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Hemorphin-7 regulates interleukin-2 promoter activity by Ca^{2+}/calmodulin/calcineurin/NFAT signaling pathway

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Hemorphin-7 (YPWTQRF, H-7) is a member of the hemorphins family, an endogenous nonclassical opioid peptides derived from hemoglobin (Hb) [for review see Ref.17]. All hemorphins, whatever their source, originated 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 [12]. By using *in vivo* microdialysis in combination with electrospray mass spectrometry processing of H-7 from LVV-hemorphin-7 in rat brain and blood was shown [21]. It should be mentioned that catepsin B participates in the generation of hemorphins-7, LVV-hemorphin-5 and hemorphins-5 from LVV-hemorphin-7 in vitro, acting both as dipeptidyl carboxypeptidase and endopeptidase [2]. H7 has been reported to be the most potent among the hemorphins in μ-opioid receptors (MOR) binding [26]. The MOR were reported to exert anti-inflammatory properties, and MOR agonists were claimed to be new therapeutic molecules for treating autoimmune and inflammatory diseases [19]. H-7 was reported to inhibit the acute inflammatory response to substance P (SP) by binding to MOR [20]. It was shown the small, but significant passage of hemorphin-7 across the blood-brain barrier [17]. Hemorphin-7 has a capacity to release β-endorphin from pituitary tissue into circulatory system [17] and, likewise β-endorphin, regulates the hypothalamo-pituitary-adrenocortical (HPA) axis activity [8]. Ca^{2+}/calmodulin (CaM) – dependent protein phosphatase 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 other cytokines and regulatory proteins via dephosphorylation (activation) and nuclear translocation of NFAT (nuclear factor of activated T cell), family members [22]. NFAT transcription factors cooperate with AP-1(activator protein-1)

proteins (Jun/Fos) in DNA binding and transactivation, and this association results in stabilization of the NFAT-DNA interaction [15]. It should be emphasized that besides AP-1, other transcription factor NFκB also plays a critical role in the induction of IL-2, TNFα and others cytokines genes [10]. Thus, AP-1 and NFκB are partners of both NFAT and calcineurin by participation in the regulation of several cytokines and otherproteins gene transcriptions. Importantly, the involvement of calcineurin and NFκB in immunological synapse formation has also been reported [25]. Earlier it has been shown that 5 hemorphins (VVYPW, VVYPWT, LVVYPWT, VVYPWTQR, LVVYPWTQRF) isolated from bovine hypothalamus are able to modulate the brain calcineurin activity by binding to CaM, demonstrating concentration-dependent biphasic response to enzyme activity [9]. Later, using synthetic LVV-hemorphin-4 the same biphasic response was observed on lymphocytes calcineurin activity at the same concentration range as in case of its action on brain calcineurin activity [4]. Because recently it has been found out that H-7 affects DNA-binding activity of transcription factors NFAT AP-1 and NFκB [5], it is suggested that H-7 may modulate lymphocytes calcineurin activity. Therefore one of the goals of the present work is to determine if H-7 is also able to modulate lymphocytes calcineurin activity. Hemorphins were shown to affect IL-2 synthesis in both physiological and pathophysiological conditions and IL-2 receptor expression in human T lymphocytes suppressed by tumor product [4, 16]. Therefore, the next goal of the present work is to examine if H-7 may regulate IL-2 promoter activity in stimulated human Jurkat T leukaemia cells. In the experiments synthetic H-7 was used.

Materials and Methods

Calcineurin activity assay. Lymphocytes were isolated from fresh venous blood of healthy volunteers (35-40 yrs. old) by Ficol-verografin gradient centrifugation (1.077 g/ml, 400g). Cells were cultured in RPMI 1640 medium (Sigma) and supplemented with 5% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate. 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-HCl, pH 7.6, 0.5 mM DTT, 1 mg/ml bovine serum albumin (BSA), 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 μM MUP, 30 x 10^6 lymphocytes (as enzyme preparation) and H-7 (at concentration ranging from 10^{-9} to 10^{-4}M). After incubation at 32°C for 1 h with stirring, the reaction was stopped by

addition of 0.25 ml of 30%- trichloracetic acid. After centrifugation at 5000x g, the pH of supernatant was adjusted to 8.0 by addition of 0.5 ml of 1 M Tris. The quantity of 4-methylumbelliferon (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. One unit of enzyme activity is defined as an amount of enzyme that caused formation of 0.1 nM of 4-MU at 32° C for 1 hr. When the mutual effect of H-7and trifluoperazine (TFP) (10^{-4} M) on calcineurin activity was studied, they were preincubated with lymphocytes for 10 min at room temperature, then substrate was added. The same approach was used to study the effect of EDTA (10-3 M) on lymphocytes calcineurin activity.

Cells and Transient DNA Transfections. Human Jurkat T leukaemia cells were cultured in RPMI medium supplemented with 5% fetal calf serum. They were transfected by IL-2 promoter (300 bp)-luciferase-reporter gene construct. Jurkat T cells were placed into 6-well dish (1,5 ml of $5x10^5$ suspension per well) and 5 μg of plasmid DNA was transfected into $5x10^5$ Jurkat T cells in the presence of "SuperFect" transcription reagent (Qiagen) according to the manufacturer's protocol. Then transfection plate was incubated for 5hrs, at 37ºC/5%CO_2. 1hr before inducing the cells with TPA (12-O-tetradecanoylphorbol-13 acetate;10 ng/ml final concentration) and ionomycin (0, 5 μM final concentration) cells were treated with H-7 (in concentrations range from 10^{-6} to 10^{-12}M). Cells were incubated for 20 hrs at 37ºC/5%CO_2 and then harvested for luciferase activity.

Luciferase Assay. Luciferase catalyzes the release of light upon addition of luciferin and ATP. The relative luciferase activity was assayed and quantified by measurement of light production. The photon production by catalytic oxidation of luciferin occurs from an enzyme intermediate, luciferyl-AMP.

Luciferin + ATP → Luciferyl-AMP + PP

Luciferyl-AMP + O_2 → oxyluciferyl + light

Relative luciferase activity was measured by luminonometer (Berthold, Germany), ising the dual luciferase reporter system from Promega.

Statistical analysis

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

Results and Discussion

To investigate the influence of synthetic H-7 on human blood lymphocytes calcineurin activity, peptide was used at concentrations of 10^{-9} to 10^{-4} M. Before using blood lymphocytes were lysated by 0.05 % Triton X-100. It should be noted that the incubation mixture did not contain exogenous CaM, because it is considered that lymphocytes contained sufficient for detection of

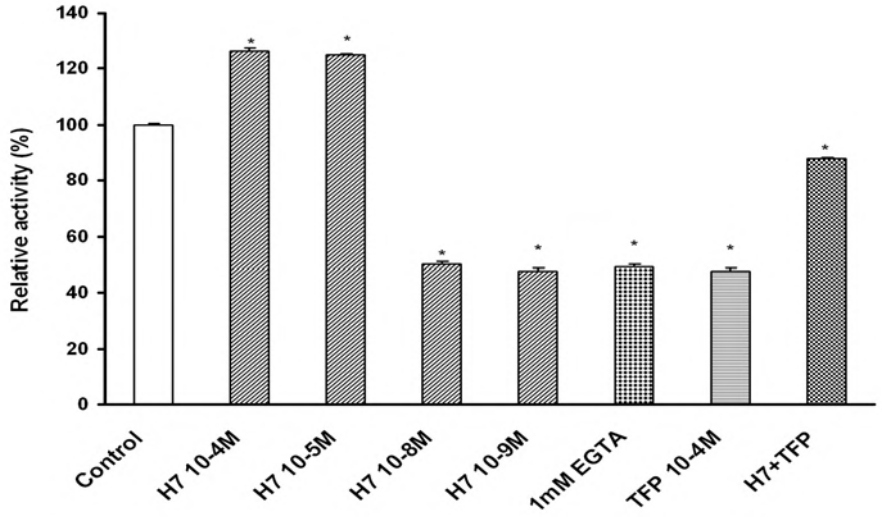

Fig.1 Effect of hemorphin-7 (H-7) on human blood lymphocytes total phosphatases activity. The phosphatase activity determined in incubation mixture, containing

50 mM Tris-HCI, pH 7.6, 0.5 mM DTT, 1 mg/ml BSA, 1 mM $MgCI_2$, 0.3 mM $CaCI_2$, 1μM 4-MUP, 30 x 10^6 lymphocytes at 32°C for 1 h was taken as 100%. H-7, TFP (10^{-4}M) or EGTA (1mM) were preincubated with lymphocytes for 10 min at room temperature, then substrate was added. Data are presented as means ± SEM. *p < 0.001.

calcineurin activity amounts of endogenous CaM. Indeed as one can see in the presence of 1 mM EGTA the total phosphatase activity of blood lymphocytes decreases by 50,7% (Fig.1.). It has been shown that H-7 at concentrations 10^{-5} to 10^{-4} M increases the blood lymphocytes total phosphatase activity (25-26, 5%). The same peptide at concentrations of 10^{-9} – 10^{-8} M induces statistically significant inhibition of blood lymphocytes total phosphatase activity (52,3-45,5%) (Fig.1). Earlier it was demonstrated that hemorphins were bound with both N- and C-terminal domain of CaM demonstrating higher affinity to N-terminal domain [6]. It seems very likely that different K_d values for binding of hemorphins to N- and C-terminal domain of CaM may explain this concentration-dependent biphasic effect of hemorphins on calcineurin activity [6]. The mutual effect of TFP (CaM antagonist and calcineurin inhibitor) and H-7 on blood total phosphatase

activity has been studied. The results obtained have revealed that when the incubation mixture contains both hemorphin (in nM concentration) and TFP (10^{-4} M), a reduction of the inhibitory effect, caused by each of these compounds separately, takes place [4]. These results may be explained by our earlier finding pointing that hemorphins change the sensitivity of CaM to TFP and other its antagonists [3]. It is likely that the binding sites for hemorphins and TFP on the surface of CaM are different, but they influence each other within the ternary complex (CaM-hemorphin-TFP).

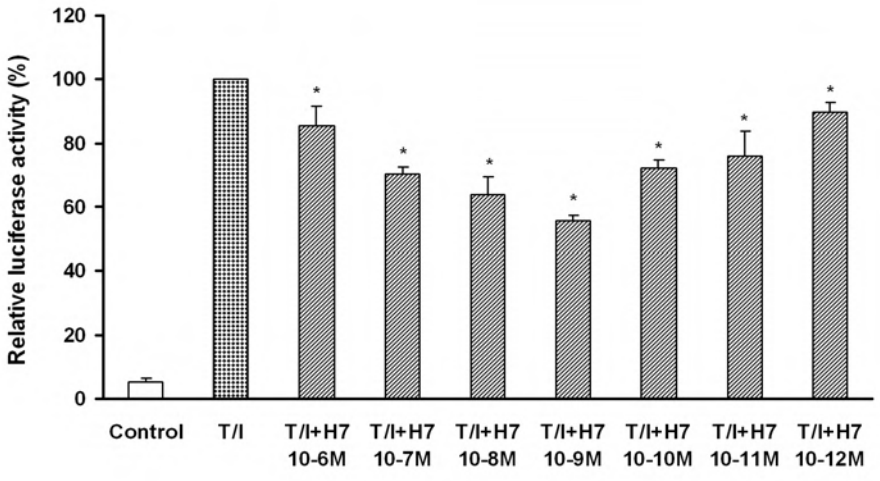

Fig.2. Hemorphin-7 negatively regulates IL-2 promoter activity in nM concentrations in human Jurkat T cells transfected with IL-2 promoter-luciferase reporter gene construct. Data are presented as mean ± SEM, *$p < 0.05$

All above mentioned indicates that modulation of total phosphatase activity of blood lymphocytes under the influence of H-7 is due to the activation/inhibition of lymphocytes calcineurin activity.

It has been demonstrated that H-7 in nM concentrations negatively regulates (by 44.5%) IL-2 promoter transcriptional activity (Fig.2). As it was mentioned above H-7 inhibits DNA binding activity of NFAT and AP-1 (by 38, 7 and 49,8% respectively) in nM concentrations [5]. It should be noted that activation of NFAT by calcineurin is sensitive to calcineurin inhibitors and immunosuppressive agents, such as cyclosporin A and FK506 [11]. Moreover, it is proposed that H-7 regulates IL-2 promoter activities by Ca^{2+}/CaM/ calcineurin/ NFAT signalling pathways. The ability of hemorphins to regulate transcriptional activity may explain their anti-cancer, anti-inflammatory and immunoregulatory properties [4, 7, 16, 20, 24].

It is known that NFAT proteins are activated under different stimuli that lead to increased intracellular calcium concentrations [22]. Besides

participation in cytokines gene expression properties, NFAT family members have been shown to regulate other genes related to cell cycle progression, cell differentiation and apoptosis, revealing a broader role for these proteins in normal cell physiology. However, it has been reported that NFAT family members participate in malignant cell transformation and tumorigenic processes as well [13-14, 23]. Importantly, calcineurin is also involved in pathophysiology of cancer [18]. It is proposed that hemorphins, demonstrate a wide spectrum of biological activity, likewise other neuropeptides. They serve as factors that switch on compensatory system in the organism. This is based on several mechanisms and implication of different signaling pathways in order to recover the homeostatic disturbance in the organism. Better understanding the mechanisms by which NFAT proteins can be regulated by members of endogenous protective system will be useful for development of a new generation of drugs without side effects for treatment of severe diseases, including cancer.

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Հեմորֆին-7-ը կարգավորում է IL-2-ի պրոմոտերի ակտիվությունը Ca^{2+}/կալմոդուլին/կալցինեյրին/NFAT-կախյալ ազդանշանային ուղիներով

Օ. Վ. Հունանյան

Վերջերս ստացվել են տվյալներ, որոնք վկայում են այն մասին, որ հեմորֆին -7-ը (H-7) ընկճում է նՄ կոնցենտրացիաների տիրույթում ԴՆԹ-ի հետ տրանսկրիպցիոն գործոններ NFAT և AP-1-ի կապումը: Ինչպես հայտնի է, կալցինեյրինը մասնակցում է մի շարք ցիտոկինների, ներառյալ ինտերլեյկին-2-ի (IL-2) սինթեզի պրոցեսում՝ համագործակցելով նշված տրանսկրիպցիոն գործոնների հետ: Այդ իսկ պատճառով առաջին անգամ ուսումնասիրվել է H-7-ի ազդեությունը լիմֆոցիտների կալցինեյրինի ակտիվության վրա: Բացահայտվել է, որ H-7-ը հանդիսանում է լիմֆոցիտների կալցինեյրինի ակտիվության կարգավորիչ՝ կապվելով կալմոդուլինի հետ: H-7-ը, ինչպես նախկինում ուսումնասիրված այլ հեմորֆինները, առաջացնում է դոզակախյալ հակառակ ազդեցություն կալցինեյրինի ակտիվության վրա: Հայտնաբերված է, որ այս պեպտիդը նՄ կոնցենտրացիաների տիրույթում ընկճում է կայցինեյրինի ակտիվությունը: Ցույց է տրված առաջին անգամ, որ H-7-ը նՄ կոնցենտրացիաների տիրույթում ընկճում է IL-2-ի պրոմոտերի տրանսկրիպցիոն ակտիվությունը Jurkat T-բջիջներում: Այսպիսով, պարզաբանվել է, որ Ca^{2+}/կալմոդուլին/կալցինեյրին/NFAT–

կախյալ ազդանշանային ուղիները պատասխանատու են H-7-ի՝ IL-2-ի պրոմոտերի տրանսկրիպցիոն ակտիվության վրա կարգավորիչ ազդեցության համար:

Այսպիսով, ստացված տվյալները վկայում են այն մասին, որ հեմորֆինները կարող են դասվել օրգանիզմի պաշտպանիչ համակարգի գործոնների շարքին, որոնց գլխավոր նպատակն է պահպանել օրգանիզմի դինամիկ հավասարակշռությունը, որը խանգարվում է տարբեր պաթոլոգիաների դեպքում, ներառյալ քաղցկեղը:

Геморфин-7 регулирует активность промотера IL-2 через Ca^{2+}/кальмодулин/кальцинейрин/NFAT сигнальные пути

О. В. Унанян

Недавно были получены данные, свидетельствующие о том, что геморфин-7 (H-7) ингибирует в нМ концентрациях связывание транскрипционных факторов NFAT и AP-1 с молекулой ДНК. Как известно, кальцинейрин контролирует синтез ряда цитокинов (IL-2 и др.) на уровне транскрипции генов, взаимодействуя с указанными транскрипционными факторами. Поэтому в данной работе впервые было изучено влияние H-7 на активность кальцинейрина лимфоцитов крови человека. Обнаружено, что H-7 регулирует активность фермента путем связывания с кальмодулином, вызывая доза-зависимый разнонаправленный эффект на активность кальцинейрина. Показано, что при нМ концентрациях H-7 ингибирует активность кальцинейрина. Впервые обнаружено, что H-7 подавляет транскрипционную активность промотера IL-2 в Jurkat Т-клетках через Ca^{2+}/кальмодулин/кальцинейрин/NFAT сигнальные пути. Полученные данные позволяют заключить, что геморфины являются членами эндогенной защитной системой организма, главная цель которых восстановить гомеостаз, нарушенный при разных патологиях, включая злокачественные опухоли.

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