Neuroprotective effects of *ganoderma lucidum* polysaccharides against oxidative stress-induced neuronal apoptosis

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Graphical Abstract

Mechanisms underlying the protective effect of *ganoderma lucidum* polysaccharides (GLPS) on cerebellar granule cells against apoptosis

**Abstract**

*Ganoderma lucidum* polysaccharides have protective effects against apoptosis in neurons exposed to ischemia/reperfusion injury, but the mechanisms are unclear. The goal of this study was to investigate the underlying mechanisms of the effects of *ganoderma lucidum* polysaccharides against oxidative stress-induced neuronal apoptosis. Hydrogen peroxide (H₂O₂) was used to induce apoptosis in cultured cerebellar granule cells. In these cells, *ganoderma lucidum* polysaccharides remarkably suppressed H₂O₂-induced apoptosis, decreased expression of caspase-3, Bax and Bim and increased that of Bcl-2. These findings suggested that *ganoderma lucidum* polysaccharides regulate expression of apoptosis-associated proteins, inhibit oxidative stress-induced neuronal apoptosis and, therefore, have significant neuroprotective effects.

**Key Words:** nerve regeneration; brain injury; H₂O₂; cerebellar granule cells; Bim; Bax; Bcl-2; cytochrome C; caspase-3; neural regeneration

**Introduction**

Oxidative stress-induced brain damage has been implicated in many neurodegenerative disorders, such as Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, Huntington’s disease and stroke (Dawson and Dawson, 2003; Veurink et al., 2003; Malkus et al., 2009). Accumulating evidence has suggested that oxidative stress associated with excessive production of reactive oxygen species profound affects neurodegenerative pathogenesis (de Vries et al., 2008; Zolezzi et al., 2013; Newland et al., 2016). Reactive oxygen species can cause lipid peroxidation, protein denaturation and DNA/RNA damage (Ye et al., 2009; Collado et al., 2012; Zou et al., 2015).
Reactive oxygen species induce several signal transduction pathways, including intrinsic and extrinsic caspase activation, which may lead to excessive cell apoptosis and expression of inflammatory genes (Chan, 2001; Allen and Bayraktutan, 2009). Oxidative stress triggers apoptosis through activation of many signaling molecules, including kinases and proteases (Tan et al., 1998; Andersen, 2004; Kaul et al., 2005). Hydrogen peroxide (H$_2$O$_2$) is used as a stressor to induce oxidative stress in experimental models (Brown et al., 2013; Sies, 2014) and to stimulate apoptotic and necrotic pathways (Clement et al., 1998).

Pharmaceutical compounds extracted from mushrooms showed benefits for a variety of conditions such as cancers, immunologic disorders and neurodegenerative diseases (Wang et al., 1997; Wasser and Weis, 1999; Cheung et al., 2000). Ganoderma lucidum (G. lucidum) belongs to the polyporaceae family of Basidiomycota, a type of mushroom widely used as a traditional medicine for thousands of years, especially in Asia (Ji et al., 2007). A variety of bioactive chemicals, such as polysaccharides, triterpenoids and proteins, can be extracted from the fruiting bodies, cultured mycelia and spores of G. lucidum (Mizushina et al., 1999). Clinical trials and other experimental studies indicated that the active compounds isolated from its fruiting body, known as "Lingzhi," participate in a variety of biological processes, showing anti-inflammatory, antioxidant, anti-tumor and immunomodulatory activities (Lakshmi et al., 2003; Lin and Zhang, 2004; Zhao et al., 2012; Pan et al., 2013; Ferreira et al., 2015). G. lucidum polysaccharides (GLPS) were shown to be neuroprotective, increasing viability in cerebral cortical neurons exposed to ischemia/reperfusion and in models for traumatic spinal cord injury (Zhao et al., 2004; Gokce et al., 2015). This evidence indicated that GLPS is a potentially promising drug candidate. However, the roles of GLPS in modulating oxidative stress-induced neuronal apoptosis have been poorly understood. The aim of our study was to investigate whether GLPS would protect cultured cerebellar granule neurons from apoptosis induced by H$_2$O$_2$.

Materials and Methods

Cell culture

Rat cerebellar granule cells (CGCs) were prepared from 7 or 8-day-old Sprague-Dawley rat pups, as previously described (D'Mello et al., 1993). All experimental procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals of the Animal Research Ethics Committee of Peking University Health Science Center (China) and
Control (no H$_2$O$_2$) 12 h  0.5%  2%  5%  GLPS (no H$_2$O$_2$)  

Figure 3 GLPS administration suppressed H$_2$O$_2$-induced apoptosis of cerebellar granule cells (light microscopy).

(A) Neurons were pretreated with H$_2$O$_2$ for 4 h. Control vehicle or GLPS at different concentrations (0.5%, 2% and 5% (w/v)) was added, together with H$_2$O$_2$, for an additional 8 hours. Representative images are shown. Scale bar: 10 μm. (B, D) Quantification of apoptotic rates. (C) Neurons were pretreated with H$_2$O$_2$ for 4 h, then cells were washed with culture medium and incubated with 5% (w/v) GLPS or control vehicle, without H$_2$O$_2$, for the indicated times. Representative images are shown. Scale bar: 10 μm. Data are expressed as the mean ± SEM (n = 3, analysis of variance and Student-Newman-Keuls post hoc test). *P < 0.05, vs. control. GLPS: Ganoderma lucidum polysaccharides; h: hours.

under the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number and suffering of the animals used in the experiments.

Briefly, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of trypsin (Life Technologies, Carlsbad, CA, USA) and DNase (Life Technologies) and then seeded at a density of 1.5 × 10$^6$ cells/mL in basal modified Eagle’s media (Life Technologies) containing 10% fetal bovine serum (Life Technologies) and potassium (Sigma-Aldrich, St. Louis, MO, USA) at concentrations causing membrane depolarization (25 mM KCl). The GLPS that was administered (0.5%, 2% or 5% (w/v); at 4, 8 or 12 hours) contained 56.9% carbohydrate and 32.45% protein and was from Lvgu Biotech (Fuzhou, China). After 7 days of culturing in vitro, CGGs were incubated in the presence or absence of 50 μM H$_2$O$_2$ (at 4, 8, 12 hours; Sigma-Aldrich), with or without GLPS, for the indicated time points. More details of specific treatments are in the figure legends. The control for both H$_2$O$_2$ and GLPS was water.

Western blot assay

Western blotting was performed as previously described (Yan et al., 2015). Briefly, neuronal lysates were separated using...
sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein bands electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline with 5% milk and 0.05% Tween-20 and then probed with primary antibodies at 4°C overnight. The following primary antibodies were used: rabbit anti-cytochrome c, rabbit anti-cleaved caspase-3, mouse anti-Bax, rabbit anti-Bim, mouse anti-Bcl-2 and mouse anti-β-tubulin, each diluted 1:1,000 (all primary antibodies from Cell Signaling Technology, Danvers, MA, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used to detect reactive bands with the enhanced chemiluminescence (ECL) and ECL-plus systems (GE Healthcare, Chalfont St. Giles, UK). To quantify bands on western blots, the average intensity of the pixels in a background-selected region was calculated and subtracted from each pixel in the sample. To correct for deviations, densitometry values obtained within the linear range of detection were normalized to those for β-tubulin.

Quantification of neuronal apoptosis
CGC apoptosis was quantified as previously described (Linseman et al., 2004). In brief, CGCs were cultured in 24-well plates and incubated with various treatments. After incubation for the indicated times, CGCs were stained with the DNA dye Hoechst 33258 (Sigma, 5 μg/mL) to visualize nuclear morphology. Apoptosis was quantified by scoring the percentage of neuronal cells with condensed or fragmented nuclei. Neurons were counted from three randomly chosen fields per well, under a light microscope (BX51WI light microscope; Olympus, Tokyo, Japan). To obtain unbiased results, experiments were performed in a blinded manner and cells were scored by investigators without knowledge of their prior treatments. All experiments were repeated at least three times and over 500 neurons were counted for each treatment group.

Statistical analysis
Statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). All statistical data are expressed as the mean ± SEM of at least three independent experiments (n ≥ 3). The statistical significance of differences was analyzed using Student’s t-test between two groups and one-way analysis of variance with Student-Newman-Keuls post hoc test for comparisons among more than two groups. A value of P < 0.05 was considered statistically significant.

Results
Oxidative stress significantly induced neuronal apoptosis in cultured CGCs
H2O2 is commonly used to induce cell apoptosis, secondary to free radicals (Medina et al., 2002). It is therefore considered a useful agent for generating models of oxidative stress (Chang et al., 2003). Accordingly, we used 50 μM H2O2 for an experimental apoptosis model. First, cultured neurons were treated with H2O2 for 4, 8 or 12 hours, then stained with Hoechst 33258 to view apoptotic cells. H2O2 administration markedly induced neuronal apoptosis, in a time-dependent manner. Apoptotic neurons were those with nuclear shrinkage, chromatin condensation or fragmentation (Figure 1A). Apoptosis began as early as 4 hours after H2O2 treatment and, 12 hours later, the apoptotic rate had reached nearly 70% (Figure 1B). Next, we investigated which proteins were activated during apoptosis. We found that cytosolic cytochrome c levels were increased, and caspase-3 was cleaved (Figure 2A). The statistical data are shown in Figure 2B. These data indicated that H2O2 activated the mitochondrial apoptotic pathway, involving BH3-only family proteins. BH3-only proteins are critical for neuronal apoptosis (Happo et al., 2012; Doerflinger et al., 2015), and can be divided into two classes: (1) pro-apoptotic proteins, such as Bax, Bad and Bim; and (2) anti-apoptotic proteins, such as Bcl-2 and Bcl-xl. As shown in Figure 2C, Bim and Bax levels were increased and those of Bcl-2 were decreased, all changing in a time-dependent manner. The statistical data are shown in Figure 2D. All findings suggested that H2O2 significantly induced neuronal apoptosis in CGCs.

GLPS markedly suppressed oxidative stress-induced apoptosis
Compounds extracted from G. lucidum showed anti-cancer, antioxidant and liver protective effects. GLPS was neuroprotective against traumatic spinal cord injury in rats (Gokce et al., 2015). However, the role of GLPS in modulating oxidative stress-induced apoptosis in cultured cerebellar granule cells remains unknown. In this study, we found that apoptosis induced by H2O2 was suppressed in a dose-dependent manner by GLPS administration (Figure 3A). The statistical data are shown in Figure 3B. Neurons were pre-treated with H2O2 for 4 hours. Cells were then washed and incubated with GLPS for an additional 4, 8 or 12 hours. The results showed that cells underwent apoptosis, even at 12 hours after H2O2 administration. However, GLPS addition significantly protected neurons from apoptosis, in a time-dependent manner (Figure 3C). The statistical data are shown in Figure 3D. We then examined whether GLPS suppressed apoptosis through inhibition of the mitochondrial pathway. As shown in Figure 4A, levels of activated caspase-3, induced by H2O2, were suppressed by GLPS (Figure 4B). Furthermore, the increased levels of Bax and Bim were also attenuated and the decreased level of Bcl-2 was increased in H2O2 treated cells also receiving GLPS (Figure 4C and D). These results indicated that GLPS suppressed H2O2-induced apoptosis.

Discussion
In this study, we demonstrated oxidative stress-induced apoptosis of cultured cerebellar granule cells. H2O2 increased cleavage and, therefore, activation of caspase-3, cytochrome c release, upregulation of the pro-apoptotic proteins Bax and Bim and downregulation of the anti-apoptotic protein Bcl-2, ultimately causing apoptosis in CGCs. GLPS administration significantly suppressed these processes, thus inhibiting H2O2-induced neuronal apoptosis. This elucidation of the neuroprotective mechanisms of GLPS may contribute to clinical use of active compounds isolated from G. lucidum.

G. lucidum has been used as a preventive medicine in Asia.
for thousands of years (Shiao, 2003). Polysaccharides, isolated from G. lucidum fruiting bodies, have antioxidant (Liu et al., 2010), immunomodulatory (Bao et al., 2001) and antitumor properties (Cao and Lin, 2006). Moreover, polysaccharides were protective against cerebral ischemic injury (Zhou et al., 2010) and traumatic spinal cord injury in rats (Gokce et al., 2015). GLPS induced neuronal differentiation of pheochromocytoma cell cultures and protected PC12 neurons from apoptosis, by the Erk1/2 and the CREB signaling pathways (Cheung et al., 2000). G. lucidum extracts decreased inflammatory mediator production by activated microglia and protected dopaminergic neurons against inflammatory and oxidative damage (Zhang et al., 2011). Furthermore, G. lucidum spores preserved injured spinal motor neurons by modulating expression of proteins important for axonal regeneration (Zhang et al., 2006). These findings suggested that polysaccharides isolated from G. lucidum had both neuroprotective and antioxidant properties. To the best of our knowledge, ours is the first report on the neuroprotective effects of GLPS against oxidative stress-induced apoptosis in cultured cerebellar granule cells.

H$_2$O$_2$ induced apoptosis in neuronal and non-neuronal cells, an effect associated with its cytotoxicity (Mailly et al., 1999; Kumar et al., 2001; Chang et al., 2003). During the late stages of apoptosis, DNA fragmentation occurs, following reactive oxygen species generation, caspase-3 activation and mitochondrial dysfunction (Yang et al., 2004). Moreover, oxidative stress-induced neurotoxicity involves a mitochondria dependent apoptotic pathway, including cytochrome c release, caspase-3 activation and changes in the Bax/Bcl-2 ratio (Brune et al., 2003; Cunha-Oliveira et al., 2007; Lai et al., 2011; Radi et al., 2014), all effects demonstrated in our study. Bcl-2 can counteract the pro-apoptotic effect of Bax/Bim by forming a heterodimer (Kobayashi et al., 1998). During apoptosis, increased Bax translocates to the mitochondria, leading to decreased mitochondrial membrane potential (Linsenman et al., 2004; Cunha-Oliveira et al., 2006). Bim forms heterodimers with Bcl-2, releasing Bax from Bcl-2, thus enabling its mitochondrial translocation (Letai et al., 2002) and Bim also directly activates Bax for apoptosis (Gavathiotis et al., 2008; Du et al., 2011). Our results showed that GLPS addition markedly suppressed all signal transduction processes induced by oxidative stress. That is, GLPS inhibited caspase-3 activation, suppressed Bax/Bim upregulation and prevented Bcl-2 downregulation, thus ultimately preventing H$_2$O$_2$-induced apoptosis in CGCs. Among the BH-3-only pro-apoptotic proteins, Bid, Noxa and Puma were reported to inhibit caspase-3 activation, suppressed Bax/Bim upregulation and prevented Bcl-2 downregulation, thus ultimately promoting H$_2$O$_2$-induced apoptosis in neurons. These findings provided potential new evidence supporting clinical use of G. lucidum to treat neurodegenerative diseases involving oxidative stress.

Author contributions: YL performed research, collected data and analyzed and interpreted data. XZS and WL performed research and wrote the paper. LMG designed research and revised the manuscript. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Research ethics: The study protocol was approved by the Ethics Committee of Peking University Health Science Center, China. The experimental procedure followed the the United States National Institutes of Health Guide for the Care and Use of Laboratory Animal (NIH Publication No. 85-23, revised 1986), and “Consensus Author Guidelines on Animal Ethics and Welfare” produced by the International Association for Veterinary Editors (IAVE). All efforts were made to minimize the number and suffering of the animals used in the experiments. The article was prepared in accordance with the “Animal Research: Reporting of In Vivo Experiments Guidelines” (ARRIVE Guidelines).

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