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Morphine preconditioning reduces lipopolysaccharide and interferon- γ -induced mouse microglial cell injury via $\delta 1$ opioid receptor activation

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Abstract

Microglial cells play an important role in the inflammatory response of a broad range of brain diseases including stroke, brain infection and neurodegenerative diseases. However, there is very little information regarding how to protect microglial cells. Here, we showed that incubation of the C8-B4 mouse microglial cells with lipopolysaccharide (LPS) plus interferon- γ (IFN γ) induced cytotoxicity as assessed by the amount of lactate dehydrogenase (LDH) released from the cells. Preconditioning the cells with morphine for 30 min concentration-dependently reduced LPS plus IFN γ -induced cell injury. This morphine preconditioning effect was abolished by naloxone, a general opioid receptor antagonist, by naltrindole, a selective δ opioid receptor antagonist and by 7-benzylidenenaltrexone maleate, a selective $\delta 1$ opioid receptor antagonist. However, this protective effect was not affected by β -funaltrexamine, a selective μ opioid receptor antagonist, nor-binaltorphimine, a selective κ opioid receptor antagonist or naltriben, a selective $\delta 2$ opioid receptor antagonist. LPS plus IFN γ induced the expression of inducible nitric oxide synthase (iNOS), which was not affected by morphine preconditioning. Our results suggest that morphine induced a preconditioning effect in microglial cells. This effect may be mediated by $\delta 1$ opioid receptors and may not be through inhibiting the expression of iNOS, a potentially harmful protein.

Keywords

microglia; morphine; neuroprotection; opioid receptors; preconditioning

Introduction

Inflammation is a pathophysiological process that is involved in many acquired human diseases. In the brain, inflammation contributes to the pathology of brain trauma, stroke, brain infection and neurodegenerative diseases (Eddleston and Mucke, 1993; Kreutzberg, 1996; Brown and Bal-Price, 2003). Microglial cells are macrophage-like cell residing in the brain

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and play a critical role in the brain inflammation reactions (Kreutzberg, 1996). Although it is well known that many factors and agents, such as bacterial products, virus and prion, induce activation of microglial cells (Kreutzberg, 1996; Xu et al., 2008; Kim et al., 2009), there is very little information regarding how to protect microglial cells.

Preconditioning-induced protection describes a phenomenon in which a prior stimulus or application of agents activates endogenous protective mechanisms against a subsequent detrimental insult (Barry and Zuo, 2005). Various stimuli, such as short episodes of ischemia and hypoxia, have been shown to induce a preconditioning effect in the brain (Fu et al., 1994; Nandagopal et al., 2001). However, preconditioning effect induced by these stimuli may have a limited clinical application because a small variation of the intensity and duration of these stimuli may cause brain injury and application of these stimuli is not easily achievable. Thus, induction of a preconditioning effect by applying commonly used drugs is more desirable.

We and others have shown that commonly used volatile anesthetics, such as isoflurane, can induce a preconditioning effect in animal brain (Kapinya et al., 2002; Zheng and Zuo, 2004). We have also shown that morphine, an opioid whose use is much more convenient than application of volatile anesthetics, induces a preconditioning effect in rat brain (Lim et al., 2004; Zhao et al., 2006). To further determine the mechanisms of morphine preconditioning-induced neuroprotection and cell type specificity of this protection, we hypothesize that morphine preconditioning can reduce microglial cell injury induced by inflammatory agents. To test this hypothesis, we used mouse microglial cultures and stimulated them by the combination of lipopolysaccharide (LPS) and interferon- γ (IFN γ). Our previous studies showed that this combination significantly stimulated microglial cells to express inducible nitric oxide synthase (iNOS), a potentially harmful protein, and induced microglial injury (Xu et al., 2008; Kim et al., 2009).

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). Fetal bovine serum (FBS) and recombinant rat IFN γ produced from *E. coli* were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Morphine sulfate was purchased from Baxter Healthcare (Deerfield, IL, USA). Nor-binaltorphimine dihydrochloride (nor-BNI) was obtained from A.G. scientific, Inc (San Diego, CA, USA) and 7-benzylidenenaltrexone maleate (BNTX) was purchased from Tocris Cookson (Ellisville, MO, USA). Lactate dehydrogenase (LDH) cytotoxicity detection kit was obtained from Clontech Laboratories, Inc. (Mountain View, CA, USA). Mammalian protein extraction reagent was purchased from Thermo Scientific (Rockford, IL, USA). Rabbit polyclonal anti-iNOS antibody whose epitope is at the C-terminus of iNOS (catalogue number: sc-650) and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (catalog number sc-2004) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A rabbit polyclonal anti-actin antibody (Catalog number A2066), the protease inhibitor cocktail (catalog number: P2714), LPS (*Escherichia coli* 0111:B4) and other general chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride membranes for Western blotting and reagents for measuring protein concentrations were purchased from Bio-Rad (Hercules, CA, USA). Enhanced chemiluminescence reagents were obtained from Pharmacia-Amersham Biotech (Piscataway, NJ, USA).

Cell culture

C8-B4 cells were cultured in Dulbecco's Modified Eagle's Medium containing 4 mM L-glutamine, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in humidified atmosphere of 95% air-5% CO₂ at 37°C. The medium was changed twice a week. The microglial cells were plated at a density of 2.0×10^4 – 4.0×10^4 /well on 6-well tissue culture plates for experiments.

Application of morphine and other chemicals and study groups

To determine dose-response of LPS and IFN γ -induced microglial cell injury as measured by LDH release, cells were incubated with various concentrations (0 – 100 ng/ml) of LPS and 10 U/ml IFN γ for 24 h at 37°C. The culture media and cells were harvested for LDH assay.

We then determined whether morphine induced a preconditioning effect. The cells were incubated with 0 – 10 µM (final concentrations in the incubation medium) morphine for 30 min at 37°C. The incubation medium was then replaced with fresh medium. The cells were kept under morphine-free culture conditions for 30 min before they were exposed to 100 ng/ml LPS plus 10 U/ml IFN γ for 24 h at 37 °C. The culture media and cells were harvested for LDH assay.

To identify the opioid receptors that were involved in the morphine-induced preconditioning effect, cells were incubated with or without 3 µM morphine in the presence or absence of naloxone (a general opioid receptor antagonist, 50 µM), s-funaltrexamine (β-FNA; a selective µ opioid receptor antagonist, 10 µM), nor-binaltorphimine (nor-BNI, a selective κ opioid receptor antagonist, 10 µM), naltrindole (a selective δ opioid receptor antagonist, 10 µM), BNTX (a selective δ₁ opioid receptor antagonist, 0.5 µM) or naltriben (a selective δ₂ opioid receptor antagonist, 0.5 µM). The antagonists were present during the period from 30 min before the addition of morphine to the end of morphine pretreatment (total 1 h). The concentrations of antagonists were chosen mainly based on previous studies (Lim et al., 2004).

To determine whether morphine preconditioning reduced iNOS expression, the C8-B4 cells were preconditioned for 30 min at 37°C with or without 3 µM morphine in the presence or absence of 50 µM naloxone. The cells were then exposed to 100 ng/ml LPS plus 10 U/ml IFN γ for 24 h at 37°C and harvested for western analysis.

Cell cytotoxicity assay

The amount of LDH released from cells was used to measure the level of cellular cytotoxicity. As we performed previously (Kim et al., 2009), LDH activity assay was based on the cleavage of a tetrazolium salt to form formazan by LDH. Briefly, 100 µl cell-free culture medium harvested from various experiments was added to a 96-well plate to mix with 100 µl of the reaction mixture from the LDH detection kit. After an incubation for 30 min at room temperature, the absorbance of samples was measured at 492 nm, with the reference wavelength of 655 nm in a spectrophotometry. The intracellular LDH activity was measured similarly after solubilizing the cells with culture medium containing 0.5% Triton X-100™ for 20 min. Background absorbance from culture medium that was not used for any cell cultures was subtracted from all absorbance measurements. The percentage of LDH released to culture medium in total LDH was calculated: $100 \times \text{LDH in the culture medium} / (\text{LDH in the culture medium} + \text{intracellular LDH released by Triton X-100}^{\text{TM}})$.

Western blot analysis

Western blot analysis was performed as we detailed before (Huang et al., 2006). Cells were homogenized in mammalian protein extraction reagent containing protease inhibitor cocktails,

1 mM EDTA and 1 μ M phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 14,000 g for 10 min at 4 °C. The resulting supernatants were used for Western blotting and 15 μ g of proteins were loaded to each lane. After incubated with the anti-iNOS or anti-actin antibody, the protein bands were visualized by the enhanced chemiluminescence reaction. The protein bands were analyzed by an ImageQuant TL 2005 densitometer (Amersham Biosciences, Piscataway, NJ, USA). The results of iNOS protein bands were normalized by the data of the corresponding actin. The results of cells after various treatments were normalized by the data of cells exposed to LPS and IFN γ only.

Statistical analysis

Data are presented as mean $\pm$ SD. They were analyzed by student t-test, one-way analysis of variance (ANOVA) followed by Tukey test for *post hoc* comparisons or Kruskal-Wallis one-way ANOVA followed by Dunn's test for *post hoc* comparisons as appropriate. The difference was regarded as statistically significant when the *P* value was less than 0.05.

Results

Incubation of cells with LPS plus IFN γ for 24 h caused a dose-dependent increase of cell cytotoxicity assessed by LDH release. The incubation with 100 ng/ml LPS plus 10 U/ml IFN γ significantly increased cell cytotoxicity compared to control group (*P* = 0.012) (Fig. 1A). Thus, this combination of experimental conditions was chosen for other experiments. Preconditioning cells with morphine for 30 min concentration-dependently reduced 100 ng/ml LPS plus 10 U/ml IFN γ -induced cell injury. This reduction was statistically significantly when 3 or 10 μ M morphine was used (Fig. 1B). We chose to use 3 μ M morphine in the rest of experiments.

The preconditioning effect of morphine was abolished by naloxone (Fig. 2A), a general opioid receptor inhibitor. Neither a selective μ opioid receptor antagonist (β -FNA) nor a selective κ opioid receptor antagonist (nor-BNI) affected the morphine preconditioning-induced cytoprotection (Fig. 2B). However, the δ opioid receptor antagonist naltrindole inhibited the morphine preconditioning-induced cytoprotection (Fig 2B). The morphine preconditioning-induced cytoprotection was also abolished by BNTX, a selective δ_1 opioid receptor antagonist, but was not affected by naltriben, a selective opioid δ_2 receptor antagonist (Fig. 3). The application of each opioid receptor antagonist alone did not affect LPS plus IFN γ -induced cytotoxicity (Figs. 2 and 3).

Western blotting study showed that the mouse C8-B4 microglial cells did not express a significant amount of iNOS under control condition and expressed a large amount of iNOS after being stimulated by LPS plus IFN γ for 24 h. Morphine preconditioning and the combination of morphine preconditioning and naloxone did not significantly affect the LPS plus IFN γ -induced iNOS expression (Fig. 4).

Discussion

Morphine is a commonly used analgesic. Morphine can also induce a protective effect in various organs, such as brain and heart (McPherson and Yao, 2001; Chao et al., 2008; Kao et al., 2008). We and others have shown that morphine can also induce a preconditioning effect in the brain (Lim et al., 2004; Ammon-Treiber et al., 2005; Zhao et al., 2006). This effect is presented as attenuation of neuronal injury/death after brain ischemia or oxygen-glucose deprivation (a condition to simulate ischemia *in vitro*). It is not known whether morphine preconditioning can protect other types of brain cells. Our current study showed that morphine preconditioning also protected microglial cells against inflammation-induced cell injury. Thus, morphine preconditioning can protect multiple types of cells in the brain.

Our results showed that morphine preconditioning-induced protection in microglial cells was abolished by naloxone, suggesting that this protective effect is mediated by opioid receptors. Our additional results suggest that the morphine preconditioning effect was mediated by δ_1 opioid receptors because this effect was abolished by naltrindole, a selective δ opioid receptor antagonist, and by BNTX, a selective δ_1 opioid receptor antagonist and was not affected by β -funaltrexamine, a selective μ opioid receptor antagonist, by nor-binaltorphimine, a selective κ opioid receptor antagonist or by naltriben, a selective δ_2 opioid receptor antagonist. Consistent with this finding, our previous studies showed that activation of δ_1 opioid receptors was involved in opioid preconditioning effect in the brain (Lim et al., 2004; Zhao et al., 2006). In addition, it has been shown that cells involved in immune functions express opioid receptors including the δ_1 opioid receptors (Bidlack, 2000).

Our previous studies showed that LPS plus IFN γ reduced microglial cell viability by inducing a large amount of iNOS expression (Xu et al., 2008). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay that can detect early cell changes after an insult. Another study has shown an important role of iNOS and nitric oxide (NO) from microglial cells in cell injury (Bal-Price and Brown, 2001). Our current results suggest that the morphine preconditioning effect as detected by reducing LDH release from cells may not be mediated by reducing iNOS expression because morphine pretreatment significantly reduced LPS plus IFN γ -induced LDH release but did not affect the LPS plus IFN γ -induced iNOS expression. Consistent with this finding, our previous study showed that preconditioning with isoflurane, a volatile anesthetic, reduced iNOS expression-dependent cell viability as detected by MTT and iNOS expression-independent cell injury as detected by LDH release in microglial cells that were exposed to LPS plus IFN γ (Kim et al., 2009). Different from MTT assay that measures mitochondrial reductase activity to reflect cell viability, an increased LDH release may require damage to the plasma membrane and signify cell injury. Future studies are needed to understand how LPS and IFN γ can increase LDH release and the mechanisms downstream of δ_1 opioid receptors for morphine preconditioning to reduce this release.

Multiple studies from Stefano and colleagues have shown that morphine induces an immediate, short duration and μ receptor-mediated NO release from immunocytes including invertebrate microglial cells. This released NO may then inhibit the activity of those immunocytes (Magazine et al., 1996; Sonetti et al., 1997). This released NO may also contribute to the inhibition of LPS plus IFN γ -induced iNOS expression by morphine pretreatment in human saphenous vein endothelium and atrium (Stefano et al., 1998; Fimiani et al., 2000). These results are different from our finding that morphine preconditioning did not change the LPS plus IFN γ -induced iNOS expression. The reasons for this discrepancy are not known. Different cell types (human endothelium and atrium vs. mouse microglia) and treatment protocols (1 μ M morphine for 5 min followed immediately by the exposure to LPS plus IFN γ vs. our preconditioning protocol) used in the previous studies and our study may contribute to the discrepancy. Along with this line of discussion, it is interesting to note that treatment with morphine alone has been shown to increase iNOS expression in rodent heart and macrophages (Singhal et al., 1998; Jiang et al., 2004).

Our results may have a broad implication. Inflammation is involved in many acquired brain diseases, such as stroke and brain infection (Eddleston and Mucke, 1993; Kreutzberg, 1996; Brown and Bal-Price, 2003). Microglial cells play a critical role in immune functions and inflammatory responses in the brain (Kreutzberg, 1996). Thus, protecting microglial cells under inflammatory condition as shown in this study may preserve immune functions in the brain. Opioids have been shown to regulate immune functions (Lysle et al., 1993). Our results provide mechanistic insight for those opioid effects. Also, opioids induce neuroprotection in various models (Zhao et al., 2006; Chao et al., 2008; Kao et al., 2008; Husain et al., 2009).

Protection of microglial cells by opioids may contribute to their neuroprotection. Finally, opioids are a class of commonly abused drugs. Drug abusing patients often have high chances of infection including brain infection. Thus, it is important to understand the effects of opioids on cells involved in immune functions.

In conclusion, we showed that morphine preconditioning induced a concentration-dependent protection against LPS plus IFN γ -induced cell injury in microglial cells. This effect may be mediated by activation of $\delta 1$ opioid receptors and may not involve inhibition of the protein expression of iNOS, a potentially harmful protein.

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Abbreviations

BNTX	7-benzylidenenaltrexone maleate
β -FNA	β -funaltrexamine
IFN γ	interferon- γ
iNOS	inducible nitric oxide synthase
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	nitric oxide
nor-BNI	nor-binaltorphimine

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Fig. 1. Morphine preconditioning-induced protection in microglial cells

(A) The mouse C8-B4 microglial cells were exposed to various concentrations of LPS and 10 U/ml IFN γ for 24 h. (B) The mouse C8-B4 microglial cells were pretreated with various concentrations of morphine for 30 min before they were exposed to 100 ng/ml LPS plus 10 U/ml IFN γ for 24 h. Results are mean $\pm$ SD (n = 17 for panel A and 21 for panel B). * P < 0.05 compared with control. $\wedge$ P < 0.05 compared with LPS plus IFN γ only.

Fig. 2. The effects of opioid receptor antagonists on morphine preconditioning-induced protection
 The mouse C8-B4 microglial cells were pretreated with 3 μ M morphine (Mor) in the presence or absence of 50 μ M naloxone (Nalo), 10 μ M s-funaltrexamine (FNA), 10 μ M nor-binaltorphimine (BNI) or 10 μ M naltrindole (NTI). Results are mean $\pm$ SD ($n = 30 - 35$). * $P < 0.05$ compared with control. ^ $P < 0.05$ compared with LPS plus IFN γ only. # $P < 0.05$ compared with morphine preconditioning and LPS plus IFN γ .

Fig. 3. The effects of δ opioid receptor antagonists on morphine preconditioning-induced protection
 The mouse C8-B4 microglial cells were pretreated with 3 μ M morphine (Mor) in the presence or absence of 0.5 μ M BNTX or 0.5 μ M naltrexone (NTB). Results are mean $\pm$ SD (n = 30 – 35).
 * P < 0.05 compared with control. ^ P < 0.05 compared with LPS plus IFN γ only. # P < 0.05 compared with morphine preconditioning and LPS plus IFN γ .

Fig. 4. Effects of morphine preconditioning on inducible nitric oxide synthase (iNOS) expression
 The mouse C8-B4 microglial cells were pretreated with 3 μ M morphine (Mor) in the presence or absence of 50 μ M naloxone (Nalo) for 30 min. The cells were then incubated with 100 ng/ml LPS plus 10 U/ml IFN γ for 24 h and harvested for Western blotting. Results are mean $\pm$ S.D. (n = 12).