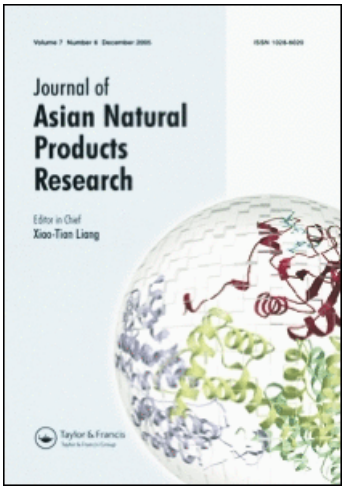

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The effect of salidroside on cell damage induced by glutamate and intracellular free calcium in PC12 cells

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Salidroside (Sald), was extracted from *Rhodiola rosea* L, a traditional Chinese medicine which has been used for long time for anti-aging, anti-cancer and anti-oxidative stress etc. In present experiment, salidroside could protect the PC12 cell against injuries caused by exposure of PC12 cells to 2 mmol/L glutamate for 15 min followed by incubation with serum-free medium for 24 h, which resembled the excitotoxin *in vivo* system. Furthermore, saldroside could decrease the $[Ca^{2+}]i$ of PC12 cells in Mg^{2+}-free Hanks' solution and D-Hanks' solution but there was no effect on basal $[Ca^{2+}]i$ in Hanks' solution. The studies also indicated that salidroside inhibited the increases of $[Ca^{2+}]i$ induced by KCl and glutamate. In conclusion, salidroside may protect PC12 cell against glutamate excitotoxic damage through suppressing the excessive entry of Ca^{2+} and the release of the calcium stores.

Keywords: Glutamate; Excitotoxicity; Salidroside; Calcium

1. Introduction

Salidroside (Sald) is extracted from Rhodiola rosea L, which has been used for a long time as a Chinese traditional medicine and was reported to have pharmacological properties including anti-aging and anti-oxidative stress [1]. Nevertheless, little was known about the protective effect of salidroside on neuronal cell.

The PC12 cell line was initially derived from a rat adrenal medullary pheochromocytoma. When grown in a serum-containing medium, PC12 cells divide and resemble precursors of adrenal chromaffin cells and sympathetic neurons. Upon addition of the nerve growth factor (NGF), cells gradually attain the phenotypic properties of sympathetic neurons [3]. It has been demonstrated that there are T-type, N-type and L-type voltage gated Ca^{2+} channels and NMDA receptor in differentiated PC12 cells [4]. Thus, the PC12 cell line is used as a neuronal cells model in order to study injury and protection of neuronal cells.

Glutamate is the primary excitatory amino acid in mammal brain. Glutamate excitotoxic damage plays a key role in nerve cell death involved in neurodegenerative disorders [2].

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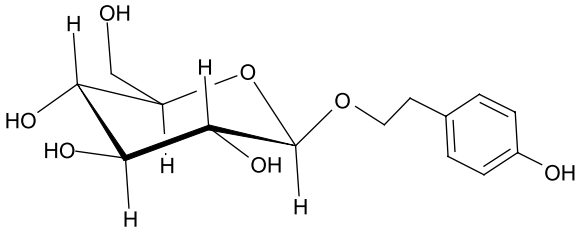

Figure 1. Chemical structure of saldroside.

In present paper, the protective effects of salidroside on PC12 cell against glutamate excitotoxic damage and possible mechanism were investigated (figure 1).

2. Results and discussion

2.1 Toxicity of glutamate in cultured PC12 cell

By using MTT assay test upon incubation of PC12 cells with different glutamate concentrations in the range of 1 mmol/L–5 mmol/L, dose-dependent glutamate toxicity was examined. As shown in Figure 2, glutamate induced a decrease in cell survival in a dose-dependent manner.

2.2 Change of the cell morphology

The cell morphology of PC12 cells was observed by microscope after treatment with glutamate. There was significant injury in PC12 cells after treatment with 2 mmol/L glutamate, exhibiting disappearance of cellular processes, decrease of the refraction and falling to pieces. The damage in group of salordside pretreated cells was greatly decreased. (see figure 3).

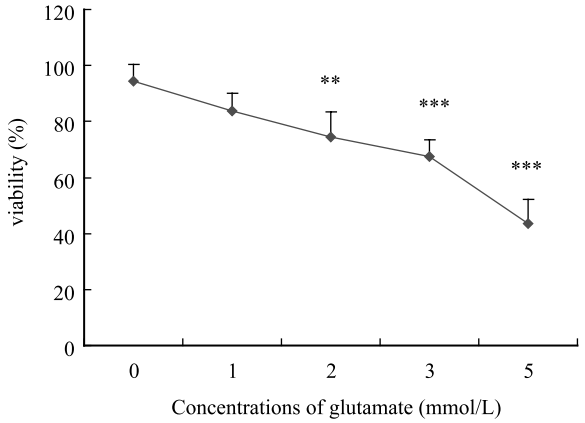

Figure 2. Dose-dependent cytotoxic effect of PC12 cells. PC12 cells were exposed in increasing glutamate concentrations (1 mmol/L-5 mmol/L) for 15 min. Then, the cells were switch to fresh medium without fetal bovine serum. Cell viability was measured using MTT. Data are the mean ± SD, $n = 4$, *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, analyzed by Student's t-test.

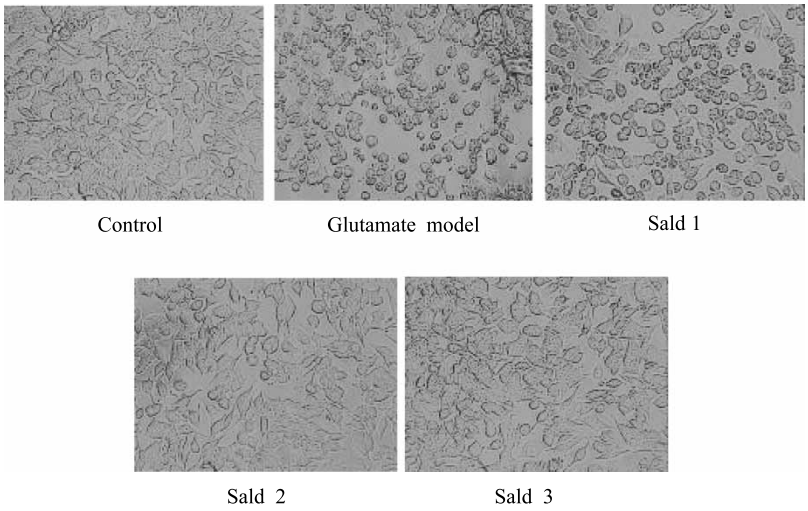

Figure 3. Effect of salidroside on glutamate-induced change of cell morphology (× 250). Sald 1: 10^{-7} mol/L salidroside, Sald 2: 10^{-6} mol/L salidroside, Sald 3: 10^{-5} mol/L salidroside.

2.3 Effect of salidroside on cell viability

The exposure of PC12 cells to glutamate 2 mmol/L for 15 min followed by incubation with serum-free medium for 24 h produced an obvious decrease in cell viability measured by MTT. Pretreated with different concentration (10^{-5} mol/L and 10^{-6} mol/L) of salidroside, the cell damage was greatly decreased (figure 4).

2.4 LDH efflux assay

After treatment as mention above, the extracellular LDH in medium in glutamate treated group was significantly increased. Salidroside could inhibit the excessive LDH efflux

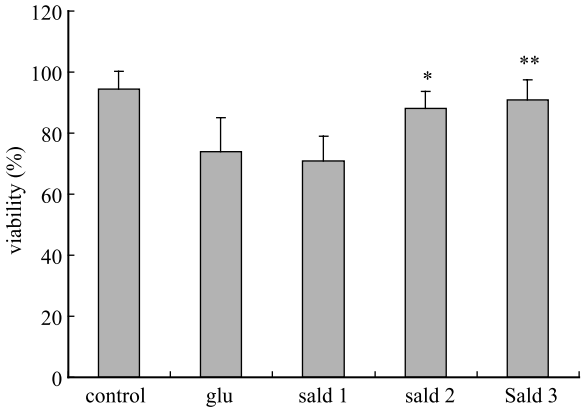

Figure 4. Neuroprotective effect of Salidroside against glutamate neurotoxicity PC12 cells were treated with glutamate for 15 min, then incubated with different concentrations of Salidroside in the fresh medium for 24 h. Control and glutamate-treated cells were analyzed for cell viability using MTT. Data were expressed as the mean ± SD, n = 4. *$P < 0.05$. ** $P < 0.01$(Sald 1: 10^{-7} mol/L salidroside, Sald 2: 10^{-6} mol/L salidroside, Sald 3: 10^{-5} mol/L salidroside).

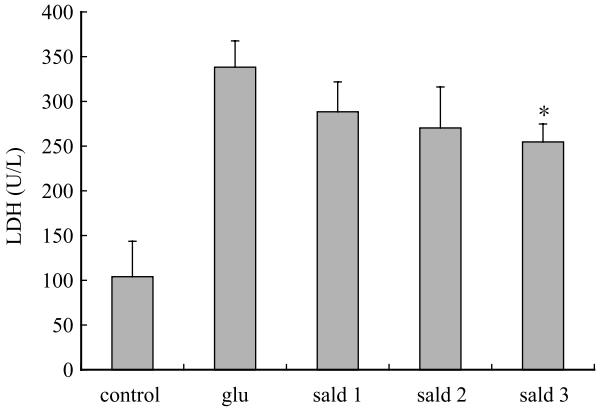

Figure 5. Effect of Salidroside on LDH release. PC12 cells were treated with glutamate for 15min, then, cells were incubated with different concentrations of Salidroside in the fresh medium. LDH release was assessed after 24 h. Data were expressed as the mean ± SD, n = 4. *P < 0.05 (Sald 1: 10^{-7} mol/L salidroside, Sald 2: 10^{-6} mol/L salidroside, Sald 3: 10^{-5} mol/L salidroside).

(figure 5). At the concentration of 10^{-5} mol/L salidroside, the LDH efflux was decreased by 24.7% than glutamate treated group.

2.5 Effect of salidroside on basal [Ca^{2+}] i of PC12 cell

The intracellular free calcium concentration ([Ca^{2+}]i) in PC12 cells was measured with Fura-2. The results showed that [Ca^{2+}]i of PC12 cells in Hank's solution was 114.4 ± 17.1 nmol/L and there was no effect of salidroside (10^{-5} mol/L) on the [Ca^{2+}]i. But salidroside could decrease the [Ca^{2+}]i of PC12 cells in Mg^{2+}-free Hanks' solution from 158.3 ± 18.7 nmol/L to 132.8 ± 11.1 nmol/L(decreased by 16.1%) and the [Ca^{2+}]i of PC12 cells in D-Hanks' solution from 93.2 ± 14.6 nmol/L to 76.0 ± 6.9 nmol/L(decreased by 18.4%) (figures 6 and 7).

2.6 Effect of salidroside on increase of [Ca^{2+}]i induced by KCl and glutamate

KCl (100 mM) markedly evoked [Ca^{2+}]i in PC12 cells in Hanks' solution from 117.7 ± 17.4 nmol/L to 221.7 ± 20.2 nmol/L (increased by 88.3%), and the rises was blocked by 10^{-5} mol/L and 10^{-6} mol/L salidorside. The results showed that salidorside inhibited the [Ca^{2+}]i increase which was stimulated by glutamate (2 mmol/L) in Mg^{2+}-free Hanks' solution in a concentration-dependent manner.

Glutamate is a major excitatory neurotransmitter in central nervous system and it may become a potent excitotoxin and contribute to the generation of neurodegeneration disease such as Alzheimer's disease, Parkinson's disease and Huntington's disease under some pathological condition [5]. The estimated content of glutamate intracellular in the brain is 10 mmol/L [6]. Attwell et al. have estimated the extracellular concentration of glutamate to be about 0.6 μmol/L [7]. In this experiment, the exposure of PC12 cells to 2 mmol/L glutamate for 15 min followed by incubation with serum-free medium for 24 h produced a late neurotoxin which resembled the excitotoxin in vivo system. The result indicated that salidroside protected PC12 cell against glutamate excitotoxic damage. Excitotoxicity is Ca^{2+}-dependent, and Ca^{2+}-over loading may be the most important factor that induces the

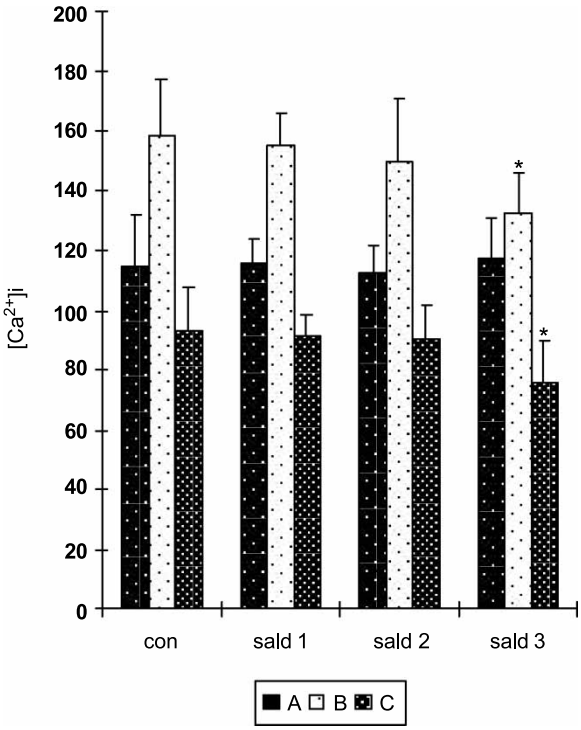

Figure 6. Effect of salidroside on basal $[Ca^{2+}]$ i of PC12 cell. A: Hanks' solution; B: Mg^{2+}-free Hanks' solution; C: D-Hanks' solution. No effect on $[Ca^{2+}]$i with different concentrations of salidroside in Hanks' solution was found; 10^{-5} M Salidroside could decrease the $[Ca^{2+}]$i of PC12 cells in Mg^{2+}-free Hanks' and D-Hanks' solution. Data were expressed as the mean ± SD, n = 4. *P < 0.05 (Sald 1: 10^{-7} mol/L salidroside, Sald 2: 10^{-6} mol/L salidroside, Sald 3: 10^{-5} mol/L salidroside).

death of neuronal cell [8]. So we measured the intracellular free calcium concentration ($[Ca^{2+}]$i) in PC12 cells with Fura-2 double-wavelength fluoremetry to investigate the mechanism of the protective effect of salidroside against glutamate excitotoxic damage. The increase in $[Ca^{2+}]$i is mediated by calcium entry through the NMDA receptor-associated

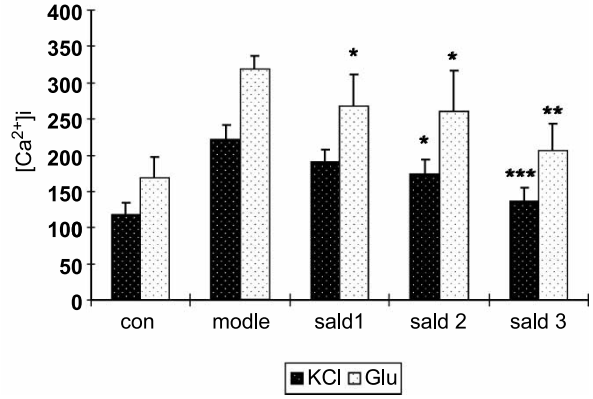

Figure 7. Effect of Salidorside on $[Ca^{2+}]$i increase stimulated by KCl and Glu. KCl: Fura-2-loaded cells by pretreatment with Sald or not were exposed to medium containing 100 mmol/L KCl. Glu: Fura-2-loaded cells by pretreated with Sald or not were exposed to medium containing 200 μmol/L GLU; Bars represent the mean ± SD of four separate experiments. *P < 0.05, ** P < 0.01, ***p < 0.001 (Sald 1: 10^{-7} mol/L salidroside, Sald 2: 10^{-6} mol/L salidroside, Sald 3: 10^{-5} mol/L salidroside).

channel and voltage-dependent channel. Excessive glutamate triggering an excessive entry of Ca^{2+} through the NMDA receptors initiates a series of cytoplasmic and nuclear processes that promote neuronal cell death. The experiment shows that $[Ca^{2+}]i$ elevation produced by K^+ depolarization was blocked by the pretreatment with salidroside, suggesting that the drug may inhibit the voltage-dependent channel. How salidroside showed significant effect on the increase of $[Ca^{2+}]i$ induced by glutamate may be related with 2 factors. One is that glutamate can't active the NMDA receptor further to form Ca^{2+}-over loading because salidroside inhibit the potential-dependent channel; the other, NMDA receptors are associated with a high-conductance Ca^{2+} channel that in resting, non-depolarising conditions is blocked by Mg^{2+} in a potential-dependent manner [9]. The result showed that Salidroside can decrease the $[Ca^{2+}]i$ of PC12 cells in Mg^{2+}-free Hanks' solution, which was higher than in the Hanks' solution, suggesting salidroside may have direct effect on NMDA receptor. Salidroside could decrease the $[Ca^{2+}]i$ of PC12 cells in Ca^{2+}-free D-Hanks solution indicated that Salidroside may have effect on release of the calcium stores. In conclusion, Salidroside may protect PC12 cell against glutamate excitotoxic damage through decreasing the excessive entry of Ca^{2+} and the release of the calcium stores, but the material mechanism need to be made sure further.

3. Experimental

3.1 Drugs and reagents

Salidroside was obtained from National Institute for the Control of Pharmaceutical and Biological Products. MTT, bovine serum albumin, EGTA and Triton X-100 were purchased from Sigma. RPMI-1640 was Gibico product. Fura-2/AM was Bio Team Laboratories Inc product. The assay kit of lactate dehydrogenase was purchased from Beijing chemistry Co. All other reagents were analytical reagents.

3.2 Cell culture and treatment

PC12 cells were grown on polystyrene tissue culture dishes in RPMI-1640 supplemented with 10% horse serum, 5% fetal bovine serum, and 100 unit/ml penicillin, 100 ug/ml streptomycin in 37°C incubator in 5% CO_2 in a humidified atmosphere. The medium was changed every 2–3 days, and PC12 cells were incubated in serum-free RPMI-1640 medium for 24 h before treatment with glutamate [10].

Cells were plated onto 96-well plate (1×10^5 cells/ml) and switched to Mg^{2+}-free-Earle's solution (mmol/L: NaCl 142.6, KCl 5.4, $CaCl_2$ 1.8, NaH_2PO_4 1.0, HEPERS 2.38, Glucose 5.6, pH7.4) containing different concentration of glutamate from 1 mmol/L to 5 mmol/L. After incubation for 15 min, the cells were switched to fresh serum-free medium with different concentration of salidroside or not, then were incubated for 24 h [11].

3.3 MTT assay

After culturing for 24 h, MTT (3-[4,5-dimethy thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (0.4 mg/ml) was added and incubated for 4 h at 37°C and

centrifuged at 400 × g for 5 min.The resulting supernatant was discarded and 100 ul of DMSO was added. After shaking the plates for 10 min, the optical density was immediately measured at 540 nm [12].

3.4 LDH efflux assay

LDH efflux assay was performed according to direction of assay kit. As a quantitative measure of cellular toxicity, the LDH efflux was estimated in 10 μL culture medium. The activity of LDH was determined spectrophotometrically.

3.5 Measurement of intracellular [Ca^{2+}]i [13]

The suspended cells were loaded with Fura-2/AM (5 μmol/L) at 37°C for 40 min, then rinsed with Hanks' solution (g/L: NaCl 8, KCl 0.4, KH_2PO_4 0.06, $MgCl_2{\cdot}6H_2O$ 0.1, $MgSO_4{\cdot}7H_2O$ 0.1, $CaCl_2$ 0.14, $NaHCO_3$ 0.35, $Na_2HPO_2{\cdot}7H_2O$ 0.09, Glucose 1) Mg^{2+}-free Hanks' solution or D-Hanks' solution containing 0.2% bovine serum albumin (g/L: NaCl 8, KCl 0.4, KH_2PO_4 0.06, $NaHCO_3$ 0.35, $Na_2HPO_2{\cdot}7H_2O$ 0.09, Glucose 1). The density of cell was 2×10^6 cell/ml. The intracellular [Ca^{2+}]i was determined by fluorescene measurement at ex 340 nm, 380 nm and the em 510 nm. TritonX-100 was added to measure the maximal fluorescene value and EGTA was added to measure the minimal fluorescence value. [Ca^{2+}]i was calculated according to formula:

$$[Ca^{2+}] = KDa \times (R - Rmin)/(Rmax - R) \times (F_{f2}/F_{b2}), \quad R = F340/F380.$$

R = F340/F380, Rmin = Fmin340/Fmin380, Rmax = Fmax340/Fmax380, KDa = 224 nmol/L, and F_{f2}/F_{b2} = Fmin380/Fmax380.

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