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Research Report

Salidroside attenuates glutamate-induced apoptotic cell death in primary cultured hippocampal neurons of rats

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ABSTRACT

Salidroside, a compound of natural origin, has displayed a broad spectrum of pharmacological properties. This study aimed to evaluate the inhibitory effects of salidroside on glutamate-induced cell death in a primary culture of rat hippocampal neurons as compared to brain-derived neurotrophic factor (BDNF), a usual positive control. MTT and LDH assays, together with Hoechst 33342 staining, terminal deoxynucleotidyl transferase dUTP-mediated nicked end labeling (TUNEL) assay and flow cytometric analysis using annexin-V and propidium (PI) label, indicated that salidroside pretreatment attenuated glutamate-induced apoptotic cell death in primary cultured hippocampal neurons, showing a dose-dependent pattern. Furthermore, caspase-3 activity assay and calcium measurements with Fluo 4-AM, respectively, revealed that salidroside pretreatment antagonized activation of caspase-3 and elevation of intracellular calcium level, both of which were induced by glutamate stimulation, thus adding to the understanding of how salidroside offered neuroprotection against glutamate excitotoxicity.

1. Introduction

Glutamate is a major excitatory amino acid neurotransmitter in the central nervous system (CNS) and its interactions with specific membrane receptors are responsible for many neurological functions, including cognition, memory, movement, and sensation. However, high concentration accumulation of glutamate in CNS and the resulting excessive stimulation of glutamate receptors induce potent neurotoxic action, which, specifically referred to as excitotoxicity, is involved in neuronal damage and degenerative disorders in CNS (Choi, 1988; Rothman and Olney, 1986; Shang et al., 2006). For the prevention and treatment of these diseases, the studies of neuroprotection against glutamate excitotoxicity, especially the search for neuroprotective drugs of natural origin, have attracted increasing research interests.

The hippocampus is responsible for many CNS functions including cognition, learning, and memory, but it is also one of the most vulnerable brain regions as regards to various neurological insults such as hypoxia–ischemia, seizure and prolonged stress. These insults contribute to excessive synaptic-glutamate accumulation, triggering a series of intracellular biochemical changes and finally inducing neuron degeneration even death (Wang et al., 2006). Based on these considerations, the primary cultured hippocampal neurons are commonly used as a culture system for the in vitro study of protection against glutamate-induced neurotoxicity.

Rhodiola rosea L. is a popular medicinal plant found in mountains at high altitudes and has long been used in traditional Tibetan medicine system as an adaptogen to enhance the body's resistance to fatigue and to extend human life. The plant displays a range of pharmacological

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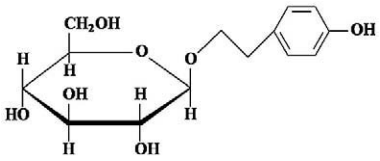

Scheme 1 – Chemical structure of salidroside (*p*-hydroxyphenethyl-β-D-glucoside).

properties, including anti-inflammation, anti-hypoxia, anti-oxidative, anti-aging, anti-cancer, and hepatoprotection activities (Díaz Lanza et al., 2001; Iaremii and Grigor'eva, 2002; Kanupriya et al., 2005; Kucinskaite et al., 2004). Salidroside, a compound with a chemical structure of phenol glycosides (see Scheme 1), is extracted from the root of *Rhodiola rosea* as one of its main active ingredients and responsible for all the documented pharmacological effects of the medicinal plant. Recently, salidroside was reported to be capable of protecting the PC12 cells against glutamate-induced excitotoxicity (Cao et al., 2006) or protecting SH-SY5Y human neuroblastoma cells against H_2O_2-induced cell apoptosis (Zhang et al., 2007). We have also found that salidroside protects PC12 cells against hypoglycemia/serum limitation-induced cytotoxicity in a previous report (Yu et al., 2008).

In order to provide a new window into the pharmacological properties of salidroside, the present study was designed to investigate neuroprotection of primary cultured hippocampal neurons of rats, induced by salidroside, against glutamate-induced neurotoxicity, another cell insult different from hypoglycemia/serum limitation-induced cytotoxicity. We hope to expand the understanding of the potential therapeutic value of salidroside for cerebral neurodegenerative diseases.

2. Results

2.1. *Effects of salidroside pretreatment on glutamate-induced decrease of cell viability in hippocampal neurons*

MTT assay revealed the dose-dependent excitotoxicity of glutamate (31.25–500 μM) on cultured hippocampal neurons (Fig. 1A). In subsequent experiments, an exposure to 125 μM glutamate for 15-min was used to induce cell insult. As illustrated in Fig. 1B, glutamate stimulation decreased the cell viability in hippocampal neurons to 69.31±1.49%, and salidroside at very low concentrations (e.g. 30 μM) was not effective for neuroprotection. Salidroside at 60 or 120 μM, however, significantly prevented cultured hippocampal neurons from glutamate-induced damage, and restored the cell survival to 75.33±2.08 or 84.18±1.28%, respectively, displaying dose-dependent protective effects.

On the other hand, glutamate stimulation significantly increased LDH release of hippocampal neurons from 18.66±1.53 to 43.64±3.05%. The protective effect of salidroside was shown by the changes in LDH release, with the most pronounced effect occurring at 120 μM salidroside that led to the decrease in LDH release from 43.64±3.05 to 36.70±8.94% (Fig. 1C). Brain-derived neurotrophic factor (BDNF, 100 ng/ml), as a usual positive control, also significantly inhibited glutamate-induced cytotoxicity according to either MTT or LDH assay (Figs. 1B and C). In addition, salidroside pretreatment alone resulted in neither cell viability loss nor LDH release change in hippocampal neurons (Figs. 1B and C).

The light micrographs confirmed the neuroprotective effects of salidroside. The untreated hippocampal neurons (control) exhibited uniformly dispersed chromatin, normal organelles and intact cell membrane. After exposure to glutamate hippocampal neurons exhibited a significant cell insult evidenced by the disappearance of cellular processes, and decrease of the refraction. However, the cell damage in cultured hippocampal neurons was greatly antagonized by salidroside pretreatment according to micrographic observation (Fig. 1D).

Taken together, the results collectively suggest that salidroside pretreatment attenuates glutamate-induced mitochondrial dysfunction and cell membrane damage in cultured hippocampal neurons.

2.2. *Effects of salidroside pretreatment on glutamate-induced apoptosis of hippocampal neurons*

Considering that MTT or LDH release assay failed to distinguish between necrosis and apoptosis, we carried out morphological examinations for determining the type of cell death induced by glutamate. Hoechst staining showed that after the excitotoxic insult of 125 μM glutamate, about 30–35% of hippocampal neurons displayed an apoptotic morphology, characterized by the condensation of chromatin, the nuclear shrinkage, and the formation of a few apoptotic bodies. Pretreatment with 60, 120 μM of salidroside or 100 ng/ml BDNF, however, reduced the excitotoxic effect of glutamate on hippocampal neurons by about 30%, 54% and 60%, respectively (Figs. 2A and B).

Fig. 1 – Cell viability determined using the conventional MTT assay and LDH release assay. Cell viability was assessed 18 h after glutamate stimulation. Although salidroside alone caused no significant cytotoxicity compared to control ($P>0.05$), salidroside attenuated glutamate-induced reduction in cell viability of cultured hippocampal neurons. (A) Dose-dependent cytotoxic effects of glutamate on the cell viability of hippocampal neurons. $^{*}P<0.05$, $^{**}P<0.01$ vs control. (B) Effects of salidroside on the cell viability of hippocampal neurons after exposure to glutamate stimulation. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs glutamate stimulation alone. $^{\#\#\#}P<0.001$ vs control. (C) Effects of salidroside on LDH release of hippocampal neurons after exposure to glutamate stimulation. Here the LDH release percentage was calculated using the formula: (absorbance of sample ÷ absorbance of maximum enzyme activity)×100. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs hippocampal neurons stimulated with glutamate alone. $^{\#\#\#}P<0.001$ vs control. (D) The light micrographs showing the cell morphology of hippocampal neurons. All data were expressed as mean±S.D. of four experiments and each included sextuplet.