УДК 577.151.04

Hemorphins exhibit protective effect on superoxide dismutase activity during endotoxin-induced stress in rats

H.H. Zakaryan, F.P. Sarukhanyan, O.V. Hunanyan, N.H. Barkhudaryan

H. Buniatian Institute of Biochemistry NAS RA
0014, Yerevan, 5/1, P. Sevag str.

Keywords: hemorphin, superoxide dismutase (SOD), calcineurin (CN), cyclosporin A (CsA), μ-opioid receptors (MOR)

Recently it has bee shown, that hemorphin-7 and LVV-hemorphin-7, which demonstrate a wide spectrum of biological activity by affecting different receptors function (e.g. μ-, δ- and κ-opioid receptors [17, 32], angiotensin (Ang) IV receptor (AT_4) [13], bombesin receptor subtype 3 (hBRS-3) [11] and corticotropin-releasing factor (CRF) receptor(s) function [1]), act as homeostatic agents in response to endotoxine (lipopolysaccharide, LPS)-induced stress [7]. They were able to regulate hypothalamo-pituitary-adrenocortical (HPA) axis activity by recovering the plasma level of corticosterone and tumor necrosis factor-alpha (TNFα). In addition, increased activity of calcineurin in both plasma and brain of rats that received intraperitoneal (ip) administration of LPS is also recovered under LPS+hemorphin treatment. It should be emphasized that CN controls gene expression of several cytokines, including IL-2, tumor necrosis factor α (TNFα) and others via dephosphorylation and nuclear translocation of NFATc (nuclear factor of activated T cell) family members [23]. Earlier we have demonstrated that hemorphins are able to modulate brain and lymphocytes calcineurin activity in vitro, exhibiting a concentration-dependent biphasic response on enzyme activity by binding with calmodulin (CaM) [3, 8]. Very recently we have shown that integration of Ca^{2+}/CaM/CN/NFAT signalling pathway with μ-opioid receptors (MOR) function is involved in the molecular mechanisms of hemorphins action in pathophysiology of LPS-induced stress, as well as in pathophysiology of diabetes and cancer [4,6,7].

Oxidant/antioxidant imbalance is involved in the pathogenesis of the inflammatory diseases [20-21]. Under the conditions of oxidative stress, SOD acts as an endogenous cellular defence system to degrade superoxide (O_2^-) into oxygen and hydrogen peroxide. Therefore, it is considered that SOD has

therapeutic potential for antioxidant therapy [24, 29]. It has been found out that CN is sensitive to oxidative stress and may be modulated by the intracellular redox potential [25]. Moreover, CN is critical regulator of mitochondrial respirarion, tissue nitration, and contractile function during acute endotoxemia [10]. It has been reported that SOD1 (Cu,ZnSOD) protects CN against inactivation [30]. It is proposed that modulation of intracellular Cu,ZnSOD activity could influence CN-dependent signalling in vascular endothelium [15]. CN was reported to be a partner of both SOD1 (Cu,ZnSOD) and mitochondrial SOD2 (Mn-SOD) [27]. By using proteomic approaches, mitochondrial proteins (Mn-SOD, aconitase, malate dehydrogenase, isocitrate dehydrogenase) were identified as partners of CN in a single multiprotein complex [27].

It should be noted that hemorphins exhibit anti-inflammatory and immunomodulatory properties [3, 19, 22]. Moreover, it has been reported that one of hemorphins (LVV-hemorphin-4), isolated from bovine hypothalamus [5] and spinal cord [16], so-called spinorphin demonstrates antioxidant properties [31].

In the given work an experimental rats model of stress was received by single ip injection of LPS (0,5 mg/kg). It is well known that many of the physiological effects associated with LPS are mediated by cytokines, including TNFα, IL-1 and IL-6, the levels of all being elevated as a result of LPS administration [9]. LPS administration activates HPA axis by increasing circulating concentration of adrenocorticotropic hormone (ACTH), which in turn induces downstream release of glucocorticoids from the adrenal cortex. It is worth to mention that LPS administration has been reported to activate calcineurin as well [26].

In the experiments there were used 2 synthetic hemorphins: LVV-hemorphin-7 (LVVH7) and hemorphin-7 (H7). It should be noted that H7 is the most potent among the hemorphins in μ-opioid receptors binding [32]. LVVH7 was shown to elicit the pressor effect and tachycardia, which are mediated by the sympathetic nervous system [14].

The aims of present study were:1) to study if hemorphin may change the effect of ip administration of LPS on plasma and brain SOD activity; 2) to examine if MOR are involved in the molecular mechanism of hemorphin action on SOD activity; 3) to determine if the inhibitory effect of cyclosporine A (CsA) on CN activity may influence the effect of hemorphins on SOD activity in stress conditions.

Materials and Methods

In the research there were used male rats of Wistar line, weighing 180-220g at the time of the experiment. Rats provided by Animal House of the Institute of Biochemistry NAS RA, were caged in groups of 5 with food and water given ad libitum. Experimental protocols were approved by Animal Care

and Use Committee at the Institute of Biochemistry NAS RA. The animals were kept at 22° C on a 12 h light-dark cycle. The experiments were conducted between 09:00 and 14:00 h. The stress was induced by a single ip injection of LPS (0.5 mg/kg). Rats were randomly divided into 6 groups (n=10 per group): the first control group received ip injection of an equivalent volume (0.5 ml) of 0.9% w/v saline; the second group received ip injection of LPS; the third and fourth groups received a simultaneous ip injection of LPS and either LVV-hemorphins-7 or hemorphin-7 (1mg/kg). Animals were decapitated 4h after ip injection of saline, LPS and LPS+hemorphin; the rats in the fifth and sixth groups received ip injection of naloxone (2mg/kg) or CsA (6 mg/kg) 30 min before injection of LPS+hemorphin. The brains were rapidly removed, frozen and stored at -70° C until use. The trunk blood was immediately collected into sodium citrate (3.2%)-coated vacutainer tubes. Then, the blood samples were centrifugated at 1500 rpm for 10 min, separated into aliquots and stored at -70° C. Brain tissue was homogenized with 2.5 volumes of 50 mM Tric-HCI pH 8, 5 buffer, containing 0.05% Triton-X-100, 0.1 mM EDTA, 1 mM ditiothreitol (DTT), protease inhibitors and centrifugated at 100 000g for 60 min at 4° C.

SOD activity in plasma and brain tissue of rats was measured using SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc., USA) according to the manufacturer's recommendation. The optical density was measured in each well at the wavelength of 450 nm using Precision microplate reader (Molecular Devices (Emax)).

Statistical analysis

The results were expressed as the means ± SEM. Data were analyzed statistically by one-way ANOVA using GraphPad Prism 4 software, statistical significance $p < 0.05$.

Results and Discussion

The effects of 2 hemorphins on brain and plasma SOD activity were studied 4 h after administration of LPS (0.5 mg/kg) in combination with hemorphin (1 mg/kg). The results obtained have demonstrated that single ip administration of LPS alone induces a decrease in plasma SOD activity by 36,4% (Fig.1). As one can see, both hemorphins participate in the recovery of SOD activity, however the effect of LVVH7 is more pronounced. Pretreatment of rats with general opioid receptors antagonist naloxone completely abrogates recovering effect of H7 on SOD activity. The famous inhibitor of calcineurin immunosuppressive drug CsA also completely abrogates the protective effect of LVVH7 on SOD activity in plasma. In the brain ip LPS injection induces very modest inhibition of SOD activity (by 17%). However, as one can see both H7 and LVVH7, when injected in combination with LPS, completely recover the activity of SOD up to the level of SOD activity in healthy rats (Fig.2). The data obtained give us reason to suggest that MOR and CN are involved in the

molecular mechanisms of hemorphins protective effect on SOD activity in the stress conditions.

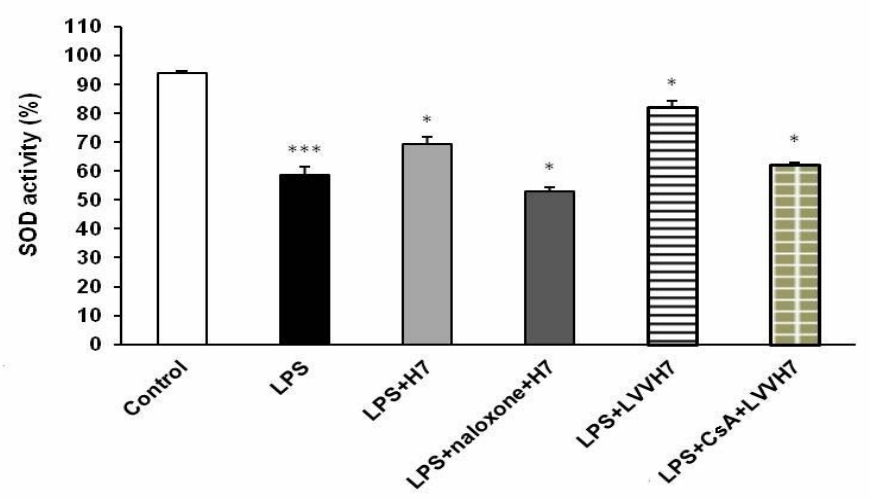

Fig. 1. SOD activity in he plasma of rats at 4 h after ip LPS and ip LPS+hemorphin injection. Naloxone and CsA were ip injected to rats 30 min before LPS+H7 or LPS+LVVH7 injection. Significantly different from control group,p <0.001. Significantly different from LPS group, p<0.01 using one-way ANOVA.

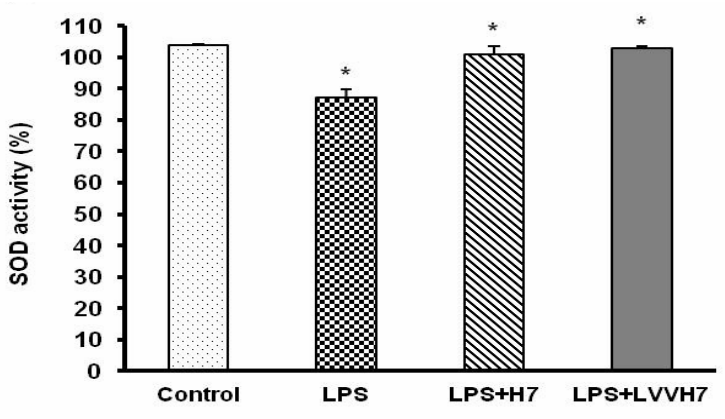

Fig.2. SOD activity in the brain of rats 4 h after ip LPS or ip LPS+hemorphin injection. Significantly different from LPS group, p<0.05 using one-way ANOVA.

Recently, by using protemic methods, we have identified for the first time peptidyl-prolyl cis-trans-isomerase A (cyclophilin A) among the list of proteins that are differently expressed/regulated in healthy mouse brain by hemorphins [7]. As it was mentioned above, CsA is an inhibitor of calcineurin activity. It

has been established that CsA exerts its inhibitory action on calcineurin activity by binding to immunophilin (cyclophilin A) [12]. It should be underscored that CsA induces an increase in production of reactive oxygen species (ROS) [28]. Hemorphins have been shown to inhibit the expression of cyclophilin A in mouse brain, and thus they may suppress the ROS production as well [7].

By using proteomic methods, we have also identified ferritin heavy chain (FHC) as protein is up-regulated by hemorphins in healthy mouse brain [2]. FCH – the primary iron storage factor, is an essential mediator of the antioxidant and protective activity of NF-kB [18]. It should be noted that suppression of reactive oxygen species by NF-κB is mediated by FCH. As mentioned above, hemorphins have been reported to exhibit antioxidant and anti-inflammatory activity, and it is suggested that the modulation of ferritin synthesis by hemorphins may be a possible mechanism directed to recover homeostasis of free iron and its storage pool in the brain.

Taking into account all of the aforementioned it is proposed that molecular mechanisms of protective effect of hemorphins on SOD activity is a complex process, which involves the interaction of hemorphins with SOD, CN, immunophilin, FHC.

Поступила 28.11.12

Հեմորֆինները ցուցաբերում են պաշտպանիչ ազդեցություն սուպերօքսիդ դիսմուտազի ակտիվության վրա առնետների մոտ էնդոտոքսինով խթանված սթրեսի պայմաններում

Հ.Հ. Զաքարյան, Ֆ.Պ. Սարուխանյան, Օ.Վ. Հունանյան, Ն.Հ. Բարխուդարյան

Ցույց է տրված, որ LVV-հեմորֆին-7-ի (LVVH7) և հեմորֆին-7-ի (H7) համատեղ ներորովայնային ներարկումները էնդոտոքսինի (լիպոպոլիսախարիդ, LPS) հետ նպաստում են LPS -ի ներարկումից ընկճված սուպերօքսիդ դիսմուտազի (SOD) ակտիվության վերականգնմանը, ինչը ավելի արտահայտված է LPS+LVVH7-ի ներարկման դեպքում: Բացահայտված է, որ երբ առնետները ստանում են μ-օպիոիդ ռեցեպտորների անտագոնիստ նալոքսոնի կամ կալցինեյրինի հայտնի ինհիբիտոր իմունասուպրեսանտ ցիկլոսպորինի (CsA) ներարկում 30 րոպե LPS+հեմորֆին ներարկումից առաջ, ապա SOD-ի ակտիվության վերականգնումը տեղի չի ունենում: Այդ փաստը վկայում է այն մասին, որ μ-օպիոիդ ռեցեպտորները և կալցինեյրինը ներգրավված են SOD-ի ակտիվության վրա հեմորֆինների կողմից դրսևորված պաշտպանիչ ազդեցության մոլեկուլային մեխանիզմերում:

Геморфины проявляют защитное воздействие на активность супероксиддисмутазы при эндотоксин-индуцированном стрессе у крыс

Э.А. Закарян, Ф.П. Саруханян, О.В. Унанян, Н.А. Бархударян

Обнаружено, что совместная внутрибрюшинная инъекция LVV-геморфина-7 (LVVH7) или геморфина-7 (H7) с эндотоксином (липополисахарид, LPS) способствует восстанавлению активности супероксиддисмутазы (SOD), подавленной инъекцией LPS. Показано, что восстанавливающее дейстие геморфина на активность SOD было выражено сильнее инъекции LPS+LVVH7. Выявлено, что инъекция антагониста μ-опиоидных рецепторов налоксона или известного ингибитора кальцинейрина иммуносуппресанта циклоспорина А (CsA) за 30 минут до инъекции LPS+ геморфин, полностью отменяет восстанавливающий эффект геморфина на активность SOD. Этот факт свидетельствует о вовлечении μ-опиоидных рецепторов и кальцинейрина в защитный механизм воздействия геморфинов на активность SOD.

References

1. *Barkhudaryan N*. In vivo microdialysis is a tool to study the mechanism of interaction between LVV-hemorphin-7 and brain serotonergic system. In: Biotechnology and health, *Yerevan, 2005, p.32-42.*
2. *Barkhudaryan N., Dosch D., Gabrielyan A., Kellermann J., Lottspeich F*. Study of the effect of hemorphins on mouse brain proteome by using different proteome analysis approaches. Proc. Int. Conf." Biotechnology and health" & DAAD Alumni seminar, Yerevan, 2008, p.36-41.
3. *Barkhudaryan N., Gambarov S., Gyulbayazyan T., Nahapetyan K*. LVV-hemorphin-4 modulates Ca^{2+}/calmodulin-dependent pathways in the immune system by the same mechanism as in the brain. J. Mol. Neurosci., 2002, 18, p.203-210
4. *Barkhudaryan N.H., Hunanyan O.V., Sarukhanyan F.P., Stepanyan F.P., Zakaryan H.H., Grigorian I.E., Dalyan E.P.* Study of molecular mechanisms of anti-tumor effect of hemorphin-7 in vivo. Med. Sci. Arm., 2012, v. LII, (N1), p. 21-32.
5. *Barkhudaryan N., Oberthuer W., Lottspeich F., Galoyan A.* Structure of hypothalamic coronaro-constrictory peptide factors. Neurochem. Res., 1992,17, p.1217-1221.
6. *Barkhudaryan N., Sarukhanyan F., Kellermann J., Lottspeich F*. Calcineurin and μ-opioid receptors are involved in the molecular mechanisms of anti-diabetic effect of LVVYPW. Medical Science of Armenia, 2011, LI (N3):33-42.
7. *Barkhudaryan N., Zakaryan H., Sarukhanyan F., Gabrielyan A., Dosch D.,Kellermann J., Lottspeich F*. Hemorphins act as homeostatic agents in response to endotoxin-induced stress. Neurochem. Res., 2010, 33, p.925-933.
8. *Barsegyan K., Barkhudaryan N., Galoyan A.* The investigation of the effect of native and synthetic coronaro-constrictory peptide factors on Ca^{2+}, calmodulin-dependent phosphoprotein phosphatase activity. Neurokhimiya (RAS & NAS RA), 1992, 11, p.141-149.

9. *Beishuizen A., Thijs L.G.* Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. J. Endotoxin Res., 2003, 9, p. 3-24.
10. *Joshi M.S., Julian M.W., Huff J.E., Bauer J.H., Xia Y., Crouser E.D.* Calcineurin regulates myocardial function during acute endotoxemia. Am.J. Respir.Crit. Care Med., 2006, 173, p.999-1007.
11. *Lammerich H.P., Busmann A., Kutzleb C., Wendland M., Seiler P., Berger C., Eickelmann P., Meyer M., Forssmann W.-G., Maronde E.* Identification and functional characterization of hemorphins VV-H-7 and LVV-H-7 as low-affinity agonists for the orphan bombesin receptor subtype 3. Br. J. Pharmacol., 2003, 138, p.1431-1440
12. *Liu J., Farmer J.D., Lane W.S. Friedman J., Weissman I., Schreiber S.L.* Calcineurin is a common target of cyclophillin-cyclosporin A and FKBP-FK506 complexes. Cell, 1991, 66, p.807-815.
13. *Moeller I., Lew R.A., Mendelsohn F.A.O., Smith A.I., Brennan M.E., Tetay T.J., Chai S.Y.* The globin fragment LVV-Hemorphin-7 is an endogenous ligand for the AT_4 receptor in the brain. J. Neurochem., 1997, 68, p. 2530-2537.
14. *Moisan S., Harvey N., Beaudry G., Forzani P., Burhop K.E., Drapeau G., Rioux F.* Structural requirements and mechanism of the pressor activity of Leu-Val-Val-hemorphin-7, a fragment of hemoglobin β-chain in rats. Peptides, 1998, 19, p.119-131.
15. *Namgaladze D., Shcherbyna I., Kienhofer J., Hofer H.W., Ullrich V.* Superoxide targets calcineurin signalling in vascular endothelium. Biochem. Biophys. Res. Commun., 2005, 334, p.1061-1067.
16. *Nishimura K., Hazato T.* Isolation and identification of an endogenous inhibitor of enkephalin-degrading enzymes from bovone spinal cord. Biochem. Biophys. Res. Comm., 1993, 194, p.713-719.
17. *Nyberg F., Sanderson K., Glamsta E.L.* The hemorphins: a new class of opioid peptides derived from the blood protein haemoglobin. Biopolymers, 1997, 43, p.147-156.
18. *Pham C.G., Bubici C., Zazzeroni F., Papa S., Jones J., Alvares K., Beaumont C., Torti F.M., Torti S.V., Franzoso G.* Ferritin heavy chain upregulation by NF-kB inhibits TNFα-induced apoptosis by suppressing reactive oxygen species.Cell, 2004, 119, p. 529-542.
19. *Petrov R.V., Mikhailova A., Fonina L.A.* Bone marrow immunoregulatory peptides (myelopeptides): isolation, structure, and functional activity. Biopolymers, 1977, 43, p. 139-146
20. *Salvemini D., Riley D.P., Lennon P.J., Wang Z.Q., Curie M.G., Macarthur H., Misko T.P.* Protective effect of a superoxide dismutase mimetic and peroxynitrite decomposition catalysis in endotoxin-induced intestinal damage. Br. J. Pharmacol., 1999, 127, p.685-692.
21. *Salvemini D., Wang Z.Q., Zweier J.L., Samouilov A., Macarthur H., Misko T.P., Currie M.G., Cuzzocrea S., Sikorski J.A., Riley D.P.* A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. Science, 1999, 286, p.304-306.
22. *Sanderson K., Nyberg F., Khalil Z.* Modulation of peripheral inflammation by locally administered hemorphin-7. Inflamm. Res., 1998, 47, p. 49-55.
23. *Serfling E., Berberich-Siebelt F., Chuvpilo S., Jankevics E., Klein-Hessling S., Twardyik T., Avots A.* The role of NF-AT transcription factors in Tcell activation and differentiation, Biochim. Biophys. Acta, 2000, 1498, п. 1-18.
24. *Sharpe M.A. Ollosson R., Stewart V.C., Clark J.B.* Oxidation of nitric oxide by oxomanganese-salen complexes: a new mechanism for cellular protection by superoxide dismutase/catalase mimetics. Biochem. J., 2002, 366, p.97-107.
25. *Sommer D., Fakata K.L., Swanson S.A., Stemmer P.M.* Modulation of the phosphatase activity by oxidants and antioxidants in vitro. Eur. J.Biochem., 2000, 267, p.2312-2322.
26. *Suzuki J., Bayna E., Li H.L., Molle E.D., Lew W.Y.* Lipopolysaccharide activates calcineurin in ventricular myocytes. J. Am. Coll. Cardiol., 2007, 49, p. 491-499.
27. *Tokheim A.M., Martin B.L.* Association of calcineurin with mitochondrial proteins. Proteins: structure, function, and bioinformatics, 2006, 64, p. 28-33.
28. *Van der torn M., Kauffman H.F., van der Deen M., Stebos D.-J., Koeter G.H., Gans R.O.B., Bakker S.J.L.* Cyclosporin A-induced osidative stress is not the consequence of increase in mitochondrial membrane potential. FEBS J., 2007, 274, p.3003-30012.

29. *Wang W., Jittikanont S., Falk S.A., Li P., Feng L., Gengaro P.E., Poole B.D., Bowler R.P., Day B.J., Crapo J.D., Schrier R.W.* Interaction among nitric oxide, reactive oxygen species, and antioxidants during endotoxemia-related acute renal failure. Am. J. Physiol. Renal Physiol., 2003, 284, p.F532-F537.
30. *Wang X., Culotta V.C., Klee C.B.* Superoxide dismutase protects calcineurin from inactivation. Nature, 1996, 383, p. 434-437.
31. *Yamamoto Y., Kanazawa T., Shimamura M., Ueki M., Hazato T.* Inhibitoty effects of spinorphin, a novel endogenous regulator, on chemotaxis, O_2^- generation, and exocytosis by N-formylmethionyl-leucyl-phenylalanine (FMLP)-stimulated neutrophils. Biochem. Pharmacol., 1997, 54, p.695-701.
32. *Zhao Q., Garreau I., Sannier F., Piot J.M.* Opioid peptides derived from haemoglobin. Hemorphins. Biopolymers, 1997, 43, p.75-98.