

Loss of Morphine-induced Suppression of NK Cell activity and T-cell Functions in μ -Opioid Receptor Knockout Mice

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Abstract: *In vivo* administration of μ -opioid receptor selective agonists to various species is known to suppress lymphocyte, NK cell, and macrophage functions, in addition to mediate pain relief and euphoria. Using a mouse model in which the μ -opioid receptor gene was disrupted by targeted homologous recombination, we explored the involvement of this receptor in natural killer (NK) cell activity and T lymphocyte function. Following peripheral morphine administration, NK cell activity was not affected in homozygous μ -opioid receptor knockout mice, heterozygous animals marginally responded to the immunosuppressive effects of the drug, while wild-type animals were significantly suppressed. In addition, splenic T-cell proliferative responses to concanavalin A, phytohemagglutinin and an antibody to T-cell receptor _{$\alpha\beta$} (TCR) plus interleukin-2 were not affected by morphine treatment in μ -opioid receptor knockout homozygous and heterozygous mice, whereas morphine significantly suppressed T-cell proliferation in wild-type mice. Taken together, these results suggest a role of the μ -opioid receptor in immunoregulation.

Keywords: Rodent, NK cells, lymphocytes, neuroimmunology, transgenic/knockout.

INTRODUCTION

Opioid agonist activities depend on binding to high-affinity receptors in the brain and on cells of the immune system, named μ , δ , and κ which prototypic ligands are morphine, the enkephalins, and the dynorphins respectively¹⁻³. Analgesic effects of opioids can be mediated by these types of opioid receptors⁴. However, *in vivo* administration of μ -opioid receptor selective agonists to various species is known to suppress a number of immune cell functions including natural killer (NK) cell cytotoxic activity, lymphocyte proliferation and production of IFN- γ , T-cell mediated cytotoxicity, antibody formation, and production of TNF- α and nitric oxide, and phagocytosis by macrophages⁵⁻¹⁶. It is recognized of the indirect effects opiate agonists elicit on leukocyte modulation through central pathways^{5,9,10-15}. However, evidence for the direct modulation of opioids on the cells of the immune system has accumulated enormously in recent years¹⁴.

The relevance of opioid receptors within the confines of immunophysiology (autocrine and paracrine

regulation) has not been fully elucidated. One recent study using μ -opioid receptor (MOR) knockout mice showed that chronic administration of morphine induced lymphoid organ atrophy, and decreased thymic CD4+CD8+ cell ratio and NK cell activity in wild-type mice, suggesting that MOR gene product represents a molecular target for morphine action on the immune system¹⁷. In addition, the MOR has been involved in regulating hematopoiesis¹⁸. The present study was conducted to further investigate the immunomodulatory effects of acute morphine action in the MOR knockout mouse.

MATERIALS AND METHODS

Reagents and culture media: Penicillin-streptomycin solution, and RPMI 1640 and AIM-V media were obtained from Life Technologies (Grand Island, NY). Morphine sulfate was a gift from Dr. Andrew Ho from our Department. Concanavalin A (Con A), phytohemagglutinin (PHA), sodium dodecyl sulfate, and HCl were purchased from Sigma Chemical Co. (St.

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Louis, MO). IgG₁ monoclonal antibodies to T-cell receptor (TCR) were obtained from Harlan Bioproducts for Science (Indianapolis, IN).

Animals: C57BL/6J female μ -opiate receptor knockout mice (20-25g) were obtained from Dr. Udi Shavit from Department of Psychology, The Hebrew University, Mount Scopus, Jerusalem, Israel. They were given water and food *ad libitum*.

Drug preparation and administration: Morphine sulfate was dissolved in pyrogen-free saline at a concentration of 30 mg/kg. Two hundred microliters of morphine (3 mg/ml) or vehicle (saline) was then administered intraperitoneally. After 3 hours following morphine or saline injection, mice were killed by cervical dislocation.

Cell preparation and culture: The spleen was immediately removed after animal death. A single-cell suspension was then prepared by disrupting the spleen in RPMI 1640 medium. Cell suspension was washed three times in this medium, and suspended and adjusted at appropriate densities with AIM-V medium (containing 0.5% penicillin-streptomycin solution). The culture medium was changed at this step to the serum-free medium AIM-V which has been observed to support cell culture¹⁹.

NK-cell assay: NK-cell cytotoxic activity was assessed by the chromium release assay using [⁵¹Cr]-labeled YAC-1 murine lymphoma cell line as reported elsewhere³. YAC-1 cells were labeled by incubating 10⁷ cells with 200 μ Ci sodium ⁵¹chromate (NEN Research Products, Boston, MA) for 2 h at 37°C, and then washed three times with RPMI 1640 medium and suspended in this medium to a density of 5 \times 10⁴ cell/ml. YAC-1 cells were added to round-bottomed 96-well plates (Becton Dickinson, Lincoln Park, NJ) containing splenic cells at various concentrations to give effector/target ratios ranging from 25:1 to 400:1. Spontaneous and maximal ⁵¹chromium release were obtained by incubating [⁵¹Cr]-labeled YAC-1 cells in AIM-V medium alone or medium containing 2% sodium dodecyl sulfate plus 0.1N HCl, respectively. After 4 h of incubation, supernatants were harvested and ⁵¹Cr release was measured in a gamma counter (Packard, Downers Grove, IL).

T-cell proliferation assay: T cell proliferation was determined by [³H]-thymidine uptake as previously reported¹². Immediately after rat death, single-cell spleen suspensions were prepared as described above and adjusted to 5 \times 10⁶ cells/ml. Cell suspensions (100 μ l) were added to round-bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 μ l) of AIM-V medium (unstimulated control) or Con A

(1 μ g/ml), and PHA (50 μ g/ml) and antiTCR (3 μ g/ml) for 48 h. After incubation for 44 h at 37°C with 5% CO₂, [³H]-methylthymidine (6.7 Ci/mmol, ICN Pharmaceuticals Inc., Costa Mesa, CA) was added (1 μ Ci/10 μ l/well), and cultures were incubated for 4 h. Cell cultures were then harvested with a semiautomatic cell harvester (Tomtec, Orange, CT), and cell-incorporated radioactivity was determined by liquid scintillation spectrophotometry using a Microbeta Plus liquid scintillation counter (model 1450, Wallac Oy, Turku, Finland) with a counting efficiency for tritium of 35%.

Statistical analysis: The results were expressed as mean \pm SEM of 5 separate rat tissue's responses to each treatment (different effector:target ratios and mitogen concentrations) (3-4 replicate determinations per treatment) per experimental group (homozygous, heterozygous, and wild-type), from a representative experiment. All experiments were repeated at least three times with similar results. Level of significance was assessed by one-way analysis of variance.

RESULTS AND DISCUSSION

Effect of morphine on NK-cell cytotoxic activity: In wild-type homozygous (+/+) mice, morphine significantly ($P < 0.001$) induced 40 \pm 4, 47 \pm 2, and 49 \pm 1 percent of reduction of NK-cell activity at effector:target ratios of 50:1, 100:1, and 200:1 respectively (Fig. 1a), which represents 30 \pm 1 percent reduction of lytic units (102570 \pm 3693 and 71485 \pm 2573 lytic units of saline and morphine treatment, respectively); in knockout heterozygous mice (+/-), morphine significantly ($P < 0.01$) induced 16 \pm 0.1, 23 \pm 0.1, and 8 \pm 0.03 percent of reduction of NK-cell activity at effector:target ratios of 50:1, 100:1, and 200:1 respectively (Fig. 1b), which represents 5.7 \pm 0.08 percent reduction of lytic units (99483 \pm 3581 and 93799 \pm 1376 lytic units of saline and morphine treatment, respectively); and in knockout homozygous mice (-/-), morphine induced ($P > 0.1$) 2.5 \pm 0.08, 0, and 3.5 \pm 0.2 percent of reduction of NK-cell activity at effector:target ratios of 50:1, 100:1, and 200:1 respectively (Fig. 1c), which represents 1 \pm 0.04 percent reduction of lytic units (113136 \pm 4072 and 111882 \pm 4027 lytic units of saline and morphine treatment, respectively), as compared with response of cells from saline-injected control.

Effect of morphine on splenic lymphocyte proliferation: Morphine caused significant ($P < 0.001$) 83 \pm 10, 70 \pm 14, and 65 \pm 3 percent reduction of splenic lymphocyte proliferation induced by Con A, PHA, and antiTCR respectively, in wild-type homozygous (+/+) mice, compared with the response of cells from saline-injected control animals (Fig. 2).

However, morphine did not significantly alter the proliferative response of splenic cells from knockout heterozygous (+/-) and homozygous (-/-) mice to these stimuli, compared with control (Fig. 2).

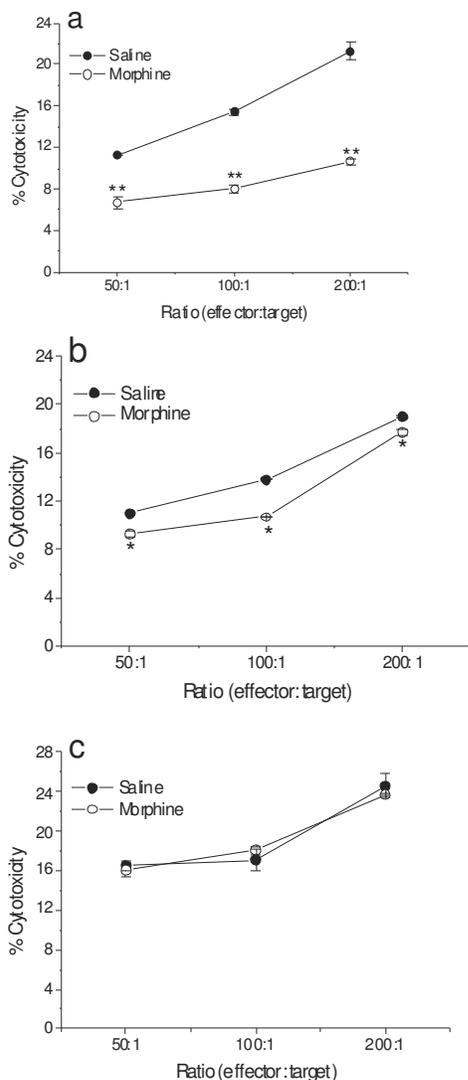


Fig. 1: Effect of morphine on NK-cell activity. Splenic NK-cell cytotoxic activities of (a) wild-type homozygous mice (+/+), (b) knockout heterozygous mice (+/-), and (c) knockout homozygous mice (-/-), were measured 3 h after intraperitoneal injection of morphine or saline as explained in the text. Data represent mean \pm SEM of cytotoxicity percent (test cpm-spontaneous cpm/maximal cpm-spontaneous cpm) \times 100 of three replicate determinations per group of animals (3 mice per group). **, $P < 0.001$; *, $P < 0.01$, as compared with response of cells from saline-injected control.

Lymphocytes, NK cells, and macrophages are very sensitive to opioid action. The role of opioid on regulating immune responses has become more significant because of the implications of drug abuse on immunity against infectious diseases and cancer. μ -Opioid receptor mediation of morphine-induced

analgesia²⁰ and immunosuppression²¹⁻²⁴ has been previously suggested. The production of μ -opioid receptor heterozygous and homozygous knockout mice displaying approximately 54% and 0% of wild-type levels of μ -opioid receptor expression, respectively, has been of significant importance to unravel the role of μ -opioid receptors in analgesia and immunoregulation¹⁷.

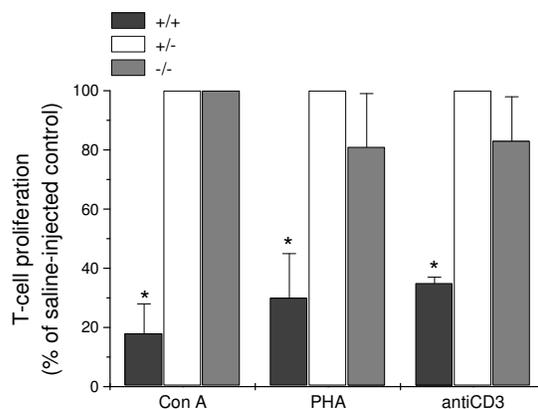


Fig. 2: Effects of morphine on T lymphocyte proliferation. Con A-, PHA-, and antiCD3-induced splenic T lymphocyte proliferation were measured 3 h after injection of morphine or saline intraperitoneally by [³H]-thymidine incorporation as explained in the text. Data represent mean \pm SEM of three replicate cpm determinations per group of animals (3 rats per group). *, $P < 0.001$, as compared with response of cells from saline-injected control.

Elimination of μ -opioid receptors in -/- animals has been previously shown to abolish morphine's effects on nociceptive responses in hot plate and tail flick tests, whereas μ receptor +/- mice were shown to display right and downward shifts in morphine dose-effect relationships, which was consistent with lower morphine potencies and efficacies in tests of both spinal and supraspinal analgesia¹⁷. It was also reported that mice lacking the μ -opioid receptor gene did not respond to the immunosuppressive effects of morphine including lymphoid organ atrophy, decrease in the ratio CD4(+)/CD8(+) cells in the thymus, and NK-cell cytotoxic activity¹⁷. In addition, it has been observed that mice lacking the μ -opioid receptor gene are unresponsive to morphine¹⁷ and heroin-induced analgesia¹⁸. Interestingly, it was reported that μ -opioid receptor knockout mice under chronic 12-h daily restraint stress for 2 days exhibited no effect in splenic lymphocyte proliferation, and IL-2, IFN-gamma and corticosterone production which were altered in wild-type mice²⁵.

In the present study, we have evidence confirming that homozygous μ -opioid receptor knockout mice were not affected in their NK-cell activity by morphine, heterozygous animals marginally

responded to the immunosuppressive effects of the drug, while wild-type animals were significantly suppressed (Fig. 1), as previously reported by others¹⁷. In addition, we showed that splenic T-cell proliferative responses to various stimuli of μ -opioid receptor knockout homozygous and heterozygous mice were not affected by morphine treatment, whereas morphine significantly suppressed T-cell proliferation in wild-type homozygous animals (Fig. 2). In conclusion, we showed that μ -opioid receptor deficient mice were not immunosuppressed by acute action of morphine.

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