Toxicology and Applied Pharmacology 292 (2016) 56–64

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Gossypol induces pyroptosis in mouse macrophages *via* a non-canonical inflammasome pathway

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ARTICLE INFO

Article history:
Received 31 July 2015
Revised 10 December 2015
Accepted 31 December 2015
Available online 4 January 2016

Keywords:
Gossypol
Pyroptosis
Inflammasome
HMGB1
Interleukin-1β

ABSTRACT

Gossypol, a polyphenolic compound isolated from cottonseeds, has been reported to possess many pharmacological activities, but whether it can influence inflammasome activation remains unclear. In this study, we found that in mouse macrophages, gossypol induced cell death characterized by rapid membrane rupture and robust release of HMGB1 and pro-caspase-11 comparable to ATP treatment, suggesting an induction of pyroptotic cell death. Unlike ATP, gossypol induced much low levels of mature interleukin-1β (IL-1β) secretion from mouse peritoneal macrophages primed with LPS, although it caused pro-IL-1β release similar to that of ATP. Consistent with this, activated caspase-1 responsible for pro-IL-1β maturation was undetectable in gossypol-treated peritoneal macrophages. Besides, RAW 264.7 cells lacking ASC expression and caspase-1 activation also underwent pyroptotic cell death upon gossypol treatment. In further support of pyroptosis induction, both pan-caspase inhibitor and caspase-1 subfamily inhibitor, but not caspase-3 inhibitor, could sharply suppress gossypol-induced cell death. Other canonical pyroptotic inhibitors, including potassium chloride and N-acetyl-L-cysteine, could suppress ATP-induced pyroptosis but failed to inhibit or even enhanced gossypol-induced cell death, whereas nonspecific pore-formation inhibitor glycine could attenuate this process, suggesting involvement of a non-canonical pathway. Of note, gossypol treatment eliminated thioglycollate-induced macrophages in the peritoneal cavity with recruitment of other leukocytes. Moreover, gossypol administration markedly decreased the survival of mice in a bacterial sepsis model. Collectively, these results suggested that gossypol induced pyroptosis in mouse macrophages *via* a non-canonical inflammasome pathway, which raises a concern for its *in vivo* cytotoxicity to macrophages.

1. Introduction

The inflammasome is a multimeric protein platform that is formed in innate immune cells, epithelial cells, and many other cell types to mount a coordinated molecular defense against pathogenic microbes and tissue injury (Lamkanfi and Dixit, 2014). The assembly of inflammasome complex is initiated upon the recognition of pathogen-associated molecular patterns (PAMPs), or danger-associated molecular patterns (DAMPs) by nucleotide-binding domain and leucine-rich repeat receptors (NLRs) or absent in melanoma 2 (AIM2)-like receptors (ALRs) (de Zoete et al., 2014). Such sophisticated mechanisms by which inflammasomes respond to danger signals lead to secretion of proinflammatory interleukin (IL)-1β and IL-18, which is crucial for clearance of infectious agents, as well as pyroptosis, an inflammatory form of cell death (Man and Kanneganti, 2015).

Pyroptosis is a programmed cell death that is characterized by cell swelling, rapid plasma-membrane rupture, and release of proinflammatory contents including IL-1β, IL-18, ATP and high-mobility group box 1 (HMGB1) (Fink and Cookson, 2006), thus facilitating recruitment of neutrophils and monocytes, and leading to clearance of pathogenic microbes (Scaffidi et al., 2002). It can be induced by a wide range of PAMPs (*i.e.*, flagellin, lipopolysaccharide (LPS), and bacterial toxins) and DAMPs (*i.e.*, ATP, uric acid crystals, and amyloid-β fibrils) and generally mediates host defenses through the release of proinflammatory components thus being beneficial to the host during an infection. On the other hand, pyroptosis also has critical roles in the development and

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; GOS, gossypol; DAMPs, danger-associated molecular patterns; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetra-acetic acid; FBS, fetal bovine serum; HMGB1, high-mobility group box 1; HRP, horse-radish peroxidase; IL-1β, interleukin-1β; LPS, lipopolysaccharide; NAC, N-acetyl-L-cysteine; PI, propidium iodide; ROS, reactive oxygen species; PAMPs, pathogen-associated molecular patterns; SD, standard deviation; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide-gel electrophoresis; siRNA, small interfering RNA; TG, thioglycolate.

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http://dx.doi.org/10.1016/j.taap.2015.12.027
0041-008X/

progression of various chronic diseases, including gout, atherosclerosis, and metabolic syndrome, demonstrating the inflammatory response as a "double-edged sword" (Man and Kanneganti, 2015).

It has recently been recognized that two distinct inflammasome activation pathways exist: the canonical and non-canonical pathways (Lamkanfi and Dixit, 2014; Man and Kanneganti, 2015). Under most circumstances, activated NLRs and ALRs recruit a bipartite protein known as apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) to induce the activation of caspase-1. This canonical inflammasome pathway is dependent on ASC for the self-proteolysis and activation of caspase-1, culminating in the cleavage of pro-IL-1β and pro-IL-18 into their secreted forms and pyroptotic cell death. In contrast to the canonical inflammasome pathway, the recently identified non-canonical pathway is mediated by caspase-11 but independent of ASC (Kayagaki et al., 2011, 2013; Hagar et al., 2013). Activation of caspase-11 by intracellular LPS induces caspase-1-independent pyroptosis as well as IL-1α/HMGB1 production and caspase-1-dependent IL-1β/IL-18 secretion. Thus, caspase-11-mediated pyroptosis may have a critical role in clearing intracellular bacteria and constitutes a potential target for modulating innate inflammatory responses (Stowe et al., 2015).

Gossypol (GOS) is a polyphenolic compound existed in cottonseed oil (Gadelha et al., 2014). Early studies of GOS mainly focused on how to reduce its toxicity as it exists at high abundance in cotton seeds used for feeding animals. Subsequent studies revealed that GOS possesses many pharmacological properties, including anti-fungal, anti-inflammatory, anti-tumor, and anti-fertility activities (Turco et al., 2007; Moon et al., 2011). Mechanical studies showed that GOS causes mitochondrial dysfunction by inhibiting cell respiration and stimulation of reactive oxygen species (ROS) generation (Keshmiri-Neghab and Goliaei, 2014). It has been reported that GOS exhibits immunosuppressive effects on mouse lymphocytes *in vitro* and suppresses delayed-type hypersensitivity *in vivo* in a mouse model, which is likely mediated by inhibiting lymphocyte proliferation and inducing apoptotic cell death (Xu et al., 2009). One recent study reported that GOS inhibits the expression of proinflammatory cytokines in mouse RAW 264.7 cells through attenuating multiple signaling pathways (Huo et al., 2013). In addition, GOS has been found to inhibit tumor necrosis factor-α-induced intercellular adhesion molecule-1 expression in breast cancer cells by suppressing the NF-κB pathway (Moon et al., 2011). In view of its potential immunomodulatory activity, it is of interest to unravel the potential effect of GOS on inflammasome activation and pyroptosis in macrophages.

In this study, we aimed to explore whether GOS could induce pyroptosis and release of proinflammatory cytokines in mouse peritoneal macrophages and RAW 264.7 cells. Our data indicated that GOS could induce a robust pyroptotic cell death but a weak release of mature IL-1β in peritoneal macrophages in a manner different from that of ATP. *In vivo* administration of gossypol had likely eliminated the peritoneal macrophages in thioglycolate-treated mice and markedly reduced the survival rate of mice in a bacterial sepsis model. Our findings highlight a concern regarding its potential cytotoxicity to macrophages by induction of pyroptosis when used *in vivo*.

2. Materials and methods

2.1. Chemicals and antibodies

Gossypol (GOS), propidium iodide (PI), dimethyl sulfoxide (DMSO), Hoechst 33342, and LPS (*Escherichia coli* O111:B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GOS was dissolved in DMSO at 60 mM, stored at −20 °C, and working solution was freshly prepared. Z-VAD-FMK (VAD), VX-765 (VX), and Z-DEVD-FMK (Z.D.) were obtained from MedChem Express (Princeton, NJ, USA). DMEM, Opti-MEM, L-glutamine, fetal bovine serum (FBS), penicillin and streptomycin were products of Invitrogen (Carlsbad, CA, USA). Thioglycollate (TG) medium (Brewer modified) was bought from Becton Dickinson (Sparks, MD, USA). Mouse anti-caspase-1 monoclonal antibody (clone 14F468) was obtained from Novus Biologicals (Littleton, CO, USA). Specific antibodies against IL-1β, caspase-11, HMGB1, β-tubulin, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse/rat IgG were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Animals

C57BL/6 mice (6–8 weeks of age) were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China). All animal experiments were performed according to the guidelines for the care and use of animals approved by the Committee on the Ethics of Animal Experiments of Jinan University.

2.3. Cell line and cell culture

The RAW 264.7 cells was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (DMEM complete medium) at 37 °C in a humidified incubator of 5% CO_2; and sub-cultured every 2–3 days.

2.4. Isolation of peritoneal macrophages

Mice were injected with 1 ml of 3% TG medium and 4 days later killed by cervical dislocation and sterilized by 75% ethanol. Peritoneal macrophages were immediately extracted by washing the peritoneal cavity with washing buffer (sterile PBS containing 5% newborn calf serum and 0.5 mM EDTA). The extracted solution was centrifuged at 300 × *g* for 10 min and isolated cells were cultured at 37 °C in DMEM complete medium. After 2-h incubation, unattached cells were discarded and attached macrophages were further cultured in fresh complete medium.

2.5. Cytotoxicity assay

Peritoneal macrophages, which were seeded in 24-well plates (3×10^5 cells/well), stimulated with 1 μg/ml LPS for 5 h, and subsequently incubated with GOS for indicated time periods. Cell death was measured by PI incorporation (Py et al., 2014). PI (2 μg/ml) was added to cell culture media at room temperature for 10 min, and cells were observed immediately by live imaging using a Zeiss Axio Observer D1 microscope equipped with a Zeiss LD Plan-Neofluar 20×/0.4 Korr M27 objective lens. Fluorescence images were captured with a Zeiss AxioCam MR R3 cooled CCD camera controlled with ZEN software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

2.6. Determination of soluble IL-1β

Soluble IL-1β in cell culture supernatants was determined by Cytometric Bead Array (CBA) Mouse IL-1β Flex Set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction. Data were acquired using CELLQuest software on a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA, USA).

2.7. Western blot analysis

Western blotting was performed as described previously (Zhang et al., 2014). Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer to polyvinylidene difluoride (PVDF) membranes (Hybond-P; GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were blocked with blocking buffer (50 mM Tris-buffered saline (pH 7.4) containing 5% nonfat milk and 0.1% Tween-20) and then incubated overnight with indicated antibodies, followed by HRP-conjugated goat anti-rabbit/mouse/rat IgG. Bands were revealed with