ELSEVIER

Cell Biology International 32 (2008) 304–311

Cell Biology International

www.elsevier.com/locate/cellbi

Aqueous extract of the Chinese medicine, Danggui-Shaoyao-San, inhibits apoptosis in hydrogen peroxide-induced PC12 cells by preventing cytochrome *c* release and inactivating of caspase cascade

Yun-fei Qian, Hua Wang, Wen-bing Yao, Xiang-dong Gao*

School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, PR China

Received 2 March 2007; revised 13 April 2007; accepted 4 October 2007

Abstract

Danggui-Shaoyao-San (DSS), a traditional Chinese medicine used for centuries for the enhancement of women's health, has been shown to display therapeutic efficacy on senile dementia. In the present study, using a rat pheochromocytoma (PC12) cell line, the effect of DSS on hydrogen peroxide (H_2O_2) induced apoptosis was studied. The apoptosis in H_2O_2-induced PC12 cells was accompanied by downregulation of Bcl-2, upregulation of Bax, the release of mitochondrial cytochrome *c* into cytosol, and sequential activation of caspase-9 and -3. DSS was able to suppress all these changes and eventually protected against H_2O_2-induced apoptosis. Taken together, these results suggest that treatment of PC12 cells with DSS can block H_2O_2-induced apoptosis by the regulation of Bcl-2 family members, as well as suppression of cytochrome *c* release and caspase cascade activation.

Keywords: Danggui-Shaoyao-San; H_2O_2; Apoptosis; PC12; Caspase; Cytochrome *c*

1. Introduction

Alzheimer's disease (AD) is a multifaceted neurodegenerative disorder characterized by the progressive deterioration of cognition and memory in association with widespread neuronal loss and deposit of senile plaques (SP). To date, the cause and the mechanism by which neurons die in AD still remain unclear, yet several lines of evidence support the involvement of oxidative stress (Markesbery, 1997; Behl, 1999). Oxidative damage, mediated by reactive oxygen species (ROS) which can be generated following cell lysis, oxidative burst, or the presence of an excess of free transition metals, has been hypothesized to play a pivotal role during the neurodegeneration of AD victims. On the other hand, studies on postmortem tissues provide direct morphological and biochemical evidence that some neurons in the brain of AD patients degenerate via an apoptotic mechanism including the presence of DNA damage, nuclear apoptotic bodies, and other markers of apoptosis (Levine, 1997). These results suggest a connection between oxidative stress and apoptosis, and therapeutic strategies aimed at preventing and delaying ROS-induced apoptosis might be a reasonable choice for the treatment of the disease.

Danggui-Shaoyao-san (DSS), a famous Chinese complex prescription, first recorded in ''JinKuiYaoLue'', consists of six Chinese herbs. Its formula is shown in Table 1. In recent years, DSS has been proved to be effective in treating climacteric period syndrome, chronic appendicitis, Parkinson's disease and Meniere's syndrome (Shang and Qiao, 2006), especially in senile dementia (Zhao et al., 2000). Previous studies showed that DSS shortened the latency of reserpine-treated mice in the water maze test (Kou et al., 2002), and inhibited the deposition of the amyloid granules in senescence-accelerated mouse brain (Li et al., 2006). In vitro, DSS also modulates cellular immune functions and attenuates

* Corresponding author. Tel.: +86 25 8327 1298; fax: +86 25 8327 1249.
E-mail addresses: suxierqyf@163.com (Y.-f. Qian), xiangdong_gao@hotmail.com (X.-d. Gao).

1065-6995/$ - see front matter © 2007 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2007.10.004

Table 1
Recipe of Danggui-Shaoyao-San (DSS) formulation

	Recipe of DSS formulation	Family	Part used	Components ratio	Harvesting places
1	*Angelica sinensis* (Oliv.) Diels.	Apiaceae	Root	3	Gansu province
2	*Paenonia lactiflora* Pall.	Paeoniaceae	Root	16	Zhejiang province
3	*Poria cocos* (Schw.) Wolf.	Polyporaceae	Fungus nucleus	4	Anhui province
4	*Astractylodes macrocephala* Koidz.	Atractylodes	Root and rhizome	4	Hubei province
5	*Ligusticum chuanxiong* Hort.	Apiaceae	Rhizome	8	Sichuang province
6	*Alisma orientale* (Sam.) Juzep.	Alismataceae	Rhizome	8	Jiangxi province

the damage caused by ischemia/reperfusion, glutamate and hydrogen peroxide in hippocampus slices of guinea pigs (Kou et al., 2003) and protects PC12 cells from damage by amyloid-$\beta_{1\text{-}42}$ (Lin et al., 2005). These results provide a pharmacological basis for an AD preventative function of DSS. The aim of the present study was to examine the protective effect of DSS on H_2O_2-induced PC12 cell damage and primarily to investigate its mechanism of anti-apoptosis.

2. Materials and methods

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT), the fluorescent DNA-binding dye Hoechst 33,258, and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louis, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Gibco Life Technologies (NY, USA). The antibody to cytochrome *c* was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DNA extraction kit and caspase-9, -8 and -3 Activity Kits were acquired from Beyotime Institute of Biotechnology (Jiangsu Province, China). All other chemicals and reagents were of analytical grade.

2.2. Plant materials

Plant materials were purchased from Nanjing Medicinal Materials Company (Jiangsu Province, China) and authenticated by Dr. Minjian Qin (College of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing, China). The vouchers are conserved at the Herbarium of College of Traditional Chinese Medicine, China Pharmaceutical University.

2.3. Extract preparation

Aqueous extract of DSS was prepared by the procedure of Tang et al. (2000). In brief, six medicinal materials were mixed in proportion and macerated for 1 h with 8× (v/w) distilled water, and decocted for 1 h, after which the filtrate was collected and the residue was decocted again for 1 h with 6×(v/w) of distilled water. The filtrates were mixed, condensed and dried by vacuum-drier at 60 °C. The yield of dried powder was 27.9% according to the original herbs. The sample was stored at 4 °C.

2.4. Determination of total phenolic compounds and sugars content in extract

Total phenolic compound contents were determined by the Folin–Ciocalteau method (Ordonez et al., 2006). 0.5 ml DSS (5 mg/ml) was mixed with 2.5 ml of 0.2 N Folin–Ciocalteau reagent for 5 min and 2 ml 75 g/L Na_2CO_3 were added. The absorbance of reaction was measured at 760 nm after 2 h incubation at room temperature. Results were expressed as gallic acid equivalents. Total sugars were determined by the phenol–sulfuric acid assay using glucose standard (Dubois et al., 1956).

2.5. Cell culture and treatment

PC12 cell line was obtained from Shanghai Institute of Cell Biology and maintained in DMEM supplemented with heat-inactivated 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells reached sub-confluence, they were treated with 0.25% trypsin in 0.02% EDTA solution, after which 1×10^5 cells/ml were seeded onto 96-well culture plate. Experiments were carried out 48 h after cells were seeded. H_2O_2 was freshly prepared from 30% stock solution prior to each experiment and added to the cells for the indicated times. To study the protective effect of DSS, different concentration of DSS was added simultaneously to the medium.

2.6. Cell viability assay

Cell survival was evaluated by MTT reduction. For our purpose, when the cells reached 80% confluence, the media were changed to those containing varying concentrations of DSS (150, 15, 1.5 μg/ml) and 0.5 mM H_2O_2. After incubation for up to 12 h, MTT solution in phosphate-buffered saline (PBS) was added with a final concentration of 0.5 mg/ml. The plates were incubated at 37 °C for an additional 4 h. Finally, the medium with MTT was removed and 200 μl dimethyl sulfoxide (DMSO) was added to each well. The amount of MTT formazan was qualified by determining the absorbance with Multiskan Spectrum (Thermo) at 570 nm, with 630 nm as a reference.

2.7. Morphological assay

PC12 cells were fixed for 10 min with 4% paraformaldehyde in PBS, and stained for 10 min with 10 μg/ml of Hoechst