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Toll-like receptor 4-mediated ROS signaling pathway involved in *Ganoderma atrum* polysaccharide-induced tumor necrosis factor-α secretion during macrophage activation

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ABSTRACT

Ganoderma atrum has been used as Chinese traditional medicine and healthful mushroom for thousands of years. The polysaccharide is regarded as the major bioactive substances in *G. atrum*. To delineate the underlying mechanism and signaling cascade involved in the immunomodulatory property of *G. atrum* polysaccharide (PSG-1). Specifically, this study is designed to examine the possibility of TLR4 as a candidate receptor interacted with *G. atrum* polysaccharide (PSG-1) and elucidate the role of reactive oxygen species (ROS) in PSG-1-induced tumor necrosis factor-α (TNF-α) production during macrophage activation. Flow cytometric and confocal laser-scanning microscopy analysis showed that fluorescence-labeled PSG-1 bind specifically to the macrophages. Moreover, PSG-1 stimulated TNF-α secretion of peritoneal macrophages from C3H/HeN mice, but not from C3H/HeJ mice. PSG-1-indcued TNF-α production was suppressed by anti-TLR4 mAb. Furthermore, ROS production was mediated by TLR4, and NADPH oxidase-derived ROS act as upstream of phosphoinositide 3-kinase(PI3K)/Akt/mitogen-activated protein kinases(MAPKs)/nuclear factor(NF)-κB signaling pathway in the regulation of PSG-1 stimulated TNF-α production. Taken together, we conclude that PSG-1 induces TNF-α secretion through TLR4/ROS/PI3K/Akt/MAPKs/NF-κB pathways during macrophage activation. Our findings provide a molecular basis for the potential of PSG-1 as a novel immunomodulatory agent.

1. Introduction

Ganoderma atrum has been used as Chinese traditional medicine and healthful mushroom for thousands of years. The polysaccharide is regarded as the major bioactive substances in *G. atrum* (Chen et al., 2007; Paterson, 2006). We recently isolated and purified a polysaccharide from *G. atrum*, named as PSG-1, with a purity of >99.8%, whose primary structural features and molecular weight were characterized (Chen et al., 2008; Zhang et al., 2012). The results have demonstrated that PSG-1 has potent antioxidation (Chen et al., 2008), antitumor (Li et al., 2011a,b; Zhang et al., 2013a,b) and cardiovascular protection (Li et al., 2010a, 2009) activities in our previous papers. Especially, it was found that PSG-1 possesses immunomodulatory effects on macrophages (Yu et al., 2013), and PI3K/Akt/MAPKs/NF-κB signaling pathway plays a key role in macrophage activation by PSG-1 (Yu et al., 2012). However, the membrane receptor and detail signaling pathway involved in the activation of macrophages in respond to PSG-1 have not yet been completely clarified.

Emerging studies have established the essential role of specific cellular receptors on macrophages in response to a wide variety of microbial pathogens with subsequent induction of intracellular signaling cascade (Ehlers, 2000; Medzhitov and Janeway, 2000; Netea et al., 2006). Toll like receptors are of a family that has been found to be crucial for innate immunity (Medzhitov, 2001; Trinchieri and Sher, 2007). To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively, with TLR1–TLR9 being conserved in both species. Mouse TLR10 is not functional because of a retrovirus insertion, and TLR11, TLR12 and TLR13 have been lost from the human genome (Akira et al., 2001). Binding of ligands to TLR leads to the formation of a complex between cytoplasmic region of TLR and the adaptor protein Myd88 and the IRAK.

Abbreviations: ATCC, American Type Culture Collection; CM-DCFH, carboxyl-DCFH; CR3, complement receptor 3; FBS, fetal bovine serum; HRP, horseradish peroxidase; IKK, IκB kinase; IRAK, interleukin-1 receptor associated kinase; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; NAC, N-acetylcysteine; NF-κB, nuclear factor-κB; PAK-1 PBD, PAK-1 p21-bindng domain agarose; PBS, phosphate buffered saline; PI3K, phosphoinositide 3-kinase; PRRs, pattern-recognition receptors; ROS, reactive oxygen species; TLRs, toll-like receptors; TLR4, toll like receptor 4; TNF-α, tumor necrosis factor α; TRAF6, tumor necrosis factor-associated factor 6.

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This is followed by the activation of TRAF6 and TRAF6 in turn activates IKK complex. The activation of IKK complex leads to the degradation of IκB and the activation of NF-κB, which regulates a wide spectrum of target genes (Karin and Ben-Neriah, 2000). TLRs are expressed by various cells of the immune system. Among them, TLR4 has been identified as an important membrane receptor of macrophages (Moresco et al., 2011), it mediates macrophage activation by transmitting a variety of extracellular signals. LPS is one of the best studied components that can interact with TLR4 (Anderson, 2000), however, the role of TLR4 in response to PSG-1 is unclear.

ROS has been traditionally regarded as toxic by-products of metabolism, which could cause damage to lipids, proteins, and DNA (Freeman and Crapo, 1982). However, more and more evidences show that ROS is not only injurious by-products of cellular metabolism but also involved in cell signaling and regulation (Finkel, 1998; Rhee, 1999). For example, numerous studies have implied that ROS is involved in MAPKs activation after cell stimulation with various agents (Iwaoka et al., 2006). Lots of cellular stimuli that induce ROS production is capable of simultaneously activating MAPKs pathways in various cell types (McCubrey et al., 2006; Rhee, 1999). The prevention of ROS accumulation by antioxidants inhibits MAPKs activation, indicating the involvement of ROS in activation of MAPKs pathways. Besides MAPKs, other signaling molecules, such as protein serine/threonine kinase, and transcriptional factors can also be activated by ROS (Thannickal and Fanburg, 2000). The PI3K, as well as its downstream serine/threonine kinase Akt, which have been implicated in a number of cellular responses, including cell migration, phagocytosis and apoptosis (Carpenter and Cantley, s1996), were reported to be activated by ROS (Ushio-Fukai et al., 1999). Moreover, NF-κB is well known as a critical transcription factor in the induction of a wide variety of genes, that are involved in regulation of immune and inflammatory responses (Hayden and Ghosh, 2004). A number of studies indicate that ROS may serve as common intracellular agents that contribute to the process of NF-κB activation in response to a diverse range of stimuli (Baeuerle and Henkel, 1994).

Many reports indicated that one of the important sources of ROS in macrophages is NADPH oxidase activation during phagocytosis (Bokoch, 1995). NADPH oxidase complex of the macrophages consists of four core protein subunits, which are cytochrome b558, along with the cytosolic components p67 phox, p47 phox, and p40 phox. Activation of the oxidase, however, requires the additional participation of Rac-1, member of the Ras superfamily of small GTP-binding proteins. During activation, Rac-1 binds GTP and migrates to the plasma membrane along with the cytosolic components to form the active oxidase complex (Bosco et al., 2009). However, little is known about the potential role of ROS in controlling PSG-1-mediated intracellular signaling pathways and the mechanism by which PSG-1 induces ROS generation in macrophages.

In this study, the possibility of TLR4 as a candidate receptor for PSG-1-mediated signaling was examined. Moreover, we investigated the role of ROS in PSG-1-mediated signal transduction involved in the regulation of TNF-α production, as well as the mechanism for PSG-1 induction ROS in macrophages.

2. Materials and methods

2.1. Mice

Female C3H/HeN and C3H/HeJ mice (6–8 weeks old) were purchased from the Shanghai Laboratory Animal Center, China. Mice were housed at nine per cage and maintained at a constant temperature (25 °C), on a 12/12 h reversed light/dark cycle. All animals used in this study were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health, Bethesda, MD (NIH Publication 85-23, 1996). All procedures were approved by the Animal Care Review Committee, Nanchang University, China.

2.2. Materials and reagents

Cell culture products were obtained from Life Technologies (Paisley, Scotland, UK). ELISA kits were from R&D Systems (Minneapolis, MN, USA). Carboxyl-DCFH (CM-DCFH) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Dextran (70 kDa), fluoresceinamine, diphenyleneiodonium (DPI) and N-acetylcysteine (NAC) were from Sigma (St. Louis, MO, USA). Antibodies against ERK1/2, phospho-ERK1/2 (p-ERK1/2), JNK1/2, phospho-JNK1/2(p-JNK1/2), p38, phospho-p38 (p-p38), Akt, phospho-Akt (p-Akt) and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Cell Signaling (Beverly, MA, USA). Anti-mouse TLR2, TLR4 and complement receptor (CR) 3 mAbs were purchased from eBiosciences (San Diego, CA, USA) and dialyzed extensively against PBS to remove NaN_3 before use in cell culture. pGL4.32 [*luc2P*/NF-κB-RE/Hygro] Vector, FuGENE® HD transfection reagent and Luciferase Assay System were obtained from Promega (Madison, WI, USA). The Rac-1 activation assay kit was purchased from Millipore Inc. (Bedford, MA, USA).

2.3. Extraction of polysaccharides from G. atrum

The polysaccharide from *G. atrum* (PSG-1) with a purity of >99.8% was isolated and purified as described previously (Chen et al., 2008). Briefly, the polysaccharide fractions were prepared from *G. atrum*, which were collected from Ganzhou, Jiangxi Province, China. All extracts were finally pooled, and the polysaccharide-enriched fractions were precipitated by the addition of 80% (v/v) ethanol. The polysaccharide fraction was further purified by gel filtration chromatography. Its primary structural features and molecular weight were characterized by infrared spectrometry, gas chromatography, size exclusion chromatography, methylation analysis and 1D/2D NMR spectroscopy (Zhang et al., 2012). Our previous study (Yu et al., 2013) showed that the quantity of endotoxin in PSG-1 was less than 0.015 EU/mg (negative), as measured by LAL assay, which demonstrated that PSG-1 is free of LPS contamination.

2.4. Cell culture

RAW264.7 cells, a macrophage-like cell line, were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in RPMI 1640 medium containing 10% heat inactivated FBS, 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.5. Isolation of mouse peritoneal macrophages

To collect peritoneal macrophages, each mouse received 5 mL ice-cold sterile PBS, the peritoneal cavity was washed carefully and 3 mL was retrieved using a Pasteur pipette. Peritoneal macrophages were aseptically collected and resuspended in RPMI-1640 containing 10% heat-inactivated FBS, 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.6. Preparation of fluorescein-labeled PSG-1 and dextran

Fluorescein-labeled PSG-1 and dextran were generated as described previously (Glabe et al., 1983). Briefly, 20 mg/mL PSG-1 or dextran solution was mixed with 200 μL 10 mg/mL CNBr in 1 mL water under magnetic stirring, and maintained at pH 11 for 15 min by the addition of 0.2 M NaOH. After dialysis against Na_2BO_7 buffer at pH 8.0 for 20 h, the CNBr-activated PSG-1 or dextran was mixed with 2 mg fluoresceinamine for 10 h in the dark at room temperature. Labeled polysaccharide was separated by gel filtration chromatography on a Sephadex G-25 column and the bright yellow fraction was collected. The amount of fluorescence-labeled polysaccharide was determined by phenol–sulfuric acid assay, and the content of fluoresceinamine was determined by measuring absorbance at 440 nm.

2.7. Flow cytometry

Cell suspensions were centrifuged, and the cell pellets (1×10^6/tube) were resuspended with 50 μL of f-PSG-1 or f-dextran (20 μg/mL) and incubated for 1 h at 4 °C. The cells were washed twice in PBS and resuspended in 500 μL of PBS for flow cytometric analysis on FACS Calibur (Becton–Dickinson, San Jose, CA, USA). To perform inhibition assays, the cells (1×10^6/tube) were treated with unlabeled PSG-1 at 160 μg/mL, anti-TLR4, TLR2 or CR3 mAb (20 μg/mL) for 1 h and then f-PSG-1 (20 μg/mL) for another hour at 4 °C. After washes, the cells were resuspended in 500 μL PBS for flow cytometric analysis.

2.8. Confocal laser microscopy

Living macrophages growing on glass cover slips were incubated with f-PSG-1 (20 μg/mL) for 1 h at 4 °C. After a brief wash with PBS, cells were observed through fluorescence confocal laser microscopy LSM 710 (Zeiss, Jena, Germany) with 450 nm excitation and 520 nm emission wavelengths.