Abstract. Ginsenoside Rb1 is generally recognized as one of the principal bioactive ingredients in ginseng and shows neuroprotective effects in various neurons. Endoplasmic reticulum (ER) stress is considered to play an important role in numerous neurodegenerative disorders. Recently, glucogen synthase kinase 3β (GSK3β) was reported to regulate ER stress-induced C/EBP homologous protein (CHOP) in neuronal cells. Therefore, in this study, we investigated the effects of ginsenoside Rb1 on GSK3β-mediated ER stress in high glucose-treated hippocampal neurons. Results from the MTT assay showed that treatment with 1 µM Rb1 for 72 h protected neurons from high glucose-induced cell injury. Using western blot analysis, we found that treatment with Rb1 effectively inhibited the phosphorylation of the high glucose-induced protein kinase RNA-like ER kinase (PERK) and of GSK3β, and reduced the level of the CHOP protein. The levels of these proteins were also decreased by treatment with the GSK3β inhibitor Licl. Rb1 also significantly decreased the mRNA expression of the gene CHOP, as shown by quantitative RT-PCR analysis. Taken together, the present results suggested that Rb1 may protect neurons from high glucose-induced cell damage by inhibiting GSK3β-mediated CHOP induction, providing a potentially new strategy for preventing and treating cognitive impairment caused by diabetes.

Introduction

Diabetes is a thriving disease throughout the world and evidence from several epidemiological studies suggests that it is an independent risk factor for cognitive dysfunction (1). Patients with type 1 and type 2 diabetes show performance decrements on measures of executive functioning (2). Substantial evidence supports that diabetic cognitive impairment is associated with dysfunctions of the hippocampus, the major area associated with learning and memory (3-5). Experimental studies in animal models and in humans have shown that hyperglycemia is the main contributor of diabetic cognitive impairment, which can alter hippocampal synaptic plasticity and cause decreased neuronal densities in the CA-1 region of the hippocampus and degeneration and apoptosis of hippocampal neurons (4,6,7).

Endoplasmic reticulum (ER) stress, often resulting from the cellular accumulation of misfolded proteins, triggers a cellular stress response known as the unfolded protein response (UPR), which is intended to protect the cell (8). UPR is characterized by translational attenuation, synthesis of ER chaperone proteins and transcriptional induction of genes including those encoding the three ER transmembrane receptors, i.e., protein kinase RNA (PKR)-like ER kinase (PERK) and inositol requiring enzyme-1 (IRE1) and activating transcription factor (ATF6) (8). If homeostasis cannot be reestablished or ER function is severely impaired, apoptosis signaling pathways are triggered by ER stress, including the C/EBP homology protein (CHOP), caspase-12 and c-Jun N-terminal kinase pathways (9,10). The CHOP protein, shown to play a critical role in ER stress-induced apoptosis, can be induced by PERK translation, and has been involved in promoting neuronal death in numerous neurodegenerative diseases (11). Recently, CHOP-dependent ER stress-mediated apoptosis was reported to be involved in hyperglycemia-induced impairment of hippocampal synapses and neurons (12). Regulation of the ER stress-induced execution phase of apoptosis is not completely understood, but clearly involves glycogen synthase kinase-3β (GSK3β), which regulates ER stress-induced CHOP expression in neuronal cells (13).

Ginseng (botanical name: Panax ginseng C.A. Meyer) is one of the most common herbal remedies and has long been
used in traditional Chinese medicine to restore and enhance benefits for patients. Ginsenoside Rbl is generally recognized as one of the principal bioactive ingredients in ginseng and has been shown to possess neuroprotective properties in numerous studies. Ginsenoside Rbl can increase the proliferation of Schwann cells and the expression and secretion of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) from these cells (14), prevent MPP⁺-induced apoptosis in PC12 cells (15) and upregulate cell genesis in hippocampal subregions, thus enhancing spatial learning and memory in rats (16). However, whether ginsenoside Rbl has neuroprotective effects on high glucose-treated hippocampal neurons, and the mechanism underlying these effects remain unclear. Therefore, in the present study, we investigated the effects of ginsenoside Rbl on high glucose-induced cytotoxicity in primary cultured rat hippocampal neurons by detecting cell viability, and further studied the underlying mechanisms by examining phosphorylated (p)-PERK, PERK, p-GSK3β (Tyr216), GSK3β and CHOP protein expression, as well as CHOP mRNA expression.

Materials and methods

Reagents. Ginsenoside Rbl standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Produces (Beijing, China). The chemical structure of ginsenoside Rbl (2-O-β-glucopyranosyl-(3β,12β)-20-[6-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-12-hydroxydammar-24-en-3-yl β-D-glucopyranoside) is shown in Fig. 1. All cell culture reagents were from the Peking Union Cell Resource Center (Beijing, China). Sprague-Dawley (SD) rats, <24 h old, were purchased from the Peking University Health Science Center (Beijing, China). Anti-p-PERK, -PERK, -p-GSK3β, -GSK3β and -CHOP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and treatments. All experimental procedures were performed in accordance with the experimental standards of the Chinese Academy of Medical Sciences, as well as with international guidelines on the ethical treatment of animals. Primary hippocampal neuronal cultures were prepared as previously described (17,18) with some modifications. The Animal Welfare Committee of Peking Union Medical College Hospital and Chinese Academy of Medical Sciences (Beijing, China) approved the animal protocols. Hippocampi were dissected from the brain on ice and minced in sterile ice-cold D-Hanks’ solution, with the blood vessels and meninges carefully removed. From these cells (14), prevented MPP⁺-induced apoptosis in PC12 cells (15) and upregulated cell genesis in hippocampal subregions, thus enhancing spatial learning and memory in rats (16). However, whether ginsenoside Rbl has neuroprotective effects on high glucose-treated hippocampal neurons, and the mechanism underlying these effects remain unclear. Therefore, in the present study, we investigated the effects of ginsenoside Rbl on high glucose-induced cytotoxicity in primary cultured rat hippocampal neurons by detecting cell viability, and further studied the underlying mechanisms by examining phosphorylated (p)-PERK, PERK, p-GSK3β (Tyr216), GSK3β and CHOP protein expression, as well as CHOP mRNA expression.

Cell viability assay. After exposure to 50 mM glucose and ginsenoside or LiCl for 72 h, cell viability was determined using the MTT assay system (Peking Union Cell Resource Center). Briefly, the cells were plated on 96-well culture plates at a density of 5×10⁴ cells/ml. Then, MTT reagent (100 µl of a 5 mg/ml solution) was added to each well (1:10) and incubated for 4 h at 37°C. Following incubation, the medium was removed and the precipitated dye was dissolved in 100 µl of dimethylsulfoxide solution for another 30 min at 37°C. The OD value of each sample was measured at 570 nm using a Multiskan 3 ELISA Reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell viability (%) was calculated as follows: ODreagent group/OD24 h control group ×100.

Western blot analysis. Following treatment as described above, total protein was extracted from cells lysed with RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 5 µg/ml aprotinin and 5 µg/ml leupeptin). The protein concentration of the supernatants was determined with the DC protein assay kit (Bio-Rad, Hercules, CA, USA) and then adjusted to the same concentration with 0.9% NaCl. A total of 40 µg of protein was resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature before overnight incubation with primary antibodies specific to p-PERK (1:100), PERK (1:100), p-GSK3β (1:200), GSK3β (1:200) and CHOP (1:200) in 5% skim milk-TBST. The membrane was then rinsed three times in TBST and incubated with horseradish peroxidase-conjugated secondary IgG (1:1,000) in TBST for 1 h, followed by washing three times with TBST before detection with ECL reagent.

Figure 1. Chemical structure of ginsenoside Rbl.
1.5 h, followed by a final series of rinses in TBST. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Roche Diagnostics GmbH, Penzberg, Germany) and were quantified using the Quantity One software (Bio-Rad).

**Real-time PCR analysis.** Total RNA was extracted from cells using the TRizol reagent according to the manufacturer’s instructions. The concentration and purity of RNA were determined spectrophotometrically at 260 and 280 nm. Single-strand cDNA was prepared from 2 µg of total RNA according to the protocol of the kit. The following primers were designed using Primer Premier 5.0 software (http://www.premierbiosoft.com/): CHOP, 5′-CCTAGCTTGGCTGACTGAGG-3′, 5′-CTGCTCCTTCTCCTCATGC-3′; GAPDH, 5′-TGGTATCGTGGAGGACTCA-3′, 5′-CCAGATGAGGCAGGGA TGAT-3′. Briefly, the PCR reaction was carried out in a 20 µl final volume containing: 10 µl of 2X SYBR®-Green I master mix, 1 µl of each primer (10 µM), 2 µl of 1 µg/µl cDNA template, and 6 µl of 0.1% diethylpyrocarbonate-treated water. The PCR conditions were as follows: initial denaturation at 95˚C for 120 sec, followed by 40 cycles of denaturation at 95˚C for 20 sec, annealing at 60˚C for 20 sec, and extension at 72˚C for 30 sec. A melting curve was obtained after amplification by holding the temperature at 65˚C for 20 sec, followed by a gradual increase in temperature to 95˚C at a rate of 0.5˚C/sec. The GAPDH mRNA level was used as an internal quantitative control, allowing to normalize the level of each target gene transcript.

**Statistical analysis.** Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using a one-way ANOVA and Dunnett’s tests. P<0.05 was considered to indicate significant differences.

**Results**

**Neuronal morphological changes.** Neuronal morphological changes were assessed under an inverted microscope. As shown in Fig. 2, hippocampal neurons treated with normal medium (control) exhibited large, vacuole-free cell bodies, with elaborate networks of neurites. However, exposure to 50 mM glucose for 72 h resulted in obvious cell loss, with the disappearance of neurites, appearance of disrupted membranes and shrinkage of cell bodies. By contrast, cultures exposed to the same concentration of glucose in the presence of ginsenoside Rb1 (1 µM) appeared markedly preserved, indicating that Rb1 has significant cytoprotective effects against high glucose insult. Licl (1 mM), which is a GSK3β inhibitor, also showed cytoprotective effects against high glucose treatment.

**Neuroprotective effects of ginsenoside Rb1 on cytotoxicity induced by high glucose.** To verify the protective effects of ginsenoside Rb1 on hippocampal neurons exposed to a high level of glucose, cell viability was evaluated by determining the percentage of MTT reduction. As shown in Fig. 3, 50 mM of glucose induced a significant decrease in cell viability
compared to the control group. However, treatment with 1 µM of ginsenoside Rb1 significantly improved cell viability in high glucose-treated hippocampal neurons.

Effects of ginsenoside Rb1 on GSK3β-mediated ER stress in high glucose-treated hippocampal neurons. To examine the activation of ER stress in high glucose-induced cell damage conditions, the phosphorylation level of PERK and GSK3β and the expression of CHOP were assessed by western blot analysis (Fig. 4A). Compared to the control, cells exposed to a high level of glucose for 72 h showed significantly increased p-PERK/P ERK and p-GSK3β/GSK3β ratios and an increased level of the CHOP protein (Fig. 4B). By contrast, treatment with ginsenoside Rb1 effectively attenuated the high glucose-induced phosphorylation of PERK and GSK3β without affecting the levels of the unphosphorylated forms of the enzymes, and reduced the level of CHOP (Fig. 4A and B).

We then investigated the effect of ginsenoside Rb1 on CHOP gene expression using quantitative RT-PCR analysis. As shown in Fig. 4C, exposure to high glucose for 72 h resulted in a significant increase in CHOP gene expression compared to the control. However, when cells were treated with ginsenoside Rb1, the CHOP mRNA level was significantly decreased compared to the high glucose group.

To investigate the hypothesis that active GSK3β is required for the increase in CHOP following ER stress, hippocampal neurons were treated with the inhibitor of GSK3β (Licl) in the presence of a high level of glucose for 72 h. Western blot analysis and RT-PCR measurements confirmed that Licl inhibits high glucose-induced phosphorylation of PERK and GSK3β, as well as CHOP protein and mRNA expression (Fig. 4). The MTT assay indicated that cell viability is also significantly improved by treatment with Licl in high glucose-treated hippocampal neurons (Fig. 3).

Discussion

Ginseng has served as an important component of numerous traditional Chinese medicine prescriptions for thousands of years and is now popular as a natural medicine worldwide. Ginsenoside Rb1 is the major pharmacologically active ingredient of ginseng, and its anti-aging and neuroprotective effects have been often reported. Cell-based studies showed that ginsenoside Rb1 protects hippocampal neurons from glutamate-induced neurodegeneration (19) and promotes cell survival, enhanced neurite outgrowth and synaptic marker expression during differentiation of human neural stem cells (20). However, the precise biological functions and underlying mechanisms of ginsenoside Rb1 are still largely unknown.

The present study evaluated ginsenoside Rb1 as a protective agent against high glucose-induced neurotoxicity. Ginsenoside Rb1 attenuated the neuronal loss and improved cell viability in high glucose-treated hippocampal neurons. We further provide evidence that this neuroprotective effect may involve the inhibition of GSK3β-mediated ER stress induced by high glucose.

Accumulating evidence suggests that diabetes is associated with cognitive deficits, and previous studies have shown that hyperglycemia has toxic effects and can lead to functional and structural abnormalities in the brain, especially the hippocampus (2,21). Hippocampal atrophy is evident early in the course of the disease, and has even been documented in elderly individuals with prediabetes (22). In animal models, diabetes is associated with changes in the hippocampal synaptic plasticity, molecular changes in hippocampal neurons, including pre- and post-synaptic structures, and degeneration of hippocampal neurons (23-25). Therefore, we used hippocampal neurons exposed to high glucose as an in vitro model to...
investigate the neuroprotective effects of ginsenoside Rb1 on high glucose-induced neurotoxicity, as well as the underlying mechanism. Consistent with a previous study (26), the results from morphological observations and the cell viability assay showed that a high level of glucose induces cell damage. Treatment of hippocampal neurons with ginsenoside Rb1 significantly attenuated the high glucose-induced morphological changes in the neurons and the decrease in cell viability, suggesting that ginsenoside Rb1 can revert the neurotoxicity induced by high glucose.

Hyperglycemia plays a critical role in hippocampus dysfunction, however, the precise cellular mechanisms underlying this effect have not yet been fully elucidated. A recent study on a murine model of type 2 diabetes suggested that ER stress is activated in the hippocampus (27), while another study indicated that CHOP-dependent ER stress-mediated apoptosis may be involved in high glucose-induced neuronal damage (12). In rats it has also been reported that diabetes exacerbates ischemic brain injury by increasing ER stress and apoptosis, associated with enhanced CHOP induction (28). CHOP is a pro-apoptotic protein that plays a major role in ER stress-induced apoptosis. In neuronal cells, GSK3β was shown to regulate ER stress-induced CHOP expression, and inhibition of GSK3β effectively reduced the expression of CHOP (13). Therefore, we hypothesized that GSK3β-mediated CHOP induction may be involved in high glucose-induced cell injury. In this study, we observed a significantly higher expression of p-PERK, p-GSK3β and CHOP in high glucose-treated hippocampal neurons, which was effectively decreased by the GSK3β inhibitor, indicating that GSK3β-mediated CHOP signaling is involved in mediating the effects of high glucose on cell morphology and structure. The results of this study showed that high glucose-induced activation of CHOP is significantly inhibited by treatment with the ginsenoside Rb1 and by the GSK3β inhibitor, which suggests that ginsenoside Rb1 can protect from high glucose-induced neurotoxicity via inhibition of the GSK3β pathway.

In summary, our study demonstrated that ginsenoside Rb1 protects hippocampal neurons from high glucose-induced cytotoxicity. The mechanisms underling this neuroprotective effect may involve, at least in part, the inhibition of GSK3β-mediated CHOP induction. To better understand diabetic cognitive impairment, the contribution of proteins downstream of CHOP in high glucose-induced neurotoxicity merit further investigation. Additional investigations on ginsenoside Rb1 is expected to further clarify the mechanisms by which this substance protects the neurons from damage, which may contribute to the development of potential therapeutical applications for this potent neuroprotective agent.

Acknowledgements

We are grateful to Professor Hongchao Yin and Bei Gu for their helpful technical assistance and scientific suggestions.

References