Department of Orthopaedics[1]; Central Laboratory for Scientific Research[2], the Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, China

Neuroprotective effects of ginsenoside Rg1 on oxygen-glucose deprivation reperfusion in PC12 cells

Sheng-Li Huang[1], Xi-Jing He[1], Zong-Fang Li[2], Lei Lin[1], Bin Cheng[1]

Received September 14, 2013, accepted October 14, 2013

Prof. Bin Cheng, Department of Orthopaedics, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University No.157 Xiwulu, Xi'an 710004, China
binchengey@sina.cn

Pharmazie 69: 208–211 (2014) *doi: 10.1691/ph.2014.3847*

The present study was aimed to investigate the protective effects of ginsenoside Rg1 (GRg1), an important component of ginseng, in oxygen-glucose deprivation/reperfusion (OGDR) and to elucidate the related mechanisms. PC12 cells were used as the model of OGDR. GRg1 administration was started 12 h before OGD and lasted for 12 h. After OGD, the cells were incubated in drug-free and full culture medium under normoxic condition for 24 h. Cell viability was then measured using MTT assay. Cell morphology was studied under a microscope. The expressions of survivin, caspase-3 and Terminal dUTP nick-end labeling (TUNEL) were measured by immunocytology. Results showed that pretreatment with GRg1 significantly increased the viability and survivin expression, and decreased the expressions of caspase-3 and TUNEL in a dose-dependent manner. In addition, it dramatically increased the number of cells and improved the cellular morphology. These results demonstrate the effect of GRg1 in preventing OGDR-induced PC12 cell apoptosis and partly reveal the mechanisms of the protective effect. It is suggested that GRg1 has potential beneficial effects in ischemic diseases.

1. Introduction

Ischemia of the central nervous system from stroke or cardiac arrest may result in considerable tissue damage. Prompt restoration of adequate blood flow is the main treatment. However, tissue damage may continue even after restoration of blood flow, partly due to reperfusion itself. Ischemia associated with reperfusion is a complex pathophysiologic process that may result in greater tissue injury than ischemia alone, and there are extremely few therapeutic approaches available so far.

Ginseng, the root of *Panax ginseng* C. A. Meyer, is a herb that has been used as a constituent of many prescriptions in traditional Chinese medicine. Ginsenoside Rg1 (GRg1) is one of the main active ingredients of ginseng. The chemical structure of GRg1 has been clarified by Wen et al. 1996. The molecular formula is $C_{42}H_{72}O_{14}$ and the molecular weight is 800. GRg1 is shown to have a great potential as neurotrophic or neuroprotective compound (Ma et al. 2013; Fang et al. 2012; Huang et al. 2012).

The rat pheochromocytoma cell line, PC12, is the most widely used cellular model for studying neuronal diseases. Although recent studies have shown that GRg1 exerts beneficial effects on neuronal cells, there is no report concerning an effectivity of GRg1 against oxygen-glucose deprivation/reperfusion (OGDR). In this study, we examined the effect of GRg1 on PC12 cells treated with OGDR.

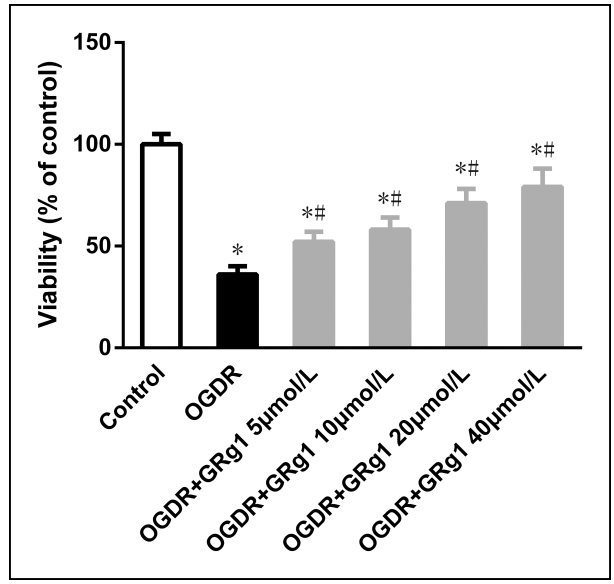

Fig. 1: Effects of GRg1 on PC12 cell survival. PC12 cells untreated or treated with GRg1 at different concentrations (5, 10, 20, 40 μmol/L) were exposed to OGD for 0.5 h followed by OGDR for 24 h. *$p < 0.05$ for the comparison with control group. #$p < 0.05$ for the comparison of cells treated with GRg1 with group.

2. Investigations and results

2.1. MTT assay

The results of MTT assay showed that the viability of cells exposed to OGDR was 36.1 ± 3.9 % of that of cells in the control group. The cell viability was increased from 51.2 ± 5.1% to 79 ± 8.7% after the use of GRg1 (5–40 μmol/L) (Fig. 1), indicating that GRg1 improved the proliferation of PC12 cells in a dose-dependent manner. However, the cell viability could not get back to the level of the control group ($P < 0.05$).

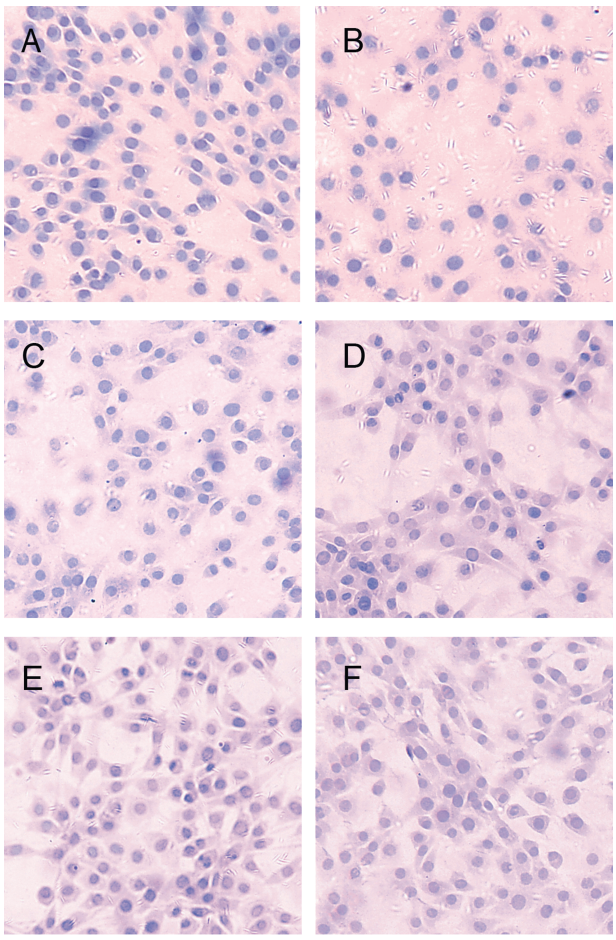

Fig. 2: Effects of GRg1 on PC12 cells treated with OGDR (HE staining, × 200). (A) Control cells. (B) Cells exposed to OGD for 0.5 h followed by reperfusion for 24 h. There is a significant decrease in the cell number, and most of the cells lose neurites and demonstrate a round shape. (C, D, E, F) Cells were pre-incubated with 5, 10, 20 and 40 μmol/L GRg1, respectively, and were then exposed to OGD for 0.5 h followed by OGDR for 24 h.

2.2. *H&E staining*

Notable changes observed in the OGDR cell group were that cells lost their neurites and appeared to be round-shaped and swollen, and lots of the nuclei turned brighter, indicating cell apoptosis. After treated with GRg1, cells exhibited improved cellular morphology, suggesting that GRg1 partially ameliorated the degenerative changes such as cell swelling. In addition, the number of PC12 cells decreased significantly after OGDR, whereas the decreasing tendency slowed down after the treatment of GRg1 (Fig. 2).

2.3. *Immunocytology*

PC12 cells treated with GRg1 for 24 h were examined by immunocytology to further confirm the above observations. Survivin-positive cells significantly increased in the GRg1-treated cell groups compared with the OGDR group (Fig. 3 A-F, Table). There was an obvious increase of caspase-3-positive cells in the OGDR group, whereas a significant concentration-dependent decrease was observed in cells exposed to GRg1 of different concentrations for 24 h (Fig. 3G-L). The percent of caspase-3-positive cells correlated negatively with the percent of survivin-positive cells ($r = -0.656$, $P < 0.01$).

2.4. *TUNEL assay*

Although the MTT assay measures cell viability, it does not differentiate between apoptotic and necrotic cell death. Therefore,

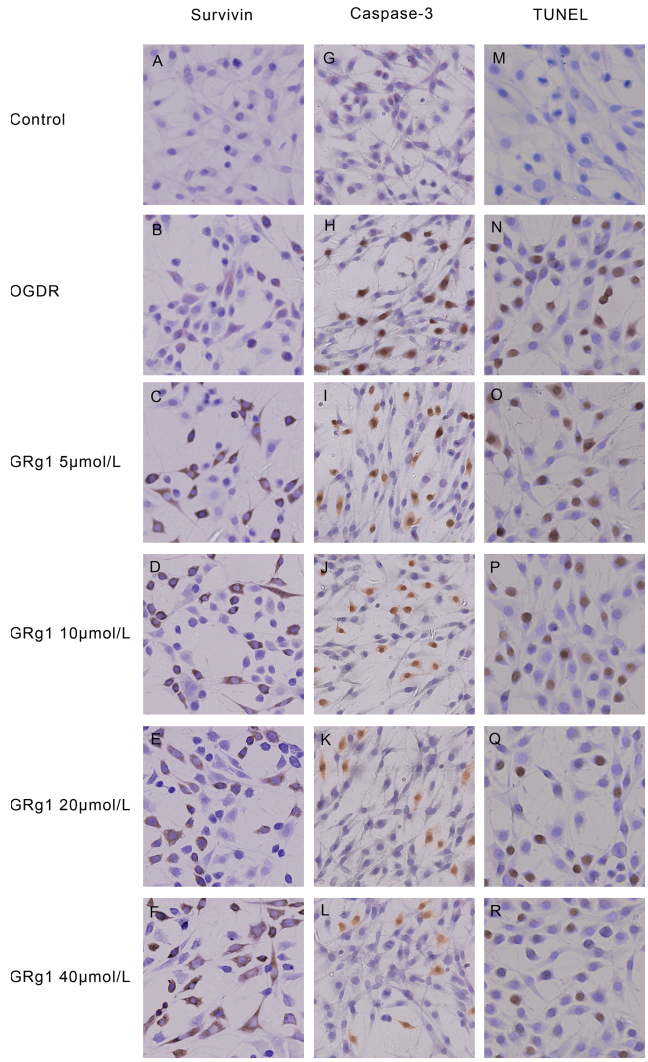

Fig. 3: Survivin, Caspase-3, TUNEL immunostaining (× 400). (A, B, C, D, E, F) Survivin staining.The plasma and processes were stained in brown. (G, H, I, J, K, L) Caspase-3 staining. The plasma and nucleus were stained in brown. (M, N, O, P, Q, R) TUNEL immunostaining. Immunoreactivity was visualized in brown.

TUNEL assay was performed to identify potential apoptotic PC12 cells (Gavrieli et al. 1992). Almost no TUNEL-positive cells were seen in the control group, whereas a large number of these cells were detected in the OGDR group. The percentage of the TUNEL-positive cells in the OGDR group was significantly higher than that in the control ($P < 0.05$). The cells had dark or dark brown nuclei and exhibited morphological features of apoptosis, including shrunken cell bodies, condensed nuclei, and formation of crescent caps of condensed chromatin at the nuclear periphery (Fig. 3M-R). GRg1 treatment resulted in a decrease in the number of TUNEL-positive cells (Table). The administration of 40 μmol/L GRg1 increased the survival rate of PC12 cells by 28% compared to the OGDR. Our findings provided strong evidence for the inhibitory effect of GRg1 on apoptosis of PC12 cells treated with OGDR. Similar to that of caspase-3, the percent of TUNE-positive cells was negatively correlated with the percent of survivin-positive cells ($r = 0.781$, $P < 0.01$).

3. Discussion

We found in the present study that pre-treatment with GRg1 may increase, in a dose-dependent manner, the viability of PC12 cells under OGDR conditions. Given that the overall pharmacology of

Table: Effects of GRg1 on OGDR-treated PC12 cells (mean ± SD, n = 5)

	Control	OGDR	OGDR + GRg1 (μmol/L)			
			5	10	20	40
Survivin	0.45 ± 0.76	52.55 ± 6.22 [a]	57.45 ± 6.08 [ab]	62.10 ± 5.97 [ab]	75.25 ± 5.72 [ab]	74.80 ± 6.83 [ab]
Caspase-3	0.70 ± 1.03	65.15 ± 5.39 [a]	56.65 ± 6.35 [ab]	46.90 ± 4.02 [ab]	38.90 ± 6.47 [ab]	39.05 ± 6.62 [ab]
TUNEL	0.50 ± 0.76	67.90 ± 4.18 [a]	60.15 ± 3.99 [ab]	56.00 ± 4.40 [ab]	49.45 ± 4.65 [ab]	49.10 ± 5.01 [ab]

a: $P < 0.05$ compared with control group. b: $P < 0.05$ compared with OGDR group.

GRg1 is complex, the effect of GRg1 on PC12 cell survival may be mediated through decreasing cell apoptosis, since OGDR can induce the apoptosis of PC12 cells. Our results demonstrate for the first time that pretreatment with GRg1 reduces the toxicity of OGDR to PC12 cells, suggesting that GRg1 has a potential protective effect on ischemia-reperfusion-promoted neural damage. In addition, toxicity of GRg1 to PC12 cells was not observed in our experiments.

The PC12 cells used in this study were derived from catecholamine-secreting pheochromocytoma of adrenal medulla in rats (Greene and Tischler 1976). Recent studies have shown that PC12 cells can be applied as a good cellular model for studying the neuronal diseases (Sharifi et al. 2009; Zhu et al. 2010). OGDR in PC12 cells has been used as a rapid and sensitive *in vitro* model of ischemia of the central nervous system for the development of potential neuroprotective agents. In our experiments, OGDR was performed *in vitro* by incubating PC12 cells in a medium in which glucose was omitted and oxygen was replaced with nitrogen. After OGD, the glucose-free medium was replaced for another 24 h, which was taken as reperfusion. This model mimics the pathological conditions of ischemia of central nervous system.

MTT assay has been widely used as an index of cell survival and proliferation, because it is a sensitive measurement of the metabolic status of cells. In our experiments, the viability of PCl2 cells was significantly decreased after OGDR, whereas the pre-incubation with GRg1 increased the cell viability significantly. These results suggest that GRg1 is capable of protecting PC12 cells from OGDR-induced damage *in vitro*.

Apoptosis is an activated cellular death process that is induced by physiological or pathological factors to eliminate redundant or damaged cells. During the process, cells undergo a variety of biochemical and morphological events, including cell shrinkage, mitochondrial degradation, and nuclear DNA fragmentation (Yang and Yu 2003). Studies of cell apoptosis have focused on the role of caspase, a class of cysteine proteases (Thornberry and Lazebnik 1998). Caspase-3, a member of the cysteine proteases family, is thought to be one of the main effectors of apoptosis (Talanian et al. 1997). It exists in the cytoplasm as an inactive proenzyme which, upon induction of apoptosis, is processed to its active form that can be detected by a protease assay (Granville et al. 1998). The apoptotic changes are often accompanied by caspase activation. In our experiments, the caspase-3 level was evidently increased in the cells after OGDR treatment, indicating that caspase-3 participated in the OGDR-induced PC12 cell apoptosis. After the treatment of GRg1, the caspase-3 level was significantly lowered, suggesting that GRg1 decreased the apoptosis at the protein level. Taken together, GRg1 might protect against OGDR-induced cell apoptosis by inhibiting the activation of caspase-3.

Survivin is the smallest protein in the inhibitors of apoptosis protein family. It has been reported to suppress the activity of caspase-3, which is a key enzyme in the apoptosis signaling pathway (Song et al. 2003). Survivin can inhibit the synthesis of caspase-3, hence blocks the apoptosis mediated by caspase-3. In our experiment, the survivin levels increased in the GRg1-treated cell groups.

GRg1 is well known to possess a wide range of biological functions, such as antioxidative, anti-inflammation and anticancer activities (Wang et al. 2010; Liao et al. 2012; Cho et al. 2012; He et al. 2012; Li et al. 2008). After treatment with GRg1, the PC12 cells exhibited improved cellular morphology. GRg1 not only significantly reduced cell death, but also improved survivin activity and suppressed caspase-3 activity. In our experiments, the survivin levels in the GRg1-treated cell groups were significantly higher than that in the OGDR group, while the caspase-3 levels in the GRg1-treated groups were significantly lower. Our results indicate that the increased expression of survivin and the decreased expressions of caspase-3 and TUNEL may be important mechanisms in the protective effects of GRg1 against OGDR-induced apoptosis.

Our results suggest GRg1 can decrease apoptosis in a concentration-dependent manner. The mechanisms underlying the GRg1 protection was unclear so far. There are two possible signaling pathways: the death receptor pathway and the mitochondria path. Because survivin plays an important role in the mitochondrial apoptosis pathway (Du et al. 2000), our results suggested that mitochondrial pathway mediates apoptosis in PC12 cells treated by GRg1.

In conclusion, GRg1 can decrease PC12 cell death, in which apoptosis plays an important role. The protective effect of GRg1 is dose-dependent. Further understanding of the underlying mechanisms may lead to novel therapeutic avenues for OGDR. It would be intriguing to know the effect of GRg1 when used at a wider range of concentrations. Overall, our findings about the effect of GRg1 may provide opportunities for novel pharmacological interventions against ischemia of central nervous system.

4. Experimental

4.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone, and 96-well plates and 6-well plates were provided by Corning Costar. Poly-L-lysine, dimethyl sulphoxide (DMSO), diaminobenzidine (DAB), paraformaldehyde, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Mouse monoclonal survivin antibody and mouse monoclonal caspase-3 antibody were obtained from Santa Cruz. Terminal dUTP nick-end labeling (TUNEL) assay was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). GRg1 was obtained from Shanghai Winherb Medical Science Co. Ltd. (Shanghai, China).

4.2. Cell culture and treatments

PC12 cells were obtained from the Culture Collection of the Chinese Academy of Science, Shanghai, China, and were incubated in high glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in humidified 5% carbon dioxide. The medium was changed every 2 days.

After reaching 60–70% confluence, cells were seeded in 96-well plates at 20,000 cells/well in 200 μL medium and in 6-well plates at a concentration of 100,000/ml with glass coverslips coated in advance with poly-L-lysine

following the manufacturer's instructions. Dissociated cells were then incubated in a 5% humidified carbon dioxide incubator at 37 °C. The cells were randomized into six groups for different treatments: control, OGDR, OGDR + GRg1 (5 μmol/L), OGDR + GRg1 (10 μmol/L), OGDR + GRg1 (20 μmol/L), and OGDR + GRg1 (40 μmol/L). The selection of GRg1 doses was based on our cell viability assay and published reports (Radad et al. 2004). After12 h of incubation, cells were treated with GRg1 of different concentrations for 12 h, except those in the control group and OGDR group. Then, the medium in the OGDR group and various OGDR + GRg1 groups was replaced with a pre-warmed (37 °C) glucose-free DMEM. After that, the cells were incubated in an oxygen-free chamber with a gas mixture of 95% nitrogen and 5% carbon dioxide for 0.5 h. OGD was terminated by removing cultures from the chamber, replacing complete culture medium, and returning cells to the incubator for 24 h under normoxic conditions for reoxygenation process. The control group, which was not subjected to OGDR, was cultured in normal DMEM in the incubator under normal conditions.

4.3. Cell viability assay

Cell viability was quantitatively evaluated at 24 h of reoxygenation using MTT assay. Briefly, MTT (0.5 mg/mL) was added into wells in the 96-well plates, and cells were incubated for 4 h at 37 °C. After the medium with MTT was removed, cells and dye crystals were dissolved in 150 mL DMSO. Optical density was then determined at 492 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). In each assay, four parallel wells were included. Results were expressed as a percentage of the optical density measured in control group cells.

4.4. Hematoxylin and eosin (H&E) staining

PC12 cells cultured on poly-L-lysine-coated glass cover slips in the 6-well plates were fixed in 4% paraformaldehyde for 10 min at room temperature. Briefly, cells were stained with hematoxylin and washed with distilled water, followed by a rinse with 0.5% hydrochloric acid in 70% ethanol and washing with tap water. After that, the cells were stained with eosin and washed again with distilled water. Finally, the slides were dehydrated, followed by xylene, and were then mounted.

4.5. Immunocytology

After the cultured cells were fixed by paraformaldehyde, the cover slips were washed with phosphate-buffered saline (PBS) three times for 5 min each time. The sections were treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, and then treated with 10% goat serum for 1 h at room temperature. Cells were subsequently incubated at 4 °C overnight with the primary antibody of mouse anti-survivin or mouse anti-caspase-3. After three washes in PBS, a secondary antibody, biotinylated goat anti-mouse IgG was added, followed by incubation for 2 h at room temperature. The cells were then rinsed and placed in avidin-peroxidase conjugate solution for 2 h. Horseradish peroxidase was detected with 0.05% diaminobenzidine. Then, the sections were counterstained with hematoxylin, dehydrated, and mounted. The plasma and processes were stained brown in survivin-positive cells, and the plasma and nucleus were stained brown in caspase-3. The negative control slides without adding primary antibody were incubated for each staining. Survivin- and caspase-3-positive cells were counted under a microscope.

4.6. TUNEL assay

Apoptotic cells were detected by a TUNEL kit. In brief, the cells fixed by paraformaldehyde were quenched in 3% hydrogen peroxide for 10 min, washed with PBS, and incubated with terminal deoxynucleotidyl transferase enzyme for 1 h at 37 °C. The reaction was stopped by washing in PBS, and anti-digoxinperoxidase was added to the slides. After PBS washing, the slides were incubated with diaminobenzidine for 10 min at room temperature. Then, counterstaining with hematoxylin was performed, and the sections were dehydrated for further microscopic study. Five microscopic fields of TUNEL-positive cells were chosen for imaging. Substitution for terminal deoxynucleotidyl transferase with distilled water was used as negative control.

4.7. Data analysis

Data were expressed as mean ± SD. For statistical evaluation, one-way analysis of variance (ANOVA) was employed followed by a t-test. Pearson correlation analysis was also performed to some index. Statistical significances were defined as a p value less than 0.05 for all analyses.

Acknowledgement: This study was supported by research grants from the National Natural Science Foundation of China (No. 81271340).

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