev.* **54**, 251–273
- Petrosillo G., Ruggiero F. M., Pistolesse M., Paradies G. (2001): Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles *via* cardiolipin peroxidation. Possible role in the apoptosis. *FEBS Lett.* **509**, 435–458
- Popović M., Popović N., Erić-Jovicić M., Jovanova-Nesić K. (2000): Immune responses in nucleus basalis magnocellularis-lesioned rats exposed to chronic isolation stress. *Int. J. Neuroscience* **100**, 125–131
- Sahin E., Gümüşlü S. (2004): Alterations in brain antioxidant status, protein oxidation and lipid peroxidation in response to different stress models. *Behav. Brain Res.* **155**, 241–248
- Sanchez M. M., Aguado F., Sanchez-Toscano F., Saphier D. (1995): Effects of prolonged social isolation on responses of neurons in the bed nucleus of the stria terminalis, preoptic area, and hypothalamic paraventricular nucleus to stimulation of the medial amygdala. *Psychoneuroendocrinology* **20**, 525–541
- Sanchez M., Aguado F., Sanchez-Toscano F., Saphier D. (1998): Neuroendocrine and immunocytochemical demonstrations of decreased hypothalamo-pituitary-adrenal axis responsiveness to restraint stress after long-term social isolation. *Endocrinology* **139**, 579–587
- Sapolsky R. M. (1992): An introduction to the adrenocortical axis. In: *Stress, the Aging Brain, and the Mechanisms of Neuron Death*. Bradford. pp. 11–27, MIT Press, Cambridge
- Sarandol A., Sarandol E., Eker S. S., Erdinc S., Vatanserver E., Kirli S. (2007): Major depressive disorder is accompanied with oxidative stress: short-term antidepressant treatment does not alter oxidative-antioxidative systems. *Hum. Psychopharmacol.* **22**, 67–73
- Slot J. W., Geuze H. J., Freeman B. A., Crapo J. D. (1986): Intracellular localization of the copper-zinc and manganese superoxide dismutases in rat liver parenchymal cells. *Lab. Invest.* **55**, 363–371
- Tanke M. A., Fokkema D. S., Doornbos B., Postema F., Korf J. (2008): Sustained release of corticosterone in rats affects reactivity, but does not affect habituation to immobilization and acoustic stimuli. *Life Sci.* **83**, 135–141
- Turrens J. F. (1997): Superoxide production by the mitochondrial respiratory chain. *Biosci. Rep.* **17**, 3–8
- Valentine J. F., Nick H. S. (1994): Glucocorticoids repress basal and stimulated manganese superoxide dismutase levels in rat intestinal epithelial cells. *Gastroenterology* **107**, 1662–1670
- Wallace D. C. (2005): A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* **39**, 359–407
- Wispe J. R., Clark J. C., Burhans M. S., Kropp K. E., Korfhagen T. R., Whitsett J. A. (1989): Synthesis and processing of the precursor for human manganese-superoxide dismutase. *Biochim. Biophys. Acta* **994**, 30–36
- Yamakura F., Taka H., Fujimura T., Murayama K. (1998): Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J. Biol. Chem.* **273**, 14085–14089
- Yang J. C., Cortopassi G. A. (1998): Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome c. *Free Radic. Biol. Med.* **24**, 624–631