Ginsenoside Rg_1 is an Anti-apoptotic Agent

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ABSTRACT

Primary neuronal culture was studied for observing effect of ginsenoside Rg_1 (Rg_1) on serum-free medium induced apoptosis. Results showed that Rg_1 at concentration of 1 μmol · L^{-1} and 10 μmol · L^{-1} could inhibit apoptosis, decrease intracellular calcium concentration in cultured cortical neurons, enhance SOD activity in both aged rat cortex and cultured cortical neurons, scavenge cytotoxic oxygen free radicals, decrease NO content and NOS activity in aged rat cortex and cultured cortical neurons, increase bcl-2 gene expression in rat brain. These results provided new data for elucidating the anti-aging effect of Rg_1. Rg_1 is considered to be a useful drug for treatment of Alzheimer's disease and brain aging.

Introduction

Ginseng has been used in China as a tonic in chinese traditional medicine for two thousad years. Ginsenoside Rg_1 (Rg_1) is one of the important active principles of ginseng. In our previous studies, Rg_1 was shown to facilitate learning and memory[1], increase central cholinergic function[2], improve immunity function and alleviate many other ailments associated with aging[3]. In order to elucidate the anti-aging mechanism of Rg_1, we concentrated our research on the relationship among aging and apoptosis, NO, NOS, Ca^{2+} and gene expression involved. The experimental results are as follows.

Methods and Results

Effect of Rg_1 on neuronal apoptosis

Apoptosis or programmed cell death (PCD) is the process by which a cell will actively commit suicide under tightly controlled circumstances. PCD is required for maintaining homeostasis and is essential in many aspects of development, differentiation, immunity and aging[4,5].

Primary neuronal culture was performed with different concentrations of Rg_1. On the 14th day, the culture was changed to serum-free medium. On the 16th day, neurons were harvested and assayed under microscope and fluorescence microscope. The result showed that serum withdrawal from the medium could induce apoptosis in primary cultured cortical neurons. It was found that Rg_1 could increase the ability to exclude Trypan blue, decrease the release of LDH, decrese the cleavage of

DNA paralleling the morphological changes of the nucleus. Rg_1 ($1\mu mol \cdot L^{-1}$ and $10\mu mol \cdot L^{-1}$) was shown to inhibit apoptosis and protect neurons against injury (Tab 1, 2 & Fig 1, 2).

Table 1. Primary neuronal culture was performed with different concentrations of Rg_1. On the 14th day, the culture was changed to serum-free medium. On the 16th day, neurons were harvested and assayed under microscope and fluorescence microscope. Number of necrotic and apoptotic neurons was calculated by trypan blue exclusion assay and Hoechst 33342 & PI stained method.

Group	Apoptotic cells(%)	Necrotic cells(%)
Control A (complete medium)	2.2 ± 0.9	1.4 ± 0.3
Control B (serum-free medium)	94.9 ± 3.6##	7.2 ± 1.0##
Rg_1 0.1 umol • L^{-1}	90.6 ± 3.2	6.8 ± 0.8
Rg_1 1 umo • L^{-1}	56.7 ± 2.1**	4.0 ± 0.9**
Rg_1 10 umol • L^{-1}	20.4 ± 0.7**	2.5 ± 0.4**

n=6, $\bar{x} \pm s$. ##P<0.01 *vs* Control A, **P<0.01 *vs* Control B

Table 2. Inhibitory effect of Rg_1 on LDH release. The activity of LDH released into culture medium was measured with LDH kit. The released LDH activity was expressed as the percentage of total LDH activity released from freeze-thawed sister cultures.

Group	LDH release(%)
Control A (complete medium)	5.160 ± 1.442
Control B (serum-free medium)	22.507 ± 4.782##
Rg_1 0.1 umol • L^{-1}	24.487 ± 4.761
Rg_1 1 umol • L^{-1}	18.855 ± 4.306**
Rg_1 10 umol • L^{-1}	10.735 ± 3.000**

n=6, $\bar{x} \pm s$. ##P<0.01 *vs* Control A, *P<0.05, **P<0.01 *vs* Control B

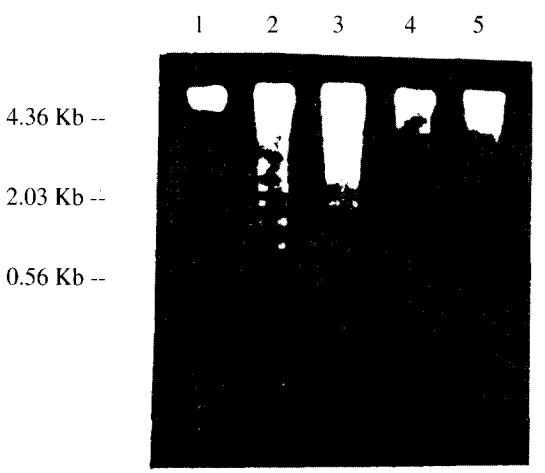


Fig. 1. DNA fragmentation in cortical neurons. Primary culture was performed and neurons were treated with different concentrations of Rg_1. On the 14th day, the medium was changed to the serum-free medium. On the 16th day, neurons were harvested. DNA extraction and electrophoresis were performed with the method described in the test. (1) Control A (complete medium). (2) Control B (serum-free medium). (3) Rg_1 0.1 · umolL^{-1}. (4) Rg_1 1 · umolL^{-1}. (5) Rg_1 10 · umolL^{-1}

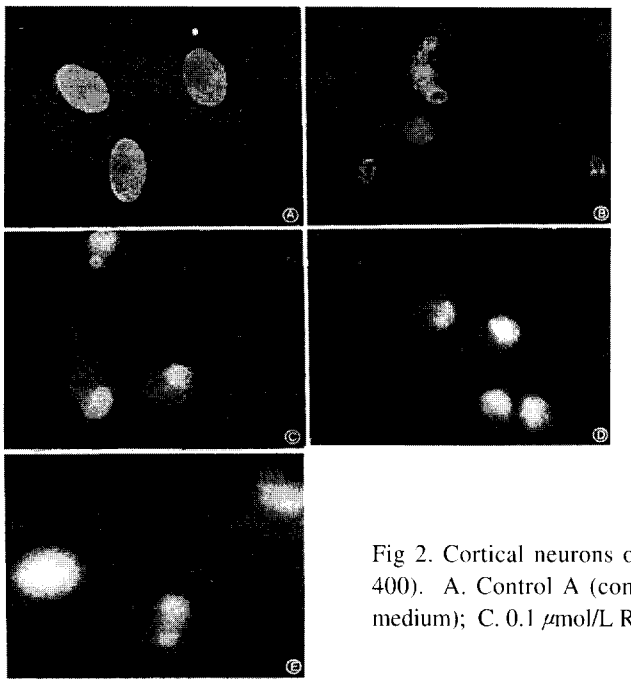


Fig 2. Cortical neurons observed under fluorescencemicroscope (× 400). A. Control A (complete medium) ; B. Control B(serum-free medium); C. 0.1 μmol/L Rg_1; D. 1 μmol/L Rg_1; E. 10 μmol/L Rg_1

Effect of Rg_1 on NO content and NOS activity

Nitric oxide (nitrite) and nitric oxide synthase (NOS) activity were measured in cerebral cortex isolated from Wistar rats of three age groups (young: 3 months; adult: 9 months; and old: 27 months). No significant differences in NO content and NOS activity between young and adult rats were found (P>0.05). The NO content and NOS activity in old rats were shown to be significantly higher than those of young and adult rats (P<0.01). When treated with Rg_1 (10, 20, 40 mg kg^{-1}), the

Table 3. Effect of age and Rg_1 on NO content and NOS activity in the rat cerebral cortex. Rats received Rg_1 0, 10, 20, 40, mg · kg^{-1} ip for 5 d. On the 5th day, 1h after ip Rg_1, rats were decapitated, and the cerebral cortexes were dissected on ice. NO was measured with Griess reaction. NOS activity was measured with the method described in the text.

Group	NO content (umol · L^{-1})	iNOS (fmol · mg^{-1} · min^{-1})	cNOS (fmol · mg^{-1} · min^{-1})
Young	1.5493 ± 0.1489	8.032 ± 0.914	234.38 ± 16.24
Adult	1.7057 ± 0.3673	7.850 ± 1.640	239.60 ± 18.90
Old	2.6112 ± 0.1001$^{**\#}$	9.522 ± 1.790	398.22 ± 21.62$^{**\#}$
Old + Rg_1 10mg · kg^{-1}	2.3946 ± 0.2319	10.195 ± 1.953	393.14 ± 16.06
Old + Rg_1 20mg · kg^{-1}	2.0071 ± 0.2315*	9.382 ± 1.004	330.85 ± 27.64*
Old + Rg_1 40mg · kg^{-1}	1.7963 ± 0.1568**	9.497 ± 1.342	279.65 ± 18.30**

n=6, $\bar{x} \pm s$. $^{\#}P<0.01$ *vs* young rats; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* adult rats; P<0.05, P<0.01 *vs* old rats; $^{*}P<0.05$, $^{**}P<0.01$ *vs* old rats.

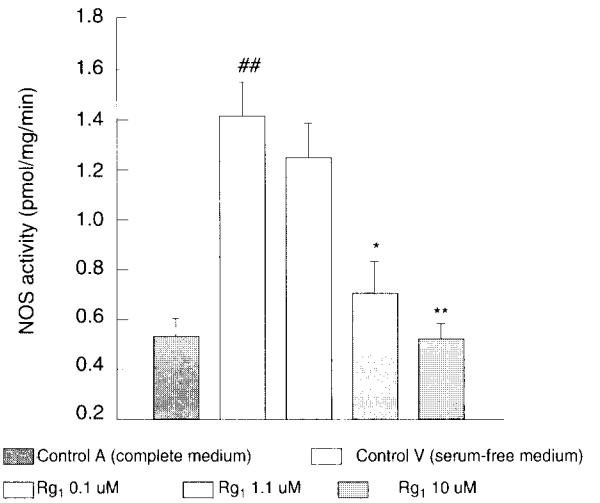


Fig. 3. Inhibitory effect of Rg_1 on NOS activity.
n=6, $\bar{x} \pm s$. ##P<0.01 *vs* Control A; *P<0.05, **P<0.01 *vs* Control B.

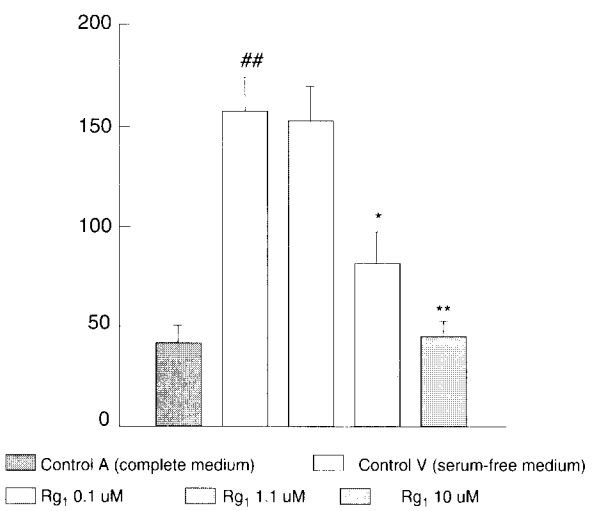


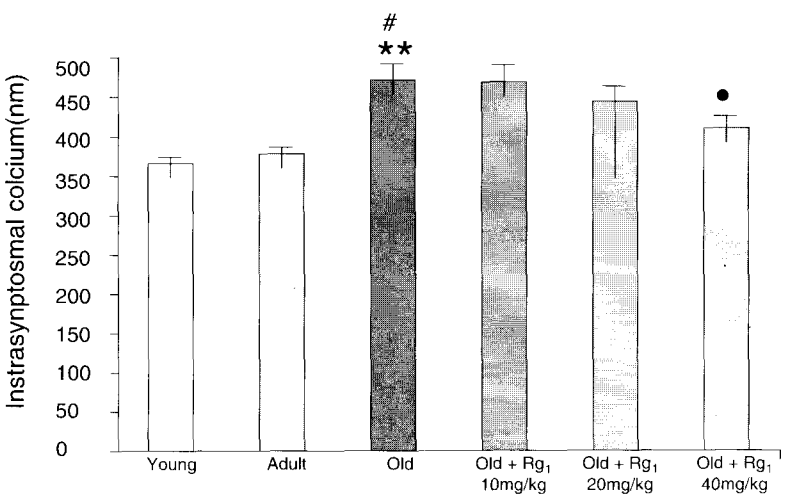
Fig. 4. Inhibitory effect of Rg_1 on NO release.
n=6, $\bar{x} \pm s$. ##P<0.01 *vs* Control A; *P<0.05, **P<0.01 *vs* Control B.

NO content and NOS activity in old rats decreased. The inhibitory effect of Rg_1 on NOS was found to be dose-dependent in the range of 10-40mg · kg^{-1}. The optimal reduction in NO content and NOS activity induced by Rg_1 occurred at 40 mg · kg^{-1} for old rats ($P<0.01$) (Tab 3, Fig 3). Rg_1 in the same concentration as mentioned above could inhibit NO release in cultured cortical neurons (Fig 4). In view of the close correlation of NO content and NOS activity with aging, the inhibitory effect of Rg_1 on NOS activity, as shown by our results, might provide an explanation for its anti-aging function.

We studied the relationship between nitric oxide (NO) and apoptosis. The concentration of NO^{2-} in culture supernatants was measured with Griess reagent. Nitric Oxide Synthase (NOS) activity was measured with ^{3}H-citrulline assay. It was found that NO concentration increased significantly after serum withdrawal, and NOS activity of apoptotic neurons was distinctly higher than control neurons. Rg_1 could decrease NO content and NOS activity in apoptotic neurons.

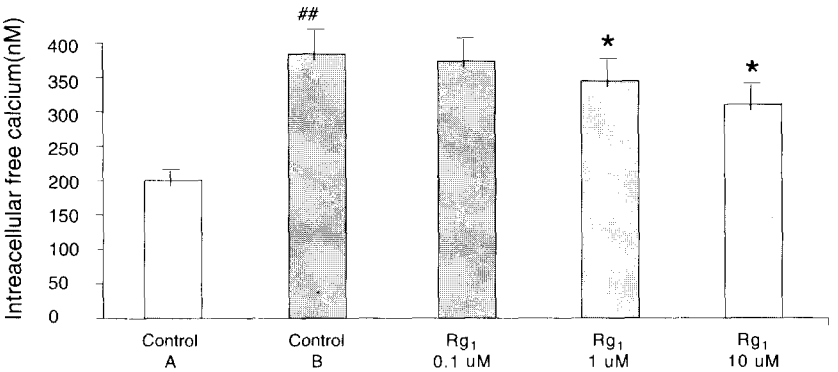
Effect of Rg_1 on intrasynaptosomal calcium concentration

Intrasynaptosomal calcium concentration was measured using double wavelength fluorescence spectrophotometer in cortex isolated from Wistar rats of three age groups (young: 3 months; adult: 9 months; and old : 30 months). Synaptosomes were loaded with the fluorescent dye, Fura-2/AM. Intrasynaptosomal free calcium concentration in old cortex was shown to be significantly higher than that in young and adult cortex. When treated with Rg_1 (10, 20, 40 mg/kg), the calcium concentration of old cortex decreased (Fig 5).



n=6, $\bar{x} \pm s$. **P<0.01 *vs* young rats; #p<0.05 *vs* adult rats; · p<0.05 vs old rats.

Fig. 5. Effect of age and Rg_1 on cortical intrasynaptosomal calcim concentration



n=6, $\bar{x} \pm s$. ##P<0.01 *vs* Control A; *p<0.05 *vs* Control B

Fig. 6. Effect of age and Rg_1 on cultured neuronal calcium concentration

Free calcium concentration was measured in cultured cortical neurons of five groups: control (complete medium); apoptosis (serum-free medium); Rg_1 0.1μM; Rg_1 1μM; Rg_1 10 μM. Neurons were enzymatically isolated and loaded with Fura-2/AM. Free calcium concentration in apoptotic cells was shown to be significantly higher than that in control cells. When treated with Rg_1 (0.1, 1, 10 μM), the calcium concentration in cultured neurons decreased (Fig 6). These findings indicate that there is a close relationship between calcium concentration and apoptosis. Rg_1 could decrease the calcium concentration in neurons so as to protect them from apoptosis.

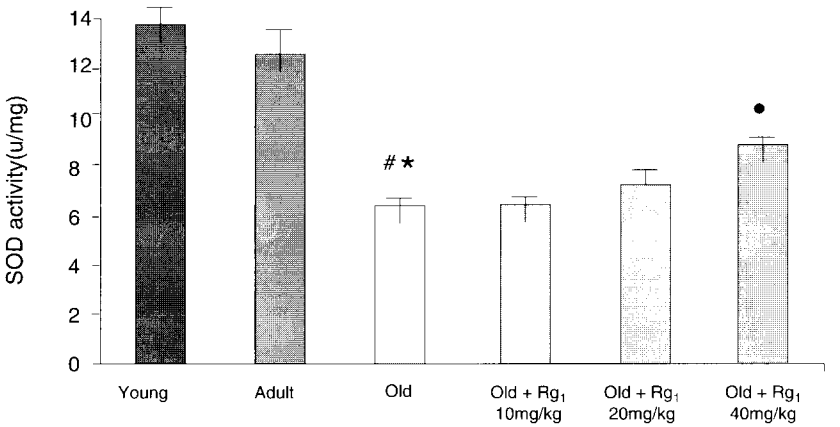
Effect of Rg_1 on neuronal SOD activity

SOD activity was measured in cerebral cortex isolated from Wistar rats of three age groups (young : 3 months; adult: 9 months; and old : 30 months). SOD activity in old cortex was shown to be significantly lower than that in young and adult cortex. There is no significant difference in SOD activity between young and adult cortex. Rg_1 could increase the SOD activity in old rat's cerebral cortex (Fig 7).

SOD activity was measured in cultured cortical neurons of five groups: control (complete medium); apoptosis (serum-free medium); Rg_1 0.1 μM; Rg_1 1μM; Rg_1 10 μM. It was found that SOD activity in apoptotic neurons was significantly lower than that in control neurons. Rg_1 could increase the SOD activity in cultured neurons (Fig 8).

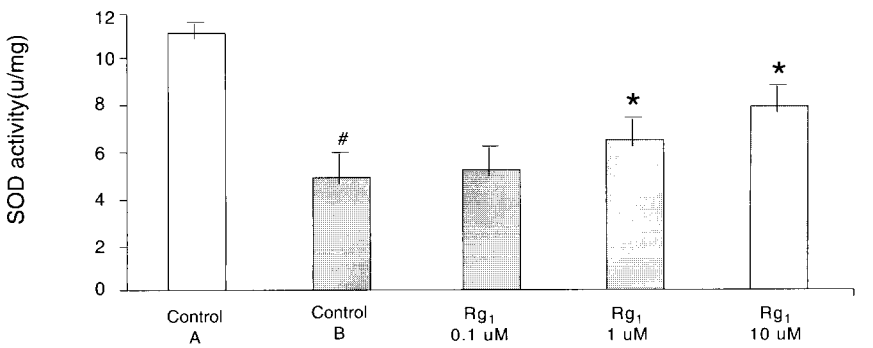
Effect of Rg_1 on free radicals

Using xanthine-xanthine oxidase system and the fenton reaction, the effect of scavenging oxygen



n=6, $\bar{x}$ ±s. *P<0.05 *vs* young rats; #p<0.05 *vs* adult rats; p<0.05 vs old rats.

Fig. 7. Effect of age and Rg_1 on cortical SOD activity



n=6, $\bar{x}$ ±s. #p<0.05 *vs* Control A; *p<0.05, **p<0.01 *vs* Control B

Fig. 8. Effect of Rg_1 on cultured neuronal SOD activity

free radical by Rg_1 was measured with electron spin resonance spectroscopy (ESR). The result indicated that the scavenging rate of hydroxyl radical of Rg_1 100 mM is 83.61%, and that of superoxide anion is 16.09%. The scavenging rate of hydroxyl radical of Rg_1 10 mM is 31.14% and that of superoxide anion is 5.14%. It suggested that Rg_1 (low concentration) may scavenge oxygen free radical through indirect way.

Effect of Rg_1 on bcl-2 and cNOS gene expression

Expression of bcl-2 and cNOS gene in rat brain was detected with *in situ* hybridization. Bcl-2 mRNA expression was found is young rat hippocampus, but not in aged rat brain. When treated with Rg_1 40 mg/kg for 5 days, there could be found significantly bcl-2 expression in aged hippocampus. This may give a clue to elucidate the anti-aging activity of Rg_1.

cNOS expression was not found in young rat brain, but it was found in aged rat hippocampus. When treated with Rg_1 40 mg/kg for 5 days, cNOS expression in aged rat hippocampus was significantly decreased. The result suggested that Rg_1 could decrease cNOS gene expression so as to lessen cNOS activity.

Discussion and Summary

In our *in vitro* and *in vivo* studies, aging or withdraw of trophic factors resulted in decrease of bcl-2 gene expression and increase of cNOS gene expression. Meanwhile, neuronal free calcium raised and SOD activity lessened. All of these events lead to the excessive formation of free radicals and damage of DNA, then induce apoptosis. Rg_1 could inhibit apoptosis and this effect can be attributed to the decrease of NO content, NOS activity and intracellular Ca^{2+} in the one hand and increase of bcl-2 gene expression and SOD activity in the other hand. As reported, apoptosis occurs in the matured nervous system especially in neuro-degenerative diseases such as Alzheimer's and Parkinson's disease or aging. Our results suggested that Rg_1 might exert anti-aging activity by preventing neuron apoptosis.

In summary, the present study provide new data to rich and develop programmed theory for understanding senescence. Our research confirmed that NO plays a role in accelerating senescence. Ca^{2+} and free radicals are widely implicated in the induction of apoptosis. In addition, this is first demonstration that Rg_1 is a useful anti-apoptotic agent.

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