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Morphine reduces mouse microglial engulfment induced by lipopolysaccharide and interferon- γ via δ opioid receptor and p38 mitogen-activated protein kinase

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Abstract

Objective: To investigate the effects of morphine on microglial phagocytosis during neuroinflammation.

Methods: C8-B4 mouse microglial cells were exposed to various concentrations of morphine after the stimulation with lipopolysaccharide and interferon- γ and then fluorescent immunostaining was performed to assess the percentage of microglia that engulfed fluorescent microspheres in total microglia. Naloxone, β funaltrexamine, or naltrindole was used with 1 μ M morphine to assess the involvement of specific opioid receptor. P38 and phosphorylated p38 were determined by Western blotting. A p38 mitogen-activated protein kinase (MAPK) activator (anisomycin 0.1 μ M) or inhibitor (SB 203580, 20 μ M) was used to determine the involvement of p38 MAPK pathway.

Results: Morphine decreased lipopolysaccharide and interferon- γ induced microglial engulfment except the highest concentration (10 μ M) and both naloxone and naltrindole (a selective δ opioid receptor antagonist) attenuated morphine effect ($P < 0.001$). The phosphorylated p38 was up-regulated in lipopolysaccharide and interferon- γ group compared with control group ($P < 0.001$). This up-regulation was decreased by 1 μ M morphine ($P < 0.001$). However, naltrindole abolished

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Disclosure statement

None declared

this morphine effect ($P = 0.015$). SB203580 blocked the increased microglial engulfment induced by lipopolysaccharide and interferon- γ ($P < 0.001$); whereas anisomycin enhanced the morphine-induced decrease of engulfment ($P < 0.001$).

Conclusion: Morphine reduced mouse microglial engulfment induced by lipopolysaccharide and interferon- γ . This morphine effect seems to be mediated by δ opioid receptor and via p38 MAPK inhibition.

Keywords

opioid effect

Introduction

Microglia are macrophage-like cells in the brain and specific phagocytes of the CNS in response to various endogenous and exogenous stimuli [1]. They survey, sample, and analyze the environment even in resting state. Once they are activated, they migrate, phagocyte and secrete inflammatory cytokines and chemokines [2]. Microglial phagocytosis is a critical host response after CNS injury since removal of degenerating neurons and axons prevents the release of proinflammatory intracellular components and contributes to restoration of a favorable environment [3]. On the other hand, excessive microglial phagocytosis may induce or exacerbate neuronal loss due to the removal of viable neuron during neuroinflammation [3, 4].

Morphine is one of the most common opioids used for postoperative pain control during perioperative period. Neuroinflammation may occur during anesthesia and surgery. Neuroinflammation is considered one of the mechanisms for postoperative cognitive dysfunction [5]. However, little is known about the effects of morphine on microglial phagocytosis during neuroinflammation. This study was designed to investigate the influence of morphine on the microglial engulfment, the major step of phagocytosis, using C8-B4 mouse microglial cells after stimulation with lipopolysaccharide (LPS) plus interferon- γ (IFN- γ). The role of specific opioid receptor during morphine-mediated modulation of microglial engulfment was also assessed using general (naloxone), selective μ (β funaltrexamine) and δ (naltrindole) opioid antagonists. Additionally, the role of p38 mitogen activated protein kinase (MAPK) in the morphine-induced effect on microglial engulfment was determined since microglial engulfment may be regulated by p38 MAPK [6, 7].

Materials and methods

Materials

C8-B4 cells (CRL-2540TM), a microglial clone isolated from 8-day old mouse cerebellum, were purchased from the American Type Culture Collection (Manassas, VA, USA). Preservative free morphine sulfate was purchased from Hospira, INC (Lake Forest, IL, USA). Heat inactivated fetal bovine serum (FBS) and fluorescent microspheres (FluoSpheres[®]) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Rabbit monoclonal anti-ionized calcium binding adaptor molecule 1 (Iba1) antibody was purchased

from Wako Chemical USA, Inc. (Richmond, VA). Chamber slides, Hoechst solution and Pierce™ BCA Protein Assay Kit were from Thermo Scientific (Waltham, MA). Precast gels for Western blots were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Lipopolysaccharide (*Escherichia coli* 0111:B4) and other general chemicals (recombinant rat IFN- γ produced from *E. coli*, anisomycin, and SB203580) except for those described below were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

C8-B4 microglial cells were cultured as described previously [8]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/l glucose, 1 mM sodium pyruvate, 1500 mg l⁻¹ sodium bicarbonate, and supplemented with 10% heat-inactivated FBS, 100 U ml⁻¹ penicillin, and 100 pg ml⁻¹ streptomycin in a humidified atmosphere of 95% air–5% CO₂ at 37°C. The medium was changed every 2 or 3 days. The cells were plated at a density of 3–4 × 10⁴ cells well⁻¹ on 8-well chamber slides for fluorescent microscopy and plated at a density of 2.5–3 × 10⁵ cells ml⁻¹ in 6-well plates for protein assay and Western blotting experiments. Cells were left to adhere overnight (24 h) before LPS and IFN- γ stimulation.

Application of the study drug and chemicals

The first experiment was designed to determine the concentrations of LPS and IFN- γ used in the subsequent experiments. Cells were incubated with various concentrations of LPS and IFN- γ for 24 h and the cells were then harvested. Preliminary study showed 100 ng/ml LPS plus 1 ng/ml IFN- γ induced a significant increase of engulfment of fluorescent microspheres and this combination was chosen for subsequent experiment. Microglial cells were plated in 8-well slide chambers for 24 h and then they were exposed to 100 ng/ml LPS plus 1 ng/ml IFN- γ for 24 h.

Morphine was administered to the incubation medium of the slide chambers at 2 h after the stimulation with LPS plus IFN- γ to assess the post-treatment effect of morphine. The cells were kept under their normal culture conditions for 22 h at 37 °C. Morphine at 1 μ M was used except for the concentration-response experiment in which 0, 1, 3, or 10 μ M morphine was administered. After concentration-response study and Western blot analysis, a p38 MAPK activator (anisomycin 0.1 μ M) or inhibitor (SB203580 20 μ M) was added just before the morphine administration to determine the involvement of p38 MAPKs in the effect of morphine post-treatment on the engulfment,

Tracers for engulfment

Engulfment was assessed using 1.0 μ m Nile red polystyrene microspheres (FluoSpheres® Fluorescent Microspheres F-8819) at a concentration of 10,000 particles/ml media. Fluorescent carboxylate-modified microspheres were added and incubated for 30 min (37°C, 5% CO₂) for detecting engulfment at 24 h after LPS plus IFN- γ stimulation. Bovine serum albumin (BSA) has been used to facilitate engulfment as an opsonizing agent.

Cell staining and fixation

C8-B4 cells in 8-well slide chambers were stained and examined under fluorescent microscopy. All washings were done in dark with 1 x phosphate buffered saline (PBS) for 3 times (5 min per wash). Medium was removed and cells were fixed with 100% MeOH for 10 min at -20°C , permeabilized with 0.1% Triton X-100 diluted in 1 x PBS for 10 min and blocked with 1% BSA for 30 min at room temperature. Cells were incubate with a primary antibody (rabbit anti-Iba1) at 4°C overnight. Incubation with secondary antibody for 1 h was performed at room temperature. Cells were washed and nuclei were counterstained with Hoechst 33342 for 5 min at room temperature. Gasket and rubber seal were removed from chamber slides and slides were covered with 2 – 3 drops of Vectashied mounting media (Vector Laboratories) and coverslip. Coverslip was sealed with clear nail polish and allowed to dry for 20 min in dark.

Cell count and engulfment assay

Images were taken using fluorescent microscopy (Olympus DP70 Digital Microscope Camera System) at X 40 after staining and fixation. Each chamber was divided into 6 compartments to take the images. Cells with or without fluorescent microspheres were counted in each compartment from at least 6 independent experiments. The percentage of microglia engulfing one or more fluorescent microspheres in total microglia was calculated (microglial cells with fluorescent microspheres $\times$ 100/total microglial cells).

Western blot analysis

Western blot analysis was performed as described before [8]. Cells for Western blot were plated at a density of $2.5 - 3 \times 10^5$ cells/mL in 6-well plates and plated cells were left to adhere overnight (24 h). After drug treatment with LPS, IFN- γ and morphine, cells were lysed and homogenized in 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) α -mercaptoethanol, 1 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 1 μM pepstatin A. The homogenates were centrifuged at $14,000 \times g$ for 10 min at 4°C . The supernatant was used for protein assay with a bicinchoninic acid protein assay kit (Pierce). The samples were loaded at 20 μg proteins per lane, run on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and then transferred to a polyvinylidene difluoride membrane (Millipore). After incubation with anti-phospho-p38 (1:1000; Cell Signaling Technology), and anti-p38 (1:500; Cell Signaling Technology), the probed protein bands were visualized by using Western blotting detection reagents (GE Healthcare, Piscataway, NJ). The protein bands were densitometrically analyzed by an ImageQuant 5.0 densitometer (Amersham Biosciences, Piscataway, NJ, USA). The results of cells treated with various conditions were then normalized by the data of control cells.

Data analysis

Each experimental condition was repeated more than 6 times (n for each condition is described in the figure legends) by using at least three different flask of cells. All statistical analyses were performed with the using SPSS software. Statistical analyses were performed by one-way analysis of variance followed by *t*-test after confirmation of normal distribution

of the data. Data are expressed as mean (S.D.). A P value < 0.05 was considered to indicate statistical significance.

Results

The effect of Morphine on microglial engulfment

Activation of C8-B4 microglial cells with 100 ng/ml LPS and 1 ng/ml IFN- γ increased the percentage of microglia with engulfment compared with control (37 [9] % for control group vs. 70 [13] % for LPS and IFN- γ group; $P < 0.001$, Figs. 1 & 2). Morphine (1 μ M and 3 μ M) applied 2 h after the initiation of LPS and IFN- γ stimulation induced down-regulation of microglial engulfment but the highest concentration (10 μ M) of morphine had no significant effect (70 [13] % for LPS and IFN- γ group vs. 32 [6] % for LPS and IFN- γ plus 1 μ M morphine; 28 [7] % for LPS and IFN- γ plus 3 μ M morphine; 48 [23] % for LPS and IFN- γ plus 10 μ M morphine, Fig 1 & 2).

Effects of opioid receptor antagonists on microglial engulfment

Experiments with various opioid antagonists revealed that 50 μ M naloxone (a general opioid receptor antagonist) and 10 μ M naltrindole (a selective δ opioid receptor antagonist) abolished morphine effect on LPS and IFN- γ induced microglial engulfment (79 [5] % for LPS and IFN- γ plus 1 μ M morphine with naloxone group and 72 [7] % for LPS and IFN- γ plus 1 μ M morphine with naltrindole group vs. 40 [7] % for LPS and IFN- γ plus 1 μ M morphine; $P < 0.001$, Fig. 3); whereas 10 μ M β funaltrexamine (a selective μ opioid receptor antagonist) had no effect on morphine-induced microglial engulfment.

The role of p38 MAPK

Western blotting showed that the phosphorylated p38 MAPK (pp38 MAPK x 100/p38 MAPK) in microglial cells was up-regulated in LPS and IFN- γ group compared with control group (100 % for control group vs. 777 [97] % for LPS and IFN- γ group, $P < 0.001$). This up-regulation was decreased in LPS and IFN- γ plus 1 μ M morphine group (398 [102] %, $P < 0.001$, Fig. 4). In addition, naltrindole (a selective δ opioid receptor antagonist, 10 μ M) abolished morphine effect (566 [138] % for LPS and IFN- γ plus 1 μ M morphine and naltrindole group vs. 398 [102] % for LPS and IFN- γ plus 1 μ M morphine group, $P = 0.015$, Fig. 4).

The effect of p38 MAPK inhibitor and activator on microglial engulfment

SB203580 (p38 MAPK inhibitor) at 20 μ M reversed the LPS and IFN- γ -induced increase in percentage of microglia with engulfment (84 [8] % for LPS and IFN- γ group vs. 30 [7] % for LPS and IFN- γ plus SB 203580 group, $P < 0.001$). In addition, anisomycin (p38 MAPK activator) at 0.1 μ M enhanced morphine-induced decrease in engulfment (45 [6] % for LPS and IFN- γ plus 1 μ M morphine group vs. 79 [8] % for LPS and IFN- γ plus 1 μ M morphine and anisomycin group, $P < 0.001$, Fig. 5).

Discussion

We set out this experiment to determine whether morphine would influence microglial engulfment. The principal finding was that morphine post-treatment decreased enhanced microglial engulfment induced by LPS plus IFN- γ and this morphine effect was reversed by naloxone (a general opioid receptor antagonist) and naltrindole (a selective δ opioid receptor antagonist). Western blot analysis also indicated that morphine decreased the phosphorylated p38/p38 ratio up-regulated by LPS and IFN- γ group and the addition of naltrindole abolished this morphine effect. SB203580 (p38 MAPK inhibitor) abolished the LPS and IFN- γ -induced increased microglial engulfment; whereas anisomycin (p38 MAPK activator) enhanced the morphine-induced decrease in engulfment.

LPS is a Gram-negative bacterial cell wall constituent and functions as a xenobiotic stimulus. LPS and IFN- γ were used to activate microglial cells to engulf fluorescent microspheres based on the previous studies [7, 9]. In the current study, incubation of microglial cell with LPS plus IFN- γ for 24 h greatly increased the engulfment and morphine post-treatment at clinically relevant concentrations (1 and 3 μ M) abolished the LPS & IFN- γ -induced engulfment activity of mouse microglia. Previous studies showed that morphine decreased the phagocytotic activity of neutrophils [10] and monocytes [2]. Shirzad et al. [11] demonstrated a significant decrease of phagocytotic activity in animals with long-term morphine treatment.

Opioid receptor has been known to be involved in the modulation of phagocytosis [12] and general (naloxone), selective μ (β funaltrexamine) and δ (naltrindole) opioid receptor antagonists were used to assess the mechanism of morphine-mediated inhibition of microglial engulfment. Naloxone and naltrindole abolished morphine effect on engulfment and the ratio of phosphorylated p38/p38. Therefore, δ opioid receptor may mediate morphine-induced inhibition of mouse microglial engulfment. Tomassini et al. [13] showed that μ and δ opioid receptors modulated morphine-induced inhibition of phagocytosis of murine peritoneal macrophages. In the other investigation by Welters et al. [10], morphine suppressed phagocytosis of human neutrophil via a nitric oxide and μ_3 opioid receptor-dependent mechanism.

The mechanism underlying microglial activation and phagocytosis is complex and remains elusive. Ninkovic et al. [2] proposed the modulation of actin polymerization and p38 MAPK signaling as key mechanisms by which morphine inhibits phagocytosis of murine macrophages. P38 MAPK, an intracellular signaling molecule, is a modulator of actin polymerization that is responsive to cellular stress and inflammatory cytokines. Long-term morphine treatment attenuated actin polymerization and membrane ruffling by an inhibition of p38 MAPK through activation of the μ opioid receptor [2]. In the present study, the phosphorylation levels of p38 MAPK were estimated by Western blotting analysis using antibodies against the nonphosphorylated and phosphorylated proteins to investigate whether p38 MAPKs are activated in activated microglia. The up-regulated phosphorylation level of p38 MAPK in LPS & IFN- γ group was decreased by 1 μ M morphine post-treatment and this morphine effect was abolished by naltrindole, a selective δ opioid receptor antagonists. A δ opioid receptor may be involved to suppress LPS-induced p38 MAPK activation in

murine macrophage [14]. The results of this study indicate that activation of p38 MAPK possibly mediate morphine effect on mouse microglial engulfment through δ opioid receptor.

This study showed that morphine decreased the microglial engulfment and the result may suggest that morphine decrease the microglial phagocytosis during neuroinflammation. The result of the current study is in line with previous investigations about the effect of morphine on phagocytosis of immune cells [10, 11, 15]. Shirzad et al. [11] compared morphine and tramadol in terms of phagocytic activity of peritoneal phagocytes and showed that phagocytosis was reduced in morphine group and increased in tramadol group. Morphine was reported to inhibit phagocytosis of *C. neoformans* by primary cultures of neonatal pig microglia [15]. Welters et al. [10] also demonstrated that morphine suppressed human neutrophil function via μ 3 opiate receptor –mediated NO release in immunocytes.

During neuroinflammation, phagocytosis may lead to death of viable neurons [4]. Considering the fact that neuroinflammation is regarded as one of the important mechanisms for postoperative cognitive dysfunction, attenuated phagocytosis might be interpreted to be beneficial and neuroprotective during neuroinflammation. However, decreased phagocytosis may also suggest morphine's immune suppression in the CNS since phagocytosis eliminates dead cells and induces an anti-inflammatory response. Long-term morphine treatment has been known to be associated with suppression of host immunity through direct and indirect actions on the cellular immunity system [12]. Therefore, long-term use of morphine might be relatively hazardous in patients with compromised or suppressed immune system [11]. Microglial engulfment and phagocytosis are complex processes. Further studies are needed to fully understand the implication of morphine effects on microglial engulfment.

This is the first study about the opioid effect on mouse microglial engulfment but the current study has a few limitations. First, phagocytosis is composed of 3 steps (recognition, engulfment and degradation) and only engulfment was evaluated. Second, only p38 MAPK pathway was assessed although phagocytosis is a relatively complex process in terms of the receptors, the mechanisms, and its consequences. Ninkovic et al. [2], proposed that μ -opioid receptor leads to an increase in intracellular cAMP, activation of protein kinase A, and inhibition of Rac1-GTPase and p38 MAPK. This process subsequently attenuated actin polymerization, which is needed for Fc γ receptors -mediated internalization. Fc γ receptors facilitate internalization of opsonized extracellular pathogen by recognizing the Fc region of the IgG antibody coating on the surface of pathogen [16]. Subsequently, bacterial phagocytosis of murine macrophages was inhibited by attenuated actin polymerization and membrane ruffling [2]. Further studies are needed to elucidate upstream and downstream pathways. Third, this study evaluated the effect of morphine on the degree of microglial engulfment during phagocytosis. Welters et al. [10] reported that morphine can stimulate the release of nitric oxide, which suppresses phagocytotic activity of human neutrophil. Further study is needed about the effect of morphine on the physiologic function parameters (inflammatory mediators such as cytokines and chemokines) for microglial phagocytosis during neuroinflammation.

Conclusion

In conclusion, this *in vitro* experiment with C8-B4 microglial cells showed that morphine post-treatment at clinical relevant concentrations decreased microglial engulfment after activation by LPS plus IFN- γ . Delta (δ) opioid receptor seems to mediate this effect and p38 MAPK may also contribute to the mechanisms of this morphine effects. These results suggest the effect of morphine on microglial phagocytosis during neuroinflammation. However, microglial phagocytosis has both advantageous and injurious consequences during neuroinflammation. The consequence of morphine effect on microglial engulfment, especially under *in vivo* conditions, remains largely unknown.

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Fig. 1.

Microscopic findings of microglial engulfment. Stimulation of microglia with 100 ng ml^{-1} LPS plus 1 ng ml^{-1} $\text{IFN}\gamma$ increased the engulfment activity (B) compared with control (A) and $1 \mu\text{M}$ morphine applied 2 h after the initiation of LPS and $\text{IFN-}\gamma$ stimulation attenuated microglial engulfment (C). Left up (blue); nuclei; Right up (green): cytoplasm; Left down (red); fluorescent microspheres; Right down; a merge of 3 images (nuclei, cytoplasm and fluorescent microspheres). Original magnifications: $\times 250$.

Fig 2.

Concentration-response of morphine effects on microglial engulfment. Morphine administered 2 h after the 100 ng ml^{-1} LPS plus 1 ng nl^{-1} $\text{IFN}\gamma$ stimulation decreased C8-B4 microglial engulfment. Data are expressed as the mean (S.D.). LI: LPS plus $\text{IFN}\gamma$ stimulation, M1: morphine at $1 \mu\text{M}$, M3: morphine at $3 \mu\text{M}$, M10: morphine at $10 \mu\text{M}$. *: $P < 0.05$ compared with control, †: $P < 0.05$ compared with LI.

Fig 3.

Effects of various morphine antagonists on microglial engulfment. Microglial cells were incubated with or without 1 μ M morphine in the presence of naloxone (a general opioid receptor antagonist, 50 μ M), β funaltrexamine (β -FNA, a selective μ opioid receptor antagonist, 10 μ M), or naltrindole (a selective δ opioid receptor antagonist, 10 μ M). Naloxone and naltrindole abolished morphine effect on LPS and IFN- γ -induced microglial engulfment. Data are expressed as the mean (S.D.). LI: LPS plus IFN γ stimulation, M1: morphine 1 μ M, *: $P < 0.05$ compared with control, †: $P < 0.05$ compared with LI, ‡: $P < 0.05$ compared with LI & M1.

Fig 4.

Western blot analysis of p38 mitogen activated protein kinase (MAPK). The phosphorylation level of p38 ($\text{pp38} \times 100/\text{p38}$) in LPS and IFN- γ stimulated microglial cells was decreased by 1 μM morphine and this effect was abolished by naltrindole (a selective δ opioid receptor antagonist, 10 μM). Data are expressed as the mean (S.D.). pp38: phosphorylated p38, LI: LPS plus IFN γ stimulation, M1: morphine at 1 μM , *: $P < 0.05$ compared with control, †: $P < 0.05$ compared with LI, ‡: $P < 0.05$ compared with LI & M1.

Fig 5. Effects of p38 mitogen activated protein kinase (MAPK) inhibitor and activator on microglial engulfment. SB203580 (p38 MAPK inhibitor) at 20 μ M abolished the LPS and IFN- γ -induced increase in percentage of microglia with engulfment; whereas anisomycin (p38 MAPK activator) at 0.1 μ M reversed morphine-induced decrease in engulfment. Data are expressed as the mean (S.D). LI: LPS plus IFN γ stimulation, M1: morphine at 1 μ M, *: $P < 0.05$ compared with control, †: $P < 0.05$ compared with LI, ‡: $P < 0.05$ compared with LI & M1.