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Chronic social isolation induces NF-κB activation and upregulation of iNOS protein expression in rat prefrontal cortex

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ABSTRACT

Exposure of an organism to stress, results in oxidative stress and increased nitric oxide (NO) production in the brain. The role of the processes caused by chronic stress in the prefrontal cortex has not been fully investigated. Considering that chronic stress increases NO production by the enzyme nitric oxide synthase (NOS), we examined the cytosolic neuronal (nNOS) or inducible (iNOS) protein levels in the prefrontal cortex of rats exposed to 21 d of chronic social isolation stress, an animal model of depression, alone or in combination with 2 h of acute immobilization or cold (4 °C) stress (combined stress). Antioxidative status via cytosolic CuZnSOD and mitochondrial MnSOD activity, cytosolic redox status via reduced glutathione (GSH) concentration were determined. Furthermore, cytosolic inducible heat shock protein 70 (Hsp70i), cytosolic/nuclear distributions of NF-κB and serum corticosterone (CORT) were also investigated to elucidate the possible mechanism involved in the cellular NOS pathway. Our results showed that both acute stressors led to increases of CORT and nNOS protein while iNOS protein expression was unaffected. In contrast to the acute stress, chronic social isolation compromised hypothalamic–pituitary-adrenal axis functioning such that the normal stress response was impaired following subsequent acute stressors. Downregulated redox GSH status as well as decreased activity of CuZnSOD and MnSOD suggests the existence of oxidative stress which remained as such following combined stressors. Changes in redox-status associated with decreased Hsp70i protein expression enabled NF-κB translocation into the nucleus, causing increased cytosolic nNOS and iNOS protein expression. Results suggest that NOS signaling pathway plays a differential role between acute and chronic stress whereby state of oxidative/nitrosative stress after chronic social isolation is caused, at least in part, by NF-κB activation and increased iNOS protein expression.

1. Introduction

Exposure of an organism to stress results in an increase of nitric oxide (NO) production in the brain (Zlatković and Filipović, 2012). As NO plays a role in synaptic plasticity, neuromodulation and other physiological functions, under pathophysiological conditions, it may induce oxidative/nitrosative damage, suggesting its involvement in neurotoxicity (Dhir and Kulkarni, 2011) and anxiety/depression (Esch et al., 2002). NO is generated by neuronal NO synthase (nNOS) or inducible NO synthase (iNOS) (Alderton et al., 2001).

Although nNOS is a constitutive enzyme that can act as classical neurotransmitters, its expression is also influenced by certain stressors (McLeod et al., 2001). In contrast, persistent activation of iNOS, mainly regulated at the transcription level, can lead to toxic levels of NO production. Once iNOS is expressed, the overproduction of oxygen and nitrogen reactive species (NRS) may cause oxidation of cellular components found after stress in the rat brain.

A main regulator of iNOS expression is the activation of nuclear factor κB (NF-κB) (Aktan, 2004), which is also involved in nNOS transcription (Napolitano et al., 2008). NF-κB is a redox-sensitive transcription factor localized in the cytoplasm as an inactive form through its interaction with the inhibitory protein I-kappaB (IκB). It can be activated by reactive oxygen species (ROS) (Li and Karin, 1999) or by neurotransmitters such as glutamate (Pizzi et al., 2005), resulting in proteolytic degradation of IκB with concomitant nuclear translocation of the liberated NF-κB heterodimer that control the NF-κB-regulated target genes (Senftleben et al., 2001). On the other hand, several studies reported that expression of stress-inducible heat shock protein 70 (Hsp70i) blocks NF-κB activation and NF-κB-dependent gene expression (Malhotra and Wong, 2002; Malhotra et al., 2002). Moreover, Hsp70i is induced by physiological, pathological and environmental stressors (Kiang and Tsokos, 1998) and its degree of induction depends on the level and duration of exposure to stressors.

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The reaction of NO with superoxide anion produces the potent oxidant peroxynitrite (Patel and Darley-Usmar, 1996). Given that concentration of NO within the biological system is regulated by the activity of NOS isoforms, while the concentration of superoxide anion is regulated by the activity of superoxide dismutases (SODs), intracellular formation of peroxynitrite depends on the activities of NOS isoforms and SODs. Thus, cells can regulate the concentrations of superoxide anion, as well as peroxynitrite *via* cytosolic CuZnSOD and mitochondrial MnSOD. Moreover, the susceptibility of brain cells to NO and peroxynitrite exposure may be dependent on intracellular reduced glutathione (GSH) and cellular stress resistance signal pathways. Through the formation of S-nitrosoglutathione, NO can cause GSH depletion and hence trigger redox-dependent changes in cellular signaling that lead to mitochondrial damage and cell death in neurons (Calabrese et al., 2000). In fact, a compromised GSH system, together with the brain oxidative stress, has been demonstrated to be a feature of depression (Zhang et al., 2009).

The role of the NOS pathway in stress and stress response is still unknown. We previously reported that 21 d of chronic social isolation (CSIS), an animal model of depression (Dronjak et al., 2007; Hall et al., 1998; Serra et al., 2007; Spasojević et al., 2007), leads to an overproduction of NO, causing a state of nitrosative stress (Zlatković and Filipović, 2012) and induces apoptosis in the prefrontal cortex (Filipović et al., 2011). Based on these findings, we hypothesized that CSIS stress may activate the NOS pathway and further compromise antioxidative capacity. Given that prefrontal cortex is a target of the maladaptive response to stress (Cerqueira et al., 2007) and that the production of NO is accompanied by the expression of NOS isoforms, the current study sought to examine whether CSIS alters the cytosolic nNOS and iNOS protein expression in rat prefrontal cortex. To elucidate the possible mechanisms involved in the cellular NOS pathway, we determined the cytosolic/nuclear distributions of NF-κB as a transcriptional factor for iNOS and nNOS synthesis, as well as protein expression of cytosolic Hsp70i as a suppressor of NF-κB activation. As an adaptive response to HPA axis induction includes the antioxidant defense systems, the cytosolic redox status via reduced glutathione (GSH) concentration and antioxidative status via CuZnSOD and MnSOD activity were also measured. Moreover, to assess the influence of CSIS on HPA axis functioning, acute immobilization (IM) or cold (C, 4 °C) stress alone or in combination with CSIS (combined stressors, CSIS + IM and CSIS + C) were also examined in order to indicate a normal adaptive response (Sapolsky et al., 2000) or potentially maladaptive response to CSIS. The intensity of the applied stressors was determined by measuring serum corticosterone (CORT) as a marker of HPA axis functioning.

2. Materials and methods

2.1. Animal subjects

Adult male Wistar rats (2–3 months old, weighing 330–400 g) were housed in groups of four per cage in a temperature-controlled environment (21–23 °C) on a 12/12 h light/dark cycle (lights on between 07:00 h and 19:00 h), with food (commercial rat pellets) and water available *ad libitum*. All experimental procedures were carried out in accordance with the Ethical Committee for the Use of Laboratory Animals of the Institute of Nuclear Sciences, "Vinča," which follows the guidelines of the registered "Serbian Society for the Use of Animals in Research and Education". Animals were randomly divided into four groups. Group I was comprised of unstressed animals (control group, $n = 6$). Group II was exposed to either 2 h of immobilization (IM) or cold (C, 4 °C) as acute stressors ($n = 6$ per stressor). IM was carried out by forcing the rats into a prone position with all four limbs fixed to a board using adhesive tape and allowing limited head movement (Kvetnansky and Mikulaj, 1970). Group III was exposed to chronic social isolation (CSIS) stress via individual housing for 21 d, according to the model of Garzón and del Rio (1981), during which animals had relatively normal auditory and olfactory experiences but no visual or tactile exposure to other animals ($n = 6$). Group IV represented the combined stressors (CSIS + IM, CSIS + C, $n = 6$ per stressor), in which rats underwent CSIS followed by a single 2 h exposure to either IM or C stress. Experiments with acute stressors were performed between 8:00 and 10:00 a.m. in order to minimize possible hormonal interference by circadian rhythms. Following the stress procedure, stressed animals as well as controls were anesthetized with ketamine/xylazine (100/5 mg/kg i.p.) and sacrificed by guillotine decapitation (Harvard Apparatus, South Natick, MA, USA). Brains were immediately removed and the prefrontal cortex was dissected on ice. Tissue samples were frozen in liquid nitrogen and kept at −70 °C until further analysis.

2.2. Serum corticosterone assay

Trunk blood was collected and the serum obtained by centrifugation at 1500×*g* for 10 min at 4 °C, and kept at −70 °C until assayed. The OCTEIA corticosterone ELISA kit (REF AC-14F1; Immunodiagnostics Systems-IDS, UK IDS) was used to measure serum CORT levels (ng/ml) in all experimental groups. All samples were analyzed in a single assay to avoid inter-assay variation. The variation between duplicates of samples was less than 7%. The lower detection limit for CORT in this assay system was 25 ng/ml.

2.3. Subcellular fractionation

To prepare nuclear/mitochondrial/cytosolic tissue protein extracts (Moutsatsou et al., 2001; Spencer et al., 2000), frozen prefrontal cortex was weighed and homogenized in 2 vol. (w/v) of cold homogenization buffer I [0.25 M sucrose, 15 mM TRIS–HCl (pH 7.9), 16 mM KCl, 15 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol (DTT), 0.15 mM spermine and 0.15 mM spermidine supplemented with following protease inhibitors: 0.1 mM 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×*g*, at 4 °C for 10 min. The pellet (P1) used for obtaining nuclear fraction was resuspended in 4 vol. buffer II [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitors], centrifuged at 4000×*g*, at 4 °C for 10 min and resulting pellet (P2) was washed by resuspension in buffer II followed by additional centrifugation at 4000×*g*, at 4 °C for 10 min. Finally, the pellets were resuspended in 1 vol. of buffer III [10 mM HEPES (pH 7.9), 0.75 mM $MgCl_2$, 0.5 M KCl, 0.5 mM EDTA, 12.5% glycerol and protease inhibitors]. The mixture was incubated on ice for 30 min with occasional vortexing. After final 30 min centrifugation at 14000×*g*, nuclear extract was obtained. Supernatant (S1) was centrifuged at 15000×*g*, at 4 °C for 20 min. The resulting mitochondrial pellet (P3) was washed by resuspension in homogenization buffer I followed by additional centrifugation at 15,000×*g*, at 4 °C for 20 min and resuspended in 250 μl of lysis buffer [50 mM TRIS–HCl (pH 7.4), 5% glycerol, 1 mM EDTA, 5 mM DTT, supplemented with mentioned protease inhibitors and 0.05% Triton X-100] to obtain mitochondrial fraction. Supernatant (S2) was further centrifuged at 100, 000×*g*, at 4 °C for 60 min and resulted supernatant was used as the cytosolic fraction. Samples are stored at −70 °C until measuring protein concentrations. Protein content in the cellular fractions was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA; Sigma Aldrich, Inc., USA) as a reference. Purity of

isolated subcellular fractions was confirmed by the absence of nuclear contaminations of the cytosolic fractions after incubation of control samples with antibody against nuclear protein- histone 2B (H2B) (Cell signaling, Inc., Beverly, MA, USA) followed by HRP-conjugated secondary goat anti-rabbit IgG antibody (A9169, Sigma Aldrich, (St. Louis, MO), as well as, the absence of mitochondrial contaminations of the cytosolic fractions after incubation of control samples with mitochondrial anti-cytochrome c oxidase (COX) subunit IV antibody (Cell Signaling) (Fig. 1).

2.4. Activity of cytosolic/nuclear CuZnSOD and mitochondrial MnSOD

Superoxide dismutase activity was measured using commercially available Biorex BXC0531 kit (Biorex Diagnostics Ltd.) on RX Daytona Clinical Chemistry Analyser (Randox Laboratories, UK). This method is based on the generation of superoxide radicals in xanthine and xanthine oxidase reactions, which further reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltrazolium chloride (INT) to form a red formazan dye. The SOD activity is measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay. First was measured total SOD activity in the samples. KCN addition during the preparation of samples for the second round of measurements blocked CuZnSOD activity, so all measured activity was derived from MnSOD. After the subtraction of these two values, CuZnSOD activity in samples was obtained. As the assay range of the kit is approximately from 0.06 to 4.52 U/ml, protein concentrations of samples for this assay were adjusted to ensure reaction linearity. SOD activity was expressed as units per milligram of total proteins.

2.5. Reduced glutathione levels

Reduced glutathione (GSH) was quantified from freshly prepared cytosolic fractions of the prefrontal cortex and estimated according to the protocol of Hissin and Hilf (1976). Briefly, 1 ml of supernatant (0.5 ml of cytosolic fraction of prefrontal cortex precipitated by 2 ml of 5% TCA) was taken and 0.5 ml of Ellman's reagent (0.0198% DTNB in 1% sodium citrate) and 3 ml of phosphate buffer (pH 8.0) were added. The color developed was read at 412 nm. Reduced glutathione level was expressed as nM per milligram of total proteins.

2.6. Electrophoresis and western blot analysis of Hsp70i, NF-κB, iNOS, nNOS

Equal amounts of cytosolic or nuclear protein fractions were separated on a 8% SDS–polyacrylamide gel using a Mini-Protean II Electrophoresis Cell (Bio-Rad, Hercules, CA, USA) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using Mini Trans-blot apparatus (Bio-Rad). Membranes were blocked for 1 h with 5% BSA in TBS-T buffer pH 7.6 (20 mM Tris–HCl, 137 mM NaCl, 0.3% Tween 20) and then cut to obtain the bottom part (for β-actin), middle (for Hsp70i), and upper (for iNOS and nNOS), and incubated overnight (4 °C) with rabbit antibody raised against Hsp70i, NF-κB, iNOS (Santa Cruz Biotechnology). After washing three times in TBS-T, the membranes were incubated for 2 h with anti-rabbit HRP-conjugated secondary antibody (Cell Signaling, Inc., Beverly, MA, USA). β-actin (primary monoclonal anti-mouse β actin antibody, Sigma St. Louis, MO, followed by HRP-conjugated secondary goat anti-mouse IgG antibody, Santa Cruz Biotechnology) was used to confirm a consistent protein loading for each lane. The blots were developed by enhanced chemiluminescence (Immobilon™ Western, Millipore Corporation, Billerica, USA) and exposed to an X-ray film. After probing with iNOS antibody, the same part of membrane was stripped and re-probed with rabbit antibody raised against nNOS (Santa Cruz Biotechnology). The signals were electronically digitized by scanning, and the image was processed for quantification using Image J analysis PC software. Protein molecular mass standards (Page Ruler™ Plus Prestained Protein Ladder, Fermentas, St. Leon-Rot, Germany) were used for calibration. Identification of this response was observed at 72 kDa for Hsp70i, 65 kDa for NF-κB, 130 kDa for iNOS, 150 kDa for nNOS and 42 kDa for β-actin. Western blot results were expressed as protein/β-actin ratio. The levels of investigated proteins in stressed animals are given as the percent change relative to control rats (100%). The data are presented as means ± S.E.M. of 6 animals per group.

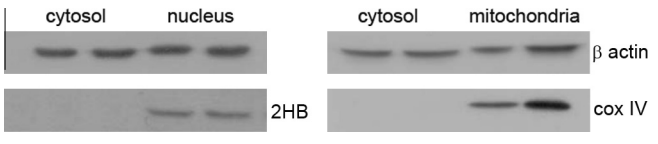

Fig. 1. Purity of the nuclear, mitochondrial and cytosolic protein fractions in the rat prefrontal cortex.

2.7. Statistical analysis

Data were analyzed by two-way ANOVA [the factors were acute (levels: none, IM and C) or chronic (levels: no stress and CSIS) stress] (STATISTICA Release 7). Duncan's post hoc test was used to evaluate differences between groups. Statistical significance was set at $p < 0.05$. The data are expressed as mean ± S.E.M. of 6 animals per group.

3. Results

3.1. Compromised HPA axis functioning under chronic social isolation stress

The results of serum CORT levels determined in controls and all stressed rat groups are presented in Fig. 2. A two-way ANOVA revealed a significant main effects of acute ($F_{2.30} = 80.63$, $p < 0.001$) and chronic ($F_{1.30} = 24.36$, $p < 0.001$) stress and an interaction of acute × chronic stress ($F_{2.30} = 5.12$, $p < 0.001$) on serum CORT secretion. In the acutely stressed animals, IM acted as an extremely potent stressor, inducing a 4-fold increase in serum CORT levels ($^{***}p < 0.001$), while C showed a 2-fold increase ($^{**}p < 0.01$) relative to the controls. CORT levels were unaltered following CSIS, as com-

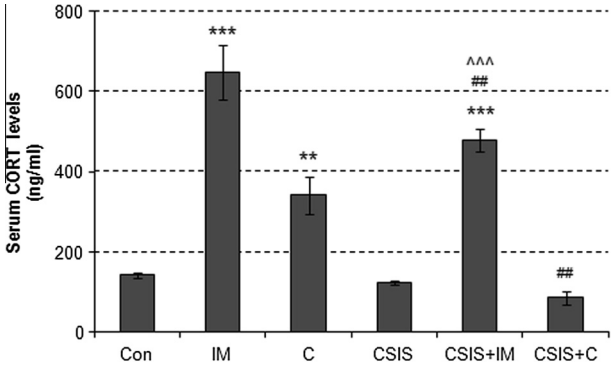

Fig. 2. Serum CORT level (ng/ml) concentrations in controls and following acute immobilization (IM) and cold (C) stress, chronic social isolation stress (CSIS), and combined stressors (SCIS + IM or SCIS + C). Asterisks indicate significantly differences between: respectively stress treatment and control ($^{**}p < 0.01$, $^{***}p < 0.001$); CSIS and combined stress ($^{\wedge\wedge\wedge}p < 0.001$); combined stressors and those respective acute stressor alone ($^{\#\#}p < 0.01$).

pared to controls ($p > 0.05$). Analysis of responsiveness to novel acute stressors (IM or C) showed a 3-fold increase in CORT levels when CSIS was followed by IM, compared to controls ($^{***}p$ (0.001) and CSIS ($^{\wedge\wedge\wedge}p < 0.001$). In contrast, consecutive exposure to acute C stress did not significantly alter CORT levels ($p > 0.05$). When the results of the combined stressors were compared to those of acute stressors a significant decrease was observed ($^{\#\#}p < 0.01$) (CSIS + IM *vs* IM and CSIS + C *vs* C).

3.2. Chronic social isolation stress compromises cytosolic CuZnSOD and mitochondrial MnSOD activity

A two-way ANOVA revealed a significant main effects of acute ($F_{2.30} = 7.1$, $p < 0.01$) and chronic ($F_{1.30} = 18.3$, $p < 0.05$) stress on cytosolic CuZnSOD activity in the prefrontal cortex. Animals exposed to acute C stress and CSIS showed a decrease in CuZnSOD activity ($^{*}p < 0.05$). Combined CSIS + IM and CSIS + C stressors also decreased cytosolic CuZnSOD activity compared to controls ($^{**}p < 0.01$, $^{***}p < 0.001$) and acute stressors alone ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$), while combined CSIS + C was significantly decreased compared to CSIS ($^{\wedge}p < 0.05$) (Fig. 3). In the nuclear fraction, a two-way ANOVA revealed a significant main effects of acute ($F_{2.30} = 9.5$, $p < 0.001$) and chronic ($F_{1.30} = 8.8$, $p < 0.01$) stress on CuZnSOD activity. Duncan post hoc tests showed decreased activity following acute C ($^{**}p < 0.01$) stress and combined CSIS + IM and CSIS + C stressors ($^{*}p < 0.05$, $^{***}p < 0.001$) compared to controls, as well as CSIS + IM compared to acute IM ($^{\#}p < 0.05$) stress and CSIS + C compared to CSIS ($^{\wedge}p < 0.05$) (Fig. 3).

In the mitochondrial fraction of the prefrontal cortex, a two-way ANOVA showed a significant main effects of acute ($F_{2.30} = 11.5$, $p < 0.001$) and chronic ($F_{1.30} = 55.2$, $p < 0.001$) stress, and a significant acute × chronic stress interaction ($F_{2.30} = 8.4$, $p < 0.01$) on MnSOD activity. Duncan post hoc tests revealed statistically significant decreases in mitochondrial MnSOD activity in animals exposed to acute C group ($^{***}p < 0.001$), CSIS group ($^{***}p < 0.001$), and both combined stressors (CSIS + IM, CSIS + C, $^{***}p < 0.001$), as compared to the controls (Fig. 3). Moreover, a significant decrease in mitochondrial MnSOD activity between combined CSIS + IM stress and acute IM stress alone ($^{\#\#\#}p < 0.001$) and CSIS ($^{\wedge}p < 0.05$) was observed.

3.3. Decreased reduced glutathione and Hsp70 levels following chronic social isolation stress

Given that antioxidant glutathione (GSH) is essential for the cellular detoxification of ROS in brain cells, its levels after acute, chronic and combined stress were determined. A two-way ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 8.3$, $p < 0.01$) on cytosolic GSH levels. Post hoc analysis showed a significant decrease in GSH levels following CSIS and the two combined stressors in the cytosolic fraction ($^{*}p < 0.05$;$^{**}p < 0.01$), while it was unchanged following both acute stressors (Fig. 4).

A two-way ANOVA of cytosolic Hsp70i showed a significant main effect of CSIS ($F_{1.30} = 10.05$, $p < 0.001$) and a significant acute - × chronic stress interaction ($F_{2.30} = 3.95$, $p < 0.05$) in the prefrontal cortex. A significant decrease in cytosolic Hsp70i was seen in both combined stressors (CSIS + IM or CSIS + C), as compared to the controls ($^{*}p < 0.05$) and acute stressors alone ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) (Fig. 5).

3.4. NF-κB activation following chronic social isolation stress

NF-κB activation and its nuclear translocation following acute, chronic or combined stressors was determined by monitoring NF-κB-p65 localization in the cytosolic and nuclear fractions of the prefrontal cortex in controls and all stressed groups (Fig. 6). In the cytosolic fraction, a two-way ANOVA showed a significant main effect of CSIS ($F_{1.30} = 27.12$, $p < 0.001$) and a significant acute-×chronic stress interaction ($F_{2.30} = 4.86$, $p < 0.05$) on NF-κB protein expression. Specifically, significant decreases in both combined stress groups compared to controls ($^{***}p < 0.001$, $^{**}p < 0.01$, respectively) as well as CSIS + IM group compared to acute IM stress alone were observed ($^{\#\#}p < 0.01$). In the nuclear fraction, a two-way ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 32.02$, $p < 0.05$) on NF-κB levels. Duncan post hoc tests showed a significant increase in NF-κB levels in CSIS ($^{*}p < 0.05$) and both CSIS + IM and CSIS + C groups ($^{**}p < 0.01$), furthermore significant differences between combined stressors and those with acute stress alone ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$) were observed.

3.5. Chronic social isolation stress upregulates nNOS and iNOS protein expression

To verify whether NF-κB activation resulted in the activation of gene transcription of downstream targets, expression of proteins such as nNOS and iNOS, with the NF-κB binding site in the promoter region, were measured by Western blot (Fig. 7). A two-way ANOVA revealed a significant main effects of acute stress and CSIS on nNOS protein in cytosolic fractions of the prefrontal cortex ($F_{1.30} = 13.84$, $p < 0.001$, $F_{2.30} = 3.94$, $p < 0.05$, respectively). Duncan's post hoc tests showed significant increase in all type of stress groups compared to the control group ($^{*}p < 0.05$, $^{***}p < 0.001$, $^{**}p < 0.01$) (Fig. 7A). With regard to cytosolic iNOS protein, a two-way ANOVA revealed a significant main effect of CSIS

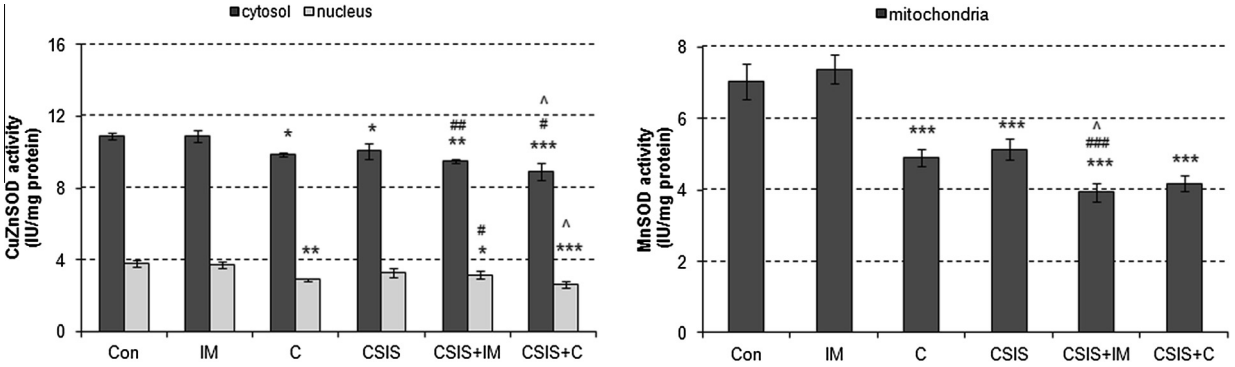

Fig. 3. Cytosolic/nuclear CuZnSOD and mitochondrial MnSOD activity (IU/mg protein) in the prefrontal cortex of control (Con) and following acute immobilization (IM) or cold (C) stress, chronic social isolation stress (CSIS) and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly differences between: respectively stress treatment and control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); CSIS and combined stress ($^{\wedge}p < 0.05$); combined stressors and those respective acute stressors alone ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$), analyzed by two-way ANOVA followed by the Duncan's post-hoc test.

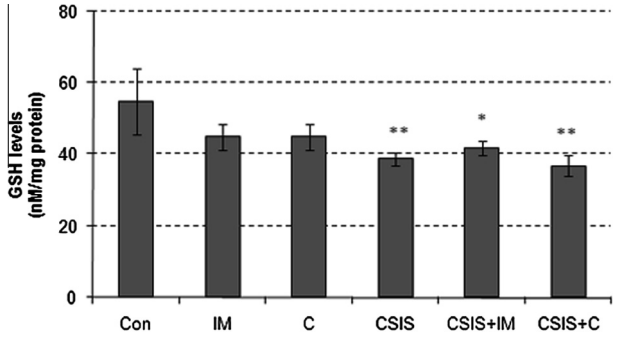

Fig. 4. GSH level (nM/mg protein) in cytosolic fraction of the prefrontal cortex in control (Con) and following acute immobilization (IM) or cold (C) stress, chronic social isolation stress (CSIS) and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly difference between respectively stress treatment compared to the control group ($^{*}p < 0.05$; $^{**}p < 0.01$), analyzed by two-way ANOVA followed by the Duncan's post-hoc test.

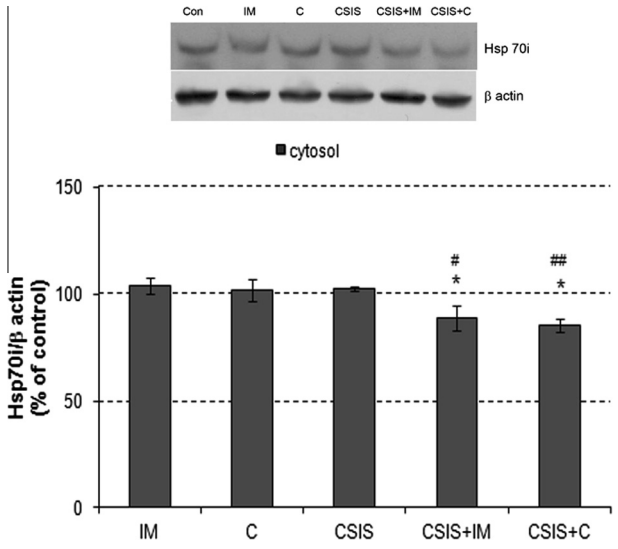

Fig. 5. Hsp70i protein levels in cytosolic fraction of the prefrontal cortex following acute immobilization (IM) and cold stress (C), chronic social isolation stress (CSIS), and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate a significant difference between: i) respective stress treatment and control ($^{*}p < 0.05$); combined stressors and those respective acute stressor alone ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$); obtained from two-way ANOVA followed by Duncan's post hoc test.

($F_{1.30} = 112.9$, $p < 0.001$) and a significant acute × chronic stress interaction ($F_{2.30} = 8.70$, $p < 0.001$) in the prefrontal cortex. CSIS induced a significant increase in cytosolic iNOS protein levels relative to the control group ($^{**}p < 0.01$). In animals exposed to combined stressors, a significant increase of this protein level in the cytosolic fraction as compared to acute stressors ($^{\#\#\#}p < 0.001$), CSIS ($^{\wedge\wedge\wedge}p < 0.001$) and controls ($^{***}p < 0.001$) (Fig. 7B) was revealed.

4. Discussion

In the present study, we have identified in an animal model of depression a possible signaling cascade in which Hsp70i downregulation associated with iNOS induction via NF-κB activation may provoke a state of oxidative/nitrosative stress.

After animals were exposed to 2 h of acute IM or C stress, increased serum CORT levels were detected in both groups. Greater responses of serum CORT after acute IM, as stronger physical and psychological stressor (Kvetnansky and Mikulaj, 1970; Scott and Dinan, 1998) than mild C stress, is consistent with the findings of

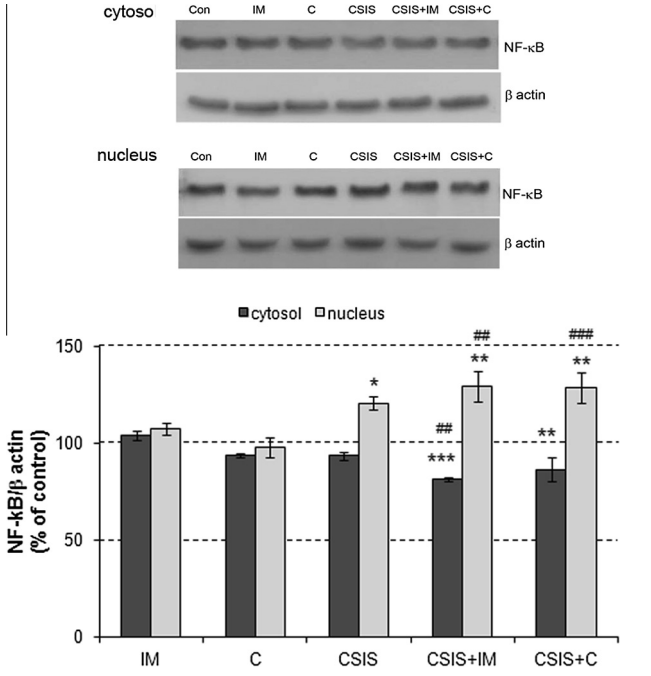

Fig. 6. NF-κB protein levels in cytosolic and nuclear fraction of the prefrontal cortex following acute immobilization (IM) and cold stress (C), chronic social isolation stress (CSIS), and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly differences between: respective stress treatment and control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); combined stressors and those acute stressors alone ($^{\#\#}p < 0.01$, $^{\#}p < 0.05$, respectively); obtained from two-way ANOVA followed by Duncan's post hoc test.

Pacák and Palkovits (2001) who reported that increases in CORT depend on the type of stressor applied. Although increased glucocorticoids (GC) may increase the basal level of ROS produced in cells (McIntosh and Sapolsky, 1996), interestingly, IM stress had no influence on CuZnSOD and MnSOD activity in subcellular fractions in the prefrontal cortex. A lack of consistency between previously reported increased protein levels of cytosolic CuZnSOD and mitochondrial MnSOD (Filipović and Pajović, 2009; Filipović et al., 2009) and their unchanged activity may depict the absence of an adequate antioxidative defense. It may be caused, at least in part, by posttranslational modifications that affect the activity of both enzymes (Hopper et al., 2006) or partially the consequence of accumulation in hydrogen peroxide (Pigeolet et al., 1990). This is supported by findings that CORT levels after acute immobilization stress are specific predictors of oxidative damage to the brain (Pérez-Nievas et al., 2007). With regard to the acute C stress, decrease in activity of cytosolic/nuclear CuZnSOD as well as mitochondrial MnSOD compared to the unaltered protein expression (Filipović and Pajović, 2009; Filipović et al., 2009), this also indicated inadequate elimination of ROS and suggests a state of oxidative stress. Moreover, increased metabolic rate after exposure to low temperatures may induce the generation of ROS (Blagojevic et al., 2011; Selman et al., 2000), and hence inactivate SOD isoforms (Hodgson and Fridovich, 1975). On the other hand, although cytosolic nNOS protein expression was elevated in both acutely stressed groups, it has been reported that NO overproduction in animals exposed to acute stress (De Oliveira et al., 2008; Krukoff and Khalili, 1997) have a predominantly neuromodulatory role via the decrease of glutamate release (Harvey, 1996; Prast and Philippu, 2001). Hence, the previously published slight increase in NO content after exposure of animals to acute stressors (Zlatković and Filipović, 2012) likely produced by increased nNOS protein may predominantly mediate physiological effects and is involved in acute response mechanisms (Esch et al., 2002). In contrast, no

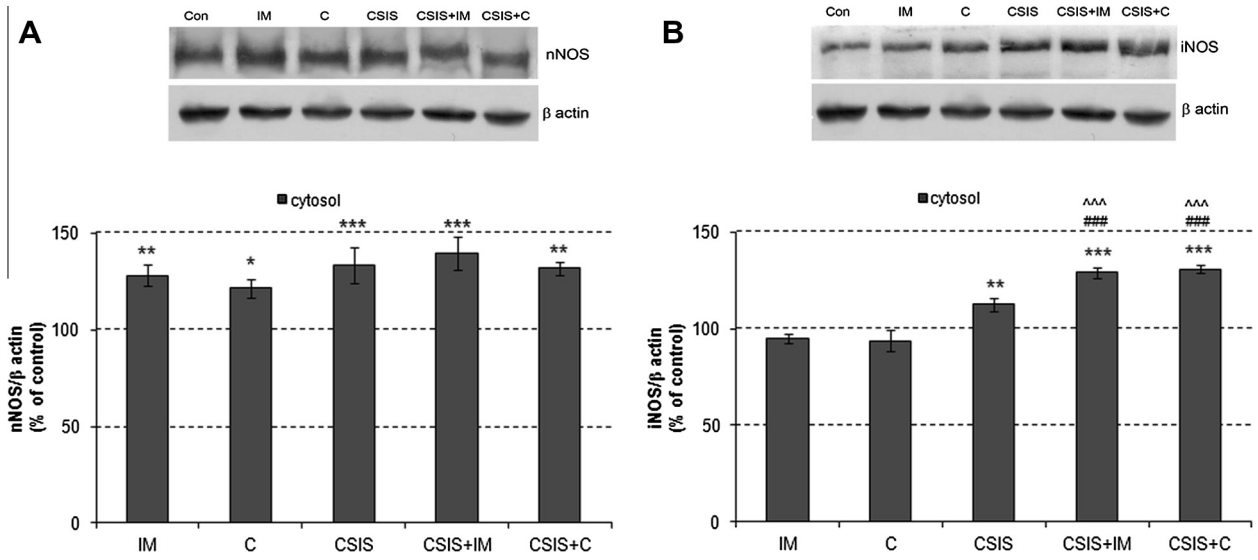

Fig. 7. Protein expression of nNOS (7A) and iNOS (7B) in cytosolic fraction of the prefrontal cortex following acute immobilization (IM) or cold stress (C), chronic social isolation stress (CSIS), and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly difference between respective stress treatment and control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), combined stressors (CSIS + IM, CSIS + C) compared to those acute stress alone ($^{\#\#\#}p < 0.001$), CSIS and both combined stressors ($^{\wedge\wedge\wedge}p < 0.001$); obtained from two-way ANOVA followed by Duncan's post hoc test.

changes in the protein expression of iNOS and Hsp70i, or the translocation of NF-κB, were detected in the prefrontal cortex after exposure to acute stressors. Although acute stress increases the production of proinflamatory cytokine (Steptoe et al., 2001) that may lead to the induction of iNOS gene expression, unaltered iNOS protein level in our study may result from mutual regulation between cytokines and glucocorticoids. Proinflammatory cytokines are potent activators of the HPA axis (Dunn, 2000), in which glucocorticoids in turn negatively control cytokine production and are able to shut down inflammatory processes to prevent damage (Besedovsky and del Rey, 2000; Sapolsky et al., 2000). Moreover, glucocorticoids may be important negative regulators of NF-κB in the brain (De Bosscher et al., 2003) which is a transcriptional factor for the iNOS gene.

In contrast to the acute stressors, 21 d of CSIS, an animal model of depression, induced a decrease in activity of cytosolic/nuclear CuZnSOD and MnSOD as well as GSH levels, indicating a state of oxidative stress. Moreover, several studies have demonstrated that lowered antioxidant defenses are probably associated with the genesis of depressive symptoms as a result of increased oxidative and nitrosative stress (Maes et al., 2011). It has been shown that exposure to repeated stress situations increases ROS generation in the brain (Lucca et al., 2009), where NO and an excess of prooxidants are responsible for both neuronal functional impairment and structural damage (Munhoz et al., 2008). Given that CSIS exhibited CORT levels similar to basal values, in the current study a state of oxidative stress may have resulted from overexpression of both nNOS and iNOS, which has previously been shown as a hallmark of nitrosative stress in the brain during chronic stress (Leza et al., 1998; Olivenza et al., 2000). In addition, overexpressed NO may act with superoxide anion and lead to the formation of peroxynitrite that may inactivate MnSOD via nitration causing a decrease in either its activity or protein levels (Nilakantan et al., 2005). The reduced CuZnSOD activity could be a consequence of high levels of superoxide anion produced during oxidative stress, which undergoes dismutation to form elevated levels of hydrogen peroxide. Moreover, if CuZnSOD activity is not coupled with the activity of respective antiperoxidative enzymes catalase, it may lead to accumulation of hydrogen peroxide which further inhibits CuZnSOD *a*ctivity (Zafir and Banu, 2009; Zafir et al., 2009).

Literature data pointed out that mild oxidative stress modifies the expression of most antioxidant enzymes, and enhances expression and DNA binding of numerous transcription factors, including NF-κB (Mattson et al., 2002). Nuclear localization of NF-κB-p65 protein subunit, which signals its activation, was detected following CSIS. Given that GSH is the major determinant of the redox status in mammalian cells (Sen, 2000), where modulation of intracellular redox equilibrium could mediate NF-κB activation in the signal transduction pathway (Haddad et al., 2000), the activation of NF-κB observed here likely resulted from GSH stress-induced depletion. Moreover, as NO can also upregulate NF-κB (Connelly et al., 2001), observed induction of both NOS protein expression isoforms in our study, will cause persistent NO production that may mediate NF-κB activation. Accordingly, activated NF-κB in the nucleus may interact with kappaB elements in the NOS2 5′ flanking region, triggering iNOS gene transcription (Chikumi et al., 2004, Davis et al., 2005, Mizel et al., 2003). Consistent with our results, it has been reported that repeated restraint stress is capable of lowering GSH levels associated with iNOS induction (Madrigal et al., 2001) by product of oxidative stress (Sahin and Gumuslu, 2004). Brain prefrontal cortical iNOS induction and sustained release of NO (and further to generation of *peroxynitrite*) has been shown to mediate stress-induced neuronal death due to oxidation of structural neuronal proteins and mitochondrial enzymes included in cell death (Brown, 2010; Olivenza et al., 2000; Sims and Anderson, 2002). Hence, increase in iNOS levels following CSIS may be related to previously reported activation of proapoptotic signaling in the prefrontal cortex (Filipović et al., 2011; Zlatković and Filipović, 2012). On the other hand, levels of cytoprotective Hsp70i protein, the effect of which might be manifested through suppression of NF-κB activation and its translocation into the nucleus (Chan et al., 2004), was unchanged allowing unhampered NF-κB translocation to the nucleus, essential for iNOS protein expression. As mitochondria have been indicated as a selective target for the protective effects of Hsp70 against oxidative injury (Calabrese et al., 2000; Polla et al., 1996), the lack of initiation of heat-shock protein response during CSIS may be a factor for previously published mitochondria-related proapoptotic cascade (Zlatković and Filipović, 2012) and finally apoptosis in the rat prefrontal cortex (Filipović et al., 2011). Moreover, reduction in GSH level has been shown as a signaling event in apoptosis (Sato et al., 1995).

To further determine whether CSIS was adaptive or maladaptive, chronically isolated animals were exposed to novel acute IM or C (combined CSIS + IM or CSIS + C) stress. The exposure of rats to combined stressors induced oxidative stress, as indicated by compromised cytosolic/nuclear CuZnSOD, mitochondrial MnSOD activity and decreased GSH levels. A decline in MnSOD activity by combined IS + IM stress compared to CSIS may be caused by translocation of p53 from the cytosol into mitochondria in response to combined stress-induced NO overproduction (Zlatković and Filipović, 2012) where p53 interacts directly with MnSOD and leads to a decrease in superoxide – scavenging activity of MnSOD (Holley and St Clair, 2009). Decreased responsiveness of the HPA axis, as determined by lesser increase in CORT levels in response to novel acute stress than to acute stressors alone, indicates compromised HPA axis activity (Filipović et al., 2005). These results may be explained by an impaired feedback inhibition of glucocorticoids, possibly due to an impaired glucocorticoid receptor shuttling between cytoplasm and nucleus and its increased cytosolic retention under CSIS (Dronjak et al., 2004; Filipović et al., 2005). Further, combined stressors induced more pronounced changes in NF-κB nuclear translocation compared to acute stress alone and consequently an increase in iNOS protein expression. Moreover, protein expression of Hsp70i in response to combined stressors was decreased in the prefrontal cortex. Consistent with our results, it has been reported that brain cells with DNA fragmentation, demonstrated by positive TUNEL assay following focal cerebral ischemia, rarely synthesize Hsp70 protein (Kinouchi et al., 1993a, 1993b). Given the occurrence of chronic isolation-induced apoptosis in the prefrontal cortex (Filipović et al., 2011), we speculate that decreased Hsp70i and increased iNOS protein levels are likely to be involved in the initiation of apoptosis. Because combined stressors intensified iNOS protein expression as compared to other groups, a more pronounced NO production (Zlatković and Filipović, 2012) together with decreased Hsp70i and GSH level may compromise the antioxidative defense system (Yamakura et al., 1998). The obvious mechanisms for attenuation of the Hsp70i response with combined stressors may include either decreased synthesis and/or increased degradation of Hsp70.

5. Conclusion

Our results showed that NOS signaling pathway plays a differential role between acute and chronic stress in the rat prefrontal cortex. Depletion of GSH together with decreased cytosolic CuZnSOD and mitochondrial MnSOD activities after CSIS indicate compromised antioxidative defense. Our data suggests a possible signaling cascade in which CSIS stress provoked oxidative/nitrosative stress through decrease in Hsp70i response associated with of NF-κB activation and subsequently iNOS protein expression. Given that CSIS of male Wistar rats is considered as an animal model of depression, future experiments targeting direct involvement of NOS inhibitors in these processes should be examined.

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References

Aktan, F., 2004. INOS-mediated nitric oxide production and its regulation. Life Sci. 75 (6), 639–653.
Alderton, W.K., Cooper, C.E., Knowles, R.G., 2001. Nitric oxide synthases: structure, function and inhibition. Biochem. J. 357, 593–615.
Besedovsky, H.O., del Rey, A., 2000. The cytokine – HPA axis feed-back circuit. Z. Rheumatol. 59 (Suppl. 2), II/26–II/30.
Blagojevic, D.P., Grubor-Lajsic, G.N., Spasic, M.B., 2011. Cold defence responses: the role of oxidative stress. Front. Biosci. (Schol Ed) 3, 416–427.
Brown, G.C., 2010. Nitric oxide and neuronal death. Nitric oxide 23, 153–165.
Calabrese, V., Copani, A., Testa, D., Ravagna, A., Spadaro, F., Tendi, E., Nicoletti, V.G., Giuffrida Stella, A.M., 2000. Nitric oxide synthase induction in astroglial cell cultures: effect on heat shock protein 70 synthesis and oxidant/antioxidant balance. J. Neurosci. Res. 60, 613–622.
Cerqueira, J.J., Mailliet, F., Almeida, O.F., Jay, T.M., Sousa, N., 2007. The prefrontal cortex as a key target of the maladaptive response to stress. J. Neurosci. 27 (11), 2781–2787.
Chan, J.Y., Ou, C.C., Wang, L.L., Chan, S.H., 2004. Heat shock protein 70 confers cardiovascular protection during endotoxemia via inhibition of nuclear factor-kappaB activation and inducible nitric oxide synthase expression in the rostral ventrolateral medulla. Circulation 110 (23), 3560–3566.
Chikumi, H., Yamamoto, H., Yoneda, K., Yamasaki, A., Sato, K., Shimizu, E., 2004. Down-regulation of inducible nitric oxide synthase by lysophosphatidic acid in human respiratory epithelial cells. Mol. Cell Biochem. 262 (1–2), 51–59.
Connelly, L., Palacios-Callender, M., Ameixa, C., Moncada, S., Hobbs, A.J., 2001. Biphasic regulation of NF-κB activity underlies the pro- and anti-inflammatory actions of nitric oxide. J. Immunol. 166, 3873–3881.
Davis, R.L., Sanchez, A.C., Lindley, D.J., Williams, S.C., Syapin, P.J., 2005. Effects of mechanistically distinct NF-kappaB inhibitors on glial inducible nitric-oxide synthase expression. Nitric Oxide. 12 (4), 200–209.
De Bosscher, K., Vanden Berghe, W., Haegeman, G., 2003. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr. Rev. 24 (4), 488–522.
De Oliveira, R.M.W., Guimarães, F.S., Deakin, J.F.W., 2008. Expression of neuronal nitric oxide synthase in the hippocampal formation in affective disorders. Braz. J. Med. Biol. Res. 41 (4), 333–341.
Dhir, A., Kulkarni, S.K., 2011. Nitric oxide and major depression. Nitric Oxide 24 (3), 125–131.
Dronjak, S., Gavrilović, L., Filipović, D., Radojčić, M.B., 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 (3), 409–415.
Dronjak, S., Spasojević, N., Gavrilović, L.J., Varagić, V., 2007. Behavioural and endocrine responses of socially isolated rats to long-term diazepam treatment. Acta vet. 57 (4), 291–302.
Dunn, A.J., 2000. Cytokine activation of the HPA axis. Ann. N.Y. Acad. Sci. 917, 608–617.
Esch, T., Stefano, G.B., Fricchione, G.L., Benson, H., 2002. The role of stress in neurodegenerative diseases and mental disorders. Neuro Endocrinol. Lett. 23, 199–208.
Filipović, D., Gavrilović, L.J., 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, D., Pajović, S.B., 2009. Differential regulation of CuZnSOD expression in rat brain by acute and/or chronic stress. Cell. Mol. Neurobiol. 29, 673–681.
Filipović, D., Zlatković, J., Pajović, S.B., 2009. The effect of acute or/and chronic stress on the MnSOD protein expression in rat prefrontal cortex and hippocampus. Gen. Physiol. Biophys. 28, 53–61.
Filipović, D., Zlatković, J., Inta, D., Bjelobaba, I., Stojiljkovic, M., Gass, P., 2011. Chronic isolation stress predisposes the frontal cortex but not the hippocampus to the potentially detrimental release of cytochrome c from mitochondria and the activation of caspase-3. J. Neurosci. Res. 89 (9), 1461–1470.
Garzón, J., del Rio, J., 1981. Hyperactivity induced in rats by longterm isolation: further studies on a new animal model for the detection of antidepressants. Eur. J. Pharmacol. 74, 287–294.
Haddad, J.J., Olver, R.E., Land, S.C., 2000. Antioxidant/pro-oxidant equilibrium regulates HIF-1alpha and NF-kappa B redox sensitivity. Evidence for inhibition by glutathione oxidation in alveolar epithelial cells. J. Biol. Chem. 275 (28), 21130–21139.
Hall, F.S., 1998. Social deprivation of neonatal, adolescent, and adult rats has distinct neurochemical and behavioral consequences. Crit. Rev. Neurobiol. 12, 129–162.
Harvey, B.H., 1996. Affective disorders and nitric oxide: a role in pathways to relapse and refractoriness? Hum. Psychopharmacol. Clin. 11 (4), 309–319.
Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74 (1), 214–226.
Hodgson, E.K., Fridovich, I., 1975. Interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide. Inactivation of the enzyme. Biochemistry 14 (24), 5294–5299.
Holley, A.K., St Clair, D.K., 2009. Watching the watcher: regulation of p53 by mitochondria. Future Oncol. 5 (1), 117–130.
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 (8), 2524–2536.
Kiang, J.G., Tsokos, G.C., 1998. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol. Ther. 80, 183–201.
Kinouchi, H., Sharp, F.R., Hill, M.P., Koistinaho, H., Sagar, S.M., Chan, P.H., 1993a. Induction of 70-kD heat shock protein and hsp70 mRNA following focal cerebral ischemia in the rat. J. Cereb. Blood Flow Metab. 13, 105–115.
Kinouchi, H., Sharp, F.R., Koistinaho, J., Hicks, K., Kamii, H., Chan, P.H., 1993b. Induction of heat shock hsp70 mRNA and HSP70 kDa protein in neurons in the "penumbra" following focal cerebral ischemia in the rat. Brain Res. 619, 334–338.

Krukoff, T.L., Khalili, P., 1997. Stress-induced activation of nitric oxide-producing neurons in the rat brain. J. Comp. Neurol. 377 (4), 509–519.
Kvetnansky, R., Mikulaj, L., 1970. Adrenal and urinary catecholamines in rat during adaptation to repeated immobilization stress. Endocrinology 87, 738–743.
Leza, J.C., Salas, E., Sawicki, G., Russell, J.C., Radomski, M.W., 1998. The effects of stress on homeostasis in JCR-LA-cp rats: the role of nitric oxide. J. Pharmacol. Exp. Ther. 286 (3), 1397–1403.
Li, N., Karin, M., 1999. Is NF-kappaB the sensor of oxidative stress? FASEB J. 13 (10), 1137–1143.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Lucca, G., Comim, C.M., Valvassori, S.S., Réus, G.Z., Vuolo, F., Petronilho, F., Dal-Pizzol, F., Gavioli, E.C., Quevedo, J., 2009. Effects of chronic mild stress on the oxidative parameters in the rat brain. Neurochem. Int. 54 (5–6), 358–362.
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.
Maes, M., Galecki, P., Chang, Y.S., Berk, M., 2011. A review on the oxidative and nitrosative stress (O&NS) pathways in major depression and their possible contribution to the (neuro) degenerative processes in that illness. Prog. Neuropsychopharmacol. Biol. Psychiatry 35 (3), 676–692.
Malhotra, V., Wong, H.R., 2002. Interactions between the heat shock response and the nuclear factor-kappa B signaling pathway. Crit. Care Med. 30, S89–S95.
Malhotra, V., Eaves-Pyles, T., Odoms, K., Quaid, G., Shanley, T.P., Wong, H.R., 2002. Heat shock inhibits activation of NF-kappaB in the absence of heat shock factor-1. Biochem. Biophys. Res. Commun. 291 (3), 453–457.
Mattson, M.P., Duan, W., Chan, S.L., Cheng, A., Haughey, N., Gary, D.S., Guo, Z., Lee, J., Furukawa, K., 2002. Neuroprotective and neurorestorative signal transduction mechanisms in brain aging: modification by genes, diet and behavior. Neurobiol. Aging 23, 695–705.
McIntosh, L., Sapolsky, R., 1996. Glucocorticoids increase the accumulation of reactive oxygen species and enhance adriamycin-induced toxicity in neuronal cultures. Exp. Neurol. 141 (2), 201–206.
McLeod, T.M., Lopez-Figueroa, A.L., Lopez-Figueroa, M.O., 2001. Nitric oxide, stress, and depression. Psychopharmacol. Bull. 35, 24–41.
Mizel, S.B., Honko, A.N., Moors, M.A., Smith, P.S., West, A.P., 2003. Induction of macrophage nitric oxide production by Gram-negative flagellin involves signaling via heteromeric Toll-like receptor 5/Toll-like receptor 4 complexes. J. Immunol. 170 (12), 6217–6223.
Moutsatsou, P., Psarra, A.M.G., Tsiapara, A., Paraskevakou, H., Davaris, P., Sekeris, C.E., 2001. Localization of the glucocorticoid receptor in rat brain mitochondria. Arch. Biochem. Biophys. 386, 69–78.
Munhoz, C.D., García-Bueno, B., Madrigal, J.L., Lepsch, L.B., Scavone, C., Leza, J.C., 2008. Stress-induced neuroinflammation: mechanisms and new pharmacological targets. Braz. J. Med. Biol. Res. 41 (12), 1037–1046.
Napolitano, M., Zei, D., Centonze, D., Palermo, R., Bernardi, G., Vacca, A., Calabresi, P., Gulino, A., 2008. NF-κB/NOS cross-talk induced by mitochondrial complex II inhibition: implications for Huntington's disease. Neurosci. Lett. 434, 241–246.
Nilakantan, V., Halligan, N.L., Nguyen, T.K., Hilton, G., Khanna, A.K., Roza, A.M., Johnson, C.P., Adams, M.B., Griffith, O.W., Pieper, G.M., 2005. Post-translational modification of manganese superoxide dismutase in acutely rejecting cardiac transplants: role of inducible nitric oxide synthase. J. Heart Lung Transplant. 24, 1591–1599.
Olivenza, R., Moro, M.A., Lizasoain, I., Lorenzo, P., Fernández, A.P., Rodrigo, J., Boscá, L., Leza, J.C., 2000. Chronic stress induces the expression of inducible nitric oxide synthase in rat brain cortex. J. Neurochem. 74 (2), 785–791.
Pacák, K., Palkovits, M., 2001. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. Endocr. Rev. 22 (4), 502–548.
Patel, R.P., Darley-Usmar, V.M., 1996. Using peroxynitrite as oxidant with low-density lipoprotein. Methods Enzymol. 269, 375–384.
Pérez-Nievas, B.G., García-Bueno, B., Caso, J.R., Menchén, L., Leza, J.C., 2007. Corticosterone as a marker of susceptibility to oxidative/nitrosative cerebral damage after stress exposure in rats. Psychoneuroendocrinology 32 (6), 703–711.
Pigeolet, E., Corbisier, P., Houbion, A., Larnbert, D., Michiels, C., Raes, M., Zachary, M.D., Remacle, J., 1990. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. Mech. Ageing Dev. 51, 283–297.
Pizzi, M., Sarnico, I., Boroni, F., Benetti, A., Benarese, M., Spano, P.F., 2005. Inhibition of IκBα phosphorylation prevents glutamate-induced NF-κB activation and neuronal cell death. Acta Neurochir. 93, 59–63.
Polla, B.S., Kantengwa, S., François, D., Salvioli, S., Franceschi, C., Marsac, C., Cossarizza, A., 1996. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. Proc. Natl. Acad. Sci. USA 93 (13), 6458–6463.
Prast, H., Philippu, A., 2001. Nitric oxide as modulator of neuronal function. Prog. Neurobiol. 64 (1), 51–68.
Sahin, E., Gumuslu, S., 2004. Alterations in brain antioxidant status, protein oxidation and lipid peroxidation in response to different stressmodels. Behav. Brain Res. 155, 241–248.
Sato, N., Iwata, S., Nakamura, K., Hori, T., Mori, K., Yodoi, J., 1995. Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. J. Immunol. 154 (7), 3194–3203.
Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.
Scott, L.V., Dinan, T.G., 1998. Vasopressin and the regulation of hypothalamic-pituitary-adrenal axis function: implications for the pathophysiology of depression. Life Sci. 62 (22), 1985–1998.
Selman, C., McLaren, J.S., Himanka, M.J., Speakman, J.R., 2000. Effect of long-term cold exposure on antioxidant enzyme activities in a small mammal. Free Radic. Biol. Med. 28 (8), 1279–1285.
Sen, C.K., 2000. Cellular thiols and redox-regulated signal transduction. Curr. Top. Cell. Regul. 36, 1–30.
Senftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krähn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.C., Karin, M., 2001. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science 293 (5534), 1495–1499.
Serra, M., Sanna, E., Mostallino, M.C., Biggio, G., 2007. Social isolation stress and neuroactive steroids. Eur. Neuropsychopharmacol. 17 (1), 1–11.
Sims, N.R., Anderson, M.F., 2002. Mitochondrial contributions to tissue damage in stroke. Neurochem. Int. 40 (6), 511–526.
Spasojević, N., Gavrilović, Lj., Varagić, V., Dronjak, S., 2007. Effects of chronic diazepam treatments on behavior on individually housed rats. Arch. Biol. Sci. 59, 113–117.
Spencer, R.L., Kalman, B.A., Cotter, C.S., Deak, T., 2000. Discrimination between changes in glucocorticoid receptor expression and activation in rat brain using western blot analysis. Brain Res. 868, 275–286.
Steptoe, A., Willemsen, G., Owen, N., Flower, L., Mohamed-Ali, V., 2001. Acute mental stress elicits delayed increases incirculating inflammatory cytokine levels. Clin. Sci. 101, 185–192.
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.
Zafir, A., Banu, N., 2009. Modulation of in vivo oxidative status by exogenous corticosterone and restraint stress in rats. Stress 12 (2), 167–177.
Zafir, A., Ara, A., Banu, N., 2009. In vivo antioxidant status: a putative target of antidepressant action. Prog. Neuropsychopharmacol. Biol. Psychiatry 33, 220–228.
Zhang, D., Wen, X., Wang, X., Shi, M., Zhao, Y., 2009. Antidepressant effect of Shudihuang on mice exposed to unpredictable chronic mild stress. J. Ethnopharmacol. 123, 55–60.
Zlatković, J., Filipović, D., 2012. Bax and Bcl-2 mediate proapoptotic signaling following chronic isolation stress in rat brain. Neuroscience 223, 238–245.