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Naloxone inhibits nod-like receptor protein 3 inflammasome

Han-Yu Lin, MD,[a] *Ya-Ying Chang, MD,*[a] *Ming-Chang Kao, MD, PhD,*[a,b] *and Chun-Jen Huang, MD, PhD*[a,b,*]

[a] *Department of Anesthesiology, Taipei Tzu Chi Hospital, Taipei, Taiwan*
[b] *School of Medicine, Tzu Chi University, Hualien, Taiwan*

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ABSTRACT

Background: Naloxone, an opioid receptor antagonist, possesses potent anti-inflammation effects. We previously confirmed the effects of naloxone on inhibiting upregulation of inflammatory cytokine interleukin-1β (IL-1β). Production of mature form IL-1β is mediated by the nod-like receptor protein 3 (NLRP3) inflammasome, a multiprotein complex composed of NLRP3, and the adaptor protein apoptosis-associated speck-like protein contains a caspase recruitment domain (ASC). We elucidated whether naloxone could inhibit the activation of NLRP3 inflammasome.

Material and methods: To induce IL-1β production and NLRP3 inflammasome activation, the human monocytic leukemia cell line THP-1 cells were first primed with lipopolysaccharide (LPS, 1 μg/mL) and then activated with adenosine triphosphate (ATP, 1 mM). For NLRP3 transcription, THP-1 cells were only treated with LPS priming.

Results: Enzyme-link immunosorbent assay data revealed that the concentration of IL-1β in THP-1 cells treated with LPS plus ATP was significantly higher than that in THP-1 cells treated with LPS plus ATP plus naloxone (0.1 μM) ($P < 0.001$). Real-time quantitative reverse transcription and polymerase chain reaction data also revealed that NLRP3 mRNA concentration in THP-1 cells treated with LPS was significantly higher than that in THP-1 cells treated with LPS plus naloxone ($P = 0.001$). ASC speck formation, that is, ASC assembles into a large protein complex, is an indicator for NLRP3 inflammasome activation. Our data revealed that the percentage of cells containing ASC specks in THP-1 cells treated with LPS plus ATP was also significantly higher than that in THP-1 cells treated with LPS plus ATP plus naloxone ($P < 0.001$).

Conclusions: Naloxone inhibits NLRP3 inflammasome activation.

Introduction

Naloxone, an antagonist of the opioid receptors, is used for opioid overdose treatment.[1] In addition, accumulating data have highlighted the anti-inflammation effects of naloxone.[2] Previous data revealed that naloxone could improve hypotension, preserve tissue perfusion, ameliorate tissue hypoxia, mitigate respiratory depression, and increase survival time in septic

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* *Corresponding author*. Department of Anesthesiology, Taipei Tzu Chi Hospital, 289, Jianguo Rd., Sindian District, New Taipei City 231, Taiwan. Tel.: +886 2 66289779x2639; fax: +886 2 66289009.
E-mail address: huangcj1112@gmail.com (C.-J. Huang).

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animals.[3-6] Our previous data that naloxone could inhibit upregulation of inflammatory mediators and mitigate activation of nuclear factor-κB (NF-κB) in endotoxin-activated murine macrophages[7] further confirmed the anti-inflammation effects of naloxone. Previous data also revealed that the mechanisms underlying the anti-inflammation effects of naloxone involve multiple pathways, including the opioid receptors, the L-type calcium channels, and the phosphoinositide 3-kinases pathway.[7-9]

Endotoxin-induced excessive release of proinflammatory mediators, such as interleukin-1β (IL-1β), plays an important role in overwhelming systemic inflammation during sepsis.[10] Endotoxin-induced production of IL-1β is tightly regulated by two separate signals.[11] The first signal is priming the pattern recognition receptor toll-like receptor 4 by lipopolysaccharide (LPS), which induces the production of pro-IL-1β.[12] The second signal is activating the caspase-1 to cleave pro-IL-1β into its bioactive form of mature IL-1β.[13] Caspase-1 activation is regulated by the nod-like receptor protein 3 (NLRP3) inflammasome, a multiprotein complex composed of NLRP3, and the adaptor protein apoptosis-associated speck-like protein contain a caspase recruitment domain (ASC).[14,15] NLRP3 inflammasome is involved in clinical conditions associated with inflammation, for example, diabetes mellitus, cardiovascular diseases, etc.[16] Forming an active NLRP3 inflammasome complex also requires "priming" and "activation."[17] Similar to pro-IL-1β, "priming" (induced by LPS) can upregulate NLRP3 transcription.[18] "Activation" (induced by adenosine triphosphate [ATP]) can lead to NLRP3 inflammasome assembly, a process involving NLRP3 oligomerization and ASC speck formation.[19]

As naloxone can inhibit IL-1β production, it is likely that naloxone can also inhibit NLRP3 inflammasome activation. To elucidate further, we designed and conducted this *in vitro* study. Our hypothesis was that naloxone can inhibit NLRP3 inflammasome activation via inhibition of NLRP3 transcription and/or NLRP3 inflammasome assembly. To facilitate investigation, an immortalized human monocytic leukemia cell line, THP-1, was employed.

Materials and methods

Cell cultures and differentiation

THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 25 mM HEPES, and 100 U/mL penicillin-streptomycin (all from Life Technologies, Carlsbad, CA). All cell cultures were incubated in a humidified chamber at 37°C in a mixture of 95% air and 5% CO_2. Confluent THP-1 cells were stimulated with 50 nM phorbol myristate acetate (PMA; Invitrogen, San Diego, CA) for 24 h to induce differentiation to macrophage.[20] Macrophage differentiation in over 90% of THP-1 cells was confirmed before these PMA-treated THP-1 cells were used for experiment.

Activation of NLRP3 inflammasome and naloxone administration

To activate the NLRP3 inflammasome, THP-1 cells were first primed with the TLR4 agonist LPS (1 μg/mL; Escherichia coli Serotype 0127:B8 endotoxin; Sigma–Aldrich) for 3 h to induce NLRP3 transcription. After priming, the cells were washed twice and preincubated with various concentrations of naloxone (0.1 and 1 μM; Genovate Biotechnology Co, Hsinchu, Taiwan) for 1 h. The dosages of naloxone were determined based on previous data that naloxone at these dosages significantly increased myocardial contractility in the presence of inotropics.[21] After preincubation with naloxone, primed THP-1 cells were activated with 1 mM ATP (Sigma–Aldrich) for 1 h to induce NLRP3 inflammasome assembly.

Enzyme-linked immunosorbent assay

The concentrations of secreted IL-1β in the cell culture supernatants were assayed using enzyme-linked immunosorbent assay (ELISA). A commercial IL-1β ELISA kit (Pierce Biotechnology, Inc, Rockfold, IL) was employed, and the assay was performed according to the manufacturer's instructions.

Real-time quantitative reverse transcription and polymerase chain reaction

Real-time quantitative reverse transcription and polymerase chain reaction (real-time qRT-PCR) was performed according to previous protocols.[22] In brief, total RNA was isolated from cell cultures with TRIzol Reagent (Invitrogen). RNA samples were then extracted by a phenol-chloroform technique. The RNA concentrations were quantified by measuring ultraviolet light absorbance at 260-nm wavelength. Thereafter, total 1.5 μg of messenger RNA (mRNA) was reverse transcribed into complementary DNA using Deoxy + HiSpec Reverse Transcriptase Kit (Yeastern Biotech Co, Taipei, Taiwan). To assay NLRP3 mRNA concentrations, real-time qRT-PCR was performed using EZtime Real-Time PCR Premix (Yeastern Biotech Co, Taipei, Taiwan) with gene-specific primers and probes on a sequence-detection system (ABI 7900HT Fast Real-Time PCR System, Applied Biosystems, Foster City, CA). The data were developed with Sequence Detector System (SDS software version 2.3, Applied Biosystems), and the threshold values (Cts) were selected according to the manufacturer's guidelines. For data normalization, an endogenous control defining complementary DNA input (glyceraldehyde-3-phosphate dehydrogenase) was used, and the relative units for gene expression were calculated by the comparative Ct method.[23] The gene-specific primers and probes were developed according to previous report.[24]

ASC speck formation

We chose to employ ASC speck formation assay for NLRP3 inflammasome assembly measurement. The activated THP-1 cells were fixed with 2% paraformaldehyde (Sigma–Aldrich) for 15 min followed by blocking and permeabilization with 0.025% Triton X-100 (Sigma–Aldrich) for 15 min at the