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Immunomodulatory activity of orphanin FQ/nociceptin on traumatic rats[1]

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KEY WORDS orphanin FQ; interleukin-1; tumor necrosis factor; *macrophages*

ABSTRACT

AIM: To explore the neuro-immune modulatory effect of orphanin FQ/nociceptin (OFQ) and opioid receptor like 1 (ORL1) receptor on the traumatic rats. **METHODS**: The quantitative method of immuno-cytochemistry and *in situ* hybridization combined with cytokine bioassay were used to detect the expression of endogenous OFQ and ORL1 and the production of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) from peritoneal macrophage. **RESULTS**: Strong signals for both OFQ immuno-reactive cells and ORL1 mRNA were detected in cerebral cortex, hippocampus, and hypothalamus in normal condition, whereas they were significantly reduced after trauma ($P < 0.05$). However, the production of IL-1 and TNF-α from peritoneal macrophage was increased, when expressed as percentage of enhancement, the increment attained to 233 % and 521 % (sample dilution 1:4), 195 % and 566 % (1:8), 233 % and 757 % (1:16), 214 % and 622 % (1:32), respectively, after trauma. After icv injection of OFQ at doses of 0.055 nmol, 0.55 nmol, and 2.75 nmol, the units of IL-1 and TNF-α were reversed ($P < 0.05$); however, the action of OFQ (0.55 nmol) was blocked by ORL1 selective antagonist [phe$^1\Psi$(CH_2-NH)Gly^2] nociceptin-(1-13)-NH_2. **CONCLUSION**: OFQ and ORL1, the new opioid peptide system, are involved in the immune response elicited by traumatic stress.

INTRODUCTION

The family of the G-protein-coupled opioid receptors comprising the μ-, δ-, κ-receptor was extended by a novel member in 1994[1]. This receptor, termed opioid receptor like 1 (ORL1), was cloned by homology-based screening and shares a sequence homology of 64 % within the transmembrane regions with the other family members. Because of its similarity to the opioid receptor family, it was expected that ORL1, like the three other opioid subtypes, would be negatively coupled to adenylyl cyclase. In the search for a natural ligand for the ORL1 receptor, inhibition of forskolin-stimulated cAMP production in ORL1-transfected cells by fractioned extracts from mammlian brain was monitored. A heptadecapeptide was purified that bound selectively the ORL1 receptor in a saturable manner and with nanomolar affinity, which therefore was assumed to be a natural ligand for the ORL1 receptor. This peptide was named Orphanin FQ (OFQ) by Reinscheid[2] or nociceptin by Meunier[3]. Recently, several studies attest to its efficacy in promoting a number of intracellular responses, including inhibition of adenylate cyclase, inhibition of calcium channels[4], and activation of inward rectifying K^+ channels[5]. Besides that, the distribution of OFQ and ORL1 receptor has been extensively investigated in rodent brain, where both the peptide and receptor were shown to be abundantly expressed[6,7], suggesting that they may play a major functional role in the central nervous system, such as locomotion[8,9] and nociception[10-12], but behavioral studies showed that which is different from that of opioids has been proposed. Furthermore, involvement of OFQ in immune functions recently has been documented and some investigations have reported a role for ORL1 in the immune system. ORL1 mRNA transcripts are present in both stimulated and unstimulated mouse splenic lymphocytes, human peripheral blood lymphocytes, human T-leukemic HSB-2, CEM-3, MOLT-4, and Raji Burkitt's lymphoma cell lines[13]. Antisense oligonucleotides of the mouse ORL1 have been shown to suppress *in vitro* polyclonal immunoglobulin production[14], and an up-regulation of human ORL1 receptor mRNA has been observed when

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human peripheral blood lymphocytes are stimulated *in vitro*[15]. To date, however, the role of OFQ system in immune function has not yet been tested directly, therefore, the aim of the present study was to examine further the effects of OFQ system in this respect, and traumatic rats were designed for screening for stress-modulating agents.

MATERIALS AND METHODS

Animal model Wistar aeult female rats (Experimental Animal Center of Chinese Academy of Sciences, D99-002, SPF, 200 – 250 g) were used. After sterilized in tincture of iodine and alcohol, dorsomyotomy and exploratory laparotomy were operated on the rats under anesthesia (sodium pentababital, 40 mg/kg) as the model of traumatic stress. Postoperative infections not occur. The rats were kept warm under standard housing condition. The trauma was performed 48 h after implanting cannula, and the rats were killed 8 h after trauma.

Icv injection of drugs Implantation of the cannula was performed stereotaxically under anesthesia (sodium pentobarbital, 40 mg/kg), and the stainless steel guide cannula (0.5 mm in diameter) with an insert cannula (0.25 mm in diameter) were implanted into right lateral ventricle (P 0.5, L 0.5, H 4.5) and fixed on the skull with dental cement. The drugs were dissolved in sterilized normal saline. Orphanin FQ (synthesized by Shanghai Institute of Biochemistry, Chinese Academy of Sciences) and ORL1 selective antagonist, [phe$^1\Psi$(CH_2-NH) Gly^2] nociceptin-(1-13)-NH_2 (Phoenix Pharmaceuticals) solution was added with protease inhibitor (1 g/L) for preventing from proteolysis. Drugs were injected in 10 s via the cannula at a volume of 20 μL. After the experiment, the rats were killed and the location of the cannula was verified.

Immunohistochemistry Tissue preparation and immunohistochemical staining were performed as described by Leng *et al*[16]. Primary antibody, rabbit anti-OFQ (Phoenix Pharmaceuticals, 1:500) was applied, whose specificity was evaluated by immunoabsorbing experiment. Secondary antibody is biotinylated-anti-rabbit IgG (1 : 200). All data were analyzed by Quantimet 570 software (Leica Q500IW).

***In situ* hybridization** The probe used in the present study were 50-mer oligonucleotide antisenses to the rat ORL1, the sequence were 5′ GGGGCAGGGATCTCCACCAGGCACTCGATCTCTTCATCTTCCACTTGTGC 3′ which were complementary to its mRNA 743 – 796 (Shanghai Sangon Company). The probe was labelled with digoxigenin according to the digoxigenin labelling and detection kit introduction (Boehringer Mannheim). Tissue sections were pre-hybridized and washed as described by Sun *et al*[17]. After that they were hybridized in the solution containing digoxigenin-labelled antisense oligonucleotide probe overnight at 37 ℃. The sections were visualized by incubating with NBT/BCIP. Control was hybridized with sense or missense oligonucleotide probe or overdose of unlabelled oligonucleotide probe. All data were analyzed by Quantimet 570 software (Leica Q500IW).

Cell cultures After sterilized in iodine and alcohol, rat macrophages from every group were collected from the peritoneal cavity. The peritoneal exudate cells implanted in plastic plate (Multi-dish 24 wells; Nunc) and cultured in RPMI 1640 (Gibco) containing 10 % fetal calf serum. Two hours later adherent cells were purified, stimulated with LPS (Sigma, 30 g/L) and continued to incubate at 37 ℃. Twenty-four hours later, the supernatant were taken for IL-1 and TNF-α analysis.

IL-1 bioassay The levels of IL-1 produced by peritoneal macrophages were determined by proliferation of C_3H/HeJ mouse (Experimental animal center of Chinese Academy of Sciences, D99-003, SPF) thymocytes (1.5×10^9/L). The thymocytes were stimulated with ConA (Sigma, 0.75 mg/L) and incubated with the appropriately diluted samples in a total volume of 200 μL for 72 h. The proliferation were measured by ^{3}H-thymidine (Shanghai Institute of Atomic Energy, Chinese Academy of Sciences, 37 μBq/well) incorporation.

TNF-α bioassay This assay was performed as previously described[18]. The levels of TNF-α produced by peritoneal macrophages were determined by the L929 bioassay. The L929 cell line (supplied by Shanghai Institute of Cell Biology, Chinese Academy of Sciences) is sensitive to the cytolytic activity of TNF-α, when treated with actinomycin D (6 mg/L). Samples were serially diluted in RPMI 1640 and incubated with the L929 cells at 37 ℃ for 24 h in a 96-well culture plate. The cell viability was determined by the addition of crystal violet. The optical density was detected by microplate reader (BioRad 450) at 570 nm.

Statistical analysis All data were given as the $\bar{x}\pm s$, and the significance of difference between two groups was assessed by analysis of variance (ANOVA) followed by *t*-test.

RESULTS

Effect of trauma on the expression of OFQ and ORL1 The expression of OFQ and ORL1 was detected in the coronary tissue sections from Bregma −0.26 mm to −0.60 mm of respective groups. Immunocytochemistry (ABC) and *in situ* hybridization were used, and the specificity of method by immunoabsorption, sense and missense oligonucleiotide controls, respectively. OFQ and ORL1 immuno-reactive cells were semi-quantified by randomly chosen three areas in photomicrograph (× 100). In the control group, the mean number of OFQ immuno-reactive cells was 116 ± 19 in cerebral cortex, 72 ± 8 in hippocampus, 64 ± 9 in hypothalamus; the mean number of ORL1 immuno-reactive cells was 104 ± 8 in cerebral cortex, 39 ± 4 in hippocampus, 143 ± 9 in hypothalamus. However, in the traumatic group, the number of OFQ immuno-reactive cells was markedly decreased in the above three regions (31 ± 11, 25 ± 5, 25 ± 5), compared with the control group (Fig 1, $P < 0.05$). The same change was occurred on anti-DIG positive cells in the same region (29 ± 9, 15 ± 4, 33 ± 7) (Fig 2, $P < 0.05$).

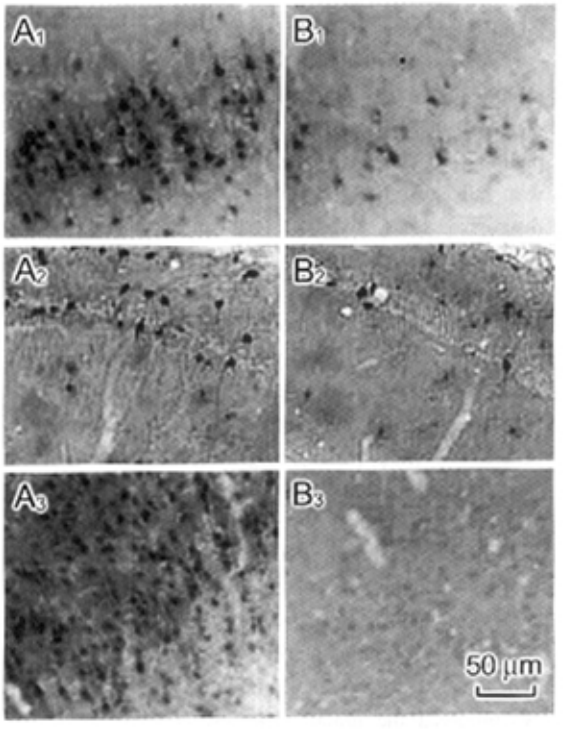

Fig 1. Orphanin FQ expression in the CNS after trauma. 1: cerebral cortex; 2: hypothalamus; 3: hippocampus. A: control; B: trauma.

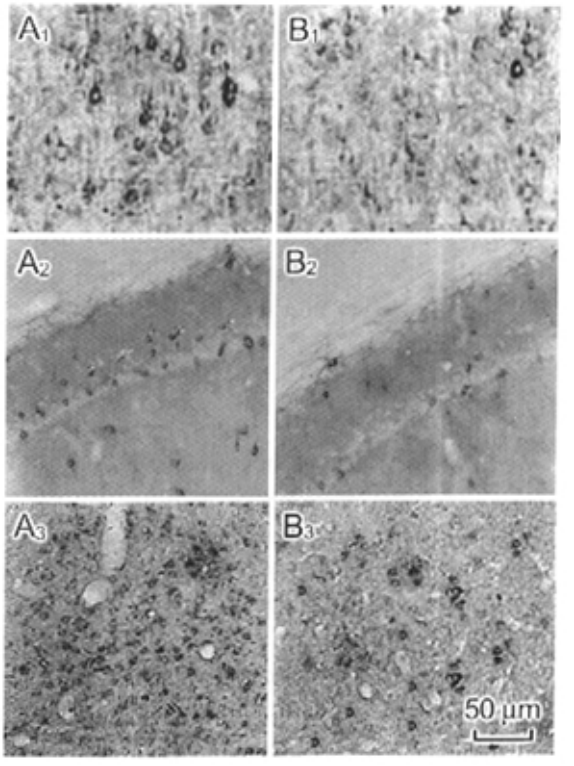

Fig 2. ORL1 expression in the CNS after trauma. 1: cerebral cortex; 2: hypothalamus; 3: hippocampus. A: control; B: trauma.

Effect of OFQ on the production of IL-1 from peritoneal macrophage in traumatic rats Peritoneal macrophage is the main source of IL-1, and acts as antigen presenting cell in immune responses. In the present study, IL-1 activity was determined by ^{3}H-TdR incorporation after stimulating peritoneal macrophage by LPS (30 mg/L). The samples were divided into four groups, and serially diluted (1:4, 1:8, 1:16, 1:32). When the ^{3}H-TdR incorporation in the control group (17 ± 5, 20 ± 5, 16 ± 4, 19 ± 3, Bq) was employed as the standard (100 %), it was shown that ^{3}H-TdR incorporation was promoted to 233 % (1:4), 195 % (1:8), 233 % (1:16) and 214 % (1:32) after trauma, which was significantly decreased by icv injection of OFQ (0.055 nmol, 0.55 nmol, 2.75 nmol). In OFQ 0.55 nmol + OFQ antagonist group, the decreased ^{3}H-TdR incorporation was again increased as compared with the OFQ 0.55 nmol group (Fig 3, $P < 0.05$).

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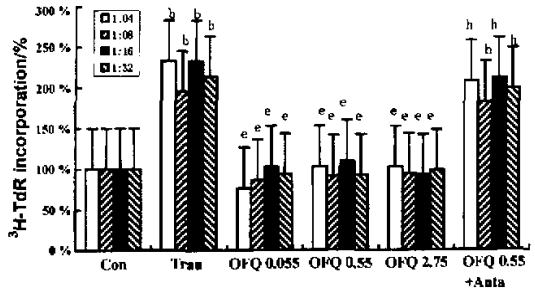

Fig 3. Effect of icv injection of OFQ (nmol) on the production of IL-1 from peritoneal macrophage. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control. $^eP < 0.05$ *vs* trauma. $^hP < 0.05$ *vs* OFQ 0.55 nmol.

Effect of OFQ on the production of TNF-α from peritoneal macrophage in traumatic rats

TNF-α is also the main product of peritoneal macrophage, whose activity was represented by L929 cell viability. The samples were divided into four groups, and serially diluted (1:4, 1:8, 1:16, 1:32). The percentage of enhancement of the optical density (*OD*) in the control group (0.95±0.17, 1.22±0.08, 1.37±0.13, 1.38±0.22) was employed as the standard (100 %). The units of TNF-α in the trauma group were increased to 521 % (1:4), 566 % (1:8), 757 % (1:16), and 622 % (1:32), compared with the control group ($P < 0.05$). However, after icv injection of OFQ (0.055 nmol, 0.55 nmol, 2.75 nmol), the units of TNF-α were all significantly decreased. In OFQ 0.55 nmol + OFQ antagonist group, the decreased units of TNF-α were reversed (Fig 4, $P < 0.05$).

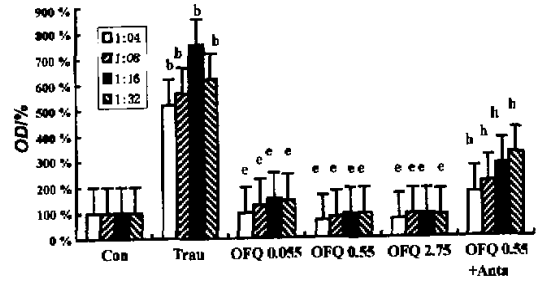

Fig 4. Effect of icv injection of OFQ on the production of TNF-α from peritoneal macrophage. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control. $^eP < 0.05$ *vs* trauma. $^hP < 0.05$ *vs* OFQ 0.55 nmol.

DISCUSSION

Our previous studies showed that operative trauma was a severe stressor, under it, the immune response could be depressed[19]. On account of this immunosuppression could be improved by central administration of naloxone, an antagonist of opioid receptor[20], endogenous opioid peptides was considered to act as an important modulator between CNS and immune system. Until now, it was showed that OFQ, as a new member of opioid family, has extensive homology to endogenous opioid peptides, and also shows broad overlap distribution with that of opioid peptides, from this standpoint, which seem to involve in some physiological functions related to opioid system[1,2]. Therefore, in the present study, the association of endogenous OFQ system with traumatic stress was firstly analyzed. The data showed that OFQ immuno-reactive cells and ORL1 mRNA transcripts in cerebral cortex, hippocampus, and hypothalamus were significantly inhibited after trauma, as well as with the decreased natural killer cell activity induced by trauma, however, both could be reversed by central administration of OFQ[21], which suggested that endogenous OFQ system might be a key factor in the trauma-induced host immune response.

Apart from the fact that sensitivity of lymphocyte proliferation and natural killer cell activity to trauma was decreased whereas the production of IL-1 and TNF-α from peritoneal macrophage, the main cell source of them, was enhanced after trauma, suggesting that monomacrophages may also be a factor in the host response to trauma. So it is therefore now necessary to test directly neuroimmune modulation of OFQ on the monomacrophage, on these cells the ORL1 was largely expressed[13,22]. Recently, the finding that OFQ's anxiolytic-like effects were observed at low nonsedating doses (0.1 – 3 nmol, icv) and were consistent across several behavioral paradigms generating different types of anxiety state in animals was reported. At these doses, stimulation of spontaneous locomotion and exploration has been reported, but no conditioned place reference or aversion reminiscent of the motivational effects of psychostimulants were detected. Conversely, high doses of OFQ (>3 nmol) interfere with normal sensori-motor function and decreased locomotion[23,24]. Accordingly, we observed the immuno-modulatory effect of OFQ at doses of 0.055 nmol, 0.55 nmol, and 2.75 nmol. The results showed that enhanced macrophage activity was reversed, but the effect was not dose-dependent, which

perhaps was due to the limited dose range. Since the blockage of the effect was induced by [phe$^1\Psi$(CH_2-NH) Gly^2] nociceptin-(1-13)-NH_2, one of ORL1 specific antagonists, the action of OFQ is postulated to be mediated by ORL1. Also, OFQ was claimed to induce hyperalgesia within 15 min after icv injection, but followed in the next 30 min by the loss of hyperalgesia and the appearance of analgesia[25], the effects at 2 h after icv injection of OFQ will be at the time range when accurately reflected the function of it.

Additionally, IL-1 is considered as one of the early factors in the host immune response upon stimulation by endotoxin or other microbial products, it synthesized within 15 min, the peak of accumulation occurs at 3 to 4 h[26]. *In vivo* experiment, Du *et al* reported that the peak level is at 8 h after trauma, then decreased and returned to steady state after 24 h[27]. Thus the inhibitory effect of OFQ on the peritoneal macrophage activity at 8 h after trauma may reflect the early host response to trauma.

In conclusion, our present results underscore the growing recognition of neuroimmune modulation of OFQ system. This system may represent one of the information channels between central nervous system and immune system.

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孤啡肽对创伤大鼠的免疫调节作用[1]

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关键词 孤啡肽；白介素-1；肿瘤坏死因子；巨噬细胞

目的：探讨孤啡肽及其受体在创伤大鼠的神经免疫调节作用. **方法**：采用免疫组织化学，原位杂交，及细胞因子的生物活性检测技术，定量分析内源性孤啡肽及其受体在中枢神经系统的表达及腹腔巨噬细胞分泌 IL-1 及 TNF-α 的能力. **结果**：在正常条件下，孤啡肽及孤啡肽受体 mRNA 免疫阳性细胞广泛分布于皮层、海马及下丘脑. 而在创伤应激作用下，阳性细胞数明显减少. 而另一方面，腹腔巨噬细胞分泌 IL-1 及 TNF-α 的能力却明显增强. 将对照组的^{3}H 掺入值及吸光度定为 100 %，则在创伤应激作用下，二者的活性分别增强至233 % 及 521 % (1:4)，195 % 及 566 % (1:8)，233 % 及 757 % (1:16)，214 % 及 622 % (1:32). 侧脑室注射三种剂量的孤啡肽(0.055 nmol, 0.55 nmol, 2.75 nmol)对 IL-1 及 TNF-α 的活性均有显著的下调作用. 而且孤啡肽(0.55 nmol)的作用能被其受体拮抗剂所阻断. **结论**：孤啡肽及其受体，作为新的内阿片肽系统，参与创伤应激介导的免疫调节.

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