Involvement of CDK4, pRB, and E2F1 in ginsenoside Rg1 protecting rat cortical neurons from β-amyloid-induced apoptosis

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KEY WORDS ginseng; saponins; amyloid beta-protein; apoptosis; cell cycle; neurons

ABSTRACT

AIM: To explore the possible mechanism of β-amyloid (Aβ)-induced apoptosis in rat cortical neurons and the protective effect of ginsenoside Rg1. METHODS: AO-EB staining was used to quantify the apoptotic cells. DNA fragmentation was observed by gel electrophoresis. The levels of cyclin-dependent kinases-4 (CDK4) and phosphorylated pRB were detected by Western blot. RT-PCR was used to examine the expression of E2F1 mRNA. RESULTS: Treatment with Aβ1-40 at the concentration of 20, 40, 80 mg/L for 48 h induced rat cortical neuron apoptosis from 12.5 %±1.5 % (control) to 22.3 %±1.4 %, 38.8 %±1.3 %, 36.7 %±1.4 %, respectively. Pretreatment with Rg1 at the dose of 0.5, 1, 2, 4, 8, 16 µmol/L for 24 h, then treatment with Aβ1-40 40 mg/L for 24 h, the percentage of apoptotic neurons decreased from 38.8 %±1.3 % to 14.5 %±1.3 %, 13.3 %±1.0 %, 11.6 %±0.29 %, 11.8 %±1.0 %, 6.2 %±0.8 %, 5.8 %±0.8 %, respectively. After treatment with Aβ1-40 40 mg/L for 24 h, there were transient increases in CDK4 and phosphorylated pRB protein level, as well as the expression of E2F1 mRNA. However, the above levels decreased markedly after pretreatment with Rg1 8 µmol/L for 24 h. CONCLUSION: Ginsenoside Rg1 attenuated Aβ1-40-induced apoptosis in rat cortical neurons via inhibiting the activity of CDK4, decreasing the phosphorylation of pRB and downregulating the expression of E2F1 mRNA.

INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disorder of the human central nervous system. Brains of AD patients are characterized by three diagnostic hallmarks: senile plaques, neurofibrillary tangles, and prominent cortical neurons loss. The major component of the senile plaques is the β-amyloid (Aβ) protein, a 38-43 amino acid polypeptide. Aggregated Aβ has been shown to trigger the pathogenesis of AD through the activation of an apoptotic pathway\(^1\). The mechanism by which Aβ causes neuronal death is not well understood.

Several groups have reported abnormal up-regulation of a variety of cell cycle protein in brains from AD patients\(^2\). The cell cycle is a tightly regulated process controlled by sequential activation of cyclin-dependent kinases (CDKs)\(^3\). Retino-blastoma protein (pRB) is a target for CDK4. It has been reported that more than 10 sites in pRB were phosphorylated in vivo.

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\(^1\) Project supported by Natural Science Foundation of Fujian Province (Ng C01100116) and Science Foundation of Fujian Provincial Science and Technology Department (Ng 2001Z037).

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Received 2002-11-22 Accepted 2003-03-28
Generally, the D-type cyclin-CDK4 complexes phosphorylated Ser780 in the G1 site of pRB[4]. Once hyperphosphorylated, pRB is released from the transcription factor complex E2F-DB. E2F1, a member of a family of six related growth regulatory transcription factors, was first recognized to promote G1 to S-phase transition by trans-activation of genes involved in DNA synthesis and cell cycle control[5]. E2F1 has been shown to induce both cell cycle progression and apoptosis in neuronal cells[6]. Giovanni et al reported that CDK4/6, pRB, E2F1 may play an important role in apoptosis as well as cycle control[7].

Ginseng has been used as a kind of medical herb for several thousands years in China. Ginseng saponins exert various important pharmacological effects with regard to the control of many diseases. Several studies have shown that ginsenoside Rg1 prevented mouse substantia nigra neurons[8] and rat pheochromocytoma cell line PC12 cells from apoptosis[9].

The study was designed to explore the mechanism of Aβ1-40-induced apoptosis in rat cortical neurons and the possibility of using ginsenoside Rg1 to treat some neurodegenerative diseases, such as AD.

MATERIALS AND METHODS

Materials Aβ1-40 was from Sigma (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and N2 supplement were from Gibco-BRL (Grand Island, NY, USA); ginsenoside Rg1, from the Department of Organic Chemistry of Bethune Medical University (Changchun, China), purity >98% ; Reverse transcription polymerase chain reaction (RT-PCR) kit from Promega (Madison, WI, USA); phosphorylated neurofilament monoclonal antibody and CDK4 polyclonal antibody from Chemicon (Temecula, CA, USA); phospho-RB polyclonal antibody from MBL (Naka-ku Nagoya, Japan).

Culture of rat cortical neurons and treatments

All experiments were performed using primary cultures of cortical neurons that were cultured from embryonic day 16 to 18 rats. The neurons were planted into 12-well dishes (approximately 1.5×10^6 cells/well) coated with poly-L-lysine (100 mg/L) in serum-medium (N2:DMEM is 1:1) supplemented with insulin 5 mg/L, transferrin 100 mg/L, progesterone 20 nmol/L, putrescine 100 µmol/L, selenite 30 nmol/L, and 10% fetal bovine serum. Multwells were incubated in a humidified atmosphere containing 5% CO2, 95% air at 37°C. After 72 h, the culture medium was exchanged for serum-free medium (N2:DMEM is 1:1). Under these conditions, cultures typically contain more than 94 % neurons as assessed by staining with antibody directed against phosphorylated neurofilament. Seven days after initial planting, the medium was exchanged with serum-free medium supplemented with ginsenoside Rg1 (0.5, 1, 2, 4, 8, 16 µmol/L) or preaggregated Aβ1-40 (20, 40, 80 mg/L). Aβ1-40, was preaggregated by incubation in medium at a concentration of 1 g/L at 37°C for up 7 d. At appropriate times of culture under the conditions described, cells were digested by 0.25% trypsin. Acriflavine (AO) 5 µL and ethidium bromide (EB) 5 µL (100 mg/L soluble in phosphate buffer saline respectively) were added to 90 µL of cells solution. Ratio of apoptotic cell was evaluated by fluorescence microscope (490 nm excitation).

DNA fragmentation Approximately 5×10^6 cells from each experimental condition were harvested, then the extraction of DNA was followed as described[10]. In brief, the cultured cells were treated with lysis buffer (1% Nonidet P-40 in edetic acid 20 mmol/L, Tris-HCl 50 mmol/L, pH 7.5, 10 µL per 1×10^6 cells) for 10 s. Then the cells were centrifugated at 1600×g for 5 min, and the supernatants were treated with 1% SDS and RNase A 5 g/L at 56 °C for 2 h, followed with proteinase K 2.5 g/L for at least 2 h at 37°C. After addition of 0.5 volume of ammonium acetate 10 mol/L, DNA was precipitated with 2.5 volume of ethanol, and separated by electrophoresis in 1.0% agarose gel containing EB 0.1 mg/L. DNA was visualized under ultraviolet light.

Western blot analysis Cellular proteins were extracted by lysis with a buffer [NaCl 150 mmol/L, Tris-Cl 50 mmol/L (pH 8.0), 0.02 % sodium azide, 1% Nonidet P-40] containing the protease inhibitor PMSF 100 mg/L, and aprotinin 1 mg/L. Equal 50 µg amounts of protein were loaded onto SDS-polyacrylamide gels, and transferred onto nitrocellulose membrane. Bolts were probed with CDK4 antibody (1:250 dilution) and phospho-Rb antibody (1:500 dilution).

Reverse transcription-PCR Total RNA was extracted from rat cortical neurons using TRIzol reagent. First strand cDNA was reverse transcribed from 4 µg of total RNA. The same amounts of cDNA were subsequently used for PCR amplification for a total of 25 cycles at 95°C for 1 min, 60°C for 45 s, and 72°C for 45 s of each cycle. These optimal amplification conditions and cycle numbers were determined experimentally to ensure specific and linear signal generation, and
expression of β-actin mRNA was used as a standard to quantify the relative amount of expression of E2F1. The mouse specific E2F1 PCR primers (AGG, CTG, GAT, CTG, GAG, ACT, GA and CTT, CAA, GCC, GCT, TAC, CAA, TC) and β-actin primers (AAC, ACC, CCA, GCC, ATG, TAC, GTA, G and GTG, TTG, GCA, TAG, AGG, TCT, TTA, CGG) were used to generate an E2F1 PCR product of 358 bp and β-actin fragment of 509 bp. The PCR products were fractionated on a 2 % agarose gel.

Statistics Data were expressed as Mean±SD and statistically compared by ANOVA.

RESULTS

Aβ1-40-induced apoptosis in rat cortical neurons
AO-EB staining was carried out to determine whether preaggregated Aβ1-40 can induce apoptosis in rat cortical neurons, to find out the best treatment dose and time. In this study, it was shown that treatment with various doses of preaggregated Aβ1-40 for 24 to 72 h could all result in cortical neurons apoptosis. But the effects of inducement by Aβ1-40 at the concentration of 40 mg/L and 80 mg/L for 48 h were the best (Tab 1).

Effect of Rg1 on Aβ1-40-induced apoptosis in rat cortical neurons After treatment with Aβ1-40, 40 mg/L for 48 h, apoptosis in rat cortical neurons was induced. However, after pretreatment with various concentrations of Rg1 (1, 2, 4, 8, 16 µmol/L) for 24 h, cell apoptosis was decreased significantly and DNA fragmentation was blocked markedly. And the protective effect of Rg1 at the dose of 8 µmol/L was the best (Fig 1, 2)

Requirement for CDK4 in apoptosis of cortical neurons evoked by Aβ1-40 After cortical neurons were treated with Aβ1-40 for 2-4 h, there was a transient increase in CDK4 protein level. However, after pretreatment with Rg1 for 24 h, the level of CDK4 decreased markedly (Fig 3).

Requirement for pRB in apoptosis of cortical neurons evoked by Aβ1-40 After cortical neurons were treated with preaggregated Aβ1-40, the treatment resulted in the increased expression level of E2F1 mRNA at 3-6 h. However, after pretreatment with Rg1 for 24 h, the expression of E2F1 mRNA decreased markedly (Fig 4).
Fig 3. Time course of CDK4 and phosphorylated pRB (ppRB) levels in rat cortical neurons after treatment with Aβ₁₋₄₀ 40 mg/L or Rg₁ 8 µmol/L. Lane 1: normal; Lane 2: 2 h; Lane 3: 4 h; Lane 4: 8 h; Lane 5: 16 h. n=3. Mean±SD. cP<0.01 vs Rg₁ 8 µmol/L group.

Fig 4. E2F1 mRNA expression in rat cortical neurons after treatment with Aβ₁₋₄₀ 40 mg/L or Rg₁ 8 µmol/L. Lane M: DNA marker; Lane 1: normal; Lane 2: 3 h; Lane 3: 6 h; Lane 4: 12 h. n=3. Mean±SD. cP<0.01, bP<0.05 vs Rg₁ 8 µmol/L group.
DISCUSSION

The dominant pathological change of AD is the occurrence of senile plaques, which are protein deposits composed primarily of the Aβ peptide. Generally, brain cells can set free a little of Aβ, which would be cleaned soon. When much Aβ is allowed to aggregate, it becomes insoluble and forms plaques. Aggregated Aβ is neurotoxic. It can enhance and enlarge the cellular injurious effect of various noxious stimulations such as excitatory toxicity and free radicals. Further, it has direct cellular toxicity. It is widely believed that aggregated Aβ plays a dominant role in the genesis of AD[1]. Studies with in vitro cultured neurons treated with toxic forms of aggregated Aβ protein[2] as well as in vivo studies utilizing transgenic mice expressing Aβ[3] demonstrated neuronal loss by an apoptotic pathway. In this study, we also showed that preaggregated Aβ1-40 could induce apoptosis in a concentration range of 20 to 80 mg/L and a time range of 24 to 72 h.

There are many signal transduction pathways in cell apoptosis. CDKs are a group of proteinases. They are activated by binding to cell cycle proteins and are phosphorylated or unphosphorylated. They regulate apoptosis by affecting the cell cycle progression and speed. CDK4 plays a required role in cell cycle. CDK4 is concerned with cyclin D. Cyclin Ds-CDK4 would gain their accessibilities to pRB through direct interaction of cyclin Ds with pRB and recognize localized sequences around the phosphorylation sites in pRB to phosphorylate the specific site(s), such as Ser 780[4].

Rb is a tumor suppressor gene. The product of Rb gene, such as pRB, play a role in regulating the progression of proliferating cells through the cell cycle. It behaves as a transcriptional repressor by binding to the transactivated domain of E2F and inhibiting its transcriptional activity, which is required for the progression of cells from G1 into S phase[5]. Meanwhile, an anti-apoptotic role has been suggested for pRB in a series of recent papers that correlated the loss of pRB with the induction of apoptosis. The appearance of apoptosis in specific neuronal compartments and in the developing lens has been observed in Rb-/- mouse embryos and these results have been interpreted as a response to the loss of pRB[6]. Moreover, the cleavage of pRB by caspase activity has been considered to be an early permissive step in the apoptosis-inducing pathway[7]. In this study, we observed a transient increase in pRB (Ser 780) phosphorylation during Aβ treatment of cortical neurons, consistent with the requirement for CDK4 activity.

E2F-responsive elements are present in the promoters of cell cycle-related genes, including E2F1’s promoter. In cycling cells, the activity of E2F1 is regulated by the retinoblastoma gene product pRB. Hypophosphorylated pRB forms a complex with E2F1 and represses transcription, perhaps by inhibition of histone deacetylase activities[8]. During G1/S transition, pRB becomes highly phosphorylated by cell cycle-dependent kinases, such as CDK4/6 and releases E2F1. An inappropriate increase in content of free E2F1 has been described as a key regulator of cell death by apoptosis in cycling cells[9]. In this study, expression of E2F1 mRNA increased 3 to 6 h after Aβ treatment of cortical neurons, however, the apoptotic cell death was induced after Aβ treatment for 24 h. It suggests that promotion of expression of E2F1 mRNA is an early event in the apoptotic process.

Ginseng is a medicinal herb widely used in Asian countries and many of its pharmacological actions are attributed to the ginsenosides. Recently, ginsenoside Rg1 has been the subject of intense study. The major target organism of Rg1 is the central nervous system. It can be used to ameliorate action and intelligence. Some studies have reported that ginsenoside Rg1 activated the expression of cyclin-kinase inhibitors, P21 and P27, arrested LNCap cells (prostate cancer line) at G1 phase and subsequently inhibited cell growth through a caspase-3-mediated apoptosis mechanism[10]. While ginsenoside Rg1 inhibited the growth of MCF-7 cells, by inducing protein expression of P21 and reduced the protein levels of cyclin D which resulted in the down-regulation of cyclin/CDK complex kinase activity, decreasing phosphorylation of pRB and inhibiting E2F release[11]. Fei et al also reported that Rh2 suppressed the growth of A375-S2 cells by inducing apoptosis[12]. However, it was not well known whether Rg1 inhibited the apoptosis of cortical neuron related to the cell cycle elements, such as CDK4, pRB and E2F1.

In this study, rat cortical neurons were first pre-treated with Rg1 8 µmol/L for 24 h, then treated with Aβ1-40 at the concentration of 40 mg/L for 48 h. It showed that not only cell apoptosis was markedly attenuated, but also that all the levels of CDK4, phosphorylated pRB, and E2F1 mRNA expression were decreased.

Taken together, it suggested that Rg1 could inhibit the activity of CDK4, decrease the phosphorylation of
pRB, downregulate the expression of E2F1 mRNA, and reduce cell apoptosis ultimately. In the other hand, we should make a thorough study in whether Rg1 only inhibited the activity of CDK4 or it affected all the above elements.

REFERENCES