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Neuroprotective effect of ginsenoside Rb1 on glutamate-induced neurotoxicity: With emphasis on autophagy

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ABSTRACT

Ginsenoside Rb1 has been demonstrated with neuroprotective effects, but the mechanisms remain unclear. This study aimed to probe the effects and mechanisms of ginsenoside Rb1 on activation of autophagy in glutamate-injured neurons. Ginsenoside Rb1 of exponential concentrations (1.2, 12, 120 μM) or autophagy inhibitor 3-methyladenine (5 mM) was added to culture medium for cortical neurons after being treated with glutamate. Cell viability was measured by MTT assay. Autophagosomes formation was observed with transmission electron microscope. Autophagy marked protein LC3 was detected with immunofluorescence and visualized under laser confocal microscopy. Changes of autophagy related protein Beclin-1 were measured with Western blot. We found that ginsenoside Rb1 protected cortical neurons from glutamate-induced cell injury. Autophagy was activated after glutamate treatment, with both autophagosomes and punctate LC3 increased significantly compared with control. Beclin-1 was elevated in glutamate-treated cells. Formation of autophagosome and punctate LC3 was attenuated by ginsenoside Rb1. The level of Beclin-1 in ginsenoside Rb1 treated cells was simultaneously decreased compared with glutamate-treated cells. These results suggested that inhibition of autophagy could be responsible for neuroprotective effects of ginsenoside Rb1 in glutamate-induced injury. Down-regulation of Beclin-1 may play an important role in this process.

Ginseng has been utilized as an invigorator for thousands of years in Far East. Pharmacological studies indicated that ginsenosides are the main active components of ginseng. More than 40 kinds of ginsenosides have been identified to date. Ginsenoside Rb1 (GRb1) has garnered much research interests, and has been proved with effects in protecting neurons from glutamate-induced injury in vitro [16,28]. As a result, the possibility of treating some central nervous system (CNS) disorders with GRb1 has been proposed [11,28].

Glutamate-induced neuron toxicity has been regarded as an ideal model for studying neurological diseases [4,7,29], because it may mimic the pathogenic process of Alzheimer and other neurodegenerative diseases [13]. The mechanisms are related to increased calcium inflow, accumulative reactive oxygen species (ROS), impaired mitochondrial function and the initiation of apoptotic and autophagic process [6,13,24,26].

Autophagy is a physiological process for degrading abnormal cytosolic macromolecules and organelles. It contributes essentially in maintaining cell metabolism and homeostasis [19]. Macroautophagy (referred as autophagy in this paper) is the most common form of autophagy. It is characterized by formation of cup-shaped preautophagosomal double membrane structure which surrounds cytoplasmic ingredients and closes to form the autophagosome. However, autophagy has also been suggested to play a role in the development of neurodegenerative diseases. It can trigger a unique cell death distinct from apoptosis and necrosis [15,19]. Recent evidences suggested that autophagy plays an important role in glutamate-mediated neuron toxicity [2,15,21], but the mechanism remains unclear.

The neuroprotective mechanisms of GRb1 against glutamate-induced neurotoxicity were related with lipid peroxidation, reducing calcium influx and antagonizing apoptosis [5,20]. To our knowledge, the effects of GRb1 on autophagy or autophagic cell death in glutamate-injury have not been investigated so far. In this study, we assessed autophagy in glutamate-injured neurons as well as the effects of GRb1 on this injury.

Standard GRb1 was obtained from Zelang Biotechnology (Nanjing, China). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Invitrogen (Carlsbad, CA, USA). The monoclonal rabbit-anti-LC3 and TRITC-goat-anti-rabbit IgG came from Cell Signalling Test (CST), and monoclonal mouse-anti-β-actin IgG was purchased from Santa Cruse Biotechnology (Santa Cruz, CA,

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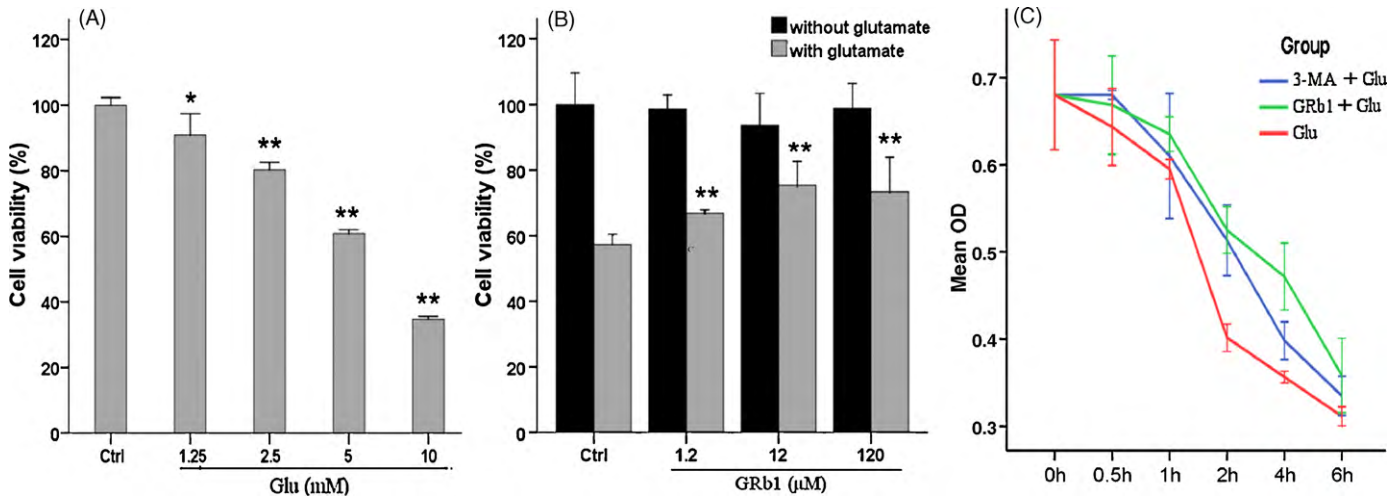

Fig. 1. Effects of GRb1 on glutamate-induced cortical neurons injury. Cell viability was evaluated by MTT assay. Optical density (OD) was observed and transformed to cell viability; mean percentage of cell survival was obtained by comparing with control group. (A) Cell viability was declined after glutamate (1.25, 2.5, 5, 10 mM, 2 h) treatment. (B) GRb1 at different concentrations (1.2, 12, 120 μM) could ameliorate neurons injury during glutamate (5 mM, 2 h) treatment. (C) GRb1 (12 μM) and autophagy inhibitor 3-MA (5 mM) preserved cortical neurons from glutamate (5 mM, 2 h)-induced cell death. Histograms represent mean ± S.D. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control. Glu = glutamate, GRb1 = ginsenoside Rb1, 3-MA = 3-methyladenine. $n = 12$ for each group.

USA), Rabbit-monoclonal Beclin-1 antibody came from Abcam Company (Cambridge, MA, USA). 3-Methyladenine (3-MA), poly-L-lysine (PLL), L-glutamate and MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] were purchased from Sigma Company (St. Louise, MO, USA). Before the subsequent treatment, GRb1 and glutamate were dissolved in DMEM, and for control, only DMEM was added to the culture medium.

Cortical neurons were prepared from new-born Sprague–Dawley (SD) rats according to the procedures described previously [3,21]. After trituration and trypsinization, the single-cell suspension was seeded in 6-well or 96-well plates coated with poly-L-lysine and incubated for 10 d in vitro, the time required for maturation of cortical neurons. Based on our preliminary experiment, in the subsequent experiments, glutamate (1.25, 2.5, 5, 10 mM) and GRb1 (1.2, 12, 120 μM) + glutamate (5 mM) or 3-MA (5 mM) + glutamate (5 mM) were added in cell culture medium, whereas only dissolved DMEM was given to the control group.

Cortical neurons were placed in 96-well plates and the cells viability was observed by MTT assay. Briefly, neurons were treated with different concentrations of glutamate (1.25, 2.5, 5, 10 mM) or GRb1 (1.2, 12, 120 μM) + glutamate (5 mM) or 3-MA (5 mM) + glutamate (5 mM) for 2 h. The effects of GRb1 (12 μM) and 3-MA (5 mM) on the survival of cortical neurons were also determined at different time points (0.5, 1, 2, 4, 6 h). After the above treatment, cortical neurons were incubated with 3-(4,5-dimethylthiazole -2-yl)-2,5-iphenyltetrazolium bromide (MTT, 0.5 mg/ml) for 4 h at 37 °C. After washing with PBS gently, the formed formazan crystals were dissolved in DMSO. Absorbance was determined at 570 nm using a microplate reader (Phenix Research Products, USA). The cell viability of the control group was defined by 100% and viability of other groups was calculated separately compared with the control group [30].

After treated with glutamate (5 mM) or GRb1 (12 μM) + glutamate (5 mM) for 2 h, cortical neurons were washed and harvested by centrifugation, cell aggregates were fixated and dissected into sections and fixed overnight. After dehydrated with cold alcohols, sections were rinsed in propylene oxide and sections were placed in embedding molds and placed in a 64 °C oven overnight. 70 nm thin sections were cut and then examined with a HITACHI H600 electron microscope.

To detect the level of autophagy after glutamate (5 mM, 2 h) and GRb1 (12 μM) + glutamate (5 mM, 2 h) treatment, the number of autophagic vacuoles (AVs) was calculated as described before [2,19], the mounted sections which were randomly sampled were placed under a copper grid (200 meshes), and every tenth grid was analyzed. Therefore, 20 grid sections per mounted sample were analyzed. And then three neurons per grid sections were randomly chosen to analyze the number of AV per cell.

After treated with GRb1 (12 μM) + glutamate (5 mM) or glutamate (5 mM) for 2 h, cortical neurons were fixed and incubated with rabbit-anti-LC3 (1:200) in 3% BSA for overnight at 4 °C, incubated with TRITC-goat-anti-rabbit IgG (1:300) for 2 h at 37 °C. The autophagy marked protein LC3 (red) and the cell nucleus (blue) were observed under a laser confocal fluorescence microscope (FV1000 Confocal Microscope, Olympus, Japan). The number of LC3 which characterized with red punctate feature was calculated as described previously [17]. $n = 6$ for each group.

Cortical neurons treated with glutamate (1.25, 2.5, 5, 10 mM, 2 h) or GRb1 (1.2, 12, 120 μM) + glutamate (5 mM) or glutamate (5 mM) or 3-MA (5 mM) + glutamate (5 mM) were harvested and incubated with lysis buffer. The mixtures were centrifuged at 12,000 × g for 5 min and the proteins (30–35 μg) in the supernatant were collected and separated by SDS-PAGE. Then proteins were transferred to PVDF membrane (Millipore) and then incubated with rabbit-anti-Beclin-1 (1:400) and mouse-anti-β-actin (1:400) in 3% BSA for overnight at 4 °C. After PBS washing, the PVDF membranes were incubated with goat-anti-rabbit IgG (1:4000) or goat-anti-mouse IgG (1:5000) in 3% BSA at 37 °C for 2 h. The protein was detected by chemiluminescence reagent (Pierce, USA) and visualized and calculated by using FluorChem FC2 system (Alpha Innotech, USA).

Parameters were described as mean ± SD. Differences were determined by one-way analysis of variance (ANOVA) and the least significant difference (LSD) post hoc tests. When time acts as a variance, differences were tested by ANOVA for repeated measures. Differences were considered significant when $p < 0.05$. All experiments were repeated more than three times, only representative diagrams are shown in this paper. The software package SPSS 16.0 was used for all statistical analysis.

As a result, exposure to increasing concentrations of glutamate yielded augmented neuronal cell injury. Cell viability declined from 90.9 ± 4.1% (Glu 1.25 mM, $p < 0.05$) to 34.8 ± 1.3% (Glu 10 mM, $p < 0.01$). The moderate concentration of glutamate-induced cell injury was observed at 5 mM (61.0 ± 1.0%, $p < 0.01$). Hence, 5 mM glutamate was chosen to perform the subsequent experiments