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Modulation of intrathecal morphine-induced immunosuppression by microinjection of naloxone into periaqueductal gray[1]

ZHANG Yu, DU Li-Na, WU Gen-Cheng, CAO Xiao-Ding[2] (*State Key Laboratory of Medical Neurobiology, Shanghai Medical University, Shanghai 200032, China*)

KEY WORDS spinal injections; morphine; periaqueductal gray; naloxone; natural killer cells; interleukin-2; tumor necrosis factor; corticotropin

AIM: To study the involvement of opioid receptor of periaqueductal gray (PAG) and hypothalamic-pituitary-adrenal (HPA) axis in the effect of intrathecal morphine on immune function. **METHODS**: Rat splenic natural killer (NK) cell activity was determined by a europium release assay; the concanavalin A-induced splenic IL-2 production, TNF-β activity, and serum TNF-α level were determined by colorimetric thiazolyl blue tetrazolium bromide (MTT) and gentian violet assay, and serum corticotrophin (ACTH) level by radio-immunological method after intrathecal injection of morphine and PAG microinjection of naloxone. **RESULTS**: Intrathecal morphine inhibited splenic NK cell activity, IL-2 production, TNF-β activity, and increased in serum ACTH level. Microinjection of naloxone 1 μg into PAG partially antagonized the inhibition of NK cell activity and the elevation of serum ACTH level by morphine. **CONCLUSION**: The opioid receptor of PAG involved in the suppression of NK cell activity by intrathecal morphine, which was accompanied by an activation of HPA axis.

Administration of morphine into epidural and intrathecal space are efficiently applied in clinic to control postoperative pain and cancer pain. Intrathecal 40 μg or epidural injection of morphine 1 mg into rodents or postoperative patients resulted in a suppression of immune function[1,2].

Although the mechanism of morphine-induced immunosuppression remains unclear, that morphine interacts directly with opioid receptor present on the surface of immunocytes might be a direct pathway contributing to the immunomodulatory effect of morphine[3]. However, evidence provided another possibility — an indirect pathway. Central nervous system played an important role in the suppressed immune status produced by morphine[4-7]. Activation of hypothalamic-pituitary-adrenal (HPA) axis might be one of other potential pathways mediate the effect of morphine[8]. To examine the involvement of opioid receptor of periaqueductal gray (PAG) and HPA axis in immunomodulatory effect of intrathecal injection of morphine, rat splenic natural killer (NK) cell activities, concanavalin A (Con A)-induced splenic interleukin (IL)-2 productions, tumor necrosis factor (TNF)-β activities, serum TNF-α, and corticotrophin (ACTH) level were investigated after intrathecal injection of morphine and PAG microinjection of naloxone.

MATERIALS AND METHODS

Reagents RPMI-1640 culture medium (Gibco Co) supplemented with 10 % fetal bovine serum. HEPES 10 mmol·L^{-1}, glutamine 2 mmol·L^{-1}, benzylpenicillin 100 kU·L^{-1}, streptomycin 100 mg·L^{-1}, 2-mercaptoethanol 50 μmol·L^{-1}, pH 7.2. Con A, recombinant human interleukin-2 (rhIL-2), dactinomycin, and gentian violet were products of Sigma Co. Recombinant murine TNF-α from Genzyme Co. Thiazolyl blue tetrazolium bromide (MTT) was from Fluka Co. Morphine was from Shenyang First Pharmaceutical Factory. Naloxone was the product of School of Pharmacy, Shanghai Medical University. Enhancement solution was from Pharmacia Co.

Rats Wistar rats (♂, n = 30, 3 - 4 month old, 280 - 300 g) were provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade Ⅱ, Certificate

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[2] Correspondence to Prof Cao Xiao-Ding. Phn 86-21-6404-1900, ext 2398. Fax 86-21-6417-4579. E-mail xdcao@shmu.edu.cn

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Cell culture YAC-1 cells, a murine T-cell lymphoma, from Shanghai Cancer Hospital, were incubated in an RPMI-1640 complete medium (15×10^8 cells $\cdot L^{-1}$), supplemented with a labeling mixture containing HEPES 25 mmol $\cdot L^{-1}$, NaCl 30 mmol $\cdot L^{-1}$, KCl 0.5 mmol $\cdot L^{-1}$, $MgCl_2$ 0.3 mmol $\cdot L^{-1}$, dextran sulfate 20 mg $\cdot L^{-1}$, pentetic acid 5 μmol $\cdot L^{-1}$, and $EuCl_3$ 1 μmol $\cdot L^{-1}$. The culture medium was renewed every 2 d. After a 4-d incubation, cells were washed by RPMI-1640 for 5 times and adjusted to 1×10^8 cells $\cdot L^{-1}$ as the target cells to examine NK cell activity. L929 cells, a TNF-sensitive murine fibroblasts, were kindly provided by Dr Wu-Ming DONG (NIEHS/NIH, USA) to determine TNF activity.

PAG implantation One week before microinjection, a stainless steel guide (OD 0.5 mm) with an inserted cannula (OD 0.28 mm) was implanted to PAG ($P_{7.6}$ $L_{0.6}$ H_6) under pentobarbital anesthesia (40 mg $\cdot kg^{-1}$, ip).

Intrathecal implantation A polyethylene catheter (PE-10, ID 0.28 mm, OD 0.61 mm, Clay Adams, Parsippany NJ) was inserted into spinal subarachnoid space between L4 and L5 vertebrae and advanced for 3 cm under pentobarbital anesthesia (40 mg $\cdot kg^{-1}$, ip) 1 wk after PAG implantation. Any rats showed neurologic deficit were excluded from studies.

Drug injection Morphine 40 μg (0.8 g $\cdot L^{-1}$) or normal saline (NS) 50 μL were injected into intrathecal space by implanted tube[1] 12 h after intrathecal implantation. Naloxone 1 μg (1 g $\cdot L^{-1}$) or NS 1 μL was injected into PAG[9] 5 min after intrathecal injection. The microinjection site were confirmed by injection of Amine Black 10B into it after experiment and comparing the brain slices with the rat brain atlas[10]. Eight hours after morphine injection, rats were decapitated, spleens were mashed to produce a single cell suspension.

NK cell activity[11] Rat splenic NK cell activity which was measured by a release of europium-labeled target cell (YAC-1) based on the time-resolved fluorometry. The splenocytes were adjusted to 1×10^{10} cells $\cdot L^{-1}$, E : T (effector : target ratio) = 100 : 1. The NK cell activities were calculated:

% lysis = (experimental release-spontaneou release)/(maximum release-spontaneous release

IL-2 production[12] **and assay** The IL-2 dependent cell line CTLL-2 was washed twice an adjusted to 6×10^7 cells $\cdot L^{-1}$. Medium (10 μL) containing no IL-2, different standard unit o IL-2 or each diluted supernatant (1 : 2, 1 : 4 1 : 8) were added in triplicate to the wel containing 25 μL CTLL-2 cells. The plates wer incubated at 37 ℃ in a humidified atmosphere o 5 % CO_2 for 20 h. MTT (5 g $\cdot L^{-1}$, dissolve in PBS 0.01 mol $\cdot L^{-1}$, pH 7.4) 10 μL wa added into each well for the last 4-h incubation Solution (100 μL) containing 50 % *N*,*N* dimethylformamide (DMF) and 20 % sodiun lauryl sulfate (SDS) were added to each well t solublize the MTT formazan. Having been mixe thoroughly for 30 min to dissolve the dark blu crystals at 25 ℃, the plate was read on th ELISA autoreader at a test wavelength of 570 nm and a reference wavelength of 655 nm. The IL-2 level of supernatant was achieved by the standar curve.

TNF production and assay TNF-β production was taken as IL-2 production, 25×10^3 L929 cells in 100 μL of complete mediun were added to wells of 96-well flat-bottome plates and incubated overnight to achiev confluence. After incubation the medium wa decanted and fresh complete medium 100 μL containing dactinomycin 6 mg $\cdot L^{-1}$ was added t each well. Following a further 30-mi incubation, 100 μL serial dilutions of th supernatants were added in triplicate to the well and the same volume of known amounts o recombinant murine TNF-α was added to reference wells to generate a standard curve in each plate. After an additional 18-h incubation at 37 ℃, the surviving cells were fixed and stained with 0.2 % gentian violet in 2 % ethanol for 15 min. The cells were washed with water and air dried. An aqueous solution of 1 % sodium dodecyl sulfate (200 μL) in distilled water was added to each well to solublize the gentian violet. The plate was read on the ELISA autoreader at a test wavelength of 570 nm and a reference wavelength of 655 nm. The serum TNF-α level was achieved on the standard curve. TNF-β activity was calculated: Cytotoxicity (%) = (Control-test)/Control × 100 %.

ACTH radioimmunoassay ACTH was measured by radioimmunoassay using an RIA kit (Diagnostic Products Co, USA). Blood samples were taken at 7:00, set at 25 ℃ for 1 h and at 4 ℃ overnight. Serum samples were stored at −70 ℃ before assay.

RESULTS

Intrathecal morphine 40 μg (50 μL) showed a suppressive effect on the splenic NK cell activity with an elevation of serum ACTH level ($P < 0.05$). The Con A-induced IL-2 production and TNF-β activity were also inhibited by intrathecal morphine, whereas serum TNF-α level was not significantly altered ($P > 0.05$). Naloxone 1 μg (1 μL) microinjected into PAG after intrathecal injection of morphine partially antagonized the inhibition of NK cell activity and the elevation of ACTH level produced by morphine ($P < 0.05$). However, naloxone failed to attenuate the suppressive effect of morphine on Con A-induced IL-2 production and TNF-β activity of splenocytes. Naloxone alone showed no significant effect on splenic NK cell activity, IL-2 production, TNF-β activity, or serum TNF-α level ($P > 0.05$), but produced a decrease of serum ACTH level ($P < 0.05$). (Tab 1)

Tab 1. Effect of microinjection of naloxone 1 μg into rat periaqueductal gray (PAG) on splenic NK cell activity, Con A-induced IL-2 production, TNF-β activity, serum TNF-α activity, and ACTH level after intrathecal injection of morphine 40 μg. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* NS + NS group. $^dP > 0.05$, $^eP < 0.05$ *vs* morphine + NS group.

Intrathecal	NS	Morphine	Morphine	NS
PAG	NS	NS	Naloxone	Naloxone
Rats	7	8	7	7
NK cell activity/%	50 ± 15	20 ± 6^b	32 ± 11^{be}	55 ± 16^a
IL-2 production/kU·L^{-1}	89 ± 22	21 ± 11^b	26 ± 24^{bd}	96 ± 37^a
TNF-β activity/%	46 ± 12	20 ± 9^b	26 ± 11^{bd}	53 ± 6^a
TNF-α activity/%	2.1 ± 1.2	2.6 ± 1.4^a	2.8 ± 0.8^{ad}	2.5 ± 1.4^a
ACTH level/ng·L^{-1}	66 ± 11	214 ± 113^b	134 ± 54^{be}	49 ± 24^b

DISCUSSION

Our present result showed that naloxone microinjected into PAG, a site was considered to have a naloxone-reversible suppressive effect on NK cell activity when morphine was microinjected directly into this site[6,9,12], partially antagonized the inhibitory effect of intrathecal morphine on splenic NK cell activity. It suggested that opioid receptor of PAG might mediate the inhibition of NK cell activity produced by intrathecal morphine. But the other sites of CNS and the pathway from CNS to immunocytes remain unclear.

The results that intrathecal injection of morphine and PAG microinjection of naloxone were accompanied by an increasing and recovering of ACTH levels, which linked the ability of morphine to elevate hypothalamic corticotrophin-releasing factor (CRF) levels[13] and in turn to induce the stimulation of adrenal corticosteroids which had an inhibitory effect on immune function. It suggested that one potential pathway (HPA axis) mediate immunosuppressive influence of morphine transmitted from action of opioid receptor of PAG to peripheral NK cell function, but there were other reports showed that acute morphine-induced suppression of blood lymphocyte activity lacked the involvement of pituitary and adrenal factors[14]. The result that naloxone alone decreased serum ACTH levels suggested that the endogenous opioid peptides might regulate normal hormone secretion.

Con A (T-cell mitogen)-induced IL-2, TNF-β production (T-cell cytokine) and serum TNF-α level (macrophage cytokine) are responses due to effects on T-lymphocytes and macrophages. The fact that differential modulating effects of the same dose naloxone microinjected into PAG on morphine-induced suppression of splenic NK cell activity, Con A-induced IL-2 production, TNF-β activity, and serum TNF-α level, indicated that PAG opioid receptor and HPA axis did not contribute to effect of morphine on T-lymphocyte and macrophage as it on NK cell with this dosage and at this time point. It suggested that the different pathway or time-course mediate the immunosuppressive effect of intrathecal morphine on different source of immunocytes.

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中脑导水管周围灰质微量注射纳洛酮对鞘内吗啡诱导的免疫抑制的调节[1]

卓 宇，杜芬娜，吴根诚，曹小定[2]（上海医科大学医学神经生物学国家重点实验室，上海 200032，中国）

关键词 鞘内注射；吗啡；导水管周围灰质；纳洛酮；自然杀伤细胞；白细胞介素-2；肿瘤坏死因子；促皮质素

目的：观察中脑导水管周围灰质(PAG)的阿片受体和促皮质素(ACTH)在鞘内吗啡影响免疫功能时的作用和变化。**方法**：用铬标的靶细胞检测大鼠脾脏自然杀伤(NK)细胞活性，MTT 和结晶紫蓝法分析 Con A 诱生的脾细胞白细胞介素-2(IL-2)，肿瘤坏死因子 TNF-β 活性和血清 TNF-α 水平，放免法测定血清促皮质素(ACTH)水平。**结果**：鞘内注射吗啡抑制脾 NK 细胞活性，Con A 诱生的 IL-2 和 TNF-β 活性，并伴有血清 ACTH 水平的上升。PAG 微量注射纳洛酮部分拮抗吗啡引起的 NK 细胞活性的抑制，伴 ACTH 水平恢复。**结论**：PAG 内的阿片受体参与鞘内注射吗啡引起的 NK 细胞活性的抑制，同时伴有 HPA 轴的激活。