Food and Chemical Toxicology 99 (2017) 199–208

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Ganoderma atrum polysaccharide ameliorates ROS generation and apoptosis in spleen and thymus of immunosuppressed mice

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ARTICLE INFO

Article history:
Received 19 March 2016
Received in revised form
9 October 2016
Accepted 28 November 2016
Available online 30 November 2016

Keywords:
Ganoderma atrum polysaccharide
Immune organ
Antioxidant enzymes
Apoptosis
Reactive oxygen specie

ABSTRACT

Ganoderma atrum polysaccharide (PSG-1) is a bioactive compound with antioxidant and immunomodulatory activities. The aim of this study was to determine the effect of PSG-1 on reactive oxygen species (ROS) generation and apoptosis in spleen and thymus of cyclophosphamide (CTX)-induced immunosuppressed mice. The results showed that PSG-1 protected mice against CTX-mediated immunosuppression, as evidenced by enhancing the ratios of thymus and spleen weights to body weight, promoting T cell and B cell survival, and increasing levels of TNF-α and IL-2. Apoptosis, ROS generation and lipid peroxidation in the immune organs of the immunosuppressed animals were ameliorated by PSG-1. The immune benefits of PSG-1 were associated with the enhancement of the activities of glutathione peroxidase, superoxide dismutase and catalase in the immune organs, implying that antioxidant activities of PSG-1 may play an important role in PSG-1-evoked immune protection. Taken together, these findings have demonstrated that PSG-1 may ameliorate CTX-induced immunosuppression through reducing apoptosis and oxidative damage in immunological system.

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1. Introduction

Ganoderma atrum, a traditional nutritional and medicinal mushroom, has recently received considerable attention due to its beneficial health effects (Xu et al., 2016). *Ganoderma atrum* polysaccharide (PSG-1) is the main component in *Ganoderma atrum* (Chen et al., 2008). The structure (Fig. 1) of PSG-1 was identified by methylation analysis and 1D/2D nuclear magnetic resonance (NMR) spectroscopy (Li et al., 2012a; Zhang et al., 2012). Previous studies have found that PSG-1 possessed prominent bioactivities such as anti-tumor, immunomodulatory, cardiovascular support, anti-diabetic and anti-aging activities (Li et al., 2011, 2012b, 2015; Zhu et al., 2016). The most attractive bioactivity of PSG-1 was reported to be its effects in immunity. Multiple mechanisms (Yu et al., 2015; Zhang et al., 2014, 2015) were involved in its immunomodulatory activities, suggesting that PSG-1 could stimulate the immunocytes and immune organs, and then activate the whole body immune system through TLR4-mediated NF-κB and MAPK signaling pathways. During the last decade, although a lot of researchers have investigated the immune responses to PSG-1 and made remarkable progress, the exact mechanism is still unclear.

Immune system is the major contributor to host defense against infection, as well as a healing process for repairing damaged tissue, which consists of immune organs, immunocytes and immune molecules. Immune organs, the main sites for immune response, execute protective responses to ensure elimination of detrimental stimuli (Blackburn and Kellems, 2005; Springer, 1990; Vitkina et al., 2016). As a byproduct of oxidative phosphorylation, reactive oxygen species (ROS) play a crucial role in maintaining immune response in immune organs. There is proof that oxidative stress, defined as a persistent imbalance between ROS generation and

Abbreviations: CAT, Catalase; CTX, Cyclophosphamide; GPx, Glutathione peroxidase; LPS, Lipopolysaccharide; MDA, Malondialdehyde; NMR, Nuclear magnetic resonance; PSG-1, *Ganoderma atrum* polysaccharide; ROS, Reactive oxygen species; SOD, Superoxide dismutase.

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http://dx.doi.org/10.1016/j.fct.2016.11.033
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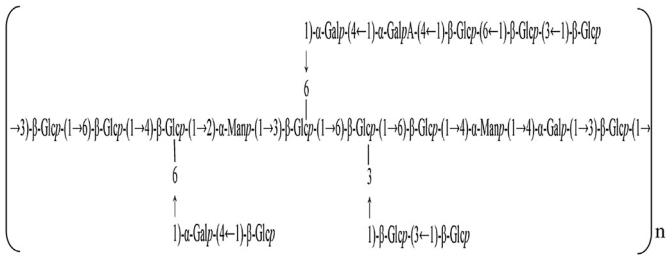

Fig. 1. The chemical structure of PSG-1. A water-soluble polysaccharide from *Ganoderma atrum* (named PSG-1) was purified and characterized in our laboratory using modern chemical analysis methods. The data showed that PSG-1 was composed of mannose, galactose and glucose. PSG-1 was comprised a backbone of 1,3-linked and 1,6-linked β-Glcp residues substituted at O-3 and O-6 position as the branch points. The residues of α-1,4-Galp, α-1,2-Manp, α-1,4-Manp were also found in the backbone. Side chains were terminated by β-Glcp, with the composition of α-1,4-Galp, α-1,4-GalpA, β-1,3-Glcp and β-1,6-Glcp.

antioxidant defense, results in tissue impairment. Briefly, the excessive ROS accumulation potentially induces damage to DNA, lipids and proteins resulting in cell damage by cellular structure alteration and biomolecule fragmentation. Therefore, Overproduction of ROS is critical for the pathogenesis of aberrant immunity (Gostner et al., 2015; Li et al., 2012b; Lessard et al., 2015).

Cyclophosphamide (CTX), an alkylating agent, is widely used to treat cancers. Additionally, CTX at a high dose (150 mg/kg body weight) was associated with increased cytotoxicity and immunosuppression, and thus used to mimic immunosuppression in mice (Chen et al., 2007). Recently, PSG-1 was found to inhibit immunodeficiency in immunosuppressed mice induced by CTX (Li et al., 2011). However, little is known about the effects of PSG-1 on ROS production and apoptosis in the immune organs. The spleen and thymus are two important immune organs. In this work, we investigated whether PSG-1 may attenuate ROS generation and apoptosis in the spleen and thymus of immunosuppressed mice.

2. Materials and methods

2.1. Experimental animals

Kunming mice (22.0 ± 2.0 g) were provided by Jiangxi College of Traditional Chinese Medicine, Nanchang University [License number: SCXK (Jiangxi) 2006-0001]. The mice were raised under the conditions of ambient temperature 20 ± 2 °C, humidity 50± 10%, 12 h/12 h light/dark cycle, and free feeding and water drinking. All animals were cared for following the guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

2.2. Reagents

PSG-1 (purity of >99.8%), isolated from *Ganoderma atrum* (Zhang et al., 2012) was dissolved in 0.9% normal saline (NS) and kept as a stock solution with a concentration of 10 mg/mL. Appropriate concentrations were diluted in NS before being used in animal treatments. DMEM and fetal bovine serum were purchased from GIBCO (USA). Lipopolysaccharide (LPS), concanavalin A (Con A) and 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolim bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Mouse TNF-α and IL-2 ELISA kits were from Shanghai Shen Xiong Technology Co. (Shanghai, China). Glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); DCFH-DA was from Molecular Probes Inc (USA). CTX was purchased from Jiangsu Hengrui Medicine Co. (Jiangsu, China). Annexin V-FITC apoptosis kit was from BD Company (USA). BCA protein assay kit was from Beyotime Biotechnology Research Institute (Jiangsu, China).

2.3. Experimental groups and treatments

The mice are randomly divided into six groups (n = 8) (Chen et al., 2007). Control group: intraperitoneal injection of an equal volume of NS once daily for 7 days. CTX group: the first day of intraperitoneal injection of CTX (150 mg/kg body weight), then an equal volume of NS was given once daily for 6 days. PSG-1 at high (PSG-1-100 + CTX), medium (PSG-1-50 + CTX) and low (PSG-1-25 + CTX) dose groups: intraperitoneal injection of PSG-1 (100 mg/kg body weight, 50 mg/kg body weight and 25 mg/kg body weight), once daily for 7 consecutive days, CTX (150 mg/kg body weight) was administered intraperitoneally 30 min before the first day of PSG-1 injection. Another PSG-1 high-dose group (PSG-1-100): intraperitoneal injection of PSG-1 (100 mg/kg body weight), once daily for 7 days, the first day of treatment by an equal volume of NS 30 min before PSG-1 was given intraperitoneally.

2.4. Determination of serum cytokines

Twenty four hours after the last treatment, the mice were injected intraperitoneally with 0.2 mL of 3.5% chloral hydrate. The eyes of the animals under anesthesia were removed to take blood samples. Blood samples were allowed to clot at room temperature for 1–2 h; the serum was then separated by centrifugation at 2,000 × g for 5 min and stored at −80 °C. In this work, concentrations of TNF-α and IL-2 in serum were measured using avidin biotin complex-ELISA with anti-mouse TNF-α and IL-2 polyclonal antibodies. Briefly, serum and standards were placed in the microtiter plates coated with a polyclonal antibody to mouse TNF-α or IL-2. Biotin-conjugate (anti-mouse TNF-α or IL-2 polyclonal antibody) and streptavidin-HRP were sequentially added into the microtiter plates. After additional enzyme substrate incubation, the reaction was terminated by stop solution. The levels of IL-2 and TNF-α were determined by measurements of the absorbance values at 492 nm by spectrophotometry, followed by construction of a standard curve.

2.5. Determination of the ratios of immune organ weights to body weight in immune suppressive mice

After removal of the eye and getting the blood, the mice were killed by cervical vertebra dislocation. Then the thymus and spleen were immediately isolated and weighed. The ratios of thymus or spleen weight (mg) to body weight (g) indicated the conditions of immune organs.

2.6. Histopathological examination of spleen and thymus

Thymus and spleen tissues were removed from the mice and used for histopathological examination. In short, the fresh tissues were cut into sections of 4–5 mm thickness. Specimens were immediately fixed in 10% formalin neutral fixative for 30 min, overnight at 4 °C with 0.1 M PBS, and then dehydrated with ethanol of gradient concentration: 75% ethanol for 90 min → 95% ethanol for 90 min → 100% ethanol 3 times each for 90 min → 100% xylene 2 times each for 60 min. Specimens were successively embedded in paraffin at 62 °C. The tissue blocks were sectioned at 6 μm thickness and attached to glass substrates. Finally, thymus and spleen tissues were stained with hematoxylin and eosin, and the histology of thymus and spleen was observed under a light microscope.

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