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MOLEKULARNI MEHANIZMI REGULACIJE ANTIOKSIDATIVNIH ENZIMA U MOZGU I JETRI AKUTNO I/ILI HRONIČNO STRESIRANIH PACOVA

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MOLECULAR MECHANISMS OF REGULATION OF ANTIOXIDANT ENZYMES IN THE BRAIN AND LIVER OF ACUTELY AND/OR CHRONICALLY STRESSED RATS

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REZIME

Molekularni mehanizmi regulacije antioksidativnih enzima u mozgu i jetri akutno i/ili hronično stresiranih pacova

Aktivacija hipolamo-hipofizo-adrenalne (HHA) ose predstavlja jedan od odgovora organizma na izlaganje stresnim uslovima. Dok aktivacija HHA ose uzrokovana kratkotrajnim stresorima ima adaptivni karakter, hroničan stres može dovesti do brojnih patoloških promena među kojima su i poremećaji raspoloženja. Uzrok deregulacije HHA ose u stresnim uslovima još uvek nije u potpunosti utvrđen, a smatra se da može biti povezan sa i pojavom oksidativnog stresa. Stoga je ispitana i okarakterisana aktivnost HHA ose, merenjem koncentracije kortikosterona (CORT) kao markera stresnog odgovora, kao i mehanizmi regulacije antioksidativnih enzima CuZnSOD i MnSOD, kao biomarkera oksidativnog stresa, u uslovima akutnog, hroničnog i kombinovanog stresa u hipokampusu i prečeonoj kori mozga i jetri mužjaka pacova Wistar soja. Cilj ove teze je bio bolje razumevanje biohemijskih i molekularnih mehanizama delovanja antioksidativnih enzima u moždanim strukturama i jetri pacova nakon izlaganja 21-dnevnoj socijalnoj izolaciji, koja predstavlja jedan od životinjskih modela depresije. Akutno izlaganje imobilizaciji ili niskoj temperaturi u trajanju od 2 sata korišćeno je kao pokazatelj "normalnog" odgovora na stres, dok je kombinacija hroničnog i akutnog stresa služila kao potvrda maladaptivnog efekta hroničnog stresa.

Rezultati istraživanja su pokazali povećanu koncentraciju CORT-a u serumu životinja izloženih akutnim stresorima, dok su kod životinja izlaganih hroničnoj socijalnoj izolaciji vrednosti ostale nepromenjene. Međutim, značajno niža koncentracija CORT-a između životinja koje su izlagane kombinovanom i odgovarajućem akutnom stresu ukazala je na poremećaj u regulaciji HHA ose koji je izazvana hroničnom socijalnom izolacijom, a što je rezultovalo u neadekvatnm odgovoru na nove stresne situacije.

Akutni stres imobilizacije doveo je do povećanja ekspresije MnSOD proteina u mitohondrijama obe moždane strukture, dok je kod životinja akutno izlaganih niskoj temperaturi ili hroničnom stresu izolacije nivo ovog proteina ostao nepromenjen. Međutim, aktivnosti CuZnSOD i MnSOD-a u prečeonoj kori nisu pratile detektovanu proteinsku ekspresiju ukazujući time na moguće stanje oksidativnog stresa. Dodatni akutni stres doveo je do opadanja nivoa MnSOD proteina u mitohondrijama prečeone kore, što uz istovremeno povećanje u citosolnoj

frakciji kao i oslobađanje citohroma c može ukazati na narušavanje integriteta mitohondrijske membrane. U hipokampusu je zabeležena smanjena aktivnost mitohondrijske MnSOD u kombinovanom stresu, ali bez uticaja na integritet mitohondrijske membrane. Ovi rezultati ukazuju na neadekvatnu antioksidativnu zaštitu ali i znatno izraženije stanje oksidativnog stresa u prečeonoj kori u odnosu na hipokampus.

Tokom izlaganja stresorima detektovane su promene na nivou proteina uključenih u produkciju azot oksida (NO), koji stvaranjem peroksinitrita može dovesti do promena na nivou antioksidativnih enzima. Obzirom da enzim azot oksid sintaza (NOS) biokatalizuje produkciju NO, odredjivana je proteinska ekspresija neuronalne (nNOS) i inducibilne (iNOS) forme ovog enzima u citosolu moždanih struktura pacova izlaganih navedenim stresnim uslovima. Takodje, odredjivana je i proteinska ekspresija inducibilne forme proteina toplotnog stresa 70 (eng. Heat shock protein 70-Hsp70i), koji ima značajnu ulogu u zaštiti ćelija od oksidativnog stresa suzbijanjem aktivacije jedarnog transkripcionog faktora kapa beta (NF-kB), što može dovesti do supresije gena za iNOS. U obe moždane strukture povećan nivo nNOS proteina zapažen je kod svih stresiranih životinja, dok je hronična socijalna izolacija dovela do porasta iNOS-a samo u prečeonoj kori. Povećanje nivoa Hsp70i u hipokampusu imalo je protektivni efekat, sprečavajući aktivaciju NF-kB a time i ekspresiju iNOS proteina. U suprotnom, u prečeonoj kori prelazak NF-kB iz citosola u jedro, koji je regulisan od strane slobodnih radikala, doveo je do povećanja nivoa iNOS proteina. NO nastao na ovaj način uzrokovao je pojačan oksidativni stres a time i aktivaciju proapoptotske signalizacije, merene na osnovu promena nivoa proteina p53, proapoptotskog Bax-a i antiapoptotskog Bcl-2, kao i citohroma c, izmedju citosolne i mitohondrijske frakcije. Prelazak p53 iz citosolne u motohondrijsku frakciju, praćen povećanjem odnosa Bax/Bcl-2 i izlaskom citohroma c iz mitohondrija u citosol potvrđuje pokrenutu proapoptotsku signalizaciju u prečeonoj kori ali ne i hipokampusu životinja izloženih hroničnom stresu izolacije. Takodje, uočene promene na nivou ovih antioksidativnih enzima kao i povećani nivo NO u jetri koji može biti uzrok smanjene aktivnosti MnSOD, doveli su do zaključka da hroničan stres socijalne izolacije utiče i na periferno tkivo.

Na osnovu dobijenih rezultata može se zaključiti da prečeona kora i hipokampus imaju različitu osetljivost na izlaganje hroničnom stresu socijalne izolacije. Stanje oksidativnog i nitrozativnog stresa može dovesti do promena u regulaciji aktivnosti i proteinske ekspresije antioksidativnih enzima kao i proapoptotske signalizacije u prečeonoj kori, dok povećana

proteinska ekspresija Hsp70i štiti hipokampus od neurotoksičnosti. Obzirom da hronična socijalna izolacija predstavlja životinjski model depresije, ustanovljene promene na nivou gore pomenutih molekula mogu ukazati na potencijalna mesta delovanja antidepresivne terapije.

Ključne reči: hronična socijalna izolacija, oksidativni stres, nitrozativni stres, proapoptotska signalizacija

NAUČNA OBLAST: Biologija

UŽA NAUČNA OBLAST: Neurobiologija

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ABSTRACT

Molecular mechanisms of regulation of antioxidant enzymes in the brain and liver of acutely and/or chronically stressed rats

The response of the organisms to stressors involves activation of hypothalamic-pituitary-adrenal (HPA) axis. HPA axis activation is adaptive in the short term, but chronic exposure to stressful stimuli may lead to pathological changes, such as mood disorders. Although precise cause of deregulation of HPA axis due to stress exposure is still unknown, it is thought that may be associated with increased oxidative stress. Therefore, we examined and characterized HPA axis activity, by measuring the concentration of serum corticosterone (CORT) as marker of stress response, and mechanisms of regulations of antioxidative enzymes CuZnSOD and MnSOD, as biomarkers of oxidative stress, in the prefrontal cortex, hippocampus and liver of male Wistar rats exposed to acute, chronic and combined stress. The aim of this thesis is better understanding the biochemical and molecular mechanisms of action of antioxidative enzymes in the brain structures and liver of rats subjected to 21-day social isolation, that represent an animal model for depresion. Acute stressors of 2h immobilization or cold are used to indicate 'normal' stress response, while combination of chronic and acute stress served as confirmation of maladaptive effect of chronic stress.

Results showed increased concentration of serum CORT level in the animals exposed to acute immobilization or cold stress, while in animals expsosed to chronic isolation these values remain unchanged. However, significantly lower CORT level detected between animals exposed to combined and appropriate acute stress showed compromised HPA axis funcioning, caused by chronic social isolation, which has resulted in inadequate response to the novel stressful situations.

Acute immobilization stress resulted in increased expression of MnSOD protein in mitochondria of both brain structures, while in animals acutely exposed to cold or chronic social isolation level of this protein remained unchanged. However, detected discrepancy between CuZnSOD and MnSOD activies and protein expression in the prefrontal cortex suggesting the possible state of oxidative stress. Additional acute stress decreased mitochondrial MnSOD protein level, which along with the increase in citosolnoj fraction and the release of cytochrome c indicate compromised integrity of the mitochondrial membrane in the prefrontal cortx. In the

hippocampus decreased activity of mitochondrial MnSOD in combined stress was observed, but without effect on the integrity of the mitochondrial membrane. These results suggest inadequate antioxidant protection, and significantly higher susceptibility of prefrontal cortex to oxidative stress.

During stress exposures changes of proteins involved in generation of nitric oxide (NO), molecule that may be involved in inactivation of antioxidative enzymes by formation of peroxinitrite, were detected. Given that nitric oxide synthase (NOS) catalyze NO production, protein expression of neuronal (nNOS) and inducible (iNOS) izoform in the cytosolic fraction of brain structures were determined. Additionaly, protein expression of inducible form of heat shock protein 70 (Hsp70i) was investigated, as this protein has protective role against oxidative stress by suppressing activation of nuclear transcriptional factor kappa beta (NF-kB) and consequently expression of iNOS gene. In both prefrontal cortex and hippocampus increased nNOS protein level was observed in all stressed groups, while chronic social isolation provoke iNOS increase only in the prefrontal cortex. Upregulation of hippocampal Hsp70i prevented NF-kB activation and reduced iNOS protein expression. On the other hand, in the prefrontal cortex nuclear translocation of NF-kB, regulated by free radicals, was accompanied with iNOS increase. iNOS-generated NO provoked state of oxidative stress that led to initiation of proapoptotic cascade, measured through changes in p53, Bax, Bcl-2 and cytochrome c protein distribution between the cytosolic and mitochondrial fractions. p53 mitochondrial translocation followed by increased mitochondrial Bax/Bcl-2 ratio and cytochrome c release into cytosol in the prefrontal cortex but not hippocampus of animal exposed to chronic social isolation confirms proapoptotic signalling. Likewise, changes of hepatic antioxidative enzymes and increased NO level that may be the cause for decreased MnSOD activity in chronically isolated animals, indicate that chronic stress affects peripheral tissues as well.

Obtained results indicate that prefrontal cortex and hippocampus have different susceptibillity to chronic social stress exposure. Oxidative and nitrosative stress may be involved in changes of protein expression and activity of antioxidative enzymes, and proapoptotic signalling in the prefrontal cortex, while upregulation of Hsp70i protect hippocampus from neurotoxicity. Given that 21-day chronic social isolation represent an animal model for depression, determined changes of the investigated molecules may indicate potential targets for antidepressant treatment.

Keywords: chronic social isolation, oxidative stress, nitrosative stress, proapoptotic signalization

RESEARCH AREA: Biology

RESEARCH FIELD: Neurobiology

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SKRAĆENICE

ACTH	adrenokortikotropni hormon (eng. *adrenocorticotropic hormone*)
Bax	(eng. *Bcl-2 associated X protein*)
Bcl-2	(eng. *B-cell lymphoma 2*)
CAT	katalaza (eng. *catalase*)
cit c	citohrom c (eng. *cytochrome c*)
CORT	kortikosteron (eng. *corticosterone*)
COX-I	ciklooksigenaza I (eng. *cyclooxygenase I*)
COX-IV	ciklooksigenaza I (eng. *cyclooxygenase IV*)
CRH	kortikotropni-oslobađajući hormon (eng. *corticotropin-releasing hormone*)
CuZnSOD	bakar-cink superoksid dismutaza (eng. *copper,zinc-superoxide dismutase*)
GAS	generalni adaptacijski sindrom (eng. *general adaptation syndrome*)
GC	glukokortikoidi (eng. *glucocorticoids*)
GSH	redukovana forma glutationa (eng. *glutathione*,
GSH-Px	glutatione peroksidaza (eng. *glutathione peroxidase*)
GSSG	oksidovana forma glutationa (eng. *glutathione disulfide*)
GR	glutation reduktaza (eng. *glutathione reductase*)
H_2O_2	vodonik peroksid (eng. *hydrogen peroxide*)
HHA osa	hipotalamo-hipofizno-adrenalna osa
Hlad	stres akutnog izlaganje niskoj temperaturi
Hsp70i	inducibilni protein toplotnog šoka 70 (engl. *inducible heat shoch protein 70*)
IM	akutni stres imobilizacije
iNOS	inducibilna azot-oksid sintaza (eng. *inducible Nitric Oxide Synthase*)
IZ	hronični stres socijalne izolacije

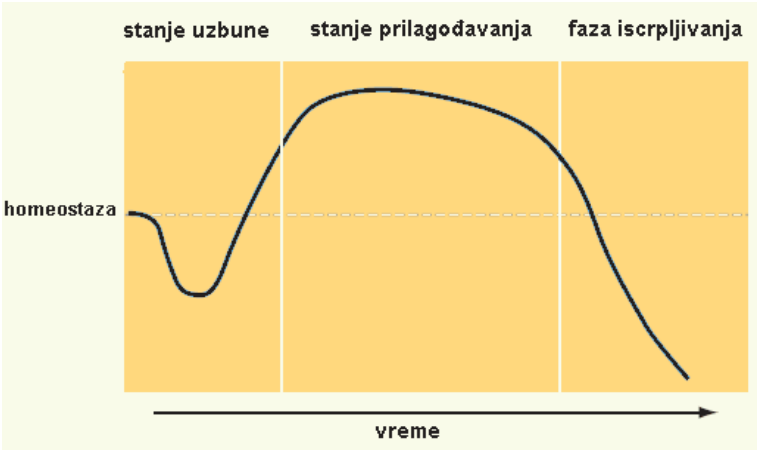
IZ+Hlad	kombinovani stres hronične izolacije i akutne hladnoće
IZ+IM	kombinovani stres hronične izolacije i akutne imobilizacije
MnSOD	mangan superoksid dismutaza (eng. *Manganese-superoxide dismutase*)
NF-κB	nukleari faktor-κB (eng. *Nuclear Factor kappa B*)
NMDA	N-metil-D-aspartat (eng. *N-methyl-D-aspartate*)
nNOS	neuronalna azot-oksid sintaza (eng. *neuronal Nitric Oxide Synthase*)
NO	azot oksid (eng. *nitric oxide*)
NOS	azot-oksid sintaza (eng. *Nitric Oxide Synthase*)
$O_2^{\cdot -}$	superoksid anjon radikal
p53	(eng. *protein 53* or *tumor protein 53*)
POMC	pro-opiomelanokortina (eng. *pro-opiomelanocortin*)
PTP	pore mitohondrijalne permeabilnosti (eng. *permeability transition pore*)
ROS	reaktivne kiseonične vrste (eng. *reactive oxygen species*)
SNS	simpatički nervni sistem
SOD	superoksid dismutaza (eng. *superoxide dismutase*)

SADRŽAJ

1. UVOD

1.1. Pojam stresa i odgovor na stres

Iako ne postoji jedinstvena definicija stresa u biološkom smislu, u osnovi objašnjenja leži koncept homeostaze, kao stanja ravnoteže koje organizam teži da dostigne. Pojam *stres* prvi je uveo Hans Selye, definišići ga kao "nespecifičan odgovor organizma na bilo koji izazov kome je izložen" (Selye, 1974), a da u stanje stresa organizam dovode različiti faktori - *stresori* koji deluju na njega. Smatrajući da organizam na sličan način reaguje na bilo koji stres, Selye je definisao generalni adaptivni sindrom (eng. general adaptation syndrome, GAS) kojim je odgovor na stres podelio na tri faze: stanje uzbune, prilagođavanja i iscrpljivanja (Slika 1).



Slika 1. **Generalni adaptivni sindrom (GAS)**

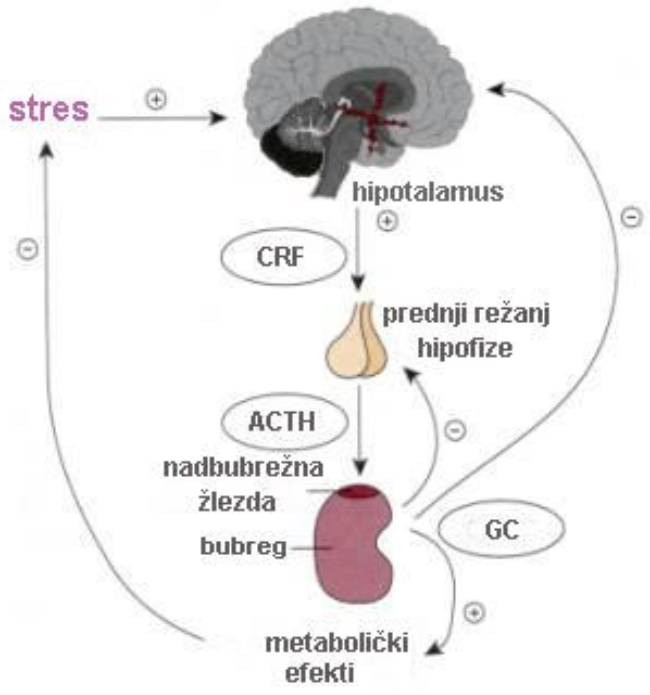
Stanje uzbune je trenutni odgovor na akutno izlaganje stresoru koji se odlikuje brzim pokretanjem procesa adaptacije na date uslove, od kojih je najznačajnije oslobađanje hormona nadbubrežne žlezde. U sledećoj fazi, fazi prilagođavanja, dolazi do adaptacije na nove uslove, pa organizam postaje otporniji na delovanje stresora. Međutim, ukoliko stresor deluje u dužem vremenskom periodu na organizam, dolazi do treće faze -iscrpljenja, gde se organizam više ne može prilagoditi uslovima sredine i gde može doći do patoloških stanja pa i smrti (Selye 1976).

Danas je Selye-ovo viđenje nespecifičnog, generalizovanog odgovora na stres prevaziđeno, jer se zna da je odgovor koji se pokreće u organizmu zavisan od tipa stresora, prethodnog iskustva sa tim stresorom kao i sposobnosti jedinke da se nosi sa stresorom (eng. *coping*) (Pacák i sar., 1998, McEwen i Wingfield, 2003; Goldstein, 2003).

Glavni učesnici odgovora na stres su simpatički nervni sistem (SNS) i hipotalamo-hipofizo-adrenalni (HHA) sistem, od kojih svaki ima različite uloge, način regulacije i uloge i čiji su odgovori različiti, u zavisnosti od intenziteta i vrste stresora (Goldstein, 2003). Nešto kasniji odgovor na stres odlikuje se oslobađanjem glukokortikoida iz kore nadbubrežne žlezde usled aktivacije HHA ose. Aktivacija ose predstavlja glavni adaptivni odgovor organizma (Munck i sar., 1984). Oslobođeni hormoni neophodni su za uspostavljanje homeostaze nakon aktivacije SNS-a i ″bori se ili odgovora beži″.

1.1.1. Regulacija HHA ose u fiziološkim uslovima

HHA osa je sistem koji čine hipotalamus, hipofiza i nadbubrežna žlezda, a koji su međusobno povezani i regulisani direktnim uticajem kao i glukokortikoidima-posredovanom negativnom povratnom spregom. Usled delovanja stresora, informacije iz kortikalnih regija i limbičkog sistema (koji reguliše emocije i memoriju- hipokampus, amigdale, hipotalamus) dolazi do paraventrikularnih jedara hipotalamusa koji luče kortikotropin-oslobađajući hormon (eng. corticotropin-releasing hormon CRH). CRH se oslobađa i portalnim krvotokom dolazi do hipofize, gde podstiče izlučivanje pro-opiomelanokortina (eng. pro-opiomelanocortin-POMC) iz bazofilnih ćelija prednjeg režnja hipofize. POMC se enzimatski iseca na više proteina od kojih je jedan i adrenokortikotropni hormon (eng. adrenocorticotropic hormone ACTH), koji deluje na koru nadbubrežne žlezde izazivajući lučenje glukokortikoidnih hormona- kortizola kod ljudi ili kortikosterona kod pacova. Kortizol ima bitnu ulogu u centralnom nervnom sistemu (CNS) usled svog efekta na učenje, memoriju i emocije. Osim toga, utiče na metabolizam regulisanjem skladištenja i korišćenja glukoze, dok u imunom sistem reguliše intenzitet i trajanje inflamatornog odgovora (Sapolsky i sar., 2000). Oslobođeni glukokortikoidi negativnom povratnom spregom deluju na hipotalamus i hipofizu, i zaustavlja dalju sintezu CRH i ACTH kada ona više nije neophodna (Slika 2).



Slika 2. HHA **osa, komponente i mehanizam regulacije**

1.1.2. Regulacija HHA ose u patofiziološkim uslovima

Dok kratkotrajna aktivacija HHA ose ima adaptivni karakter, prekomerna produkcija hormona stresa ili nemogućnost zaustavljanja aktivirane HHA ose dovode do nastanka patološkog stanja. Tada možemo govoriti o štetnim posledicama stresa i njegovom maladaptivnom efektu. Posledice preteranog ili preslabog fiziološkog odgovora na stres mogu doprinositi razvoju već postojećih ili nastanku novih patoloških stanja među kojima su psihički poremećaji, kao što su depresija ili šizofrenija (McEwen i Stellar, 1993; Mayer, 2000; Reiche i sar., 2004). Povećan broj CRH neurona (Raadsheer i sar., 1994), nivoa CRH i kortizola kao i uvećanje hipotalamusa, hipofize i nadbubrežne žlezde uočen je kod osoba sa psihičkim poremećajima (Holsboer, 2000; Pariante i Lightman, 2008), što može biti, između ostalog, uslovljeno poremećajem glukokortikoidima posredovane negativne povratne sprege HHA ose (Gold i sar., 1988; Nemeroff, 1996; Juruena i sar., 2003; Filipović i sar., 2005). Pored melanholične depresije, sa hiperfunkcijom HHA ose povezani su i opsesivno-kompulzivni poremećaj i napadi panike (Tsigos i Chrousos, 2002). Sa druge strane, hipofunkcija HHA ose javlja se kod pacijenata

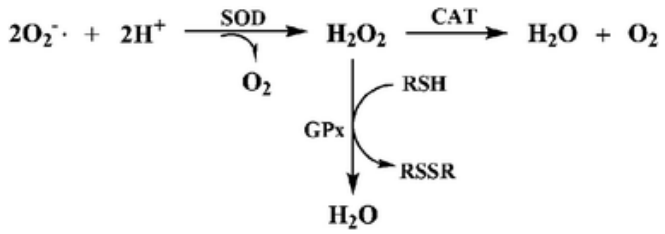
obolelih od post-traumatskog stresnog poremećaja, atipične i sezonske depresije i sindroma hroničnog umora (Tsigos i Chrousos, 2002).

1.2. Uloga antioksidativnih enzima u oksidativnom stresu

Aerobni organizmi konstantno su izloženi reaktivnim kiseoničnim vrstama (eng. reactive oxygen species- ROS). Iako su u fiziološkim koncentracijama, ROS uključene u brojne procese u ćeliji kao što je imunski odgovor, ćelijska signalizacija, regulatorni mehanizmi, njihov nivo mora biti strogo kontrolisan jer povišene koncentracije mogu izazvati oštećenja DNK, lipida i proteina i biti povezane sa brojnim patološkim stanjima (Alfadda and Sallam, 2012). U slučaju neravnoteže između stvaranja i eliminacije ROS nastaje stanje oksidativnog stresa.

Najznačajnije unutarćelijsko mesto nastanka ROS su mitohondrije, tačnije elektronski transportni lanac, gde se mali procenat molekula kiseonika kontinuirano pretvara u superoksidni anjon. Pored toga, reaktivne kiseonične vrste mogu nastati kao nusproizvodi aktivnosti enzima, kao što su NADPH oksidaza, ksantin oksidaza, azot oksid sintaza (Dröge, 2002). I odgovor organizma na delovanje stresora dovodi do stvaranja ROS, a izlaganje hroničnom stresu ili visokim koncentracijama glukokortikoida dovodi do povećavanja ovih štetnih molekula za 10% (McIntosh and Sapolsky, 1996). Mehanizam kojim slobodni radikali oštećuju ćeliju nije u potpunosti proučen, s obzirom na to da su to kratkoživeći molekuli što otežava njihovu detekciju i kvantifikaciju. Zbog toga se, kao indirektni pokazatelji uloge ROS u patološkim stanjima koriste markeri oksidativnog oštećenja makromolekula (protina, lipida, DNK), kao i promene u aktivnosti i nivou antioksidativnih enzima ili neenzimskih antioksidanasa.

Prvu liniju odbrane od ROS-a čini superoksid dismutaza (SOD), enzim koji katalizuje dismutaciju superoksid-anjon radikala u vodonik peroksid (H_2O_2) (Fridovich 1995), a koji se dalje metaboliše putem katalaze (CAT, lokalizovana prvenstveno u peroksizomima) ili glutation peroksidaze (GSH-Px, lokalizovana u mitohondrijama i citosolu) do H_2O (Slika 3). GSH-Px zahteva prisustvo redukovane forme glutationa (GSH), koji se u ovoj reakciji oksiduje. Oksidovani glutation (GSSG) se uz pomoc glutation reduktaze (GR) ponovo redukuje u GSH, uz prisustvo redukovanog NADPH. Neenzimski antioksidansi su brojni, i u ovu grupu se ubraja GSH, vitamin A, vitamin E, koenzim Q_{10}, itd.

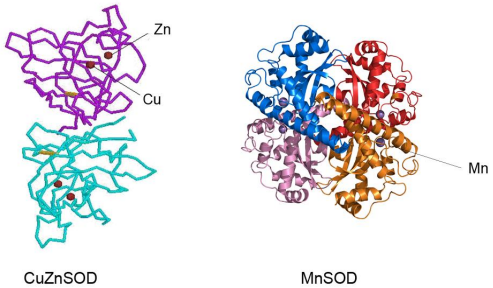


Slika 3 Odbrambeni mehanizmi od ROS-a. Superoksid dismutaza (SOD) uz dejstvo katalaze ili glutation peroksidaze eliminišu štetne vrste kiseonika.

1.2.1. Superoksid dismutaza

Superoksid dismutaze su porodica enzima čiji se članovi razlikuju na osnovu vrste metala koji se nalazi u aktivnom mestu enzima, a unutar ćelije nalaze se bakar-cink superoksid dismutaza (CuZnSOD, SOD1) i mangan superoksid dismutaza (MnSOD, SOD2).

CuZnSOD je homodimer molekulske mase od ~32 kDa koji je lokalizovan većinom u citoplazmi, ali može biti detektovan i u jedru (Crapo i sar., 1992; Okado-Matsumoto i Fridovich, 2001). Svaka subjedinica je sastavljena od 151 aminokiselinskog ostatka, a monomerne subjedinice su povezane disulfidnim mostom. U obe subjedinice nalaze se Cu^{2+}, od kojeg zavisi katalitička aktivnost enzima, i Zn^{2+}, koji stabilizuje kvaternernu konformaciju enzima (Slika 4, levo) (McCord i Fridovich, 1969; Rotilio i sar.,1972). CuZnSOD je veoma stabilan (McCord i Fridovich, 1969; Forman i Fridovich, 1973; Rotilio i sar., 1972) i otporan na toplotnu denaturaciju, delovanje proteaza i denaturišućih reagenasa (Forman i Fridovich, 1973), ali se može inaktivirati cijanidom (Rotilio i sar., 1972), što se zasniva na vezivanju i odstarnjivanju Cu^{2+} iz aktivnog mesta enzima. Takođe, može se inaktivirati izlaganjem vodonik-peroksidu, pogotovu na višim pH vrednostima (Hodgson i Fridovich, 1975; Sinet i Garber, 1981; Salo i sar., 1988).



Slika 4. Struktura CuZnSOD i MnSOD enzima.

MnSOD je tetramer molekulske mase ~80 kDa sa 2-4 atoma Mn u katalitičkom mestu (Fridovich 1975) (Slika 4, desno). Ovaj mitohondrijski enzim kodiran je od strane jedarnih gena i sintetiše se u citosolu u formi pro-enzima. Za transport kroz mitohondrijske membrane neophodno je posttranskripciono dodavanje 24-aminokiselinske signalne sekvence na N-kraj proenzima, koja ga upućuje ka mitohondrijama u čijem je matriksu i lokalizovana kao funkcionalan enzim (Ishikawa i sar., 1987, Wispé i sar., 1989). Za razliku od CuZnSOD, MnSOD se ne može inhibirati cijanidom (Ishikawa i sar., 1987) ali je osetljiv na hemijsku i toplotnu denaturaciju (Weisiger i Fridovich, 1973). Prekomerna ekspresija može imati neuroprotektivnu ulogu sprečavajući apoptozu, dok produžena izloženost ROS može dovesti do inaktivacije mitohondrijskih enzima, što može pokrenuti nastanak brojnih patoloških stanja (Wallace, 2005). Gubitkom Mn, gubi se i katalitička aktivnosti enzima.

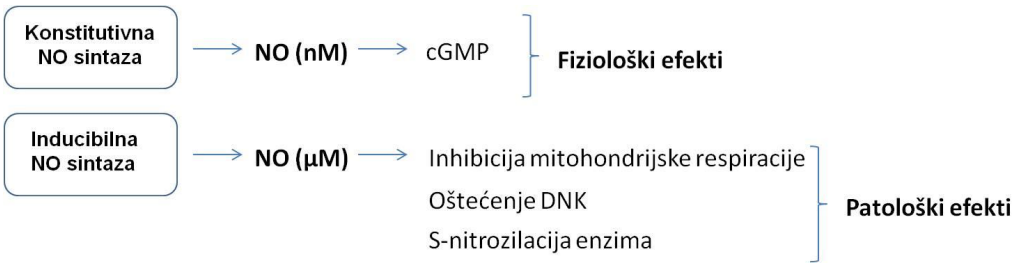
Glukokortikoidi su uključeni u regulaciju ekspresije antioksidativnih enzima. Snižena aktivnost antioksidativnih enzima uočena je u mozgu pacova koji su tretirani glukokortikoidima (McIntosh et al. 1998a), kao i kod pacova izloženih imobilizaciji, što dovodi do stvaranja oksidativnog stresa u mozgu, usled snižavanja aktivnosti SOD i ostalih antioksidativnih enzima (Zaidi i Banu, 2004). Sa druge strane, povećana aktivnost antioksidativnih enzima zabeležena je u jetri pacova tretiranih GC (McIntosh i sar., 1998a). Takođe, pokazano je da se kod životinja izloženim hroničnoj imobilizaciji ili niskoj temperaturi aktivnost CuZnSOD povećava u jetri (Sahin i Gumuslu, 2004; Sahin i Gumuslu, 2007a; Sahin i Gumuslu, 2007b), dok je tokom hroničnog ili akutnog stresa fizičkog ograničavanja (engl. restraint stress) detektovano sniženje SOD aktivnosti (Zafir i Banu, 2009). McIntosh i saradnici (1998a,b) pokazali su da GC snižavaju aktivnost antioksidativnih enzima u hipokampusu i korteksu, bez obzira na to da li postoji oksidativni stres. Takođe, deksametazon (sintetički glukokortikoid koji poseduje antiinflamatorno i imunosupresivno dejstvo), može smanjiti ekspresiju MnSOD iRNK, što potvrđuje glukokortikoidnu regulaciju MnSOD gena (Valentine i Nick 1994; Antras-Ferry i sar., 1997).

1.3. Ćelijska signalizacija u oksidativnom/nitrozativnom stresu

1.3.1. Uloga azot-oksida u ćelijskoj signalizaciji

Azot oksid (NO) je gasoviti molekul koji zbog lipofilne prirode nesmetano difunduje kroz ćelijske membrane i ima ulogu unutarćelijskog glasnika. Kada se nalazi u nanomolarnim

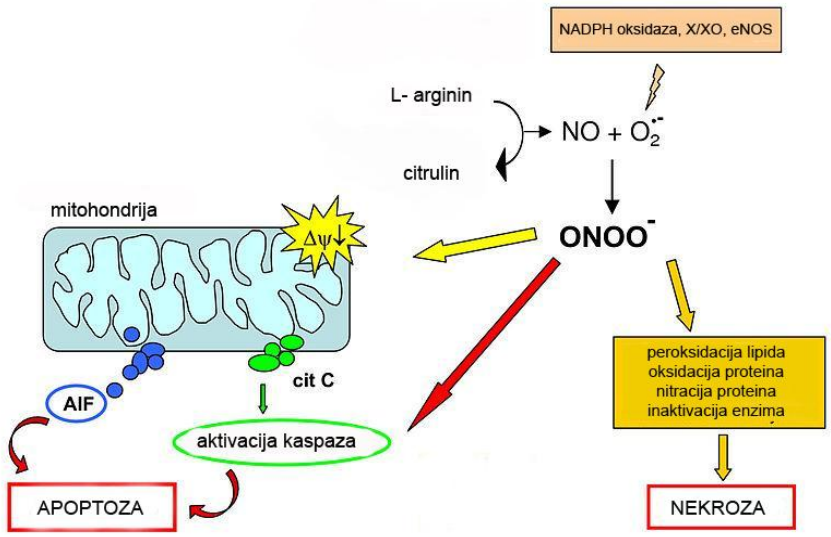
koncentracijama NO uključen u regulaciju odbrane organizma i imunom odgovoru, nervnoj komunikaciji, regulaciji vaskularnog tonusa itd. Sa druge strane, u mikromolarnim koncentracijama NO postaje citotoksičan i dovodi do oštećenja u različitim tkivima delujući na različite procese ili strukturne komponente ćelije (Slika 5).



Slika 5. **Efekti NO u ćelijama zavise od koncentracije u kojima je prisutan.**

U uslovima visoke koncentracije superoksid-anjon radikal ($O_2^{\bullet -}$) reaguje sa NO stvarajući peroksinitritni radikal. On se brzo protonuje u peroksinitritnu kiselinu, koja se dalje razlaže na vrlo reaktivni hidroksilni radikal ($OH^{\bullet}$) i azot dioksid radikal. Toksičnost NO potiče upravo usled mogućnosti stvaranja ovako potentnih oksidanasa (Slika 6). Reakcija $O_2^{\bullet -}$ i NO u in vitro uslovima tri puta je brža od reakcije dismutacije $O_2^{\bullet -}$, koju katalitzuje SOD (Ischiropoulos i Beckman, 2003), pa kada je prisutan u odgovarajućim koncentracijama NO može efikasno da se takmiči sa SOD za $O_2^{\bullet -}$. NO može direktno vršiti nitraciju i hidroksilaciju aromatičnih prstenova aminokiselina, i snažan je oksidans koji lako reaguje sa sulfhidrilnim grupama proteina, lipidima, DNK (Ischiropoulos i Beckman, 2003).

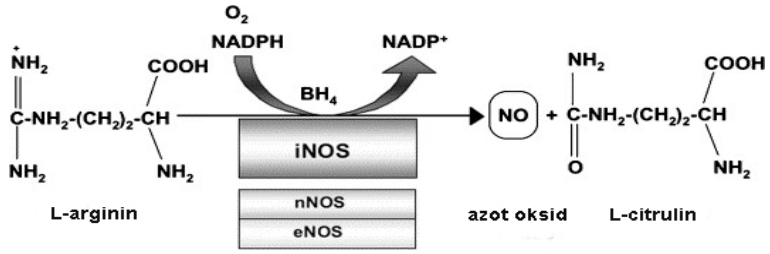
Ćelijski nivo azot oksida kontrolisan je sistemima koji sintetišu i metabolišu NO. Zahvaljujući svojoj visokoj sposobnosti difuzije, NO odlazi u okolna tkiva gde se deaktivira uz pomoć O_2-zavisnih mehanizama i uklanjanjem (eng. scavenging) od strane eritrocita (Santos i sar., 2012). NO uglavnom nastaje delovanjem enzima, ali postoje i neenzimski izvori NO koji najčešće podrazumevaju redukciju nitrita u uslovima povećene kiselosti sredine u tkivima kao posledica patoloških stanja (Luiking i sar., 2010). Poluživot NO je veoma kratak (<1s) usled njegove oksidacije oksihemoglobinom do nitrata i nitrita (NO_x), reakcijama sa molekulima koji se nalaze u ćeliji ili usled njegovog hvatanja. Zbog toga se kao indirektan pokazatelj nivoa NO u ćeliji mere njegovi metaboliti (NO_x) u plazmi ili unutar ćelije



Slika 6. Toksičnost NO (Novo i Parola, 2008).

1.3.1.1. Azot-oksid sintaze

Azot-oksid sintaze (eng. Nitric Oxide Synthase- NOS) su enzimi čijim se katalitičkim delovanjem aminokiselina L-arginin konvertuje u L-citrulin i oslobađa se NO i (Moncada i Higs, 1993) (Slika 7).



Slika 7. Sinteza NO.

U sisarskim ćelijama prisutne su tri različite vrste, neuronalna (nNOS, NOS1), inducibilna (iNOS, NOS2) i endotelna (eNOS, NOS3) NOS. Ovi enzimi su proizvodi različitih gena, poseduju različitu tkivnu lokalizaciju, regulaciju, katalitička svojstva i osetljivost na inhibitore. Neuronalna NOS je konstitutivno eksprimirana primarno u nervnim, ali je pronađena i u mišićnim ćelijama, dok se eNOS konstitutivno eksprimira u endotelnim ćelijama krvnih sudova.

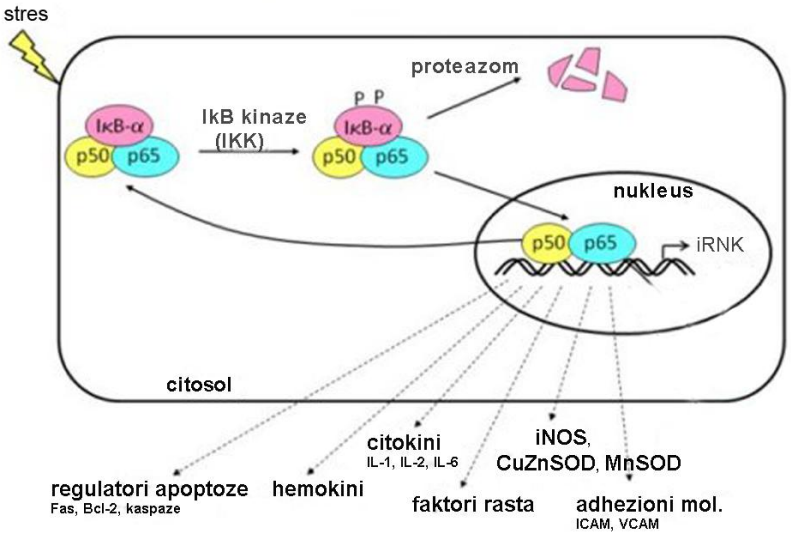
Inducibilna izoforma se sintetiše u mnogim ćelijama organizma i za razliku od nNOS i eNOS, iNOS vezuje kalmodulin (CaM) pri svim fizološkim koncentracijama kalcijuma (Ca^{2+}), tako da, za razliku od ostale dve izoforme, nije Ca^{2+}-zavisna već je ekspresija iNOS uglavnom regulisana na nivou transkripcije. Iako je zbog ovakvog načina regulacije, iNOS je potrebno nekoliko sati kako bi nagradila NO, količine koje se stvaraju delovanjem ove izoforme su velike (uM) i vezuju se za citotoksične efekte koje ispoljava ovaj molekul. Ekspresija nNOS i eNOS stimulisana je ulaskom Ca^{2+} u ćeliju i vezivanjem za CaM. Izlaganje stresorima pojačava oslobađanje glutamata (Moghaddam, 1993), ekscitatornog neurotransmitera koji, vezivanjem za N-metil-D-aspartat (NMDA) receptore na postsinaptičkoj membrani, dovodi do ulaska Ca^{2+} u ćeliju, aktiviranja nNOS enzima i povećanja produkcije NO (Samdani i sar., 1997; Boucher i sar., 1999; Bredt i Snyder,1989; Garthwaite i sar., 1989; Kornau i sar., 1997; Christopherson i sar., 1999). NO zauzvrat utiče na smanjenje daljeg oslobađanja glutamata, pa na taj način ima ulogu neuromodulatora. Aktivnost NOS enzima regulisana je različitim mehanizmima, uključujući fosforilaciju, nitrozilaciju, interakciju sa drugim proteinima, dostupnošću supstrata/kofaktora i promenama u transkripciji (Wang i Marsden, 1995; Alderton i sar., 2001; Daff, 2010).

Brojne studije pokazale su vezu između izlaganja stresorima i aktivacije NOS izoformi. Promene na nivou nNOS uočene su nakon izlaganja pacova akutnoj imobilizaciji, hroničnom stresu fizičkog ograničavanja (Leza i sar., 1998) kao i hroničnom blagom stresu koji je povezan sa nastankom ponašanja nalik depresiji kod pacova (Zhou i sar., 2011). iNOS je aktivirana u korteksu pacova usled akutnog stresa fizičkog ograničavanja (Madrigal i sar., 2001) dok je hronična imobilizacija u trajanju od 21 dana takođe dovela do aktiviranja iNOS izoforme (Olivenza i sar., 2000).

1.3.2. Uloga jedarnog faktora kapa B (NF- κB) u ćelijskoj signalizaciji

Jedarni faktor-κB (NF-κB) je redoks-osetljivi transkripcioni faktor koji se nalazi u gotovo svim tipovima ćelija. S obzirom na to da pripada kategoriji brzo-delujućih transkripcionih faktora u ćelijama se nalazi u neaktivnom (inaktiviranom) stanju, i njegova aktivacija ne zahteva *de-novo* sintezu proteina, u mogućnosti je da prvi odgovori na štetne stimuluse kojima je izložena ćelija pa je važan činilac u regulisanju ćelijskih odgovora. U neaktivnom stanju, NF-κB se nalazi u kompleksu sa proteinima IkB familije, što omogućava njegovo zadržavanje u citoplazmi (Baldwin, 1996). Aktivaciju NF-κB može stimulisati prooksidativni status ćelije, tj. ROS/RNS

(reaktivne azotne vrste) ili nizak nivo GSH (Schreck i sar., 1992; Mihm i sar., 1995), koji dovodi do fosforilacije IkB (Traenckner i sar., 1995; Mercurio i sar., 1997), što omogućava disocijaciju NF-κB iz kompleksa, degradaciju inhibitora i aktivaciju NF-κB što dalje vodi ka translokaciji p65 subjedinice (NF-κB-p65) u jedro (Baldwin, 1996, Karin, 1999) gde indukuje transkripciju gena koji poseduju κB region.



Slika 8. **Put aktivacije NF-κB transkripcionog faktora**. NF-κB aktivnost kontrolisana je putem negativne (prekinuta aktivacija usled povećane transkripcije IkB gena) i pozitivne (intenziviranje početkog inflamarornog signala) povratne sprege. (http://redox.fc.ul.pt/research.html)

Usled velikog broja različitih gena koji su aktivirani od strane NF-κB, ovaj protein može imati i protektivnu i štetnu ulogu. Zahvaljujući ulozi medijatora odgovora na stres (Pahl, 1999, Li i Stark, 2002) aktivacija NF-κB deluje antiapoptotski (Li i sar., 1999), ali poremećen NF-κB signalni put može dovesti do pojave apoptoze u određenim tkivima tokom patloških stanja, kao što je depresija (Koo i sar., 2010). Među brojnim genima koji su pod kontrolom ovog transkripcionog faktora nalazi se i iNOS (Xie i sar., 1994), CuZnSOD (Rojo i sar., 2004), MnSOD (Xu i sar., 2007) (Slika 8).

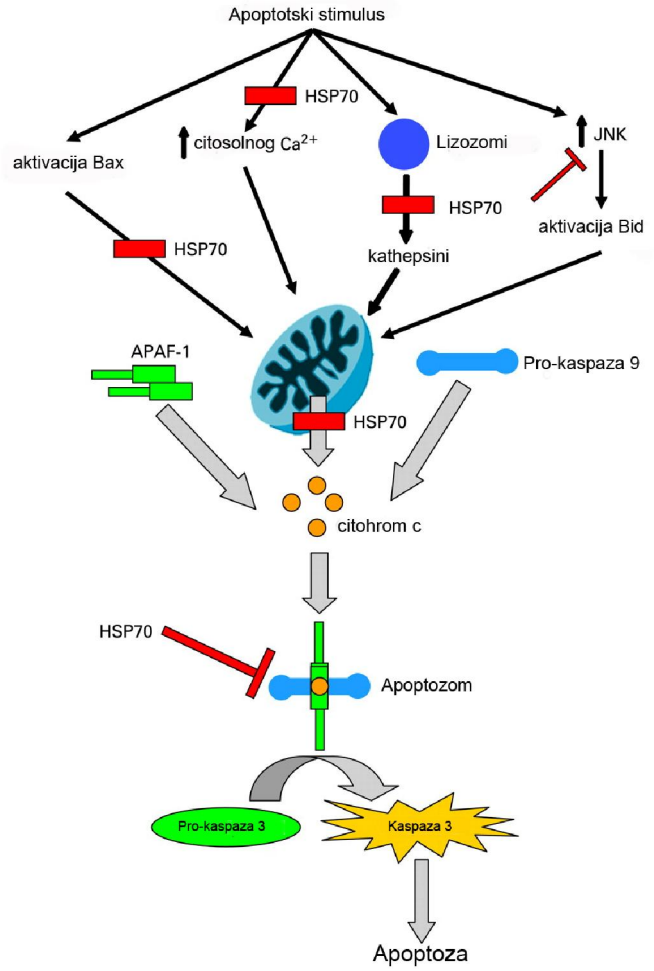
1.3.3. Zaštitna uloga proteina toplotnog stresa

Jedan od odgovora organizma na izlaganje stresnim događajima je i aktivacija proteina toplotnog stresa (Belay i Brown, 2006). Proteini toplotnog stresa (eng. Heat Shock Proteins- Hsps) prisutni su u većini ćelija, i na osnovu svoje veličine podeljeni su u familije. "Heat shock" protein70

(Hsp70) familija obuhvata konstitutivno eksprimirane Hsc70 i stresom indukovane Hsp70 (Hsp70i) forme proteina molekulske mase 70kDa. Hsc70 ima ulogu molekulskog šaperona i učestvuje u ispravnom sklapanju i unutarćelijskom transportu novosintetisanih proteina (Lindquist i Craig 1988; Becker i Craig 1994). Sa druge strane, pod stresnim uslovima, dolazi do aktiviranja inducibilne forme Hsp70 proteina koja je uključena u popravke oštećenja koja mogu nastati u ćeliji, pa stoga ima zaštitnu ulogu (Lindquist i Craig 1988; Georgopoulos i Welch 1993; Morimoto i sar., 1994; De Maio 1999).

Hsp70i je neophodan za zaštitu ćelija od različitih faktora koji dovode do strukturnih promena makromolekula (Parsell i Lindquist 1994), u šta spada i oksidativni stres (Calabrese i sar., 1996; Polla i sar., 1996), a stepen njegove indukcije zavisi od nivoa i dužine izlaganja stresoru (Kiang, 2004). Pored toga, povećana ekspresija Hsp70i proteina povećava otpornost na citotoksičnost izazvanu NO (Bellman i sar., 1996).

U skladu sa protektivnom ulogom, utvrđeno je da pokretanje Hsp70i odgovora štiti neurone od programirane ćelijske smrti (Kelly i sar., 2002; Arieli i sar., 2003; Belay i Brown 2003;) najverovatnije zahvaljujući zajedničkom delovanju sa Bcl-2 proteinom (Beere i Green 2001), inhibiciji aktivacije kaspaze-3 (Oleana i sar., 1998; Beere i sar., 2000; Beere i Green, 2001), ili oslobadjanja citohroma c iz mitohondrija (Mosser i sar., 2000). Takođe, Chan i saradnici (2004) su pokazali da se Hsp70 protein može vezivati za NF-κB/IκB kompleks što onemogućava aktivaciju NF-κB proteina i sprečava njegovu translokaciju u jedro, a što može dovesti do supresije aktivacije brojnih gena među kojima je i gen za iNOS (Guix i sar., 2005). Stoga pokretanje Hsp70i odgovora ne treba smatrati samo kao posledicu izlaganja stresorima, već kao način da se poveća otpornost na ćelijsku smrt (Fleshner i sar., 2004). Utvrđeno je da Hsp70i može inhibirati proapoptotske promene koje se dešavaju na nivou mitohondrija, kao i događaje koji prethode ili su posledica promena na nivou mitohondrija (Beere i sar., 2000; Li i sar., 2000; Steel i sar., 2004; Stankiewicz i sar., 2005) (slika 9), a takođe može delovati na više nivoa istovremeno.



Slika 9. Hsp70i može inhibirati komponente unutrašnjeg puta apoptoze na pre-mitohondrijalnom, mitohondrijalnom i post-mitohondrijalnom nivou (Dudeja i sar., 2009)

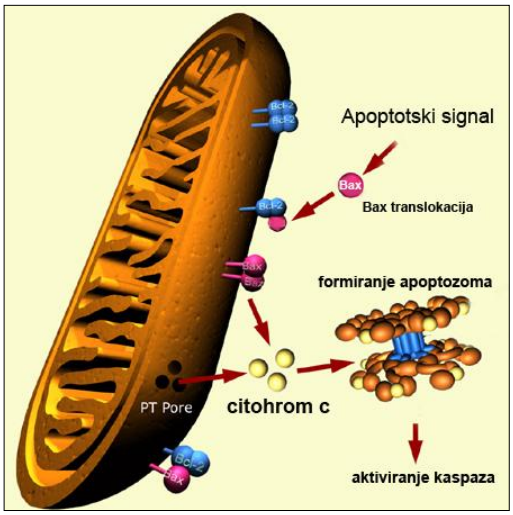
1.4. Proapoptotska signalizacija

Apoptoza ili "programirana ćelijska smrt" je jedan od mehanizama kojim ćelija aktivno uz utrošak energije i sintezu određenih proteina pokreće vlastitu smrt kao sastavni deo fizioloških procesa ili kao odgovor na određena patološka stanja (Kerr i sar., 1972). Postoje dva glavna puta koja ćeliju vode do apoptoze. Spoljašnji put vezan je za aktivaciju Fas i TNF receptora odgovarajućim ligandima, dok unutrašnji put podrazumeva oslobađanje apoptogenih faktora (npr. citohroma c) iz mitohondrija kao odgovor na ćelijski stres uzrokovan DNK oštećenjem, slobodnim radikalima, radijacijom, citokinima, hormonima. Bez obzira na to kojim putem se

preneo signal za apoptozu, on dovodi do aktiviranja proteolitičkih enzima- kaspaza, čiji su supstrati različiti funkcionalni i strukturni ćelijski proteini.

1.4.1. Unutrašnji put apoptoze - uloga Bax i Bcl-2 proteina

Unutrašnji put apoptoze regulisan je B-cell-lymphoma 2 (Bcl-2) porodicom proteina, koja se sastoji od antiapoptotskih (Bcl-2, Bcl-x_L) i proapoptotskih (Bax, Bak, Bid) članova (Shimizu i sar., 1999; Donovan i Cotter, 2004). Inicijacija ovog puta dovodi do oslobađanja pro-apoptotskih proteina iz mitohondrija koji će aktivirati citosolne kaspaze, a njegova regulacija je pod uticajem anti-apoptotskih molekula Bcl-2 familije proteina (Slika 10).



Slika 10. **Šema unutrašnjeg (mitohondrijskog) puta apoptoze.** Kao odgovor na oštećenje, pro-apoptotski članovi Bcl-2 familije (Bax, Bim) se aktiviraju i i odlaze do mitohondrija gde "neutrališu" anti-apoptotske proteine (Bcl-2,,Bcl-xL). Ovo rezultuje u narušavanju integriteta mitohondrijalne membrane i oslobađanju pro-apoptotskih molekula iz mitohondrijskog intermembranskog prostora.

Bax je solubilni protein koji se pretežno nalazi u citosolu, a tokom pokretanja apoptotskog puta prebacuje se i ugrađuje u mitohondrijsku membranu. Bilo da sam formira kanale u spoljašnjoj mitohondrijskoj membrani kroz koje citohrom c izlazi u citoplazmu ili da stupa u interakciju sa već postojećim kanalima, kao sto su prolazne mitohondrijske pore (PTP, eng. permeability transition pore), Bax dovodi do remećenja mitohondrijskog membranskog potencijala, što rezultuje u influksu fluida u matriks, bubrenja mitohondrije, pucanja membrane i oslobađanja pro-apoptotskih proteina iz matriksa u citosol.

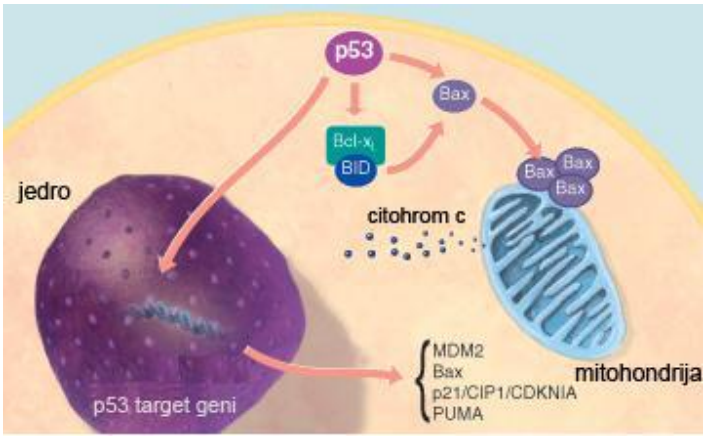
Bcl-2 protein je lokalizovan na spoljašnjoj mitohondrijskoj membrani i svoju protektivnu ulogu ispoljava sprečavanjem aktivacije pro-apoptotskih članova familije (Lindsay i sar., 2011). Bcl-2 inhibira translokaciju i promene Bax-a i drugih pro-apoptotskih članova kao i Bax/Bak oligomerizaciju u mitohondrijskoj membrani, i favorizuju zatvaranje PTP kanala.

Odnos pro-apoptotskih i anti-apoptotskih članova Bcl-2 familije (Bcl-2/Bax ili Bax/Bcl-2) u mitohondrijama određuju kakav će biti odgovor ćelije na "signale ćelijske smrti" (Desagher i Martinou, 2000; Hengartner, 2000). Povećana ekspresija anti-apoptotskog Bcl-2 znači i veći odnos Bcl-2/Bax što štiti ćeliju od apoptoze. Prelazak Bax iz citosola u mitohondrije i veći Bax/Bcl-2 odnos dovodi do oslobađanja citohroma c iz mitohondrija i pokretanje događaja koji mogu voditi u apoptozu (Hsu i sar., 1997). Unutrašnji put rezultira oslobađanjem citohroma c i Smart/Diablo iz mitohondrija. Citohrom c asocira sa Apaf-1 i prokaspazom-9 u kompleks koji se zove apoptosom. Dimerizacijom ovog kompleksa dolazi do aktivacije kaspaze-9 koja dalje aktivira efektorske kaspaze, uglavnom kaspazu-3.

1.4.2. Uloga p53 u proapoptotskoj signalizaciji

p53 (protein 53) je tumor supresorski protein koji u višećelijskim organizmima ima bitnu ulogu u regulaciji ćelijskog ciklusa. Zbog svoje uloge u prevenciji oštećenja često se opisuje kao "zaštitnik genoma" (Lane, 1992). Ključno za njegovu ulogu kao tumor supresora je sposobnost da aktivira apoptozu preko vise različitih puteva (Fridman i Lowe, 2003).

p53 posreduje u apoptozi preko transkripcione aktivacije pro-apoptotskih gena kao što su Bax, Noxa, Puma, kao i preko supresije anti-apoptotskih Bcl-2 i IAPS gena (detaljnije opisano u Johnstone i sar., 2002).



Slika 11. putevi p-53 zavisne apoptoze. p53 utiče na apoptozu putem aktivacije gena koji regulišu ćelijski ciklus, DNK reparaciju i apoptozu. Transkripciono-nezavisni put apoptoze može uključivati direktan efekat p53 koji ima na Bax, ili oslobađanje BID iz kompleksa sa Bcl-xL (http://www.rndsystems.com/cb_detail_objectname_SP04_p53.aspx).

Iako se najčešće pominje njegova uloga kao transkripcionog faktora, p53 može pokrenuti apoptozu nezavisnu od njegove transkripcione aktivnosti (Caelles i sar., 1994; Gao i Tsuchida 1999). Translokacija p53 iz citoplazme u mitohondrije (Chipuk i Green, 2003; Manfredi, 2003; Murphy i sar., 2004; Moll i sar., 2005) dovodi do promena permeabilnosti mitohondrijalne membrane i oslobađanja citohroma c, aktivacije kaspaze-3 (Marchenko i sar., 2000; Erster i sar., 2004) i konačno apoptoze (Liu i sar., 1996; Green i Reed, 1998). Takođe, p53 može da gradi komplekse sa članovima Bcl-2 familije, pa usled fizičke interakcije sa Bcl-xL i Bcl-2 onemogućava njihovo anti-apoptotsko delovanje (Mihara i sar., 2003; Chipuk i sar., 2004). Preko uticaja na oligomerizaciju Bak-a, pro-apoptotskog člana Bcl-2 familije, p53 takođe može posredovati u pokretanju apoptoze (Mihara i sar., 2003) (Slika 11). Ustanovljeno je da se translokacija p53 u mitohohondrije dešava veoma brzo nakon apoptotskog signala (Erster i sar., 2004) i prethodi translokaciji u jedro i transkripcionoj aktivnosti ovog proteina (Zhao i sar., 2005). Literaturni podaci pokazuju da je NO jedan od mogućih inicijatora prelaska p53 u mitohondrije (Nakaya i sar.,2000; Hofseth i sar.,2003).

1.5. Životinjski modeli depresije

Depresija predstavlja najčesći oblik psihijatrijskih poremećaja od koje, prema podacima Svetske zdravstvene organizacije, boluje oko 5-7% stanovništva. Istraživanja u ovoj oblasti su veoma intenzivna, međutim životinjski model koji idealno prezentuje većinu simptoma koje ispoljavaju depresivni pacijenti je veoma teško uspostaviti. Manjak samosvesnosti i samopoštovanja kao i suicidne misli neki su od simptoma depresije koji se ne mogu izazvati kod eksperimentalnih životinja, pa posmatranje i ocenjivanje tkz. endofenotipova (fizioloških, endokrinoloških, neuroanatomske promene) predstavlja jedan od načina za ispitivanje depresije u animalnim modelima.

Životinjski modeli depresije koriste se za ispitivanje neurobiologije depresije i mehanizma delovanja antidepresiva. Temelje se na izlaganju životinja stresu, na genetskoj manipulaciji i selektivnom uzgoju. Mnoge vrste, uglavnom glodara i sisara, koriste se u ove svrhe ali u pretkliničkim istraživanjima dugi niz godina primat imaju pacovi, obzirom da se, osim u genomu, sličnost sa ljudima javlja i u organizaciji mozga, neurotransmiterima, receptorima i signalim mehanizmima. Takođe, pacovi su jednostavni su za rukovanje i gajenje sa relativno malim troškovima, dok brzi reproduktivni ciklus omogućava dobijanje velikog broja

jedinki za kratko vreme. McKinney i Bunney (1969) su postavili, a Willner je dopunio (Willner, 1984) minimum kriterijuma koji životinjski model mora da zadovolji da bi bio korišćen u eksperimentalnim studijama. Na prvom mestu je fenomenološka sličnost između ponašanja životinjskog modela i simptoma u obolelog čoveka (eng. face validity); ove promene moraju da budu izazvane događajima koje izazivaju poremećaje kod ljudi (eng. etiological validity), kao i da se primenom antidepresanata koji se koriste u terapijama ove promene gube (eng. predictive validity). Takođe, modeli treba da izazivaju slične neurohemjske procese (eng. construct validity) i da budu reproducibilni od strane drugih istraživača.

Postoje različite vrste životinjskih modela depresije (Deussing, 2006). Pojava transgenih miševa smatrana je poslednjih godina kao veoma značajan pomak u istraživanju depresivnih poremećaja, obzirom da je omogućila proučavanje specifičnih funkcija jednog gena a time i otkrivanje potencijalnih kandidata za buduću farmakološku terapiju. Genetičkim modelima je ukazano da terapija može, između ostalog, biti usmerena na glukokortikoidni i CRH receptor (Grigoriadis, 2005; DeBattista i Belanoff, 2006), međutim kompleksne promene u koje se javljaju u depresiji zahtevaju unapređenje ovih životinjskih modela i istovremenu ciljanu deregulaciju više gena (Urani i sar., 2005). Tako npr. bilateralna olfaktorna bulbektomija dovodi do promena na nivou kore, hipokampusa i amigdala što rezultuje u promenama u ponašanju, endokrinom i imunskom sistemu kao i na nivou neurotransmitera, koje su zabeležene kod pacijenata koji boluju od depresije (Song i Leonard, 2005). Ipak, velika invazivnost kao i činjenica da se ove lezije ne spadaju u uzroke nastanka depresije kod ljudi veliki su nedostaci ovog animalnog modela.

Takođe, životinjski modeli depresije zasnivaju se i na izlaganju životinja akutnim ili hroničnim stresorima, što stvara promene u ponašanju koji podsećaju na simptome depresije kod čoveka a koje mogu biti vraćeni antidepresivima (Deussing, 2006). Naučena bespomoćnost (eng. learned helplessness) se zasniva na zapažanju da životinje kada su izložene ponovljenim neizbežnim šokovima (footshock) razvijaju kognitivni deficit, anhedoniju i smanjenu želju za bekstvom. Ovo ponašanje vezano je za činjenicu da izloženost stresnim životnim događajima koji se ne mogu kontrolisati čini da se ljudi osećaju kao da gube kontrolu, što konačno dovodi do depresivnog stanja. Međutim, iako je model delimično zasnovan na realnoj situaciji u kojoj se čovek može naći, veliki nedostatak je potreba za primenom jakih stresora na životinjama (pitanje etičnosti), kao i kratkotrajni efekat, obzirom da se životinje nekoliko dana nakon prestanka

neizbežnih šokove oporave (Deussing, 2006). Životinjski modeli zasnovani na izlaganju hroničnom stresu odlikuju se izuzetnom sposobnošću da istovremeno dovedu do niza promena vezanih za depresiju (izražen face validity). Oni su bazirani na kliničkim dokazima da su stresni životni događaji koji značajno povećavaju rizik od depresivnih epizoda uglavnom hroničnog karaktera (razvod, finansijski problemi) (Krishnan I Nestler, 2011). Model hroničnog blagog stresa uključuje primenu različitih kratkotrajnih fizičkih stresora tokom dužeg vremenskog perioda (1-7 nedelja), dok se modeli psihosocijalnog stresa kao što su socijalna izolacija ili odnos dominantnog-potčinjenog (eng. social defeat) zasnivaju na remećenju urođenog ponašanja životinja (Krishnan i Nestler, 2011).

1.5.1. Hronični stres socijalne izolacije kao životinjski model depresije

Socijalna izolacija je psihosocijalni stres koji je najzastupljeniji kod sisara i može predtsatvljati model depresije obzirom da je osećaj usamljenosti vezan je za određene tipove depresije kod ljudi (Costello i sar., 2000; Steptoe et al., 2004; Heinrich i sar., 2006). Anksioznost (Barrot i sar., 2005), povećana konzumacija alkohola (Ehlers i sar., 2007) diazepama i kokaina (Blanchard i sar., 2001), anhedonija izražena kroz smanjeni unos zaslađenih rastvora (Krishnan i Nestler, 2011) i promenjen odgovor na potkrepljenje ('rewarding stimuli') (Barrot i sar., 2005), smanjena higijena (eng. grooming, Spasojević i sar., 2007), smanjena mobilnost u testu prinudnog plivanja (eng. forsed swimmed test, Karim i Arslan, 2000) neke su od promena u ponašanju koje su zabeležene kod pacova izloženih hroničnoj socijalnoj izolaciji, a koje su nalik depresivnom ponašanju kod ljudi. Brojne studije su pokazale da se hroničnom primenom različitih klasa antidepresiva kod pacova gubi ponašanje slično depresiji, što sve zajedno ukazuje da je socijalna izolacija validan životinjski model depresije.

Utvrđeno je da hronična socijalna izolacija dovodi i do promena na nivou HHA ose, a depresivna stanja karakterišu se kako hiperfunkcijom (Holsboer i Ising, 2009), tako i hipofunkcijom ove ose (Krishnan i Nestler 2008). Takođe, hronična socijalna izolacija životinja dovodi do povećanja proinflamatornih citokina i stanja oksidativnog stresa koje se inače javljaju kod ljudi koji boluju od depresije (Pugh i sar., 1999; Schiepers i sar., 2005). Promene u sivoj masi i smanjena neurogeneza u hipokampusu koja je zabeležena u post-mortem studijama pacijenata obolelih od depresije (Wainwright i Galea, 2013) može biti izazvana kod pacova

hroničnom socijalnom izolacijom (Dong i sar., 2004; Westenbroek i sar, 2004; Stranahan i sar., 2006).

Dvadesetjednodnevna socijalna izolacija kod pacova Wistar soja prouzrokuje brojne fiziološke poremećaje i promene u ponašanju, uključujući smanjenje telesne težine, povećanu defekaciju i uriniranje, smanjeno uspravljanje (eng. vertical rears) i smanjenu higijenu (Spasojević i sar., 2007). Povećano oklevanje za silazak sa uzdignute platforme (eng. elevated platforme test, Spasojević i sar., 2007), ukazuje na anksiozno ponašanje izolovanih životinja, dok je smanjena mobilnost u testu prinudnog plivanja (Mohale i Chandewar, 2013) ukazala na odlike depresivnog ponašanja. Dodatno, naša grupa sprovela je neinvazivne testove ponašanja nakon dvadesetjednodnevne socijalne izolacije kako bi dodatno potvrdila validnost ovog stresa kao životnjskog modela za proučavanje depresije. Povećano zakopavanje klikera u istoimenom testu (eng. marble burying test) odlika je anksioznosti dok je smanjen unos saharoze (eng. sucrose preference test) karakteristično ponašanje nalik depresiji. Hroničan tretman niskim dozama anksiolitika diazepama (Spasojević i sar., 2007), antipsihotika olanzapina, kao i nekoliko antidepresiva u istraživanjima naše grupe dovelo je do vraćanja uočenih promena na fiziološke vrednosti (manuskript u pripremi). Na osnovu pomenutih pokazatelja može se reći da je socijalna izolacija pacova soja Wistar u trajanju od 21 dana, korišćena i u našem radu, validan životinjski model sistem depresije

2. CILj ISTRAŽIVANjA

Osnovni cilj disertacije bio je ispitivanje molekulskih mehanizama regulacije antioksidativnih enzima u prečeonoj kori, hipokampusu i jetri mužjaka pacova Wistar soja izloženih akutnim stresnim uslovima i hroničnoj socijalnoj izolaciji, koji predstavlja jedan od često primenjivanih modela ispitivanja depresije kod čoveka. Budući da je u našim rezultatima prethodno pokazano da hroničan stres socijalne izolacije u trajanju od 21 dan dovodi do poremećaja u regulaciji HHA ose usled delimične deregulacije mehanizma negativne povratne sprege glukokortikoidne signalizacije u višim centrima HHA ose tj. hipokampusu i prečeonoj kori, u okviru navedenog cilja, ispitana je i okarakterisana aktivnost HHA ose, merenjem koncentracije kortikosterona (CORT) u krvnom serumu životinja. Akutni stres imobilizacije ili izlaganja niskoj temperaturi u trajanju od 2 sata korišćeni su kao pokazatelji uobičajenog odgovora na stres, dok je kombinacija hroničnog i akutnog stresa služila kao potvrda maladaptivnog efekta hroničnog stresa socijalne izolacije. U našim prethodnim istraživanjima takođe su uočene promene proteinske ekspresije CuZnSOD u navedenim moždanim strukturama (Filipović i Pajović, 2009) kao i proteinske ekspresije CuZnSOD i MnSOD u jetri pacova (Filipović i sar., 2010). U skladu sa tim definisani su i sledeći neposredni zadaci istraživanja:

1. Ispitati uticaj pomenutih stresnih uslova na proteinsku ekspresiju MnSOD u citosolnoj i mitohondrijskoj frakciji, kao i aktivnost CuZnSOD i MnSOD u citosolnoj, mitohondrijskoj i jedarnoj frakciji prečeone kore, hipokampusa i jetre pacova.

2. Ispitati da li u pomenutim moždanim strukturama i jetri pacova izloženih datim stresnim uslovima dolazi do promene u nivou azot monoksida (NO) merenjem produkcije njegovih stabilnih metabolita nitrata/nitrita (NO_x^-), gde NO nitrozilacijom može izazvati oštećenja/inaktivacuju SOD, pa nitrozativni stres može predstavljati jedan od uzroka oksidativnog stresa.

3. Ispitati promene u ekspresiji inducibilne i neuronalne forme azot oksid sintaze (iNOS, nNOS) u citosolnoj frakciji prečeone kore i hipokampusa, koje su zaslužne za stvaranje azot monoksida u mozgu.

4. Ispitati da li dolazi do promene inducibilne forme proteina toplotnog stresa (Hsp70i) u citosolnoj frakciji pomenutih moždanih struktura, kao potencijalnog protektivnog molekula koji može uticati na tkivnu specifičnost odgovora na stres.

5. Ispitati promene na nivou p53, Bax, Bcl-2 i citohroma c, proteina uključenih u proapoptotsku signalizaciju i unutrašnji put apoptoze, kao krajnjeg efekta koji može imati stres na organizam.

3. MATERIJAL I METODE

3.1. Tretiranje eksperimentalnih životinja

U eksperimentima su korišćeni mužjaci pacova soja Wistar, starosti 3 meseca i težine 250-300 g. Sve životinje su odgajane u vivarijumu Instituta za nuklearne nauke ″Vinča″, Laboratorije za molekularnu biologiju i endokrinologiju u standardnim laboratorijskim uslovima (konstantna temperatura od 22 ± 2°C, vlažnost od 55 % i svetlosni režim 12 h svetlost/12 h mrak) i *ad libitum* režimu ishrane. Rad sa eksperimentalnim životinjama je izveden u skladu sa propisima Etičkog komiteta za upotrebu laboratorijskih životinja Instituta za nuklearne nauke ″Vinča″, koji prati smernice registrovanog " Srpskog udruženja za upotrebu životinja u istraživanju i obrazovanju", dozvola br 02/11.

3.2. Eksperimentalni uslovi

Eksperimentalne grupe životinja izlagane su sledećim eksperimantalnim uslovima:

Akutnom stresu u trajanju od 2 sata pri čemu su korišćene dve vrste akutnog stresa:

- **Imobilizacija (IM)**, koja predstavlja kombinaciju fizičkog i psihološkog stresa. Životinja se u ležećem položaju fiksira za metalno postolje vezivanjem ekstremiteta za metalne nosače, dok se glava fiksira u vratnom regionu provlačenjem kroz metalni prsten (Kvetnansky i Mikulaj, 1970);
- **Niska temperatura (Hlad),** odnosno izlaganje životinja temperaturi od 4°C, što predstavlja fizički stres umerenog intenziteta;

Hroničnom stresu u trajanju od 21 dana:

- **Izolacija (IZ),** gde držanje po 1 životinje u kavezu, koji su međusobno odvojeni pregradama kako bi se onemogućio vizuelni kontakt, predstavljajući psihosocijalni stres (Garzón i Del Rio, 1981)

Kombinovanom stresu koji predstavlja kombinaciju prethodno opisanih stresova:

- **Izolacija i imobilizacija (IZ+IM)**
- **Izolacija i niska temperatura (IZ+Hlad)**

Kontrolna grupa životinja odgajana je u standardnim uslovima- 4 životinje u kavezu.

3.3. Žrtvovanje životinja i priprema seruma i tkiva

Životinje su žrtvovane dekapitacijom pomoću giljotine (Harvard Apparatus, Holliston, MA, SAD). Nakon izolacije celih mozgova, izolovani su hipokampus i prečeona kora, koji su nakon zamrzavanja u tečnom azotu čuvani na -70°C do početka eksperimnta, dok je izolovanje unutarćelijskih frakcija jetre rađeno na svežim uzorcima. U toku žrtvovanja u cilju merenja koncentracije kortikosterona (CORT) u serumu uzimana je krv pojedinačnih životinja. Puna krv pacova dobijena iskrvavljenjem sakupljana je u polipropilenske epruvete, ostavljena da koaguliše na sobnoj temperaturi 30 min, nakon čega je centrifugirana 15 min na 3000 rpm (Sorvall GLC-3) a dobijeni serumi su čuvani na -20°C do ELISA eseja.

3.4. Određivanje koncentracije kortikosterona u serumu

Nivo CORT-a u serumu određen je ELISA testom, uz primenu OCTEIA Corticosterone ELISA kit (ref AC-14F1; Immunodiagnostics Systems IDS, UK). Standardi i uzorci kontrolnih životinja i životinja izloženih stresu su u duplikatu nanošeni na ploču i inkubirani preko noći na 2-8 °C sa antitelom koje je konjugovano sa HRP-om. Ploča je zatim ispirana, dodavan je hromogeni supstrat TMB (tetrametilbenzidin) koji dovodi do razvijanja boje. Enzimska reakcija je prekidana dodavanjem STOP rastvora (0.5 M HCl). Apsorbanca je očitavana na 450 i 650 nm (korekciona OD). Merenje je vršeno na ELISA čitaču (WALLAC 1420-Victor^2 Multilabel Counter, LKB). Koncentracija CORT u uzorcima očitava se sa standardne krive, pri čemu su vrednosti CORT -a izražene u ng/ml. Intra- i interesejske varijacije su bile ispod 7%.

3.5. Priprema jedarne, mitohondrijske i citosolne frakcije homogenata prečeone kore, hipokampusa i jetre

Hipokampus i prečeona kora su izolovani disekcijom iz celog mozga. Tkivo je homogenizovano u staklenom Potter-Elvehjem homogenizeru sa teflonskim tučkom u dve zapremine hladnog (4ºC) pufera I [0.25 M saharoza, 15 mM TRIS-HCl (pH 7.9), 16 mM KCl, 15 mM NaCl, 5 mM etilen-diamin tetrasirćetna kiselina (EDTA), 1 mM etilenglikol tetrasirćetna kiselina (EGTA), 1 mM ditiotreitol (DTT), 0.15 mM spermin i 0.15 mM spermidin sa dodatkom proteaznih inhibitora: 0.1 mM fenilmetilsulfonil fluorid (PMSF), 2 µg/ml leupeptina, 5 µg/ml aprotinina, 5 µg/ml antipaina] a zatim centrifugirani na 2.000 × *g,* 4°C u trajanju od 10 min.

Supernatant je dalje centrifugiran na 15.000 × *g*, 4°C, 20 min, i dobijeni talog od koga će biti dobijene mitohondrije resuspendovan je u homogenizacionom puferu I, centrifugiran na 15.000 × *g*, 4°C, 20 min i u talog je dodato 250 µl pufera za liziranje mitohondrija [50 mmol/l TRIS-HCl (pH 7.4), 5% glicerol, 1 mmol/l EDTA, 5 mmol/l DTT, sa dodatkom pomenutih proteaznih inhibitora i 0.05% Triton X-100]. Nakon inkubiranja na ledu 30 min na šejkeru sa povremenim vrtloženjem, uzorci su finalno centrifugirani (60 min, 14000 × *g*, 4°C) i dobijeni supernatant predstavlja mitohondrijsku frakciju. "15.000 × *g*" supernatant je dalje centrifugiran na 100.000 × *g* u trajanju od 60 min, prilikom čega se dobija čista citosolna frakcija.

Talog dobijen centrifugiranjem homogenata ("2.000 × *g*" talog) je resuspendovan u 4 zapremine hladnog (4ºC) pufera II [10mM HEPES, pH 7.9, 1.5mM MgCl2, 10mM KCl sa dodatkom proteaznih inhibitora] i podvrgnut centrifugiranju na 4000 × *g*, 4°C, u trajanju od 10 min. Dobijeni talog je ispran dva puta puferom II, što je praćeno centrifugiranjem na malim brzinama (4.000 × *g*, 4°C, 10 min). Finalno, talog je resuspendovan u 1 vol (w/v) pufera III [10mM HEPES, pH 7.9, 0.75mM MgCl2, 0.5M KCl, 0.5mM EDTA, 12.5% glicerol sa proteaznim inhibitorima] i inkubiran na ledu 30 min na mešalici sa povremenim vrtloženjem. Supernatant koji predstavlja jedarni ekstrakt dobijen je finalnim centrifugiranjem (30 min, 14000 × *g*, 4°C).

U slučaju jetre, nakon perfundovanja *in situ* hladnim (4°C) puferom A [10 mM Tris-HCl pH 7.5, 1 mM (EDTA), 250 mM saharoza, 10 mM Na_2MoO_4, 3 mM $MgCl_2$], odgovarajući režanj je isečen i homogenizovan u 4 zapremine (w/v) pufera sa dodatkom proteaznih inhibitora [20 mM Tris-HCl pH 7.0, 1 mM (EDTA), 10 % glycerol, 50 mM NaCl, 2mM DTT, 20 mM Na-fosfatni pufer] i centrifugiran na 3.000 × *g*, 15 min, 4 °C. Za dobijanje citosolne i mitohondrijske frakcije, "3.000 × *g*" supernatant je dalje centrifugiran 25 min, na 11.000 × *g*, 4°C na SS-34 Sorvall centrifugi. Dobijeni talog je ispran u homogenizacionom puferu A, dodatno centrifugiran 20 min na 12.000 × *g*, 4 °C i resuspendovan u 250 µl pufera za liziranje mitohondrija. Nakon inkubacije od 30 min na 4°C i centrifugiranja 20 min na 15.000 × *g* dobijena je čista frakcija mitohondrija. "11.000 × *g*" supernatant je dodatno centrifugirana na 100.000 × *g* na 4 °C u trajanju od 60 min na Beckman L8-M Ultracentrifuge Ti50 čime je dobijena čista frakcija citosola.

3.5.1. Priprema prečišćene jedarne frakcije ćelija jetre

Za dobijanje prečišćene jedarne frakcije talogu dobijenom centrifugiranjem homogenata ("3.000 × *g*" talog,) dodat je pufer za izolovanje jedara [5 mM Tris-HCl pH 7.2, 2.2 M saharoza, 5 mM $CaCl_2$], i nakon resuspendovanja naliven je u kivete u kojima je prethodno dodata 2.2 M saharoza. Tako naneti uzorci centrifugirani su 60 min na 24.000 rpm (Beckman SW 27 rotor). Dobijeni beli talog je ispiran tri puta u puferu za pranje jedara [5 mM Tris-HCl pH 7.9, 1 mM $MgCl_2$] što je praćeno centrifugiranjem na malim brzinama (10 min, 4.000 × *g*, 4°C). Opranom talogu je dodata jedna zapremina pufera za liziranje jedara [10 mM Tris-HCl pH 8.0, 0.3 M NaCl, 1 mM β-merkaptoetanol, 1 mM EDTA, 0.5 mM $MgCl_2$], suspenzija je provučena pet puta kroz špric (25G) i sat vremena ostavljena na ledu na mešalici, uz povremeno vrtloženje. Suspenzija je nakon toga centrifugirana 30 min (14.0000 × *g*, 4°C) a dobijeni supernatant predstavlja prečišćenu jedarnu frakciju jetre.

3.6. Određivanje koncentracije proteina

Koncentracija proteina u uzorcima je određivana po metodi Lowry i saradnika (1951), koja se koristi kada je očekivana koncentracija proteina u uzorku u opsegu od 1 mg/ml do 10 mg/ml. Standard za određivanje koncentracije proteina je goveđi serum albumin (BSA), od koga se priprema štok koncentracije 10 mg/ml i dalje serija razblaženja za standardnu krivu raspona finalnih koncentracija 1-10 mg/ml.

U metodi su korišćeni su sledeći reagensi:

Reagens A: 2% Na_2CO_3 +0.16% Na-K-tartarat + 1% SDS

Reagens B: 4% $CuSO_4$ x 5 H_2O

Reagens C: Reagens A: Reagens B (99:1), pravi se neposredno pre upotrebe

Reagens D: Folin & Ciocalteu's phenol reagens se razblažuje u destilovanoj vodi (dH_2O) u odnosu 1:1.

Slepa proba (blank) je sadržala 900 µl ddH_2O, bez proteina, dok je u ostale epruvete dodavano po 10 µl rastvora BSA/uzorka i dH_2O do finalne zapremine od 900 µl. U epruvete je potom dodavano po 100 µl 1M NaOH, dobro promešano i smeša je inkubirana 10 min na sobnoj temperaturi (RT). Nakon toga je dodavano 2 ml rastvora C, ponovo promešano i ostavljeno da stoji 15 min na RT. Po isteku tog vremena, dodavano je 0.3 ml reagensa D, promešano i

inkubirano 45 min na RT, do razvijanja boje. Optička gustina (OD) je merena na talasnoj dužini od 750 nm na spektrofotometru S-30 Boeco. Na osnovu OD_{750nm} očitanih vrednosti za BSA je konstruisana prava $OD750_{nm}$= a x cP (μg/mL) + b. Sa prave je određivana koncentracija proteina u uzorku na osnovu pročitane OD_{750nm} za uzorak.

3.7. Nivo metabolita azot oksida (NOx^-): nitrita i nitrata (NO_2^- i NO_3^-) u citosolnoj frakciji prečeone kore, hipokampusa i jetre pacova

Merenje nivoa NO je vršeno na osnovu merenja nitrita (NO_2^-) u citosolnoj frakciji prečeone kore, hipokampusa i jetre, gde su nitrati (NO_3^-) prethodno prevedeni u NO_2^- u prisustvu kadmijuma (Cd) (Cortas i Wakid, 1990). Nivo NO_2^- je određen kolorimetrijskim esejem uz korišćenje Griess-ovog reagensa [1% sulfonamid, 2.5% H_3PO_4, 0.1% N-(1-naftil) etilenediamine dihidrohlorid] (Navarro-Gonzálvez i sar., 1998), pri čemu nitriti reaguju sa sulfanilamidom u kiselom rastvoru naftil etilenediamina formirajući bojeno azo-jedinjenje. Reakcija je otpočinjala dodavanjem 100 μl uzorka (u kome su prethodno nitrati uz pomoć Cd prevedini u nitrite) u 100 μl reakcione smeše koja je sadržala Reagens A [0.1% N-(1-naftil) etilenediamin dihidrohlorid u dejonizovanoj vodi] i Reagens B [1% sulfonamid u 2.5% H_3PO_4] u odnosu 1:1. OD na 550 nm je merena na ELISA čitaču (WALLAC 1420-Victor^2 Multilabel Counter, LKB), a koncentracija nitrita se očitava sa standardne krive koja je dobijena iz serije poznatih koncentracija $NaNO_2$ u rasponu 0.5-10 μm. Finalna koncentarcija nitrita izražena je kao nmol/mg proteina.

3.8. SDS-poliakrilamid gel elektroforeza (SDS-PAGE)

Koncentracije proteina u uzorcima su svedene na istu koncentraciju (mg/ml) pomoću pufera za izolaciju. Neposredno pred upotrebu alikvoti su pomešani sa puferom za pripremu uzoraka proteina za SDS-PAGE (62.5 mM TRIS-Hcl, pH 6.8, 10% glicerol, 2% SDS, 0.002% bromfenol plavo, 5% β-merkaptoetanol) u odnosu 1:2/1:3 (mitohondrijska i jedarna frakcija/citosolna frakcija). Posle kuvanja na 100 ºC u trajanju od 1 min, uzorci su ohlađeni i nanošeni na SDS-poliakrilamid gel. Proteini su razdvajani na osnovu molekulske mase korišćenjem denaturišuće SDS-poliakrilamid gel elektroforeze. Elektroforeza je rađena na sistemu Mini Protean II (Bio Rad). U zavisnosti od molekulske mase koju imaju, proteini su razdvajani na 8%, 10% i 12% poliakrilamidnim denaturišućim gelovima u puferu za elektroforezu koji sadrži 0.25 M TRIS bazu, 0.192 M glicin, 0.1 % SDS. Na gel je nanošeno oko 60-80 μg proteina po uzorku.

Elektroforeza je trajala oko 90 min pri konstantnom naponu od 100V. Posle elektroforeze gelovi su korišćeni za Western blot analizu.

3.9. ″Western blot″ analiza

Western blot predstavlja prenos proteina sa gela na polivinilidendifluorid (PVDF) membranu (BioRad, Corporation, SAD) a zatim, uz pomoć primarnih i sekundarnih antitela spregnutih sa enzimom, detekciju specifičnih proteina. Nakon završene elektroforeze, gelovi su potopljeni u transfer pufer [20% metanol, 0.025 M TRIS HCl, 0.192 M glicin, pH 8.3]. PVDF membrana (veličine pora 0.45 μm) prvo je aktivirana 1 min u 100 % metanolu, zatim potopljena u transfer pufer. Nakon 5-10 min, formiran je sendvič koji se sastojao, redom, od gel blot papira Whatman GB003 (Whatman, Inc, UK), polakrilamidnog gela, PVDF membrane i papira Whatman (svi natopljeni transfer puferom). Ovako napravljen sendvič postavljen je u aparat za vlažni transfer (Trans-Blott Cell, Bio Rad) i potopljen u pufer za transfer. Prenos proteina sa gelova na membrane odvija se pri konstantnoj struji od 13 mA, 4°C, 120 min.

Nakon transfera, membrane su inkubirane 2 h u puferu za blokiranje, 5 % nemasnom mleku u prahu ili 5% BSA rastvorenom u TBS-T puferu [50 mM TRIS HCl pH 7.4, 150 mM NaCl, 0.1 % Tween 20], na sobnoj temperaturi uz blago mešanje. Nakon blokiranja membrana je presecana na osnovu Page Ruler^{TM}Plus Prestained Protein Ladder markera opsega 10-250 kDa (Fermentas, St. Leon-Rot, Germany) na određenim molekulskim težinama i inkubirana preko noći sa primarnim antitelima na 4°C (Tabela 1).

Nakon toga membrane su ispirane 3 puta po 15 min TBS-T puferom i inkubirane 2 h uz mešanje sa sekundarnim anti-mišjim odnosno anti-zečijim antitelom konjugovanih sa HRP. Nakon ponovnog ispiranja u TBS-T puferu nanošen je supstrat za hemiluminiscenciju (ECL eng. *enhanced chemiluminescence*). Membrane su inkubirane u ECL supstratu 5 min i detekcija traka je vršena na filmovima (ORTHO CP-GU X-ray film, AGFA, Belgium) pri čemu je dužina ekspozicije varirala od 1 do 10 min u zavisnosti od proteina. Intezitet signala na filmu je odgovorao količini specifičnog proteina u analiziranim uzorcima. Kvantifikacija signala je vršena u programu za analizu slike ImageJ.

Tabela 1. Podaci o primarnim antitelima korišćenim u westrern blot metodi

Antigen	Molekulska masa (kDa)	Poreklo	Proizvođač	Razblaženje
MnSOD	25	zečje	Stressgene	1:4000
NF-κB	65	zečje	Santa Cruz Biotechology	1:1000
iNOS	130	zečje	Santa Cruz Biotechology	1:500
nNOS	150	zečje	Santa Cruz Biotechology	1:500
β-aktin	42	mišje/zečje	Sigma/Santa Cruz Biotechnology	1:1000
Bax	23	zečje	Santa Cruz Biotechology	1:1000
Bcl-2	26	zečje	Santa Cruz Biotechology	1:1000
Hsp70i	72	zečje	Santa Cruz Biotechology	1:1000
p53	53	zečje	Stressgene	1:1000
citohrom c	15	mišje	Santa Cruz Biotechology	1:1000

3.10. Određivanje aktivnosti enzima SOD

Za određivanje aktivnost superoksid dismutaze korišćen je Biorex BXC0531 kit (Biorex Diagnostics Ltd., UK), koji se zasniva na stvaranju superoksid radikala preko ksantina i ksantin oksidaze. Superoksid radikal dalje reaguje sa 2-(4-jodofenil)-3-(4-nitrofenol)-5-feniltrazolijum hlorid (I.N.T.) i formira formazan crvenu boju. Aktivnost superoksid dismutaze se određuje na osnovu stepena inhibicije ove reakcije. Jedna jedinica SOD je ona koja izaziva 50 % inhibicije stope smanjenja INT u uslovima eseja. Prvo je merena totalna SOD aktivnost u uzorcima. Dodavanjem 8 mM KCN tokom pripremanja uzoraka za drugu turu merenja (uzorak:KCN=1:1) blokirana je aktivnost CuZnSOD, pa sva izmerena aktivnost potiče od MnSOD. Oduzimanjem vrednosti totalne SOD i MnSOD dobija se aktivnost CuZnSOD u uzorcima. Koncentracija

proteina u uzorcima je podešena tako da zadovoljava linearnost reakcije. Merenje je vršeno na RX Daytona™ aparatu.

3.11. Statistička obrada rezultata

Kvantifikacija rezultata dobijenih Western blotom je izvršena denzitometrijskom analizom signala u programu za analizu slike ImageJ (Image Processing and Analysis in Java). Svi eksperimenti su ponovljeni šest puta, svaki put sa novom grupom kontrolnih jedinki i životinja izloženih stresorima. Izmerene vrednosti za optičku gustinu (OD) signala su korigovane u odnosu na pozadinu i izražene su u arbitrarnim jedinicama (count). Za Western blot sve vrednosti su izražene u odnosu na optički gustinu trake koja odgovara β-aktinu sa istog blota, a rezultati su potom predstavljeni kao procenat od odgovarajuće kontrole.

Svi rezultati su izraženi kao srednja vrednost ± standardna greška (SEM). Obrada rezultata je urađena u softverskom programu STATISTICA 7.0 (StatSoft, Inc. SAD). Za procenu statističke značajnosti je korišćena dvofaktorijalna ANOVA (Two-way ANOVA) [faktori su akutni stres (nivoi: bez stresa, IM and Hlad) ili hronični stres (nivoi: bez stresa i IZ stres], praćena *Duncan's post hoc* testom. Za sve testove pretpostavljena statistička značajnost je $p< 0.05$.

4. REZULTATI

4.1. Promene koncentracije CORT-a u krvnom serumu akutno, hronično i kombinovano stresiranih pacova

Glukokortikoidni hormon CORT se ubraja u najvažniji hormon koji se pojačano sintetiše kao odgovor organizma na stresne uslove. On se sintetiše u srži nadbubrežne žlezde i ima značajnu ulogu u ponovnom uspostavljanju homeostaze. Njegova sinteza se nalazi pod regulacijom HHA ose, pa je stoga dobar pokazatelj njene funkcije. Analiza rezultata metodom dvofaktorijalne ANOVA-e pokazala je značajan efekat akutnog ($F_{2.30}$=80.63, $p < 0.001$), hroničnog ($F_{1.30}$=24.36, $p < 0.001$) i interakcije akutnoi × hronični stres ($F_{2.30}$=5.12, $p < 0.001$) na koncentarciju CORT-a u krvom serumu pacova (Tabela 2).

Tabela 2. Nivoi CORT-a (ng/ml) u serumu kontrolnih životinja i životinja izloženih akutnom stresu imobilizacije (IM) ili niske temperature (Hlad), hroničnoj socijalnoj izolaciji (IZ) kao i njihovim kombinacijama (IZ+IM, IZ+Hlad). U poređenju sa kontrolnom grupom, Duncan post hoc test pokazao je značajno povećanje CORT-a kod obe grupe akutno stresiranih životinja ($^{***}p < 0.001$, $^{**}p < 0.01$) kao i kod IZ+IM grupe ($^{***}p < 0.001$). Značajno niži nivo CORT-a zabeležen je kod životinja izloženih kombinovanim stresnim uslovima u poređenju sa životinjama iz odgovarajuće akutno stresirane grupe ($^{\#\#}p < 0.01$)

	AKUTNI STRES	IZOLACIJA
KONTROLA	142.905±14.16	123.39±10.89
IMOBILIZACIJA	648.89±166.78***	477.98±74.03$^{***\ \#\#\ \wedge\wedge\wedge}$
NISKA TEMPERATURA	342.112±112.53**	86.41±37.68$^{\#\#}$

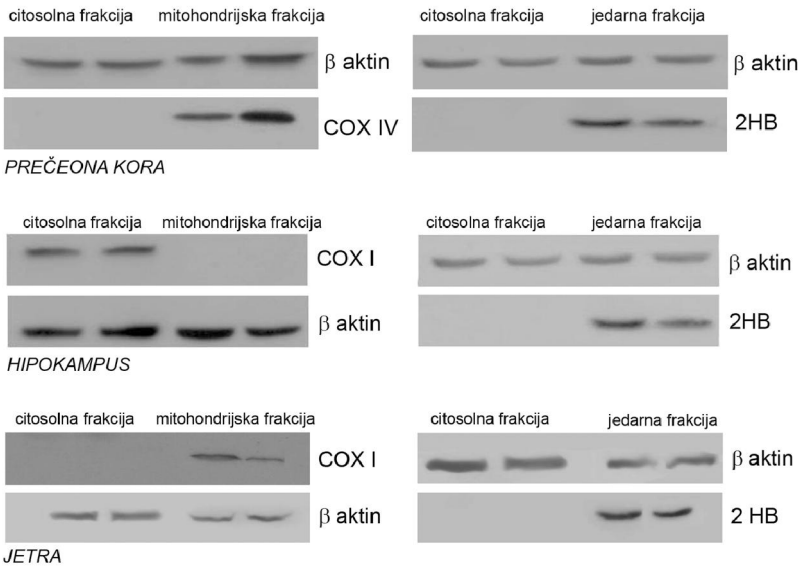
Akutno stresirane životinje su pokazale značajno povećanje CORT-a u odnosu na kontrolnu grupu životinja. Na osnovu četvorostrukog povećanja nivoa CORT-a u serumu, može se zaključiti da stres imobilizacije predstavlja izrazito jak stres ($^{***}p < 0.001$), jači od akutnog stresa niske temperature, čija je vrednosti CORT-a bila dva puta veća u odnosu na kontrolu ($^{**}p < 0.01$).

Nasuprot akutno stresiranim životinjama, kod životinja izlaganih hroničnom stresu socijalne izolacije u trajanju od 21 dana nije došlo do promena u koncentraciji CORT-a u odnosu na kontrolnu grupu ($p > 0.05$), što ukazuje da je ovaj stres blagog intenziteta.

Nakon izlaganja hronično stresiranih životinja dodatnom akutnom stresu imobilizacije (IZ+IM) nivo serumskog CORT bio je viši u poređenju sa kontrolnom grupom ($^{***}p < 0.001$), ali niži u poređenju sa životinjama izlaganih samo akutnom stresu IM ($^{\#\#}p < 0.01$). Dodatni akutni stres niske temperature kod hronično stresiranih životinja (IZ+Hlad) nije značajno promenio nivo CORT-a u odnosu na kontrolnu grupu ($p > 0.05$), dok je značajan pad zabeležen u odnosu na sam akutni stres niske temperature ($^{\#\#}p < 0.01$).

4.2. Čistoća unutarćelijskih frakcija homogenata prečeone kore, hipokampusa i jetre

Stepen prečišćenosti citosolne, jedarne i mitohondrijske frakcije su proverene metodom Western blota korišćenjem antitela na subjedinice I ili IV citohrom c oksidaze (COX I, Molecular Probe®, Life Technologies ili COX IV, Cell Signalling) i histona (2HB) (Cell Signalling).



Slika 12. Čistoća unutraćelijskih frakcija prečeone kore, hipokampusa i jetre

Pomenuta antitela su specifično detektovala proteine u mitohondrijskoj i jedarnoj frakciji prečeone kore, hipokampusa i jetre, a njihovo odsustvo u citosolnoj frakciji potvrđuju ispravnost

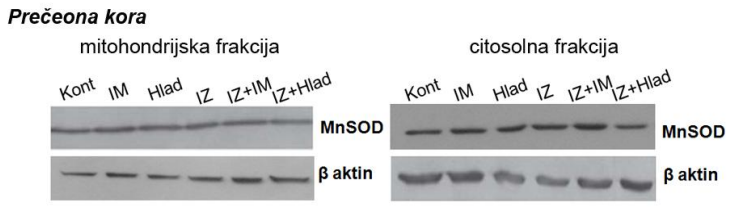
protokola za izdvajanje ćelijskih frakcija. Detektovanje traka koja odgovaraju β aktinu u sva tri kompartmana potvrđuju konzistentnost nanošenja proteina (Slika 12).

4.3. Uticaj stresa na proteinski nivo MnSOD u ćelijskim frakcijama prečeone kore i hipokampusa pacova

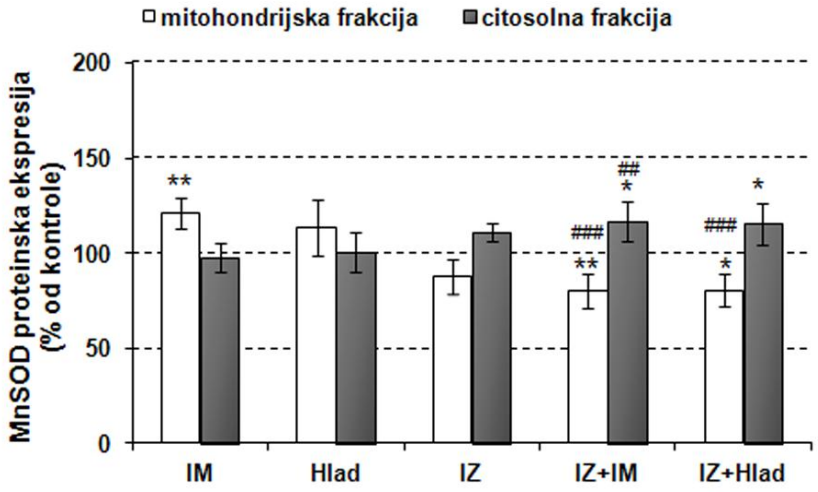
4.3.1. Kombinovani stres doveo je do opadanja mitohondrijskog i porasta citosolnog nivoa MnSOD u prečeonoj kori

Dvofaktorijalna ANOVA analiza ukazala je na značajan efekat hroničnog ($F_{1.30}$=87.01, $p < 0.001$) i interakcije akutni × hronični stres ($F_{2.30}$=7.64, $p < 0.01$) na nivo MnSOD proteina u mitohondrijskoj frakciji prečeone kore. Post-hoc test pokazao je povećanje nivoa MnSOD proteina nakon akutnog stresa IM ($^{**}p < 0.01$) (Slika 13) u odnosu na kontrolnu grupu, za razliku od akutnog izlaganja niskoj temeperaturi koja nije dovela do promena nivoa ovog proteina. Hroničan stres socijalne IZ nije izazvao promene u nivou mitohondrijskog MnSOD ($p > 0.05$). Ipak, dodatni akutni stres IM ili Hlad nakom hronične socijalne IZ doveo je do značajnog smanjenja mitohondrijske MnSOD i to u poređenju sa kontrolnom grupom ($^{**}p < 0.01$, $^{*}p < 0.05$, redom), kao i grupom izloženom akutnim stresorima IM ili Hlad ($^{###}p < 0.001$).

U citosolnoj frakciji prečeone kore dvofaktorijalna ANOVA analiza ukazala je na postojanje efekta hroničnog stresa ($F_{1.30}$=27.89, $p < 0.001$) na nivo MnSOD proteina. Duncan's post-hoc test nije pokazao promene citosolnog nivoa MnSOD usled izlaganja akutnim stresorima ili hroničnoj socijalnoj IZ ($p > 0.05$) u odnosu na kontrolnu grupu životinja. Ipak, dodatno izlaganje akutnoj IM ili niskoj temperaturi životinja koje su bile podvrgnute socijalnoj IZ dovelo je do značajnog povećanja citosolnog nivoa MnSOD proteina ($^{*}p < 0.05$). Takođe, povećani nivo MnSOD je zabeležen u grupi IZ+IM u odnosu na akutni stres IM ($^{##}p < 0.01$, Slika 13).



Imunoreaktivna traka na ~25 kDa koja odgovara molekulskoj masi subjedinice MnSOD-a proteina detektovana je u mitohondrijskoj i citosolnoj frakciji prečeone kore

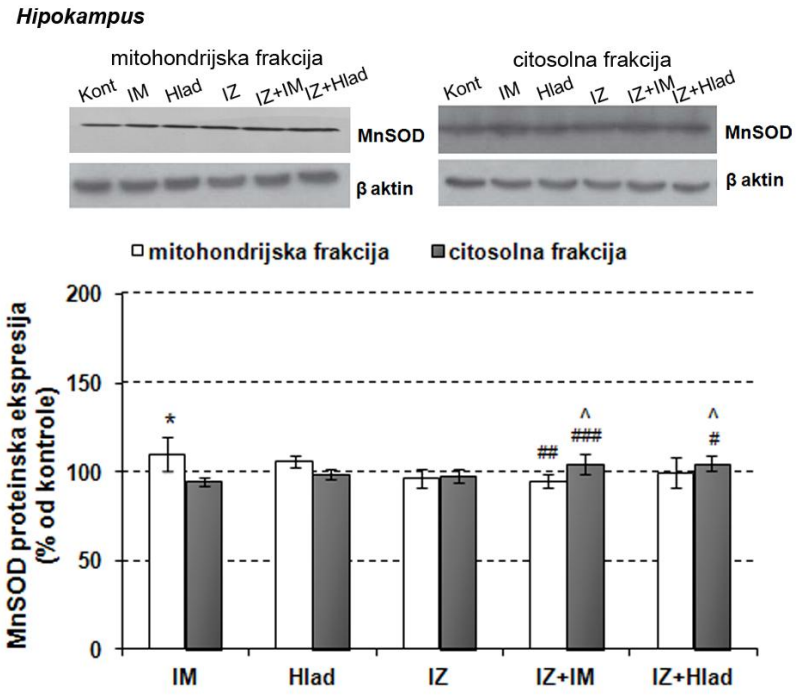


Slika 13. **Relativne promene u nivou MnSOD proteina u mitohondrijskoj i citosolnoj frakciji prečeone kore nakon akutnog stresora imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole. Simboli ukazuju na značajne razlike između: životinja izlaganih stresorima i kontrolnih ($^{*}p < 0.05$; $^{**}p < 0.01$); kombinovanog i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$).

4.3.2. Stres ne dovodi do promene proteinskog nivoa MnSOD-a u hipokampusu

U mitohondrijskoj frakciji hipokampusa dvofaktorijalna ANOVA pokazala je značajan efekat hroničnog stresa na proteinski nivo MnSOD ($F_{1.30}=17.66$, $p < 0.001$). Značajno povećanje mitohondrijskog proteina, u poređenju sa kontrolnom grupom, post-hoc test pokazao je nakon izlaganja akutnom stresu IM ($^{*}p < 0.05$) (Slika 14). Akutno izlaganje niskoj temperaturi kao i hroničnoj socijalnoj IZ nije dovelo do promena u proteinskoj ekspresiji ($p > 0.05$). U poređenju sa IM, grupu životinja izložena kombinovanom stresu IZ+IM karakteriše značajno niži nivo MnSOD ($^{\#\#}p < 0.01$).

U citosolnoj frakciji hipokampusa pokazan je značajan efekat interakcije akutni × hronični stres ($F_{2.35}=8.7$, $p < 0.01$) i porast proteinske ekspresije MnSOD nakon izlaganja kombinovanim stresorima u poređenju sa hroničnom socijalnom IZ ($^{\wedge}p < 0.05$) ili odgovarajućim akutnim stresorima ($^{\#\#\#}p < 0.001$, $^{\#}p < 0.05$, redom).



Slika 14. **Relativne promene u nivou MnSOD proteina u mitohondrijskoj i citosolnoj frakciji hipokampusa nakon akutnog stresora imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest/sedam nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičkih gustina ± SEM i izražena kao procenat od odgovarajuće kontrole. Simbol ukazuje na značajne razlike između: životinja izlaganih stresorima i kontrolnih životinja ($^{*}p < 0.05$); hroničnog i kombinovanog stresa ($^{\wedge}p < 0.05$); kombinovanog i odgovarajućeg akutnog stresa ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). Imunoreaktivna traka na ~25 kDa koja odgovara molekulskoj masi subjedinice MnSOD-a proteina detektovana je u mitohondrijskoj i citosolnoj frakciji hipokampusa

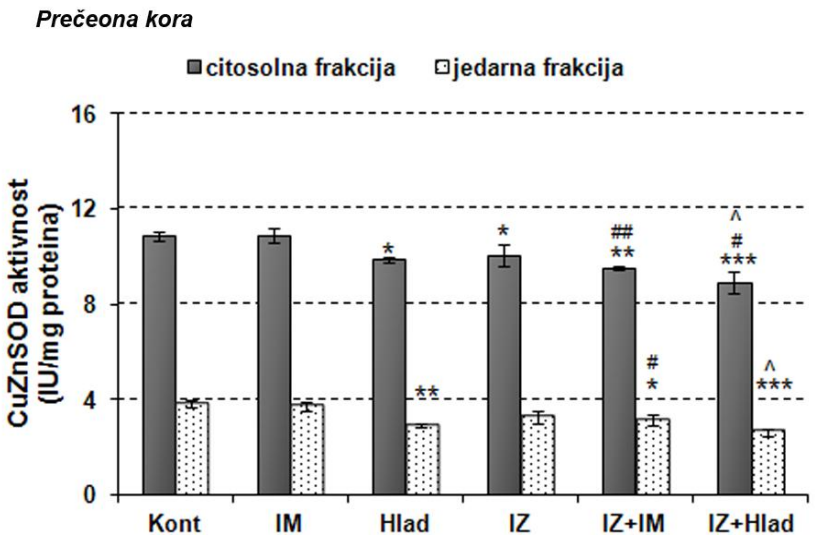
4.4. Uticaj stresa na aktivnost superoksid dismutaza u prečeonoj kori i hipokampusu pacova

Superoksid dismutaza predstavlja prvu liniju odbrane organizma od štetnih kiseoničnih vrsta pa je očuvana aktivnost ovog enzima bitna za normalno funkcionisanje. Prethodna istraživanja naše grupe su pokazala da akutni stres IM dovodi do povećanja u nivou CuZnSOD proteina u citosolnoj frakciji prečeone kore i hipokampusa. Međutim, povećan nivo proteina ne podrazumeva i povećanu aktivnost što najverovatnije ukazuje na posttranslacione modifikacije.

4.4.1. Smanjena aktivnost CuZnSOD i MnSOD nakon akutnog stresa niske temperature ili hroničnog stresa socijalne izolacije u prečeonoj kori pacova

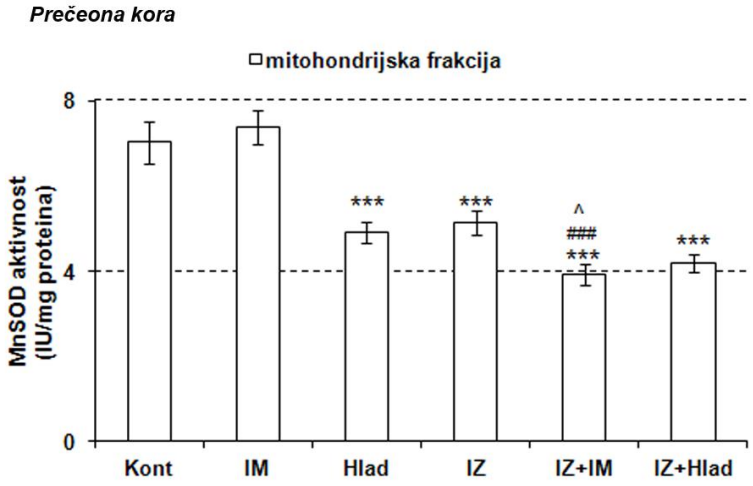
Dvofaktorijalna ANOVA analiza pokazala je značajan efekat akutnog ($F_{2.30}$=7.1, $p < 0.01$) i hroničnog ($F_{1.30}$=18.3, $p < 0.05$) stresa na aktivnost CuZnSOD u citosolnoj frakciji prečeone kore. Post-hoc test je pokazao da životinje izložene akutnom stresu niske temperature i hroničnoj socijalnoj IZ imaju značajno niži nivo aktivnosti ovog enzima u poređenju sa kontrolnim životinjama ($^{*}p < 0.05$). Takođe, kombinovani stres doveo je do sniženja aktivnosti CuZnSOD u poređenju sa kontrolom ($^{**}p < 0.01$, $^{***}p < 0.001$) i odgovarajućim akutnim stresom ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$), dok je u IS+IM grupi ova aktivnost bila značajno niža i u poređenju sa hroničnom socijalnom IZ ($^{\wedge}p < 0.05$) (Slika 15).

U jedarnoj frakciji prečeone kore utvrđen je značajan efekat akutnog ($F_{2.30}$= 9.5, $p < 0.001$) i hroničnog ($F_{1.30}$= 8.8, $p < 0.01$) stresa na aktivnost CuZnSOD, dok je post-hoc test pokazao smanjenu aktivnost nakon akutnog izlaganja niskoj temperaturi ($^{**}p < 0.01$) i kombinovanim stresnm uslovima ($^{*}p < 0.05$, $^{***}p < 0.001$) (Slika 15).



Slika 15. Aktivnost CuZnSOD u citosolnoj / jedarnoj frakciji prečeone kore kontrolnih (Kont) i stresiranih životinja. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajne razlike između: stres tretmana i kontrole ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); hroničnog i kombinovanog stresa ($^{\wedge}p < 0.05$); kombinovanog i odgovarajućeg akutnog stresa ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

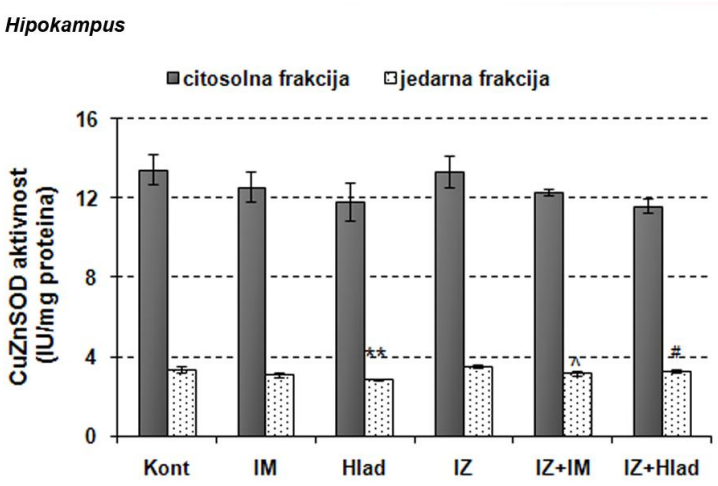
U mitohondrijskoj frakciji prečeone kore, dvofaktorijalna ANOVA pokazala je značajan efekat akutnog ($F_{2.30}$=11.5, $p < 0.001$) i hroničnog ($F_{1.30}$=55.2, $p < 0.001$) stresa, kao i njihove interakcije tj. akutnog × hroničnog stresa ($F_{2.30}$=8.4, $p < 0.01$) na aktivnost MnSOD. Opadanje aktivnosti utvrđeno je post-hoc testom kod životinja izloženih akutnom stresu Hlad ($^{***}p < 0.001$), hroničnoj socijalnoj IZ ($^{***}p < 0.001$), kao i kombinovanim stresnim uslovima (IS+IM, IS+Hlad, $^{***}p < 0.001$) (Slika 16). Takođe, značajno niža aktivnost zabeležena je u IS+IM u poređenju sa IM grupom ($^{###}p < 0.001$) i IZ ($^{\wedge}p < 0.05$).



Slika 16. Aktivnost MnSOD u mitohondrijskoj frakciji prečeone kore kontrolnih (Kont) i stresiranih životinja. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajne razlike između: stres tretmana i kontrole ($^{***}p < 0.001$); hroničnog i kombinovanog stresa ($^{\wedge}p < 0.05$); kombinovanog i odgovarajućeg akutnog stresa ($^{###}p < 0.001$).

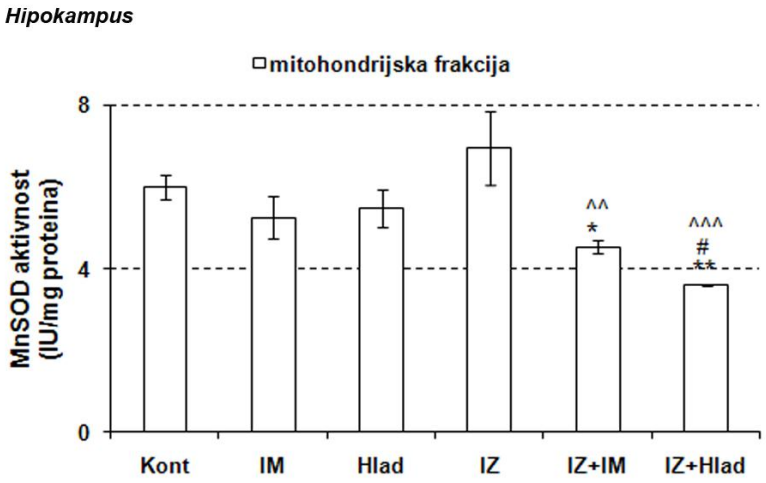
4.4.2. Dodatni akutni stres imobilizacije ili niske temperature snižava aktivnost MnSOD u hipokampusu hronično stresiranih pacova

Dvofaktorijalna ANOVA pokazala je da stres nema značajan uticaj na aktivnost citosolne CuZnSOD u hipokampusu ($p > 0.05$) pacova. Nasuprot tome, u jedarnoj frakciji postoji značajan efekat akutnog ($F_{2.30}$=6.169, $p < 0.01$) i hroničnog ($F_{1.30}$=6.582, $p < 0.05$) stresa na CuZnSOD aktivnost. Kod životinja akutno izloženih niskoj temperaturi, CuZnSOD aktivnost u jedru znatno je smanjena u odnosu na kontrolne ($^{**}p < 0.01$) i životinje izložene IZ+Hlad ($^{#}p < 0.05$) (Slika 17).



Slika 17. Aktivnost CuZnSOD u citosolnoj / jedarnoj frakciji hipokampusa kontrolnih (Kont) i stresiranih životinja. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajne razlike između: stres tretmana i kontrole ($^{**}p < 0.01$); hroničnog i kombinovanog stresa ($^{\wedge}p < 0.05$); kombinovanog i odgovarajućeg akutnog stresa ($^{\#}p < 0.05$).

Dvofaktorijalna ANOVA pokazala je značajan efekat interakcije akutni × hronični stres ($F_{2,30}$=4.4, $p < 0.05$) na aktivnost MnSOD. Post-hoc test pokazao je smanjenju MnSOD aktivnosti kod životinja izloženih IZ+IM i IZ+Hlad, u odnosu na kontrolne ($^{*}p < 0.05$ i $^{**}p < 0.01$, redom) i životinje izložene hroničnoj IZ ($^{\wedge\wedge}p < 0.01$ i $^{\wedge\wedge\wedge}p < 0.001$) (Slika 18), kao i u IZ+Hlad grupi u odnosu na grupu izloženu akutnom stresu niske temperature ($^{\#}p < 0.05$).



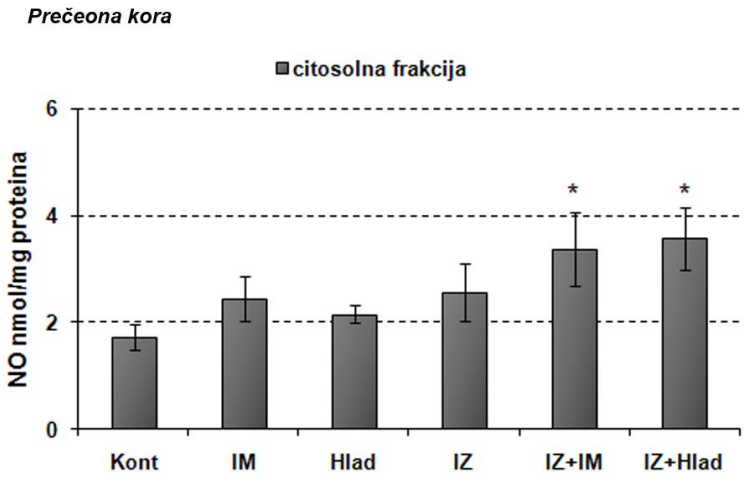
Slika 18. Aktivnost MnSOD u mitohondrijskoj frakciji hipokampusa kontrolnih (Kont) i stresiranih životinja. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajne razlike između: tretmana stresa i kontrole ($^{*}p < 0.05$; $^{*}p < 0.01$); hroničnog i kombinovanog stresa ($^{\wedge\wedge}p < 0.01$; $^{\wedge\wedge\wedge}p < 0.001$); kombinovanog i odgovarajućeg akutnog stresa ($^{\#}p < 0.05$).

4.5. Nivoi citosolnog azot monoksida (NO) u prečeonoj kori i hipokampusu pacova

Nitriti i nitrati (NO_x^-) predstavljaju stabilne metabolite NO, koji se u praksi koriste za indirektno merenje produkcije azot monoksida u biološkim sistemima. Takođe, pokazano je da je merenje nivoa NO_x^- pouzdan pokazatelj sposobnosti NOS za stvaranje NO u mozgu (Salter i sar., 1996).

4.5.1. Kombinovani stres dovodi do povećanja nivoa citosolnog NO u prečeonoj kori

Dvofaktorijalna ANOVA otkrila je značajan efekat hroničog stresa u prečeonoj kori ($F_{1.30}$=7.49, $p < 0.01$) na nivo NO_x^-, a post-hoc test je ukazao na značajan porast metabolita kod životinja izloženih kombinovanim stresorima u odnosu na kontrolne životinje ($^*p < 0.05$) (Slika 19).

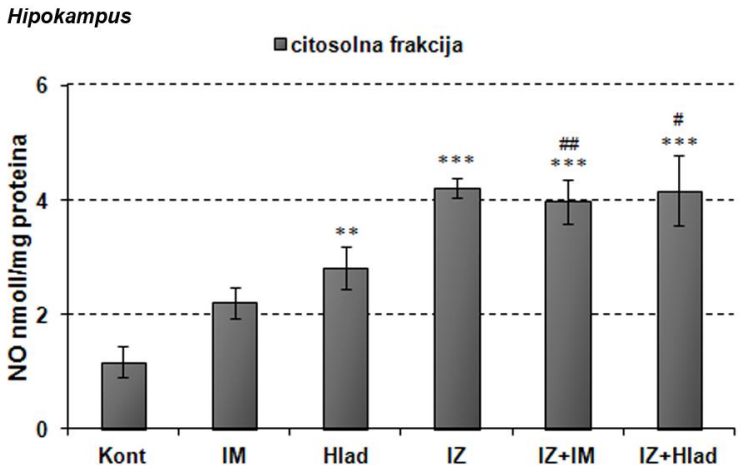


Slika 19. **Nivo NO metabolita (NO_x^-) (nmol/mg proteina) u citosolnoj frakciji prečeone kore kontrolnih (Kont) i stresiranih životinja.** Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajno povećanje (NO_x^-) kod životinja izloženih kombinovanim stresnim uslovima u odnosu na kontrole ($^*p < 0.05$).

4.5.2. Akutno izlaganje životinja niskoj temperaturi ili hroničnoj socijalnoj izolaciji dovodi do povećanog nivoa citosolnog NO u hipokampusu pacova

Dvofaktorijalna ANOVA pokazala je značajan efekat hroničnog stresa na nivo NO_x^- metabolita u hipokampusu ($F_{1.30}$= 46.44, $p < 0.001$). Post hoc Duncan test pokazao je značajan porast nivoa NO_x^- nakon akutnog stresa niske temperature ($^{**}p < 0.01$) kao i hroničnoj socijalnoj izolaciji i kombinovanim stresnim uslovima u odnosu na kontrolnu grupu ($^{***}p < 0.001$). Takođe, nivo

NO_x^- u hipokampusu bio je značajno viši kod životinja izloženih kombinovanom stresu u odnosu na akutno stresirane životinje ($^{\#\#}p < 0.01$ i $^{\#}p < 0.05$, redom) (Slika 20).



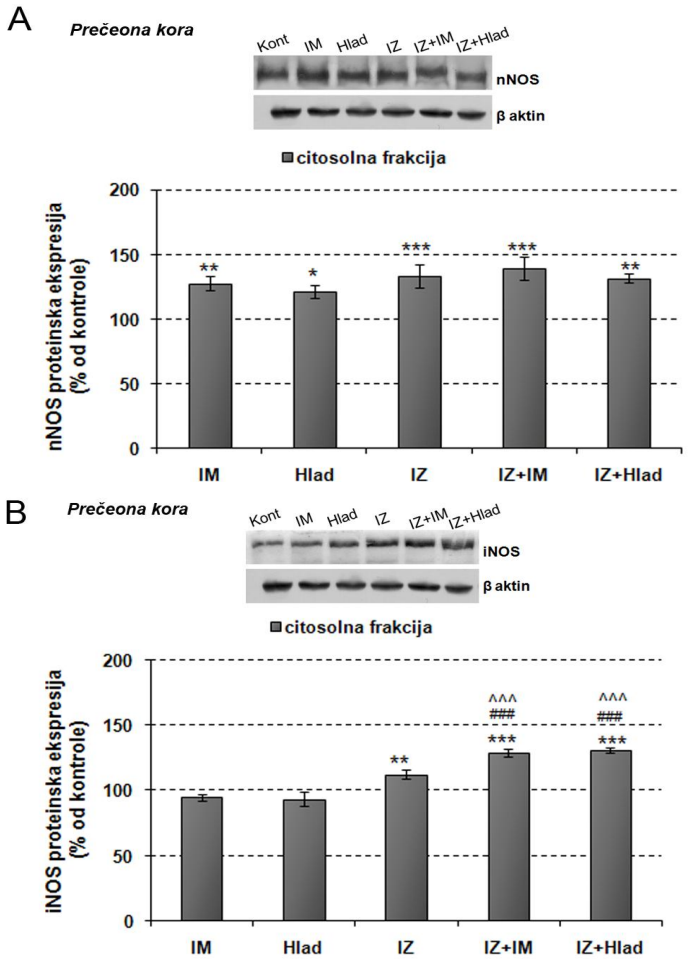
Slika 20. **Nivo NO metabolita (NO_x^-) (nmol/mg proteina) u citosolnoj frakciji hipokampusa kontrolnih (Kont) i stresiranih životinja.** Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajno povećanje (NO_x^-) između tretmana stresa i kontrola ($^{**}p < 0.01$, $^{***}p < 0.001$) kao i između kombinovanog stresa i odgovarajućeg akutnog stresa (IM vs IZ+IM; Hlad vs IZ+Hlad) ($^{\#\#}p < 0.01$, $^{\#}p < 0.05$).

4.6. Uticaj stresa na proteinsku ekspresiju NOS-a u citosolnoj frakciji prečeone kore i hipokampusa pacova

4.6.1. Akutni ili/i hronični stres socijalne izolacije povećava ekspresiju nNOS-a u prečeonoj kori pacova

Dvofaktorijalna ANOVA pokazala je značajan efekat akutnog stresa ($F_{2.30} = 3.94$, $p < 0.05$) i hronične IZ ($F_{1.30} = 13.84$, $p < 0.001$) na nivo nNOS proteina u citosolnoj frakciji prečeone kore (Slika 21 A). Post hoc test ukazuje na značajan porast u svim eksperimentalnim grupama u odnosu na kontrolnu grupu ($^{*}p < 0.05$, $^{***}p < 0.001$, $^{**}p < 0.01$).

U slučaju iNOS proteinske ekspresije, ustanovljen je značajni efekat hroničnog ($F_{1.30}$=112.9, $p < 0.001$) stresa i interakcije akutni × hronični stres ($F_{2.30} = 8.70$, $p < 0.001$) na nivo ovog proteina. Hronični stres socijalne IZ doveo je do značajnog porasta iNOS proteina u odnosu na kontrolnu grupu ($^{**}p < 0.01$). Kod životinja izloženih kombinovanim stresnim uslovima značajan porast nivoa proteina u citosolnoj frakciji pokazan je u odnosu na akutne stresne uslove ($^{\#\#\#}p < 0.001$), hronični stres ($^{\wedge\wedge\wedge}p < 0.001$) i kontrolu ($^{***}p < 0.001$) (Slika 21 B).



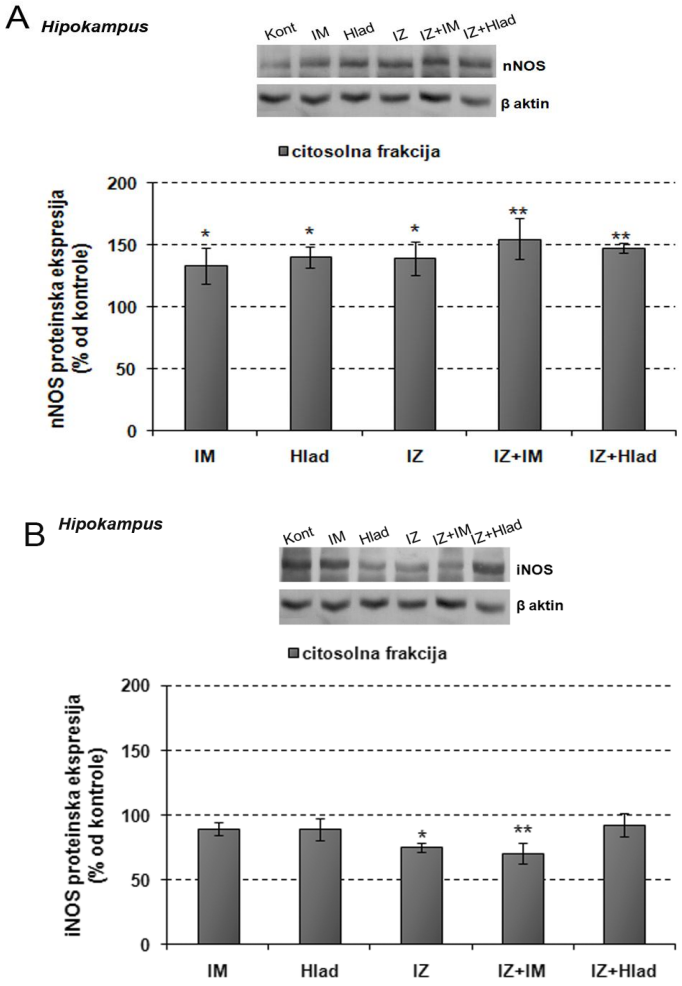
Slika 21. Relativne promene u nivou nNOS (A) i iNOS (B) proteina u citosolnoj frakciji prečeone kore nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad). Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simboli ukazuju na značajnu razliku izmedju stresnih grupa i kontrola ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$), kombinovanih stresnih grupa i odgovarajućeg akutnog stresa (IZ+IM *vs* IM i IZ+Hlad *vs* Hlad) ($^{\#\#\#}p < 0.001$) kao i kombinovanih stresnih grupa i hronične socijalne IZ ($^{\wedge\wedge\wedge}p < 0.001$). Reprezentativne trake na 150kDa i 130kDa koje odgovaraju nNOS i iNOS proteinima, redom, pokazuju ekspresiju ovih proteina u citosolnoj frakciji prečeone kore.

4.6.2. Hronični stres socijalne izolacije dovodi do povećanja ekspresije nNOS proteina i smanjenja iNOS proteina u hipokampusu pacova

Dvofaktorijalna ANOVA analiza pokazala je značajan efekat hroničnog stresa ($F_{1.30} = 6.42$, $p < 0.05$) na nivo nNOS proteina u citosolnoj frakciji hipokampusa. Kao i u prečeonoj kori, Duncan's post-hoc test je i u hipokampusu pokazao značajno povećanu nNOS proteinsku

ekspresiju kod životinja u svim stresiranim grupama u odnosu na kontrolnu grupu ($^{*}p < 0.05$; $^{**}p < 0.01$) (Slika 22 A).

Dvofaktorijalna ANOVA analiza takođe je pokazala značajan efekat hroničnog stresa na ekspresiju iNOS proteina ($F_{1.30}$=5.970, $p < 0.05$) u citosolnoj frakciji hipokampusa. Post-hoc test ukazao je na značajan pad nivoa ovog proteina kod životinja izloženih hroničnoj socijalnoj IZ ($^{*}p < 0.05$) kao i IZ+IM ($^{**}p < 0.01$) (Slika 22 B) u odnosu na životinje iz kontrolne grupe.

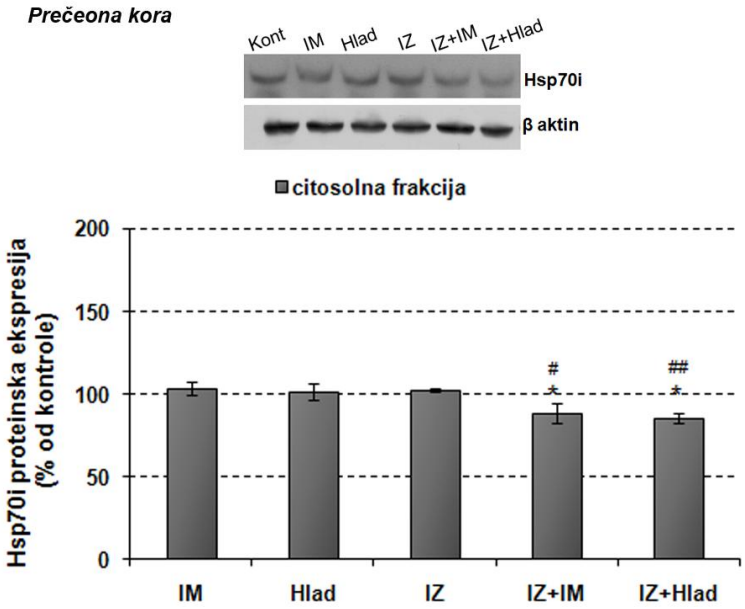


Slika 22. **Relativne promene u nivou nNOS (A) i iNOS (B) proteina u citosolnoj frakciji hipokampusa nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole. Simboli ukazuju na značajnu statističku razliku izmedju odredjenih stresnih grupa i kontrola ($^{*}p < 0.05$; $^{**}p < 0.01$). Reprezentativne trake na 150kDa i 130kDa koje odgovaraju nNOS i iNOS proteinima, redom, pokazuju ekspresiju ovih proteina u citosolnoj frakciji hipokampusa.

4.7. Uticaj stresa na proteinsku ekspresiju Hsp70i u citosolnoj frakciji prečeone kore i hipokampusa pacova

4.7.1. Sniženi nivoi Hsp70i proteina u prečeonoj kori pacova nakon izlaganja kombinovanim stresorima

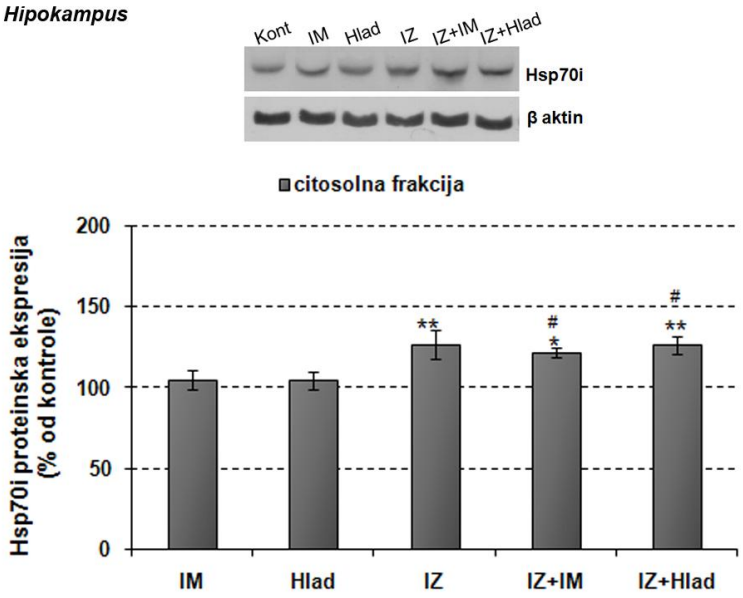
Dvofaktorijalna ANOVA analiza ukazala je na značajan efekat hroničnog stresa ($F_{1.30}$=10.05, $p < 0.001$) kao i interakcije akutni × hronični stres ($F_{2.30}$=3.95, $p < 0.05$) na Hsp70i proteinsku ekspresiju u citosolnoj frakciji prečeone kore pacova. Duncan's post-hoc test pokazao je značajno niži nivo ovog proteina kod životinja izloženih kombinovanim stresnim uslovima (IZ+IM i IZ+Hlad) u odnosu na kontrolne životinje ($^{*}p < 0.05$) kao i u odnosu na životinje izložene odgovarajućim akutnim stresorima IM ili niske temperature ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) (Slika 23).



Slika 23. Relativne promene u nivou Hsp70i proteina u citosolnoj frakciji prečeone kore nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad). Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole. Simboli ukazuju na značajnu razliku između životinja izloženih kombinovanom stresu i kontrola ($^{*}p < 0.05$) kao i izmedju kombinovanog stresa i odgovarajućeg akutnog stresa ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). Prikazana traka na 72kDa odgovara Hsp70i proteinu u citosolnoj frakciji prečeone kore.

4.7.2. Hronična socijalna izolacija dovodi do povećane ekspresije Hsp70i proteina u hipokampusu pacova

Nasuprot prečeonoj kori, u citosolnoj frakciji hipokampusa, dvofaktorijalna ANOVA pokazala je značajan efekat hroničnog stresa ($F_{1.30}$=22.948, $p < 0.001$) na nivo Hsp70i proteina. Duncan's post-hoc test ukazao je na značajan porast nivoa Hsp70i proteina kod životinja izloženih hroničnoj socijalnoj IZ ($^{**}p < 0.01$) i kombinovanim stresorima IZ+IM ($^{*}p < 0.05$) i IZ+Hlad ($^{**}p < 0.01$) u odnosu na kontrolne životinje ili životinje izložene odgovarajućem akutnom stresu ($^{\#}p < 0.05$) (Slika 24).



Slika 24. **Relativne promene u nivou Hsp70i proteina u citosolnoj frakciji hipokampusa nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole. Simboli ukazuju na značajnu razliku između odredjenih stresnih grupa i kontrola ($^{*}p < 0.05$; $^{**}p < 0.01$) kao i između kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#}p < 0.05$). Reprezentativna traka na 72 kDa odgovara Hsp70i proteinu u citosolnoj frakciji hipokampusa.

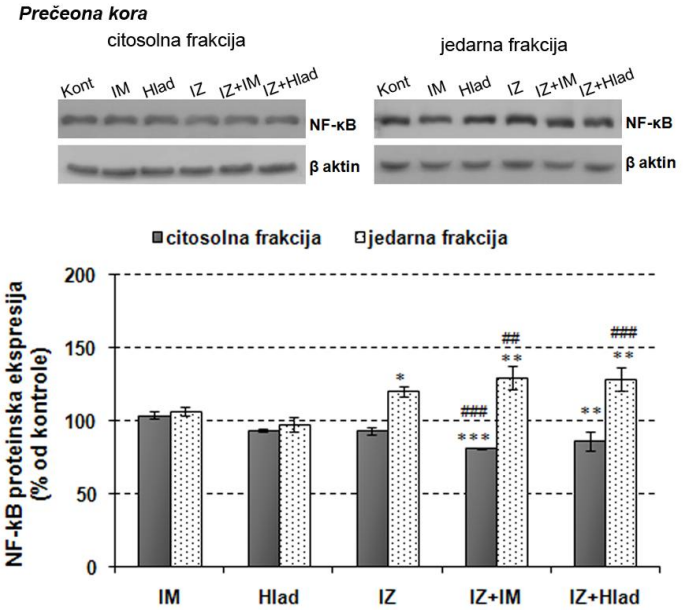
4.8. Uticaj stresa na redistribuciju proteina NF-kB izmedju citosolne i jedarne frakcije u prečeonoj kori i hipokampusu pacova

Aktivacija NF-kB dovodi do translokacije NF-kB-p65 subjedinice ovog proteina iz citoplazme u jedro gde ima ulogu transkripcionog faktora utičući, između ostalih, i na aktivnost gena za iNOS

i nNOS. Detekcijom p65 subjedinice u citosolnoj i jedarnoj frakciji prečeone kore i hipokampusa može se utvrditi potencijalna aktivacija NF-kB proteina u kontroli i nakon izlaganja stresorima.

4.8.1. Hronični stres socijalne izolacije aktivira NF-kB u prečeonoj kori pacova

U prečeonoj kori uočen je značajan efekat hroničnog stresa u citosolnoj ($F_{1.30}$=27.12, $p < 0.001$) i jedarnoj ($F_{1.30}$=32.02, $p < 0.05$) frakciji, i interakcije akutni × hronični stres ($F_{2,30}$=4.86 $p < 0.05$) u citosolnoj frakciji na NF-kB proteinsku ekspresiju. Značajan pad nivoa NF-kB zabeležen je u kombinovanom stresu u poređenju sa kontrolom ($^{***}p < 0.001$, $^{**}p < 0.01$) kao i u IZ+IM u odnosu na akutni stres IM ($^{\#\#}p < 0.01$). Duncan's post-hoc test je pokazao značajan porast jedarnog proteina u hroničnoj socijalnoj IZ ($^{*}p < 0.05$) i u oba kombinovana stresa IZ+IM i IZ+Hlad u odnosu na kontrolu grupu ($^{**}p < 0.01$). Značajna statistička razlika je pokazana izmedju kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$) (Slika 25).

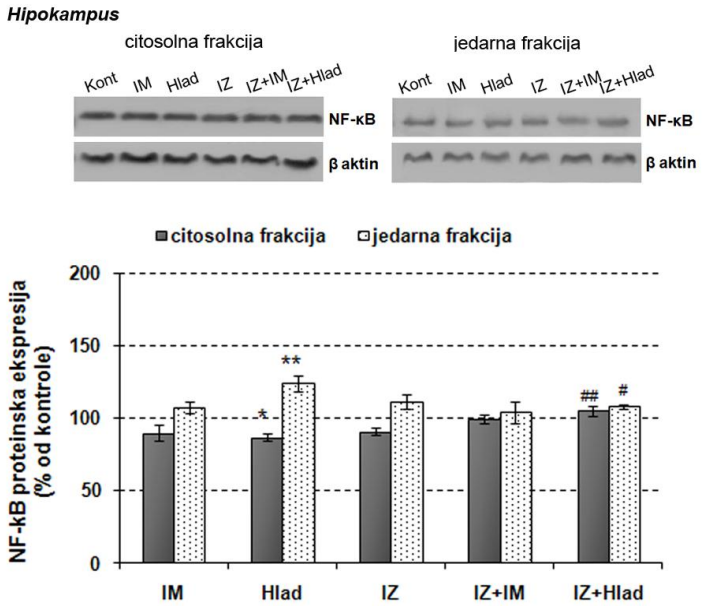


Slika 25. **Relativne promene u nivou NF-kB proteina u citosolnoj i jedarnoj frakciji prečeone kore nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simboli ukazuje na značajne razlike između: odredjenih stresnih grupa i nestresiranih kontrola ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, redom). Prikazana traka na 65kDa odgovara NF-kB proteinu u citosolnoj i jedarnoj frakciji prečeone kore

4.8.2. Translokacija NF-kB iz citosola u jedro nakon izlaganja akutnom stresu niske temperature u hipokampusu pacova

Dvofaktorijalna ANOVA ukazala je na postojanja značajnog efekta hroničnog ($F_{1.30}$=5.124, $p < 0.05$) stresa i interakcije akutni × hronični stres ($F_{2.30}$=8.884, $p < 0.001$) na NF-κB proteinsku ekspresiju u citosolnoj frakciji hipokampusa. U odnosu na kontrolu, post hoc test je pokazao značajno smanjenje NF-kB ($^{*}p < 0.05$) usled akutnog izlaganja Hlad, dok je značajan porast u IZ+Hlad grupi uočen u odnosu na akutni stres Hlad ($^{\#\#}p < 0.01$) (Slika 26).

U jedarnoj frakciji pokazan je značajni efekat akutnog ($F_{2.30}$=3.675, $p < 0.05$) i interakcije akutni × hronični stres ($F_{2.30}$=4.56, $p < 0.05$) na NF-κB proteinsku ekspresiju. Značajan porast nivoa proteina detektovan je kod životinja izlaganih akutnom stresu niske temperature u odnosu na kontrolnu grupu ($^{**}p < 0.01$), kao i IZ+Hlad grupe u odnosu na akutni stres Hlad ($^{\#}p < 0.05$).



Slika 26. **Relativne promene u nivou NF-kB proteina u citosolnoj i jedarnoj frakciji hipokampusa nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simbol ukazuje na značajne razlike između: odredjenih stresnih grupa i kontrola ($^{*}p < 0.05$, $^{**}p < 0.01$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$, $^{\#}p < 0.05$, redom). Prikazana traka na 65kDa odgovara NF-kB proteinu u citosolnoj i jedarnoj frakciji hipokampusa.

4.9. Uticaj stresa na nivoe Bax i Bcl-2 proteina u citosolnoj i mitohondrijskoj frakciji prečeone kore i hipokampusa

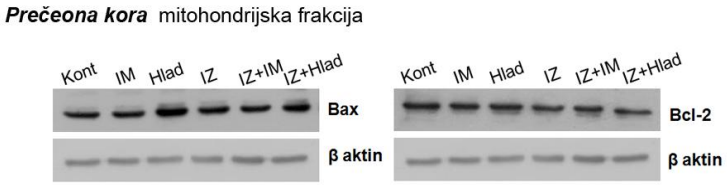
Da bismo utvrdili da li stres može dovesti do promene integriteta mitohondrijske membrane, analizirali smo nivo pro-apoptotskog Bax i anti-apoptotskog Bcl-2 proteina i njihovu unutarćelijsku distribuciju između mitohondrijske i citosolne frakcije u obe moždane strukture. Povećan Bax/ Bcl-2 odnos u mitohondrijskoj frakciji smatra se proapoptotskim događajem i može ukazivati na mogućnost pokretanja unutrašnjeg (mitohondrijskog) puta apoptoze.

4.9.1. Hronični stres socijalne izolacije povećava odnos Bax/Bcl-2 proteina u mitohondrijskoj frakciji prečeone kore

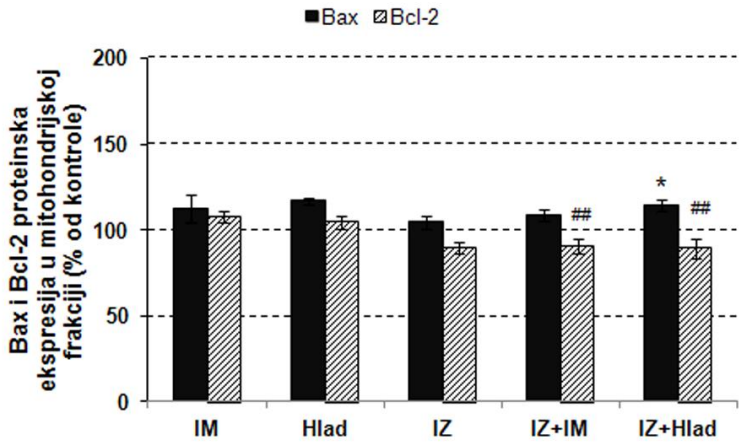
Dvofaktorijalna ANOVA analiza pokazala je značajan efekat akutnog stresa na nivo Bax ($F_{2.30}$=4.88, $p < 0.05$) i hroničnog stresa na nivo Bcl-2 proteina ($F_{1.30}$=21.59, $p < 0.001$) u mitohondrijskoj frakciji prečeone kore.

Post-hoc Duncan's analiza pokazala je značajan porast nivoa mitohondrijskog Bax nakon akutnog izlaganja životinja niskoj temperaturi i kombinovanom stresu IZ+Hlad, u poređenju sa kontrolnim životinjama ($^{*}p < 0.05$).

Nasuprot tome, značajno smanjenje nivoa Bcl-2 proteina opaženo je u mitohondrijskoj frakciji životinja izloženih kombinovanim stresnim uslovima IZ+IM i IZ+Hlad u odnosu na životinje koje su bile izložene samo odgovarajućem akutnom stresu ($^{\#\#}p < 0.01$) (Slika 27).



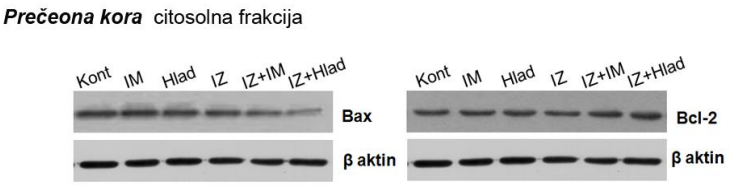
Imunoreaktivna traka na 23 kDa odgovara Bax a na 26 kDa Bcl-2 proteinu u mitohondrijskoj frakciji prečeone kore.



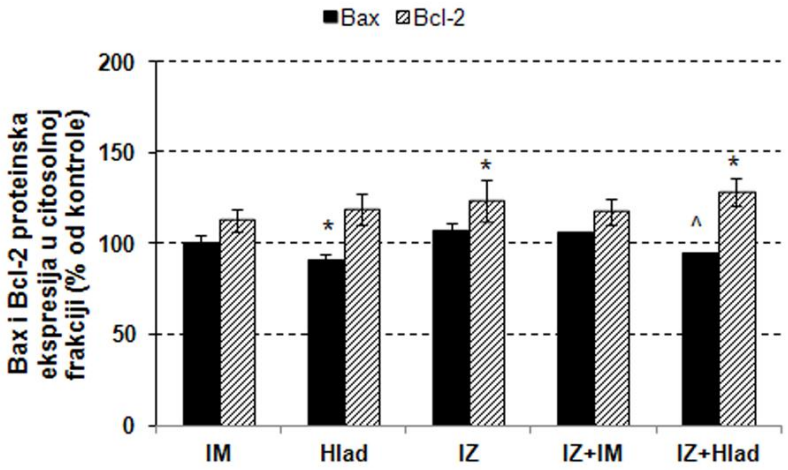
Slika 27. **Relativne promene u nivou Bax i Bcl-2 proteina u mitohondrijskoj frakciji prečeone kore nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simboli ukazuju na značajne razlike između: odredjene stresne grupa i kontrola ($^{*}p < 0.05$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$).

U citosolnoj frakciji, dvofaktorijalna ANOVA ukazala je na značajan efekat akutnog ($F_{2.30}$=4.89, $p < 0.05$) i hroničnog ($F_{1.30}$=8.53, $p < 0.01$) stresa na nivo Bax i hroničnog stresa ($F_{1.30}$=4.14, $p \leq 0.05$) na nivo Bcl-2 proteina u prečeonoj kori pacova. Duncan's post-hoc test je pokazao značajno smanjenje Bax-a kod životinja akutno izloženih niskoj temperaturi u odnosu na kontrolne životinje ($^{*}p < 0.05$), i kombinovanom stresu IZ+Hlad u odnosu na hroničnu socijalnu IZ ($^{\wedge}p < 0.05$).

Hronična socijalna IZ i kombinovani stres IZ+Hlad doveli su do značajnog povećanja citosolnog nivoa Bcl-2 proteina u prečeonoj kori (Slika 28).



Imunoreaktivna traka na 23 kDa odgovara Bax a na 26 kDa Bcl-2 proteinu u citosolnoj frakciji prečeone kore

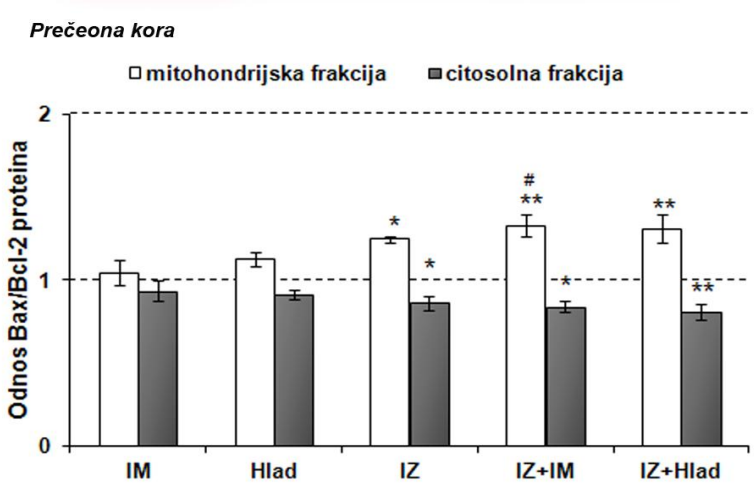


Slika 28. **Relativne promene u nivou Bax i Bcl-2 proteina u citosolnoj frakciji prečeone kore nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simboli ukazuju na značajne razlike između: odredjenih stresnih grupa i kontrola ($^{*}p < 0.05$); životinja izlaganih kombinovanim stresnim uslovima i hronične socijalne izolacije ($^{\wedge}p < 0.05$).

Pored merenja pojedinačnih nivoa ovih proteina, izračunat je i odnos nivoa Bax i Bcl-2 (Bax/Bcl-2) u mitohondrijskoj i citosolnoj frakciji (Slika 29).

Dvofaktorijalna ANOVA pokazala je značajan efekat hroničnog stresa ($F_{1.30}=20.86$, $p < 0.001$; $F_{1.30}=11.61$, $p < 0.01$, redom) na odnos Bax/Bcl-2 u mitohondrijskoj i citosolnoj frakciji prečeone kore. Ovaj odnos bio je povećan u mitohondrijskoj frakciji nakon hronične socijalne IZ i oba kombinovana stresa (IZ+IM i IZ+Hlad) ($^{*}p < 0.05$, $^{**}p < 0.01$), kao i u IZ+IM u odnosu na akutni stres IM ($^{\#}p < 0.05$) (Slika 30).

Istovremeno Bax/Bcl-2 odnos bio je snižen u citosolnoj frakciji prečeone kore u istim stresnim uslovima ($^{*}p < 0.05$, $^{**}p < 0.01$).

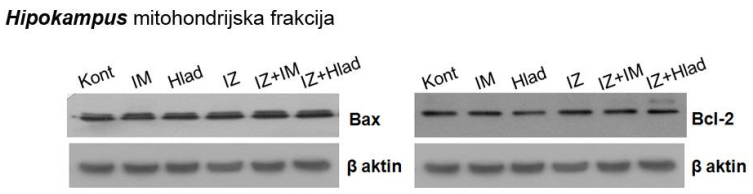


Slika 29. **Odnos Bax/Bcl-2 proteina u prečeonoj kori nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simbol ukazuje na značajne razlike između: odredjenih stresnih grupa i kontrola($^{*}p < 0.05$; $^{**}p < 0.01$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#}p < 0.05$, redom)

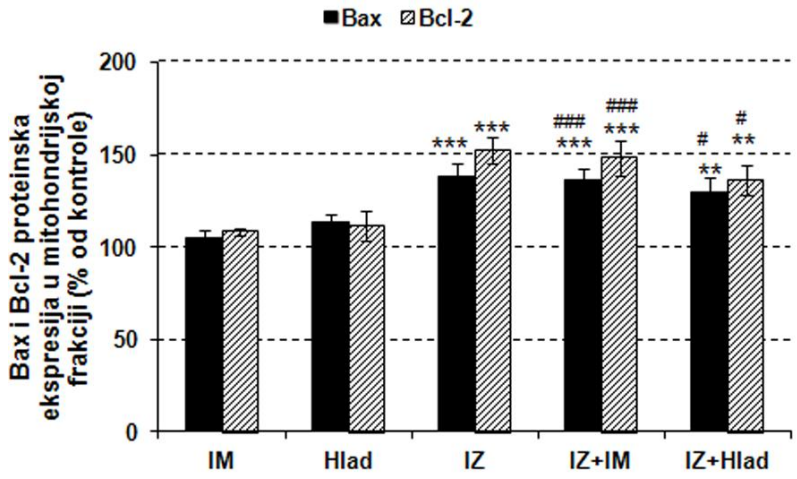
4.9.2. Nepromenjen odnos Bax/Bcl-2 proteina u hipokampusu pacova nakon izlaganja stresnim uslovima

U hipokampusu, dvofaktorijalna ANOVA pokazala je značajan efekat hroničnog stresa ($F_{1.30}$=37.78, $p < 0.001$; $F_{1.30}$=51.04, $p < 0.001$) na Bax i Bcl-2 proteinsku ekspresiju u mitohondrijskoj frakciji.

Post-hoc analiza pokazala je značajan porast nivoa Bax i Bcl-2 proteina nakon hronične socijalne izolacije i oba kombinovana stresora ($^{**}p < 0.01$, $^{***}p < 0.001$) (Slika 30). Takođe, utvrđen je značajan porast ovih proteina usled izlaganja kombinovanim stresorima u odnosu na akutni stres ($^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$).



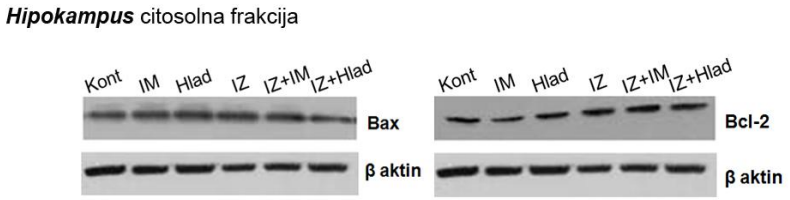
Prikazana traka na 23 kDa odgovara Bax a na 26 kDa Bcl-2 proteinu u mitohondrijskoj frakciji hipokampusa



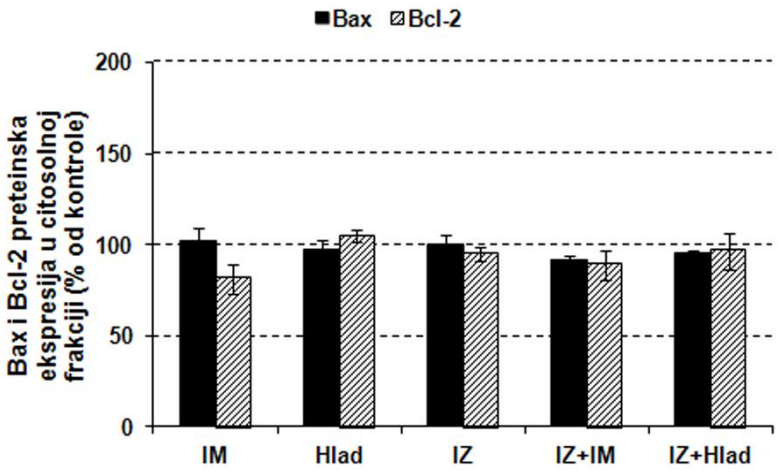
Slika 30. Relativne promene u nivou Bax i Bcl-2 proteina u mitohondrijskoj frakciji hipokampusa nakon akutnog stresa imobilizacije (IM) ili niske temperaure (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad). Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole. Simboli ukazuje na značajne razlike između: odredjenih stresnih grupa i kontrola ($^{**}p < 0.01$; $^{***}p < 0.001$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{###}p < 0.001$, $^{#}p < 0.05$, redom).

U citosolnoj frakciji hipokampusa dvofaktorijalna ANOVA nije ukazala na značajan uticaj stresa na nivo Bax proteina, dok je značajan efekat akutnog stresa ($F_{2.30}=3.96$, $p < 0.05$).uočen na nivou Bcl-2 proteina.

Ipak, Duncan's post-hoc test nije pokazao značajne razlike nivoa citosolnog Bax i Bcl-2 proteina između eksperimentalnih grupa životinja (Slika 31).

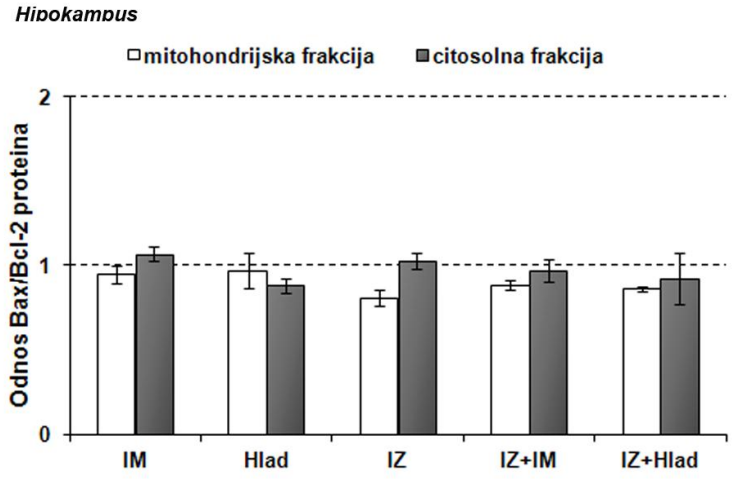


Prikazana traka na 23 kDa odgovara Bax a na 26 kDa Bcl-2 proteinu u citosolskoj frakciji hipokampusa



Slika 31. Relativne promene u nivou Bax i Bcl-2 proteina u citosolnoj frakciji hipokampusa nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad). Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.

Konačno, izlaganje stresorima nije uticalo na Bax/Bcl-2 odnos u mitohondrijskoj ili citosolnoj frakciji hipokampusa (Slika 32)

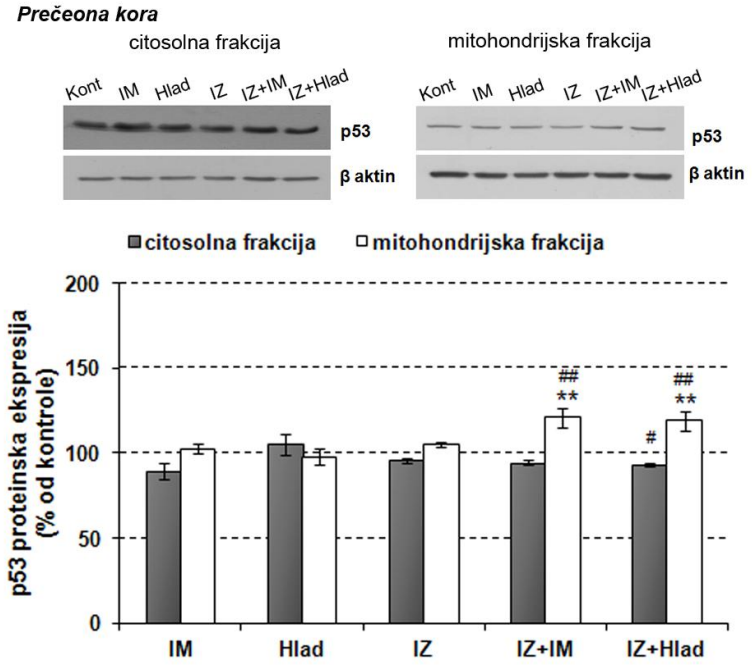


Slika 32. Odnos Bax/Bcl-2 proteina u hipokampusu nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i i njihovim kombinacijama (IZ+IM ili IZ+Hlad). Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM.

4.10. Uticaj stresa na nivo p53 proteina u citosolnoj i mitohondrijskoj frakciji prečeone kore i hipokampusa

4.10.1.Translokacija p53 proteina nakon kombinovanog stresa u prečeonoj kori pacova

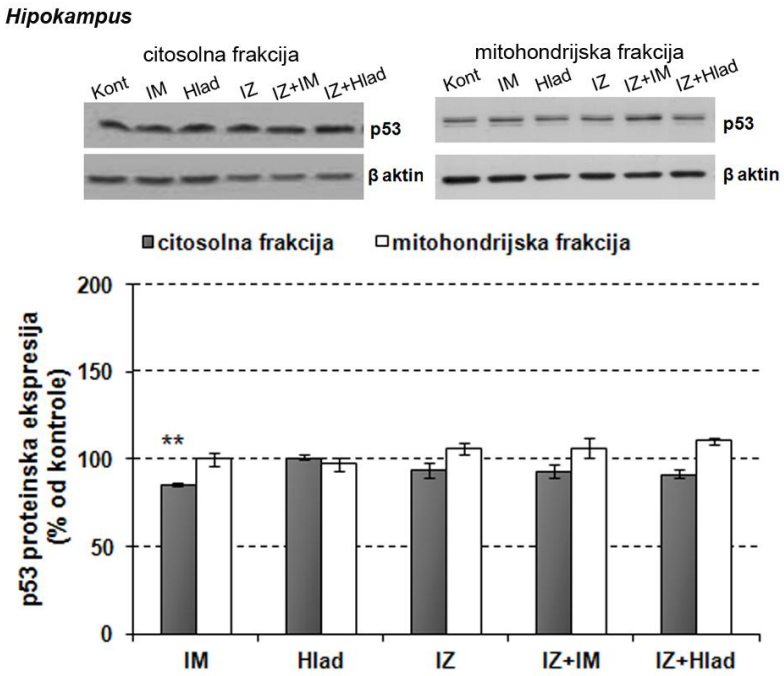
Dvofaktorijalna ANOVA analiza ukazala je na značajan glavni efekat hroničnog stresa socijalne IZ ($F_{1.30}$=22.1, $p < 0.001$) na nivo mitohondrijskog p53 proteina kao i interakcije akutni × hronični stres ($F_{2.30}$=3.6, $p < 0.05$) na p53 proteinski nivo u citosolskoj frakciji prečeone kore. Kombinovani stresni uslovi (IZ+IM i IZ+Hlad) doveli su do povećanja nivoa p53 u mitohondrijskoj frakciji u poređenju sa kontrolom ($^{**}p < 0.01$) i akutnim stresnim uslovima ($^{\#\#}p < 0.01$).Nasuprot tome, citosolni nivo p53 proteina bio je značajno smanjen u IZ+Hlad u poređenju sa Hlad ($^{\#}p < 0.05$) (Slika 33).



Slika 33. Relativne promene u nivou p53 proteina u citosolnoj i mitohondrijskoj frakciji prečeone kore nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad). Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simbol ukazuje na značajne razlike između: odredjenih stresnih grupa i kontrola ($^{**}p < 0.01$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$, $^{\#}p < 0.05$, redom). Prikazana traka na 53 kDa odgovara p53 proteinu u mitohondrijskoj i citosolnoj frakciji prečeone kore

4.10.2.Nepromenjeni nivoi p53 proteina u hipokampusu pacova

Nasuprot prečeonoj kori, dvofaktorijalna ANOVA analiza ukazala je da izlaganje stresnim uslovima nije dovelo do promena nivoa p53 proteina u citosolnoj i mitohondrijskoj frakciji hipokampusa. Duncan's post-hoc test pokazao je značajno niži nivo ovog proteina u citosolnoj frakciji životinja izloženih akutnom stresu IM u poređenju sa kontrolnom grupom ($^{**}p < 0.01$), dok promene p53 proteinske ekspresije na nivou mitohondrijeske frakcije nisu zabeležene (Slika 34).

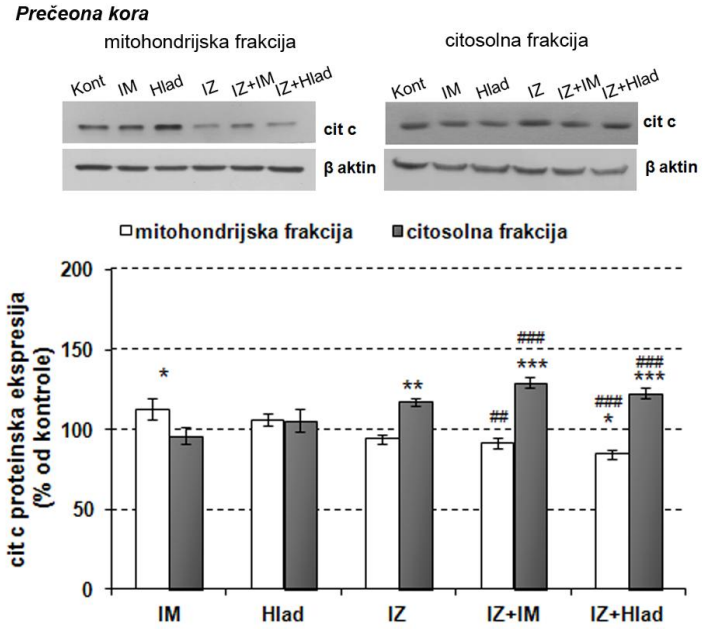


Slika 34. **Relativne promene u nivou p53 proteina u citosolnoj i mitohondrijskoj frakciji hipokampusa nakon akutnog stresa imobilizacije (IM) ili niske temperaure (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simbol ukazuje na značajne razlike između akutnog stresa IM i kontrole ($^{**}p < 0.01$). Prikazana traka na 53 kDa koja odgovara p53 proteinu u mitohondrijskoj i citosolnoj frakciji hipokampusa

4.11. Uticaj stresa na nivo citohroma c u citosolnoj i mitohondrijskoj frakciji prečeone kore i hipokampusa

4.11.1.Hronična socijalna izolacija narušava integritet mitohondrijske membrane u prečeonoj kori pacova

Značajan efekat hroničnog stresa na nivo citohroma c u mitohondrijskoj ($F_{1.30}$=26.8, $p < 0.001$) i citosolnoj ($F_{1.32}$=53.8, $p < 0.001$) frakciji prečeone kore je pokazan dvofaktorijalom ANOVA analizom. Post-hoc test pokazao je značajno veći nivo mitohondrijskog citohroma c nakon akutnog stresa IM ($^{*}p < 0.05$) u odnosu na kontrolne životinje. Hronična socijalna IZ dovela je do povećanja nivoa citohroma c u citosolnoj frakciji ($^{**}p < 0.01$). U kombinovanim stresnim uslovima zabeleženo je značajno sniženje nivoa citohroma c u mitohondrijskoj frakciji i povećanje u citosolnoj frakciji u poređenju sa kontrolnim ($^{**}p < 0.05$, $^{***}p< 0.001$) i životinjama izloženih akutnom stresu ($^{###}p < 0.001$, $^{##}p < 0.01$) (Slika 35).



Slika 35. Relativne promene u nivou cit c proteina u citosolnoj i mitohondrijskoj frakciji prečeone kore nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad). Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest/sedam nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Simboli ukazuje na značajne razlike između: odredjenih stresnih grupa i kontrola ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{##}p < 0.01$, $^{###}p < 0.001$, redom). Prikazana traka na 15 kDa odgovara citohromu c u citosolnoj i mitohondrijskoj frakciji prečeone kore

4.11.2.Nepromenjeni nivoi citohroma c nakon stresnih uslova u hipokampusu pacova

Dvofaktorijalna ANOVA analiza pokazala je da izlaganje stresnim uslovima nije imalo uticaja na nivo citohroma c u mitohondrijskoj i citosolnoj frakciji hipokampusa, a post-hoc test na nepromenjen nivo ovog proteina između eksperimantalnih grupa životinja ($p > 0.05$, Slika 36)

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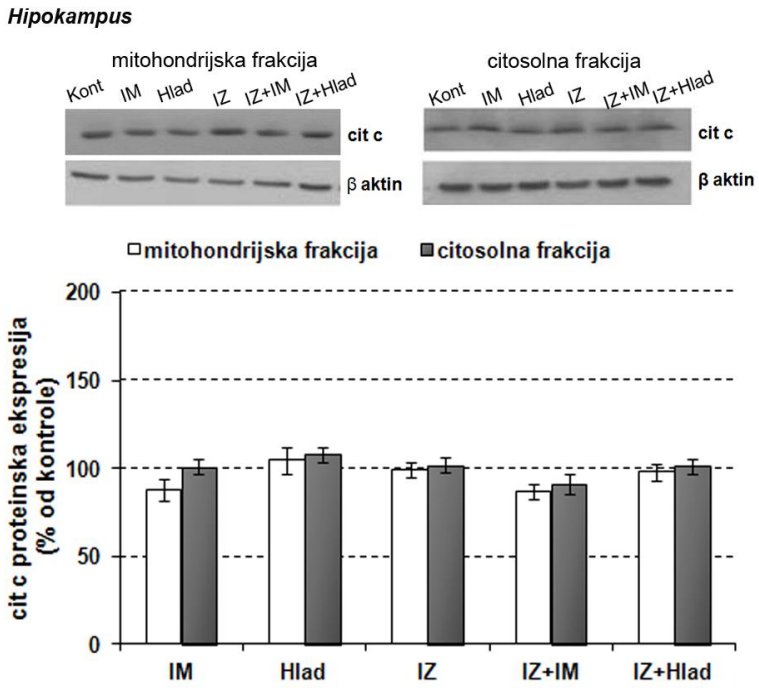


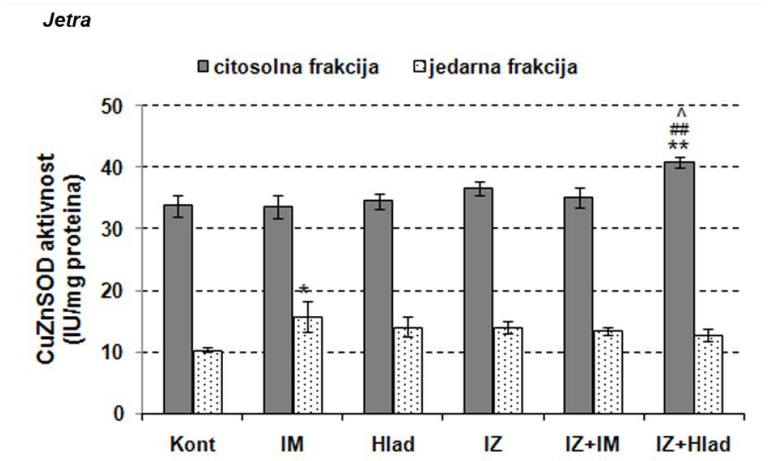
Slika 36. **Relativne promene u nivou cit c proteina u citosolnoj i mitohondrijskoj frakciji hipokampusa nakon akutnog stresa imobilizacije (IM) ili niske temperature (Hlad), hronične socijalne izolacije (IZ), kao i njihovim kombinacijama (IZ+IM ili IZ+Hlad).** Intenzitet traka određen je denzitometrijski, primenom ImageJ softverskog paketa, a optička gustina normalizovana je u odnosu na optičku gustinu β-aktina. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost optičke gustine ± SEM i izražena kao procenat od odgovarajuće kontrole.Imunoreaktivne trake na 15 kDa ukazuju na prisustvo citohroma c u mitohondrijskoj i citosolnoj frakciji hipokampusa.

4.12. Akutno izlaganje niskoj temperaturi ili hroničnom stresu socijalne izolacije smanjuje aktivnost MnSOD u jetri pacova

U citosolnoj frakciji ćelija homogenata jetre dvofaktorijalna ANOVA ukazala je na značajan efekat hroničnog stresa na aktivnost CuZnSOD ($F_{1.30}$=8.776 $p < 0.05$). Post-hoc Duncan's test

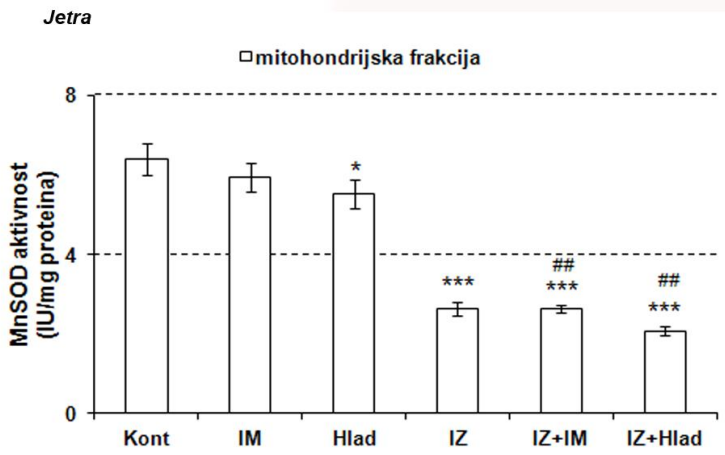
pokazao je značajan porast aktivnosti ovog enzima u IZ+Hlad grupi u odnosu na kontrolne životinje ($^{**}p < 0.01$) kao i životinje akutno izložene niskoj temperaturi ($^{\#\#}p < 0.01$) (Slika 37).

Sa druge strane, u jedarnoj frakciji ćelija homogenata jetre dvofaktorijalna ANOVA nije ukazala na uticaj stresa na aktivnost CuZnSOD. Duncan's post-hoc test pokazao je statistički značajan porast CuZnSOD aktivnosti kod životinja izloženih akutnom stresu IM ($^{*}p < 0.05$) u odnosu na kontrolnu grupu.



Slika 37. Aktivnosti CuZnSOD u citosolnoj / jedarnoj frakciji jetre kontrolnih (Kont) i stresiranih životinja. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajne razlike između: odredjenih stresnih grupa i kontrola ($^{*}p < 0.05$, $^{**}p < 0.01$); hroničnog i kombinovanog stresa ($^{\wedge}p < 0.05$); kombinovanog i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$).

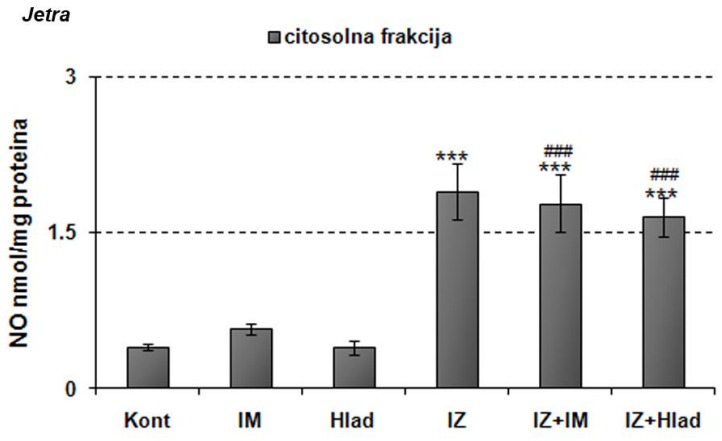
Dvofaktorijalna ANOVA analiza ukazala je na značajan efekat akutnog ($F_{2.30}=3.86$, $p < 0.05$) i hroničnog ($F_{1.30}=263.58$, $p < 0.001$) stresa na aktivnost mitohoindrijske MnSOD u ćelijama homogenata jetre. Duncan's post- hoc test pokazao je pad aktivnosti ovog enzima kod životinja akutno izloženih niskoj temperaturi ($^{*}p < 0.05$), kao i kod životinja izoženih hroničnom stresu socijalne IZ i kombinovanim stresovima IZ+IM ili IZ+Hlad ($^{***}p < 0.001$) u odnosu na kontrolnu grupu (Slika 38). Takođe, kod životinja izloženih kombinovanim stresnim uslovima IZ+IM ili IZ+Hlad zabeležena je značajno niža aktivnost mitohondrijalne MnSOD u odnosu na životinje izložene samo akutnim stresnim uslovima IM ili niske temperature ($^{\#\#\#}p < 0.001$).



Slika 38. Aktivnost MnSOD u mitohondrijskoj frakciji jetre kontrolnih (Kont) i stresiranih životinja. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajne razlike između: odredjenih stresnih grupa i nestresiranih kontrola ($^{*}p< 0.05$; $^{***}p< 0.001$); kombinovanih stresnih grupa i odgovarajućeg akutnog stresa ($^{\#\#}p < 0.01$).

4.13. Hronična socijalna izolacija povećava nivo citosolnog NO u jetri pacova

Dvofaktorijalna ANOVA pokazala je značajan efekat hroničnog stresa na nivo citosolnog NO_x^- metabolita ($F_{1.30}$= 79.72, $p < 0.001$). Post-hoc test ukazao je na veći nivo NO kod životinja izloženih socijalnoj IZ i kombinovanim stresovima u odnosu na kontrolnu grupu životinja ($^{***}p < 0.001$), kao i kombinovano stresiranih životinja u odnosu na odgovarajuću grupu životinja izlaganu akutnom stresu ($^{\#\#\#}p < 0.001$) (Slika 39).



Slika 39. Nivo NO metabolita (NO_x^-) (nmol/mg proteina) u citosolnoj frakciji jetre pacova kod kontrolnih (Kont) i stresiranih životinja. Rezultati iz šest nezavisnih eksperimenta predstavljeni su kao srednja vrednost ± SEM. Simboli ukazuju na značajno povećanje (NO_x^-) između stresiranih životinja i kontrole ($^{***}p < 0.001$) kao i odgovarajućih kombinovanih i akutnih stresora (IM *vs* IZ+IM; Hlad *vs* IZ+Hlad) ($^{\#\#\#}p < 0.001$).

5. DISKUSIJA

5.1. Hroničan stres socijalne izolacije dovodi do deregulacije HHA ose

Izlaganje organizma različitim stresnim uslovima dovodi do aktivacije HHA ose (Vazquez, 1998), a njeno normalno funkcionisanje rezultat je precizne regulacije koja se odvija na nekoliko nivoa neuroendokrinog sistema. Poremećena aktivnost HHA ose može biti povezana sa nastankom brojnih patoloških stanja među kojima su i afektivni poremećaji u koje spada depresija. Kod životinja izloženih akutim stresorima IM ili Hlad u trajanju od 2 sata zabeleženo je značajno povećanje nivoa serumskog CORT-a, što se može smatrati normalnom aktivacijom HHA ose (McEwen, 1998). U skladu sa stresor-specifičnim odgovorom HHA ose, u IM grupi skoro četvorostruko povećanje u odnosu na kontrolne životinje ukazuje da je ovaj fizički i psihološki stres (Kvetnansky i McCarty, 2000) mnogo jačeg intenziteta od fizičkog stresa Hlad, koja je izazvala dvostruko povećanje CORT-a. Poznato je da stres IM može aktivirati kako HPA tako i SNS sistem koji su uključeni u odgovor na izlaganje stresnim situacijama, što za posledicu ima povećanje ACTH i kateholamina. Sa druge strane, efekat niske temperature primarno je vezan za aktivaciju SNS-a (Goldstein i sar., 1996) što može biti razlog manjeg povećanja CORT i detektovane razlike u odgovoru HHA ose.

Sa druge strane, među studijama postoji neslaganje vezano za efekat hroničnog stresa socijalne IZ na nivo kortikosterona. Olivenza i sar. (2000) detektovali su povećan nivo ovog parametra, dok su druge grupe zabeležile smanjeni (Holson i sar., 1991) ili nepromenjeni (Malkesman i sar., 2006; Gamallo i sar., 1986) nivo CORT-a. Ove razlike mogu nastati usled različite dužine trajanja izolovanosti životinja, životnog doba jedinki, postojanja resocijalizacije pre uzorkovanja. U našoj studiji nakon 21 dana socijalne IZ nije zabeležena promena CORT-a u serumu u poređenju sa kontrolnim životinjama. Ipak, ovaj bazalni nivo ne treba se smatrati adaptacijom, s obzirom na to da je hroničan stres povezan sa brojnim patološkim stanjima kao što su depresija i šizofrenija (Walker i Diforio, 1997; McEwen, 2000; Yadid i sar., 2000). Da bismo potvrdili hipotezu da hronični stres socijalne IZ ima maldaptivni efekat i da utiče na sposobnost životinja da zadrže "normalni" odgovor na stres, životinje držane u hroničnoj socijalnoj IZ izlagane su dodatnom jednokratnom akutnom stresu IM ili Hlad. Nedostatak

socijalne intrakcije može uticati na adaptivnu sposobnost životinja na nove stresne situacije (Ishida i sar., 2003). Hronično stresirane životinje pokazale su manje povećanje nivoa serumskog CORT-a u odgovoru na dodatni akutni stres u odnosu na one koje su bile izložene samo akutim stresorima (IZ+IM *vs* IM i IZ+Hlad *vs* Hlad). Ovo ukazuje da je hronična socijalna IZ kompromitovala normalno funkcionisanje HHA ose, najverovatnije usled delimičnog poremećaja negativne povratne sprege u višim centrima mozga (Filipović i sar., 2005). Zadržavanje glukokortikoidnog receptora (GR) u citosolnoj frakciji i onemogućavanje njegove translokacije u jedro gde ima ulogu transkripcionog faktora može dovesti organizam u stanje u kome nema "isključivanja" prenosa stresnog signala i time izazvati alostatsko opterećenje. Takođe, smanjena sekrecija CRH koja je zabeležena kod pacova u dugotrajnoj izolaciji (Sánchez i sar., 1995) može biti razlog nepromenjenog nivoa CORT-a. U poređenju sa kontrolnim životinjama, povećani nivo CORT-a zabeležen je samo u IZ+IM grupi, dok IZ+Hlad nije izazvala promene ovog parametra. Kao što je pomenuto, Hlad kao blag stresor koji prevashodno deluje na SNS nije uspela da izazove odgovor poremećene HHA ose, dok je jači stresor IM uspeo da izazove odgovor, ali slabijeg intenziteta. Ovim je potrvđeno da hronični stres socijalne IZ, iako se karakteriše nepromenjenim nivoom CORT-a, ima maladaptivni efekat.

Deregulacija HHA ose ima značajnu ulogu u patologiji depresivnih poremećaja (Holsboer i Barden, 1996; Nemeroff i Vale, 2005). Kliničke studije su pokazale da kod pacijenata koji boluju od teških oblika depresije i psihotičnih stanja, deregulacija HHA ose nastaje usled neefikasnog mehanizma povratne sprege usled poremećene funkcije GR (Pariante i Miler, 2001) i povećanog osobađanja CRH koji drži HHA osu u aktivnom stanju (Holsboer i Ising, 2009; Vreeburg i sar., 2009). Međutim, treba imati u vidu da se stanja depresije ne karakterišu samo povećanim nivoom kortizola. Atipična depresija (povezana sa povećanim snom i apetitom), posttraumatski stresni poremećaj, sindrom hroničnog umora povezani su sa smanjenom koncentracijom cirkulišućeg glukokortikoida (Van Den Eede i sar., 2007; Krishnan i Nestler, 2008), što pokazuje da promene aktivnosti HHA ose u oba smera (hiper- i hipofunkcija) mogu dovesti do depresije.

5.2. Hronični stres socijalne izolacije dovodi do deregulacije mitohondrijske membrane u prečeonoj kori

S obzirom na to da se aktivacija HHA ose i promena nivoa glukokortikoida karakteriše povećanom produkcijom ROS-a (McEwen, 2001), antioksidativna zaštita mora da bi se organizam sačuvao od oštećenja koja mogu nastati u takvom okruženju. Akutni stres IM doveo je do povećanja ekspresije MnSOD proteina u mitohondrijskoj frakciji prečeone kore i hipokampusa. Međutim, iako se povećanje nivoa MnSOD i CuZnSOD (Filipović i Pajović, 2009) može smatrati protektivnim efektom, aktivnost CuZnSOD i MnSOD nije bila promenjena. Odsustvo korelacije između proteinske ekspresije i aktivnosti ovih enzima može biti zbog posttranslacionih modifikacija koje utiču na aktivnost enzima (Seo i Lee, 2004; Hopper i sar., 2006). Tokom akutnog izlaganja Hlad, iako zabeležen trend rasta, nivo MnSOD proteina u mitohondrijskoj frakciji obe moždane strukture nije bio statistički značajno povećan. Međutim, smanjena citosolna i jedarna CuZnSOD aktivnost kao i aktivnost mitohondrijske MnSOD u prečeonoj kori ukazuje na neadekvatnu antioksidativnu zaštitu koja je karakteristična za stanje oksidativnog stresa. Poznato je da izlaganje niskim temperaturama vodi ka ubrzavanju metabolizma (Krog i sar., 1955; Bravo i sar., 2001), što dalje prouzrokuje stvaranje ROS (Selman i sar., 2000; Blagojevic i sar., 2011), gde ove reaktivne vrste mogu inaktivirati SOD (Hodgson i Fridovich 1975; Pigeolet i sar., 1990). Ipak, dvočasovno izlaganje niskoj temperaturi u hipokampusu nije izazvalo intenzivne promene antioksidativne zaštite kao u prečeonoj kori. Smanjenje aktivnosti CuZnSOD u jedarnoj frakciji hipokampusa može ukazivati na neadekvatnu zaštitu DNK i drugih molekula koji se nalaze u jedru. Za razliku od prečeone kore, aktivnost CuZnSOD u citosolnoj frakciji kao i MnSOD aktivnost je u slučaju hipokampusa ostala nepromenjena i u skladu sa proteinskom ekspresijom, ukazujući da u je ovoj strukturi antioksidativna zaštita očuvana.

Iako je MnSOD klasifikovan kao isključivo mitohondrijski enzim, prisustvo ovog proteina detektovano je i u citosolnoj frakciji ispitivanih moždanih struktura kako kontrolnih (nestresiranih) tako i akutno stresiranih životinja. Odsustvo traka za COX-I i COX-IV u citosolnoj frakciji prečeone kore i hipokampusa kontrolnih životinja (Slika 13) ukazuje da postupak izolacije mitohondrijske frakcije nije uzrok prelaska MnSOD-a u citosol. Objašnjenje može ležati u činjenici da subjedinice MnSOD koje se sintetišu u citoplazmi, dobijanjem signalne sekvence prelaze u matriks mitohondrija gde u prisustvu Mn^{2+} formiraju aktivan

tetramer (Wispé i sar., 1989). Obzirom da odsustvo citohroma c u citosolnoj frakciji pokazuje očuvanost mitohondrijske membrane i fiziološko stanje ćelije, detektovni MnSOD u citosolnoj frakciji nestresiranih i akutno stresiranih životinja najverovatnije predstavlja proteine koji su "na putu" ka mitohondrijama (Jin i sar., 2005).

Hronični stres socijalne IZ nije doveo do promena u nivou MnSOD proteina u mitohondrijskoj i citosolnoj frakciji prečeone kore, međutim, zabeležena je smanjena aktivnost citosolnog i jedarnog CuZnSOD kao i mitohondrijskog MnSOD. Ovi podaci mogu biti očekivani obzirom da izlaganje stresnim situacijama tokom dužeg vremenskog perioda prouzrokuje povećano stvaranje ROS u mozgu (Lucca i sar., 2009), a oksidativni stres može izazvati neurotoksičnost koja se javljaju u neuropatološkim promenama (Bilici i sar., 2001; Michel i sar., 2007). Podaci sugerišu da oksidativni mehanizmi zastupljeni u patologiji psihijatrijskih poremećaja predstavljaju i ciljna mesta delovanja terapije (Sarandol i sar., 2007; Ng i sar., 2008). Iako je za nastanak ROS-a u stresu "krivac" povećan nivo GC, dobijeni podaci ukazuju da stanje oksidativnog stresa može postojati i u uslovima nepromenjenog CORT-a pa uzrok štetnog prooksidativnog stanja može biti u osnovi drugih procesa. Povećan nivo NO može biti odgovoran za strukturne i funkcionalne promene koje se dešavaju na mozgu (Bolaños i sar., 1997; Munhoz i sar., 2008). Tako usled reakcije NO sa $O_2^{\cdot-}$ dolazi do stvaranja peroksinitrita (Patel i Darley-Usmar, 1996) koji može dovesti do inaktivacije SOD putem nitracije (Nilakantan i sar., 2005). Takođe, uzrok smanjene aktivnosti SOD dodatno može biti nesinhronizovanost SOD aktivnosti sa aktivnošću ostalih antioksidativnih enzima, gde nagomilani ROS može dovesti do smanjenja aktivnosti antioksidativnih enzima hemijskom modifikacijom subjedinica (Pigeolet i sar.,1990). Dodatni akutni stres IM ili Hlad je u prečeonoj kori doveo do značajnog opadanja MnSOD u mitohondrijama i istovremenog povećnja nivoa ovog proteina u citosolnoj frakciji. Kako je u prečeonoj kori zabeležen izlazak citohroma c, koji se pod fiziološkim uslovima nalazi isključivo u mitohondrijama dok se u citosolu nalazi samo kao učesnik mitohondrijskog puta apoptoze, možemo tvrditi da je i povišeni nivo MnSOD proteina u citosolnoj frakciji životinja izloženih kombinovanom stresu posledica gubljenja integriteta mitohondrijalne membrane. Izlazak citohroma c može dovesti do promena na respiratornom lancu i daljem stvaranju ROS (Cai i Jones 1998; Cruthirds i sar., 2003) što je ogleda u smanjenoj aktivnosti CuZnSOD i MnSOD.

Sa druge strane, u hipokampusu nisu detektovane značajne promene u nivou mitohondrijskog i citosolnog MnSOD kao ni promene aktivnosti CuZnSOD i MnSOD u hroničnoj socijalnoj IZ. Opadanje aktivnosti MnSOD zabeleženo je tek nakon dodatnog akutnog stresa hronično izolovanih životinja, ali bez izlaska citohroma c. Ovo ukazuje da nema promena u integritetu mitohondrijske membrane, kao i na veću otpornost hipoakmpusa na oksidativni stresa nakon izlaganja hroničnoj socijalnoj IZ.

5.3. Smanjena aktivnost SOD u prečeonoj kori može biti posledica nitrozativnog stresa posredovanog povećanom ekspresijom iNOS proteina

U obe moždane strukture zabeležen je trend povećanja NO usled izlaganja stresnim uslovima. Obzirom da NO ne mora imati samo patološku ulogu već može između ostalog imati ulogu u komunikaciji između neurona, neuromodulatora (Rose, 2000; Sekaran i sar., 2005), potrebno je razlikovati da li NO potiče od aktivnosti konstitutivno eksprimirane nNOS ili inducibilne iNOS forme.

Osim stvaranja ROS, stres i GC mogu dovesti i do povećanog oslobađanja glutamata (Sapolsky, 2000) koji vezivanjem za NMDA receptore može aktivirati nNOS protein a na taj način nastao NO može imati ulogu neuromodulatora. U prečeonoj kori i hipokampusu akutno stresiranih životinja detektovan je povećani nivo nNOS proteina. Iako aktivnošću ove izoforme nastaju fiziološke količine NO, zabeleženo je statistički značajno povećanje NO u citosolu hipokampusa nakon izlaganja Hlad. Ovo može biti uzrok prelaska NF-kB iz citosolne u jedarnu frakciju budući da NO može aktivirati ovaj transkripcioni faktor (Lander i sar., 1993; Mühl i Pfeilschifter, 1995; Zouki i sar., 2001). Ipak, povećanje NO koji najverovatnije vodi poreklo od nNOS-a može se smatrati fiziološkim i protektivnim odgovorm (Oosthuizen i sar., 2005), obzirom da je aktivnost citosolnog CuZnSOD kao i MnSOD u mitohondrijama nakon izlaganja niskoj temperaturi u hipokampusu ostala nepromenjena.

Iako se u akutnom stresu smatra protektivnim odgovorom, povećana ekspresija nNOS-a nakon izlaganja hroničnom stresu povezuje se sa nastankom depresije (McLeod i sar., 2001), a visok nivo nitrita u krvnoj plazmi (Suzuki i sar., 2001) i nNOS ekspresija u hipokampusu zabeleženi su kod pacijenata koji su bolovali od depresivnog poremećaja (De Oliveira i sar., 2008).Takođe, pojedini antidepresivi koji se primenjuju u terapiji upravo deluju na NOS (Finkel

i sar., 1996; Wegener i sar., 2003). Stoga NO koji vodi poreklo od nNOS izoforme u obe moždane strukture nakon izlaganja hroničnom stresu socijalne IZ može biti povezan sa stanjem depresije. Međutim, hronični stres (bilo u grupi socijalne IZ ili kombinovanog stresa) ponovo je doveo do značajnih razlika u odgovoru između moždanih struktura. U prečeonoj kori je, pored povećanog nivoa nNOS, detektovan i povećan nivo iNOS-a, izoforme koja se može smatrati patološkom odlikom i obeležjem nitrozativnog stresa. Takođe, uočen je trend rasta NO, ali njegov nivo nije dostigao statističku značajnost. Obzirom da se aktivnost iNOS karakteriše produkcijom velike količine NO, razlog njegovog smanjenog nivoa može biti formiranje peroksinitrita usled povećane koncentracija $O_2^{\cdot-}$, na čiju oslabljenu eliminaciju indirektno može ukazivati smanjena aktivnost SOD-a u hroničnoj socijalnoj IZ (Fridovich, 1997). Na ovaj način formirani peroksinitrit može dalje inhibirati SOD aktivnost (Kamat, 2006; Mastrocola i sar., 2005; Nilakantan i sar., 2005), pa NO produkovan od strane iNOS-a može u prečeonoj kori doprineti nastanku oksidativnog stresa i imati citotoksičan efekat. Sa druge strane, u hipokampusu nastali NO može nastati jedino zbog povećane ekspresije nNOS-a. Otpornost hipokampusa na citotoksične efekte hronične socijalne IZ može biti posredovano aktiviranjem protektivnog mehanizma Hsp proteina. Povećana produkcija NO kao i sniženi nivo GSH (Zlatković and Filipović, 2013, Zlatković i sar., 2013) su ključni faktori u pokretanju Hsp70i odgovora (Hao i sar., 1999, Calabrese i sar., 2000) koji stabiliše kompleks NF-κB/IκB u citoplazmi, sprečavajući na taj način aktivaciju NF-κB i njegovu translokaciju u jedro gde može imati ulogu transkripcionog faktora za iNOS (Heneka i sar., 2000). Povećani nivo Hsp70i u citosolu i nepromenjeni nivo NF-κB u citosolu i jedru, ukazuju upravo da se NF-κB u hipokampusu nalazi u stabilnom kompleksu u citosolu i da na taj način Hsp70i štiti hipokampus od potencijalnih oštećenja. U prečeonoj kori, nivo ovog protektivnog proteina je značajno niži u odnosu na kontrolu pa se NF-κB nesmetano aktivira i translocira u jedro. Posledica tog signalnog puta može biti transkripcija iNOS gena, što se može indirektno zaključiti na osnovu povećane ekspresije ovog proteina.

5.4. Hronična socijalna izolacija dovodi do pokretanja proapoptotske signalizacije u prečeonoj kori ali ne i u hipokampusu pacova

Da bismo utvrdili da li stanje oksidativnog stresa izazvano izlaganjem hroničnoj socijalnoj IZ može imati citotoksični efekat i biti povezana sa apoptotskim dešavanjima u ćeliji posmatrani su

proteini koji su uključeni u unutrašnji (mitohondrijski) put apoptoze. Translokacija p53 proteina iz citosola u mitohondrije kao i preraspodela proapoptotskog Bax i antiapoptotskog Bcl-2 proteina na nivou mitohondrijske membrane vodi ka oslobađanju citohroma c iz mitohondrija i aktivaciji proapoptotske kaskade.

Izlaganje akutnim stresnim uslovima IM ili Hlad nije doveo do redistribucije p53 proteina izmedju citosolne i mitohondrijske frakcije u prečeonoj kori. Ipak, u citosolnoj frakciji hipokampusa detektovan je smanjeni nivo p53 nakon akutnog stresa IM. Obzirom da je njegov nivo u mitohondrijama ostao nepromenjen, može se pretpostaviti da je došlo do premeštanja iz citosola u jedro gde p53 ima ulogu transkripcionog faktora (Zhao i sar., 2000). Takođe, u obe moždane strukture, nivo citosolnog Bcl-2 i mitohondrijskog Bax-a ostao je nepromijenjen, osim blagog porasta mitohondrijskog Bax i njegovog paralelnog smanjenja u citosolnoj frakciji prečeone kore usled izlaganja akutnom stresu Hlad. Razlog za pokretanje preraspodele Bax može biti detektovano stanje oksidativnog stresa, uzrokovano inaktivacijom CuZnSOD i MnSOD, za koje je pokazano da može prethoditi apoptotskim događajima. Ipak, nepromenjena distribucija Bcl-2 proteina kao i nepromenjen Bax/Bcl-2 proteinski odnos ukazuje na to da je integritet mitohondrijske membrane ostao netaknut nakon akutnih stresora u obe moždane strukture. Ovo je dodatno potvrđeno i nepromenjenim nivoom citohroma c u citosolnoj frakciji. Iako je premeštanje Bax u mitohondrije označeno kao jedan od početnih događaja u unutrašnjem putu apoptoze, pokazano je da se Bax može prebaciti u mitohondrijalnu membranu i u odsustvu apoptoze, a da sama oksidacija Bax promoviše njegovu translokaciju (D'Alessio i sar., 2005)

Nasuprot tome, u hroničnom stresu socijalne IZ detektovan je statistički značajan porast Bcl-2 proteina u citosolnoj frakciji prečeone kore i njegovo istovremeno smanjenje u mitohondrijskoj frakciji dok je nivo Bax bio nepromenjen. Ovo ukazuje na smanjenje odnosa mitohondrijskog Bax/Bcl-2, koje je praćeno oslobađanjem citohroma c iz mitohondrija što predstavlja marker inicijacije mitohondrijskog apoptotskog puta. Iako je ranije ustanovljena značajna uloga premeštanja Bax-a u mitohondrijsku membranu, naši podaci ukazuju na zanimljivu činjenicu da proapoptotski događaji tokom 21-dnevne socijalne IZ mogu biti pokrenuti prvenstveno gubljenjem Bcl-2 iz mitohondrija. Ovo je u skladu sa podacima Tamatani-ja i sar. (1998) koji su ustanovili da apoptozu indukovanu NO karakteriše nishodna regulacija Bcl-2 koja je praćena oslobađanjem citohroma c iz mitohondrija u citosol. Sa druge strane, povećan NO nastao zbog povećane ekspresije nNOS u hipokamusu nije doveo do translokacije

p53 niti re-aranžmana Bax i Bcl-2 proteina. Ovi podaci mogu potvrditi pretpostavku da NO/nNOS ima protektivnu/fiziološku ulogu, dok je iNOS ″zadužen″ za ćelijska oštećenja koja su zabeležena u prefrontalnom korteksu.

Dodatni akutni stresni uslovi IM ili Hlad doveli su do izraženijih proapoptotskih promena u prečeonoj kori – povećan nivo NO pokrenuo je ulazak p53 u mitohodrije kao i značajnije povećanje Bax/Bcl-2 odnosa koje je praćeno je izlazakom citohroma c iz mitohondrija u citosol. Sa druge strane, proapoptotski događaji nisu pokrenuti u hipokampusu ni nakon dodatnog izlaganja akutnim stresnim uslovima. Iako je u kombinovanom stresu (IZ+IM i IZ+Hlad) došlo do porasta mitohondrijskog Bax-a, to je bilo praćeno i istovremenim rastom mitohondrijskog Bcl-2, pa je finalno njihov odnos bio nepromenjen. Ulazak p53 u mitohondrije kao i izlazak citohroma c iz mitohondrija u hipokampusu nije zabeležen. Ovi rezultati, zajedno sa odsustvom apoptoze koja nije detektovana TUNEL esejem (Filipović i sar., 2011) nedvosmisleno pokazuju da je, u odnosu na prečeonu koru gde je apoptoza potvrđena, hipokampus otporniji na delovanje hronoične socijalne IZ.

5.5. Promene aktivnosti superoksid dismutaze u jetri nakon izlaganja akutnim, hroničnim i kombinovanim stresnim uslovima

Glukokortikoidi koji se oslobađaju tokom odgovora na stres u glavnom perifernom "ciljnom" organu, jetri, pokreću anaboličke procese (Rizza i sar., 1982; Lecocq i sar., 1964) koji iako omogućavaju normalno funkcionisanje organizma, mogu dovesti i do povećanja ROS (Rolo i Palmeira, 2006). Do danas je objavljen veliki broj studija koje se tiču aktivnosti SOD-a u celokupnom ćelijskom ekstraktu jetre, dok su podaci koji se tiču ćelijskih kompartmana malobrojni. Sahin i Gumuslu su u svojim studijama (2004; 2007a; 2007b) pokazali da životinje izložene hroničnoj imobilizaciji ili niskoj temperaturi imaju povišenu aktivnost CuZnSOD.

Akutni stresni uslovi IM ili Hlad kao ni hronična socijalna IZ nisu doveli do promena u aktivnosti citosolnog CuZnSOD-a. Međutim, poređenjem sa ranije detektovanom povećanom proteinskom ekspresijom (Filipović i sar., 2010) neslaganje promena ovih vrednosti mogu ukazivati na posttranslacione modifikacije (Furukawa i O'halloran, 2006). Blagi porast aktivnosti koji je zabeležen u jedarnoj frakciji usled izlaganja IM, a koji prati povećanu proteinsku ekspresiju, može ukazivati na viši nivo superoksid anjon radikala u ovom ćelijskom kompartmanu ali i efikasnu zaštitu DNK i drugih jedarnih makromolekula.

Međutim, uočena je veća osetljivost mitohondrija na izlaganje stresnim uslovima jer je osim snižene ekspresije MnSOD proteina koja je zabeležena usled izlaganja akutnom ili hroničnom stresu (Filipović i sar., 2010) značajno smanjena i aktivnost MnSOD, što može sugerisati ugrožen kapacitet detoksikacije u mitohondrijama. Obzirom da smo detektovali značajno viši nivo NO, a reaktivni metaboliti azota mogu biti povezan sa oštećenjem funkcije antioksidativnih enzima (Lawler i Song, 2002) postizanje stanja oksidativnog stresa može biti preko NO signalnog puta. NO lako difunduje u ostale kompartmane, pa tako i u mitohondrije, gde može biti uzrok inaktivacije MnSOD enzima nitrozilacijom. Smanjena aktivnost MnSOD vodi u ciklus dodatnog stvaranja ROS i dalje inaktivacije antioksidativne zaštite. Dodatni akutni stresni uslovi nakon hronične socijalne IZ nisu u mnogome promenili antioksidativnu zaštitu u jetri. Aktivnost CuZnSOD ostala je nepromenjena, dok je MnSOD imao značajno nižu aktivnost. Dodatno, hronična socijalna IZ povezana je sa gubitkom integriteta mitohondrijalne membrane i izaskom MnSOD proteina u citosol (Filipović i sar., 2010), što nedvosmisleno ukazuje na patološko stanje koje nastaje u ovom kompartmanu ćelije usled izlaganja hroničnom stresu socijalne IZ.

6. ZAKLJUČAK

Rezultati dobijeni u okviru ove doktorske disertacije ukazali su na specifične efekte koje stres ima na prečeonu koru, hipokampus i jetru pacova. Dok izlaganje akutnim stresnim uslovima ne dovodi do ozbiljnih oštećenja, značajne promene uočene na nivou prečeone kore usled izlaganja hroničnoj socijalnoj izolaciji ukazuju na patološko stanje gde dolazi do pokretanja proapoptotske signalizacije.

U skladu sa prethodno definisanim ciljevima disertacije došli smo do sledećih zaključaka:

1. Izlaganje akutnom stresu imobilizacije ili niskoj temperaturi prouzrokuje fiziološki odgovor HHA ose, a porast nivoa kortikosterona zavisi od vrste primenjenog stresa. Hronična socijalna izolacija, iako se karakteriše nepromenjenim nivoom kortikosterona, dovodi do poremećaja u aktivnosti HHA ose, što se ogleda u neadekvatnom odgovoru hronično stresiranih životinja na dodatni akutni stres.

2. Povećanje proteinskog nivoa MnSOD u mitohondrijskoj frakciji prečeone kore i hipokampusa usled izlaganja akutnom stresu imobilizacije predstavlja potrebu organizma za odstranjivanje ROS-a koji može nastati u uslovima visokog nivoa kortikosterona, dok je nepromenjena aktivnost MnSOD najverovatnije posledica posttranslacionih modifikacija koje mogu uticati na aktivnost ovog enzima. Izlaganje akutnom stresu niske temperature nije uticalo na proteinski nivo MnSOD, a smanjenje aktivnosti CuZnSOD i MnSOD u prečeonoj kori može nastati usled povećanog metabolizma na niskim temperaturama. Sa druge strane, hronična socijalna izolacija u prečeonoj kori dovodi do smanjenja mitohondrijskog a povećanja citosolnog nivoa MnSOD, opadanja aktivnosti mitohondrijske MnSOD kao i aktivnosti CuZnSOD u citosolu i jedru što ukazuje na značajan podatak da stanje oksidativnog stresa može postojati i u uslovima nepromenjenog CORT-a. U hipokampusu je detektvano sniženje MnSOD aktivnosti u kombinovanom stresu ali bez efekta na nivo MnSOD proteina.

3. Obzirom na nepromenjeni nivo CORT-a, uzrok nastanka oksidativnog stresa u prečeonoj kori može biti nitrozativni stres. Povećan nivo NO u hipokampusu može imati predominantno ulogu u fiziološkim procesima u ćeliji.

4. Gubitak integriteta mitohondrijske membrane i nastanak oksidativnog/nitrozativnog stresa u prečeonoj kori može biti prouzrokovano aktivacijom NF-κB praćeno povećanom proteinskom ekspresijom iNOS, gde povećan nivo NO, ulazak p53 proteina iz citosola u mitohondrije, porast mitohondrijskog odnosa Bax/Bcl-2 dovodi do pokretanja proapoptotske signalizacije. Povećana proteinska ekspresija Hsp70i u hipokampusu sprečava aktivaciju NF-κB štiteći hipokampus od proapoptotskih događaja usled izlaganja hroničnom stresu socijalne izolacije.

Dobijeni rezultati ukazuju da je prečeona kora osetljivija na izlaganje hroničnom stresu socijalne izolacije, koje u ovoj strukturi dovodi do značajnih promena koje utiču na ćelije i mogu voditi do apoptoze. Sa druge strane, u hipokampusu se pokreće protektivni mehanizam posredovan Hsp70i proteinom pa je stoga ova moždana struktura otpornija na hronično izlaganje socijalnoj izolaciji. Literaturni podaci pokazuju da hroničan stres dovodi do promena na nivou sinapsi, tj. reorganizacije i distribucije sinaptičkih vezikula i morfologije hipokampusa pre nego do ćelijske smrti (Magariños i sar., 1997; Blanchard i sar., 2001).

5. Povećani nivoi NO i smanjenja aktivnosti MnSOD u jetri pacova nakon izlaganja hroničnom stresu socijalne izolacije ukazuje na postojanje oksidativnog i nitrozativnog stresa pa se može se reći da periferija i centralni nervni sistem na hronični stres odgovaraju slično

Rezultati ove disertacije ukazuju na uzroke promena proteinske ekspresije i aktivnosti superoksid dismutaze kod pacova izloženih hroničnoj socijalnoj izolaciji. Iako se u stresu CORT i GC smatraju glavnim "krivcem" za nastanak ROS i oštećenjima koji oni prouzrokuju, naši rezultati ukazuju da je u uslovima hroničnog stresa i nepromenjenog nivoa CORT-a uzrok ovih promena nitrozativni stres. Takođe, tkivno-specifičan odgovor na hroničnu izolaciju koji je primećen u moždanim strukturama ukazuje na mnogo veću osetljivost prečeone kore u odnosu na hipokampus. Obzirom da 21 dan socijalne izolacije može predstavljati životinjski model za izučavanje depresije, dalji cilj istraživanja je ispitivanje efekata antidepresiva i antipsihotika na vraćanje ovih parametara na bazalne vrednosti, kako u obe moždane strukture, tako i u jetri, organu u kome se oni metabolišu. Takođe, dobijeni podaci mogu ukazati na potencijalna mesta delovanja antidepresivne terapije.

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8. BIOGRAFIJA

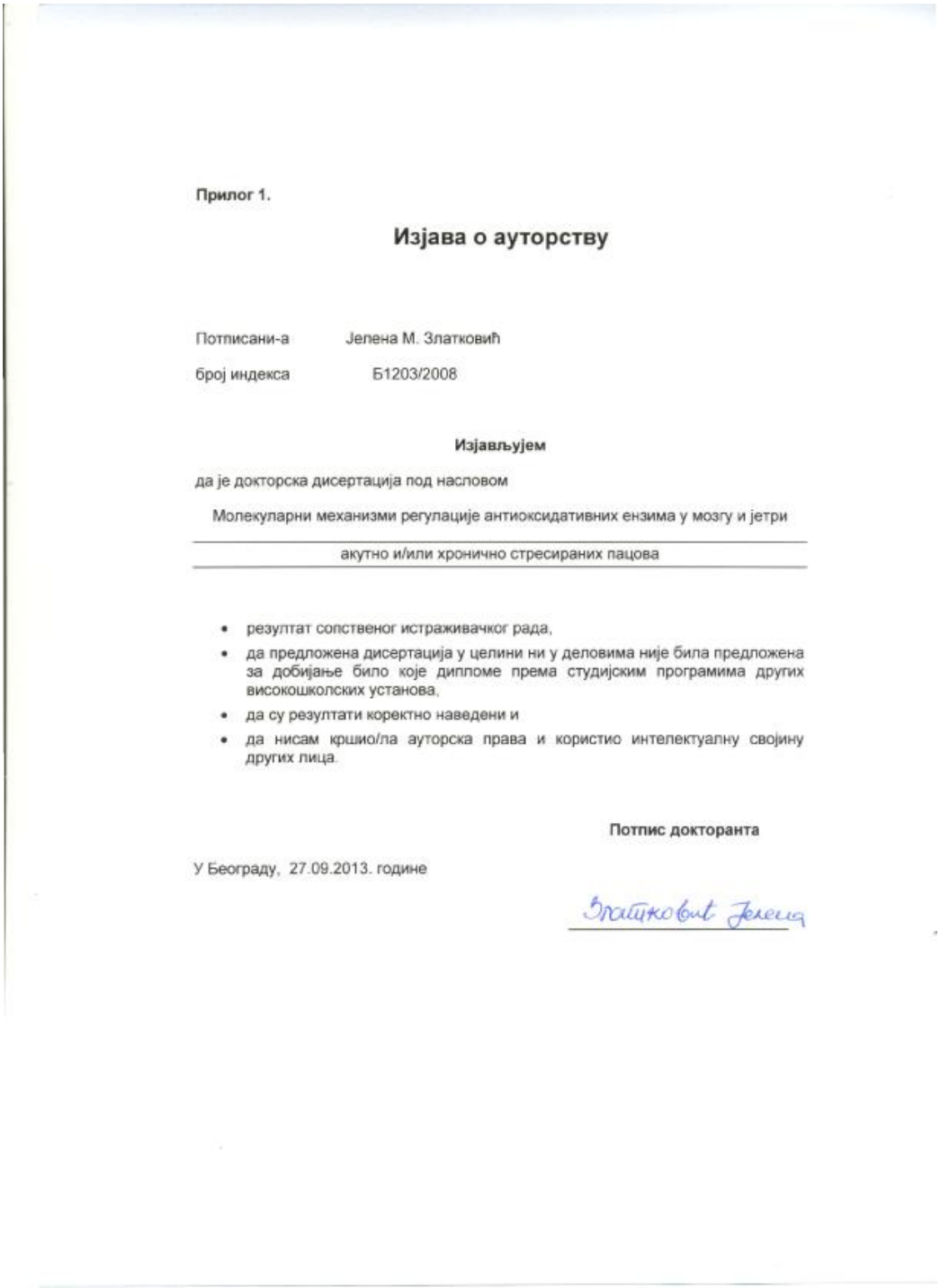
Jelena Zlatković je rođena 16.11.1981. godine u Pirotu. XIII beogradsku gimnaziju završila je 2000. godine i iste godine upisala je Biološki fakultet, Univerziteta u Beogradu, smer Molekularna biologija i fiziologija, odsek Eksperimentalna biomedicina. Diplomirala je 30.11.2007. godine sa srednjom ocenom 8.66 i ocenom 10 na diplomskom ispitu. Doktorske studije na Katedri za Neuronauke (modul Eksperimentalna neurobiologija: od gena do ponašanja) Biološkog fakulteta je upisala oktobra 2008. godine.

01.07.2008. godine je zaposlena kao istraživač-pripravnik u Laboratoriji za molekularnu biologiju i endokrinologiju 090, Instituta za nuklearne nauke "Vinča" na projektu No 143044B finansiranom od strane Ministarstva za nauku i tehnološki razvoj Republike Srbije u periodu 2006-2010. godine. Trenutno je angažovana na projektima Ministarstva prosvete, nauke i tehnološkog razvoja "Molekularni mehanizmi patofizioloških promena u ćelijama centralnog nervnog sistema i perifernog tkiva kod sisara" No 173044 pod rukovodstvom dr Anice Horvat i "Efekat metaboličkih i nemetaboličkih stresora na ekspresiju i delovanje neuroendokrinih regulatora energetske homeostaze" No 173023 pod rukovodstvom dr Jelene Đorđević, vanrednog profesora Biološkog fakulteta, Univerziteta u Beogradu. Zvanje istraživač saradnik stekla je 13.05. 2010.godine.

Naučno-istraživački rad Jelene Zlatković, iz oblasti neurobiologije odvija se u Laboratoriji za molekularnu biologiju i endokrinologiju 090, Instituta za nuklearne nauke "Vinča" pod mentorstvom dr Dragane Filipović, višeg naučnog saradnika. Istraživanja Jelene Zlatković su usmerena na ispitivanje molekularnih mehanizama koji leže u osnovi promene proteinske ekspresije i aktivnosti antioksidativnih enzima u mozgu i jetri laboratorijskih životinja izloženih hroničnoj socijalnoj izolaciji.

Autor je četiri i koautor četiri rada u vodećim međunarodnim časopisima, kao i brojnih međunarodnih i domaćih kongresnih saopštenja štampanih u celini i izvodu.

9. PRILOZI



Прилог 1.

Изјава о ауторству

Потписани-а Јелена М. Златковић

број индекса Б1203/2008

Изјављујем

да је докторска дисертација под насловом

Молекуларни механизми регулације антиоксидативних ензима у мозгу и јетри акутно и/или хронично стресираних пацова

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанта

У Београду, 27.09.2013. године

Златковић Јелена

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Јелена М. Златковић

Број индекса Б1203/2008

Студијски програм Неуронауке (модул Експериментална неуробиологија: од гена до понашања

Наслов рада Молекуларни механизми регулације антиоксидативних ензима у мозгу и јетри актутно и/или хронично стресираних пацова

Ментор др Драгана Филиповић, др Надежда Недељковић

Потписани/а Јелена М. Златковић

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанта

У Београду, 27.09.2013. године

Златковић Јелена

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић" да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Молекуларни механизми регулације антиоксидативних ензима у мозгу и јетри актутно и/или хронично стресираних пацова

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство
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(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанта

У Београду, 27.09.2013. године

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

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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 negligable 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 NH_2-terminal leader sequence by specific proteases (Wispe et al. 1989). In the presence MnSOD, superoxide radical (O_2^-) can be converted to the hydrogen peroxide (H_2O_2) which can then diffuse out of mitochondria in the cytoplasm. In the presence of high iron concentrations, H_2O_2 can form the highly reactive hydroxyl radical (HO) *via* the Fenton reaction. O_2^- can also react with nitric oxide to form the highly reactive peroxynitrite anion ($ONOO^-$), as it has been demonstrated in brain after stress (Olivenza

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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 ± 2°C, humidity 55 ± 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 ± 2°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 × *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 × *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 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 × *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 ± 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 ± 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

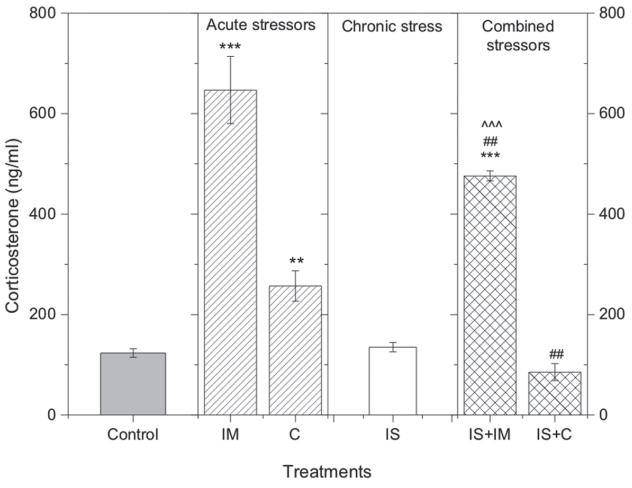

Figure 1. Changes in serum CORT levels (ng/ml) of adult Wistar male rats in unstressed control and rats exposed to acute immobilization (IM) or cold (C) stressors, chronic isolation (IS) or their combinations, as indicated. The results are expressed as mean ± S.E.M. of 6 animals per group. Symbols indicate 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) $^{\wedge\wedge\wedge}$ $p < 0.001$, 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 ($^{\wedge\wedge\wedge}$ $p < 0.001$). On the other hand, consecutive exposure to acute C did not significantly alter serum CORT level ($p > 0.05$) compared of 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 × 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).

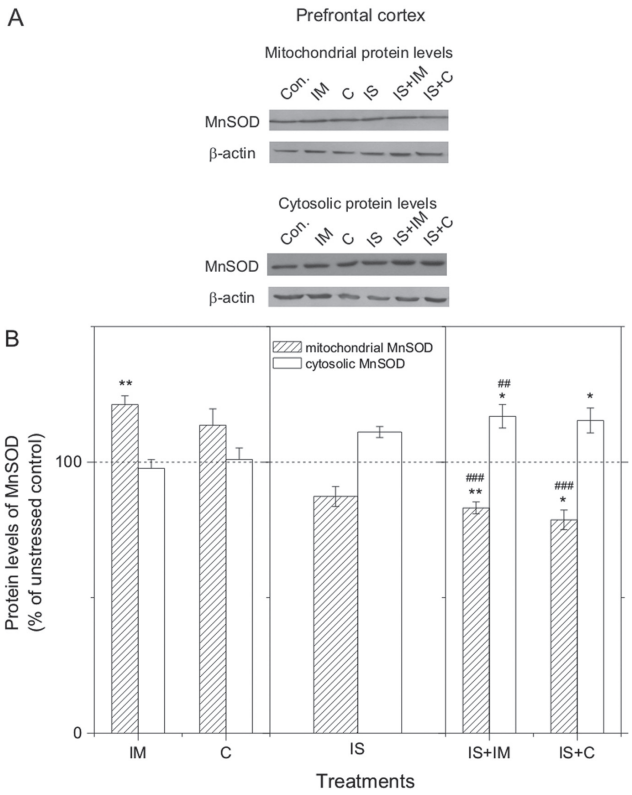

Figure 2. MnSOD protein level in mitochondrial or cytosolic fractions of the prefrontal cortex. **A.** Representative Western blots of MnSOD protein normalized to β-actin protein. **B.** Relative changes in MnSOD protein levels of rats exposed to acute immobilization (IM) or cold (C) stressors, chronic isolation (IS) or their combinations in both fractions, as indicated. The values are expressed as means ± 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

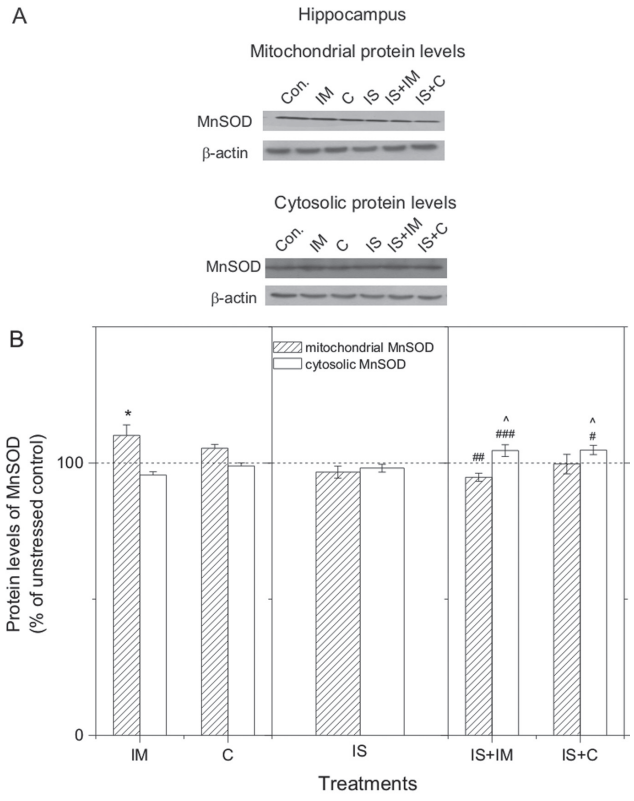

Figure 3. MnSOD protein level in mitochondrial or cytosolic fractions of the hippocampus. **A.** Representative Western blots of MnSOD protein normalized to β-actin protein. **B.** Relative changes in MnSOD protein levels of rats exposed to acute immobilization (IM) or cold (C) stressors, chronic isolation (IS) or their combinations in both fractions, as indicated. The values are expressed as means ± 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 × 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 reprobed 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

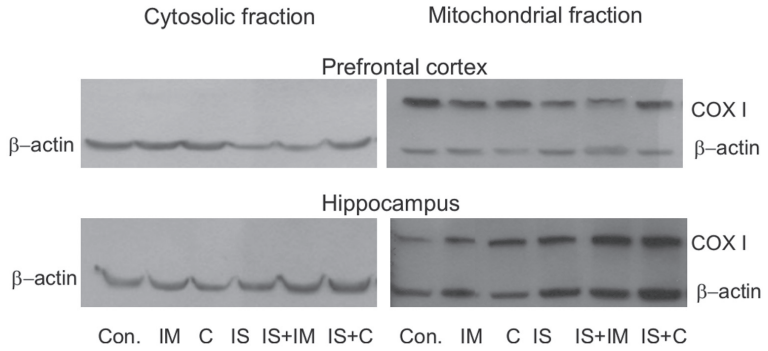

Figure 4. Cytochrome c oxidase subunit I (COX I) Western blots of mitochondrial or cytosolic fractions in unstressed control and all stressed groups from prefrontal cortex and hippocampus. β-actin was used as loading control. COX I was expressed in the mitochondrial fractions but not in the cytosolic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

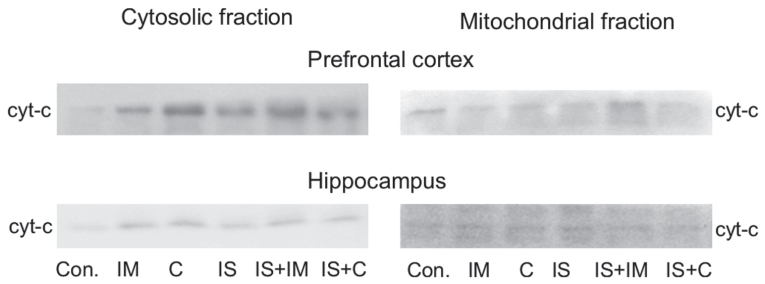

Figure 5. Cytochrome c (cyt-c) Western blots of mitochondrial and cytosolic fractions in unstressed control and all stressed groups from prefrontal cortex and hippocampus. cyt-c was detected in mitochondrial and cytosolic fractions in all stressed groups of both brain structures, whereas it was barely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

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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

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Mitochondria are central integrators and transducers of proapoptotic signals for neuronal apoptosis. The tumor suppressor protein p53 can trigger apoptosis independently of its transcriptional activity, through subcellular translocation of cytochrome c and caspase activation. To define better the proapoptotic role of p53 under various stress conditions, we investigated the protein levels of p53 and cytochrome c in mitochondrial and cytosolic fractions, as well as caspase-3 activation and apoptosis, in the prefrontal cortex and hippocampus of male Wistar rats subjected to acute, chronic, or combined stressors. Mitochondrial p53 can suppress the antioxidant enzyme MnSOD, so its activity was also determined. In the prefrontal cortex, but not in hippocampus, increased protein levels of p53 were found in mitochondria, leading to cytochrome c release into cytoplasm, activation of caspase-3, and apoptotic cell death following combined stressors. Decreased mitochondrial MnSOD activity following combined stressors in both brain structures indicated a state of oxidative stress. This suggests that chronic isolation stress compromises mitochondrial MnSOD activity in both the prefrontal cortex and the hippocampus but likely results in mitochondrial-triggered proapoptotic signaling mediated by a transcription-independent p53 mechanism only in the prefrontal cortex. Thus, our data demonstrate a tissue-specific (prefrontal cortex vs. hippocampus) response to applied stressors. © 2011 Wiley-Liss, Inc.

Key words: p53; proapoptotic proteins; MnSOD; stress; rat brain

Exposure of an organism to physical and/or physiological stressors leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis and the increased release of glucocorticoids (GCs; Herman et al., 2003). The effects of GCs are largely protective against the negative consequences of acute stressful situations, thus preserving homeostasis. Moreover, the acute stress response allows an organism to adapt to and overcome acute stressful situations. However, continued exposure to stressors and a prolonged stress response can have maladaptive consequences (Selye, 1956). Specifically, elevated levels of GCs during chronic stress may lead to an increase in the generation of reactive oxygen species (ROS) that can directly induce mitochondrial dysfunction, disruption of energy pathways (Papadopoulos et al., 1997), damage to neuronal precursors, and impairments in neurogenesis (Kroemer, 1997), thus inducing signaling events that result in apoptotic cell death (Cregan et al., 2002). Previous studies have shown that mitochondria and mitochondria-generated ROS contribute to the apoptotic process, whereby p53-mediated apoptotic signaling still remains an important target (Li et al., 1999; Dhar et al., 2006). The p53 is a tumor suppressor protein and transcription activator that can be activated upon various stress signals in neurons (Somasundaram, 2000; Ryan et al., 2001). After activation, p53 rapidly translocates from the cytoplasm to the mitochondria (Manfredi, 2003; Chipuk and Green, 2003; Murphy et al., 2004; Moll et al., 2005), causing changes in mitochondrial permeability that lead to the release of apoptotic proteins such as cytochrome c, activation of caspase 3 (Li et al., 1999; Marchenko et al., 2000; Erster et al., 2004), and

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eventually apoptosis (Liu et al., 1996; Green and Reed, 1998). Manganese superoxide dismutase (MnSOD) is an important antioxidant enzyme found in mitochondria, which catalyzes the dismutation of the highly reactive superoxide anion to molecular oxygen and hydrogen peroxide, protecting cells from damage induced by reactive oxygen species (ROS; Slot et al., 1986). Moreover, it has been shown that mitochondrial p53 interacts with MnSOD, leading to inactivation of its enzymatic activity and the propagation of oxidative stress (Holley et al., 2010). On the other hand, MnSOD can inhibit apoptosis by reducing the formation of superoxide radicals (Slot et al., 1986) and preventing the release of mitochondrial cytochrome c into the cytosol (Fujimura et al., 1999). Keller et al. (1998) have reported that neuronal apoptosis was significantly reduced in transgenic mice that overexpress MnSOD.

It has been postulated that the regulation of adaptive vs. maladaptive central nervous system (CNS) responses involves different cellular pathways (Chrousos and Kino, 2007). Given that the mitochondrial p53 signaling pathway may affect mitochondrial function, the present study was designed to determine whether 2 hr of acute immobilization or cold stress, 21 days of chronic isolation stress, or a combination of the two resulted in mitochondrial dysfunction, as measured by altered expression levels of p53 and cytochrome c in the mitochondrial and cytosolic fractions of rat prefrontal cortex and hippocampus. The consequence could be increased protein levels of p53 in the mitochondrial fraction and cytochrome c in the cytosolic fraction. Because the primary antioxidant defense enzyme, MnSOD, is located in the mitochondrial matrix, where p53 negatively regulates MnSOD activity, we postulated that mitochondria-localized p53 may target MnSOD and inhibit its superoxide scavenging activity. The presence of cleaved caspase-3 and apoptotic cell death was also investigated. Serum corticosterone (CORT) levels were monitored as an indicator of the stress response.

MATERIALS AND METHODS

Animal Treatments

Adult male Wistar rats (2–3 months old, body weight 330–400 g) were housed in groups of four per cage in a temperature-controlled environment (21–23°C) on a 12-hr/12-hr light/dark cycle, with food and water 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 comprised the unstressed controls (n = 6–8). Group II was exposed to one of two acute stressors, either 2 hr of immobilization (IM) or cold (C; 4°C; n = 6–8 per stressor). Group III was exposed to chronic isolation via individual housing for 21 days (IS; n = 6–8), according to the model of Garzon and del Rio (1981), in which animals had relatively normal auditory and olfactory experiences but no visual or tactile exposure to other animals. Group IV was exposed to chronic IS followed by a single 2-hr exposure to acute stress, either IM or C (4°C), representing the combined stressors (IS + IM, IS + C; n = 6–8, respectively). Experiments with acute stressors were performed between 8:00 and 10:00 AM, consistent with CORT circadian rhythmicity. Rats were exposed to IM by placing them into a prone position with all four limbs fixed to a board with adhesive tape. The head was fixed with a metal loop over the neck area to limit head movement (Kvetnansky and Mikulaj, 1970). Animals exposed to C stress were initially kept at an ambient temperature (20°C ± 2°C) and then carefully transferred to a cold room at 4°C. After the stress procedure, stressed animals and controls were anesthetized deeply with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and then sacrificed by guillotine decapitation (Harvard Apparatus, South Natick, MA). The brain was immediately removed, and the prefrontal cortex and hippocampus were dissected on ice. Tissue samples were frozen in liquid nitrogen and kept at −70°C until further use.

Serum CORT Assay

Trunk blood was collected, and the serum obtained by centrifugation at 1,500*g* for 10 min at 4°C was kept at −70°C until assayed. The OCTEIA Corticosterone ELISA kit (REF AC-14F1; Immunodiagnostics Systems; IDS) was used for measuring serum CORT levels (ng/ml) in all experimental groups. The variation between the samples was less than 7%. The lower detection limit for CORT in this assay system was 25 ng/ml.

Cytosol/Mitochondria Fractionation

To prepare cytosol and mitochondria tissue protein extracts, frozen brain prefrontal cortex and hippocampus were weighed and homogenized in 2 vol (w/v) of ice-cold homogenization buffer I (0.25 mol/liter sucrose, 15 mmol/liter Tris-HCl [pH 7.9], 16 mmol/liter KCl, 15 mmol/liter NaCl, 5 mmol/liter ethylenediaminetetraacetic acid [EDTA], 1 mmol/liter ethylene glycol tetraacetic acid, 1 mmol/liter dithiothreitol [DTT], 0.15 mmol/liter spermine, and 0.15 mmol/liter spermidine, supplemented with following protease inhibitors: 0.1 mmol/liter phenylmethanesulfonyl fluoride, 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 2,000*g* at 4°C for 10 min. The supernatant was centrifuged at 15,000*g* at 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* at 4°C for 20 min, and resuspended in 250 μl lysis buffer (50 mmol/liter Tris-HCl [pH 7.4], 5% glycerol, 1 mmol/liter EDTA, 5 mmol/liter 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. 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, St. Louis, MO) as a reference.

Electrophoresis and Western Blot Analysis

Equal amounts of cytosolic or mitochondrial protein fractions were separated on a 10% SDS-polyacrylamide gel using a Mini-Protean II Electrophoresis Cell (Bio-Rad, Hercules, CA) 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 p53 antibody (Stressgene Biotechnologies, Victoria, British Columbia, Canada) or anti-mouse cytochrome c antibody (6H2; Santa Cruz Biotechnology, Santa Cruz, CA). After washing three times in TBS-T, the membranes were incubated for 2 hr with anti-rabbit HRP-conjugated secondary antibody (No. 7074; Cell Signaling, Beverly, MA) or goat anti-mouse IgG (SC-2005; Santa Cruz Biotechnology). To confirm a consistent protein loading for each lane, the membranes were stained for β-actin (primary monoclonal anti-mouse β-actin antibody; A5316; Sigma; 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, United Kingdom) and exposed to an X-ray film. The signals were electronically digitized by scanning, and the image was processed for quantification in Image 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 53 kDa for p53, 15 kDa for cytochrome c, and 42 kDa for β-actin. The results are given as p53/β-actin and cytochrome c/β-actin ratios. The levels of p53 and cytochrome c in stressed animals are given as the percentage change relative to control rats (100%). The data are given as mean ± SEM of six to eight animals per group.

MnSOD Activity

Enzyme activity was measured using the commercially available Biorex BXC0531 kit for determination of superoxide dismutase activity (Biorex Diagnostics). This method is based on generation of superoxide radicals in xanthine and xanthine oxidase reactions, which further react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltrazolium chloride to form a red formazan dye. SOD activity is measured by the degree of inhibition of this reaction. Total SOD activity was first measured in the samples. The addition of KCN during the preparation of samples for the second round of measurements blocked CuZnSOD activity, so all measured activity derived from MnSOD.

Immunohistochemistry

Animals were anesthetized with an overdose of sodium pentobarbital as described above and then transcardially perfused with 0.9% saline solution, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. The brains were removed quickly and stored in fresh buffer containing 0.5% PFA. The brains were cut into coronal sections of 40 μm thickness on a vibratome (VT 100 S; Leica, Bensheim, Germany), and the sections were processed by using immunofluorescence. After three washes in PBS, pH 7.4, the sections were successively incubated with 5% donkey serum in PBS, pH 7.4, for 60 min. Sections were exposed to rabbit polyclonal antibody against cleaved caspase-3 (1:1,000; No 9661; Cell Signaling) at 4°C overnight, followed by incubation with Alexa-Fluor 555-conjugated anti-rat secondary antibodies (Molecular Probes, Invitrogen, Eugene, OR) for 2 hr at room temperature. After three washes in PBS, pH 7.4, the sections were mounted on gelatin-coated glass slides and coverslipped with fluorescent mounting medium (Dako, Glostrup, Denmark).

TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling (TUNEL) assay was performed by using the In Situ Cell Death Detection Kit POD (Roche, Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the sections were washed in 0.1 M PBS, pH 7.4, three times for 10 min at room temperature (RT) and then treated with 3% Triton X-100 in methanol for 10 min at RT. After washing several times in 0.1 M PBS, the sections were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) at 4°C, and nonspecific binding of the antibody was reduced by blocking in 3% BSA in 0.1 M PBS for 30 min at RT. Then, sections were incubated in TUNEL assay mixture containing terminal deoxynucleotidyl transferase (TdT) for 1 hr at 37°C in the dark. Sections were then mounted, and TUNEL-positive cells were detected with a fluorescence microscope.

Statistical Analysis

The 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, whereas for the chronic stress they were no stress and IS). The Duncan post hoc test was used to evaluate the differences between groups. Statistical significance was set at $P < 0.05$. All data are given as mean ± SEM.

RESULTS

HPA Axis Function After Different Types of Stress

To assess the impact of the stressors used here on the responsiveness of the HPA axis, serum CORT concentrations were measured in rats exposed to acute, chronic, or combined stressors (Fig. 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 ($F_{2,31} = 5.51$, $P < 0.001$) stress. Consistently with previous studies (Wren et al., 2002), the serum CORT concentration in the control group was 143 ng/ml. In the acutely stressed animals, IM acted as an extremely potent stressor, inducing a fourfold increase in CORT ($P < 0.001$), and C resulted in an approximately twofold increase ($P < 0.001$) compared with the control group. The serum CORT concentration in chronically isolated animals was similar to the control value ($P > 0.05$). However, chronically stressed animals exposed to

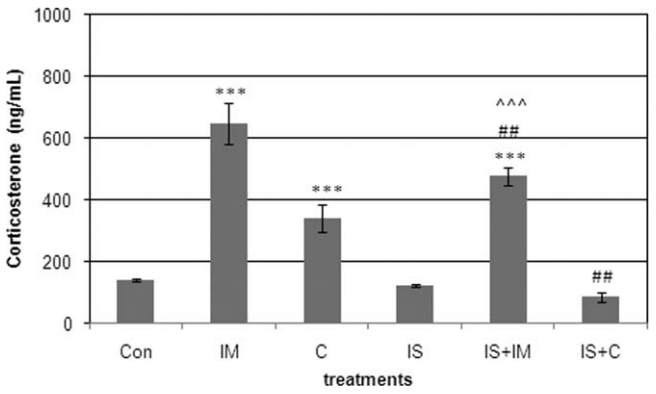

Fig. 1. Serum CORT concentrations in control rats (Con) and groups exposed to different stressors. Symbols indicate significant differences among 1) acute immobilization (IM) stress, acute cold (C) stress, combined stress isolation followed by immobilization (IS + IM), and control, $^{***}P < 0.001$; 2) combined IS + IM or IS + C stress and acute IM or C stressors, $^{\#\#}P < 0.01$; and 3) combined IS + IM stress and chronic isolation (IS) stress, $\hat{P} < 0.001$.

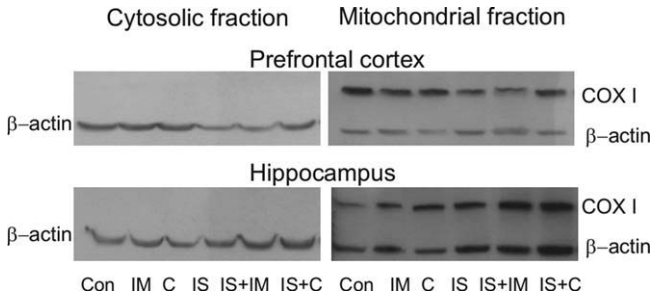

Fig. 2. Purity of the extracted cytosolic and mitochondrial fractions verified by COX I expression.

the novel acute stressor IM showed a significant increase of CORT compared with controls ($P < 0.001$) or chronic isolation alone ($P < 0.001$). In contrast, the exposure of chronically isolated animals to acute C stress did not significantly alter CORT concentrations compared with either controls or chronic IS stress alone ($P > 0.05$). The combined stressors evoked significantly lower CORT concentrations than the single acute stressors alone ($P < 0.01$).

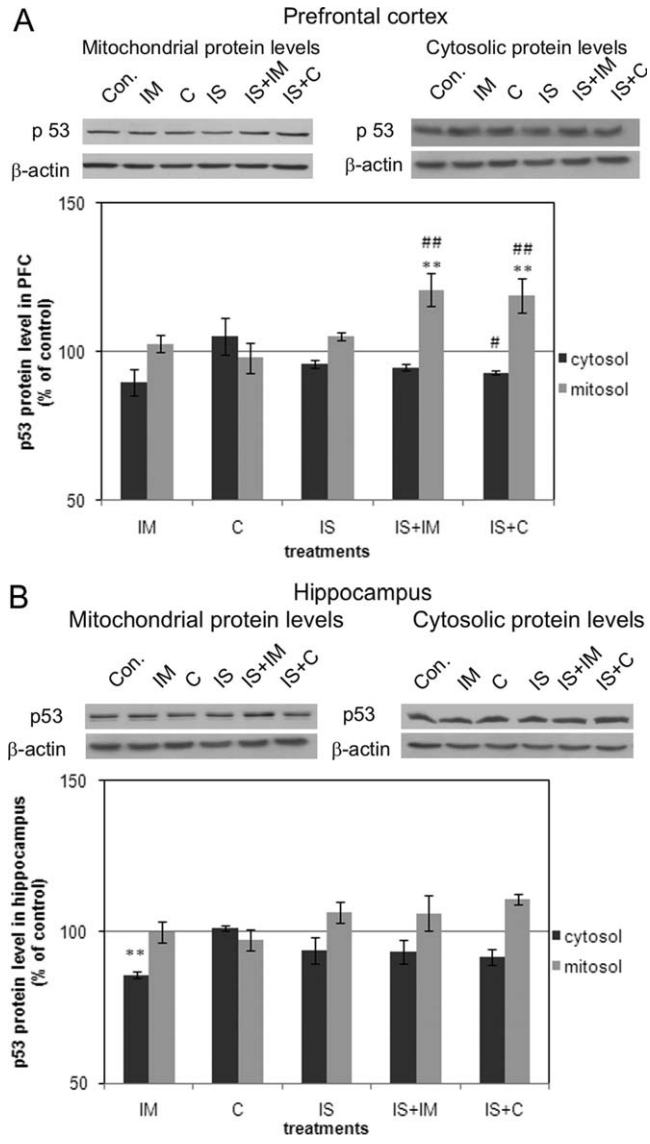

Fig. 3. Subcellular distribution of p53 in prefrontal cortex (PFC) (**A**) and hippocampus (**B**). Western blot analysis of p53 from the cytosolic and mitochondrial fractions in PFC showed a mitochondrial significant increase of p53 after combined IS + IM and IS + C stressors. Symbols indicate significant differences among 1) combined stressors and control, $^{**}P < 0.01$; 2) combined IS + IM, IS + C stressors, and acute IM or C stressors, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$.

Mitochondrial Translocation of p53 in the Prefrontal Cortex After Chronic Isolation Stress

To confirm the purity of our subcellular protein fractions, in particular the absence of mitochondrial contaminations of the cytosolic fractions, the PVDF membranes from mitochondrial and cytosolic samples were incubated with mouse anticytochrome c oxidase (COX) subunit I antibody (Molecular Probes, Invitrogen; 1:500). COX I is a mitochondrial marker tightly bound to the inner mitochondrial membrane (Jin et al., 2005). COX I was strongly expressed in the mitochondrial fraction, whereas no immunoreactive band was seen in the cytosolic fraction (Fig. 2).

p53 Immunoreactivity was evident as a single band with a molecular mass of 53 kDa in both cytosolic and mitochondrial fractions from the prefrontal cortex and hippocampus (Fig. 3). A two-way ANOVA of mitochondrial p53 protein levels revealed a significant effect ($F_{1,30} = 22.1$, $P < 0.001$) of chronic stress on mitochondrial p53 protein levels as well as an acute × chronic stress interaction ($F_{2,30} = 3.6$, $P < 0.05$) on cytosolic p53 protein levels in the prefrontal cortex.

A significant increase in mitochondrial p53 was seen in both groups of combined stressors (IS + IM or IS + C) compared with both the control group ($P < 0.01$) and the groups of single acute stressors ($P < 0.01$; Fig. 3A). At the same time, cytosolic p53 protein levels were decreased in combined IS + C stress compared with acute C stress alone ($P < 0.05$; Fig. 3A) in the prefrontal cortex. In the hippocampus, however, p53 immunoreactivity was not significantly different between the stressed groups, except in acute IM stress, in which cytosolic p53 protein levels were decreased compared with the control group ($P < 0.01$; Fig. 3B).

Release of Cytochrome c From Mitochondria to the Cytosol After Chronic Isolation Stress in the Prefrontal Cortex

The subcellular distribution of cytochrome c was confirmed by Western blot analyses as a single band of molecular mass 15 kDa in the cytosolic and mitochondrial fractions in the prefrontal cortex in control and treated groups (Fig. 4A). A two-way ANOVA revealed a significant effect of chronic IS stress on cytochrome c levels in both mitochondrial and cytosolic fractions in the prefrontal cortex ($F_{1,30} = 26.8$, $P < 0.001$, and $F_{1,32} = 53.8$, $P < 0.001$, respectively). Post hoc Duncan test showed a significant increase in mitochondrial cytochrome c protein level after acute IM stress compared with the control group ($P < 0.05$). Chronic IS stress induced a significant increase of the cytosolic cytochrome c protein level relative to the control group ($P < 0.01$). In animals exposed to combined stressors, a significant decrease of the cytochrome c protein level in the mitochondrial fraction compared with acute stressors ($P < 0.001$, $P < 0.01$) and controls ($P < 0.05$; Fig. 4A) was revealed. In contrast, cytosolic cytochrome c was increased after combined stressors compared with controls ($P < 0.001$) and acute stressors ($P < 0.001$), which confirms the release of cytochrome c from mitochondria into the cytosol (Fig. 4A). The presence of cytochrome c protein levels in the mitochondrial and cytosolic fraction of the hippocampus in control and treated animals was also confirmed (Fig. 4B). However, none of the applied stressors caused changes in cytochrome c protein levels in mitochondrial or cytosolic fractions of the hippocampus compared with controls ($P > 0.05$; Fig. 4B).

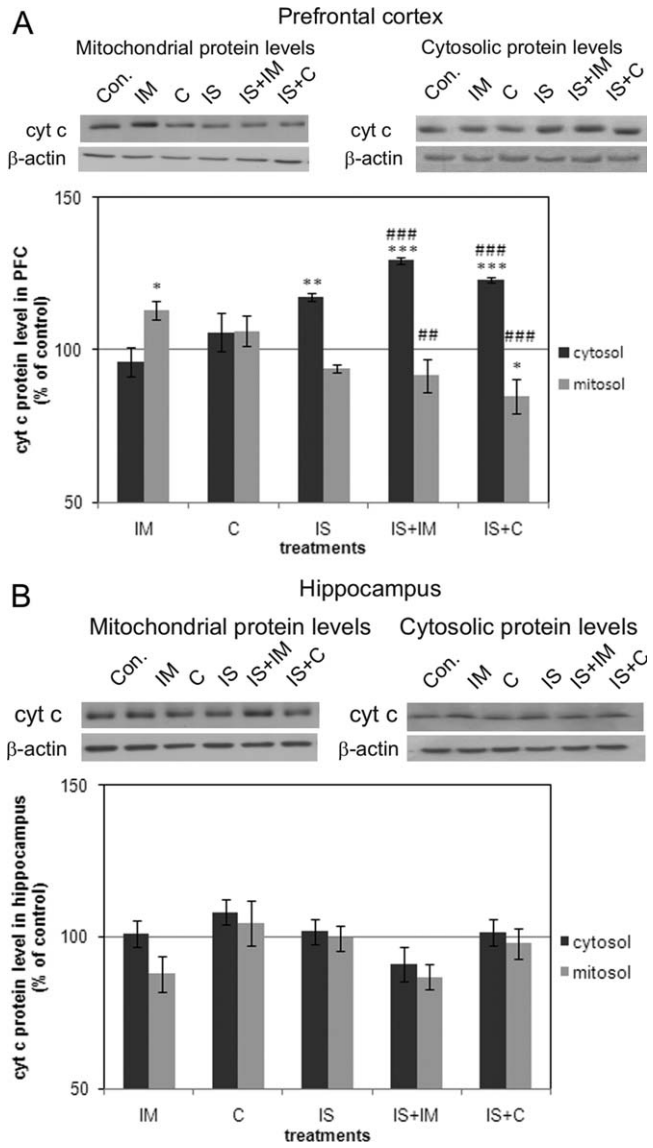

Fig. 4. Subcellular distribution of cytochrome c (cyt c) in PFC (**A**) and hippocampus (**B**). Western blot analysis of cytochrome c from the cytosolic and mitochondrial fractions in PFC showed its cytosolic-dominant distribution after chronic isolation (IS) and combined IS + IM, IS + C stress. Symbols indicate significant differences among 1) stress treatment and control, $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$; 2) combined stressors and acute stressors, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

Chronic Isolation Stress Compromises Mitochondrial MnSOD Activity in Both Prefrontal Cortex and Hippocampus

For the mitochondrial fraction of prefrontal cortex, a two-way ANOVA showed significant effects of acute ($F_{1,30} = 11.5$, $P < 0.001$) 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. Post hoc Duncan tests showed significant decreases in MnSOD activity in the acute C group ($P < 0.001$), the chronic IS group ($P < 0.001$), and both combined stressed groups (IS + IM, IS + C, $P < 0.001$), compared with the control group (Fig. 5A). Combined IS + IM stress resulted in significantly lower MnSOD activity than the single acute IM stress alone ($^{\#\#\#}P < 0.001$). Stress treatment (either acute or chronic) had no effect on MnSOD activity in the mitochondrial fraction of hippocampus (Fig. 5B). A two-way ANOVA showed a significant acute × chronic stress interaction ($F_{2,30} = 4.4$, $P < 0.05$) on MnSOD activity in the hippocampus. Duncan post hoc tests revealed statistically significant decreases in mitochondrial MnSOD activity of animals

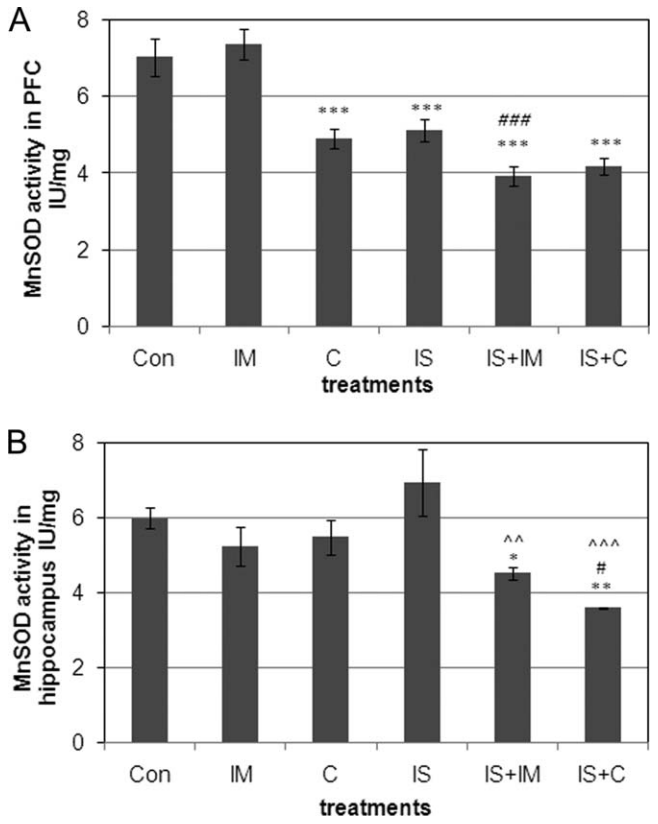

Fig. 5. Mitochondrial MnSOD activity in PFC (**A**) and hippocampus (**B**) of animals exposed to acute, chronic, or combined stress. Symbols indicate significant differences among 1) stress treatment and control, $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$; 2) combined stressors and acute stressors, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$; 3) combined IS + IM or IS + C stressor and chronic isolation (IS) $\hat{}P < 0.01$, $\hat{}\hat{}P < 0.001$, respectively.

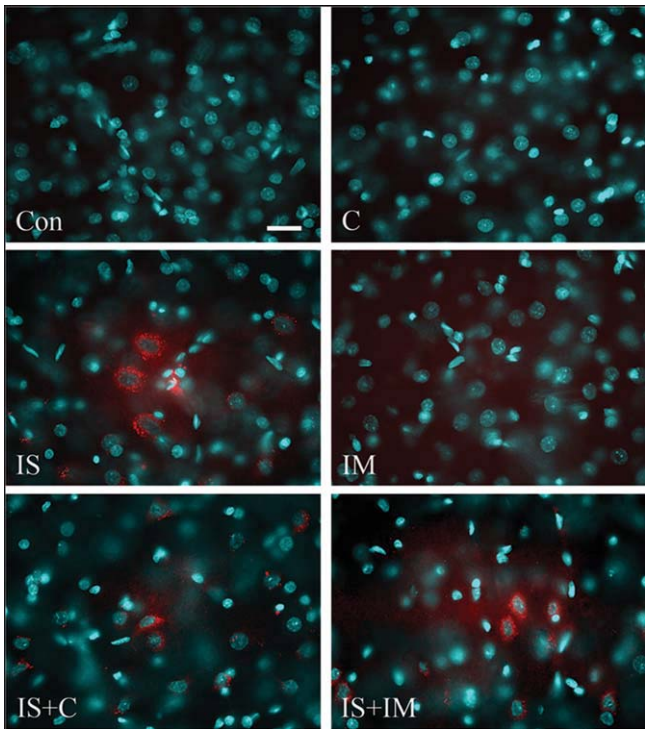

Fig. 6. Representative photomicrographs of fluorescent double staining of cleaved caspase-3 (red) and DAPI (blue) in control (Con), acute immobilization (IM) or cold (C) stress, chronic isolation (IS), and combined IS + IM or IS + C stress of prefrontal cortex. Cleaved caspase-3 staining was detected in the rat brain exposed to chronic IS stress and both combined IS + IM, IS + C stressors. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

exposed to either IS + IM or IM + C, compared with controls ($P < 0.05$, $P < 0.01$) or chronic isolation ($P < 0.01$, $P < 0.001$; Fig. 5B). Also, a significant decrease in MnSOD activity between combined IS + C stress and acute C stress alone in hippocampus was observed ($P < 0.05$).

Chronic Isolation Stress Triggered Cells To Undergo Apoptosis by Activating Caspase-3 in the Prefrontal Cortex but Not Hippocampus

To investigate further the proapoptotic events initiated by the release of cytochrome c, we carried out immunohistochemistry studies of cleaved caspase-3, a key downstream apoptotic protease that is extensively implicated in normal and pathological functions in the CNS, in control and treated rat brains. Immunohistochemistry experiments revealed that chronic IS stress, as well as the application of an additional acute stress, caused activation of caspase-3 in the prefrontal cortex (Fig. 6). We were not able to detect a cleaved caspase-3 in the hippocampus, so photomicrographs of fluorescent staining are not shown. To assess whether apoptotic cell death occurred under various stress conditions, TUNEL assays were performed on tissue sections from control, acute, chronic, and combined stressed rat brains. We found apoptotic cells in chronic IS stress and both combined stressors (IS + IM, IS + C) only in the prefrontal cortex (Fig. 7).

DISCUSSION

Mitochondrial dysfunction resulting from damage to the mitochondrial electron transport chain has been suggested to be an important factor in the pathogenesis of a range of neuropsychiatric disorders, such as bipolar disorder, depression, and schizophrenia (Bowling and Beal, 1995). Recent studies have shown that ROS generated from mitochondria can influence p53-mediated apoptotic signaling. Thus, in this study, we analyzed the subcellular distribution of p53 and cytochrome c protein levels in isolated cytosolic and mitochondrial fractions, mitochondrial MnSOD activity, activation of caspase-3, and apoptotic cells in the prefrontal cortex and hippocampus of male Wistar rats subjected to different types of stress. Animals subjected to acute IM or C stress exhibited significant increases in serum CORT concentrations, which represent normal activation of the HPA axis (McEwen, 1998). IM was found to be a strong

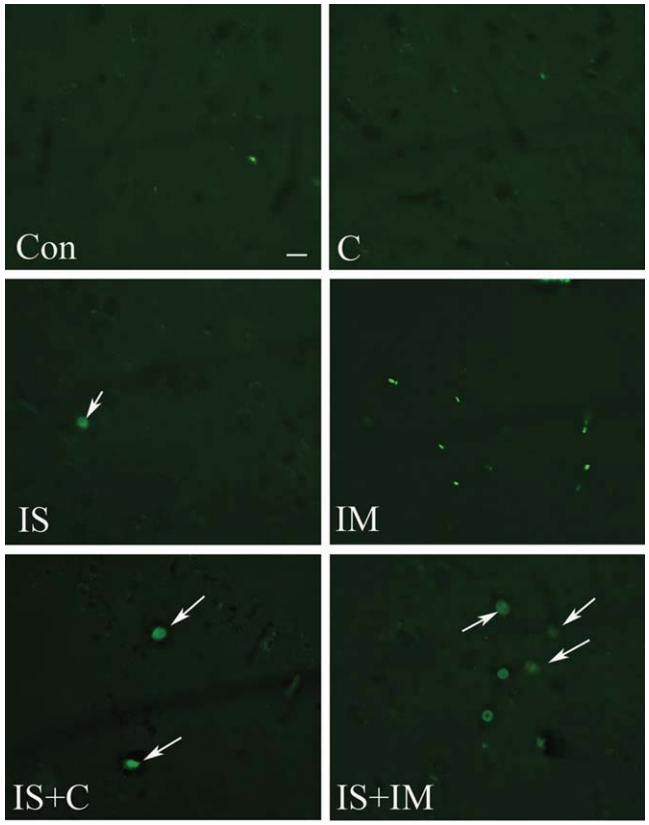

Fig. 7. Immunofluorescent photomicrographs of apoptotic cells evaluated by TUNEL assay in control (Con), acute immobilization (IM) or cold (C) stress, chronic isolation (IS), and combined IS + IM or IS + C stress of prefrontal cortex. TUNEL-positive cells were detected under chronic IS stress and both combined IS + IM, IS + C stressors. Arrows show positive green markings for apoptotic cells. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stressor, whereas C was found to be a mild stressor, as judged by serum CORT concentrations. The cytosolic and mitochondrial levels of p53 and cytochrome c remained unaltered by acute stressors in both the prefrontal cortex and the hippocampus, with the exception of a decreased level of cytosolic p53 without its translocation to mitochondria in the hippocampus and an increased level of mitochondrial cytochrome c after acute IM stress in the prefrontal cortex. It has been shown that increased levels of CORT after acute IM stress are directly proportional to increased levels of oxidative mediators (Pérez-Nievas et al., 2007). Thus, it seems tempting to speculate that, in response to acute IM stress, hippocampal p53 translocates to the nucleus, where it functions as a transcription factor (Zhao et al., 2000), whereas increased levels of mitochondrial cytochrome c without a corresponding increase in cytosolic levels could be a consequence of up-regulation of oxidative phosphorylation and protection against mitochondrial oxidative stress (Borniquel et al., 2006). MnSOD activity after acute stressors likely reflects a typical adaptive response to acute stress resulting in brain adaptation (i.e., restoration of homeostasis via oxidative balance). However, decreased MnSOD activity after acute C stress in the prefrontal cortex could partially be the consequence of inactivation by hydrogen peroxide (Pigeolet et al., 1990) generated by the catalytic reaction of MnSOD. It has been shown that exposure to environmental factors such as low temperatures might modulate the redox balance by activating ROS signaling (Blagojević, 2007). Although MnSOD activity is primarily protective, if not coupled to the respective peroxidase activity it may lead to the accumulation of toxic H_2O_2. Thus, the interaction of H_2O_2 with metal ions, such as Fe^{2+}, can result in the formation of hydroxyl radicals that are highly reactive and can suppress MnSOD activity (Pigeolet et al., 1990). We did not observe activation of caspase-3 as a potential regulatory candidate activating apoptotic signaling in rat brain in either the prefrontal cortex or the hippocampus. If we assume that p53, cytochrome c, and the activation of caspase-3 are proapoptotic, our findings suggest that either of the acute stressors employed here was unable to induce the specific subcellular alternations necessary for apoptotic signaling.

In contrast to acute IM stress, chronically stressed animals showed a significant decrease of mitochondrial MnSOD activity in the prefrontal cortex, suggesting that its detoxifying capacity was compromised by oxidative stress (Filipović et al., 2009). It is possible that chronic IS acting either directly or indirectly may shift the antioxidant/prooxidant balance toward a more prooxidant state, with more oxidative stress being produced in mitochondria in the prefrontal cortex. It has been shown that increased CORT levels, which may also occur during chronic stress, lead to a decline in antioxidative enzymes in rat brain, indicating a direct effect of CORT on the induction of oxidative stress (Pérez-Nievas et al., 2007; Zafir and Banu, 2009). The fact that chronic IS stress did not change serum CORT concentrations compared with the controls (Malkesman et al., 2006) may illustrate the mechanism underlying the glucocorticoid paradox, whereby a state of oxidative stress might also exist under CORT concentrations similar to basal values. Moreover, a prooxidant state could result, at least in part, from the sustained overproduction of nitric oxide (NO) caused by chronic stress, via the neurotoxic action of glutamate through N-methyl-D-aspartate (NMDA) receptors (Madrigal et al., 2001).

The transcription-independent induction of apoptosis by p53 involves cytochrome c release and caspase activation, suggesting that p53 has the capacity to engage the apoptotic pathway directly from the cytoplasm (Schuler and Green, 2001; Chipuk et al., 2004). Thus, cytosolic p53 can directly induce permeabilization of the outer mitochondrial membrane by forming an inhibitory complex with protective Bcl-2 family proteins, resulting in cytochrome c release and caspase activation triggering apoptotic cell death (Mihara et al., 2003; Chipuk et al., 2004). In our study, increased protein levels of cytosolic cytochrome c following chronic IS stress in the

prefrontal cortex suggest that chronic IS might induce proapoptotic signaling in the prefrontal cortex as a result of compromised mitochondrial membrane integrity and loss of mitochondrial function (Cruthirds et al., 2003). The absence of COX subunit I from the cytosolic fraction of controls showed that the mitochondria fractionation procedure itself was not responsible for the release of mitochondrial cytochrome c protein to the cytosolic compartment (see Fig. 2). Immunohistochemistry showed caspase-3 activation only in chronically treated rat brain compared with control and acute stressors, indicating that cytochrome c release together with subsequent activation of caspase-3 may form an essential component of neuronal apoptosis. Moreover, detection of apoptosis with TUNEL staining confirms an apoptotically mediated pathway induced by chronic IS in the prefrontal cortex. We have already reported unchanged mitochondrial MnSOD protein expression in the prefrontal cortex of animals exposed to chronic IS stress (Filipović et al., 2009). In this study, decreased mitochondrial MnSOD activity could be due to generated ROS (Pigeolet et al., 1990; Madrigal et al., 2001), because the release of cytochrome c from the mitochondria provokes changes in the respiratory chain that could result in the generation of superoxide anion, known to react rapidly with NO to form the stable peroxynitrite anion (Cai and Jones, 1998). Also, high levels of reactive nitrogen species such as nitic oxide (NO) and peroxynitrite have been shown to inhibit MnSOD activity, typically via nitration (Yamakura et al., 1998; Lawler and Song, 2002). Taken together, our results show that the prefrontal cortex appears to be more sensitive to chronic IS stress than the hippocampus; it exhibited increased levels of cytosolic cytochrome c, cleaved caspase-3 activation, decreased MnSOD activity, and apoptotic cell death.

A combined stress model, chronic IS followed by a single exposure to acute IM or C stress, was used to investigate the potentially maladaptive effects of chronic IS stress. Thus, when exposed to an additional acute stressor, the chronic IS group showed a lesser increase of CORT concentrations compared with acutely stressed animals, whereas additional acute IM stress produced a greater increase in serum CORT concentrations in chronically isolated rats compared with the acute C stress. This suggests that the response of the chronically stressed animals subjected to an additional acute stressor depends on the applied stressful stimuli (Pacak and Palkovits, 2001; Gavrilović and Dronjak, 2005). The decreased response of CORT concentrations to a novel challenge of chronically isolated animals may indicate that HPA axis activity was terminally compromised by the previous experience of chronic stress and could not be resumed after a subsequent acute stressor, as a consequence of impaired glucocorticoid receptor (GR)-mediated feedback inhibition in the hippocampus and prefrontal cortex (Filipović et al., 2005). In fact, we found previously that cytosolic protein levels of GR were significantly decreased after both acute stressors used here, whereas chronic IS stress led to negligible changes in GR and caused a reduced responsiveness to a novel acute stressor. Thus, incomplete nuclear translocation of GR and its cytosolic retention in chronic and combined stress may suggest that deregulation of the HPA axis induced by chronic IS stress results from a partial disruption of GR negative feedback control in the hippocampus and prefrontal cortex (Mizoguchi et al., 2003; Filipović et al., 2005). On the other hand, the proapoptotic signaling initiated by the chronic IS stress in the prefrontal cortex enhanced the proapoptotic response to subsequent acute stress, as indicated by increased p53 immunoreactivity in the mitochondrial fraction and mitochondrial cytochrome c released into the cytoplasm. Because we found cleaved caspase-3 expression and apoptosis after chronic IS, as well as with both combined stressors, these data confirm that chronic IS stress is responsible for triggering cell apoptosis. Moreover, a decreased mitochondrial MnSOD activity in the prefrontal cortex following combined stressors was a consequence of chronic induced oxidative stress, but, because both additional acute stressors led to cytosolic p53 translocation to mitochondria, p53-related inactivation of MnSOD could be a complementary pathway (Drane et al., 2001; Zhao et al., 2005; Holley and St. Clair, 2009). Nevertheless, in the hippocampus, an additional acute stressor did not lead to changes in p53, cytochrome c protein distribution, or caspase-3 activation, but MnSOD activity was decreased. (Filipović et al., 2009). Decreased MnSOD activity observed in this region could indicate oxidative stress without the redistribution of p53 and cytochrome c.

In summary, the present study provides evidence that chronic IS causes mitochondrial dysfunction in the prefrontal cortex. Compared with the hippocampus, the prefrontal cortex was more sensitive to chronic stress; it exhibited initiation of mitochondrial apoptotic signaling via relocation of p53, cytochrome c, activation of caspase-3, and apoptotic cell death. Hence, chronic stress may result in a reorganization of the distribution of synaptic vesicles and hippocampal morphology (Magariños et al., 1997; Blanchard et al., 2001) rather than cell death. Decreased mitochondrial MnSOD activity after chronic IS suggests that a state of oxidative stress may also exist under CORT concentrations similar to the basal value. Moreover, the neurotoxic action of glutamate through NMDA receptors has been implicated in the pathogenesis of stress-induced brain injury (Sapolsky, 1992), demonstrating that NMDA receptor activation in particular leads to the production of damaging NO radicals that contribute to cell death (Bowling and Beal, 1995; Leza et al., 1998; Olivenza et al., 2000) Thus, future work will characterize the possible role of NO in mitochondrial function caused by prolonged stress exposure.

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Stress-induced alternations in CuZnSOD and MnSOD activity in cellular compartments of rat liver

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Abstract Exposure to different stressors initiates generation of reactive oxygen species (ROS), which create harmful environment for cellular macromolecules. Superoxide dismutases (SODs) represent the first line of antioxidant defense. Hence, any alternation in their function might be potentially damaging. To better define the role of SODs, we investigated the CuZnSOD activity in cytosolic and the nuclear fraction as well as mitochondrial MnSOD activity in the liver of Wistar male rats after exposure to 2 h of acute immobilization (IM) or cold (4°C) stress, 21 days of chronic social isolation (IS) or their combination (chronic stress followed by acute stress). Serum corticosterone (CORT) was monitored as an indicator of the stress response. Acute IM stress, with elevated CORT level, led to increased hepatic CuZnSOD activity in the nuclear fraction. Chronic isolation stress, where CORT was close to control value, did not change the CuZnSOD activity either in nuclei or in cytosolic fraction, while combined stress IS+Cold led to increased cytosolic CuZnSOD activity. MnSOD activity in mitochondrial fraction was decreased in all treated groups. Data have shown that different stressors have diverse effect on hepatic CuZnSOD and MnSOD activity as well as on serum CORT level. Increased nuclear CuZnSOD activity after acute stress represents physiological response since the named activity protects cells against oxidative stress, while chronic IS stress compromises CuZnSOD function, suggesting an inefficient defense against ROS. Observed decrease of MnSOD activities indicate inadequate elimination of ROS after acute or chronic stress, which is characteristic of the oxidative stress.

Keywords Stress · CuZnSOD · MnSOD · Corticosterone · Liver

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Introduction

The living organisms in today's environment are excessively exposed to stress of various origin. Current research indicates that the most detrimental effects to an organism result from social and psychological factors. Also, the link between detrimental effects caused by oxidative stress and neuroendocrine response is not known. When an organism is exposed to acute stress, mechanisms that represent physiologic response to stress are activated in order to preserve cells from damage that can be caused. However, under conditions of chronic stress, the same systems could be dysregulated and, therefore, could promote and exacerbate pathophysiological processes [1, 2].

It is known that stress activates hypothalamic–pituitary–adrenal (HPA) axis, which results in an increased secretion of glucocorticoids (GCs) that affect not only brain but peripheral organs as well [3]. In their main peripheral target organ, the liver, GCs initiate anabolic processes of gluconeogenesis and glycogenesis [4, 5]. Although these high-energy-dependent processes are beneficial, they might lead to an increased generation of reactive oxygen species (ROS) [6], creating potentially harmful environment for cellular macromolecules. In order to protect it from oxidative damage, antioxidative system removes highly reactive ROS. One of the key enzymes is superoxide dysmutase (SOD) which catalyzes dismutation of superoxide anion radical (O_2^-) into oxygen and hydrogen peroxide (H_2O_2).

Copper–zinc SOD (CuZnSOD) is dimer predominantly located in cytosol, whereas tetrameric manganese SOD (MnSOD) represents mitochondrial isoform of enzyme. Importance of these enzymes is illustrated by the severe pathologies evident in humans and animal models that are initiated under conditions of altered SOD function [7–9].

GCs have been implicated as regulatory factors for antioxidative enzymes in peripheral tissues that express the glucocorticoid receptor [10, 11], and increased activities of the antioxidant enzymes have been observed in the liver of rats treated with GCs [12]. Concerning effects of various stressors such as exercise, cold or restrain stress on hepatic antioxidative enzyme activity, changes of total SOD could be found [13–17]. These data on total SOD are generalized, as does not indicate the changes of its specific isoforms, CuZnSOD and MnSOD. Hence, to better define the role of SOD under stress, we examined whether 2 h exposure to acute immobilization or cold stress, with or without previous 21 day exposure to social isolation could alter both CuZnSOD and MnSOD activity. Sahin and Gumuslu previously showed that animals exposed to chronic immobilization or cold stress displayed increased CuZnSOD activity in the liver [18–20]. Pajović et al. reported increased CuZnSOD and MnSOD activity in rat hippocampus following long-term isolation [21]. Up to date we have found no research conducted in order to examine discrete cellular compartments, only the ones referring to whole cell extract. Therefore, determination of CuZnSOD activity in cytosolic and nuclear fraction as well as mitochondrial MnSOD activity would provide more precise information about changes in enzymatic activity under stress conditions. Our laboratory has previously demonstrated that during chronic isolation stress there is an increase in oxidative stress in rat liver as evidenced by increased cytosolic CuZnSOD and decreased mitochondrial MnSOD protein levels [22]. To further elucidate the role of stress on SOD, our goal was to investigate whether the same stress conditions also changed CuZnSOD and MnSOD activities leading to the formation of a state of oxidative stress. Pearson's correlation test was used to examine whether serum corticosterone (CORT) level had an effect on SOD activity changes.

Materials and methods

Animal treatments

Adult male Wistar rats (2–3 months old, body weight 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, with food and water 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 comprised the unstressed controls ($n = 6$–8). Group II was exposed to one of two acute stressors, either 2 h of immobilization (IM) or 2 h of cold (4°C) ($n = 6$–8 per stressor). Group III was exposed to chronic isolation by individual housing for 21 days (IS, $n = 6$–8), according to the model of Garzon and del Rio [23], in which animals had relatively normal auditory and olfactory experiences but could no visual or tactile exposure to other animals. Group IV was exposed to chronic IS followed by a single 2 h exposure to acute stress, either IM or C (4°C), representing the combined stressors (IS+IM, IS+Cold, $n = 6$–8, respectively). Experiments with acute stressors were performed between 8:00 and 10:00 a.m., consistent with CORT circadian rhythmicity. Rats were exposed to IM by introducing them into a prone position with all four limbs fixed to a board with adhesive tape. The head was fixed with a metal loop over the neck area to limit head movement [24]. Animals exposed to cold stress were initially kept at an ambient temperature (20 ± 2°C) and then carefully transferred into a cold room at 4°C. Following the stress procedure, stressed animals and controls were sacrificed by guillotine decapitation (Harvard Apparatus, South Natick, MA, USA).

Serum CORT assay

Trunk blood was collected and the serum, obtained by centrifugation at 1,500×g for 10 min at 4°C, was kept at −70°C until assayed. The OCTEIA corticosterone ELISA kit (REF AC-14F1; Immunodiagnostics Systems-IDS, UK IDS) was used for measuring serum CORT levels (ng/ml) in all experimental groups. The variation between the samples was less than 7%. The lower detection limit for CORT in this assay system was 25 ng/ml.

Subcellular fraction preparation

After trunk blood collection, the livers were rapidly perfused in situ with ice-cold buffer A (20 mM Tris–HCl buffer pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 50 mM NaCl, 2 mM dithiothreitol (DTT)), excised, placed on ice, rinsed with ice-cold buffer, weighed, and homogenized in 4 vol (w/v) of buffer A supplemented with a protease-inhibitor cocktail (0.1 mM PMSF, 5 μg/ml aprotinin) by 8 strokes in a Potter-Elvehjem teflon–glass homogenizer. The pellets obtained after centrifugation of the homogenates (10 min, 2,000×g, 4°C, SS-34 Sorvall centrifuge) were used to prepare purified nuclei [25]. Briefly, the

crude nuclear pellets were resuspended in ice-cold buffer B (5 mM Tris–HCl pH 7.2, 2.2 M sucrose, 5 mM $CaCl_2$), layered over cold buffer B and centrifuged for 120 min in a Beckman SW 27 rotor at 24,000 rpm. The resulting pellet of nuclei was resuspended in cold buffer C (5 mM Tris–HCl buffer pH 7.9, 1 mM $MgCl_2$) washed three times in this buffer and the nuclei were kept at 4°C in the same buffer supplemented with 25% glycerol until use. The 2,000×*g*-supernatants were further centrifuged 20 min, at 12,000×*g*, 4°C in an SS-34 Sorvall centrifuge. The resulting mitochondrial pellets were washed by resuspension in homogenization buffer A, followed by additional centrifugation at 12,000×*g,* 4°C for 20 min and resuspended in 250 µl of 50 mM Tris–HCl lysis buffer pH 7.4 containing 5% glycerol, 1 mM EDTA, 5 mM DTT, supplemented with the above-mentioned protease inhibitors and 0.05% Triton X-100. The suspensions were incubated at 4°C for 30 min in an end-over-end mixer and centrifuged at 15,000×*g* for 20 min to eliminate insoluble material. The obtained supernatants were further centrifuged at 100,000×*g* for 60 min at 4°C in a Beckman L8-M Ultracentrifuge Ti50, to obtain the pure cytosolic fractions. The protein content in the nuclear, mitochondrial, and cytosolic fractions was determined by the method of Lowry et al. [26], using bovine serum albumin (BSA, Sigma-Aldrich) as the reference. The samples were kept at −70°C before analysis.

Purity of the subcellular fractions

Purity of obtained fraction was confirmed by western blot technique, using primary antibodies against proteins specific for appropriate cellular fraction. Control samples for hepatic nuclear, mitochondrial, or cytosolic protein fractions were separated on a 10% SDS polyacrylamide gel using a Mini-Protean II Electrophoresis Cell (BioRad Laboratories California, USA) and electrophoretically transferred to a poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Hercules, CA) using a Mini Trans-blot apparatus (BioRad, Hercules, CA). 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% nonfatted milk, and incubated overnight (4°C) with mouse anti-cytochrome *c* oxidase (COX) subunit I antibody (Molecular Probe 1:500, Invitrogen) for mitochondrial, anti-rabbit Histone H2B (Cell Signalling, #2722) for nuclear fraction, and primary monoclonal anti-mouse *β*-actin antibody (Sigma-Aldrich, A5316) for each fraction. Overnight incubation in primary antibodies was followed by 2 h membranes incubation in HRP-conjugated secondary goat anti-mouse IgG antibody (SC 2005) or goat anti-rabbit IgG (Cell Signaling, #7074). The blots were developed by enhanced chemiluminescence (ECL; Amersham) and exposed to an X-ray film. Protein lanes for specific mitochondrial and nuclear proteins were detected only in an adequate subcellular fraction (Fig. 2), which confirms purity of prepared samples. Detected *β*-actin signal in all three fractions confirms correct isolation and steady protein content in obtained fractions.

SOD activity

Enzyme activity was measured using commercially available Biorex BXC0531 kit for determination of superoxide dismutaze activity (Biorex Diagnostics Ltd.). 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 preparation of samples for second round of measurements blocked CuZnSOD activity, so all measured activity derived from MnSOD. After subtraction of these two values, CuZnSOD activity in samples was obtained. Protein concentrations of samples were chosen in order to ensure reaction linearity.

Statistical analysis

For data analysis Two-way ANOVA was used, following Duncan post-hoc test for analyzing difference of the serum CORT level and SOD activity between the animal groups. Results were represented as mean ± S.E.M. For statistical significance, *P* value of obtained results should be less then 0.05 ($P < 0.05$). Serum CORT and SOD activity correlation were performed using Pearson's correlation test.

Results

Serum CORT levels

Obtained results for serum CORT levels are presented in Fig. 1. Significant effect of acute ($F_{2,30} = 80.63$, $P < 0.001$), chronic ($F_{1,30} = 24.36$, $P < 0.001$), and an interaction of acute × chronic stress ($F_{2,30} = 5.12$, $P < 0.001$) was showed by Two-way ANOVA test. Animals exposed to acute IM stress showed increase of serum CORT level from 142.905 ng/ml, as measured in a control group, up to the 648.89 ng/ml (***$P < 0.001$), while cold stress showed less increase to the 342.112 ng/ml (***$P < 0.001$). In animals exposed to chronic isolation, serum CORT level was not significantly changed ($P > 0.05$), relative to control group. After appliance of novel acute IM stress in

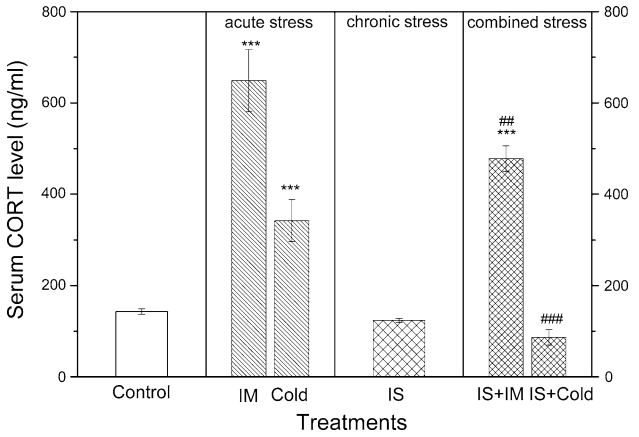

Fig. 1 Serum CORT level (ng/ml) in unstressed control and animals exposed to acute immobilization (IM) or cold stressors, chronic isolation (IS) or combinations of these stressors (IS+IM, IS+Cold). Duncan post-hoc test revealed significant increase in both acute IM ($^{***}P < 0.001$) and cold ($^{***}P < 0.001$) group as well as IS+IM ($^{***}P < 0.001$) compared to unstressed control group. Statistically lower CORT level in animals exposed to IS+IM ($^{\#\#}P < 0.01$) and IS+Cold ($^{\#\#\#}P < 0.001$) compare to those exposed to apropriate acute stress was detected

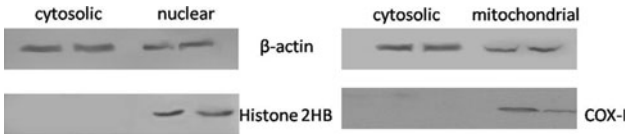

Fig. 2 Purity of the cytosolic, nuclear, and mitochondrial fractions. The results were obtained by western blot technique using specific antibodies against histone 2HB for nuclear and cytochrome *c* oxidase subunit I (COX I) for mitochondrial fraction. All protein fractions were obtained from control samples of rat liver. The data showed no presence of other protein fraction in control samples

chronically isolated animals, CORT level was 477.98 ng/ml, which is higher compared to control (142.905 ng/ml, $^{***}P < 0.001$), but lower than in cases when isolation did not precede acute IM (648.89 ng/ml, $^{\#\#}P < 0.01$). Additional cold stress did not significantly change serum CORT level ($P > 0.05$) compared to unstressed control. Significant decrease ($^{\#\#\#}P < 0.001$) was shown when animals exposed to combined stress were compared to those acutely stressed.

CuZnSOD activity

In cytosol fraction of rat liver, Two-way ANOVA showed effect of chronic stress on CuZnSOD enzymatic activity ($F_{1,30} = 8.766$, $P < 0.05$). Post-hoc Duncan test reported significant increase in SOD activity of IS+Cold group in comparison to the unstressed control ($^{**}P < 0.01$) and acutely stressed group ($^{\#\#}P < 0.01$) (Fig. 3a). Two-way ANOVA showed no effects of acute ($F_{2,30} = 1.7055$, $P > 0.05$), chronic ($F_{1,30} = 0.0009$, $P > 0.05$) nor acute × chronic ($F_{2,30} = 2.8524$, $P > 0.05$) stress on CuZnSOD activity in nuclear fraction of rat liver. Duncan post-hoc test revealed statistically significant increase of CuZnSOD activity in nuclear fraction of animals exposed to acute immobilization ($^{*}P < 0.05$) when compared to control group (Fig. 3b).

MnSOD activity

Two-way ANOVA showed significant effect of acute ($F_{2,30} = 3.857$, $P < 0.05$) and chronic stress ($F_{1,30} = 263.581$, $P < 0.001$) on MnSOD activity in mitochondrial fraction. Duncan post-hoc test revealed significant decrease in acute cold ($^{*}P < 0.05$), chronic, and combined stressed groups ($^{***}P < 0.001$) in comparison to the unstressed

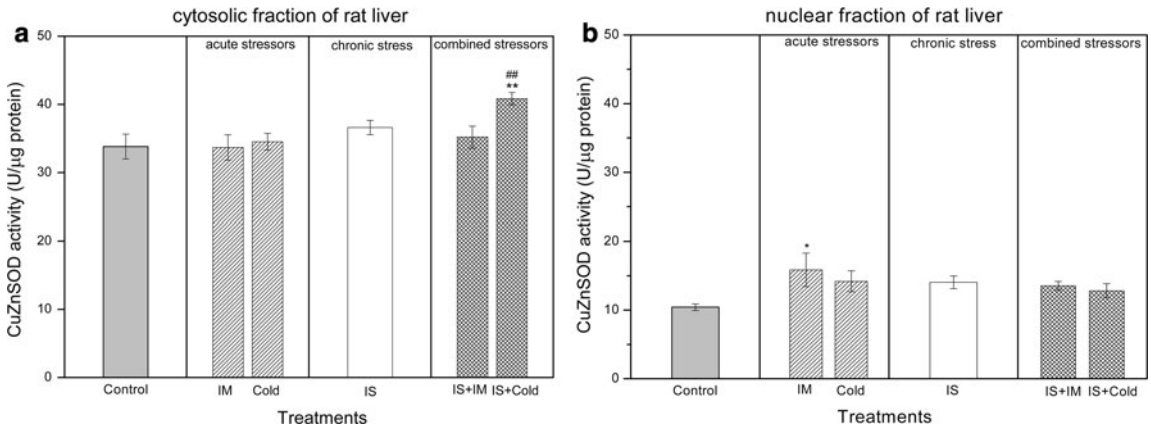

Fig. 3 Hepatic CuZnSOD activity in cytosolic and nuclear fractions of rats exposed to acute immobilization (IM) or cold stressors, chronic isolation (IS) or their combinations (IS+IM, IS+Cold). In cytosolic fraction (**a**), significant increase of CuZnSOD activity in IS+Cold group was compared to unstressed control group ($^{**}P < 0.01$), and acute cold stress ($^{\#\#}P < 0.01$) was confided by Duncan post-hoc test. In nuclear fraction (**b**), statistically significant increase of CuZnSOD activity in IM compared to unstressed control group ($^{*}P < 0.05$) was revealed

control group, as seen in Fig. 4. Also, animals exposed to combined IS+IM or IS+cold stress had lower MnSOD activity compared to those acutely stressed ($^{###}P < 0.001$).

Correlation analysis

Pearson's test reveal no correlation between serum CORT level and CuZnSOD activity in cytosol or nuclear fraction ($P > 0.05$). Positive correlation was found between CORT level and MnSOD activity in mitochondrial fraction of animals exposed to chronic isolation ($r = 0.964166$, $P < 0.01$) and those exposed to chronic isolation and additional cold stress ($r = 0.938281$, $P < 0.05$) (Fig. 5).

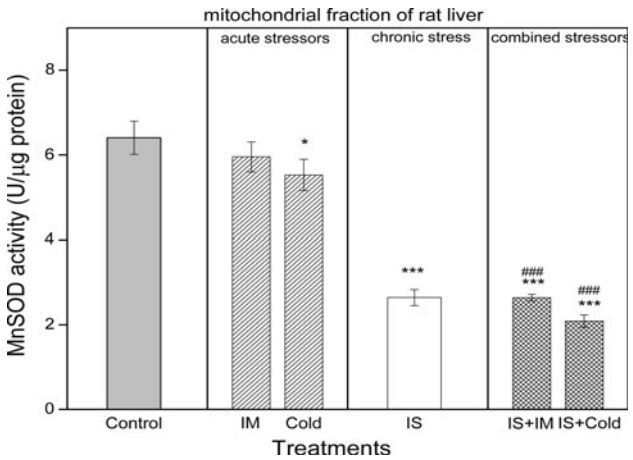

Fig. 4 Hepatic MnSOD activity in mitochondrial fraction of rats exposed to acute immobilization (IM) or cold stressors, chronic isolation (IS) or their combinations (IS+IM, IS+Cold). *Symbols* indicate, according to Duncan post-hoc test, significant decrease of MnSOD activity in animals exposed to 2 h of cold stress ($^{*}P < 0.05$), chronic IS ($^{***}P < 0.001$), and chronic IS followed by both IM and cold stress ($^{***}P < 0.001$) compared to the control group. Decreased MnSOD activity was also evident between acutely stressed animals (IM, Cold) and those previously exposed to chronic isolation (IS+IM, IS+Cold) ($^{###}P < 0.001$)

Discussion

Previous data have shown that acute, chronic, and combinations of these two stressors have different effect on CuZnSOD and MnSOD protein expression in liver subcellular fractions [22]. Since the lack of appropriate response to the high ROS might lead to formation of harmful cellular environment and consequently to pathological conditions, the present study was undertaken to investigate whether the same stressors alter CuZn- and MnSOD-enzymatic activities as well, giving the more precise depiction about antioxidative protection under these stress conditions.

Adequate stress response involves activation of HPA axis that results in an increased release of glucocorticoids (GCs). GCs during acute stress, because of mobilization of energy reserves and regulation of physiological processes, could mediate adaptation to stress and maintenance of homeostasis. Hence, it is emphasized that acute stress response has a protective effect on an organism [27, 28]. However, it is not clear whether chronic stress results in further elevations in circulating GCs levels or if there is some kind of adaptation in the release of GCs under chronic stress. Monitoring changes in the CORT level may serve as an indicator of stress response [19, 29]. In animals exposed to 2 h of acute stressors, significant increase in CORT levels was observed, indicating that animals were under stressful situation. Markedly elevated CORT value in IM group showed that this physical and psychological stressor [30, 31] has higher intensity than moderate cold stress, which should not be surprising as the immobilization has an influence on both adrenocorticotropic hormone (ACTH) and catecholamines, whereas effects of cold stress were seen only on noradrenaline levels [32]. In the present study, unchanged CuZnSOD activity in cytosol fraction in both acutely stressed groups was found, indicating that this enzyme mediates in restoration of homeostasis (redox

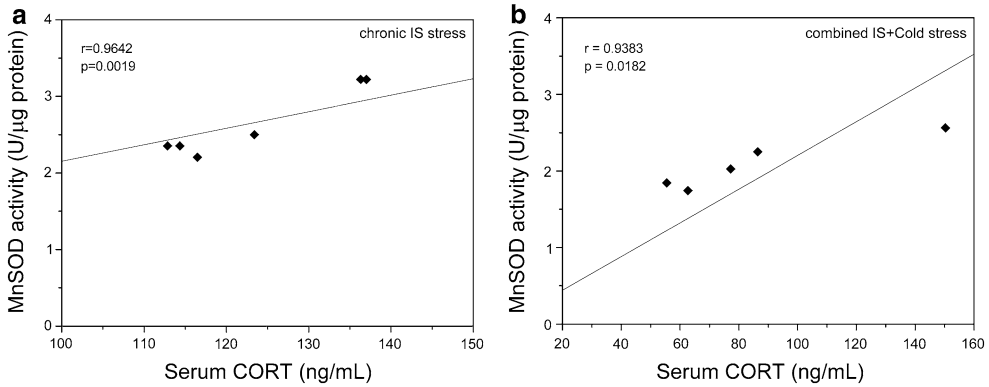

Fig. 5 Correlation between serum CORT level and mitochondrial MnSOD activity in animals exposed to chronic isolation stress (IS) (**a**) and chronic isolation followed by acute cold stress (IS+Cold) (**b**)

balans). In nuclear fraction of animals exposed to acute IM stress, CuZnSOD activity was increased. The high CuZnSOD activity may suggest increased superoxide anion levels, which was in accordance with its increased nuclear protein expression [22], indicating protective effect of CuZnSOD on DNA and other nuclear macromolecules. Concerning MnSOD activity in mitochondrial fraction, decreased activity in cold group compared to the control group was detected, which was followed by decreased protein expression in mitochondrial fraction [22]. This could have been expected since exposure to the low temperatures elevate metabolic rate in active tissues [33] and might modulate the redox balance by activating ROS signaling [34]. Therefore, the proximity of the main site for ROS generation (i.e. respiratory chain) might have an influence on lower MnSOD activity, enabling MnSOD inactivation by generated H_2O_2 [35]. This shows that MnSOD is not only the first line of antioxidative defense [22, 36, 37], but also the main target for generated ROS as well.

After rats' exposure to 21 days of social IS stress, when serum CORT returned to the basal level, CuZnSOD activities in cytosolic and nuclear fractions were unchanged when compared to the control group. Since the unchanged CuZnSOD activity did not correspond to increased protein expression under the same chronic IS stress [22], this could indicate the lack of effective CuZnSOD response. Discrepancy between protein expression and activity could, at least in part, appear because of post-translational modification, which could be independent of its protein synthesis [38]. Moreover, although CuZnSOD activity is primary protective, if it is not matched with corresponding peroxidase activity of catalase or glutathione peroxidase [39], it may lead to the SOD-driven accumulation of toxic H_2O_2 causing prooxidative state in the liver. Several studies have demonstrated that increased levels of CORT in chronic stress induce a decrease in levels of antioxidative enzymes, indicating a direct effect of CORT on the induction of oxidative stress [13, 15]. However, the effects of social isolation stress on CORT level in adult rats are not consistent among studies: increased CORT level has been reported [40], whereas other groups reported no changes [41, 42] or reduced CORT level [43]. In our study, chronically stressed animals showed unchanged serum CORT level, relative to control, as well as decreased mitochondrial MnSOD, which was in accordance with its protein expression [22], suggesting that its detoxifying capacity was compromised by the oxidative stress [35]. Decrease of mitochondrial MnSOD activity is in positive correlation with CORT level (Fig. 5a), suggesting that the state of oxidative stress may also exist under unchanged CORT level compared to the control group. Alternative way for reaching the state of oxidative stress could be via nitric oxide (NO) signaling pathway, since it was reported that chronic immobilization increases activity of NO-synthase [44], and NO can promote superoxide formation from the respiratory chain [45]. Furthermore, reactive nitrogen species could be related with impaired antioxidant enzyme function [46].

In order to determine whether chronic stress affected the animal's ability to retain "normal" stress response, chronically stressed animals were subjected to additional 2 h of IM or cold stress. Chronically stressed animals showed a lesser increase in serum CORT level in response to novel acute stressors compared to acute IM or cold-stressed animals alone, which indicates that HPA axis activity was compromised by chronic isolation stress and could not be resumed after subsequent acute stressors [47]. Under these conditions, CuZnSOD activity in cytosolic and nuclear fraction was close to the control values, except in IS+Cold group, where increased activity was observed. Although cytosolic CuZnSOD protein expression was increased in animals exposed to combined stress [22], its unchanged activity confirms that chronic IS stress compromises CuZnSOD activity, which might lead to inefficient defense against ROS. Since CORT level in IS+Cold group remained unchanged compared to the control group, increased CuZnSOD activity may be a protective response to elevated levels of ROS that could arise from some other source [45, 46]. Again, assumption about alternative ROS source was supported by positive correlation in combined IS+Cold stress between unchanged CORT levels when compared to the control group and decreased MnSOD activity. In mitochondrial fraction of both combined stressed groups, there was a decrease in MnSOD activity, which closely followed the changes in its protein expression [17]. It should not be surprising, since combined stress was related to the loss of mitochondrial membrane integrity and MnSOD protein release into cytosol [14], which is more of a pathological than a physiological process.

Presented results show that different stressors exert a different degree of influence on hepatic CuZnSOD and MnSOD activity as well as on serum CORT level. In acute stress, when the serum CORT level was elevated, increased nuclear CuZnSOD activity was observed, protecting cells against oxidative stress. Opposite to that, chronic IS stress compromises the CuZnSOD function, judging by its unchanged activity after appliance of novel acute stress, suggesting inefficient defense against ROS. Moreover, decreased MnSOD activity observed in all stressed groups indicate compromised elimination of ROS, which is characteristic for oxidative stress. Probably, stress-related ROS increase in mitochondria alters enzyme activity, creating a harmful environment and, therefore, could be associated with pathological conditions. The data provide significant information about different consequences that stress has on

hepatic CuZn- and MnSOD-activity, and therefore, different susceptibility of cellular compartments to applied stressful stimuli.

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

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

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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

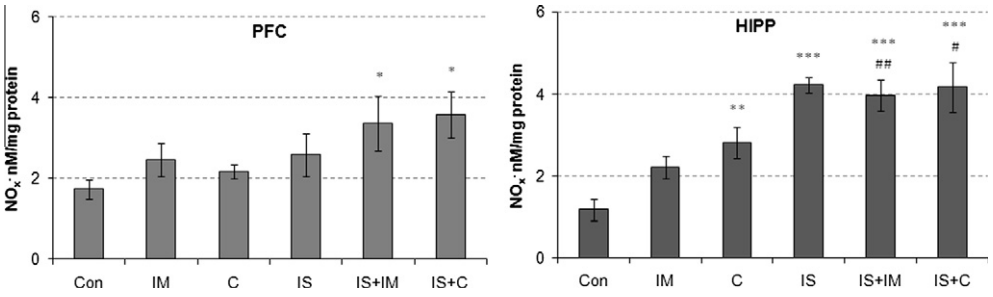

Fig. 1. The effect of acute immobilization (IM) stress, cold stress (C), chronic isolation (IS) and combined stressors (IS + IM or IS + C) on NO metabolites (NO_x^-) (nM/mg protein) in the prefrontal cortex (PFC) and hippocampus (HIPP). Data are presented as mean + S.E.M. Symbols indicate a significant 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.

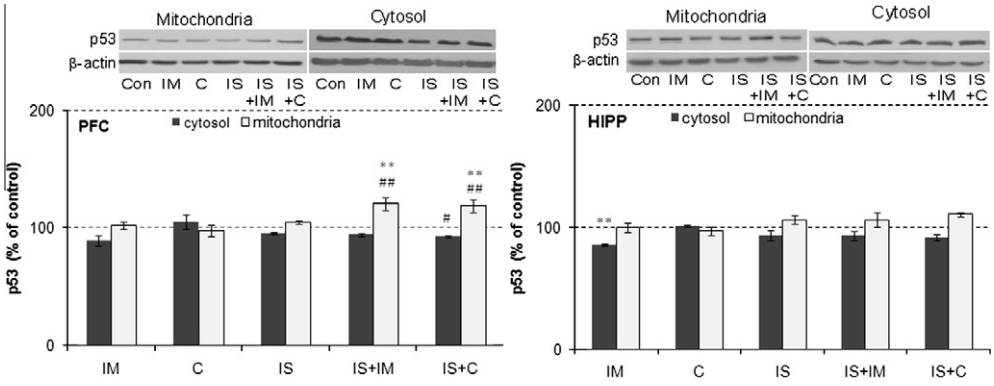

Fig. 2. Relative quantification of p53 protein level in the cytosolic and mitochondrial fraction of the prefrontal cortex (PFC) and hippocampus (HIPP) following acute immobilization (IM) stress, cold stress (C), chronic isolation (IS), and combined stressors (IS + IM or IS + C). Data are presented as 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.

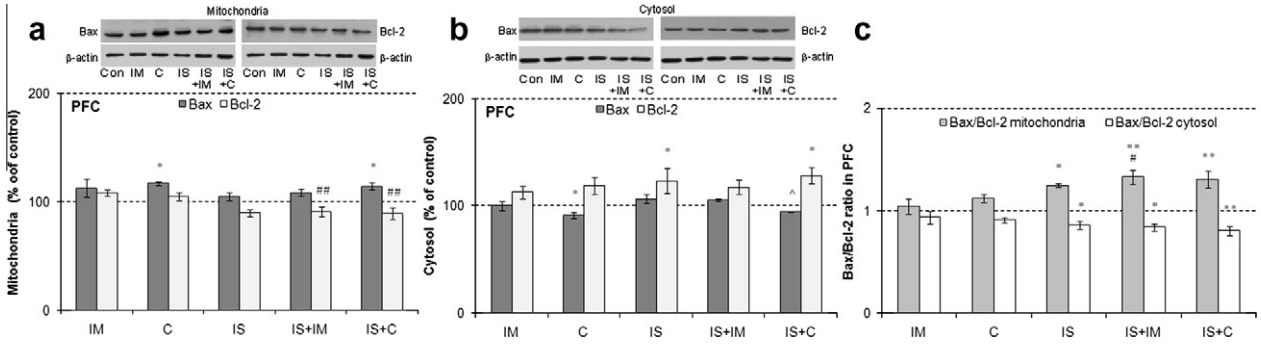

Fig. 3. Protein expression of Bax and Bcl-2 in the mitochondrial (3a) and cytosolic fraction (3b) and the Bax to Bcl-2 protein ratio (3c) in prefrontal cortex (PFC) following acute immobilization (IM) stress, cold stress (C), chronic isolation (IS), and combined stressors (IS + IM or IS + C). Symbols 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

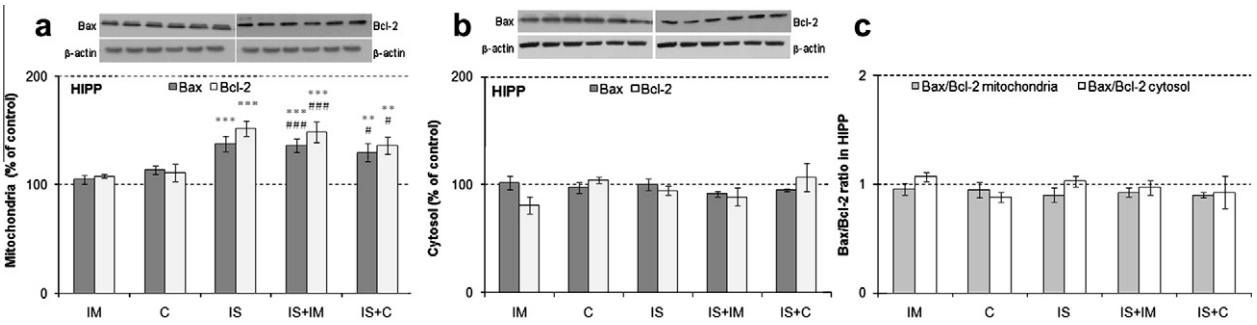

Fig. 4. Protein expression of Bax and Bcl-2 in the mitochondrial (4a) and cytosolic fraction (4b) and the Bax to Bcl-2 protein ratio (4c) in the hippocampus (HIPP) following acute immobilization (IM) stress, cold stress (C), chronic isolation (IS), and combined stressors (IS + IM or IS + C). Symbols 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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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.

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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.

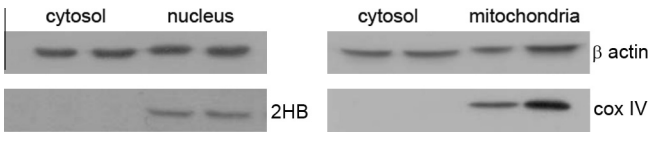

Fig. 1. Purity of the nuclear, mitochondrial and cytosolic protein fractions in the rat prefrontal cortex.

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-

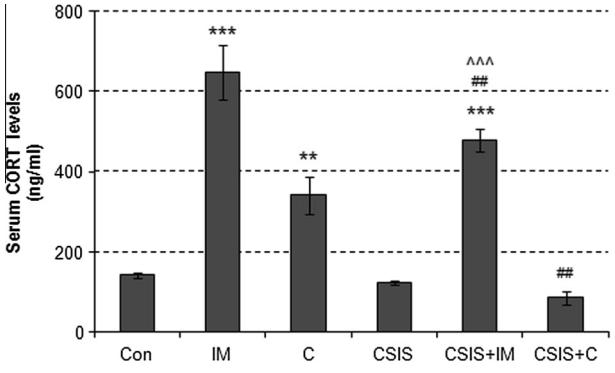

Fig. 2. Serum CORT level (ng/ml) concentrations in controls and following acute immobilization (IM) and cold (C) stress, chronic social isolation stress (CSIS), and combined stressors (SCIS + IM or SCIS + C). Asterisks indicate significantly differences between: respectively stress treatment and control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

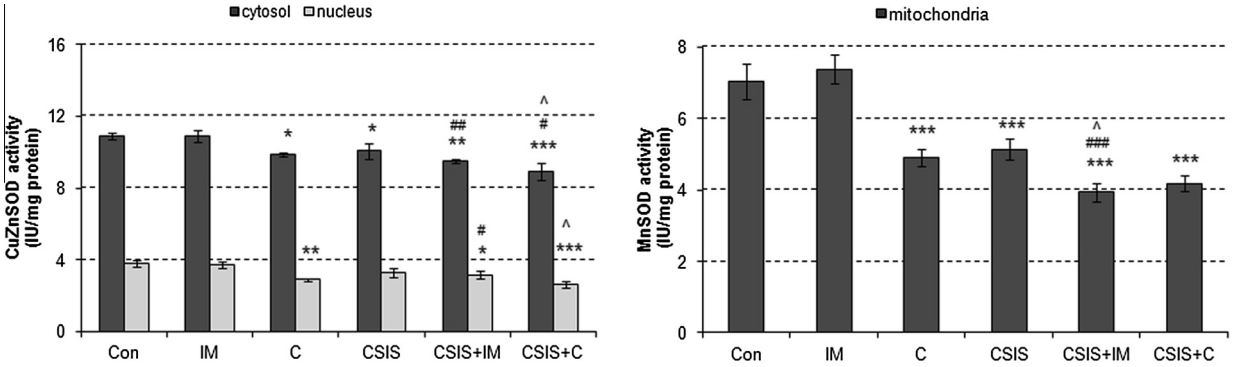

Fig. 3. Cytosolic/nuclear CuZnSOD and mitochondrial MnSOD activity (IU/mg protein) in the prefrontal cortex of control (Con) and following acute immobilization (IM) or cold (C) stress, chronic social isolation stress (CSIS) and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly 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.

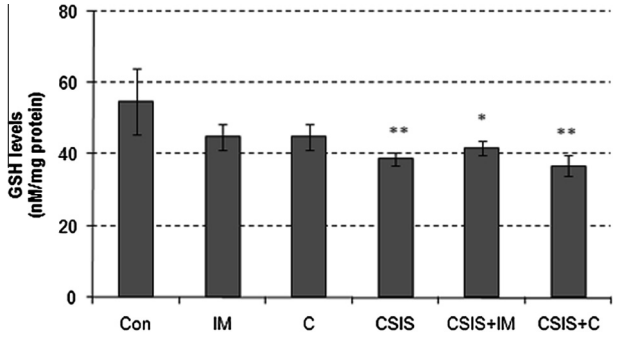

Fig. 4. GSH level (nM/mg protein) in cytosolic fraction of the prefrontal cortex in control (Con) and following acute immobilization (IM) or cold (C) stress, chronic social isolation stress (CSIS) and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly difference between respectively 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.

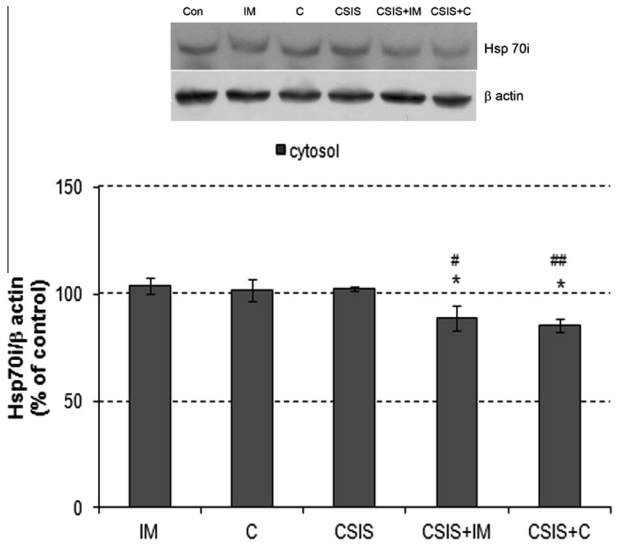

Fig. 5. Hsp70i protein levels in cytosolic fraction of the prefrontal cortex following acute immobilization (IM) and cold stress (C), chronic social isolation stress (CSIS), and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate a significant difference between: i) respective stress treatment 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

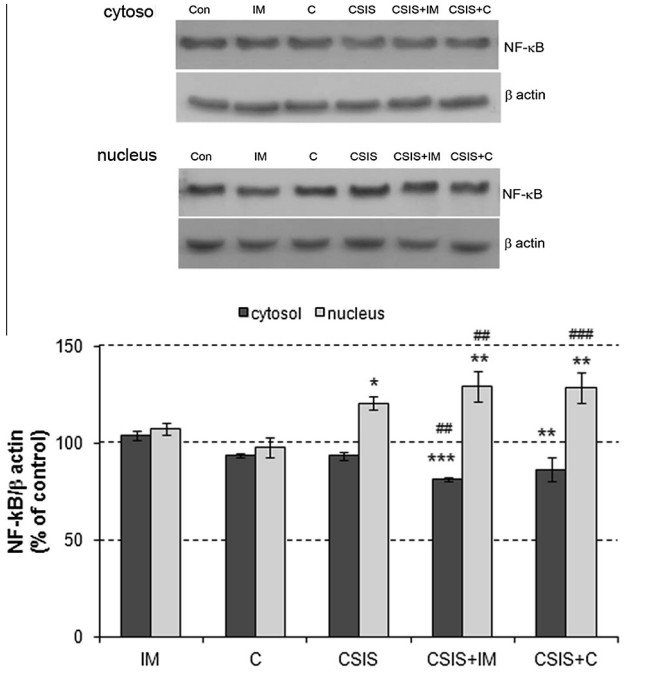

Fig. 6. NF-κB protein levels in cytosolic and nuclear fraction of the prefrontal cortex following acute immobilization (IM) and cold stress (C), chronic social isolation stress (CSIS), and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly differences between: respective stress 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

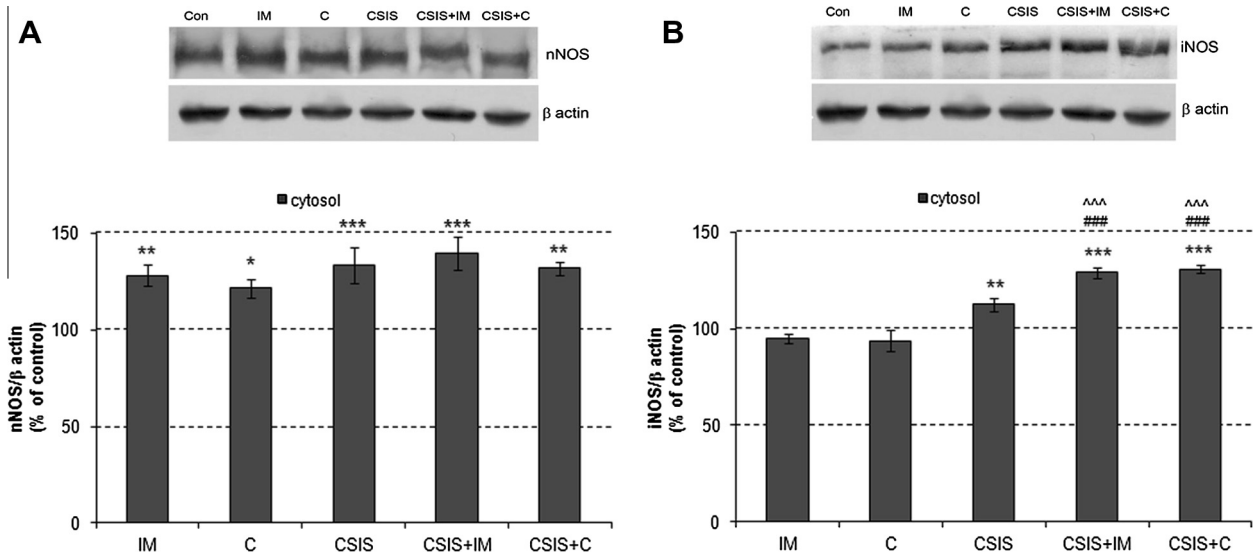

Fig. 7. Protein expression of nNOS (7A) and iNOS (7B) in cytosolic fraction of the prefrontal cortex following acute immobilization (IM) or cold stress (C), chronic social isolation stress (CSIS), and combined stressors (CSIS + IM or CSIS + C). Asterisks indicate significantly difference between respective 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.

Acknowledgments

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Protective effect of Hsp70i against chronic social isolation stress in the rat hippocampus

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Abstract Stress-related glucocorticoids and glutamate release has been implicated in depression. Glutamate neurotoxicity is mediated, in part, by the production of nitric oxide via nitric oxide synthase (NOS) isoforms and mitochondrial damage. We previously reported that chronic social isolation stress triggers proapoptotic signaling in the rat prefrontal cortex, but not in the hippocampus. Given that the hippocampus is highly sensitive to stress, we examined signaling cascades underlying the hippocampal cellular protection through the NOS pathway, antioxidant capacity and heat shock protein (Hsp) expression. We investigated neuronal (nNOS) and inducible (iNOS) protein levels, subcellular protein distributions of nuclear factor-κB (NF-κB), CuZnSOD and MnSOD activity, reduced glutathione (GSH), stress-inducible Hsp70 (Hsp70i) protein expression and serum corticosterone (CORT) levels of rats exposed to 21 days of chronic social isolation, an animal model of depression, alone or in combination with 2 h of acute immobilization or cold stress (combined stress). Both acute stressors elevated CORT, with lesser magnitude increase in chronically isolated rats exposed to novel acute stress as compared to acute stressors alone, indicating compromised HPA axis activity. Acute cold decreased nuclear CuZnSOD activity and stimulated NF-κB nuclear translocation. Chronic social isolation resulted in no activation of NF-κB, but led to decreased GSH, iNOS and increased nNOS and Hsp70i levels, alterations that remained following combined stressors. Decreased mitochondrial MnSOD activity after combined stressors suggests compromised detoxifying capacity. These data indicate that Hsp70i upregulation may provide hippocampal cellular protection against chronic social isolation stress mediated by downregulation of iNOS protein expression through suppression of NF-κB activation.

Keywords Chronic social isolation · Hippocampus · Hsp70i · NF-κB · Nitric oxide synthases

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Introduction

A physiological response to physical or psychosocial stress involves the activation of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in the release of glucocorticoids (GCs). The daily variation in GCs are critical for the homeostatic regulation of neural processes (De Kloet et al. 2005) and dysregulation of GCs, such as hyper- or hypoactivity of the HPA axis, plays a role in the development of anxiety and depression (Esch et al. 2002). The stimulation of the HPA axis by stress may enhance the production of reactive oxygen species (ROS) relevant to cytotoxicity (Kasckow et al. 2001). To neutralize ROS, cells use enzymatic superoxide dismutases (SODs) localized within specific cellular compartments, such as cytosolic copper–zinc superoxide dismutase (CuZnSOD) and mitochondrial manganese superoxide dismutase (MnSOD), as the first line of cellular defense against oxidative stress. SODs catalyze the dismutation of superoxide anion to oxygen and hydrogen peroxide, which is further detoxified by catalase

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and glutathione peroxidase (Fridovich 1995). It has been found that stress may impair antioxidant defenses, leading to oxidative damage (Liu and Mori 1994) whereby the extent of stress-triggered effects is related to the duration and type of stress (Pacak et al. 1998). We previously reported that acute immobilization stress increases cytosolic CuZnSOD and MnSOD protein levels, which may reflect preventive action against oxidative stress, while chronic social isolation compromises SODs protein levels (Filipović and Pajović 2009; Filipović et al. 2009). Moreover, chronic stress may also affect levels of reduced glutathione (GSH), the non-enzymatic component of antioxidant defense (Madrigal et al. 2001a; Ahmad et al. 2010). Importantly, some mood disorders are characterized by GSH depletion in the brain and the accumulation of oxidative and nitrosative damage (Pal and Dandiya 1994).

One factor that may link oxidative stress and brain damage is the redox-sensitive transcription factor nuclear factor-κB (NF-κB), which stimulates the expression of a variety of genes, such as inducible iNOS (Xie et al. 1994), neuronal nNOS (Hall et al. 1994, Li et al. 2007) and the CuZnSOD gene (Meyer et al. 1993). In the inactive state, the NF-κB complex is retained in the cytoplasm via interaction with the IkB protein family. Activation of NF-κB requires the dissociation of the NFkB–IkB complex, which causes translocation of the NF-κB-p65 protein to the nucleus (Baldwin 1996). ROS/RNS (reactive nitrogen species) and GSH levels may be critical determinants of NF-κB activation (Schreck et al. 1992; Mihm et al. 1995).

Prolonged activation of the glutamate receptor by stress (Musazzi et al. 2011) activates nNOS or iNOS, resulting in NO production that, together with the increased ROS formation due to NADPH oxidase activation and mitochondrial respiration, can provoke oxidative damage. In addition, regionally selective activation of microglia by chronic stress in rats (Tynan et al. 2010) subsequently releases high concentrations of NO, promoting nitrosative stress independent of glutamate receptor activation (Cassina et al. 2002). The overexpression of nNOS has been associated with stress and depression, including in the hippocampus (McLeod et al. 2001), while iNOS is associated with pathological inflammatory processes (Brown 2007) and may also be responsible for stress-induced depression (Haroon et al. 2012). For example, rats exposed to 6 h of acute immobilization stress showed increased nNOS activity and protein levels, while chronic immobilization stress (21 days for 6 h) induced iNOS in rat neurons (Olivenza et al. 2000; Madrigal et al. 2001b). Furthermore, animals exposed to chronic mild stress develop some depression-like characteristics (De Vry and Schreiber 1997; Willner 2005; Dronjak et al. 2007; Grippo et al. 2007), whereby overexpression of both NOS isoforms, associated with overproduction of NO in the hippocampus, may be partly responsible for stress-induced depression (Zhou et al. 2007).

To adapt to environmental changes and survive injury, cells synthesize heat shock proteins (Hsps). While Hsp70 is involved in cellular repair and protective mechanisms (Lindquist and Craig 1988; Georgopoulos and Welch 1993; Morimoto et al. 1994), the degree of the inducible form Hsp70i depends on the type and duration of exposure to stressors (Kiang 2004). It has been reported that Hsp70i induction protects neurons from apoptosis (Arieli et al. 2003). The protective effect of Hsp70i has been demonstrated through suppression of NF-κB activation and its nuclear translocation, which causes the suppression of iNOS gene transcription (Guix et al. 2005). Moreover, the Hsp70 response also suppresses microglial activation (Heneka et al. 2000), which upon chronic stress exposure represents a significant source of ROS (Tynan et al. 2010; Hinwood et al. 2012).

Recently, we reported that exposure to 21 days of chronic social isolation (CSIS) stress triggers proapoptotic signaling (Zlatković and Filipović 2012) and leads to programmed cell death in the rat prefrontal cortex but not in hippocampus (Filipović et al. 2011), indicating the existence of some form of protection in the hippocampus. As these changes may be mediated by NF-κB activation and increased iNOS protein expression accompanied by a decrease in Hsp70i levels (Zlatković and Filipović, 2013), we hypothesized that hippocampal resistance to CSIS-induced changes may be achieved by increased Hsp70i and its disabling of NF-κB translocation into the nucleus. Hence, we measured cytosolic Hsp70i, nNOS and iNOS protein expression and cytosolic/nuclear distributions of NF-κB as a transcriptional factor for NOS isoforms. The activity of cytosolic CuZnSOD and mitochondrial MnSOD enzymes, together with GSH levels, served as indirect markers of a state of oxidative/nitrosative stress. Corticosterone (CORT) levels were used as a neuroendocrine marker of the stress response.

CSIS is a variety of chronic mild stress that may represent a more natural stressor in rodents, as it has been shown to produce behavioral changes that are similar to human depression (Heim and Nemeroff 2001; Heinrich and Gullone 2006) and therefore considered a valid 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). To reveal the influence of CSIS on HPA axis activity and stress-induced changes in the hippocampus, we also examined additional animal groups exposed to either 2 h of immobilization (IM) or cold (C, 4 °C), as acute stressors, alone or in combination with CSIS stress. Acute stressors were intended to indicate a normal adaptive response (Munck et al. 1984; Sapolsky 2000), while CSIS stress

followed by a single acute stress (combined stress, CSIS + IM and CSIS + C) was used to examine whether chronic stress resulted in irreversible alterations in the examined parameters, which may indicate a potentially maladaptive effect. Given that HPA axis functionality depends on the type and combination of the stressor (Pacak and Palkovits 2001), two different acute stressors [i.e., IM, as a combination of physical and psychological stress (Kvetnansky and Mikulaj 1970; Scott and Dinan 1998), vs. C, as a mild physical stress (Pacak and Palkovits 2001)] were used to identify stressor-specific HPA responses. The hippocampus was chosen for examination because chronic, but not acute, stress produces significant alterations in hippocampal neurochemistry, morphology and function (Moghaddam 1993; Anisman and Matheson 2005).

Materials and methods

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 access to food (commercial rat pellets) and water 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 comprised unstressed animals (control group $n = 6$). Group II was exposed to either 2 h of immobilization (IM) or cold (C) stress (at 4 °C) ($n = 6$ per stressor). Group III was exposed to chronic social isolation (CSIS) stress via individual housing for 21 days ($n = 6$), according to the model of Garzón and del Rio (1981). Group IV represented the combined stressors (CSIS + IM, CSIS + C, $n = 6$ per stressor), in which rats underwent CSIS stress 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. to minimize possible hormonal interference by circadian rhythms. Rats were exposed to IM by placing them in a prone position with all four limbs fixed to a board with adhesive tape. The head was fixed with a metal loop over the neck area to limit head movement (Kvetnansky and Mikulaj 1970). Animals exposed to C stress were initially kept at an ambient temperature (20 ± 2 °C) and then carefully transferred into a cold room at 4 °C. Following the stress procedure, animals were anesthetized with ketamine/xylazine (100/5 mg/kg i.p.) and killed by guillotine decapitation (Harvard Apparatus, South Natick, MA, USA). Brains were immediately removed and hippocampi were dissected on ice, frozen in liquid nitrogen and kept at −70 °C until further analysis.

Serum corticosterone assay

Trunk blood was collected and the serum obtained by centrifugation at 1,500*g* for 10 min at 4 °C was kept at −70 °C until assayed. The blood from the controls and all experimental groups was collected within 3 min of touching the cage. The OCTEIA corticosterone ELISA kit (AC-14F1; Immunodiagnostics Systems-IDS, UK IDS) was used for measuring 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 <7 %. The lower detection limit for CORT in this assay system was 25 ng/ml.

Subcellular fractionation

The extraction of nuclear, mitochondrial and cytosolic fractions was performed as previously described (Spencer et al. 2000; Moutsatsou et al. 2001). Each fraction was stored at −70 °C until the measurement of protein concentrations (Lowry et al. 1951). The purity of subcellular protein fractions of controls was examined by immunoblotting of nuclear/mitochondrial and cytosolic probes with antibodies against nuclear protein-histone 2B (H2B) (Cell Signalling, Inc., Beverly, MA, USA) or mitochondrial anti-cytochrome c oxidase (COX) subunit I antibody (Molecular Probe Invitrogen, Carlsbad, CA, USA) (Jin et al. 2005). The absence of bands for both H2B and COX subunit I in the cytosolic fraction of controls confirmed a lack of contamination of nuclear or mitochondrial fraction in the cytosolic compartment. The presence of bands for β-actin confirmed consistent protein loading (Fig. 1).

Activity of cytosolic/nuclear CuZnSOD, mitochondrial MnSOD and levels of reduced GSH

Enzyme activity of SODs was measured using the Biorex BXC0531 kit (Biorex Diagnostics Ltd.). First, total SOD

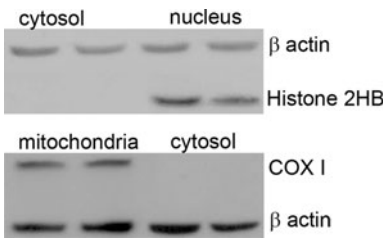

Fig. 1 Purity of the nuclear, mitochondrial and cytosolic protein fractions in the hippocampus

activity in the samples was measured. The addition of KCN during the preparation of samples for a second round of measurements blocked CuZnSOD activity, such that all measured activity derived from MnSOD. After subtraction of these two values, CuZnSOD activity in samples was obtained. Reduced GSH was quantified using freshly prepared cytosolic fractions of the hippocampus and estimated according to the method of Hissin and Hilf (1976) using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid, DTNB), which reacted with the aliphatic thiol compounds in a Tris–HCl buffer (0.2 mol, pH-8.2), thus forming a yellow-colored p-nitrophenol anion. The intensity of color was used for the spectrophotometric measurement of GSH concentration at 412 nm, expressed as nM/mg protein. The concentration of GSH in unknown samples was determined by reference to the standard GSH curve (range 50–1,000 μM).

Electrophoresis and Western blot analysis of, NF-κB, nNOS, iNOS and Hsp70i

Equal amounts of cytosolic or nuclear protein fractions were separated on SDS–polyacrylamide gel and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked in a TBS-T buffer of pH 7.6 containing 5 % BSA and incubated overnight (4 °C) with rabbit antibody raised against NF-κB, iNOS, nNOS and Hsp70i, (Santa Cruz, CA, USA), followed by 2 h with anti-rabbit HRP-conjugated secondary antibody (Cell Signaling, Inc., Beverly, MA, USA). To confirm a consistent protein loading, β-actin (Sigma St. Louis, MO), followed by HRP-conjugated secondary goat anti-mouse IgG antibody (Santa Cruz, CA,) was used. The blots were developed by enhanced chemiluminescence (Immobilon™ Western, Millipore Corporation, USA) and exposed to an X-ray film. The signals were processed for quantification using Image J analysis PC software. Protein molecular mass standards (Page Ruler™Plus Prestained Protein Ladder, Fermentas, Germany) were used for calibration. Identification of this response was observed at 65 kDa for NF-κB, 130 kDa for iNOS, 150 kDa for nNOS, 72 kDa for Hsp70i and 42 kDa for β-actin. Western blot results were expressed as protein/β-actin ratio. The levels of investigated proteins are presented as the percent change relative to control rats (100 %). The data are presented as mean ± S.E.M. of six animals per stressor.

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 presented as mean ± S.E.M. of six animals per stressor.

Results

Chronic social isolation stress compromises HPA axis

A two-way ANOVA revealed 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 a significant acute × chronic stress interaction ($F_{2.30} = 5.12$, $p < 0.001$) on serum CORT secretion (Table 1). Animals exposed to acute IM stress showed an approximately fourfold increase in serum CORT levels, while C stress showed a twofold increase as compared to controls ($^{***}p < 0.001$, $^{**}p < 0.01$). In chronically isolated animals, serum CORT levels were similar to control levels ($p > 0.05$). In combined CSIS + IM stress, a significant elevation of serum CORT levels occurred, reaching a threefold increase relative to controls ($^{***}p < 0.001$) and a fourfold increase compared to CSIS alone ($^{\wedge\wedge\wedge}p < 0.001$). In contrast, exposure to acute C following CSIS did not significantly alter serum CORT levels ($p > 0.05$) as compared to either controls or CSIS alone. When combined stressors were compared to acute stressors, a significant decrease was observed ($^{\#\#}p < 0.01$).

Table 1 Serum corticosterone (CORT) concentrations in controls and following acute immobilization (IM) and cold (C) stress, chronic social isolation (CSIS), and combined stressors (CSIS + IM or CSIS + C)

Stress	Control	Acute IM	Acute C	CSIS	Combined CSIS + IM	Combined CSIS + C
Serum CORT (ng/ml)	142.91 ± 4.6	648.9 ± 166.8***	342.1 ± 112.5**	123.4 ± 10.9	477.8 ± 74.0$^{***\ \#\#\ \wedge\wedge\wedge}$	86.4 ± 37.7$^{\#\#}$

Statistical analyses were performed using two-way ANOVA followed by Duncan's post hoc test

** $p < 0.01$

*** $p < 0.001$ stress versus control

$^{\#\#}$ $p < 0.01$ acute versus combined stress

$^{\wedge\wedge\wedge}$ $p < 0.001$ chronic versus combined stress

Chronic social isolation stress decreases MnSOD, but not CuZnSOD activity

A two-way ANOVA revealed no significant effect of stress on cytosolic CuZnSOD activity in the hippocampus. In contrast, a significant main effects of acute ($F_{2.30} = 6.169$, $p < 0.01$) and chronic ($F_{1.30} = 6.582$, $p < 0.05$) stress were revealed in the nuclear fraction on CuZnSOD activity. In animals exposed to acute C stress, nuclear CuZnSOD activity was significantly decreased compared to controls ($^{**}p < 0.01$) and the CSIS + C group ($^{\#}p < 0.05$) (Fig. 2b). With regard to mitochondrial MnSOD activity, a two-way ANOVA revealed a significant acute × chronic stress interaction ($F_{2.30} = 4.4$, $p < 0.05$) in the hippocampus. Post hoc tests revealed statistically significant decreases in mitochondrial MnSOD activity in animals exposed to both CSIS + IM and CSIS + C, as compared to controls ($^{*}p < 0.05$ and $^{**}p < 0.01$, respectively) and CSIS stress ($^{\wedge\wedge}p < 0.01$ and $^{\wedge\wedge\wedge}p < 0.001$, respectively) (Fig. 2c). Furthermore, a significant decrease in MnSOD activity following CSIS + C stress relative to acute C stress alone was observed ($^{\#}p < 0.05$).

Chronic social isolation stress decreases reduced GSH levels

A two-way ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 18.1$, $p < 0.001$) on cytosolic GSH levels. Post hoc analysis showed a significant decrease in GSH following CSIS and both combined stressors in the cytosolic fraction ($^{*}p < 0.05$; $^{**}p < 0.01$), but no change following acute stressors (Fig. 3).

Lack of NF-κB activation following chronic social isolation stress

NF-κB activation and its nuclear translocation were examined by monitoring NF-κB-p65 localization in cytosolic and nuclear fractions in the hippocampus (Fig. 4). In the cytosolic fraction, a two-way ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 5.124$, $p < 0.05$) and a significant acute × chronic stress interaction ($F_{2.30} = 8.884$, $p < 0.001$) on NF-κB protein levels. Specifically, in comparison to controls, acute C stress resulted in a significant decrease in NF-κB protein expression ($^{*}p < 0.05$). In contrast, a significant increase in the combined CSIS + C group, as compared to acute C alone, was observed ($^{\#\#}p < 0.01$). In the nuclear fraction, a two-way ANOVA revealed a significant main effect of acute stress ($F_{2.30} = 3.675$, $p < 0.05$) and a significant acute × chronic stress interaction ($F_{2.30} = 4.564$, $p < 0.05$) on NF-κB protein levels. Significant increases in NF-κB protein in the acute C group relative to controls ($^{**}p < 0.01$) and CSIS + C group compared to acute C alone ($^{\#}p < 0.05$) were found.

Chronic social isolation stress upregulates nNOS and downregulate iNOS protein expression

A two-way ANOVA revealed a significant main effect of acute stress ($F_{2.30} = 3.27$, $p \leq 0.05$) and CSIS ($F_{1.30} = 6.42$, $p < 0.05$) on nNOS protein levels. Duncan's

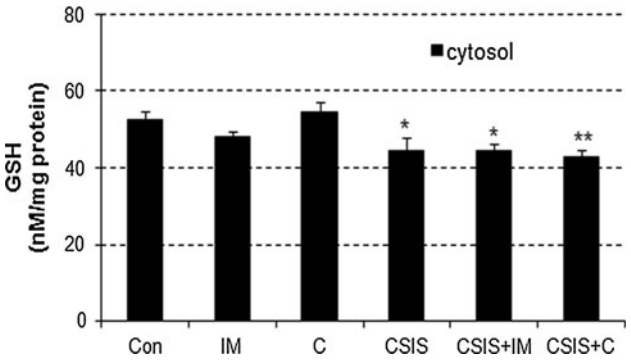

Fig. 3 GSH levels (nM/mg protein) in the cytosolic fraction of the hippocampus in controls (Con) and following acute immobilization (IM) or cold (C) stress, chronic social isolation (CSIS) and combined stressors (CSIS + IM or CSIS + C). *Asterisks* indicate significant differences between the respective stress treatment and controls ($^{*}p < 0.05$; $^{**}p < 0.01$)

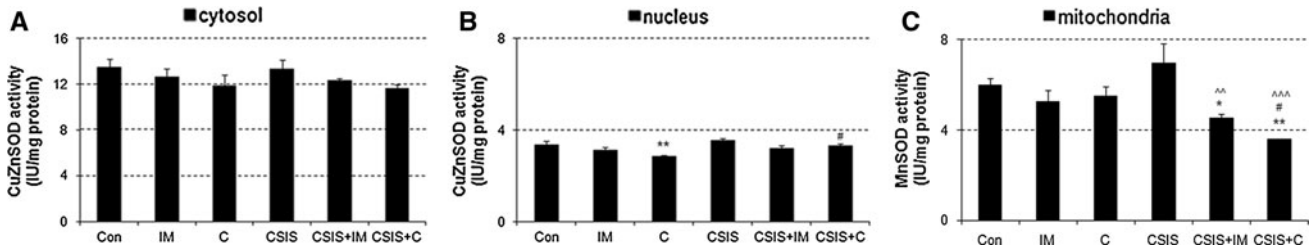

Fig. 2 Cytosolic (**a**), nuclear (**b**) CuZnSOD and mitochondrial (**c**) MnSOD activity (IU/mg protein) in the hippocampus of control (Con) and following acute immobilization (IM) and cold (C) stress, chronic social isolation (CSIS) and combined stressors (CSIS + IM or CSIS + C). *Symbols* indicate significant differences between: respective stress treatment and controls ($^{*}p < 0.05$, $^{**}p < 0.01$); CSIS and combined stress ($^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$); combined stressors and the respective acute stress alone ($^{\#}p < 0.05$)

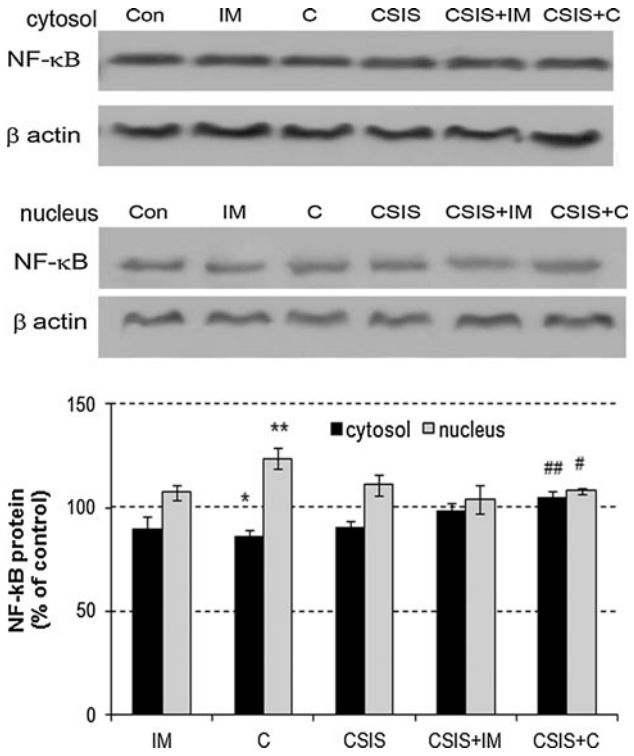

Fig. 4 NF-κB protein levels in cytosolic and nuclear fraction in the hippocampus following acute immobilization (IM) and cold stress (C), chronic social isolation (CSIS) and combined stressors (CSIS + IM or CSIS + C). *Symbols* indicate significant differences between: the respective stress treatment and controls ($^{*}p < 0.05$, $^{**}p < 0.01$) and combined CSIS + C group compared to acute C alone in both cytosolic and nuclear fractions ($^{\#\#}p < 0.01$ and $^{\#}p < 0.05$, respectively)

post hoc test revealed increased nNOS protein expression in animals exposed to acute IM and C ($^{*}p < 0.05$), CSIS ($^{*}p < 0.05$), and the combined CSIS + IM and CSIS + C ($^{**}p < 0.01$) compared to controls (Fig. 5). With regard to iNOS protein expression, a two-way ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 5.970$, $p < 0.05$). In comparison to control rats, significant decrease in iNOS levels in animals exposed to CSIS ($^{*}p < 0.05$) and CSIS + IM ($^{**}p < 0.01$) was detected (Fig. 5).

Hsp70i protein upregulation following chronic social isolation stress

A two-way ANOVA revealed a significant main effect of CSIS ($F_{1.30} = 22.948$, $p < 0.001$) on Hsp70i protein in the cytosolic fraction in the hippocampus. Post hoc tests revealed a significant increase in Hsp70i protein levels in animals exposed to CSIS ($^{**}p < 0.01$) and both combined CSIS + IM ($^{*}p < 0.05$) and CSIS + C ($^{**}p < 0.01$) stressors as compared to controls or the respective acute stressors alone ($^{\#}p < 0.05$) (Fig. 6).

Discussion

In the present study, we have identified in an animal model of depression a possible signaling cascade in which hippocampal Hsp70i upregulation may exert a protective role through iNOS reduction via NF-κB attenuation. A number of studies have shown that stressful stimuli lead to activation of the HPA axis and the elevation of GCs (Munck et al. 1984). We found that serum CORT levels were increased in both acutely stressed groups. Acute IM stress evoked higher responses in CORT levels, indicating that IM stress is stronger than C. Furthermore, the adaptive response to psychological stress via HPA axis induction includes the antioxidant defense systems. Although high serum CORT levels were observed following both acute stressors, changes in cytosolic CuZnSOD and mitochondrial MnSOD activity in the hippocampus were not detected.

Previously reported increases in CuZnSOD mRNA expression and cytosolic protein levels, as well as an increase in mitochondrial MnSOD protein levels in the hippocampus following acute IM stress (Filipović and Pajović 2009; Filipović et al. 2009), suggest that the hippocampus is susceptible to stressful stimuli. A lack of consistency between protein levels of SOD isoforms and their activities may suggest posttranslational protein modifications affecting activity (Hopper et al. 2006) or partially non-coupled activity of another hydrogen peroxide-removing enzyme such as catalase (Pajović et al. 2006), which may lead to the accumulation of hydrogen peroxide, resulting in inhibition of SOD isoforms. Our data are consistent with those of Stojiljković et al. (2005), who also reported unchanged hippocampal CuZnSOD activity after both acute IM and C stressors in whole cell extract. In addition to the cytosol, CuZnSOD is also located in the nucleus, at levels that are 50–60 % of those in the cytosol (Slot et al. 1986). An increased metabolic rate, observed during exposure to cold (Bravo et al. 2001), has been shown to induce the generation of ROS (Selman et al. 2000; Blagojevic et al. 2011) that could in turn inactivate SOD (Pigeolet et al. 1990). Despite unchanged hippocampal CuZnSOD mRNA and protein expression following acute C stress (Filipović and Pajović 2009), decreased CuZnSOD activity in the nuclear fraction may indicate inadequate protection of DNA and other nuclear molecules.

In contrast, the nuclear translocation of NF-κB, indicated by an increase in protein levels concurrent with a decrease in the cytosolic fraction, suggests that NF-κB is activated by acute C stress. Its activation may be a consequence of the increased nNOS expression that is accompanied by a marked increased in NO content (Zlatković and Filipović 2012), as NF-κB activation partially depends on RNS (Schreck et al. 1992). Moreover, 2 h

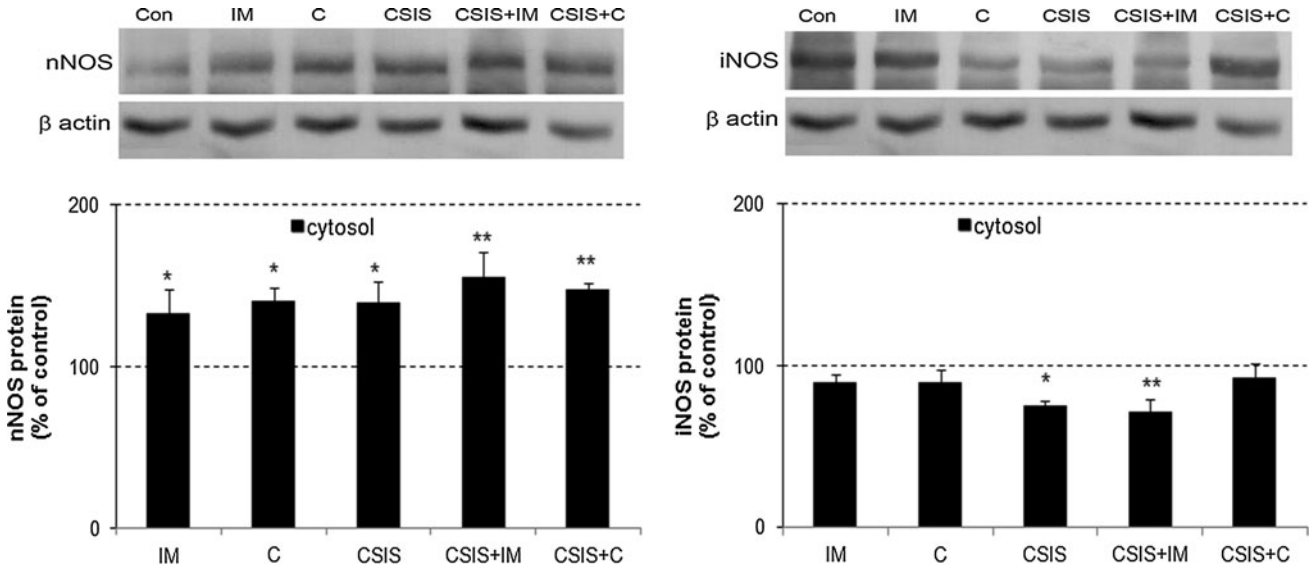

Fig. 5 nNOS and iNOS protein expression in cytosolic fractions in the hippocampus following acute immobilization (IM) and cold stress (C), chronic social isolation (CSIS) and combined stressors (CSIS + IM or CSIS + C). *Asterisks* indicate significant differences between the respective stress treatment and controls ($^{*}p < 0.05$, $^{**}p < 0.01$)

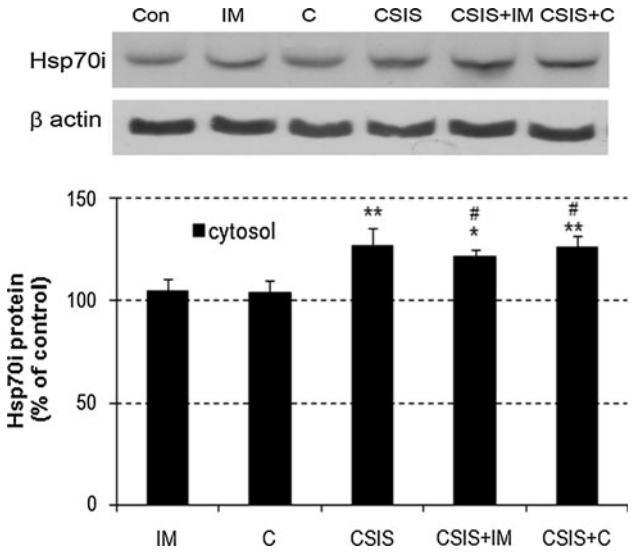

Fig. 6 Hsp70i protein levels in the cytosolic fraction of the hippocampus following acute immobilization (IM) and cold stress (C), chronic social isolation (CSIS) and combined stressors (CSIS + IM or CSIS + C). *Symbols* indicate significant differences between: respective stress treatment and controls ($^{*}p < 0.05$, $^{**}p < 0.01$); combined stressors and respective acute stressors ($^{\#}p < 0.05$)

of cold stress has been reported to result in microglial activation (Sugama et al. 2011), which may contribute to an increase in NO production (Boje and Arora 1992). The nuclear translocation of NF-κB after acute stress was also confirmed in experiments in which rats were subjected to acute restraint stress (Madrigal et al. 2001b). But whether NF-κB can act as a transcription factor is as yet unknown, as Fujita (1999) found that no genes were upregulated during exposure to temperatures below 5 °C without recovering at higher temperatures.

Given that moderate increases in ROS/RNS often lead to NF-κB activation required for nNOS or iNOS induction, we examined cytosolic nNOS and iNOS protein levels following all types of stressors. Increased cytosolic nNOS protein levels followed by an increase in NO content (Zlatković and Filipović 2012) after acute stressors may represent a component of the normal cellular stress response involved in neuromodulation and neurotransmission (Boissel et al. 1998). In contrast, iNOS is considered to be an indicator of stress-induced impairment in the brain (Madrigal et al. 2006). Moreover, changes in GSH and the protein expression of iNOS and Hsp70i were not detected in the hippocampus after exposure of rats to acute stressors, indicating a neuroprotective effect of nNOS-derived NO (Calabrese et al. 2007).

Whereas acute stress provokes a physiological stress response that has a protective role, chronic stress may represent a significant cause of depression (Lee et al. 2010). In our study, CSIS was characterized by unchanged CORT levels as compared to control animals. Interestingly, controversy remains as to the effects of social isolation on CORT levels. Increased CORT levels have been reported in response to social isolation (Gamallo et al. 1986), whereas other groups observed no changes (Holson et al. 1991; Malkesman et al. 2006) or reduced CORT levels (Sanchez et al. 1998). As the glucocorticoid receptor (GR) shuttling between cytoplasm and nucleus is essential for proper HPA axis activity, an unchanged CORT response

during CSIS in our study may result from reduced nuclear translocation of cytosolic GR and increased cytosolic retention, as previously reported (Dronjak et al. 2004; Filipović et al. 2005), suggesting diminished GR-negative feedback control in the hippocampus. In addition, decreased secretion of corticotrophin-releasing hormone found following long-term isolation (Sanchez et al. 1998) may also account for unaltered CORT levels.

In an attempt to ascertain the molecular basis by which CSIS alters functional activity within the hippocampus, we examined the activity of antioxidative enzymes, markers of a state of oxidative/nitrosative stress and important hallmarks of stress and a depressive-like state (Rao et al. 2010). We found that unchanged cytosolic/nuclear CuZnSOD and mitochondrial MnSOD activity after CSIS was consistent with previously published unaltered protein levels (Filipović and Pajović 2009; Filipović et al. 2009). Furthermore, the observed hippocampal decrease in GSH levels may be due to chronically upregulated nNOS levels that overproduce NO (Zlatković and Filipović 2012) and oxidize intracellular reduced GSH, altering the antioxidant levels within the cell. Our results are consistent with reports demonstrating that activation of glutamate receptors enhanced nNOS activity, producing NO capable of inhibiting GSH by nitration or nitrosylation (Rauhala et al. 1998). Furthermore, upregulated nNOS may be related to psychiatric dysfunction, as elevated plasma nitrate levels and increased nNOS expression have been found in the hippocampus of depressed patients (De Oliveira et al. 2008). Stress-induced iNOS expression, however, mediates cytotoxicity primarily via the oxidative/nitrosative effects produced by the extensive and prolonged release of NO (Munhoz et al. 2008). The decreased iNOS protein expression following CSIS observed in the current study may be a consequence of increased Hsp70i protein levels.

Moreover, overproduction of NO as well as a depleted redox GSH status are critical factors in the induction of cytoprotective Hsp70 (Hao et al. 1999; Calabrese et al. 2000). Thus, decreased GSH and an increase in Hsp70i expression following CSIS are in agreement with the data of Bellia et al. (2009), who suggested a positive correlation between decreased redox status and the induction of Hsp70i as a cellular stress response. It was noted that Hsp70i stabilizes cytoplasmatic NF-κB/IκB complex (Malhotra and Wong 2002) and prevents NF-κB translocation into the nucleus (Zheng et al. 2008), where it acts as a transcription factor for iNOS (Heneka et al. 2000). Thus, in the current study, unchanged NF-κB levels in the nucleus may be due to unaltered CORT levels or a feedback loop of the Hsp70i pathway in the chronic stress state that stabilizes NF-κB. Given that GSH may first be consumed by ROS/RNS (Masella et al. 2005), its decreased levels demonstrate its ability to reduce stress-induced free radicals, preserving antioxidative enzyme activity and thus preventing chronic stress-induced NF-κB activation in the hippocampus. Future experiments targeting the direct involvement of Hsp70 in the relationship between GSH and NF-kB should be examined. Moreover, glutamate receptor activation leads to the activation of nNOS, which may play a protective role via the blocking of caspases (Khaldi et al. 2002). Thus, nNOS stimulation, together with increased Hsp70i, may have anti-apoptotic effects via the absence of cleaved caspase-3 and apoptosis (Kiang 2004), characteristics previously demonstrated in the hippocampus (Filipović et al. 2011).

The ability of brain structures to retain a 'normal' stress response following CSIS stress was examined by exposing previously isolated animals to novel acute IM or C stress. Reduced responsiveness to novel acute stress in chronically isolated animals compared to acute stress alone, as determined by CORT levels, may indicate terminally compromised HPA axis activity. Consistent with our results, it has been reported that individuals with major depressive disorder show blunted cortisol responses to novel psychosocial stressors (Simeon et al. 2007). Moreover, novel acute IM stress was strong enough to provoke HPA axis reaction in chronically isolated rats, while acute C did not alter serum CORT levels. These results are not surprising, since IM represents the strongest stress that results in increases of plasma catecholamine and CORT levels accompanied by the activation of the sympatho-adrenomedullary system and HPA axis (Pacak et al. 1998), while C as a mild stress is specific only to the sympatho-neural system (Fukuhara et al. 1996; Kvetnansky et al. 1998). Our results corroborate the findings of Pacak et al. (1998), who demonstrated that the response of chronically stressed animals to novel stressors was dependent on the type of stressor, indicating that acute exposure to decreased temperature (metabolic stress) was not as strong a stressor as immobilization (physical and psychological stress).

In contrast to the negligible responsiveness of cytosolic CuZnSOD to CSIS and combined stressors, mitochondrial MnSOD activity was compromised by both combined stressors, suggesting that CSIS specifically affected the activity of the mitochondrial isoform of SOD, but not the cytosolic isoform. Furthermore, a likely "cumulative" effect of CSIS and novel acute stressors that generates excessive NO production (Zlatković and Filipović 2012) may result in decreased MnSOD activity via nitration (Yamakura et al. 1998). Increased Hsp70i protein levels after both combined stress suppressed NF-κB activation, as measured by its unchanged level in the nucleus, and led to decreased or unchanged iNOS protein expression. We previously demonstrated that CSIS induced apoptosis in the

prefrontal cortex, but not hippocampus (Filipović et al. 2011). Studies have indicated that Hsp70i induction protects neurons from apoptosis (Kelly et al. 2002; Arieli et al. 2003; Belay and Brown 2003) through its ability to increase Bcl-2 stability during oxidative stress (Jiang et al. 2011) and suppress mitochondrial cytochrome c release (Didelot et al. 2006) or activity of c-Jun N-terminal kinase (Gabai et al. 1998; Kumar and Tatu 2003). Consistent with these data, we previously reported that CSIS increased mitochondrial Bcl-2 expression (an anti-apoptotic protein), where an unchanged Bax/Bcl-2 ratio (Zlatković and Filipović 2012) resulted in the inhibition of release of mitochondrial cytochrome c to the cytosol (Filipović et al. 2011). Furthermore, the activation of stress-activated protein kinase SAPK/c-Jun N-terminal kinase was inhibited in chronically isolated animals (Filipović et al. 2012) in the hippocampus. Given that Hsp70i was increased in our study, we suggest that Hsp70i may be involved in the inhibition of hippocampal apoptosis in conditions of either CSIS or intense stress (combined stressors) (Yao et al. 2007). In addition, a decrease in iNOS levels after combined CSIS + IM stress was probably due to the prevention of the de novo protein synthesis or decreased protein stability via a post-transcriptional mechanism (Chiou et al. 2000).

Conclusion

The data presented here suggest that CSIS depletes redox GSH status and affects mitochondrial MnSOD activity, compromising the antioxidative defense system. The induction of Hsp70i protein expression may be regulated by decreased GSH due to nitrosative stress that may be derived from increased nNOS protein expression. Our data suggest a possible signaling cascade in which CSIS stress provokes an Hsp70i response, a protective effect that may be manifested through suppression of NF-κB activation and reduced iNOS expression. These data suggest a possible cellular pathway of stress tolerance that preserves the hippocampus from molecular damage and apoptosis under CSIS. Given that hippocampal nNOS overexpression in chronically stressed animals has been shown to suppress neurogenesis (Zhou et al. 2007), which is implicated in the pathogenesis of anxiety and depression (Dhir and Kulkarni 2011), further work should be directed toward the relationship between nNOS, adult hippocampal neurogenesis and depression following CSIS stress.

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Conflict of interest The authors declare that they have no conflict of interest.

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