УДК 577.152.313.16+616.379-008.64

The regulatory influence of LVVYPW on immune-neuroendocrine changes in streptozotocin-induced diabetes

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Keywords: hemorphin, streptozotocin-induced diabetes, calcineurin, TNFα, corticosterone

LVVYPW (myelopeptide-2, LVV-hemorphin-3, LVV-H3) is a member of hemorphins family, the endogenous nonclassical opioid peptides, derived from hemoglobin (Hb) [for review see Ref. 21]. All hemorphins, irrespective of their source, originate from the same region of the β-chain of Hb (residue 31-40 of bovine and residue 32-41 of human Hb), named LVV-hemorphin-7 (LVV-H7) [15]. By using *in vivo* microdialysis in combination with electrospray mass spectrometry it has been shown that LVV-H7 (LVVYPWRQRF) metabolized into shorter fragments and several hemorphins, including LVV-H3, are formed in the striatum of rats [25]. LVV-H3 originally has been isolated from the supernatant of porcine bone marrow cell culture and has been shown to display immunoregulatory and anti-tumor effect by restoring the activity of $CD4^+$ T cells suppressed by tumor cell metabolites [23]. [^{3}H] LVV-H3 has been shown to bind to human blood T lymphocytes in dose-dependent manner [12].

Earlier we have shown that LVV-H3 like other hemorphins *in vitro* modulate Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase calcineurin activity in the brain and immune system by binding with CaM [4, 8, 14], exhibiting a concentration-dependent biphasic response to enzyme activity. Recently, by using the experimental rat models of endotoxin-induced stress [6], and sarcoma-45 [5], we have demonstrated for the first time that hemorphins are able to regulate calcineurin activity *in vivo* as well. Calcineurin is known as a key enzyme in the signal transduction cascade leading to T cell activation. This enzyme 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 [28]. In the brain calcineurin regulates synaptic plasticity and synaptic development and participates in neurotransmitters (serotonin (5-HT), noradrenalin, dopamine, glutamate), neuropeptides and neurohormones (ACTH)

release [32, 17]. Thus, by affecting cytokines and ACTH production, calcineurin may regulate immune-neuro-endocrine interactions. It is very likely that hemorphins, by modulation of calcineurin activity, may also be involved in these processes. Indeed, very recently it has been shown that hemorphins (hemorphins-7 and LVV-hemorphin-7) act as homeostatic agents in response to endotoxin-induced stress and they have a capacity to modulate the hypothalamo-pituitary-adrenocortical (HPA) axis activity by recovering increased calcineurin activity in the brain and plasma and increased corticosterone and TNFα levels in the plasma of rats [6]. Calcineurin/NFAT signaling pathway was shown to play an important role in the regulation of insulin gene expression, modulation of insulin receptor (IR), as well as insulin-like growth factor-1 (IGF-1) functions [19, 30]. Moreover, insulin receptor substrate-1 (IRS-1) is considered as direct substrate for calcineurin *in vivo* [16]. Thus, calcineurin interacts with all components of insulin system (insulin, IR, IGF-1, IRS-1) both at transcription level and posttranslational modification.

The involvement of cytokines, such as IL-2 and TNFα in insulin signalling were also reported [11]. It should be underscored that excessive TNFα expression in diabetes often contributes to several complications in diabetes, such as retinopathy, nephropathy and neuropathy [18, 29, 27]. Increased activity of HPA axis resulting in elevated circulating glucocorticoid levels has been described in patients with diabetes mellitus [2] and in streptozotocin-treated rats as well [9].

The reduced levels of hemorphin-7 peptides in the plasma of diabetic patients [13] indicate to involvement of these peptides in pathophysiology of diabetes. Indeed, recently, we have demonstrated the plasma glucose-lowering effect of LVV-H3 in streptozotocin (STZ)-induced diabetic rats [26].

Taking into account all of aforementioned we considered advisable to study the role of hemorphin on immune-neuro-endocrine changes in streptozotocin-induced diabetes. In the experiments synthetic LVVYPW was used.

Thus, the aims of the present study were:

- to study the effect of single intraperitoneal injection of LVV-H3 on increased plasma and brain calcineurin activity in streptozotocin-induced diabetic rats,
- to determine the effect of LVV-H3 on elevated plasma levels of TNFα and corticosterone in streptozotocin-induced diabetic rats.

Materials and Methods

In the experiments Wistar line rats (180-220g) of both sexes were used. 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. Diabetes was induced by single intraperitoneal (i.p.) injection of 60 mg/kg body weight streptozotocin (STZ) made in fresh 0.1 M citrate buffer, pH 4.5. The rats in control group (n=5) received i.p. injection of an equivalent volume (0.5 ml) of 0.9% w/v saline. Blood was obtained from the tail vein of fasted rats and the blood glucose concentration was measured using One Touch Ultra Easy glucometer (Life scan, Inc., Milpitas, CA, USA). Animals were considered diabetic if they had blood glucose concentrations >20 mmol/l in addition to polyuria and other diabetic features. 14 days after treatment with STZ rats were randomly divided into 2 groups: 1) untreated diabetic group (n=5) received i.p. injection of vehicle; 2) LVV-H3-treated diabetic group (n=5) received daily single i.p. injections of 1mg/kg synthetic LVV-H3 dissolved in saline. On the 16-th day of experiment animals were decapitated between 8:00-10:00 a.m. 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, plasma separated into aliquots and stored at -70◦ C.

Calcineurin activity assay. Brain tissue was homogenized with 2.5 volumes of 50 mM Tric-HCI, pH 7.5 buffer, containing 0.05% Triton-X-100, 0.1 mM EDTA, 1 mM ditiothreitol (DTT), protease inhibitors and centrifugated at 100 000×g for 60 min at 4◦ C. Calcineurin activity was measured by spectrofluorimetric assay using 4-methylumbelliferyl phosphate (4-MUP) as a substrate [1]. A typical enzyme assay was performed in 1 ml of incubation mixture, containing 50 mM Tris-HCI, pH 7.5, 0.5 mM DTT, 1 mg/ml bovine serum albumin (BSA), 1 mM $MgCI_2$, 0.3 mM $CaCI_2$, 1 μM MUP, and necessary amount of enzyme (100 000×g soluble brain protein fraction or plasma). One unit of enzyme activity is defined as amount of enzyme that caused the formation of 0.1 nM of 4-methylumbelliferon (4-MU) at 32◦ C for 1 h. It is important to emphasize that incubation mixture did not contain exogenous calmodulin (CaM), since brain preparation and plasma contained enough endogenous CaM for determination of enzyme activity. After incubation the reaction was stopped by addition of 0.25 ml of 30% trichloroacetic acid. After centrifugation at 5000×g, the pH of supernatant was adjusted to 8.0 by addition of 0.5 ml of 1 M Tris. The quantity of 4-MU was determined fluorimetrically using a Perkin-Elmer MPF-44A spectrofluorimeter. The fluorescence was measured at 445 nm (Exitation at 365 nm). As control the substrate and enzyme were incubated separately.

TNFα and corticosterone assays. TNFα and corticosterone levels in plasma were measured using rat TNFα ELISA kit (DRG International Inc., USA) and Cortisol ELISA kit (DRG Instruments GmbH, Germany) according to the manufacturer's recommendation. The optical density was measured in each well at the wavelength of 450 nm using Stat Fax 303+ Microstrip reader (Awareness Technology, Inc., USA).

Data were analyzed statistically by one-way ANOVA using GraphPad Prism 4 software. Statistical significance - $p<0.05$. All data were expressed as mean ±SEM.

Results and Discussion

The effect of LVV-H3 on brain and plasma calcineurin activity in streptozotocin-induced diabetic rats was studied 24 h after single ip injection of peptide. The results obtained revealed that in streptozotocin-induced diabetes a significant increase took place in both brain (1,75 fold) and plasma (1,58 fold) calcineurin activity (Fig.1A, 1B), respectively in comparison with the control group receiving ip injection of saline.

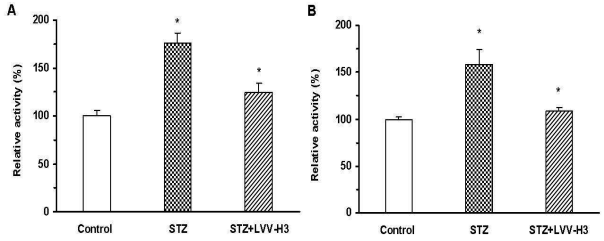

Fig.1. Calcineurin activity in rat brain (A) and plasma (B) after ip STZ and ip STZ+LVV-H3.

*Significantly different from control group received ip saline ($P<0.05$ (A, B)) for STZ group and significantly different from STZ group ($P<0.05$ (A, B)) for STZ+LVV-H3 group (one-way ANOVA).

Treatment of diabetic rats with hemorphins induced significant decrease of calcineurin activity in both brain (1,42 fold) and plasma (1,45 fold) in comparison with not treated by LVV-H3 diabetic group. As one can see (Fig.1A, 1B) ip administration of LVV-H3 (1mg/kg) induces neutralization of the effect of streptozotocin-induced diabetes on calcineurin activity. Earlier we have shown that hemorphins modulate calcineurin activity by binding with CaM [3, 8]. Studying direct interactions between CaM and with μ-opioid receptor (MOR) a possible CaM binding motif in third intracellular loop of MOR has been revealed and a novel Ca^{2+}/CaM signalling pathway of opioid receptors in the regulation of transcriptional activity was suggested. Based on our own data received [3-4, 6, 8] and the latter mentioned finding we proposed that molecular mechanisms of hemorphins action on immune and nervous system involve integration of Ca^{2+}/calmodulin /calcineurin/NFAT signalling pathway with MOR function. Our recent finding, indicating that hemorphin-7 inhibits the DNA binding activity of NFAT transcription factor [7], supports this hypothesis. It has been shown that hemorphin-7 have a capacity to release of MOR agonist β-endorphin [21]. Importantly, the plasma glucose lowering action of anti-diabetic drug metformin involves increase of β-endorphin

secretion from adrenal gland to stimulate MOR, leading to glucose trasporter 4 (GLUT-4) gene expression [10]. Taking into consideration above mentioned we conclude that our hypothesis can be valid for plasma glucose-lowering effect of hemorphin as well.

Because calcineurin/NFAT pathway is responsible for production of TNFα on gene transcription level [28], we expected that LVV-H3 would inhibit significantly the increased plasma level of TNFα (4,1 fold) in streptozotocin-induced diabetic rats, in comparison with the control group, received saline. However, ip administration of LVV-H3 induced modest (1,25 fold), but statistically significant inhibition of increased plasma level of TNFα in diabetic rats (Fig.2).

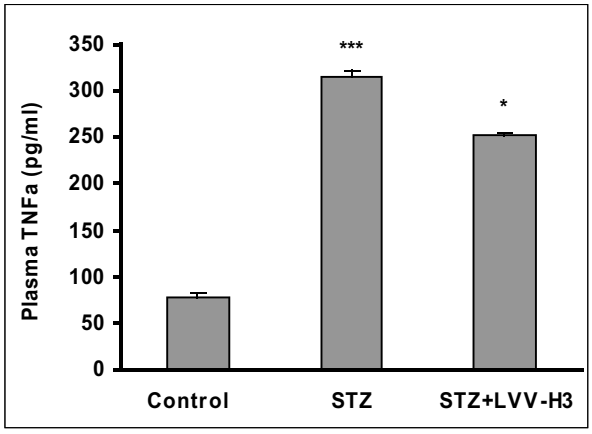

Fig.2. Plasma TNFα levels in STZ-induced diabetic rats and LVV-H3-treated diabetic rats.
***Significantly different from control group ($p<0.001$) for STZ group; *significantly ifferent from STZ group ($p<0.05$) for STZ+LVV-H3 group (one-way ANOVA).

It is suggested that other endogenous compounds may possibly be responsible for modest effect of LVV-H3 on increased plasma level of streptozotocin-induced diabetic rats.

The plasma levels of corticosterone in streptozotocin-induced diabetic rats were increased significantly (2,8 fold) in comparison with plasma levels of corticosterone in control group received saline administration. The *in vivo* administration of LVV-H3 induced significant inhibition of increased plasma level of corticosterone (1.61 fold) in comparison with plasma levels of diabetic rats, which were not treated by LVV-H3 (Fig.3).

Recently, 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, using two different quantitative proteomic methods: ICPL (isotope-coded protein label) and 2-D DIGE (2-dimensional fluorescence difference gel electrophoresis) [6]. It is

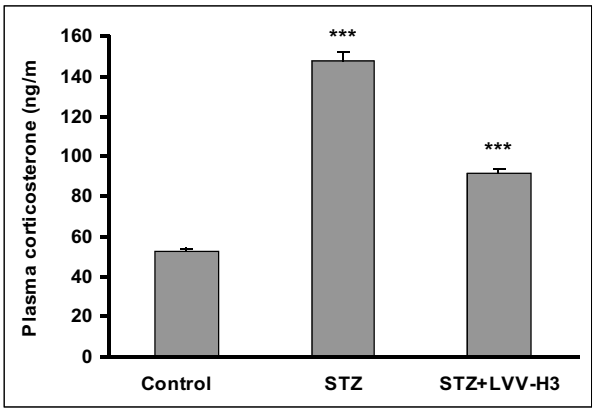

Fig.3. Plasma corticosterone in STZ-induced diabetic and LVV-H3-treated diabetic rats. *** Significantly different from control group ($p<0.001$) for STZ group and significantly different from STZ group ($p<0.001$) for STZ+LVV-H3 group (one-way ANOVA).

worthy of mentioning that the immunosuppressive drugs cyclosporin A (CsA) and FK506 are inhibitors of calcineurin. They are natural, but not endogenous compounds. Both these compounds exert their inhibitory action on calcineurin activity by binding to immunophilins (cyclophilin A and FKBP12, respectively) [20]. It is suggested that hemorphins may be endogenous ligands for immunophilins (Iphs). Iphs interact with glucocorticoid receptor (GR), regulate nuclear translocation of GR and steroid receptor signalling [24]. It is suggested that by modulation of immunophylin expression, hemorphins may affect plasma corticosterone levels.

Because immune-neuroendocrine challenge studies demonstrate increased responsiveness of HPA axis in type 2 diabetes mellitus [22], the significant down regulatory effect of hemorphins on increased plasma levels of corticosterone in diabetic rats may have a significant therapeutic potential.

Поступила 17.12.10

LVVYPW-ի կարգավորիչ ազդեցությունը իմունանեյրոէնդոկրինային փոփոխությունների վրա ստրեպտոզոտոցինով խթանված շաքարախտի ժամանակ

Ֆ. Պ. Սարուխանյան, Ն. Հ. Բարխուդարյան

Ուսումնասիրվել է սինթետիկ LVV-հեմորֆին-3-ի (LVV-H3) ազդեցությունը հիպոթալամո-հիպոֆիզար-մակերիկամային համակարգի ակտիվության վրա ստրեպտոզոտոցինով խթանված դիաբետիկ առնետների մոտ: Շաքարախտը առաջացվել է առնետների մոտ ստրեպտոզոտոցինի (60 մգ /կգ) ներորովայնային մեկ ներարկման միջոցով: Ցույց է տրվել, որ դիաբետիկ առնետների պլազմայում զգալի չափով ավելանում են ուռուցքի նեկրոզի գործոն ալֆայի (TNFα) (4,1 անգամ) և կորտիկոստերոնի (2,8 անգամ) քանակները: LVV-H3-ի (1մգ/կգ) ներորովայնային ներարկման հետևանքով դիաբետիկ առնետների պլազմայում տեղի է ունենում TNFα -ի (1,25 անգամ) և կորտիկոստերոնի (1,61 անգամ) քանակների նվազում: Կալցինեյրինի խթանված ակտիվությունը դիաբետիկ առնետների ուղեղում (1,75 անգամ) և պլազմայում (1,58 անգամ) զգալի չափով չեզոքացվում է հեմորֆինի ազդեցության տակ: Քննարկված են LVV-H3-ի թերապևտիկ հնարավորությունները և ազդեցության մոլեկուլային մեխանիզմները:

Регуляторное воздействие LVVYPW на иммуно-нейроэндокринные изменения при стрептозотоцин-индуцированном диабете

Ф.П. Саруханян, Н.А. Бархударян

Было исследовано воздействие синтетического геморфина LVVYPW (LVV-геморфин-3, LVV-H3) на активность гипоталамо-гипофизарно-адреналовой системы. Диабет был индуцирован разовой внутрибрюшинной инъекцией стрептозотоцина (60 мг/кг). У диабетических крыс были зарегистрированы повышенные концентрации в плазме фактора некроза опухолей-альфа (TNFα) (4,1 раза) и кортикостерона (2,8 раза). Внутрибрюшинная инъекция LVV-H3 (1 мг/кг) привела к снижению концентрации в плазме как TNFα (1,25 раза), так и кортикостерона (1,61 раза). Повышенная активность кальцинейрина в мозгу (1,75 раза) и в плазме (1,58 раза) у диабетических крыс была нейтрализована в результате инъекции LVV-H3. Обсуждаются терапевтический потенциал LVV-H3 и механизмы его действия при патофизиологии диабета.

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