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BAX AND B-CELL-LYMPHOMA 2 MEDIATE PROAPOPTOTIC SIGNALING FOLLOWING CHRONIC ISOLATION STRESS IN RAT BRAIN

J. ZLATKOVIĆ AND D. FILIPOVIĆ *

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

Abstract—Mitochondrial dysfunction has been implicated in several psychiatric disorders, including depression. Given that the B-cell-lymphoma 2 (Bcl-2) protein family plays a *role* in the regulation of *mitochondrial apoptotic* pathway, we hypothesized that *ratio* of *proapoptotic* to *antiapoptotic* proteins (e.g., Bcl-2-associated X protein (*Bax*)/*Bcl-2*) may determine prosurvival/proapoptotic intracellular signaling under stress. *We tested* this hypothesis by *examining the effects of* 2 h of acute stress immobilization (IM) or cold (C), 21 days of social isolation as chronic stress and combined stress (chronic stress followed by acute stress) on cytosolic/mitochondrial levels and ratios of Bax and Bcl-2 proteins in relation to cytosolic nitric oxide (NO) metabolites (nitrates and nitrites) and p53 protein redistribution between cytosolic and mitochondrial compartments in the prefrontal cortex (PFC) and hippocampus (HIPP) of male Wistar rats. The stress-induced changes in serum corticosterone (CORT) concentrations were also followed. Acute stressors resulting in an elevated CORT level did not change the Bax/Bcl-2 ratio in either brain region. However, chronic isolation, resulting in CORT levels similar to basal values, led to a translocation of mitochondrial Bcl-2 to the cytosol in the PFC. Furthermore, the *Bax*/*Bcl-2 ratio in the PFC was significantly increased following chronic isolation and* remained elevated after combined stressors. NO metabolites were increased by chronic isolation and the two combined stressors in the HIPP and following the combined stressors in the PFC. Translocation of p53 and proapoptotic Bax from the cytosol into mitochondria in response to NO overproduction following combined stressors was detected only in the PFC. These data indicate that chronic isolation stress exerts opposing actions on p53 and NO mechanisms in a tissue-specific manner (PFC vs. HIPP), triggering proapoptotic signaling via *Bcl-2 translocation* in the PFC.

Key words: depression, Bax, Bcl-2, p53, nitric oxide, rat brain.

*Corresponding author. Tel/fax: +381-(11)-2455-561.
E-mail address: dragana@vinca.rs (D. Filpović).
URL: http://www.vinca.rs (D. Filipović).

Abbreviations: ANOVA, analysis of variance; Bax, Bcl-2-associated X protein; Bcl-2, B-cell-lymphoma 2; CORT, corticosterone; COX, c oxidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GCs, glucocorticoids; HIPP, hippocampus; HPA, hypothalamic–pituitary–adrenal; HRP, horseradish peroxidase; IM, immobilization; iNOS, inducible NO synthase; IS, isolation; MnSOD, manganese superoxide dismutase; *NMDA*, N-methyl-D-aspartate; nNOS, neuronal NO synthase; NO, nitric oxide; PFC, prefrontal cortex; S.E.M., standard error of the mean.

INTRODUCTION

Stressful experiences have been implicated in the pathogenesis of mood disorders. Alterations of stress hormones, such as glucocorticoids (GCs), and specifically dysfunctions in the hypothalamic–pituitary–adrenal (HPA) axis, play a role in the development of depressive disorders (McEwen, 2005). Recent studies in animals and humans suggest that abnormalities in mitochondria may also be involved in major depression (Sarandol et al., 2007). B-cell-lymphoma 2 (Bcl-2) protein family is comprised of antiapoptotic and proapoptotic members which regulate the apoptosis mediated by mitochondria (Shimizu et al., 1999). *Compromised mitochondrial* membrane integrity includes rearrangement of proapoptotic Bax and antiapoptotic Bcl-2 molecules in its membrane. Bax is a soluble protein present predominantly in the cytosol (Brady and Gil-Gómez, 1998; Gross et al., 1999; Kroemer and Reed, 2000), whereby during the induction of apoptosis, it shifts to mitochondrial membranes. Bcl-2 is present in mitochondria and functions as a repressor of apoptosis (Reed et al., 1998). The ratio of Bcl-2/Bax in mitochondria determines the cellular response to death signals transmitted by mitochondria (Desagher and Martinou, 2000; Hengartner, 2000). While overexpression of Bcl-2 (a higher Bcl-2/Bax ratio) protects cells from apoptosis, the translocation of Bax to the mitochondria induces cytochrome c release that can trigger apoptosis (Hsu et al., 1997).

Under acute or chronic stress, excessive activation of glutamate receptors, *such* as the N-methyl-D-aspartate (*NMDA)* receptor, causes *oxidative stress* that can lead to a number of deleterious consequences, including *mitochondrial* dysfunction (Coyle and Puttfarcken, 1993). Overproduction of nitric oxide (NO) by NMDA receptor stimulation primarily activates the mitochondrial apoptotic pathway by modulating the expression of apoptosis-associated proteins such as Bax and Bcl-2 (Brüne, 2005; Pacher et al., 2007). Moreover, NO-dependent signaling pathways that initiate cell death may also involve the tumor suppressor protein p53 (Nakaya et al., 2000; Hofseth et al., 2003). This tumor suppressor protein can induce apoptosis independent of its transcriptional activity by rapid translocation from the cytoplasm to the mitochondria (Chipuk and Green, 2003; Manfredi, 2003; Murphy et al., 2004; Moll et al., 2005), causing changes in mitochondrial permeability that result in the release of apoptotic protein cytochrome c, the activation of caspase 3 (Marchenko et al., 2000; Erster et al., 2004), and eventually apoptosis (Liu et al., 1996; Green and Reed, 1998).

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Previous data from our laboratory have shown that 21 days of chronic isolation stress, an animal model of depression (Spasojević et al., 2007; Dronjak et al., 2007), compromises HPA axis activity by deregulating glucocorticoid negative feedback control in the central nervous system (CNS), especially in the hippocampus (HIPP) and cortex, due to impaired cytoplasmic/nuclear GR shuttling (Dronjak et al., 2004; Filipović et al., 2005). Furthermore, we have recently demonstrated that chronic isolation stress-induced apoptosis in the rat prefrontal cortex (PFC) was accompanied by the release of cytochrome c and the activation of caspases-3 (Filipović et al., 2011). In the light of these findings, we hypothesized that this induction of apoptosis may be caused by mitochondrial-dependent proapoptotic pathway initiated by changes in the Bax/Bcl-2 ratio. Also, the effect of NO in the initiation of *mitochondrial proapoptotic signals* has not yet been fully addressed in depression. We tested our hypothesis by measuring the production of NO and p53, Bax and Bcl-2 *protein* expression and their mitochondrial/cytosolic redistribution in the PFC and HIPP of male Wistar rats exposed to acute, chronic and combined stressors. Serum corticosterone (CORT) levels, as the main hormonal parameter of the stress response of the HPA axis, were monitored.

EXPERIMENTAL PROCEDURES

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 h/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). Group II was exposed to either 2 h of immobilization (IM) or cold (C) stress (at 4 °C). Immobilization 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 stress via individual housing for 21 days, according to the model of Garzón and Del Río (1981), during which animals had relatively normal auditory and olfactory experiences but no visual or tactile exposure to other animals. Group IV represented the combined stressors (IS + IM, IS + C) rats underwent chronic social isolation stress followed by a single exposure to 2 h of 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 and controls were anesthetized with ketamine/xylazine 100/20 mg/kg (*i.p.*) and sacrificed by guillotine decapitation (Harvard Apparatus, South Natick, MA, USA). Brains were immediately removed and the PFC and HIPP were dissected on ice. Tissue samples were frozen in liquid nitrogen and kept at −70 °C until further analysis.

Serum CORT 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 assay. 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.

Brain NO metabolites (NO_x^-): nitrite–nitrate (NO_2^- and NO_3^-) levels in PFC and HIPP

NO is a highly *reactive molecule* that is rapidly converted to more stable nitrates/nitrites (NO_x^-), which are good markers for NO activity/ levels. Therefore, for NO assay estimation, NO_x^- levels were estimated in the cytosol of the PFC and HIPP, where NO_3^- was previously transformed into NO_2^- in the presence of Cd (Cortas and Wakid, 1990). NO_2^- was determined by colorimetric assay using Griess reagent (1% sulfanilamide, 2.5% H_3PO_4, 0.1% N-(1-naphthyl) etylenediamine dihydrochloride) (Navarro-Gonzálvez et al., 1998). The optical density of 550 nm was *measured* using an ELISA *microplate* reader. The standard was prepared with several concentrations of $NaNO_2$ (ranging from 0.5 to 10 μM) and was expressed as μM. The measurement of NO_x^- levels has been found to be a reliable technique to determine the synthesizing capacity of NOS in the brain (Salter et al., 1996).

Cytosol/mitochondria fractionation

To prepare the cytosol and mitochondria tissue protein extracts, frozen brain PFC and HIPP were weighed and homogenized in 2 vol. (w/v) 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 the 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*, 4 °C for 10 min. The supernatant was centrifuged at 15,000*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*g*, 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]. The supernatant was further centrifuged at 100,000*g* for 60 min to obtain the pure cytosolic fraction. The protein fractionation procedure was assessed by immunoblotting the fractions with anti-cytochrome c oxidase (COX) subunit I antibody (Molecular Probe 1:500, Carlsbad, California.) as a mitochondrial marker (Filipović et al., 2009). The absence of COX I in the cytosolic fraction confirmed the purity of the cytosolic and mitochondrial fractionation. Protein content in the cytosolic and mitochondrial fractions was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA; Sigma Aldrich, Inc., USA) as a reference.

Western blot analysis of mitochondria/cytosolic p53, Bax, Bcl-2 proteins

Equal amounts of cytosolic or mitochondrial protein fractions of PFC and HIPP were separated on an 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 a Mini Trans-blot apparatus (Bio-Rad). The membranes were blocked in a TBS-T buffer pH 7.6 (20 mM Tris–HCl, 137 mM NaCl, 0.3% Tween 20) containing 5% BSA and incubated overnight (4 °C) with either polyclonal anti-rabbit Bax, Bcl-2 (Santa Cruz Biotechnology Santa Cruz, CA, USA), or p53 antibody (Stressgene Biotechnologies, Victoria, BC, Canada). After washing three times in TBS-T, the membranes were incubated for 2 h with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling, #7074, Inc., Beverly, MA, USA). To confirm a consistent protein loading for each lane, the membranes were stained for -actin (primary monoclonal anti-mouse -actin antibody A5316, Sigma St. Louis, MO, followed by HRP-conjugated secondary goat anti-mouse IgG antibody, SC 2005, Santa Cruz Biotechnology). After a further wash in TBS-T, the blots were developed by enhanced chemiluminescence (ECL; Amersham, Bucks, UK) and exposed to an X-ray film. The signals were electronically digitized by scanning and the image was processed for quantification using Image software. Protein molecular mass standards (Page Ruler^{TM}Plus Prestained Protein Ladder, Fermentas, St. Leon-Rot, Germany) were used for calibration. Identification of this response was observed at 23 kDa for Bax, 26 kDa for Bcl-2, 53 kDa for p53 and 42 kDa for β-actin. The levels of Bax/Bcl-2 ratio in stressed animals are given as the percent change relative to control rats (100%).

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) [the factors were acute (levels: none, IM and C) or chronic (levels: no stress and isolation (IS)) stress]. 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 ± standard error of the mean (S.E.M.) of 6–7 animals per group.

RESULTS

Chronic isolation stress compromised HPA axis activity

Serum CORT levels, a marker of the neuroendocrine stress response, are presented in Table 1. A two-way ANOVA revealed a significant effect of acute ($F_{2.31} = 85.18$, $p < 0.001$), chronic ($F_{1.31} = 25.78$, $p < 0.001$) and combined stress ($F_{2.31} = 5.51$, $p < 0.001$). In acutely-stressed animals, IM acted as an extremely potent stressor, resulting in a 4-fold increase in serum CORT levels ($p < 0.001$), while C led to a 2-fold increase in CORT levels ($p < 0.01$), as compared to the control group. Chronic isolation for 21 days did not alter the serum CORT level ($p > 0.05$), as compared to the control group. Analysis of the responsiveness to an additional stressor (IM or C) revealed an increase in CORT levels in the isolation + immobilization group, approximately 3-fold relative to the controls ($p < 0.001$) and 4-fold relative to chronic isolation ($^{\wedge\wedge\wedge}p < 0.001$). In contrast, repeated exposure to acute C did not significantly alter CORT levels ($p > 0.05$) as compared to either the control or chronic isolation stress groups. Moreover, serum CORT levels in animals exposed to the combined stressors (IS + IM and IS + C) were significantly lower than those exposed to each respective acute stressor alone ($p < {}^{\#\#}0.01$).

Table 1. Serum corticosterone (CORT) concentrations (mean ± S.E.M. of 6–7 animals per group)in control rats following acute immobilization (IM) stress, cold stress (C), chronic isolation and combined stressors (IS + IM or IS + C). Statistical analysis was performed using two-way ANOVA followed by Duncan's post hoc test ($^{***}p < 0.001$, stress *versus* control; $^{\#\#}p < 0.01$ acute *versus* combined stress; $^{\wedge\wedge\wedge}p < 0.001$ chronic *versus* combined stress)

Serum CORT concentrations (ng/ml)		
Acute stress	Control	Isolation
	142.91 ± 14.16	123.39 ± 10.89
Immobilization	648.89 ± 166.78***	477.98 ± 74.03$^{***,\#\#}$ $^{\wedge\wedge\wedge}$
Cold	342.11 ± 112.53**	86.41 ± 37.68$^{\#\#}$

NO metabolites (NO_x^-) in PFC and HIPP

A two-way ANOVA revealed significant effects of chronic IS stress in both the PFC and HIPP ($F_{1.30} = 7.49$, $p < 0.01$; $F_{1.30} = 46.44$, $p < 0.001$, respectively). Post hoc Duncan's tests showed a significant increase of NO_x^- levels following acute C and IS stress as compared to the control group in HIPP ($p < 0.01$; $p < 0.001$, respectively), while it remained unchanged in the PFC (Fig. 1). In animals exposed to the combined stressors, a significant increase in NO_x^- levels was revealed in HIPP and PFC ($p < 0.001$ and $p < 0.05$). When the subsequent acute IM or C stressors were applied to chronically-isolated animals, NO_x^- levels increased above those of the acute stressors alone ($^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ respectively), but only in the HIPP.

p53 response in PFC and HIPP

A two-way ANOVA indicated a significant main effect of chronic IS stress ($F_{1.30} = 22.1$, $p < 0.001$) on mitochondrial p53 protein levels, and a significant interaction of acute and chronic stress ($F_{2.30} = 3.6$, $p < 0.05$) on cytosolic p53 protein levels in the PFC. Post hoc analysis showed that the two combined stressors (IS + IM or IS + C) increased mitochondrial p53 levels as compared to the control group ($p < 0.01$) and to the acute stressors alone ($^{\#\#}p < 0.01$) (Fig. 2). In contrast, cytosolic p53 protein levels were decreased following combined IS + C stress as compared to acute C stress alone ($^{\#}p < 0.05$) (Fig 2). However, none of the applied stressors had any effect on p53 immunoreactivity in the HIPP, except in acute IM stress when compared to the control group ($p < 0.01$) (Fig. 2).

Protein expression of Bax, Bcl-2 in PFC and HIPP

To determine whether stress may lead to alterations in mitochondrial membrane integrity, the protein expression of proapoptotic Bax and antiapoptotic Bcl-2 and their intracellular distribution in the mitochondrial and cytosolic fractions was analyzed in the PFC and HIPP. A two-way ANOVA revealed a significant main effect of acute stress on Bax ($F_{2.30} = 4.88$, $p < 0.05$) and chronic stress on Bcl-2 ($F_{1.30} = 21.59$, $p < 0.001$) in the mitochondrial fraction of the PFC. Post hoc

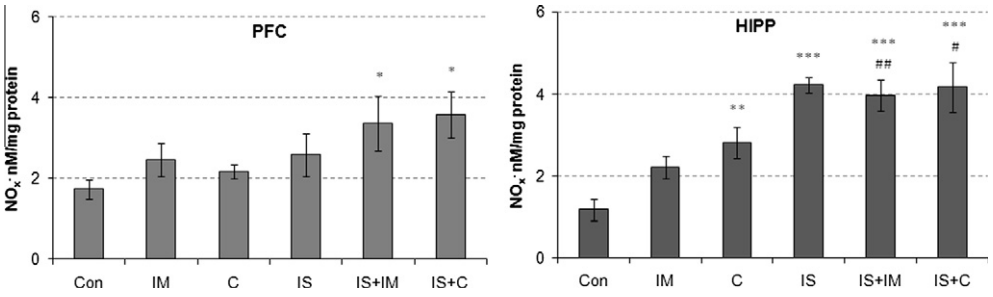

Fig. 1. The effect of acute immobilization (IM) stress, cold stress (C), chronic isolation (IS) and combined stressors (IS + IM or IS + C) on NO metabolites (NO_x^-) (nM/mg protein) in the prefrontal cortex (PFC) and hippocampus (HIPP). Data are presented as mean + S.E.M. Symbols indicate a significant difference between: (i) respective stress treatment and control $^{*}p < 0.05$, $^{***}p < 0.001$; (ii) combined IS + IM, IS + C stressors and acute IM or C stressor, respectively $^{\#\#}p < 0.01$, $^{\#}p < 0.05$; obtained from two-way ANOVA followed by Duncan's post hoc test.

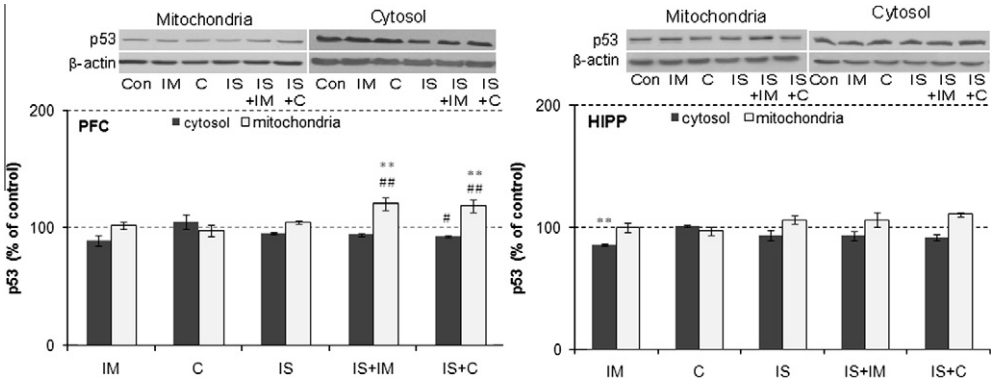

Fig. 2. Relative quantification of p53 protein level in the cytosolic and mitochondrial fraction of the prefrontal cortex (PFC) and hippocampus (HIPP) following acute immobilization (IM) stress, cold stress (C), chronic isolation (IS), and combined stressors (IS + IM or IS + C). Data are presented as mean + S.E.M. Symbols indicate a significant difference between: (i) respective stress treatment and control $^{**}p < 0.01$; (ii) combined IS + IM, IS + C stressors and acute IM or C stressor, respectively $^{\#\#}p < 0.01$, $^{\#}p < 0.05$; obtained from two-way ANOVA followed by Duncan's post hoc test.

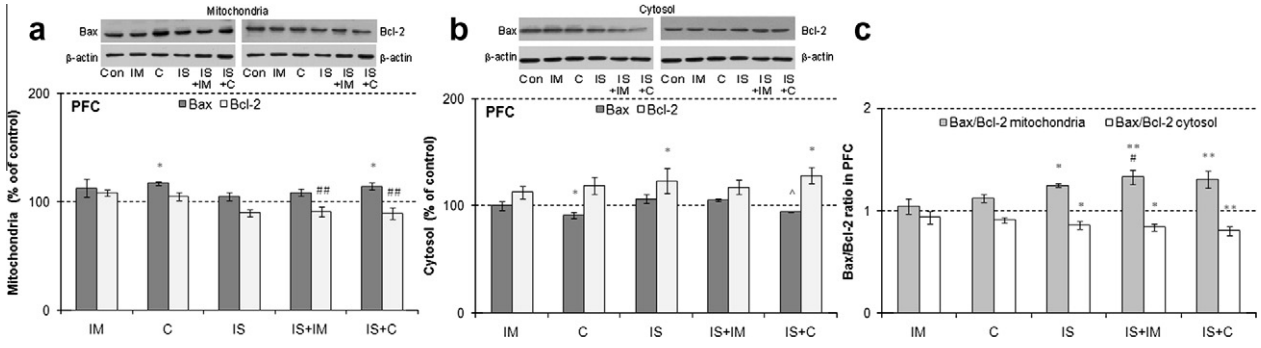

Fig. 3. Protein expression of Bax and Bcl-2 in the mitochondrial (3a) and cytosolic fraction (3b) and the Bax to Bcl-2 protein ratio (3c) in prefrontal cortex (PFC) following acute immobilization (IM) stress, cold stress (C), chronic isolation (IS), and combined stressors (IS + IM or IS + C). Symbols indicate a significant difference between: 9i) respective stress treatment and control $^{*}p < 0.05$, $^{**}p < 0.01$; (ii) combined stressors and respective acute stressors $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; (iii) combined IS + C stress and chronic isolation (IS) stress, $\hat{}\ p < 0.05$; obtained from two-way ANOVA followed by Duncan's post hoc test.

analysis demonstrated a significant increase of mitochondrial Bax protein following acute C stress and the combined IS + C stress ($p < 0.05$). In contrast, a significant decrease in mitochondrial Bcl-2 following the two combined IS + IM and IS + C stressors compared to acute stress alone was revealed ($^{\#\#}p < 0.01$) (Fig. 3a). With regard to the cytosolic fraction of the PFC, a two-way ANOVA revealed a significant main effect of acute ($F_{2.30} = 4.89$, $p < 0.05$) and chronic ($F_{1.30} = 8.53$, $p < 0.01$) stress on Bax in the PFC. As shown in Fig. 3b, cytosolic Bax was significantly decreased by acute C stress as compared to control ($p < 0.05$) and by combined IS + C stress as compared to chronic IS stress ($\hat{}\ p < 0.05$), while chronic IS and combined IS + C stress led to a significant increase in cytosolic Bcl-2. Moreover, we calculated the relative ratios of Bax to Bcl-2 in mitochondrial and cytoplasmic compartments (Fig. 3c). A two way ANOVA

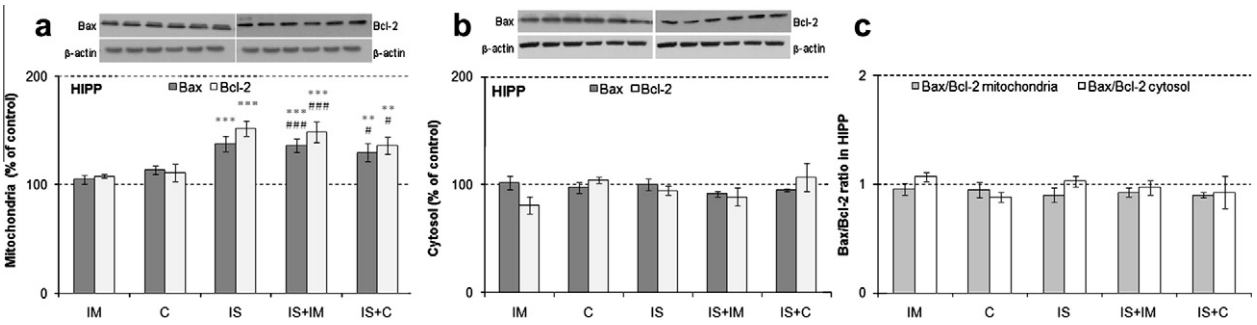

Fig. 4. Protein expression of Bax and Bcl-2 in the mitochondrial (4a) and cytosolic fraction (4b) and the Bax to Bcl-2 protein ratio (4c) in the hippocampus (HIPP) following acute immobilization (IM) stress, cold stress (C), chronic isolation (IS), and combined stressors (IS + IM or IS + C). Symbols indicate a significant difference between: (i) respective stress treatment and control $^{**}p < 0.01$, $^{***}p < 0.001$; (ii) combined stressors and respective acute stressors $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$; obtained from two-way ANOVA followed by Duncan's post hoc test.

showed a significant main effect of chronic IS stress ($F_{1.30} = 20.86$, $p < 0.001$; $F_{1.30} = 11.61$, $p < 0.01$) on the Bax/Bcl-2 ratio in mitochondrial and cytosolic fractions of the PFC. The Bax/Bcl-2 ratio was elevated in mitochondria following chronic IS and the two combined (IS + IM and IS + C) stressors ($p < 0.05$, $p < 0.01$), as well as in IS + IM as compared to acute IM alone ($^{\#}p < 0.05$) (Fig. 3c). The Bax/Bcl-2 ratio was simultaneously downregulated in the cytosolic fraction of the PFC under the same stress conditions ($p < 0.05$, $p < 0.01$).

In the HIPP, a two-way ANOVA revealed a significant main effect of chronic IS stress ($F_{1.30} = 37.78$, $p < 0.001$; $F_{1.30} = 51.04$, $p < 0.001$) on Bax and Bcl-2 in the mitochondrial fraction. Post hoc analysis showed a significant increase of Bax and Bcl-2 following chronic isolation and the two combined stressors in the mitochondrial fraction ($p < 0.01$; $p < 0.001$) (Fig. 4a). Moreover, a significant increase of these proteins under the combined stressors compared to those of acute stress alone was also found ($^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$). Regarding the cytosolic fraction of the HIPP, a two-way ANOVA revealed a significant main effect of acute ($F_{2.30} = 3.96$, $p < 0.05$) stress on Bcl-2. The Bax/Bcl-2 ratio was unchanged under all stress conditions in both the mitochondrial and cytosolic fractions of the HIPP (Fig. 4c).

DISCUSSION

In this study we examined whether acute, chronic or combined stress would initiate mitochondrial-dependent proapoptotic pathway through a rearrangement of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins in mitochondrial membrane in relation with the serum CORT level, NO production together with protein expression and subcellular distribution of p53 in the PFC and HIPP of male Wistar rats. Exposure to acute stress has been shown to release glucocorticoids from the adrenal cortex (Bremner, 1999). As a model of acute stress, we used IM because it has been demonstrated to be a strong stressor that produces inescapable physical and mental stress (Kvetnansky and Mikulaj, 1970; Dronjak and Gavrilovic, 2006), while C represents mild stress (Dronjak et al., 2004). In the current study, CORT levels were higher in the IM group than in the C group, confirming that IM was stress of high intensity (Garcia et al., 2000). Increased CORT levels following both acute stressors, act on prefrontal and hippocampal glucocorticoid receptors to reduce circulating levels of CORT (Filipović et al., 2005). Moreover, stress and glucocorticoids have been found to increase glutamate concentrations in the hippocampal synapse (Sapolsky, 2000), resulting in the production of NO, which possesses both neuroprotective and neurodestructive properties (Dawson et al., 1991; Dawson and Dawson, 1995; McCaslin and Oh, 1995). Brain NO is primarily generated by either neuronal NO synthase (nNOS) or inducible (iNOS) that is associated with pathological processes (Brown, 2007). Moreover, it has been shown that NO overproduction in animals exposed to acute stress is caused by nNOS expression (Krukoff and Khalili, 1997; De Oliveira et al., 2000) acting predominantly as a neuromodulator by decreasing glutamate release. In the current study, the increased hippocampal NO_x^- level following acute C may be due to increased hippocampal nNOS protein expression (Zlatković and Filipović, 2012a), suggesting a normal physiological and protective role (Oosthuizen et al., 2005). With regard to p53, the cytosolic/mitochondrial distribution remained unaltered by acute stressors in both the PFC and HIPP. However, a decreased level of cytosolic p53 without its translocation to mitochondria in the HIPP could be due to its translocation to the nucleus, where it functions as a transcription factor (Zhao et al., 2000). To examine effects of acute stressors on mitochondrial membrane integrity, we analyzed the translocation of both Bax and Bcl-2 between the cytosolic and mitochondrial fractions. In both brain structures, cytosolic Bcl-2 and mitochondrial Bax remained unchanged, except for a slight increase in mitochondrial Bax with a parallel decrease in the cytosolic fraction under acute C stress in the PFC. As the Bax/Bcl-2 ratio determines sensitivity to apoptotic stimuli, our results indicate that the mitochondrial membrane integrity still remains intact following acute stressors in both brain structures.

To better understand the effects of a more naturalistic type of chronic stress, we used 21 days of social isolation, chronic mild stress, as it has been shown to produce

behavioral changes that are similar to human depression (Heim and Nemeroff, 2001; Heinrich and Gullone, 2006) and considered to be a valid and useful experimental model of depression (Hall, 1998; Liu et al., 2005; Fuchs and Flügge, 2006; Dronjak et al., 2007; Serra et al., 2007; Spasojević et al., 2007; Carnevali et al., 2012). Social isolation precludes the social stimuli necessary to modulate adaptive responses to new situations and has been demonstrated to be a risk factor in human depression (Ishida et al., 2003). In contrast to the effect of acute stressors, chronically-stressed animals exhibited CORT values similar to the basal (control) levels (Malkesman et al., 2006). Given that chronic IS stress compromises the primary antioxidant defense enzyme manganese superoxide dismutase (MnSOD) activity in the PFC (Filipović et al., 2011), the current data illustrates the glucocorticoid paradox, in which a state of oxidative stress may exist under CORT levels similar to the basal value. Moreover, sustained overproduction of NO_x^- following chronic IS and the combined stressors indicates a prooxidant state in both the PFC and HIPP. Since extensive and prolonged release of NO_x^- in the PFC may result from high levels of iNOS but not nNOS protein expression (Zlatković and Filipović, 2012b), elevated NO_x^- levels in our study may be in part responsible for the stress-induced decline in MnSOD activity (Mastrocola et al., 2005) or due to a reduction in MnSOD protein levels (Filipović et al., 2009). Our data are in agreement with the results of Olivenza et al. (2000), who reported that after chronic immobilization stress (21 days for 6 h), iNOS was induced in rat cortical neurons. Furthermore, previous reports have suggested that sustained NO overproduction via iNOS can induce apoptosis via mitochondrial Bax translocation. Ghatan et al. (2000) reported that NO induced Bax translocation to mitochondria in both cortical neurons and a neuroblastoma cell line, and Bax-deficient cortical neurons were resistant to NO-induced cell death. A novel finding in our study is that the effects of IS stress were not mediated by the regulation of Bax as a proapoptotic factor, but rather by increased cytosolic Bcl-2 protein caused by its translocation from mitochondria in the PFC (Cao et al., 2001). Since we previously demonstrated chronic stress-induced apoptosis in the PFC (Filipović et al., 2011), our results corroborate the findings of Tamatani et al. (1998), who found that NO-induced apoptosis is accompanied by a down-regulation of Bcl-2 that is linked to the release of apoptotic factors such as cytochrome *c* from mitochondria into the cytosol.

Previous studies have indicated that NO overproduction may possess both proapoptotic and antiapoptotic effects. Antiapoptotic effects include an increase in Bcl-2 and NF-κB activation (Li et al., 2000), while proapoptotic effects include the inhibition of NF-κB, decreased Bcl-2 expression, and increased p53 expression (Matthews et al., 1996; Marshall and Stamler, 2002; Yung et al., 2004). Our data revealed that NO overproduction following chronic IS stress did not alter the Bax/Bcl-2 ratio in the HIPP. As we have previously reported increased nNOS protein expression in all stressed groups in the HIPP (Zlatković and Filipović, 2012a), we confirmed that nNOS-derived NO overproduction has a primarily protective effect on cells (Nicotera et al., 1997). Since redistribution of Bcl-2 family members caused an increase in the prefrontal Bax/Bcl-2 ratio, our results suggest that the PFC appears to be more sensitive to chronic IS stress than the HIPP. These results are not unexpected, as stress has been shown to increase the number of apoptotic cells in the temporal cortex, but decrease the number in the hippocampal pyramidal cell layers (Lucassen et al., 2001). The precise balance between apoptosis and neurogenesis in the HIPP drives the continuous turnover of cells in this region (Biebl et al., 2000; Heine et al., 2004).

To investigate the potentially maladaptive effects of chronic IS stress, we used a combined stress model (chronic IS followed by a single exposure to acute IM or C stress). Decreased CORT levels in response to an additional IM or C stress in chronically-isolated animals compared to acute stressors alone indicates a compromised HPA axis activity resulting from prior chronic IS stress that could not recover after subsequent acute stressors. (Filipović et al., 2005). With the combined stressors, NO overproduction together with mitochondrial translocation of p53 in the PFC was observed. Nevertheless, p53 mitochondrial translocation was followed by mitochondrial Bax translocation following IS + C stress. Furthermore, a decrease in mitochondrial Bcl-2 levels, with its *concomitant increase* in cytosolic fraction, resulted in an increase in the mitochondrial Bax/Bcl-2 ratio in the PFC, a combination that has been shown to accelerate apoptotic cell death (Oltvai et al., 1993). Again, these changes are consistent with our previous findings that combined stress causes apoptosis via cytochrome c release and caspase-3 activation (Filipović et al., 2011). p53 has also been implicated in mediating NO-induced apoptosis by directly targeting the mitochondria of the cell (Murphy et al., 2004; Moll et al., 2005). Furthermore, Bax translocation from the cytosol to mitochondria has been demonstrated to be a critical step in p53-mediated apoptosis (Deng and Wu, 2000; Wu and Deng, 2002; Mihara et al., 2003). Thus, our data suggest that combined stressors that upregulate NO production in the PFC may lead to p53-mediated apoptosis by inducing Bax translocation from the cytosol to mitochondria.

CONCLUSION

The results presented here indicate that chronic IS stress triggers proapoptotic signaling in the PFC via the translocation of antiapoptotic Bcl-2 from the mitochondria to the cytosolic fraction. In chronically isolated animals *subsequently* subjected to an *acute* stressor, sustained NO overproduction accompanied by the *translocation* of *p53* and Bax to *mitochondria augment the initiation of proapoptotic response* and suggest that mitochondrial p53 localization participates

in proapoptotic signaling. Given that the Bax/Bcl-2 ratio was unchanged in the HIPP and upregulated in the PFC, it is likely that chronic IS stress exerts opposing actions on Bax and Bcl-2 in a tissue-specific manner (PFC *versus* HIPP), indicating that the PFC is a key target of the maladaptive response to stress (Cerqueira et al., 2007).

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