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Ephedrine hydrochloride protects mice from LPS challenge by promoting IL-10 secretion and inhibiting proinflammatory cytokines☆

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ABSTRACT

Sepsis and its derivative endotoxic shock are still serious conditions with high mortality in the intensive care unit. The mechanisms that ensure the balance of proinflammatory cytokines and anti-inflammatory cytokine production are of particular importance. As an active α- and β-adrenergic agonist, ephedrine hydrochloride (EH) is a widely used agent for cardiovascular diseases, especially boosting blood pressure. Here we demonstrate that EH increased Toll-like receptor 4 (TLR4)-mediated production of interleukin 10 (IL-10) through p38 MAPK activation. Simultaneously, EH negatively regulated the production of proinflammatory cytokines. Consistently, EH increased lipopolysaccharide (LPS)-induced serum IL-10 and inhibited tumor necrotic factor-α (TNFα) production in vivo. As a result, EH treatment protected mice from endotoxic shock by lethal LPS challenge. In brief, our data demonstrated that EH could contribute to immune homeostasis by balancing the production of proinflammatory cytokines and anti-inflammatory cytokine in TLR4 signaling. This study provides a potential usage of EH in autoimmunologic diseases or other severe inflammations.

1. Introduction

Lipopolysaccharide (LPS), the ligand of Toll-like receptor 4 (TLR4), is the normal component of the cell wall of gram-negative bacteria and has been recognized for many years as a key risk factor in the development of septic shock syndrome [1,2]. Sepsis remains a major cause of morbidity and mortality in hospitalized patients [3]. LPS ligation initiates innate immune responses by activating the mitogen-activated protein kinases (MAPKs), the transcription factor nuclear factor κB (NF-κB), and phosphatidylinositol (PI) 3-kinase (PI3K) signaling pathways and sequentially inducing the production of proinflammatory and anti-inflammatory cytokines, the balance of which determines the extent of host immune response [4]. Lethal dose of LPS elicits a systemic inflammatory process of the immune system to release proinflammatory cytokines, such as interleukin 6 (IL-6), IL-1β, IL-8, TNF-α, prostaglandins (PGE_2), nitric oxide, etc. These overwhelming proinflammatory cytokines result in hepatic and renal failure, acute respiratory distress syndrome, myocardial dysfunction and disseminated intravascular coagulation, which led to death [5]. Inhibition of the proinflammatory cytokines especially TNF-α, and IL-1β, is regarded as effective methods to manage septic shock [6].

Anti-inflammatory interleukin 10 (IL-10) plays a crucial role in limiting inflammatory responses and preventing host damage [7]. Enhancement of IL-10 production is also effective in the clinical treatment of septic shock [7]. Effective drug is eagerly wanted to specifically increase IL-10 while inhibit proinflammatory cytokines production in response to LPS.

Ephedrine hydrochloride (EH) is a compound from ephedrine and more stable than ephedrine, which is one of the main active components of *Ephedra sinica* (known in Chinese as Ma Huang). As a α- and β-adrenergic agonist, ephedrine stimulates the central nerve system, dilates bronchial tubes, elevates blood pressure, and increases heart rate [8]. *E. sinica* has pharmacological functions including anti-inflammatory effects. As recently reported, two active constituents of *E. sinica*, ephedrannin A and B, inhibited LPS-induced TNF-α and IL-1β

Abbreviations: EH, ephedrine hydrochloride; LPS, lipopolysaccharide; TLR, Toll-like receptor; DCs, dendritic cells; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol (PI) 3-kinase; IL-10, interleukin 10; ERK1/2, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; DXM, dexamethasone; NS, normal saline.

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through NF-κB and p38 MAPK in vitro [9]. To date, no reports have appeared to concern whether EH has an anti-inflammatory effect or not.

In this study, we demonstrated that EH promoted LPS-triggered IL-10 production and inhibited the production of proinflammatory cytokines IL-6, TNF-α, and IL-12p70, and chemokine MIP-2 in primary peritoneal macrophages and macrophage-like cell line Raw264.7. A similar anti-inflammatory role was observed in other immune cells, for example, dendritic cells. EH decreased LPS-mediated ERK phosphorylation and facilitated p38 phosphorylation, the latter of which accounted for EH-enhanced IL-10 production. Consistently, the anti-inflammatory role of EH was observed in vivo, and EH treatment protected mice from endotoxic shock triggered by lethal LPS challenge.

2. Materials and methods

2.1. Mice and reagents

Female C57BL/6J mice, 4 to 8 weeks of age, were obtained from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China). All mice were housed in a pathogen-free facility. All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China). Anti-β-Actin, and horseradish peroxidase-coupled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-antibodies against the extracellular signal-regulated kinase p44/p42 (ERK1/2, Thr202/Tyr204), c-Jun N-terminal kinase/stress-associated protein kinase (JNK/SAPK, Thr183/Tyr185), p38 (Thr180/Tyr182), Akt (Ser473), and p65 subunit (Ser536) of NF-κB, and corresponding antibodies against total signaling proteins were from Cell Signaling Technology (Beverly, MA). SB203580 was from Calbiochem (San Diego, CA). Anti-mouse IL-10 neutralizing antibody (AB-417-NA) was from R&D Systems (Minneapolis, MN). LPS (0111: B4) and DMSO were purchased from Sigma (St. Louis, MO). Ephedrine hydrochloride (Molecular formula: $C_{10}H_{15}NO{\cdot}HCl$; Molecular weight: 202) (30 mg/ml) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dexamethasone (Molecular formula: $C_{22}H_{29}FO_5$; Molecular weight: 392) (1 mg/ml) was obtained from Sigma (St. Louis, MO). Recombinant mouse GM-CSF and IL-4 were purchased from PeproTech (London, United Kingdom). CD4 and CD19 mAb-coated microbeads were obtained from Miltenyi Biotechnology (Bergisch Gladbach, Germany).

2.2. Cell culture

Thioglycolate-elicited mouse primary peritoneal macrophages were prepared from female C57BL/6J mice (6–8 weeks old) and cultured in endotoxin-free DMEM with 10% FCS, as described previously [10]. Mouse macrophage cell line RAW264.7, obtained from ATCC (Manassas, VA), was cultured as described previously [11]. Bone marrow-derived dendritic cells (DCs) from C57BL/6J mice (4 weeks old) were generated as described [11]. Splenic T or B cells were purified separately using CD4 or CD19 mAb-coated microbeads as described [12].

2.3. Detection of cytokine production

Enzyme-linked immunosorbent assay (ELISA) kits for murine interleukin 6 (IL-6), TNF-α, IL-12p70, IL-10 and murine macrophage inflammatory protein-2 (MIP-2) were purchased from R&D Systems (Minneapolis, MN). IL-6, TNF-α, IL-12p70, MIP-2 and IL-10 in the supernatants were measured. IL-6, TNF-α, and IL-10 in the sera were also measured by ELISA.

2.4. Nitric oxide measurement

Nitric oxide (NO) production was assayed by measurement of the nitrite concentration with the Griess assay (Sigma, St. Louis, MO). Supernatants (100 μl) were added to 100 μl of a 1:1 mixture of 1% sulfanilamide dihydrochloride and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4. Plates were incubated at 25 °C for 10 min, and absorbance at 550 nm was measured with a microplate reader. Nitrite concentrations were calculated with a sodium nitrite standard curve as reference [13].

2.5. Western blot analysis

Cells were lysed with M-PER™ Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (Calbiochem, San Diego, CA), then protein concentrations were measured with BCA assay (Pierce) and made equal with extraction reagent. Equal amounts of extracts were separated by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously [10].

2.6. Detection of cell apoptosis by fluorescence-activated cell sorting (FACS)

Peritoneal macrophages were treated with indicated concentrations of EH for 24 h, then harvested and labeled with Annexin-V-FITC and propidium iodide (PI) provided by Calbiochem (San Diego, CA), following manufacturer's instructions. Samples were examined by a flow cytometer (FACS Calibur, BD Bioscience, NJ) as described previously [10]. Data were analyzed using CellQuest software (Becton Dickinson, San Jose, USA).

2.7. In vivo LPS (with or without EH) injection and serum cytokines detection

Female C57BL/6J mice (6–8 weeks of age) were injected intraperitoneally with LPS (10 mg per kg body weight; Sigma) with or

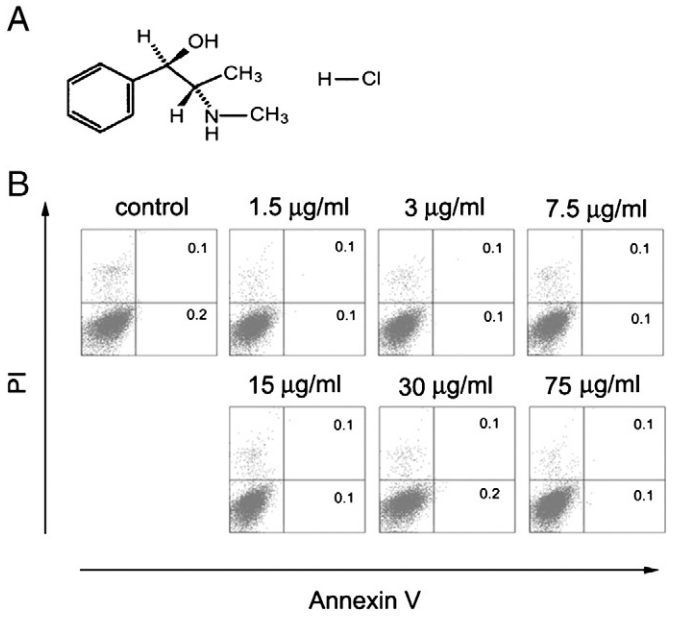

Fig. 1. Clinical concentrations of EH do not induce apoptosis of mouse peritoneal macrophages. (A) Chemical structure of ephedrine hydrochloride (EH). (B) Mouse primary peritoneal macrophages were treated with various concentrations of EH, as indicated, for 24 h. Cells were then harvested and labeled with Annexin V and PI, and analyzed by FACS. Similar results were obtained in three independent experiments.