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Cellular and molecular mechanisms underlying the action of ginsenoside Rg1 against Alzheimer's disease☆

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Abstract

Ginsenoside Rg1 inhibits oxidation, aging and cell apoptosis, and improves cognitive function. In this study, we pretreated rat brain tissue sections with ginsenoside Rg1, and established brain slice models of Alzheimer's disease induced by okadaic acid. The results revealed that ginsenoside Rg1 pretreatment suppressed the increase in phosphorylated Tau protein expression induced by incubation with okadaic acid, and reduced brain-derived neurotrophic factor expression. These results suggest that ginsenoside Rg1 upregulates brain-derived neurotrophic factor expression and inhibits Tau protein phosphorylation in brain slices from a rat model of Alzheimer's disease.

Key Words

Alzheimer's disease; ginsenoside Rg1; okadaic acid; phosphorylated Tau protein; brain-derived neurotrophic factor; traditional Chinese medicine; neural regeneration

Research Highlights

(1) In this study, we incubated rat brain slices with okadaic acid, prepared brain slice models of Alzheimer's disease, and selected phosphorylated Tau protein (Ser^{202}) for detection of Tau protein phosphorylation, providing theoretical evidence for identifying the target site of okadaic acid-induced excessive Tau protein phosphorylation.
(2) Ginsenoside Rg1 upregulated brain-derived neurotrophic factor expression and inhibited Tau protein phosphorylation in brain slices from a rat model of Alzheimer's disease.

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INTRODUCTION

Ginsenoside Rg1, one of the biologically active ingredients of ginseng, can increase memory and cognitive functions[1]. A previous study confirmed that ginsenoside Rg1 obviously improved learning and memory ability in an animal model of Alzheimer's disease, and its mechanism of action is probably associated with cholinergic function elevation, an increase in the number of M-cholinoceptors and receptor protein synthesis[2]. Ginsenoside Rg1 inhibits oxidation, removes free radicals, and suppresses aging and apoptosis[3-5]. The inhibitory effect of ginsenoside Rg1 on Tau protein phosphorylation is probably associated with its effects on decreasing glycogen synthase kinase-3β activity[6] and inhibiting cyclin-dependent kinase-5 expression[7].

Brain-derived neurotrophic factor is associated with learning and memory ability and plays an important role in the repair and reconstruction of neurons in Alzheimer's disease. Brain-derived neurotrophic factor is mainly used in the early treatment of Alzheimer's disease. Development of drugs that stimulate brain-derived neurotrophic factor secretion for the treatment of Alzheimer's disease is a new research direction. At present, such research is embryonic, and it remains unclear whether ginsenoside Rg1 can upregulate brain-derived neurotrophic factor expression.

In the present study, we used okadaic acid to prepare brain slice models of Alzheimer's disease in rats with Tau protein phosphorylation, and investigated the effects of ginsenoside Rg1 on phosphorylated Tau protein and brain-derived neurotrophic factor expression.

RESULTS

Quantitative analysis of rat brain slices

Wistar rat brain slices (400 μm-thick) containing cortex and hippocampus were prepared and randomly assigned to a blank control group (no other treatment), a model group (okadaic acid was used), and low-dose, moderate-dose and high-dose ginsenoside Rg1 groups (n = 10 in each group). In the ginsenoside Rg1 groups, ginsenoside Rg1 (final concentrations of 60, 120 and 240 μM) was slowly injected using a microsyringe for 2 hours of pretreatment; then, okadaic acid was added for model induction.

Effects of okadaic acid on phosphorylated Tau protein and brain-derived neurotrophic factor protein expression in rat brain slices

The results of immunohistochemical staining revealed that phosphorylated Tau protein expression was significantly increased ($P < 0.01$), and brain-derived neurotrophic factor expression was significantly decreased ($P < 0.01$) in the model group compared with the blank control group (Table 1).

Ginsenoside Rg1 effects on phosphorylated Tau protein expression in brain slices from a rat model of Alzheimer's disease

Phosphorylated Tau protein was mainly distributed in the cytoplasm and processes of neurons, in the granular cells and pyramidal cells in cortex layers II, III and V, and in the pyramidal cells of the hippocampus.

Phosphorylated Tau protein expression was reduced in ginsenoside Rg1 groups compared with the model group with the exception of the CA1, CA3 and dentate gyrus in the low-dose ginsenoside Rg1 group ($P < 0.05$ or $P < 0.01$). Phosphorylated Tau protein expression was lower in the high-dose ginsenoside Rg1 group than in the moderate-dose and low-dose ginsenoside Rg1 groups ($P < 0.05$, $P < 0.01$; Table 2, Figure 1).

Table 1 Effects of okadaic acid on phosphorylated tau protein and brain-derived neurotrophic factor protein expression (grayscale value for staining intensity) in brain slices

Group	Phosphorylated Tau protein			
	CA1	CA3	Dentate gyrus	Cortex
Blank control	163.3±2.7	159.5±1.9	157.8±1.9	165.3±2.1
Model	153.4±1.4[a]	152.1±1.4[a]	151.6±1.3[a]	151.4±1.4[a]
Group	**Brain-derived neurotrophic factor**			
	CA1	CA3	Dentate gyrus	Cortex
Blank control	147.5±1.9	148.3±1.9	146.8±1.0	147.6±1.1
Model	156.1±2.3[a]	157.4±1.0[a]	153.8±1.5[a]	154.5±2.1

[a]$P < 0.01$, *vs.* blank control group. Data were expressed as mean ± SD, n = 10. Variance homogeneity was tested using the Levene test. Normality was tested using the Shapiro-Wilk test. Mean values of various groups were compared using one-way analysis of variance. Paired comparisons of mean values were made using the least significant difference t-test. Grayscale value was collected in each group using an image processing and analysis system. The lower the gray value, the higher was the level of protein expression.

Table 2 Effects of ginsenoside Rg1 on phosphorylated tau protein expression (grayscale value) in brain slices from a rat model of Alzheimer's disease

Group	CA1	CA3	Dentate gyrus	Cortex
Blank control	163.3±2.6	159.4±1.9	157.8±1.8	165.3±2.1
Model	153.4±1.4[a]	152.1±1.4[a]	151.6±1.4[a]	151.4±1.4[a]
Low-dose ginsenoside Rg1	154.0±1.9[df]	152.4±1.4[df]	152.1±1.5[e]	153.4±1.8[bdf]
Moderate-dose ginsenoside Rg1	157.1±2.7[cf]	154.9±1.4[c]	153.3±1.8[b]	159.7±1.9[cf]
High-dose ginsenoside Rg1	160.2±1.4[c]	156.4±1.9[c]	153.9±1.9[c]	163.2±1.3[c]

[a]$P < 0.01$, *vs.* blank control group; [b]$P < 0.05$, [c]$P < 0.01$, *vs.* model group; [d]$P < 0.01$, *vs.* moderate-dose ginsenoside Rg1 group; [e]$P < 0.05$, [f]$P < 0.01$, *vs.* high-dose ginsenoside Rg1 group. Data were expressed as mean ± SD, n = 10. Data were analyzed using one-way analysis of variance and least significant difference t-test. The lower the gray value, the higher was the the level of protein expression.

Ginsenoside Rg1 effects on brain-derived neurotrophic factor protein expression in brain slices from a rat model of Alzheimer's disease

Brain-derived neurotrophic factor was mainly distributed in the cell membrane, cytoplasm and processes; nuclei were not stained or were only lightly stained.

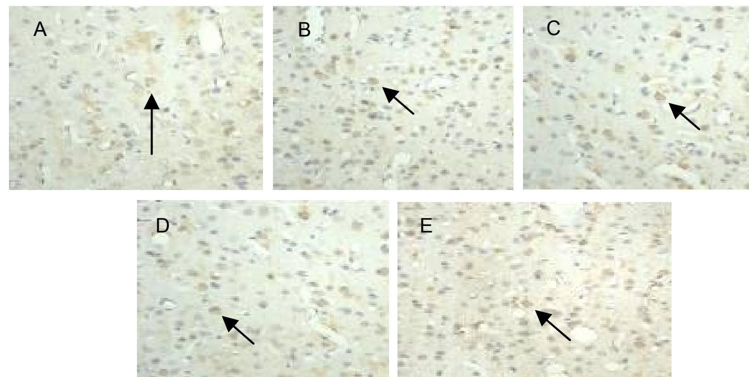


Figure 1 Effects of ginsenoside Rg1 on phosphorylated Tau protein expression in brain slices from a rat model of Alzheimer's disease (immunohistochemistry, light microscope, × 400).

(A) Blank control group; (B) model group; (C) high-dose ginsenoside Rg1 group; (D) moderate-dose ginsenoside Rg1 group; (E) low-dose ginsenoside Rg1 group. Phosphorylated tau protein expression was highest in the model group, followed by the high-dose ginsenoside Rg1 group, and lowest in the blank control group. Arrows show phosphorylated tau protein-positive cells.

Brain-derived neurotrophic factor expression was increased, but grayscale values[5] for immunohistocheministry staining were decreased in the ginsenoside Rg1 groups compared with the model group with the exception of the CA3, dentate gyrus and cortex in the low-dose ginsenoside Rg1 group (P < 0.05 or P < 0.01). Significant differences were detectable between different ginsenoside Rg1 groups except in the dentate gyrus between moderate-dose and low-dose ginsenoside Rg1 groups (P < 0.05 or P < 0.01). Brain-derived neurotrophic factor protein expression was greater in the high-dose ginsenoside Rg1 group than in the moderate-dose and low-dose ginsenoside Rg1 groups (P < 0.05 or P < 0.01; Table 3, Figure 2).

DISCUSSION

Imbalances in the protein kinase and phosphatase system can induce Tau protein phosphorylation, leading to the formation of abnormally phosphorylated Tau protein[8-11]. Phosphorylated Tau protein participates in the formation of neurofibrillary tangles, resulting in the occurrence of Alzheimer's disease; moreover, the number of neurofibrillary tangles is strongly associated with the degree of dementia in Alzheimer's disease patients[12-14]. Okadaic acid, a specific protein phosphatase inhibitor[15], has been extensively used to induce excessive phosphorylation of Tau protein[16], but its target site and its location in cells remain unclear. Tau has 45 sites of excessive phosphorylation, including Ser^{202}[17]. Obvious excessive phosphorylation is seen in the brains of Alzheimer's disease patients by Tau protein detection[17-18]. Okadaic acid injection in the rat brain induced Tau protein phosphorylation on Ser^{198}/Ser^{199}/Ser^{202}, Ser^{396}/Ser^{404} sites, resulting in spatial memory deficits[19]. Thus, in the present study, we assessed the levels of phosphorylation at this site.

Table 3 Effects of ginsenoside Rg1 on brain-derived neurotrophic factor protein expression (gray value) in brain slices of a rat model of Alzheimer's disease

Group	CA1	CA3	Dentate gyrus	Cortex
Blank control	147.53±1.92	148.34±1.96	146.78±0.96	147.61±1.09
Model	156.11±2.31^{a}	157.36±0.98^{a}	153.78±1.45^{a}	154.48±2.10^{a}
Low-dose ginsenoside Rg1	154.24±1.40^{bdg}	156.55±1.21^{eg}	152.81±1.52^{g}	153.80±1.40^{dg}
Moderate-dose ginsenoside Rg1	152.47±0.84^{cg}	153.52±1.16^{cg}	151.64±1.84^{cf}	151.93±2.11^{cg}
High-dose ginsenoside Rg1	149.31±1.40^{c}	150.21±1.60^{c}	149.88±1.46^{c}	149.37±1.82^{c}

^{a}P < 0.01, *vs.* blank control group; ^{b}P < 0.05, ^{c}P < 0.01, *vs.* model group; ^{d}P < 0.05, ^{e}P < 0.01, *vs.* moderate-dose ginsenoside Rg1 group; ^{f}P < 0.05, ^{g}P < 0.01, *vs.* high-dose ginsenoside Rg1 group. Data were expressed as mean ± SD, n = 10. Variance homogeneity was tested using the Levene test. Normality was tested using the Shapiro-Wilk test. Mean values of various groups were compared using one-way analysis of variance. Paired comparisons of mean values were made using the least significant difference t-test. The lower the gray value, the higher the protein expression.

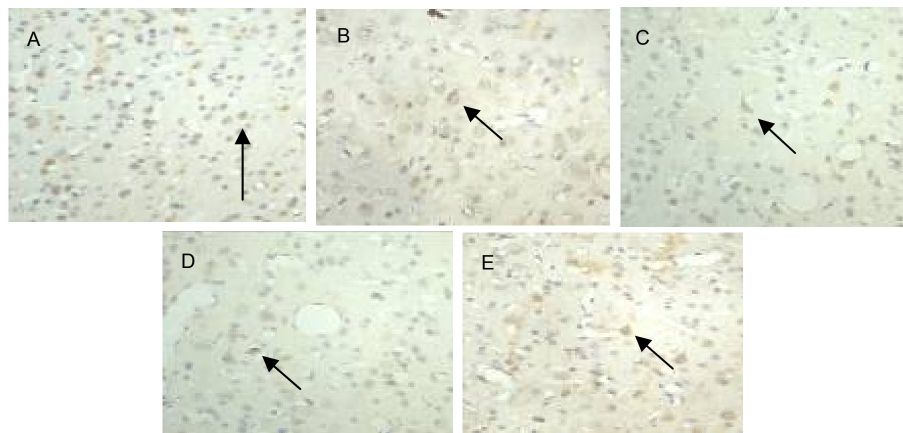


Figure 2 Effects of ginsenoside Rg1 on brain-derived neurotrophic factor expression in brain slices from a rat model of Alzheimer's disease (immunohistochemistry, light microscope, × 400).

(A) Blank control group; (B) model group; (C) high-dose ginsenoside Rg1 group; (D) moderate-dose ginsenoside Rg1 group; (E) low-dose ginsenoside Rg1 group. Brain-derived neurotrophic factor protein expression was greatest in the blank control group, followed by the high-dose ginsenoside Rg1 group, and lowest in the model group. Arrows show brain-derived neurotrophic factor positivity.

The results of immunohistochemical staining suggested that phosphorylated Tau protein expression is increased in the model group compared with the blank control group, which suggests that Ser^{202} is probably an important target site during okadaic acid induction of excessive Tau protein phosphorylation. Following ginsenoside Rg1 pretreatment, phosphorylated Tau protein expression was significantly lower in the ginsenoside Rg1 groups compared with the model group, and high-dose ginsenoside Rg1 was the most effective at reducing phosphorylated Tau protein expression. Thus, it is likely that ginsenoside Rg1 reduces neurofibrillary tangle formation by preventing okadaic acid-induced excessive Tau protein phosphorylation.

Brain-derived neurotrophic factor plays an important role in synaptic plasticity and in protecting injured neurons. It can improve the learning and memory functions of dementia animal models[20], and participates in long-term potentiation, as well as learning and memory formation[21]. Nagahara *et al* [22] proposed that brain-derived neurotrophic factor could protect neurons in the nervous circuitry of Alzheimer's disease patients. Brain-derived neurotrophic factor mRNA levels and protein content were found to be decreased in the hippocampus and cortex of Alzheimer's disease patients with mild cognitive impairment[23]. The significant decrease in brain-derived neurotrophic factor expression led to progressive atrophy of the cholinergic system in the basal forebrain and Tau protein phosphorylation in the brain[24], suggesting that brain-derived neurotrophic factor downregulation might be a mechanism of inducing Alzheimer's disease. We added okadaic acid to artificial cerebrospinal fluid that was used for incubation of rat brain slices, and found that, compared with the blank control group, brain-derived neurotrophic factor expression was diminished in the model group, consistent with the decreased brain-derived neurotrophic factor expression in the brains of Alzheimer's disease models. Thus, we confirmed that okadaic acid could inhibit brain-derived neurotrophic factor expression. Increased brain-derived neurotrophic factor content in the brain can improve neuronal survival[25-27], resulting in a delay in or prevention of the progression of Alzheimer's disease. Glycogen synthase kinase-3β is a Tau protein kinase. Excessive expression of glycogen synthase kinase-3β in cell or animal models could induce Tau protein hyperphosphorylation.

Brain-derived neurotrophic factor inhibited Tau protein phosphorylation by suppressing glycogen synthase kinase-3β activity[28-30]. However, brain-derived neurotrophic factor has a high molecular weight. Exogenous brain-derived neurotrophic factor taken by oral administration can be easily damaged by gastric acid. Brain-derived neurotrophic factor delivered by other means of peripheral administration cannot cross the blood-brain barrier. Therefore, promoting the production or release of endogenous brain-derived neurotrophic factor could be an effective treatment for Alzheimer's disease.

In summary, ginsenoside Rg1 decreased phosphorylated Tau protein expression in brain slices from a rat model of Alzheimer's disease, slowed down the formation of neurofibrillary tangles, upregulated brain-derived neurotrophic factor expression, and/or

contributed to the production or release of endogenous brain-derived neurotrophic factor.

MATERIALS AND METHODS

Design

A random controlled animal study.

Time and setting

Experiments were performed at the Biomedicine Experimental Center, College of Medicine, Xi'an Jiaotong University, China from July 2008 to May 2009.

Materials

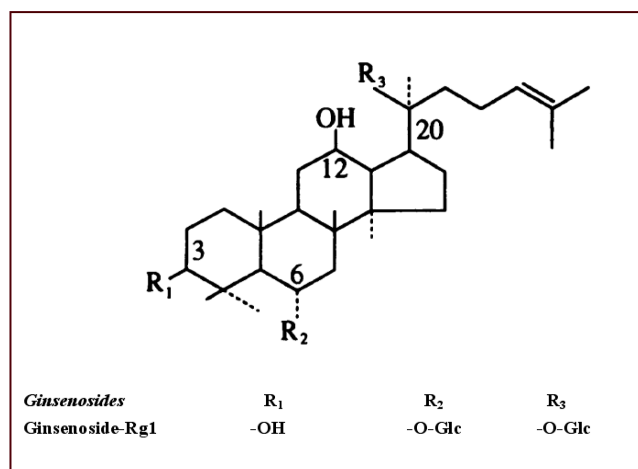
Experimental animals

Healthy, clean, male Wistar rats aged 5 weeks and weighing 110–130 g were supplied by the Experimental Animal Center, College of Medicine, Xi'an Jiaotong University (license No. SCXK (Shan) 2007-001).

Protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China[31].

Traditional Chinese medicine

Ginsenoside Rg1 is one of the biologically active ingredients of ginseng, molecular formula $C_{42}H_{72}O_{14}$, molecular weight 801.01. The chemical structural formula is as follows:



Ginsenoside Rg1 was purchased from Jilin Hongjiu Biological Technology Co., Ltd., with a purity of ≥ 98% (high performance liquid chromatography). In accordance with a previous method[32], brain slices from a rat model of Alzheimer's disease with Tau protein phosphorylation were pretreated with artificial cerebrospinal fluid containing 60, 120, 240 μM of ginsenoside Rg1.

Methods

Preparation, grouping and intervention for brain slices of a rat model of Alzheimer's disease with Tau protein phosphorylation

In accordance with a previous method[33], the rats were intraperitoneally anesthetized with 6% chloral hydrate (400 mg/kg), decapitated within 1 minute, and placed in an artificial cerebrospinal fluid ice water mixture supplemented with 150 mM NaCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 mM KH_2PO_4, 1.5 mM K_2HPO_4 and 10 mM glucose (pH 7.4) for 5 minutes at 4°C.

Fascia on the brain tissues and unrelated tissues were removed. Treated brain tissues were fixed on a microtome, and coronally sliced into 400 μm-thick sections. Each section contained cortex and hippocampus. Brain slices with good appearance were placed in 6-well plates containing artificial cerebrospinal fluid. Mixed gas (95% O_2 + 5% CO_2) was continuously given in artificial cerebrospinal fluid at 35°C. Following 1 hour of incubation, ginsenoside Rg1 of 98.99% purity (dissolved in analytical grade methanol) was added to the ginsenoside Rg1 groups (concentrations of 60, 120, 240 μM[32]).

After 2 hours of pretreatment, okadaic acid (ENZO, NY, USA) (dissolved in dimethyl sulfoxide) was added to the model and ginsenoside Rg1 groups to a final concentration of 1 μM for 3 hours[32]. Blank control group was not administered.

Immunohistochemical staining of phosphorylated Tau protein and brain-derived neurotrophic factor protein expression in rat brain slices

Brain slices were washed with artificial cerebrospinal fluid, fixed in 4% paraformaldehyde for 4 hours, immersed in 30% sucrose solution, and sliced into 10-μm-thick frozen sections. Each section contained cortex and hippocampus.

Brain tissue sections were blocked with normal goat serum antibody, incubated in rabbit anti-rat phosphorylated Tau protein and brain-derived neurotrophic factor polyclonal antibody (Boster, Wuhan, China; 1:1 000, 1:600) at 4°C overnight, in biotin-labeled goat anti-rabbit secondary antibody for 40 minutes, developed using 3,3'-diaminobenzidine, counterstained with hematoxylin, dehydrated, permeabilized, and mounted. PBS in the place of primary antibody served as a negative control.

Data processing

Positive judgment and quantitative analysis of grayscale

values for immunohistochemical staining were performed. Image characteristics were quantitatively analyzed using an image collection and analysis system. Grayscale analysis was done using a Qwin550CW image processing and analytical system (Leica, Hessen, Germany). Six fields from each section in the same region were randomly selected, and the detection area was the same size. The average value for the six groups of data was considered as the average gray value of the target area of the section. The lower the gray value, the higher was the level of protein expression.

Statistical analysis

Measurement data are expressed as mean ± SD, and were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Homogeneity of variance was tested using the Levene test, and normality was tested using the Shapiro-Wilk test. Mean values for various groups were compared using one-way analysis of variance. Paired comparisons of mean values were made using the least significant difference *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Xi Li participated in the study conception and design and obtained funding, provided technical and data support, and served as the principal investigator. Ming Li and Xi Li were in charge of study implementation, provided data and ensured the integrity of the data. Ming Li, Yuan Li, Qiankun Quan and Juan Wang wrote the manuscript, and were responsible for data analysis and statistical processing. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, College of Medicine, Xi'an Jiaotong University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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