International Immunopharmacology 15 (2013) 442–449

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

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

Suppression of LPS-induced inflammatory responses by gossypol in RAW 264.7 cells and mouse models

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ARTICLE INFO

Article history:
Received 19 October 2012
Received in revised form 17 December 2012
Accepted 7 January 2013
Available online 23 January 2013

Keywords:
Gossypol
NF-κB
MAPK
Cytokines
Acute lung injury

ABSTRACT

Gossypol, a yellowish polyphenolic compound originally from cotton plant, has been known to exert a potential for anti-cancer, anti-inflammatory and other important therapeutic activities. The purpose of this investigation was to determine the protection of gossypol on inflammation in Lipopolysaccharide (LPS) stimulated RAW 264.7 cells and LPS induced in vivo lung injury model. The effects of gossypol on pro-inflammatory cytokines and signaling pathways were evaluated by enzyme-linked immunosorbent assay and Western blot. The results showed that gossypol significantly inhibited the production of LPS-induced TNF-α, IL-6 and IL-1β both in vitro and vivo. Furthermore, gossypol blocked the phosphorylation of IκBα protein, p65, p38, c-Junterminal kinase (JNK) and extracellular signal-regulated kinase (ERK) in LPS stimulated RAW 264.7 cells. From the in vivo study, it was observed that gossypol attenuated lung histopathologic changes in mouse models. The present data suggest that gossypol suppresses the inflammation in vitro and vivo, and may be a potential therapeutic candidate for the treatment of inflammatory disorders.

1. Introduction

Inflammation, an evolutionarily conserved host reaction, is a response to injury, infection and stress. Acute lung injury is an inflammatory disorder of lung and commonly characterized by the development of hypoxemia, damage to the alveolar capillary membrane barrier, pulmonary edema and the resultant respiratory failure [1–3]. Increasing evidence has revealed that lipopolysaccharide (LPS) derived from gram-negative bacteria can induce the inflammatory response by activating numerous inflammatory cells and result in acute lung injury [4,5]. Cytokines, such as tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) have been known to play important roles in pro-inflammatory responses. Cytokines are released from activated macrophages [6].

The transcription factor Nuclear factor-kappaB (NF-κB) has been of interest for inflammatory-mediated responses [7]. NF-κB plays a central role in the regulation of many genes being responsible for the generation of inflammation mediators, and it is required for the maximal transcription of numerous cytokines, including TNF-α and IL-6 [8]. The most predominately characterized NF-κB complex is a p50/p65 heterodimer, which is associated with IκB, and is kept inactive in the cytoplasm of resting cells [9]. IκBα degradation results in the translocation of p65 NF-κB to the nucleus. The tyrosine phosphorylation of p65 NF-κB efficiently modulates the transcription activity [10]. Mitogen-activated protein kinases (MAPKs) regulate various inflammatory and immune responses [11]. Recently, a number of in vitro studies have shown that the production of inflammatory mediators is strongly affected by MAPKs, such as p38 MAPK, c-Junterminal kinase (JNK) and extracellular signal-regulated kinase (ERK) [12,13]. The suppression of these MAPK family members blocks the production of pro-inflammatory cytokines. In addition, p38 MAPK has been proposed to regulate the activation of NF-κB [14]. Because NF-κB and MAPKs play critical roles in the responses of cells to various cytokines or stresses [15], the inhibition of signal transduction proteins in the pathways leading to activation of NF-κB and MAPK is now recognized as a valid strategy to cure inflammatory diseases.

Gossypol (Fig. 1), a compound originally isolated from cottonseed, inhibits the growth of several tumor cell lines in vitro. It has been suggested to be a potential antitumor drug [16,17]. Recently, gossypol has been found to significantly inhibit tumor metastasis and invasion in human cancer cells [18,19]. However, the information on the anti-inflammatory effects and mechanism of gossypol is still limited. The purpose of this study is to examine the anti-inflammatory effects of gossypol in a mouse model of LPS-induced acute lung injury and

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http://dx.doi.org/10.1016/j.intimp.2013.01.008

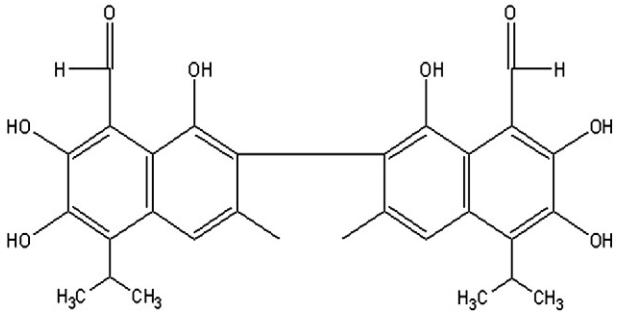

Fig. 1. The chemical structure of gossypol.

the anti-inflammatory mechanism of gossypol in LPS-stimulated RAW 264.7 macrophages through inflammatory mediators, NF-κB and MAPK pathway.

2. Materials and methods

2.1. Chemicals and reagents

Gossypol was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China). Dimethyl sulfoxide (DMSO), lipopolysaccharide (*Escherichia coli* 055:B5), and 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were obtained from Invitrogen-Gibco (Grand Island, NY). Mouse TNF-α, IL-6 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (CA, USA). Mouse monoclonal phospho-specific p42-p44 ERK antibodies, mouse monoclonal phospho-specific p46-p54 JNK antibodies, mouse monoclonal phospho-specific p38 antibodies, mouse mAb Phospho-IκBα, rabbit mAb IκBα and rabbit mAb Phospho-NF-κb p65 (Ser536) were purchased from Cell Signaling Technology Inc (Beverly, MA). HRP-conjugated goat anti-rabbit and goat-mouse antibodies were provided by GE Healthcare (Buckinghamshire, UK). All other chemicals were of reagent grade.

2.2. Animals

Male BALB/c mice were purchased from the Center of Experimental Animals of Baiqiuen Medical College of Jilin University (Jilin, China). The mice were housed under a pathogen-free condition at a mouse facility and received food and water ad libitum. The laboratory temperature was 24 ± 1 °C, and relative humidity was 40–80%. Mice (18–20 g) were housed for 2–3 days to adapt the environment before experiment. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. In vitro study

2.3.1. Cell culture and treatment

The RAW 264.7 mouse macrophage cell line was purchased from the China Cell Line Bank (Beijing, China) and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3 mM Glutamine, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), 10% heat-inactivated fetal bovine serum, at 37 °C under a humidified atmosphere of 5% CO2. In all experiments, macrophages were incubated in the presence or absence of various concentrations of gossypol which were always added 1 h prior to LPS (1 μg/mL) stimulation.

2.3.2. Cell viability assay

Cell viability was evaluated by MTT assay. Briefly, cells were plated at a density of 4×10^5 cells/ml onto 96-well plates in a 37 °C, 5% CO2 incubator for 1 h. Then the cells were treated with 50 ul gossypol of different concentrations (0–50 ug/ml) for 1 h, followed by stimulation with 50 ul LPS (4 ug/ml). After 18 h of LPS stimulation, 20 ul MTT reagent (5 mg/ml) was added to each well, and incubation continued for 4 h. The supernatant was removed and the formation of formazan was resolved with 150 ul/well of DMSO. The optical density was measured at a wavelength of 570 nm using a microplate reader (TECAN, Austria). Concentrations were determined for three wells of each sample, and this experiment done in triplicate.

2.3.3. Cytokine assay (TNF-α, IL-6 and IL-1β)

Gossypol solubilized with DMSO was diluted with DMEM prior to treatment. RAW 264.7 cells were plated onto 24-well plates (4×10^5 cells/well), and incubated in the presence of either LPS alone 1 μg/ml, or LPS plus gossypol (15 g/ml, 30 μg/ml and 45 μg/ml) for 18 h. Cell-free supernatants were subsequently employed in pro-inflammatory cytokine assays using a mouse enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), according to the manufacturer's instructions (BioLegend, Inc, Camino Santa Fe, Suite E, San Diego, CA, USA).

2.3.4. Western blot analysis

RAW 264.7 macrophages were plated onto 6-well plates and incubated for 24 h, then pretreated with gossypol for 1 h and after LPS (1 μg/ml) stimulation for 30 min, the cells were collected and washed twice with cold PBS. The cells were lysed in lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.6% NP-40, 1 mM Na3VO4, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM p-nitrophenyl phosphate, and 1:25 Complete Mini Protease Inhibitor cocktail (Boehringer, Mannheim, Germany) and maintained on ice for 30 min. The cell lysates were washed via dilution and centrifugal concentration. The total protein of RAW 264.7 cells was extracted by Thermo Scientific M-PER Mammalian Protein Extraction Reagent. Protein concentrations were determined using a BCA™ protein assay kit. Aliquots of the lysates were separated on 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD) with a glycine transfer buffer [192 mM glycine, 25 mM Tris–HCl (pH 8.8), 20% methanol (v/v)]. After blocking the membrane with 5% non-fat milk in TBST buffer for 2 h at room temperature, the membrane was incubated overnight with specific primary antibody at 4 °C. The membrane was then incubated for an additional 60 min with a peroxidase-conjugated secondary antibody at room temperature. Membranes were reacted with ECL detection reagent to visualize immunoreactive bands by X-ray film exposure.

2.4. In vivo study

2.4.1. Experimental design

Mice were randomly allocated into the following groups: Control, LPS, LPS + Gossypol and LPS + Dexamethasone (LPS + DEX). Mice from the control and LPS groups received an equal volume of PBS. Gossypol (15 mg/kg) and DEX (5 mg/kg) were given intraperitoneally. LPS + DEX group serves as positive control. 15 mg/kg was in the non-toxic concentrations range of gossypol for the vivo experiment [20,21]. 1 h later, 10 ug LPS dissolved in 50 ul PBS was instilled intranasally to induce lung injury. Control mice were given 50 μL PBS without LPS. All the mice were alive after 6 h LPS treatment, the collection of bronchoalveolar lavage fluid (BALF) was performed three times through a tracheal cannula with autoclaved PBS [22].

2.4.2. Measurement of cytokines

The concentrations of TNF-α, IL-6, and IL-1β in the BALF were determined using sandwich enzyme-linked immunosorbent assay (ELISA)