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ORIGINAL ARTICLE

(+)-Naloxone inhibits morphine-induced chemotaxis via prevention of heat shock protein 90 cleavage in microglia

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Background/Purpose: Microglia have a crucial role in maintaining neuronal homeostasis in the central nervous system. Immune factors released from microglia have important roles in nociceptive signal transduction. Activation of microglia seems to be a shared mechanism in pathological pain and morphine tolerance because pharmacological attenuation of microglia activation provides satisfactory management in both situations.
Methods: In the present study, we investigated the effect of 1nM (+)-naloxone, which is not an opioid receptor antagonist, on morphine-induced activation of microglia EOC13.31 cells.
Results: Our results showed that 1μM morphine enhanced microglia activation and migration, decreased α-tubulin acetylation, and induced heat shock protein 90 (HSP90) fragmentation and histone deacetylase 6 (HDAC6) expression. Morphine-induced α-tubulin deacetylation and HSP90 fragmentation were HDAC6-dependent. Pretreatment with (+)-naloxone (1nM) inhibited morphine-evoked microglia activation and chemotaxis and prevented α-tubulin deacetylation and HSP90 fragmentation by inhibiting HDAC6 expression.
Conclusion: Based on the findings of the present study, we suggest that (+)-naloxone inhibits morphine-induced microglia activation by regulating HDAC6-dependent α-tubulin deacetylation and HSP90 fragmentation.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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Introduction

The clinical use of opioids, which are fundamental in the treatment of pain, is limited by their adverse effects such as tolerance and hyperalgesia. To explore new pain therapies, it is necessary to have a clear understanding of the development of opioid tolerance and neuropathic pain, which share the same feature of diminished morphine analgesia.[1] We previously reported that (−)-ultralow dose naloxone inhibits spinal microglia reactivity and thus attenuates morphine tolerance.[2] We and others have shown it also inhibits neuropathic pain.[3,4] A recent study demonstrated that the glia-modulating agents minocycline and propentofylline attenuate the development of morphine tolerance by inhibiting rat spinal microglia reactivity.[5] These results suggest the involvement of microglia activation in the development of morphine tolerance and/or hyperalgesia.

Under normal conditions, microglia have a small soma that bears thin-branched processes.[6] In the injured central nervous system, ramified microglia rapidly transform from a resting state to an active state, release a variety of cytokines, proliferate, migrate, and become phagocytic.[7] Lamellipodia, filopodia, and membrane ruffles are essential for cell migration and phagocytosis,[8] and membrane protrusion is dependent on cytoskeletal rearrangement such as the formation of lamellar networks, polymerization of actin filaments, and tubulin assembly.[9] Recent studies have suggested that microglia activation during neuropathy leads to abnormal pain signal processing[10]; however, the mechanism is poorly understood. We have previously demonstrated that chronic morphine treatment enhances microglia reactivity, which induces the release of proinflammatory cytokines and directly affect the interaction between microglia and nociceptive neurons,[11] and that ultralow dose (−)-naloxone (15 pg) cotreatment with morphine inhibits microglia activation and reverses antinociceptive effect of morphine in morphine-tolerant rats.[12,13] These results suggest that ultralow dose (−)-naloxone may be clinically valuable in pain management. Microglia constitute only 5—12% of all cells in the central nervous system (CNS), which leads to some important role of microglia been ignored. In addition, chronic morphine treatment changes opioid receptor conformation and sensitization, and decreases available receptors. It is unclear whether ultralow dose (−)-naloxone suppresses neuroinflammation via opioid receptors or by other signal pathways. The aim of the present study was therefore to examine the pathways affected by ultralow dose (+)-naloxone, which does not act through opioid receptors.[14,15] We found that it does not inhibit microglia activation by acting on opioid receptors. It instead downregulates the histone deacetylase 6 (HDAC6)-dependent α-tubulin deacetylation signaling pathway.

Methods

Cell culture

EOC13.31 mouse microglia cells (CRL-2468; American Type Culture Collection; Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan) were grown in 70% Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS), and 20% conditioned medium from cultures of the LADMAC cell line (American Type Culture Collection; Bioresource Collection and Research Center).[16] The medium was changed every 3—4 days. Cells were collected and used immediately for chemotaxis experiments or were plated for Western blot analysis or immunocytochemistry.

Drug delivery for culture cells

Before the experiment, the cells were washed three times with serum-free DMEM, starved for 4 hours in the same medium, and incubated for 30 minutes with the medium and 1nM (+)-naloxone (a generous gift from Dr. Jau-Shyong Hong, National Institutes of Health, Research Triangle Park, NC, USA) or 1nM tubacin (Sigma—Aldrich, St. Louis, MO, USA). The medium or 1μM morphine (Sigma—Aldrich) was then added. Incubation continued for 2 hours. The cells were thereafter gently harvested in phosphate-buffered saline (PBS)/1mM EDTA and were used for migration, Western blotting, or immunocytochemical studies.

Chemotaxis assay

Before the chemotaxis assay, the cells were washed three times with serum-free DMEM, starved for 4 hours in the same medium, and then gently harvested in PBS/1mM EDTA, and counted. They were resuspended in serum-free medium at 1×10^4 cells per 100 μL, and placed in siliconized low-adhesion microcentrifuge tubes for drug treatment. They were then incubated for 30 minutes in serum-free DMEM with or without 1nM (+)-naloxone. The medium or 1μM morphine was then added and incubation continued for 2 hours. The cells were counted using Trypan blue to estimate survival (>99% viability). The treated cells were added (1×10^3 cells in 50 μL) to the upper wells of a 48-well microchemotaxis Boyden chamber (Neuro Probe; Cabin John, MD, USA). The lower wells were filled with DMEM containing 10% FBS. The chambers were then incubated for 1 hour in a humidified incubator at 37°C in 5% carbon dioxide (CO_2). After removing any cells remaining on top of the membrane, the number of EOC13.31 cells that had migrated to the underside of the filter (25 mm × 80 mm polycarbonate membrane, 3.0-μm pore size; Osmonics, Livermore, CA, USA) was examined. The membrane was rinsed with PBS and the migrated cells were fixed with methanol and stained with crystal violet (Sigma-Aldrich). The membrane was then rinsed twice with distilled water, dried, inverted, and mounted on a microscope slide for analysis. Images of six random fields for each well were captured using an Olympus BX 50 fluorescence microscope (Olympus, Optical, Tokyo, Japan; 20× objective). The cells were counted by averaging the results for all six fields. All experiments were performed at least three times with $n = 18$ per trial.

Western blotting

EOC13.31 cells at a density of 5×10^5 cells/cm^2 were plated on poly-L-lysine-coated culture dishes (Nunc, Roskilde,