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Anti-inflammation effects of naloxone involve phosphoinositide 3-kinase delta and gamma

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ABSTRACT

Background: Phosphoinositide 3-kinase (PI3K) delta and gamma (the p110δ and p110γ isoforms of PI3K) actively participate in the process of inflammation. We sought to elucidate the possible roles of PI3Kδ and PI3Kγ in mediating the anti-inflammation effects of naloxone.

Materials and methods: Murine macrophages were treated with endotoxin, endotoxin plus naloxone, or endotoxin plus naloxone plus the PI3K inhibitors (the PI3Kδ inhibitor IC87114, the PI3Kγ inhibitor AS252424, or IC87114 plus AS252424) and denoted as the LPS, LPS + N, LPS + N + IC, LPS + N + AS, and LPS + N + IC + AS group, respectively. Differences in inflammatory molecules and levels of nuclear factor-κB (NF-κB) activation and Akt activation (indicator of PI3K activity) among these groups were compared.

Results: The concentrations of inflammatory molecules (macrophage inflammatory protein 2, tumor necrosis factor-α, interleukin-1β, and cyclooxygenase-2/prostaglandin E_2) and the levels of NF-κB activation (p-NF-κB p65 and p-inhibitor-κB concentrations and NF-κB-DNA binding activity) of the LPS + N group were significantly lower than those of the LPS group (all $P < 0.001$). These data confirmed the anti-inflammation effects of naloxone. Moreover, the anti-inflammation effects of naloxone could be counteracted by the inhibitors of PI3Kδ and PI3Kγ, as the concentrations of inflammatory molecules and the levels of NF-κB activation of the LPS + N group were significantly lower than those of the LPS + N + IC, LPS + N + AS, and LPS + N + IC + AS groups (all $P < 0.05$). In contrast, the concentration of phosphorylated Akt of the LPS + N group was significantly higher than those of the LPS, LPS + N + IC, LPS + N + AS, and LPS + N + IC + AS groups (all $P < 0.05$).

Conclusions: PI3Kδ and PI3Kγ play crucial roles in mediating the anti-inflammation effects of naloxone.

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1. Introduction

Naloxone is a potent opioid receptor antagonist and is traditionally used for opioid overdose treatment in clinical settings [1]. Moreover, accumulating data have highlighted the anti-inflammation effects of naloxone and suggested that naloxone could exert certain therapeutic effects in clinical situations associated with endotoxemia. For instance, naloxone could increase blood pressure, preserve tissue perfusion, improve tissue hypoxia, mitigate respiratory depression, and increase survival time in septic animals [2–5]. This concept is further supported by our recent data that naloxone could inhibit upregulation of inflammatory molecules and mitigate activation of the upstream transcription factor nuclear factor-κB (NF-κB) in endotoxin-activated murine macrophages [6]. It is generally believed that the mechanisms underlying the anti-inflammation effects of naloxone mainly involve its effects on antagonizing the endogenous opioid system [7]. However, our recent data demonstrated that the underlying mechanisms also involve the effects of naloxone on inhibiting the L-type calcium channels [6]. These data provide clear evidence to indicate the complexity of the mechanisms underlying the anti-inflammation effects of naloxone.

The signaling pathway of phosphoinositide 3-kinases (PI3K) and its downstream target Akt is essential in maintaining physiological homeostasis and preserving integrity of the immune system [8,9]. Activation of PI3K has been shown to significantly enhance endogenous anti-inflammation capacity and, in turn, limit the endotoxin-induced upregulation of inflammatory molecules [10,11]. To date, three classes of family members (class I, II, and III) have been identified in the PI3K family [8]. Among the identified members of PI3K family, the class IA p110δ isoform (PI3Kδ) and the class IB p110γ isoform (PI3Kγ) are predominantly expressed in leukocytes and have been shown to actively participate in the process of inflammation [12–16].

Considering the crucial role of PI3K in modulating the immune response [9], it is likely that PI3K may actively participate in mediating the anti-inflammation effects of naloxone. In addition, PI3Kδ and PI3Kγ are two predominant PI3K isoforms that actively involved in immune response [12–16]. To elucidate further, we thus conducted this cellular study with the hypothesis that naloxone inhibits endotoxin-induced inflammatory response in macrophages through modulation of PI3Kδ and/or PI3Kγ.

2. Methods

2.1. *Endotoxin-induced cell activation protocols*

This study used a widely used endotoxin-induced macrophages activation model [17–19] to facilitate investigation. The immortalized murine macrophage-like cell line RAW264.7 cells were plated in culture dishes (10-cm cell culture dishes and six-well cell culture plates with coverslips; BD Biosciences, San Jose, CA) and cultured with the Dulbecco modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with a mixture of fetal bovine serum (10%, Life Technologies) and penicillin/streptomycin (1%, Life Technologies). All culture dishes were placed in a humidified chamber (37°C) supplied with a gas mixture (95% air and 5% CO_2). Then, confluent RAW264.7 cells were activated with endotoxin (i.e., lipopolysaccharide (LPS), 100 ng/mL; *Escherichia coli* Serotype 0127:B8; Sigma–Aldrich, St. Louis, MO).

2.2. *Experimental protocols*

Six endotoxin groups and six control groups of RAW264.7 cells were used. Two of the endotoxin groups were randomly allocated to receive LPS or LPS plus naloxone (10 μM; Genovate Biotechnology Co, Hsinchu, Taiwan), and denoted as the LPS and the LPS + N group, respectively. To facilitate investigation of the possible roles of PI3K, PI3Kδ, and PI3Kγ, the other four endotoxin groups were treated with LPS plus naloxone plus the nonselective PI3K inhibitor LY294002 (10 μM, Sigma–Aldrich), the PI3Kδ inhibitor IC87114 (1.0 μM, Sigma–Aldrich), the PI3Kγ inhibitor AS252424 (0.1 μM, Sigma–Aldrich), or LPS plus naloxone plus IC87114 (1.0 μM) plus AS252424 (0.1 μM), and denoted as the LPS + N + LY, the LPS + N + IC, the LPS + N + AS, and the LPS + N + IC + AS group, respectively. Each group contained 12 cell culture dishes and one six-well cell culture plate with coverslips. In groups receiving LPS and naloxone, naloxone was added immediately after LPS administration. In groups receiving LPS, naloxone, and the PI3K inhibitors, the PI3K inhibitors were added 30 min before naloxone. The dosages of naloxone [6] and LY294002 [20] were determined according to previous studies. Moreover, the dosages of IC87114 [21] and AS252424 [22] were determined according to the half maximal inhibitory concentration (IC_{50}) of IC87114 and AS252424 in inhibiting PI3Kδ and PI3Kγ, respectively. To serve as the controls of additives, the six control groups were allocated to receive phosphate-buffered saline (PBS, Life Technologies), naloxone (10 μM), PBS plus naloxone plus LY294002 (10 μM), PBS plus naloxone plus IC87114 (1.0 μM), PBS plus naloxone plus AS252424 (0.1 μM), or PBS plus naloxone plus IC87114 (1.0 μM) plus AS252424 (0.1 μM), and denoted as the PBS, the N, the N + LY, the N + IC, the N + AS, and the N + IC + AS group, respectively. The timings for additive administrations in the control groups were compatible with those in the endotoxin groups.

For inflammatory molecules assays, the culture media in six culture dishes from each group were collected after endotoxin exposure for 6 h or comparable duration in the control groups. For NF-κB and Akt assays, the cell culture in another six culture dishes and the six-well culture plate from each group were collected after endotoxin exposure for 30 min or comparable duration in the control groups.

2.3. *Assays of inflammatory molecules*

Enzyme-linked immunosorbent assay was performed to measure the concentrations of inflammatory molecules, including macrophage inflammatory protein 2 (MIP-2), tumor

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