Neuroscience Research 64 (2009) 306–310

Contents lists available at ScienceDirect

Neuroscience Research

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

Neuroprotective effects of ginsenoside Rd against oxygen-glucose deprivation in cultured hippocampal neurons

Ruidong Ye [a,1], Nanlin Li [b,1], Junliang Han [a], Xiangwei Kong [c], Rong Cao [d], Zhiren Rao [d], Gang Zhao [a,*]

[a] Department of Neurology, Xijing Hospital, Fourth Military Medical University, No. 15, Changle West Road, Xi'an 710032, China
[b] Department of Vascular and Endocrine Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China
[c] College of Stomatology, Fourth Military Medical University, Xi'an 710032, China
[d] PLA Institute of Neuroscience, Fourth Military Medical University, Xi'an 710032, China

ARTICLE INFO

Article history:
Received 31 December 2008
Received in revised form 26 March 2009
Accepted 31 March 2009
Available online 10 April 2009

Keywords:
Ginsenoside Rd
Neuroprotection
Oxygen-glucose deprivation

ABSTRACT

We previously found that ginsenoside Rd (GSRd), one of the main active ingredients in *Panax Ginseng*, attenuates H_2O_2-induced oxidative injury in PC12 cells. Mounting evidence suggests that the oxidative stress is crucially involved in the pathophysiologic process of ischemia. In the present study, we examined the protective role of GSRd to attenuate ischemic neuronal injury in vitro. Cultured hippocampal neurons were exposed to oxygen-glucose deprivation (OGD) for 2 h followed by a 24-h reoxygenation. GSRd exhibited remarkable neuroprotection when presented during OGD and reoxygenation, which may be ascribed to its antioxidative properties by reducing the intracellular reactive oxygen species and malondialdehyde production; increasing glutathione content; and enhancing the antioxidant enzymatic activities of catalase, superoxide dismutase and glutathione peroxidase. Additionally, GSRd could stabilize the mitochondrial membrane potential and attenuate apoptotic death of hippocampal neurons after OGD exposure. These findings suggested that GSRd may be a potential neuroprotective agent for cerebral ischemic injury and should encourage further in vivo studies on stroke to explore the potential neuroprotective efficacy of GSRd.

1. Introduction

Ginseng, the root of *Panax Ginseng* C.A. Meyer (Araliaceae), is a traditional Chinese herbal medicine widely used in the Far East. Extensive studies proved that the molecular components responsible for the pharmacological effects of ginseng are ginseng saponins, namely ginsenosides. Currently, over 30 different ginsenosides have been identified and isolated from ginseng. Dammar-24(25)-ene-3β,12β,20(*S*)-triol-(20-*O*-β-D-glucopyranosyl)-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (ginsenoside Rd, GSRd; Fig. 1) is one of the main active components of ginsenosides. There is evidence indicating that GSRd exerts antioxidant effects in kidney injury models and in senescence-accelerated mice (Yokozawa et al., 1998, 2004; Yokozawa and Owada, 1999). In the central nervous system, GSRd was reported to be effective in decreasing reactive oxygen species (ROS) formation in cultured astrocytes (Lopez et al., 2007). Consistently, we demonstrated that GSRd can protect PC12 cells from H_2O_2-induced oxidative stress (Ye et al., 2008). As the oxidative stress has been suggested to be crucially involved in the pathophysiologic process of ischemia (Doyle et al., 2008), we postulated that GSRd probably possess an ability to protect neurons from ischemic damage. Therefore, we examined the protective effects of GSRd against neuronal insult induced by oxygen-glucose deprivation (OGD) in hippocampal neuron cultures.

2. Materials and methods

2.1. Materials

GSRd with a purity of 98% was obtained from Tai-He Biopharmaceutical Co. Ltd. (Guangzhou, China). GSRd stock solutions were prepared in saline containing 10% 1,3-propanediol (v/v). Hoechst 33342, rhodamine 123 (Rh 123), and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma–Aldrich Inc. (St. Louis, Mo, USA). The commercial kit for the detection of lactate dehydrogenase (LDH) was procured from Promega (Madison, WI, USA). Other commercial kits for the detection of malondialdehyde (MDA), glutathione (GSH), oxidized glutathione (GSSG), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other

* Corresponding author. Tel.: +86 29 84775361; fax: +86 29 83251302.
E-mail address: zhaogang@fmmu.edu.cn (G. Zhao).
[1] These authors contributed equally.

0168-0102/$ – see front matter © 2009 Published by Elsevier Ireland Ltd and the Japan Neuroscience Society.
doi:10.1016/j.neures.2009.03.016

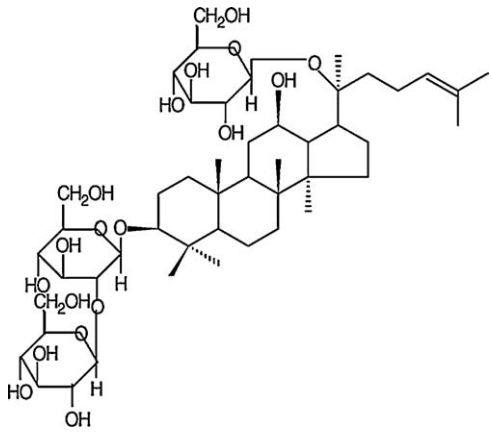

Fig. 1. The chemical structure of GSRd. The molecular formula for GSRd is $C_{48}H_{82}O_{18}{\cdot}3H_2O$ and the molecular weight is 1001.

reagents were from commercial suppliers and of standard biochemical quality.

2.2. Primary culture of hippocampal neurons

Hippocampi were isolated from embryonic day 18 Sprague–Dawley rat embryos. Animal procedures were in accordance with NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All efforts were made to minimize animal number and their suffering. After trituration and trypsinization, the single-cell suspension was seeded in 6-well or 96-well plates coated with poly-L-lysine (50 μg/ml) at the density of $(2–2.5) \times 10^5\ cm^{-2}$. Cells were maintained in Neurobasal medium supplemented with 2% B-27, 0.5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. OGD

OGD was carried out after 7 days in vitro (DIV) cultures. Briefly, the medium was replaced with pre-warmed Dulbecco's-modified eagle medium (DMEM) without glucose. The cell cultures were then transferred into an anaerobic chamber equilibrated with 95% N_2 and 5% CO_2. The chamber was kept in a 37-°C incubator. Sham OGD cultures were maintained in a normal oxygenated DMEM containing 25 mM glucose. After 2 h, cultures were placed back to the normoxic incubator with normal culture medium.

For concurrent treatment, GSRd was present in the culture medium during OGD and reoxygenation; for control, only vehicle (saline containing 10% 1,3-propanediol) was added to the culture medium.

2.4. MTT and LDH assay

At 24 h post-OGD, cell survival was quantitatively assessed by MTT assay as described before (Schabitz et al., 2003).

The cell damage was determined based on the release of LDH into the incubation medium. The assay was in accordance with the manufacturer's instructions. LDH leakage was expressed as the percentage of the total cell LDH activity. The total LDH amount corresponding to complete cell death was measured in sister cultures treated with 0.1% Triton X-100 for 30 min.

2.5. Hoechst 33342 staining

To detect the characteristic features of apoptotic nuclei, the cultured hippocampal neurons were stained with 5 μM Hoechst 33342 dye for 10 min, followed by observation under a DMR fluorescence microscope (Leica Microsystems, Germany). The hippocampal neurons with fragmented, condensed DNA or normal DNA were counted, respectively. The ratio of apoptotic neurons to total neurons was calculated (Chen et al., 2008; Eleuteri et al., 2008).

2.6. Measurement of ROS

Formation of ROS was determined by use of fluorescent probe DCFH-DA. Cell-permeant nonfluorescent DCFH-DA has been shown to be oxidized to the high fluorescent 2,7-dichlorofluorescin in the presence of ROS. At the end of the experiments, the medium was removed and about 1×10^6 cells were harvested by trypsinization. After washing twice with phosphate-buffered saline (PBS), the cells were incubated with DCFH-DA (10 μM) for 30 min at 37 °C in the dark. The cells were harvested and suspended in PBS. The fluorescence intensity was measured by flow cytometry (Coulter, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.7. Measurement of cellular MDA, GSH, GSSG levels and antioxidant enzyme activities

Cellular MDA, GSH, GSSG levels and antioxidant enzyme activities were determined by spectrophotometry using commercial kits. The assay was in accordance with the manufacturer's instructions.

2.8. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was evaluated by use of the fluorescent dye Rh 123 (Zhao et al., 2007a,b). Rh 123 accumulates in normal mitochondria due to its high negative charge, and the reduction of MMP will lead to the release of Rh 123 and reduction of its fluorescence intensity. At the end of the experiments, the medium was removed, and about 1×10^6 cells were harvested by trypsinization. After washing twice with PBS 0.01 M, the cells were incubated with Rh 123 (5 μM) for 30 min at 37 °C in the dark. The cells were harvested and suspended in PBS. The MMP was measured based on the fluorescence intensity of 2×10^4 cells on the flow cytometer.

2.9. Statistical analysis

Data were expressed as mean ± S.D. Differences were determined by one-way analysis of variance (ANOVA) and subsequently the least significant difference (LSD) post hoc tests. The software package SPSS 12.0 was used for calculations. Values were considered to be significant when $P < 0.05$.

3. Results

3.1. GSRd ameliorated OGD-induced cytotoxicity

First, we compared the neuronal injury induced by OGD (1.5, 2, and 3 h) with that induced by removal of glucose alone (2 h) by using MTT and LDH assay. The cell survival in the control group was standardized to 100%. There was no significant difference between the control and sham OGD group. OGD exposure of 1.5–3 h yielded graded neuronal damage. By contrast, glucose deprivation for 2 h in the presence of atmospheric oxygen caused less cell damage (Fig. 2A).

Second, it was demonstrated that incubation with GSRd alone does not induce cytotoxicity in hippocampal neurons (Fig. 2B and C). Cells were incubated with GSRd at different concentrations