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Methionine-enkephalin augments interleukin-6 production and gene expression[1]

ZHONG Fei, LI Xiao-Yu[2], YANG Sheng-Li[3] (*Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, [3]Shanghai Research Centre of Biotechnology, Chinese Academy of Sciences, Shanghai 200233, China*)

AIM: To study the effect of methionine-enkephalin (met-enk) on the production and gene expression of interleukin-6 (IL-6) both *in vivo* and *in vitro*. **METHODS:** IL-6 was assayed using its dependent cell line MH60·BSF2 and measured by MTT spectrophotometry. IL-6 gene expression was proceeded by RNA isolation and hybridization with IL-6 cDNA. **RESULTS:** Met-enk stimulated IL-6 mRNA expression and increased its stability. Met-enk 0.1 and 1 mg·kg^{-1} ip for 6 d enhanced serum IL-6 levels. **CONCLUSION:** An up-regulation of IL-6 by met-enk was mediated through an increase in transcriptional activity and stability of IL-6 mRNA.

KEY WORDS methionine-enkephalin; interleukin-6; gene expression; messenger RNA; cultured cells

Interleukin-6 (IL-6) is a pluripotent cytokine that promotes B-cells to produce antibody, stimulates growth of hybridomas and plasmacytomas as well as the growth and differentiation of T-cells. Its spectrum of activities includes the hematologic and neurologic fields. IL-6 is similar, in structure and function, to a family of neuropoieitic cytokines capable of modulating the expression of a number of neurotransmitter synthetic enzymes and neuropeptide hormones in post-mitotic neurons. This cytokine is released from a variety of cell types such as monocytes/macrophages, fibroblasts, endothelial cells, T-cells, and astrocytes. Depending on the cell type, its expression can be induced by a wide variety of agents including interleukin 1 (IL-1), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), platelet-derived growth factor, neuropeptides such as substance P, lipopolysaccharides (LPS), and viruses[1].

As a link between the neuroendocrine and immune system, the endogenous opioid peptides met-enk has been found as a modulator on several immunologic functions both *in vitro* and *in vivo* such as enhancing the proliferation of peripheral blood lymphocytes and splenocytes when stimulated with or without mitogens, increasing the production of cytokines such as IL-1, IL-2, IFN-γ from macrophages or splenocytes[2,3].

In this study the effects of met-enk on the production and gene expression of IL-6 from macrophages and L929 cells *in vitro* and mouse splenocytes *in vivo* were investigated to obtain more evidence on the neuro-immunomodulating network.

MATERIALS AND METHODS

Mice BALB/c and C57BL/6 mice (20±*s* 2 g) were purchased from Shanghai Animal Center, Chinese Academy of Sciences.

Cell lines L929 cell (tumorigenic murine fibroblast) was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. MH60·BSF2 cell line was kindly supplied by Dr TIAN Zhi-Gang, Shandong Medical Academy of Sciences, cultured with 10 % newborn bovine serum RPMI 1640 medium con-

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[2]Correspondence to Prof LI Xiao-Yu.
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taining human recombinant IL-6 20 kU·L^{-1}.

Reagents Met-enk, LPS, and trypsin were purchased from Sigma Co. Dactinomycin (Dac), diethyl pyrocarbonate and MTT were from Fluka Co. Guanidine isothiocyanate and *N*-lauroyl sarcosin Na were from Amersham Co. Medium RPMI 1640 (Gibco Lab)-containing media was supplemented with HEPES buffer10 mmol·L^{-1}, penicillin 100 kU·L^{-1}, streptomycin 100 mg·L^{-1}, *L*-glutamine 2 mmol·L^{-1}, 2-mercaptoethanol 50 mmol·L^{-1} and 10 % newborn bovine serum, pH 7.2. [α-^{32}P]dATP was the product of Beijing Furui Company of Biotechnology. Random primer labeled kit was from Boehringer Mannheim Co. IL-6 cDNA plasmid was constructed in our lab.

Peritoneal macrophages Thioglycollate-elicited peritoneal macrophages were obtained d 4 after an ip injection of 1 mL 3 % thioglycollate medium, and resuspended in RPMI 1640 medium at a concentration of 2×10^6 cells·L^{-1}. The cell suspension (1 mL) was seeded to 24-well plate. After 2-h incubation at 37 ℃ in a 5 % CO_2 atmosphere, nonadherent cells were washed off twice with RPMI 1640 medium.

Bioassay for IL-6[4] The proliferation bioassay for IL-6 was performed using an IL-6-dependent B-cell hybridoma, MH60·BSF2. The MH60·BSF2 cells were cultured at a density of 1×10^4 cells/well in the presence of macrophage and L929 cell supernatants collected after 20-h met-enk treatment or the serum from mice after ip met-enk for 6 d. After 24 or 48 h, depending on the growth condition of MH60·BSF2, MTT 20 μL (10 g·L^{-1}) were added for 5 h and then 100 μL of 10 % SDS—HCl 0.01 mol·L^{-1} were added to each well after mixing on a plate mixer. The culture plates were incubated overnight at 37 ℃ in a 5 % CO_2 incubator. The absorbance at 570 nm was measured by ELISA spectrophotometer.

RNA isolation and analysis Total cellular RNA was isolated from L929 cells or mouse splenocytes by the guanidine isothiocyante method[5] and quantitated by absorbance at 260 nm. RNA samples (30 μg) denatured with formaldehyde were applied onto a nitrocellular filter in the presence of 10×standard saline citrate (SSC) and immobilized by heat treatment at 80 ℃ for 2 h. Prehybridization (at least 2 h, 68 ℃) and hybridization (overnight at 68 ℃) were carried out in a mixture containing 5×Denhardt's Solution, 5×SSC, 0.1 % SDS, and salmon sperm DNA 200 mg·L^{-1}. The final washing steps were carried out with solutions containing 2×SSC 0.1 % SDS (2×15 min), 0.1×SSC 0.1 % SDS (2×15 min), and 0.1×SSC (2×15 min). The hybridization probe was prepared by digestion of murine IL-6 cDNA-containing plasmid pGEM 3Zf(+) IL-6 with EcoR I and BamH I, and labeling of the appropriate DNA fragment (670 bp) with [α-^{32}P]dATP using random primers. Dot blots were exposed to X-ray film for variable times at −70 ℃. Autoradiograms were scanned with a Laser densitometer (LKB Ultrascan XL).

RESULTS

IL-6 production in murine peritoneal macrophages IL-6 production from murine peritoneal macrophages was enhanced after treatment with met-enk ranged from 10^{-9} to 10^{-5} mol·L^{-1} for 20 h. LPS is a potent inducer of IL-6 expression and it cooperated with met-enk in stimulating IL-6 synthesis and secretion from macrophages (Tab 1).

Tab 1. Enhancement of IL-6 production in mouse peritoneal macrophages by met-enk for 20 h *in vitro*. $n=6$, $\bar{x}\pm s$, $^bP<0.05$, $^cP<0.01$ *vs* control; $^fP<0.01$, *vs* control without LPS.

Met-enk (mol·L^{-1})	A_{570} MTT incorporation without LPS	with LPS (10 mg·L^{-1})
0	0.627±0.039	0.877±0.086^f
10^{-10}	0.688±0.079	0.852±0.033
10^{-9}	0.768±0.042^c	1.095±0.053^c
10^{-8}	0.787±0.056^c	1.108±0.063^c
10^{-7}	0.838±0.021^c	1.122±0.070^c
10^{-6}	0.882±0.021^c	1.118±0.040^c
10^{-5}	0.802±0.045^c	0.998±0.085^b

IL-6 production from L929 cell Well growing L929 cells were treated with met-enk for 20 h. Met-enk (10^{-8} — 10^{-7} mol·L^{-1}) markedly stimulated L929 cells in producing IL-6 as compared to the control wells (Tab 2). An optimal concentration (10^{-8}—10^{-7} mol·L^{-1}) of met-enk may exist because lower or higher concentrations on the promoting IL-6

production were not significant.

Tab 2. Activity of met-enk on IL-6 production from L929 cells for 20 h *in vitro*. $n=6$, $\bar{x}\pm s$, $^cP<0.01$ *vs* control.

Met-enk ($mol\cdot L^{-1}$)	MTT incorporation A_{570}
0	0.728±0.026
10^{-10}	0.773±0.047
10^{-9}	0.738±0.035
10^{-8}	0.883±0.033^c
10^{-7}	0.915±0.034^c
10^{-6}	0.724±0.064

IL-6 gene expression in L929 cell Met-enk $10^{-8}-10^{-5}$ $mol\cdot L^{-1}$ resulted in an elevated background level of IL-6 mRNA after 2 h. These changes were most remarkable at 6 h after treatment with met-enk 10^{-6} $mol\cdot L^{-1}$. Lower concentration (10^{-10} $mol\cdot L^{-1}$) showed a stimulating activity. However, IL-6 gene expression did not elevate further with increased concentration of met-enk (Fig 1).

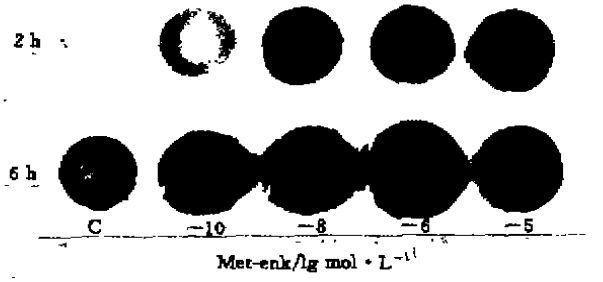

Fig 1. Dot blot analysis of IL-6 mRNA induction L929 cells were grown to confluency and treated with met-enk for 2 or 6 h. Total RNA (30 μg) was isolated and immobilized onto nitrocellulose filters. The filters were probed with [α-^{32}P] labeled IL-6 cDNA from mice. C: control.

Production of IL-6 and IL-6 mRNA *in vivo* The IL-6 levels in mouse serum after ip met-enk 0.1 or 1 $mg\cdot kg^{-1}$ were markedly higher than that of saline group and met-enk 1 $mg\cdot kg^{-1}$ was comparable to that after 2-h treatment with ip LPS 5 $mg\cdot kg^{-1}$ (Tab 3).

Tab 3. Influence of met-enk on IL-6 levels in mouse serum *in vitro*. $\bar{x}\pm s$, $^bP<0.05$, $^cP<0.01$ *vs* saline.

Group	dose/$mg\cdot kg^{-1}$	n	MTT incorporation A_{570}
Saline		4	0.573±0.045
Met-enk	0.1×6 d	5	0.658±0.034^b
Met-enk	1.0×6 d	5	0.900±0.029^c
LPS	5.0×2 h	4	1.005±0.031^c

Both 0.1 and 1 $mg\cdot kg^{-1}$ doses of met-enk induced IL-6 mRNA transcription evidently and the action of met-enk at the higher dose was similar to that of LPS-treated group's (Fig 2).

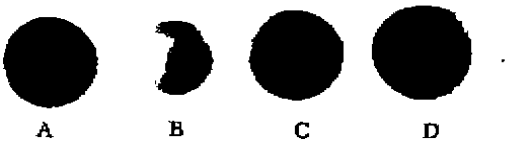

Fig 2. Met-enk induces IL-6 gene expression in mice. RNA was isolated from murine splenocytes, and 30 μg RNA was blotted and hybridized with [α-^{32}P]labeled IL-6 cDNA probe specific for IL-6. A) LPS 5 $mg\cdot kg^{-1}\times 2$ h; B) Saline; C) Met-enk 0.1 $mg\cdot kg^{-1}\times 6$ d; D) Met-enk 1.0 $mg\cdot kg^{-1}\times 6$ d.

Stability of IL-6 mRNA The increase in IL-6 mRNA caused by met-enk was consistent with the possibility of an increased stabilization of IL-6 mRNA. The results after IL-6 mRNA hybridization and autoradiation showed that met-enk at higher concentrations ($10^{-8}-10^{-6}$ $mol\cdot L^{-1}$) increased the stability of IL-6 mRNA (Fig 3).

DISCUSSION

In the present study, met-enk not only directly induced IL-6 production from mouse peritoneal macrophages and L929 cells *in vitro* and elevated the serum levels of IL-6 in mice *in vivo*, but also markedly increased LPS-induced production of IL-6 from macrophages.

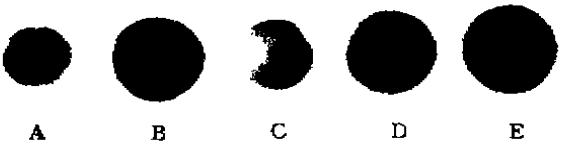

Fig 3. Met-enk increases the stability of IL-6 mRNA. L929 cells were induced with met-enk for 6 h. Dactinomycin (1 mg·L^{-1}) was added to cultures to inhibit funther RNA synthesis for 1 h. Total RNA 30 μg of L929 cells was analyzed per sample. A) control with Dac; B) control; C) met-enk 10^{-10} mol·L^{-1}; D) met-enk 10^{-8} mol·L^{-1}; E) met-enk 10^{-6} mol·L^{-1}.

Furthermore, met-enk caused the transcriptional activation of the IL-6 gene in L929 cells as well as in mouse splenocytes, a significant increase in IL-6 mRNA stability was observed. Like IL-6, the synthesis and secretion of other cytokines such as IL-1, IL-2, and IFN-γ were up-regulated by met-enk[6,7], suggesting that met-enk is an important immunomodulator during immune responses and inflammatory process.

The involvement of met-enk in IL-6 production may be of physiological relevance in stress and inflammatory responses and may play a role in the communication between neuroendocrine system and immune system. The physiological source of endo-opioids may be generated by cells of the immune system beside neuoendocrine origin. However, IL-6 was generated not only by immunocompetent cells but also by astrocytes and fibroblast cells[8]. Therefore, met-enk may be one of the mediated messengers which mediate the primary or secondary responses when the body were stimulated by mitogens or antigens.

The increased expression of IL-6 mRNA involved both transcriptional and post-transcriptional regulatory mechanisms. Previous studies have displayed that induction of IL-6 expression is mainly the results of transcriptional gene activation. The 5, flanking sequences upstream of IL-6 gene contains several response elements that confer gene inducibility by the transcription factors such as AP-1, NF-κB and NF-IL6. Induction of IL-6 transcription by LPS and IL-1 has been shown to be linked to the activation of NF-κB and NF-IL6[9,10]. As met-enk enhanced LPS-induced IL-6 and IL-1 production, it is thus considered that met-enk may exert potential effect on IL-6 expression through enhancing activation of NF-κB and NF-IL6.

In addition, mRNA stability was controlled by both RNA specific sequence and RNA binding proteins. A conserved A + U rich sequence in the 3, untranslated region (UTR) has been identified among labile mRNA for cytokines such as IL-6, GM-CSF, and IL-2. This kind of 3,UTR could be a target for endonucleases or transacting factors regulating IL-6 mRNA degradation[11]. It is not clear which AU binding proteins were involved in the increased stabilization of IL-6 mRNA induced by met-enk.

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甲硫氨酸脑啡肽增强白细胞介素-6的产生及其基因表达[1]

钟 飞, 李晓玉[2], 杨胜利[3]
(中国科学院上海药物研究所, 上海 200031; [2]中国科学院上海生物工程研究中心, 上海 200233, 中国)

目的: 研究甲硫氨酸脑啡肽对白细胞介素-6的产生及其基因表达的影响. **方法**: 用依赖株 MH60·BSF2和 MTT 法测定 IL-6、分离 RNA 和 IL-6 cDNA 杂交后测定其基因表达. **结果**: 甲啡肽体外诱导小鼠 IL-6 mRNA 的表达并提高其稳定性. 腹腔注射甲啡肽0.1和1 mg·kg^{-1} 也能明显提高 IL-6水平并促进脾细胞 IL-6 mRNA 的表达. **结论**: 甲啡肽能通过提高转录活力并增加其 mRNA 稳定性上调 IL-6.

关键词 甲硫氨酸脑啡肽; 白细胞介素-6; 基因表达; 信使 RNA; 培养的细胞

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Effects of tetrandrine on rabbit platelet aggregation and platelet activating factor generation

ZHANG Min, ZHANG Le-Zhi, LU Jin-Sheng
(*Department of Pharmacology, Third Military Medical College, Chongqing 630038, China*)

AIM: To study the effects of tetrandrine (Tet) on platelet aggregation and platelet activating factor (PAF) generation in rabbit platelet-rich plasma (PRP). **METHODS**: The aggregation rate of platelets induced by calcimycin (Cal) and PAF and the inhibition rate of Tet on platelet aggregation were measured. The amount of PAF in PRP stimulated with Cal and treated with Tet was also measured. **RESULTS**: Cal 1—8 μmol·L^{-1} and PAF 9.5—190.5 pmol·L^{-1} induced platelet aggregation. At the final concentrations of 4—64 μmol·L^{-1}, Tet inhibited the aggregation induced by Cal 4 μmol·L^{-1} and PAF 142.9 pmol·L^{-1}. The IC_{50}(95 % confidence limits) were 8.6 (6.0—12.2) μmol·L^{-1} for Cal and 14.0 (6.4—30.4) μmol·L^{-1} for PAF. In the PRP aggregation by Cal, there was a marked increase in PAF content. Tet dependented the release of PAF from platelets by Cal in a concentration-dependent manner, with IC_{50} of 21 (8—54) μmol·L^{-1}. **CONCLUSION**: The inhibition effect of Tet on platelet aggregation might be concerned with the reduction of endogenous PAF generation.

KEY WORDS tetrandrine; platelet aggregation; platelet activating factor; calcimycin

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