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Calcineurin and μ-opioid receptors are involved in the molecular mechanisms of anti-diabetic effect of LVVYPW

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LVVYPW (LVV-H3, LVV-hemorphin-3, mielopeptide-2) is a member of hemorphins family, the nonclassical endogenous opioid peptides, derived from hemoglobin (Hb) [for review see Ref 34]. LVV-H3 originally has been isolated from the supernatant of porcine bone marrow cell culture and was shown to display anti-tumor effect by restoring the activity of $CD4^+$ T cells suppressed by tumour cell metabolites [32, 35]. In addition, it has been shown that this peptide inhibits tumor growth in rat bearing sarcoma-45 by binding to DNA [6].

A growing body of data demonstrates that hemorphins exhibit pleiotropic effects by affecting different receptors function (e.g. μ-, δ-, κ-opioid receptors [11, 41], angiotensin (Ang) IV receptor (AT_4) [33], bombesin receptor subtype 3 (hBRS-3) [26] and corticotropin-releasing factor (CRF) receptor(s) [3]). Interestingly, all of the mentioned receptors and their ligands have a good functional link with components of insulin system. The increase in β-endorphin secretion activates μ-opioid receptors (MOR) resulting in an increased expression of insulin-responsive glucose transporter 4 (GLUT4). This is one of the mechanisms of action of anti-diabetic drugs [14]. AT_4 is a transmembrane insulin-regulated aminopeptidase (IRAP) [1]. There is a high degree of overlap between the distribution of IRAP and GLUT4. It has been shown that IRAP is co-localized with GLUT 4 in the mouse hippocampus [18]. hBRS-3 was shown to modulate the plasma

insulin concentration [30]. Agonist of CRF receptor(s) urocortin 3 elevates insulin and glucagon secretion [29]. Finally, the existence of functional link between hemorphins and β-endorphin was reported [34]. It has also been shown that Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase calcineurin is a key enzyme underlying the molecular mechanisms of hemorphins action in the brain and immune system [4]. Very recently we have demonstrated that LVV-H3 recovers increased calcineurin activity in plasma and brain of streptozotocin (STZ)-induced diabetic rats *in vivo* [37]. Calcineurin was reported to control gene expression of several biologically active proteins, including cytokines (tumour necrosis factor alpha (TNFα), IL-2, IL-4, etc.) via dephosphorylation and nuclear translocation of NFATc (nuclear factor of activated T cell) family members [39]. It is necessary to underscore that calcineurin participates in insulin and insulin-like growth factor-I (IGF-1) genes expression as well [2, 28]. It should be noted that calcineurin/NFAT signalling regulates pancreatic β-cell growth and function [24]. Hemorphin-7 containing peptides concentrations were shown to be down regulated in plasma of patients with diabetes and cancer [16, 20]. It should be emphasized that hemorphins are inhibitors of dypeptidyl peptidase IV (DPPIV) and angiotensin-converting enzyme (ACE) [15, 42]. It is known that inhibitors of both ACE and DPPIV have been already used as both anti-diabetic and anti-tumor drugs [17, 25, 31, 40]. These facts support the view that hemorphins, demonstrating anti-tumour properties [6, 10, 32, 35], have also good prospects for applied medicine as anti-diabetic drugs. Very recently it has been shown that LVV-H3 recovers the immune-neuroendocrine changes in STZ-induced diabetic rats, demonstrating homeostatic response to STZ-induced diabetes [37].

Taking into account all of aforementioned, we considered advisable to study if LVV-H3 has a capacity to induce anti-diabetic properties by lowering blood glucose concentrations in STZ-induced diabetic rats. In the experiments synthetic LVV-H3 was used.

Thus, the goals of the present study were:

-to determine if LVV-H3 may demonstrate plasma glucose-lowering effect in STZ-induced diabetic rats and to study the molecular mechanisms underlying this effect;

- to reveal the changes in the expression levels of some proteins regulated in the brain of diabetic rats by treatment with LVV-H3, using quantitative proteome analysis approaches.

Materials and Methods

In the experiments male rats (Wistar line) were used, weighing 180-220g at the time of experiment. Rats provided by UNESCO Chair-Life Science International Postgraduate Educational Center (LSIPEC, Yerevan, Armenia), 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. Diabetes was induced by single intraperitoneal (i.p.) injection of STZ (60 mg/kg) (Sigma-Aldrich, Inc. USA), made in fresh 0.1 M citrate buffer, pH 4.5. Blood samples were collected under halothane anesthesia from the tail vein for measurement of glucose concentrations, by using the GlucoPlus glucometer (GlucoPlus Inc., Canada). Animals were considered diabetic if they had plasma 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 (n=10 per group): 1) untreated diabetic group; 2) LVV-H3-treated diabetic group. The untreated diabetic group received i.p. injection of vehicle, while the second group received daily a single i.p. injection of synthetic LVV-H3 (1mg/kg), dissolved in saline. The rats in control group (n=5) received i.p. injection of an equivalent volume (0.5 ml) of 0.9% w/v saline. The effect of LVV-H3 on plasma glucose level was determined using blood samples collected 1h, 2h and 24 h after injection. We have also studied i.p. injection of different doses of LVV-H3 (0.1 mg/kg, 0.5mg/kg, 1mg/kg, 2mg/kg) to examine if LVV-H3 acts in dose-dependent manner.

β-endorphin-like immunoreactivity (BER). Determination of BER was performed (2h after hemorphin injection) by using Beta-Endorphin (Rat) ELISA commercial kit (Phoenix Pharmaceuticals, Inc., USA) according to manufacturer's recommendation. For determination of the role of μ-opioid receptors (MOR) in glucose-lowering effect of hemorphins MOR antagonist naloxone (2mg/kg) (Sigma Aldrich Inc. USA) was i.p. injected to rats 30 min before i.p. injection of LVV-H3.

Sample preparation. For preparation of brain samples from control rats, STZ-induced diabetic rats and hemorphin-treated diabetic rats the brains were homogenized with 6M Guanidine-HCI and 0.1 M HEPES containing buffer, pH 8.5 with protease inhibitors (0.5 mM PMSF, 1 μM pepstatin, 100 μM leupeptin, 1 μg/ml aprotinin). The homogenate was sonicated 3 times 10 sec with 30 sec intervals. After incubation 1 h at room temperature the homogenate was centrifugated for 35 min at 100 000×g and the supernatant was frozen at -70° C until use.

Protein labelling by ICPL isotopes and mass spectrometry. In order to reveal the proteins that are differently regulated in STZ-induced diabetic rat

brain under the treatment with hemorphin the ICPL (isotope-coded protein label) technology was used in its triplex version. Isotopic labelling of proteins with ICPL was done according to the method of Schmidt et al. [38]. Brain proteins of 3 rats (from each of the 3 groups, received different treatment: vehicle, STZ and STZ+hemorphin, was taken 1 rat) pooled and labelled with the light, medium and heavy version of ICPL label (ICPL0, ICPL4 and ICPL10 respectively). The labelled samples were mixed together. An aliquot (50 μg protein) was digested by combination of endoproteinase Glu-C (5 μg in 50 mM Tris buffer, pH 8.0, 5 h incubation at 25ºC) and trysin (1 μg in 50 mM Tris buffer, pH 8.0 at 37 ºC overnight). Then the sample was acidified by adding TFA to the final concentration 1%. The resulting peptides were separated by reversed phase HPLC and analyzed online by ESI-MS and MS/MS using ORBITrap mass spectrometer. The experiments were repeated 3 times. Data were analyzed using ICPLQuant software [12].

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

Results and Disscussion

After i.p. administration of LVV-H3 into fasting STZ-induced diabetic rats at doses 1mg/kg, it has been observed a significant decrease in plasma glucose concentrations from 28,86±1,6 mmol/l to 18,08±2,3 mmol/l. The mentioned dose was selected as optimal after finding that the decrease in plasma glucose concentrations took place in dose-dependent manner (Fig.1), and we did not observe any essential differences in glucose lowering effect of LVV-H3 injection at doses 1mg/kg or 2 mg/kg in 2 h. It should be noted that glucose level reached the plateau after 1h by using i.p. injections of both doses (1mg/kg and 2 mg/kg). It is worth to mention that in case of LVV-H3 injection at doses 0.5 mg/kg, 1mg/kg and 2 mg/kg some glucose-lowering effect of LVV-H3 was observed in 24 h as well (Fig.1). In parallel to plasma glucose-lowering effect,

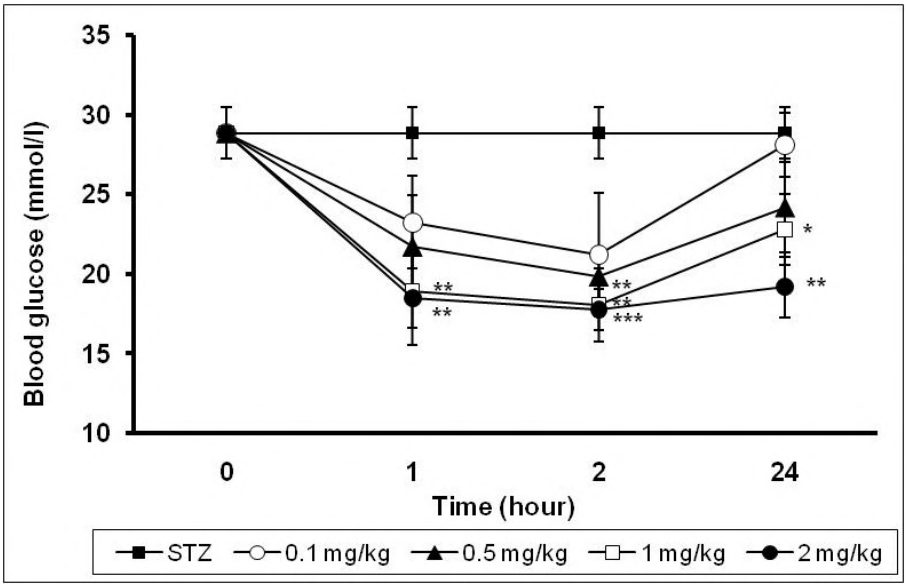

Fig.1. Time courses of changes in plasma glucose concentrations in fasted STZ-diabetic rats after i.p. administration of different doses of LVV-H3.
*p<0.05, **p<0.01, ***p<0.001-significantly different from group received single i.p. injection of STZ (60 mg/kg) (one-way ANOVA).

LVV-H3 induced an increase in plasma β-endorphin-like immunoreactivity (BER) (Fig.2). As one can see, LVV-H3 completely recovered the plasma BER

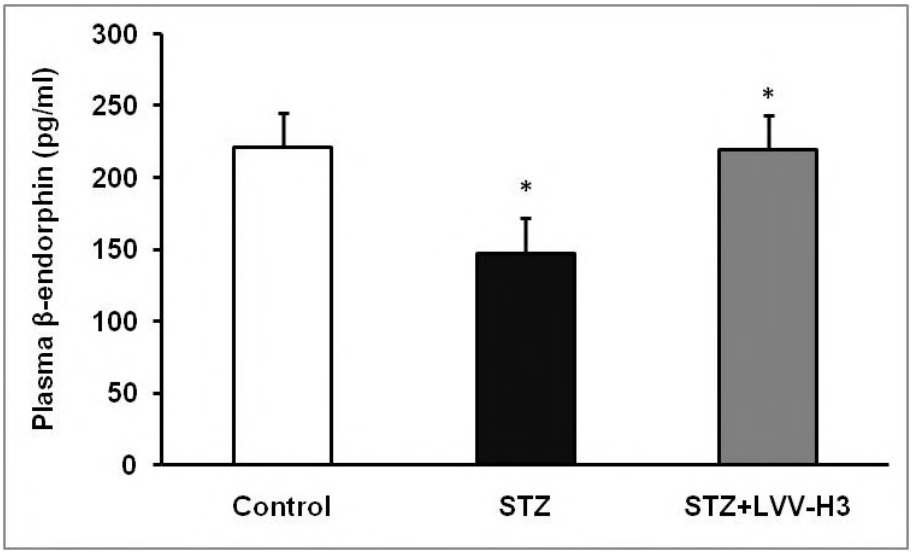

Fig.2. The influence of treatment of diabetic rats with LVV-H3 (1mg/kg) on plasma level of β-endorphin-like immunoreactivity.
*p<0.05-significantly different from control group for STZ group and from STZ group for LVV-H3 treated group (one-way ANOVA).

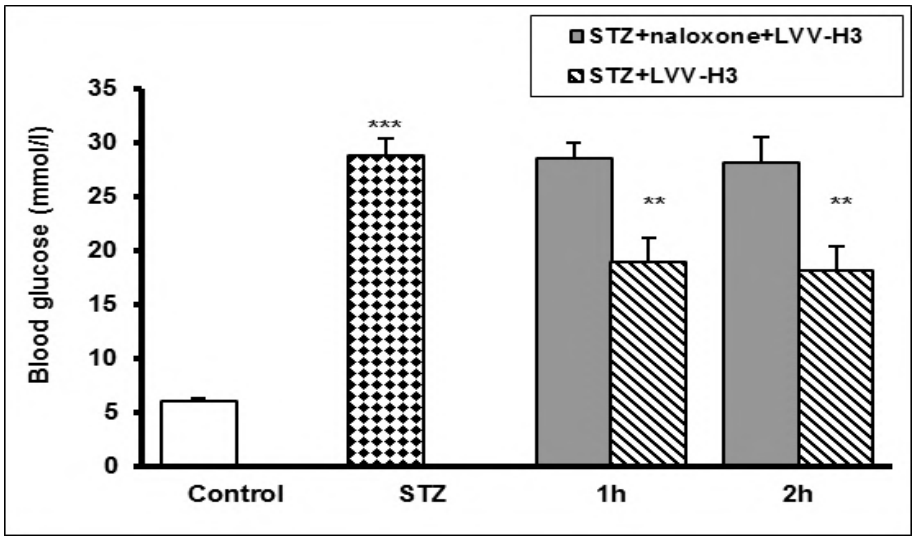

Fig.3. Effect of μ-opioid receptor antagonist naloxone on LVV-H3-induced changes in plasma glucose levels in STZ-induced diabetic rats.

***p<0.001-significantly different from control group, received 0.9% w/v saline, for STZ group; **p<0.01-significantly different from STZ group for groups received i.p. injection of LVV-H3 (one-way ANOVA).

level in diabetic rats. The i.p. administration of MOR antagonist naloxone completely abolished the plasma glucose-lowering effect of LVV-H3 (Fig.3). Thus, the data obtained in the present study demonstrated that hemorphin induces a decrease in plasma glucose concentrations of STZ-induced diabetic rats by activation of MOR and stimulation of β-endorphin release from pituitary gland into circulation. It should be noted that earlier it has been demonstrated the functional link between hemorphin activity and release of β-endorphin in physiological conditions [23, 34]. It is necessary to emphasize that metformin, which was successfully used in clinical treatment of type 2 diabetes, caused a significant parallel increase in insulin sensitivity and plasma β-endorphin level in human subject [14].

Recently, we have found out that calcineurin *in vivo* is involved in the molecular mechanisms of hemorphins action in pathophysiology of severe diseases (stress, cancer and diabetes) [6-7, 37]. Earlier, in 1992 we showed that hemorphins modulate the activity of Ca^{2+}/CaM-dependent enzyme activity, including calcineurin, by binding to CaM, demonstrating concentration-dependent biphasic response to enzymes activity *in vitro* [4-5, 8]. Moreover, taking into account our data obtained, we came to the conclusion that hemorphins may modulate brain calcineurin activity not only by binding with catalytic CaM-binding subunit A, but also by binding with regulatory subunit B [8], which is well known to contain 35% structural homology with CaM. Interestingly, mice with β-cell-specific deletion of calcineurin regulatory subunit B1 (CNB1), develop age-dependent diabetes characterized by decrease in β-cell proliferation and mass, reduced pancreatic insulin content and hypoinsulinemia [24].

By using LC-ESI mass spectrometric analysis CNB1 was found as regulated protein by LVV-H3 in diabetic rat brain. Rats received i.p. injection with STZ only demonstrated up- regulation (1.62 fold) of CNB1 expression level. In diabetic rat brain treated with i.p. administration of LVV-H3 the expression level of CNB1 became close to the expression level of CNB1 in healthy rat brain (1.1 fold). Thus, LVV-3 recovers the expression level of CNB1, acting as homeostatic agent. It should be noted that among the regulated proteins in diabetic rat brain was also found FK-506- binding protein-1A (up-regulated in STZ-treated rat brain – 1.62 fold) and partially down-regulated under the treatment of LVV-H3 (1.2 fold up-regulation). Interestingly, chronic FK506 treatment revealed the diabetogenic effect of

immunosuppressant therapy involving FK506 [28]. The carcinogenic potential of tacrolimus (FK506) was also reported [9]. Next protein, regulated in diabetic rat brain under the treatment with LVV-H3, was S-100 calcium-binding protein A13 (S-100A13). This protein was down-regulated in diabetic rat brain (0,635 fold). The expression level of S-100A13 was changed under the treatment of diabetic rats with LVV-H3 and became close to the expression level of this protein in healthy rats brain (up-regulation 1,125 fold). Importantly, the involvement of

S-100A13 in pathophysiology of tumor was also reported [27]. It should be noted that there is a functional link between S-100A13 and synaptotagmin-1, a Ca^{2+} sensor, that couples local rise in Ca^{2+} to neurotransmitter release [19]. It has been shown that S-100A13 regulates the release of p40 synaptotagmin 1 [13], which was proposed to be involved in pathophysiology of diabetes [21]. Importantly, synaptotagmin 1 also was found to be regulated in diabetic rat brain under the treatment with LVV-H3 (data not shown).

At present, epidemiologic evidence suggests that cancer incidence is associated with diabetes as well as certain diabetes risk factors and treatment. The question arises whether diabetes treatment influences the risk of cancer or cancer prognosis. Because hemorphins exhibit anti-cancer effect [6, 10, 32, 35] and taking into consideration the data obtained in present study, pointing out on their anti-diabetic effect, it is suggested that they have good prospects for applied medicine for creation of new effective complex anti-diabetic drugs without side effect.

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The authors have declared no conflict of interest.

Кальцинейрин и μ-опиоидные рецепторы вовлечены в молекулярные механизмы антидиабетического эффекта LVVYPW

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Получены новые данные о глюкозопонижающемт эффекте LVVYPW (LVV-геморфин-3, LVV-H3) в плазме у стрептозотоцин (STZ)-индуцированных диабетических крыс. После внутрибрюшинной инъекции различных доз LVV-H3 STZ-индуцированным диабетическим крысам мы обнаружили дозозависимое снижение концентрации глюкозы в плазме. Параллельно было обнаружено повышение β-эндорфиноподобной иммунореактивности в плазме в ответ на внутрибрюшинную инъекцию LVV-H3 (1мг/кг). Антагонист μ-опиоидных рецепторов (MOR) налоксон (2 мг/кг) полностью отменял глюкозопонижающий эффект геморфина. Так как недавно нами было показано регуляторное воздействие геморфинов на активность Ca^{2+}/кальмодулин (CaM)-зависимой протеинфосфатазы кальцинейрина в плазме и в мозгу у диабетических крыс, то мы заключили, что молекулярние механизмы глюкозопонижающего эффекта геморфина сложный процесс, включающий интеграцию Ca^{2+}/CaM/кальцинейрин сигнального пути с функцией MOR. Используя LC-ESI масс-спектрометрический анализ, было обнаружено, что в мозгу диабетических крыс LVV-H3 регулирует òàêæå экспрессию таких белков как FK506-связывающийся белок 1, S-100-связывающийся белок A13.

Կալցինեյրինը և μ-օպիոիդ ռեցեպտորները ներգրավված են LVVYPW-ի հակադիաբետիկ ազդեցության մոլեկուլային մեխանիզմներում

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Ստացված են նոր տվյալներ ստրեպտոզոտոցինով (STZ) խթանված դիաբետիկ առնետների պլազմայում LVVYPW-ի (LVV-հեմորֆին-3, LVV-H3)՝ գլյուկոզը նվազեցնող ազդեցության վերաբերյալ: LVV-H3-ի տարբեր դոզաների ներորովայնային ներարկումից հետո հայտնաբերվել է պլազմայում գլյուկոզի կոնցենտրացիայի նվազում դոզակախյալ եղանակով: Միաժամանակ՝ LVV-H3-ի (1 մգ/կգ) ներորովայնային ներարկումից հետո պլազմայում հայտնաբերվել է β-էնդորֆինանման իմունառեակտիվության աճ: μ-օպիոիդ ռեցեպտորների (MOR) անտագոնիստ նալոքսոնը (2 մգ/կգ) ամբողջությամբ վերացնում է հեմորֆինի գլյուկոզը նվազեցնող ազդեցությունը: Քանի որ վերջերս մենք ցույց ենք տվել հեմորֆինների կարգավորիչ դերը պլազմայի և ուղեղի Ca^{2+}/կալմոդուլին (CaM)-կախյալ պրոտեին ֆոսֆատազի կալցինեյրինի ակտիվության վրա դիաբետիկ առնետների մոտ, ապա մեր կողմից առաջ քաշվեց այն տեսակետը, որ հեմորֆինի գլյուկոզի կոնցենտրացիան նվազեցնող ազդեցության մոլեկուլային մեխանիզմները բարդ պրոցեսներ են, որոնք ներառում են Ca^{2+}/CaM/կալցինեյրին ազդանշանային ուղիների ինտեգրացումը MOR-ի գործունեության հետ: Կիրառելով LC-ESI մաս-սպեկտրոմետրիկ անալիզը ցույց է տրվել, որ դիաբետիկ առնետների ուղեղում LVV-H3-ը կարգավորում է այնպիսի սպիտակուցների էքսպրեսիան, ինչպիսիք »Ý FK506-կապվող սպիտակուց 1-ը, S-100- կապվող սպիտակուց A13-ը: