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Prolonged pretreatment of mice with cholera toxin, but not isoproterenol, alleviates acute lethal systemic inflammatory response

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ABSTRACT

Isoproterenol, a synthetic non-selective β-adrenergic agonist, is often used during the immediate postoperative period after open heart surgery for its chronotropic and vasodilatory effects. It has been demonstrated that isoproterenol pretreatment followed by immediate LPS administration leads to reduced tumor necrosis factor-α (TNF-α) response *in vivo*. However, sepsis never happens immediately after the surgery, but rather severe immune dysfunction occurs at least 24 h later. It remains elusive what effects isoproterenol might exert to innate immunity during the period. In this scenario, we investigated the effects of 24-h isoproterenol pretreatment on septic shock induced by experimental endotoxemia and bacterial peritonitis, with cholera toxin as another cAMP elevator. Unexpectedly, we found that isoproterenol and cholera toxin exhibited distinct effects in acute lethal systemic inflammatory response. Isoproterenol worsened liver injury without enhancing NK/NKT activity. Meanwhile, cholera toxin but not isoproterenol showed dramatically reduced TNF-α response in LPS induced septic shock. Our data provide a caution for the clinical use of isoproterenol and suggest that isoproterenol has cAMP-independent functions.

1. Introduction

Isoproterenol, a synthetic non-selective β-adrenergic agonist, is often used during the immediate postoperative period after open heart surgery for its chronotropic and vasodilatory effects [1]. Isoproterenol elicits its pharmacological actions by stimulation of the G-protein (Gs)-coupled membrane receptors, which leads to the activation of the intracellular adenylate cyclase-cyclic adenosine monophosphate (cAMP) system [1]. cAMP elevators such as cholera toxin, which causes constitutive activation of Gs by stimulating ADP-ribosylation of its α-subunit, as well as isoproterenol suppress proinflammatory cytokine production from monocytic cells in response to lipopolysaccharide (LPS) challenge *in vitro* [2–5]. Furthermore, isoproterenol pretreatment (30 min) followed by immediate LPS administration leads to reduced tumor necrosis factor-α (TNF-α) response *in vivo* [3]. It has been demonstrated that the induction of transcription factor c-Fos is responsible for the cAMP-mediated suppression of inflammatory cytokine production [6].

Sepsis is a characteristic set of systemic reactions to overwhelming infection. The incidence of sepsis is increasing, and the mortality rate of severe sepsis is very high (up to 70%), especially in neonatal and medical intensive care units [7,8]. Endotoxin or LPS from gram-negative bacteria plays a major role in the pathogenesis of sepsis [7,8]. Endotoxin is known to provoke the release of various proinflammatory cytokines including TNF-α via the cooperative action of CD14 and Toll-like receptor 4 (TLR4) [7,8]. TNF-α has been identified as a critical mediator of endotoxin-induced injury to many tissues, including the liver, to a large extent through its pro-apoptotic activity [9,10]. The sensitivity of hepatocytes to TNF-α killing depends on interferon-γ (IFN-γ) levels, with NK and NKT cells as the major source of IFN-γ during sepsis [11–13]. Despite continuing progress in the development of antibiotics and other supportive care therapies, there is a lack of effective means of prevention of or therapy for sepsis [7,8].

Mediastinal sepsis following open heart surgery is a significant cause of death [14]. The suppression of LPS-induced production of inflammatory cytokines from monocytic cells by isoproterenol suggests that isoproterenol infusion might lead to beneficial effects against sepsis. However, sepsis never happens immediately after the surgery, but rather severe immune dysfunction occurs at least 24 h later [15]. It remains elusive what effects isoproterenol might exert to innate immunity during the period. In this scenario, we investigated the effects of 24-h isoproterenol pretreatment on septic shock induced by experimental

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endotoxemia and bacterial peritonitis, with cholera toxin as another cAMP elevator. Unexpectedly, we found that isoproterenol and cholera toxin exhibited distinct effects in acute lethal systemic inflammatory response. Isoproterenol worsened liver injury without enhancing NK/NKT activity. Our data provide a caution for the clinical use of isoproterenol.

2. Materials and methods

2.1. Reagents

Isoproterenol, cholera toxin, foskolin, LPS (serotype O111:B4), and type I collagenase were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). PE- or FITC-conjugated anti-F4/80, anti-TNF-α, anti-TCRβ, anti-CD49b antibodies, brefeldin A solution, and fixation/permeabilization kit were from eBioscience (San Diego, CA). ELISA kits for IL-6, IL-17, IL-12, IL-1β, TNF-α and IFN-γ were purchased from eBioscience (San Diego, CA) and ELISA kit for IL-10 was from DAKEWE (Beijing, China). cAMP assay kit and ELISA kits to detect aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from R&D systems (Minneapolis, MN). Antibodies against actin, p38, and c-Fos were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against phospho-p38 was from Cell Signaling Technology (Beverly, MA). Percoll gradient and DNase I were obtained from GE Healthcare (Buckinghamshire, UK) and Roche (Indianapolis, IN), respectively. TUNEL staining kit was purchased from Promega (Madison, WI, USA).

2.2. Mice

Female C57 BL/6 mice (6–8 weeks old) were purchased from Institutes of Experimental Animals, Academy of Chinese Medical Sciences. All mice were maintained under specific pathogen-free conditions. All experiments were performed in accordance with institutional guidelines for animal care. Mice were ip injected with 50 mg/kg isoproterenol, 250 μg/kg cholera toxin, or same volume of 0.9% saline 24 h before they were subjected to septic shock induction as described previously [16]. For LPS-induced endotoxemia, mice were ip injected with LPS (1 mg/mouse). For bacterial peritonitis, mice were given ip injections of heat-killed (95 °C for 30 minutes) *Escherichia coli* (DH5α, Invitrogen, Carlsbad, CA; 5×10^8/mouse).

2.3. Induction of bone marrow-derived macrophages

Bone marrow-derived macrophages were obtained by culturing the nonadherent bone marrow cells in RPMI 1640 medium containing 15% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mM 2-ME with 100 ng/ml M-CSF for 7 days, as described previously [17].

2.4. Isolation of peritoneal macrophages

Cells were collected from normal, unstimulated mice by repeatedly injecting 3 ml of tissue culture medium intraperitoneally and aspirating the fluid contents with a syringe. Harvests from 10 to 24 mice were pooled, pelleted by centrifugation (2000 rpm for 5 min) at 4 °C, washed and resuspended in tissue culture medium. Cells were then added to each 35-mm culture dish and cultures were incubated for 1 h at 37 °C. After gentle agitation, non-adherent leukocytes were removed by aspirating the supernatant with a sterile Pasteur pipette and by washing monolayers two times with tissue culture medium, as previously described [18].

2.5. ELISA for detection of serum cytokines and aminotransferases

Serum were collected and stored at −70 °C until used. Levels of cytokines and AST/ALT in the serum were determined by using ELISA kits according to the manufacturer's instructions.

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of mice liver tissues

The liver tissues of mice were removed and fixed overnight in 4% paraformaldehyde after LPS induced endotoxemia for 12 h. The tissues were dehydrated, infiltrated with paraffin and sectioned at 5 μm. TUNEL assays were performed according to the manufacturer's instructions.

2.7. Immunoblotting analysis for proteins from bone marrow-derived macrophages

Whole cell lysates were harvested and prepared as previously described [19] and were resolved by SDS-PAGE before being transferred to nitrocellulose membranes. The membranes were then probed with various primary antibodies followed by peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using an ECL chemiluminescence kit.

2.8. Flow cytometry for separation of cell populations and cell counting

Erythrocytes were depleted by hypotonic lysis. The leukocytes were washed with FACS washing buffer (2% FBS, 0.1% NaN_3 in PBS) twice and were then incubated with specific antibodies for 30 min on ice in the presence of 2.4G2 mAb to block FcγR binding. Isotype antibodies were included as negative controls. For intracellular cytokine staining, single-cell suspensions were stimulated with 1 μg/ml LPS in the presence of brefeldin A solution for 4 h. After stimulation, cells were stained with FITC-anti-F4/80 antibody, fixed and permeabilized using a fixation/permeabilization kit and stained with PE-anti-TNF-α antibody in accordance with the manufacturer's instructions. Flow cytometry was performed on a Becton Dickinson FACS Calibur machine.

2.9. Measurement of intracellular cAMP

Cells were washed twice with cold PBS and lysed in 250 μl cell lysis buffer provided by a Parameter cAMP assay kit with three cycles of freeze–thaw. The cell lysates were used to measure intracellular cAMP according to the manufacturer's instructions.

2.10. Isolation of liver mononuclear cells

Isolation of liver mononuclear cells was performed as previously described [20]. Briefly, livers were removed and minced into small pieces, which were shaken in the digestion buffer (0.05% collagenase in HBSS with 0.01% trypsin inhibitor) at 37 °C for 30 min, and filtered through a #200 gauge stainless steel mesh. For elimination of hepatocytes, the cells were overlaid by 33% Percoll solution and centrifuged at 800 *g* for 30 min at room temperature with no break. The supernatant was aspirated, and the cell pallet was washed with PBS. Then the cells were suspended by RBC lysing buffer for 3 min at room temperature, and followed by PBS washing for 3 times. Liver mononuclear cells were collected.

2.11. Statistical analysis

The survival curves were compared by Kaplan–Meier analysis. Other statistical analysis was performed using the Student's *t*-test. The difference was considered statistically significant when $p < 0.05$.