International Immunopharmacology 39 (2016) 377–382

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Astragaloside IV alleviates *E. coli*-caused peritonitis via upregulation of neutrophil influx to the site of infection

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ARTICLE INFO

Article history:
Received 22 June 2016
Received in revised form 4 August 2016
Accepted 11 August 2016
Available online 17 August 2016

Keywords:
Astragaloside IV
Peritonitis
Neutrophils
Migration
CXCR2

ABSTRACT

Astragaloside IV (AS-IV), an active saponin purified from Astragali Radix, has been identified with broad biological and pharmacological activities. In the present study, we continue to explore the potential effect of AS-IV on antibacterial response using an acute *E. coli* peritoneal infection model. Our findings implied that administration of AS-IV decreases mortality in mice challenged by lethal *E. coli* infection. The protection of AS-IV was related to promotion of neutrophil extravasation into the peritoneum and bacterial clearance. Toll-like receptor (TLR) activation in neutrophils has been reported to reduce CXCR2 expression and subsequent neutrophil migration. Our data indicated that AS-IV prevented the reduction of CXCR2 expression and neutrophil migration induced by LPS, the activator for TLR4. Moreover, we found that AS-IV blocks LPS-induced suppression of CXCR2 on neutrophils by inhibiting the expression of G protein-coupled receptor kinase-2 (GRK2), an agonist that regulates desensitization and internalization of chemokine receptors. Taken together, these data propose that AS-IV, through modulating GRK2-CXCR2 signal in neutrophils, offers an essential efficacy on host antibacterial immunity.

1. Introduction

Neutrophils are the pivotal effector cells participating in innate immunity to microorganism infection [1,2]. The migration of neutrophils to an inflammatory site is mainly relied on chemokines, especially CXC chemokines containing a tripeptide glutamate-leucine-arginine (CXC-ELR chemokines). The primary CXC-ELR chemokines include CXCL1 and CXCL2/3 in mice, and their homologues of growth-related oncogenes (GROα, -β, and -γ) and CXCL8 in humans [3]. These chemokines through binding to CXCR2 activate neutrophils and then facilitate their extravasation into the infection focus. There is a diversity of evidence to show that mice deficient in CXCR2 exhibited a reduced neutrophil infiltration into the inflammatory site and a high mortality rate during serious sepsis [4,5]. There is no difference in *Aspergillus fumigatus*-caused pneumonia following by either CXCR2 blockade or neutrophil depletion, which is more serious than in the presence of neutrophils [6]. As known that defects in neutrophil recruitment usually enhance host sensitivity to infection, an increased comprehension of potential signal of neutrophil migration is essential for defending against infection.

Astragali Radix, as a traditional Chinese medicine, has been extensively employed for more than twenty centuries in therapy of a various diseases including atherosclerosis, tumor and diabetic nephropathy [7,8]. Astragaloside IV (AS-IV), an active ingredient isolated from Astragali Radix, has been identified with broad biological and pharmacological actions [9,10]. Several studies found that AS-IV show a range of immunoregulatory, anti-inflammatory and antioxidant effects on atherosclerosis, myocardial ischemia and Alzheimer's disease (AD) [11–13]. Notably, the new pharmacological effect of AS-IV in tissue repair has also been reported, the mechanism by which is related to promotion of the production of numerous growth factors, collagens and matrix metalloproteinases, acceleration of the recruitment of dermal fibrocytes and keratinocytes [14,15]. AS-IV shows a beneficial effect on diabetic wound damage and vascular endothelial dysfunction and has demonstrated elevated wound healing [16,17].

However, the anti-inflammatory response of AS-IV on bacterial infection has not been clearly clarified. In the present study, we testify the potential effect of AS-IV in a mouse model of *E. coli*-infected peritonitis. Our findings show that administration of AS-IV apparently contributes to clearance of *E. coli* infection in mice. The protection of AS-IV is related to promotion of CXCR2 expression and neutrophil influx into the site of infection. Additionally, AS-IV facilitates the upregulation of CXCR2 on the surface of neutrophils involved in inhibition of G protein-coupled receptor kinase-2 (GRK2) expression. All together, we

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http://dx.doi.org/10.1016/j.intimp.2016.08.011
1567-5769/

determine AS-IV as a new modulator of neutrophil function in anti-*E. coli* infection.

2. Materials and methods

2.1. Bacterial strain

E. coli strain ATCC 25922 was gained from China Center of Industrial Culture Collection (Beijing, China). *E. coli* cultures were incubated in 5 ml of tryptic soy broth (TSB) at 37 °C overnight, and then diluted 1:100 in fresh TSB. A log-phase culture was rinsed 3 times by centrifugation in phosphate-buffered saline (PBS; pH 7.4), and diluted in PBS.

2.2. Mice and Astragaloside IV

Male BALB/c mice, 6–8 wk. of age, were purchased from the Center of Experimental Animals of Guangdong Province, and bred under 12-h light/dark cycles with free access to standard chow and water. All experiments were conducted according to the guidelines of the Animal Care and Use Committee at the Guangdong Pharmaceutical University. AS-IV was obtained from Aladdin Industrial Inc. (Pico Rivera South of Los Angeles, California). 10 mg/kg AS-IV was administered intragastrically to mice for 3 consecutive days before bacterial infection. The dosage of AS-IV was chosen in accordance with previous literature [18].

2.3. Bacterial peritonitis

E. coli were injected intraperitoneally (i.p.) into mice at a lethal dose of 2×10^8 CFU in a total volume of 200 μl. Survival was monitored every 12 h for 7 d. In some experiments, mice were sacrificed at 6 or 24 h by bleeding and their peritoneal cavities were lavaged with 3 ml of sterile PBS. Peritoneal lavage fluid was collected and organs were removed and mechanically homogenized in 1 ml sterile PBS prior to CFU. The number of colonies was counted and expressed as the $\log_{10}$ (CFU/ml) or $\log_{10}$ (CFU/organ).

2.4. Elisa

Concentrations of mouse cytokine and chemokine in serum and peritoneal lavage fluid were measured by ELISA using antibodies from eBioscience (San Diego, CA).

2.5. Neutrophil isolation

Mouse neutrophils were isolated from bone marrow (BM) and blood by Percoll density gradient centrifugation, as previously described [19]. Mouse femurs and tibias were removed and flushed with PBS containing 0.5% FBS. The BM neutrophils were purified through a 2-layer gradient of 72 and 65% Percoll (Sigma, St. Louis, USA). After centrifuged at 1200*g* for 30 min, mature neutrophils were recovered at the interface of the 65–72% fractions. The blood neutrophils were purified through a 3-layer gradient of 78, 69 and 52% Percoll. After centrifuged at 1200*g* for 30 min, the neutrophils were collected from the 69%–78% interface fractions.

2.6. In vitro migration

Purified neutrophils were preincubated with 30 μg/ml AS-IV for 1 h, and then stimulated with 1 μg/ml LPS for additional 1 h. In some experiments, CXCR2 inhibitor SB225002 (0.4 μM; Selleckchem) and GRK2 inhibitor (150 μM; Calbiochem) were added 30 min before challenge with AS-IV or LPS. Neutrophils (1×10^6 cells/ml) were seeded in the upper chamber and allowed to migrate toward 30 ng/ml CXCL2 (PeproTech) in the lower chamber for 1 h. After the membrane was fixed and stained, neutrophils that migrated through the membrane were counted with a light microscope (Carl Zeiss, Oberkochen, Germany).

2.7. Flow cytometry

Neutrophils were pretreated with Fc Block (Biolegend) and then stained with FITC-conjugated anti-Ly6G mAb, PE-conjugated anti-CD11b and PerCP/Cy5.5-conjugated anti-CXCR2 mAb for 30 min in the dark. After a washing step, fluorescence of the cells was measured by a FACSCalibur cytometer (BD Biosciences, San Jose, CA) and analyzed by the CELLQUEST v3.3 software. In some experiments, intracellular staining was performed by incubating with rabbit anti-GRK2 antibody (GeneTex, Irvine, CA), followed with Dylight 488-conjugated anti-rabbit IgG.

2.8. Statistical analysis

All data are expressed as mean ± SEM. Statistical differences between groups were determined by the Student's *t*-test or one way ANOVA. Values of $P < 0.05$ were considered significant.

3. Results

3.1. AS-IV protects mice from E. coli infection

To investigate the effect of AS-IV in acute bacterial sepsis, we established a mouse peritonitis model by i.p. injection of 2×10^8 CFU *E. coli*. Mice that received AS-IV administration for 3 days displayed obviously elevated survival rate in comparison with untreated controls (Fig. 1a). The number of *E. coli* in the peritoneal cavity and blood markedly decreased in the AS-IV-treated mice at 6 and 24 h postinfection. Meantime, the burden of bacteria in various organs was also reduced in the AS-IV-treated mice at 24 h postinfection (Fig. 1d). These results suggested that AS-IV provide a protective effect against *E. coli* infection.

3.2. AS-IV lessens inflammatory response in E. coli-infected mice

To observe the influence of AS-IV on inflammatory response postbacterial infection, proinflammtory mediators in blood and peritoneal exudates were measured 6 h after infection. The elevated serum levels of TNF-α, IL-6 and CXCL2 challenged by *E. coli* infection were significantly lowered in the AS-IV-treated mice (Fig. 2a). On the contrary, the concentrations of IL-6 and CXCL2, but not TNF-α, in the peritoneal exudates did not show a statistical difference between AS-IV-treated and untreated mice after *E. coli* infection (Fig. 2b). These findings indicated that the protection of AS-IV is related to suppression of systemic inflammation.

3.3. AS-IV facilitates i.p. neutrophil migration in E. coli-infected mice

The prompt infiltration of neutrophils into the infection sites is essential for controlling bacterial infections. The quantity of $CD11b^+$-$Ly6G^{hi+}$ neutrophils markedly enhanced at 6 h postinfection in AS-IV-treated mice when compared with that in untreated mice (Fig. 3a). The expression of CXCR2 on neutrophils was decreased in mice when challenged with *E. coli* infection (Fig. 3b), and the reduction was related to a lower chemotactic ability to CXCL2 (Fig. 3c), which was obviously reversed by AS-IV administration (Fig. 3b and c). These data demonstrated that the protection of AS-IV from bacterial infection is related to acceleration of neutrophil recruitment.

3.4. AS-IV modulates neutrophil chemotaxis involved in CXCR2 in vitro

TLR activation in neutrophils has been reported to suppress the expression of CXCR2 and subsequent cellular migration [20]. As shown in Fig. 4a, resting neutrophils expressed high levels of CXCR2. When stimulated by LPS, CXCR2 expression on neutrophils was decreased, and the reduction was accompanied with lowered chemotaxis to CXCL2 (Fig. 4a and b). AS-IV treatment blocked the decrease of CXCR2