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Ganoderma atrum polysaccharide improves aortic relaxation in diabetic rats via PI3K/Akt pathway

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ABSTRACT

A newly identified polysaccharide (PSG-1) has been purified from *Ganoderma atrum*. The study was to investigate the protective effect of PSG-1 on diabetes-induced endothelial dysfunction in rat aorta. Rats were fed a high fat diet for 8 weeks and then injected with a low dose of streptozotocin to induce type 2 diabetes. The diabetic rats were orally treated with PSG-1 for 4 weeks. It was found that administration of PSG-1 significantly reduced levels of fasting blood glucose, improved endothelium-dependent aortic relaxation, increased levels of phosphoinositide 3-kinase (PI3K), phospho-Akt (p-Akt), endothelial nitric oxide synthase (eNOS) and nitric oxide in the aorta from diabetic rats, compared to un-treated diabetics. These results suggested that the protective effects of PSG-1 against endothelial dysfunction may be related to activation of the PI3K/Akt/eNOS pathway.

1. Introduction

Ganoderma, one of the most popular medicinal fungi, has been used as a folk remedy to promote health and longevity in oriental countries for centuries. The polysaccharides isolated from *Ganoderma* have been reported to exhibit diverse biological properties (Nie, Zhang, Li, & Xie, 2013). Recently, a new bioactive polysaccharide (PSG-1) has been isolated from *Ganoderma atrum* in our laboratory. PSG-1 has been found to exhibit anti-oxidant (Chen, Xie, Nie, Li, & Wang, 2008), anti-tumor (Li et al., 2011), immunomodulatory (Yu et al., 2012), hypoglycemic and hypolipidemic effects (Zhu et al., 2013). PSG-1 was also reported to protect cardiomyocytes from injury induced by anoxia/reoxygenation (Li, Nie, Yan, Zhu, & Xie, 2009).

Cardiovascular complications are the major causes of mortality and morbidity in patients with diabetes (He, Naruse, & King, 2005). While, endothelial dysfunction which is characterized by reduced activity of endothelial nitric oxide synthase (eNOS) and bioavailability of nitric oxide (NO) (Gupta, Toruner, Chung, & Groszmann, 2003; Kim et al., 2002), plays a key role in the pathogenesis of diabetic cardiovascular complications (Kobayashi, Oishi, Hayashi, Matsumoto, & Kamata, 2006). Previous studies have indicated that the endothelium-dependent relaxation to acetylcholine (Ach) was weaker in the aortic rings from streptozotocin (STZ)-induced diabetic rats (Topal et al., 2012).

Apoptosis of endothelial cells is reported to be a primary event in the process of diabetes-associated macrovascular and microvascular diseases (Xu, Zhong, Zeng, & Ge, 2008). The apoptosis of endothelial cells is related to hyperglycemia, hyperlipidemia and insulin resistance in type 2 diabetes (Adler et al., 2002). Hyperglycemia accelerates apoptosis of endothelial cells by multiple proteins inside the cells, including the pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins of Bcl-2 family (Lian, Ren, & Gao, 2011). To our knowledge, there have been few reports about the protective effects of polysaccharides on diabetes-induced endothelial dysfunction. Therefore, the aim of this study was to investigate the effect of PSG-1 on endothelium-dependent relaxation of the aorta from high fat diet and STZ-induced diabetic rats, an animal model of type 2 diabetes in humans.

2. Materials and methods

2.1. Chemicals and reagents

STZ, Ach and norepinephrine (NE) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Cyclosporine A (CSA, purity 99.3%) was obtained from Taizhou Creating Chemical Co. *N*-Acetyl-L-cysteine (NAC) was purchased from Beijing Solarbio Science and Technology Co. One touch glucometer (Accu-chek Performa) was

Abbreviations: CSA, cyclosporine A; eNOS, endothelial nitric oxide synthase; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; PSG-1, polysaccharide from *Ganoderma atrum*; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

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obtained from Roche Diagnostics (Mannheim, Germany). All other chemicals and solvents were of analytical grade.

PSG-1 was prepared from the fruiting bodies of *G. atrum* by boiling water according to the method of Chen et al. (2008). The chemical components were analyzed by the phenol-sulphuric acid method. PSG-1 was found to compose of glucose, mannose, galactose and galacturonic acid in a molar ratio of 4.91:1:1.28:0.71, with an average molecular weight of approximately 1013 kDa, and the preliminary structure of PSG-1 was characterized by using methylation analysis and 1D/2D nuclear magnetic resonance (NMR) spectroscopy (Zhang et al., 2012).

2.2. Animals and treatments

Ninety male Wistar rats (180–200 g) were obtained from Shanghai Slaccas Laboratory Animal Company (Certificate Number: SCXK (hu) 2007-0005, Shanghai, China). Before starting the experiments, all the animals were housed at an ambient temperature of 25 ± 2 °C, 12/12 h of light-dark cycle with ad libitum food and water for 1 week. Then, 10 rats were fed a standard normal chow diet consisting of 12% fat, 60% carbohydrate and 28% protein (as a percentage of total kcal), the others were fed with high fat diet consisting of 40% fat, 42% carbohydrate and 18% protein. After 8 weeks of dietary manipulation, rats fed on high fat diet were fasted for 12 h and injected with a single low dose of STZ [30 mg/kg body weight (BW), in 154 mmol/L isotonic saline, pH 7.2] into the tail vein to induce type 2 diabetes, while those fed on standard normal chow diet received an equivalent volume of saline. Hyperglycemia was confirmed by the levels of fasting blood glucose (FBG) higher than 11.1 mmol/L at Day 4 and Day 7 after STZ injection.

Fifty type 2 diabetic rats were randomly divided into the following groups: un-treated diabetic, diabetic treated with PSG-1 (200 and 400 mg/kg BW) (Zhu et al., 2013), diabetic treated with CSA (8 mg/kg BW) (Wakita, Tomimoto, Akiguchi, & Kimura, 1995) and diabetic treated with NAC (100 mg/kg BW) (Karageorgos et al., 2006). Ten rats fed on standard normal chow diet served as a non-diabetic control group, received the same volume of vehicle solution. All treatments were conducted once daily over a 4-week period. FBG levels were measured at the end of experimental period. 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 U.S. National Institutes of Health (NIH Publication 85-23, 1996). All procedures were approved by the Animal Ethnics Committee, Nanchang University.

2.3. Preparation of aortic ring

All animals were anesthetized with 10% chloralic hydras intraperitoneally, then sacrificed by exsanguination after 4 weeks of treatment. The aorta was removed, cleaned of excess fat and connective tissue with extra care to avoid any damage to the endothelium. Aortic rings of approximately 4 mm in length were prepared and suspended in 10-mL organ bath filled with Krebs–Henseleit solution (KHS) [composition (mmol/L): NaCl 118.0, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, and glucose, 11.0, oxygenated with 95% O_2 + 5% CO_2 (pH 7.4, 37 °C)] for measurement of relaxation of the aorta. The remaining portion of the aorta was fixed in 10% neutral buffered formalin and frozen in liquid nitrogen, respectively.

2.4. Endothelium-dependent relaxation of aorta

To determine the vasoactive effects of PSG-1 on endothelium-dependent relaxation, the responses to Ach were examined in aortic rings from diabetic rats. Aortic rings were mounted on stainless steel hooks and suspended in 10-mL organ bath filled with KHS (95% O_2 + 5% CO_2, pH 7.4, 37 °C), then stretched to a 1.5-g initial tension and equilibrated for 60 min. The Krebs buffer was replaced every 15 min, tension being readjusted each time. At the end of the equilibration period, the functional integrity of the endothelium was verified as described by Nguelefack et al. (2009).

After the verification, the aortic rings were washed with KHS to its initial tension, pre-contracted with NE (2×10^{-7} mol/L) and then relaxed with Ach (10^{-8} mol/L to 10^{-5} mol/L). After each addition, the concentration of Ach was maintained for 10 min to allow the tension to develop. The tension was first amplified by a high input impedance amplifier (JZ100, Chengdu Instrument Factory, Chengdu, China), and then processed by multiple channel physiological signal collecting and processing system (RM6240BD, Chengdu Instrument Factory, Chengdu, China). Relaxation (%) = [(Tension Ach − Basal Tension)/(Tension at steady pre-contracted state × Basal Tension)] × 100%.

2.5. Determination of eNOS and NO levels

The eNOS protein concentration in the aorta was determined with an ELISA kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the manufacturer's instructions. NO levels were measured as total nitrite with the spectrophotometric Griess reaction (Cortas & Wakid, 1990). Total protein concentration in tissue homogenates of aorta was determined by Bradford method using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

2.6. Histopathological study

The formalin-fixed aortic tissues were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax. The specimens were then cut into 5 μm-thick sections using a rotary microtome. Sections were stained with hematoxylin and eosin dye, and photomicrographs were obtained under light microscope (Ti series inverted microscope, Nikon, Japan).

2.7. Analysis of apoptosis

The apoptosis of endothelium cells in the aorta was examined with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (Gavrieli, Sherman, & Ben-Sasson, 1992). Briefly, 5 μm-thick sections after deparaffinization were treated with 3% H_2O_2 to quench endogenous peroxidase, permeated with 1% Triton X-100 for 10 min, followed by treatment of 10 mg/ml proteinase K at room temperature for 30 min. Sections were then incubated with terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes a template-independent addition of fluorescein-dUTP (FITC-12-dUTP) to the free 3′-OH ends resulting from DNA degradation. The sections were observed with a Nikon Ti series inverted microscope equipped with a standard fluorescent filter to view the fluorescence produced by FITC-12-dUTP.

2.8. Immunohistochemical analysis

The aortic tissue samples collected for histology were subjected to immunohistochemical analysis to determine the expression of Bax and Bcl-2 proteins according to our procedures (Zhu et al., 2013).

The positive signals of these proteins were observed with a Nikon Ti series inverted microscope equipped with bright-field high-quality objectives. Images were acquired by a digital camera (Nikon Digital Sight DS-Fi1c) and image acquisition software.