BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1996 Mar; 17 (2): 182 - 185

Augmentation of TNF-α production, NK cell activity and IL-12 p35 mRNA expression by methionine enkephalin[1]

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KEY WORDS methionine enkephalin; tumor necrosis factor; natural killer cells; interleukin-12; gene expression

AIM: To study the host immune surveillance functions by the neuropeptide methionine enkephalin (met-enk). **METHODS**: To measure the effects of met-enk on NK activity, the production and gene expression of anti-tumor cytokines tumor necrosis factor α (TNF-α) and interleukin-12 (IL-12) *in vitro* and *in vivo*. **RESULT**: Met-enk promoted NK activity at $1 \times 10^{-8} - 1 \times 10^{-5}$ mol·L^{-1}, increased TNF-α production both *in vitro* and *in vivo*, and enhanced IL-12 p35 gene transcription after ip 0.1 mg·kg^{-1} for 6 d. **CONCLUSION**: The up-regulating effects of met-enk contribute to the host neuro-immunomodulating mechanism against tumors and invading antigens.

Enkephalins and endorphins were originally described as the endogenous ligands for the morphine receptors in the brain. In addition to their central nervous activity, an immunomodulating action of the enkephalins was first reported in 1979[1]. Methionine enkephalin (met-enk) regulated a variety of immunological responses, including enhancing the proliferation of human peripheral lymphocytes and mouse spleen cells, regulating antibody production, natural killer cells (NK) activity and synthesis of a number of cytokines[2,3]. These results suggested that met-enk might be used to elevate the body resistance to cancer and other diseases, or diminish autoimmune responses which are detrimental to host. To elucidate that met-enk has the activity of anti-tumor through related immune regulating responses, this study is to investigate the effects of met-enk on immune surveillance and immune defence functions.

[1] Project supported by the Fund for the 8th Five-Year Plan Key Project of Chinese Academy of Sciences, No S852015
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Received 1994-07-15 Accepted 1995-06-05

MATERIALS AND METHODS

Mice BALB/c and C57BL/6 mice, 6 - 8 wk old, 19 - 23 g were purchased from Shanghai Animal Center, Chinese Academy of Sciences.

Cell lines L929 cell line (tumorigenic murine fibroblast) and YAC-1 cell line (a Moloney leukemia virus-induced mouse T-lymphoma) were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences.

Agents RPMI 1640 medium (Gibco Laboratories) was supplemented with 10 % newborn bovine serum (NBS, Shanghai Shisheng Co), HEPES 10 mmol·L^{-1} (Fluka AG, Switzerland), *L*-glutamine 2 mmol·L^{-1}, 2-mercaptoethanol 50 μmol·L^{-1} (Fluka), penicillin 100 kU·L^{-1} and streptomycin 100 mg·L^{-1}. Met-enk, lipopolysaccharides (LPS), deoxyribonuclease (DNase), and trysin were purchased from Sigma Co Dactinomycin (Dac), diethyl pyrocarbonate (DEPC), and MTT were from Fluka Co. Guanidine isothiocyanate was from Gibco Lab and *N*-lauroyl sarcosin Na from Amersham Co. d[α-^{32}P]ATP was the product of Beijing Furui Company of Biotechnology. [^{3}H]TdR was from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. Random primer labeled kit was from Boehringer Mannheim Co. Plasmid pUC BM20-IL12 p35 cDNA was constructed in our Lab. Hind Ⅲ and EcoR Ⅰ were from Promega Co. Crystal violet was from Shanghai Chougmin Chemical Reagent Factory.

Culture of macrophages Murine peritoneal macrophages were harvested from BALB/c mice 4 d after an ip injection of 1 mL 3 % thioglycollate broth. Cells were harvested by peritoneal lavage and purified by adherence to 24-well culture plate for 2 h (37 ℃, 5 % CO_2). After removing nonadherent cells, macrophages were cultured with different agents for 20 h and all supernatants were frozen at −20 ℃.

TNF-α activity assay The TNF-α activities were determined by cytotoxicity assay against L929 cells[4].

NK cell activity assay[5] YAC-1 target cells (1×10^9 cells·L^{-1}) were labeled with [^{3}H]TdR 370 MBq·L^{-1} at 37 ℃ for 2 h, washed with a large excess of medium thrice and suspended at a final density of 1×10^8 cells·L^{-1} in RPMI 1640. Mouse splenocytes suspensions were prepared at 2 ×

10^9 viable cell·L^{-1} in RPMI 1640 media as effectors. 100 μL of spleen effector cells at ratio of 100:1 (E:T ratio) were mixed in triplicate wells with 100 μL of 1×10^4 target cells in 96-well plates. RPMI 1640 (0.1 mL) in blank group, 2.5 % Triton × 100 (0.1 mL) in maximal release group. After incubation at 37 ℃ in 5 % CO_2 for 8 h, the culture cells were treated with 0.15 % trypsin and 0.0125 % DNase for 30 min. The cells were collected onto glass fiber papers and detected by liquid scintillation. Results were expressed: Specific release (%) = (Blank-Test)/(Blank-Maximum) × 100 % (dpm).

RNA isolation and analysis Two hours after mice were ip met-enk, total RNA was extracted from mouse splenocytes by the acid-guanidium-thiocyanate-phenol-chloroform method[6]. RNA samples (30 μg) denatured with formaldehyde were applied on nitrocellulose filter in the presence of 5 × stardard saline citrate (SSC) and fixed by heat (80 ℃ for 2 h). Prehybridization (68 ℃ for 2 - 4 h) and hybridization (overnight at 68 ℃) were carried out in a mixture containing 5 × Denhardt's solution, 5 × SSC 0.1 % SDS, 200 mg·L^{-1} denatured sperm DNA. The membranes were washed twice with 2 × SSC 0.1 % SDS for 30 min at 65 ℃, 0.1 × SSC 0.1 % SDS (2 × 15 min) and 0.1 SSC (2 × 15 min). The hybridization probe was prepared from murine IL-12 p35 cDNA and labeled with d[α-^{32}P] ATP using random primers. Dot blot filters were exposed to X-ray film at −70 ℃. Autoradiograms were scanned with a laser densitometer.

RESULTS

TNF-α production TNF-α production from murine peritoneal macrophages was enhanced by met-enk only at high concentration (5 μmol·L^{-1}), when macrophages were induced by LPS, no synergetic effect of met-enk at different concentrations (1×10^{-12} - 5×10^{-6} mol·L^{-1}) was observed (Tab 1).

However, after C57BL/6 mice were ip met-enk 0.1 and 1.0 mg·kg^{-1} for 6 d, the serum TNF-α level of the lower dose group was markedly stronger than control, but less strong than that of LPS (after 2 h) ip with 5 mg·kg^{-1} (Tab 2).

NK cell activity Mouse non-adherent splenocytes treated with met-enk exhibited a marked increase in NK activity. Met-enk 1×10^{-8} - 1×10^{-5} mol·L^{-1} directly augmented the NK activity *in vitro* (Tab 3). The change was correlated with an increased concentration of met-enk.

IL-12 p35 gene expression in mice *In vitro* the IL-12 p35 gene expression of L929 cells was not affected by met-enk although different concentrations were administrated (data not shown). However, met-enk ip 0.1 mg·kg^{-1} and 1.0 mg·kg^{-1} for 6 d increased IL-12 p35 mRNA expression from murine splenocytes, the effect of higher dose was more markedly (Fig 1).

Tab 1. Effect of met-enk on TNF-α production from mouse peritoneal macrophages stimulated with or without LPS *in vitro*. $n = 4$ wells for 1 homogenate (pooled from 15 mice), $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* without LPS control.

Met-enk/ mol·L^{-1}	TNF-α activity (specific lysis %) without LPS	with LPS 10 mg·L^{-1}
0	10.5 ± 5.0	56.9 ± 2.0^f
10^{-12}	6.9 ± 4.3	59.2 ± 2.4
10^{-10}	11.4 ± 5.1	59.8 ± 2.9
10^{-8}	11.1 ± 1.7	58.0 ± 1.6
10^{-6}	9.1 ± 3.7	57.5 ± 1.3
5×10^{-6}	55.6 ± 9.2^c	56.3 ± 2.2

Tab 2. Effect of ip met-enk on TNF-α levels in mouse serum. $n = 5$ mice for each group. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control; $^fP < 0.01$ *vs* LPS group.

	mg·kg^{-1}·d^{-1}	TNF-α activity (specific lysis %)
Saline	-	26.5 ± 6.9
Met-enk	0.1 × 6 d	40.2 ± 7.6^{bf}
Met-enk	1.0 × 6 d	30.9 ± 8.3
LPS	5.0 × 1	59.2 ± 6.4^c

Tab 3. Effect of met-enk on NK cell activity of splenocytes (effector : target = 100 : 1) *in vitro*. $n = 3$ wells for 1 homogenate (pooled from 15 mice), $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Met-enk/ mol·L^{-1}	NK activity (specific cytotoxicity %)
0	40.6 ± 5.7
10^{-10}	50.6 ± 4.6
10^{-9}	51.0 ± 8.3
10^{-8}	53.7 ± 6.0^b
10^{-7}	60.3 ± 3.8^c
10^{-6}	54.4 ± 5.6^b
10^{-5}	61.3 ± 4.0^c

DISCUSSION

TNF-α plays a role in immune surveillance, it

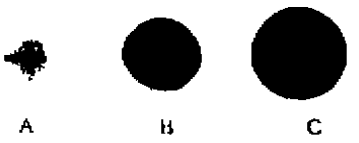

Fig 1. Met-enk enhances mouse IL-12 p35 gene expression *in vivo*. A) Control; B) Met-enk 0.1 mg·kg^{-1} × 6 d; C) 1.0 mg·kg^{-1} × 6 d.

was likely to be a factor of inherent anti-neoplastic activity *in vivo*[7]. Our work demonstrated that met-enk (5×10^{-6} mol·L^{-1}) stimulated directly the TNF-α production from mouse peritoneal macrophages. The result that met-enk at lower dose regulated the production of TNF-α *in vivo* suggested that this opioid peptide as an effective mediator in the neuroendocrine and immune network would contribute to immune surveillance. As higher dose of met-enk did not exert any influence on TNF-α *in vivo*, it may be due to an involvement of other secondary messenger systems[8] which abolished its initial effect on TNF-α production indirectly.

In vitro studies showed that met-enk at higher concentrations ($1 \times 10^{-8} - 1 \times 10^{-5}$ mol·L^{-1}) was effective in potentiating NK cell activity from normal mice, suggesting that there might be a direct stimulating effect on NK cell. Numerous observations showed that met-enk could modulate NK cell activity *in vitro* and *in vivo*. Nevertheless, some studies revealed opposite effects of met-enk *ie*, stimulation, suppression or both[9,10]. These data suggest that the regulatory mechanisms governing the response of immune cells to enkephalins be rather complex.

IL-12 is a heterodimeric cytokine that can act as a growth factor for activated human T- and NK cells, enhance the cytolytic activity of human NK/LAK cells and stimulate the production of IFN-γ[11]. The expression of IL-12 p35 mRNA was increased by met-enk *in vivo*. It may be closely related to neuro-immunomodulating mechanism of enkephalin, however, no obvious changes of IL-12 gene expression in L929 cells *in vitro* were observed, suggesting that the up-regulating effect of met-enk on IL-12 gene expression not be a direct action.

In a word, met-enk enhanced the function of immune defense against tumors and invading antigens through elevating NK activity, stimulating the production and gene expression of some cytokines such as TNF-α, IL-2, IFN-γ and IL-12.

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甲硫氨酸脑啡肽增强 TNF-α 产生，NK 细胞活性和 IL-12 p35 mRNA 的表达[1]

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关键词 甲硫氨酸脑啡肽；肿瘤坏死因子；自然杀伤细胞；白细胞介素-12；基因表达

目的：研究甲硫氨酸脑啡肽对机体免疫监督功能的影响. **方法**：测定 met-enk 对 NK 活性，抗癌细胞因子如：TNF-α 和 IL-12 的产生和基因表达的影响. **结果**：Met-enk (1×10^{-8} - 1×10^{-5} mol·L^{-1})能增强 NK 细胞活性. 体外，体内均能刺激 TNF-α 的产生. ip 0.1 及 1 mg·$kg^{-1}\times6$ d 可增强 IL-12 p35 mRNA 的表达. **结论**：Met-enk 上调抗癌细胞因子及 NK 活性，在癌症监督中起一定的作用.

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BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1996 Mar; 17 (2): 185 - 189

氯代斯阔任对多巴胺受体的作用[1]

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Effect of (±)12-chloroscoulerine on brain dopamine receptors

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KEY WORDS (±)12-chloroscoulerine; dopamine receptors; (−) stepholidine; catalepsy; stereotyping

AIM: To assess potencies of tetrahydroprotoberberines (THPB) and hydrobenzyltetrahydroisoquinolines (HBTI) on DA receptors. **METHODS**: The receptor binding assay with calf striatum to D_1 and D_2 receptors, and the animal behavior tests were used. **RESULTS**: (±) 12-Chloroscoulerine (CSL) was the most potent one among the THPB and HBTI. The affinities of CSL to D_1 and D_2 receptors were 13 and 51 nmol·L^{-1}, respectively. In rats, CSL showed an antagonistic effect on the stereotypy and induced catalepsy. In the 6-OHDA lesioned rats, however, CSL exerted the agonistic effect to DA receptors. **CONCLUSION**: CSL had dual actions to DA receptors and its effects were similar to that of (−)stepholidine.

[1] Supported by the National Natural Science Foundation of China, No 39130091 and the State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, No K016.
Received 1995-04-11 Accepted 1995-06-12

关键词 (±)12-氯代斯阔任；多巴胺受体；左旋千金藤立定；强直性木僵；刻板

目的：比较四氢原小檗碱同类物(THPB)和氢化苄基-四氢异喹啉类(HBTI)两类化合物对 DA 受体的作用强度，从而找出对 DA 受体作用更有效的化合物. **方法**：用小牛纹状体膜蛋白对 D_1 和 D_2 受体进行放射受体结合分析并进行大鼠行为实验. **结果**：(±)12-氯代斯阔任(CSL)对 D_1 和 D_2 受体的亲和力分别为 13 和 51 nmol·L^{-1}，与先导化合物左旋千金藤立定[(−)stepholidine, SPD]在同一水平，动物行为实验表明它对 DA 受体有阻滞作用，但在超敏条件下，出现激动作用，这些特点与 SPD 的作用类似. **结论**：CSL 是目前 THPB 中对 DA 受体作用最强者，与 SPD 类似是对 DA 受体阻滞剂兼有激动作用.

左旋千金藤立定(SPD)是四氢原小檗碱同类物(THPB)的先导化合物，经生化药理，神经药理，电生理等十多种试验，证明它为多巴胺(DA)受体阻滞剂[1-4]并优先作用于 D_1 受体[2]. 用 6-羟基多巴胺(6-OHDA)损毁大鼠单侧黑质，使 DA 受体超敏，SPD 则引起旋转行为的激动作用[5-7]. 这种特性很引人注意，因为在 DA 受体阻滞剂中，尚未见报道这种现象，颇有研究的价值. SPD 已用于临床治疗血管性头痛和偏头痛，多动症等疾病[8]，并有降低眼内压作用[9]. 为了找到更有效的化合物，化学合成了 THPB 和 HBTI 化合物，