

Ginsenoside Rb1 promotes PC12 cell cycle kinetics through an adenylate cyclase–dependent protein kinase A pathway

Shin-Da Lee^{a,c}, Ming-Jae Lo^{b,*}

^aDepartment of Physical Therapy, Graduate Institute of Rehabilitation Science, China Medical University, Taichung, Taiwan, ROC

^bDepartment of Early Childhood Education, National Taichung University, Taichung 403, Taiwan, ROC

^cDepartment of Healthcare Administration, Asia University, Taichung, Taiwan, ROC

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Abstract

Ginsenoside Rb1 (G-Rb1), a constituent of ginseng, bears various beneficial effects on neuroendocrine cells. Previous studies have indicated that G-Rb1 can enhance glutamate release in undifferentiated and differentiated PC12 cells via the protein kinase A (PKA)–dependent signaling pathway. We hypothesized that G-Rb1 stimulates rat adrenomedullary chromaffin cell line PC12 (PC12 cells) proliferation and mitosis by promoting the cell cycle at all regulatory points. This mechanism is partly mediated via the adenylate cyclase–dependent PKA signaling pathway. In the present study, we investigated the mechanism by which G-Rb1 promotes cell cycle kinetics from the PC12 cells. The cell cycle kinetics of these cells were determined using flow cytometric DNA analysis. Analysis of the PC12 cell cycle revealed that G-Rb1 may affect all phases of the cell cycle and accelerate cell cycle kinetics by stimulating G0G1 phase transiting to S and G2M phases. The cell cycle kinetics were decreased by coincubating with the adenylate cyclase inhibitor SQ22536. Compared with the G-Rb1–treated group, the PKA inhibitor H89 produced a marked decrease in the G-Rb1–stimulated cell cycle kinetics by inhibiting G0G1 phase from transiting to the S phase. These results support the position that G-Rb1 exerts a stimulatory effect on cell cycle kinetics to promote PC12 cell proliferation. The result also suggests that the division rate is mediated via the adenylate cyclase–dependent PKA signaling pathway.

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Keywords: Ginsenoside Rb1; PC12 cells; Cell cycle kinetics; Adenylate cyclase; Protein kinase A

Abbreviations: cAMP, 3′-5′-cyclic adenosine monophosphate; FBS, fetal bovine serum; G-Rb1, ginsenoside Rb1; PC12 cells, rat adrenomedullary chromaffin cell lines PC12; PKA, protein kinase A.

1. Introduction

Ginseng is a well-known and popular herbal medicine used worldwide. Purified ginsenoside Rb1 (G-Rb1), one of the active ingredients of ginseng, plays a key role in regulating neurotransmitter release in neuroendocrine cells [1–4]. Significant improvements in learning and memory have been observed in normal mice and in brain-damaged and aged rats after administration of crude ginseng extract or

G-Rb1 [5–8]. Rat adrenal–derived pheochromocytoma (PC) 12 cells are neurosecretory (dopamine, norepinephrine, glutamate, and/or acetylcholine) and contain many membrane-bound and cytosolic macromolecules associated with neurons [3,9,10]. We used PC12 cells in this study because these cells produce dopamine and have been used extensively as model “dopaminergic” cell lines for the study of Parkinson disease [11–14]. It has been shown that G-Rb1 promotes glutamate release in undifferentiated and differentiated PC12 cells by increasing the phosphorylation of synapsins (as regulators of neurotransmitter release) through a 3′-5′-cyclic adenosine monophosphate (cAMP)–protein

* Corresponding author. Tel.: +886 4 22183858; fax: +886 4 22183400.
E-mail address: mjlo@ms3.ntcu.edu.tw (M.-J. Lo).

kinase A (PKA) pathway [3,4]. Therefore, we hypothesized that the proliferative effect of G-Rb1 stimulates PC12 cell mitosis by promoting the cell cycle at all regulatory points. We also hypothesized that part of this mechanism is mediated via the adenylate cyclase–dependent PKA signaling pathway.

The measurement of DNA is one of the first and most widespread applications of flow cytometry. The content of DNA also gives information about cell cycles [15,16]. The involvement of the adenylate-cyclase-dependent PKA pathway in PC12 cell cycle kinetics induced by G-Rb1 is still unclear. To test our hypothesis that G-Rb1 is stimulatory, the specific objective was to determine whether the direct stimulatory role of G-Rb1 on PC12 cell cycle kinetics was accompanied by an increase in adenylate cyclase and PKA enzyme activities. It has been shown that G-Rb1 can influence activities of PKA, which may increase the release of glutamate in neuroendocrine cells [3,4]. To the extent that this occurs, it suggests that G-Rb1 may provide a nutritional benefit for humans in the prevention of aging-related disorders such as Parkinson disease or memory decline.

2. Methods and materials

2.1. Experimental design

Ginsenoside Rb1 (C₅₄H₉₂O₂₃; molecular weight, 1109.29; purity, 97.5%) was isolated from the ginseng root of *Panax quinquefolius* (American ginseng). The RPMI 1640 medium was obtained from HyClone (Logan, Utah). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, Calif). Ginsenoside Rb1, SQ22536, H89, Hanks balanced salt solution, penicillin, streptomycin, and L-glutamine were purchased from Sigma Chemicals (St Louis, Mo). Collagen IV protein was from Abcam (Cambridge, Mass). Flask preparation was carried out using a Nunc cell scraper, which spread the 125 μ L collagen IV over the growing surface of the 25-cm² tissue culture flask (25T flask). It was allowed to dry completely under sterile conditions before use.

2.2. PC12 cell culture

The PC12 rat pheochromocytoma cells were from Food Industry Research and Development Institute (Culture Collection and Research Center; CCRC60048). The PC12 cells used for culture were processed following a method described by Kitazawa et al [10] in 2002 with minor modification. The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, 50 μ g/mL streptomycin, and 2 mmol/L L-glutamine and maintained at 37°C in a 5% CO₂ humidified atmosphere. The PC12 cells were grown in 25-cm² tissue culture flasks (Greiner Bio-One Laboratory, Taipei, Taiwan) coated with collagen IV (5 μ g/cm² 25 T flasks). The medium was renewed every 2 days, and the cells were subcultured once or twice a week when they reached 70% to 80% confluence. The medium was

changed to RPMI 1640 and supplemented with 1% FBS before treatment.

2.3. Cell cycle analysis

We determined the cell cycle profile of these cells by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) [17–19]. After harvest of PC12 cells, DNA stain was processed by Cell Cycle Analysis Kit (Gene Research Laboratory, Taipei, Taiwan). After harvesting and washing, a 5 mL solution A (fix buffer, FC0031) was added while vortexing. It was then kept at 4°C for 20 minutes and spun at 300g to 400g for 5 minutes. The supernatant was discarded gently, and the pellet was washed twice with 5 mL solution B (10-fold wash buffer, FC0032) and mixed well. The supernatant was discarded after spinning at 1200 rpm for 5 minutes. The pellet was then added to 1 mL of solution C (propidium iodide and RNase, FC0033), mixed well, incubated at 37°C for 10 minutes, and then spun at 300g to 400g for 5 minutes. The supernatant was discarded gently. The pellet was added to 1 mL of solution D (propidium iodide, FC0034) and mixed well following incubation at 4°C for 30 minutes. After incubation with the DNA stain, the cells were analyzed on a FACSCalibur flow cytometer. The percentage of cells in G0G1, S, and G2M phases can be determined. Typical DNA analysis histograms obtained with flow cytometry are shown in Fig. 1.

2.4. Statistical analyses

Data are presented as means \pm SEM of 3 independent experiments performed in duplicate. The treatment mean values were tested for homogeneity by a 2-way analysis of variance, and the difference between specific mean values was tested for significance using Duncan multiple-range test [20]. For single comparisons, the significance of differences between mean was determined by Student *t* test. A difference between 2 mean values was considered statistically significant if *P* was < .05.

3. Results

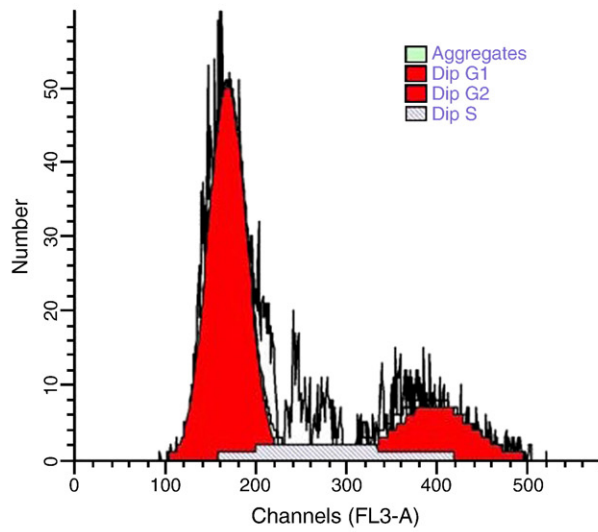
3.1. DNA analysis of histograms obtained with flow cytometry

Flow cytometric analysis was used to determine the percentages of cells in different phases (G0G1, S, and G2M) of the cell cycle. Effects of the vehicle and G-Rb1 (10⁻⁹ and 10⁻⁶ mol/L) on PC12 cell cycle profile for 72 hours are shown in Fig. 1.

3.2. Effects of G-Rb1 on PC12 cell cycle kinetics

Compared with vehicle-treated cells, the proportion of G-Rb1 (10⁻⁷ and 10⁻⁶ mol/L)-treated cells in G0G1 decreased at an average of 27.5% to 35.3% and 32.3% to 36.1%. Moreover, there was a concomitant increase in the proportion of cells in S and G2M phase after 48 and 72

A: Vehicle, 72h.



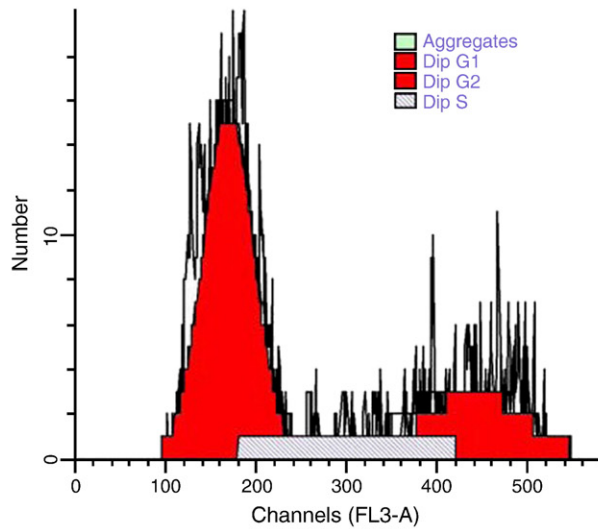
Diploid: 100%

Dip G1: 70.07% at 167.94

Dip G2: 20.32% at 396.66

Dip S: 9.61%

%CV: 12.58

B: G-Rb1 (10^{-9} M), 72h.

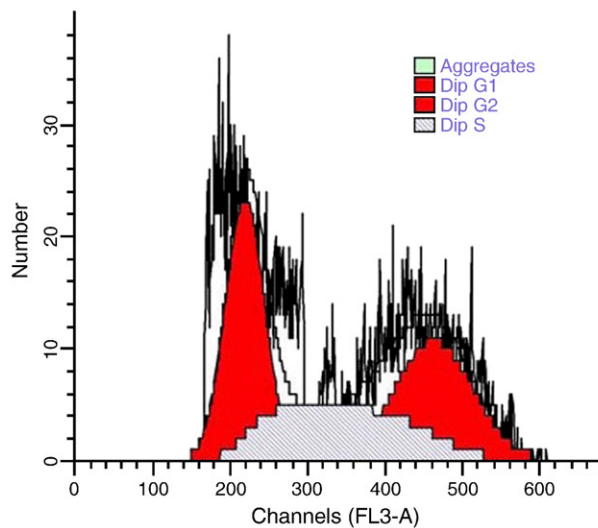
Diploid: 100%

Dip G1: 63.74% at 168.94

Dip G2: 23.98% at 440.96

Dip S: 12.29%

%CV: 16.49

C: G-Rb1 (10^{-6} M), 72h.

Diploid: 100%

Dip G1: 36.93% at 219.42

Dip G2: 33.95% at 462.77

Dip S: 29.12%

%CV: 11.52

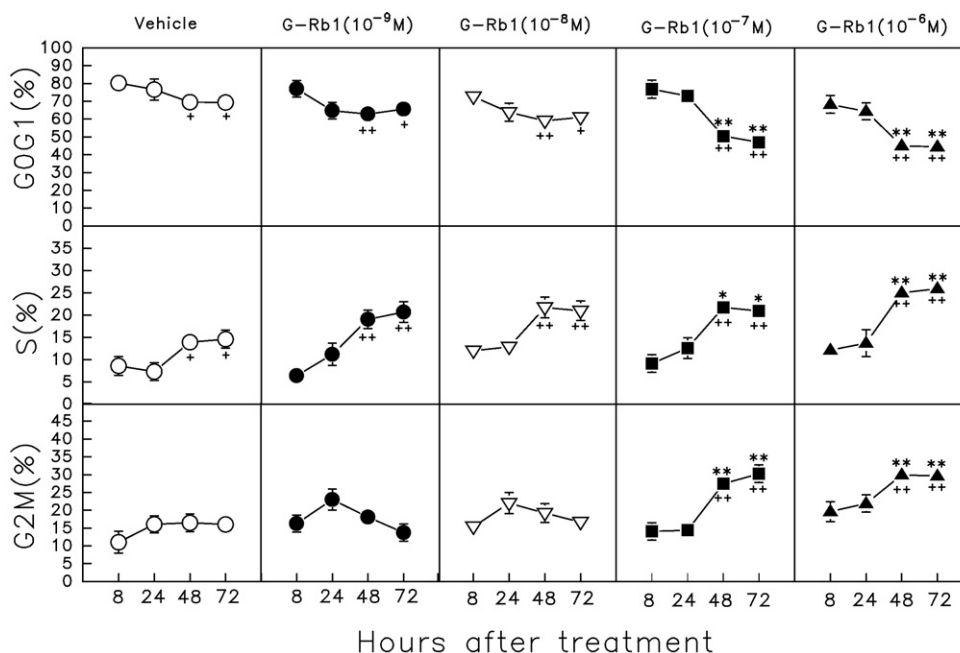


Fig. 2. Quantitative analysis of G-Rb1 (10^{-9} – 10^{-6} mol/L) on PC12 cell cycle kinetics (G0G1, S, and G2M phase) by flow cytometry for 8, 24, 48, and 72 hours. Each point represents the mean of 3 independent experiments performed in duplicate ($n = 6$), and error bars indicate the SEM. The cell numbers in different phases (G0G1, S, and G2M) of the cell cycle were expressed as relative percentage. * $P < .05$ or ** $P < .01$ as compared with the vehicle-treated group, respectively. + $P < .05$ or ++ $P < .01$ as compared with the 8-hour-treated group, respectively.

hours ($P < .05$ and $P < .01$; Fig. 2). Flow cytometric analysis showed G-Rb1-treated PC12 cells leaving the resting phase (G0G1) and undergoing DNA replication and mitosis.

In the time course experiments, PC12 cells were cultured in the vehicle or presence of G-Rb1 for 8, 24, 48, and 72 hours. Compared with 8 hours, the gradual time-dependent elevation in the percentage of either the vehicle or G-Rb1 (10^{-9} – 10^{-6} mol/L)-treated PC12 cells in the S phase was reflected by a decrease in the percentage of cells in the resting G0G1 phase at 48 and 72 hours ($P < .05$ and $P < .01$; Fig. 2, upper and middle panels). However, the fraction of PC12 cells in the G2M phase showed significant time-dependent difference only with higher concentrations of G-Rb1 (10^{-7} and 10^{-6} mol/L)-treated cells for 48 and 72 hours, as compared with 8 hours (Fig. 2, lower panel).

3.3. Effects of SQ22536 on G-Rb1-stimulated PC12 cell cycle kinetics

Flow cytometric analysis showed that adenylate cyclase inhibitor SQ22536 (10^{-4} and 10^{-3} mol/L) produced a marked decrease in the G-Rb1 (10^{-7} mol/L)-stimulated cell cycle kinetics by inhibiting the G0G1 phase from transiting to the S phase ($P < .01$; Fig. 3).

Compared with 8 hours, the gradual time-dependent elevations in the percentage of either G-Rb1 (10^{-7} mol/L)-

or G-Rb1 plus SQ22536 (10^{-5} mol/L)-treated PC12 cells in the S and G2M phases were reflected by a decrease in the percentage of cells in the resting G0G1 phase at 48 and 72 hours ($P < .01$; Fig. 3). However, there were no time differences in the percentages of G-Rb1 plus SQ22536 (10^{-4} and 10^{-3} mol/L)-treated PC12 cells in the S and G2M phases at 24, 48, and 72 hours, compared with 8 hours (Fig. 3).

3.4. Effects of H89 on G-Rb1-stimulated PC12 cell cycle kinetics

To examine the effects of H89 on G-Rb1-stimulated PC12 cell proliferation, we examined cell cycle kinetics in PC12 cells after various treatments. H89 (10^{-5} – 10^{-3} mol/L) plus G-Rb1 (10^{-7} mol/L) decreases the proportion of cells in the S and G2M phase at 24, 48 and 72 hours. We also observed a concomitant increase in the proportion of cells in the G0G1 phase, compared with the G-Rb1-treated cells ($P < .05$ and $P < .01$; Fig. 4). Flow cytometric analysis showed the PKA inhibitor H89 produced a marked decrease in G-Rb1-stimulated cell cycle kinetics by inhibiting the G0G1 phase from transiting to the S and G2M phases.

The gradual time-dependent elevation in the percentage of either G-Rb1 (10^{-7} mol/L)- or G-Rb1 plus H89 (10^{-5} mol/L)-treated PC12 cells in the S and G2M phases was reflected

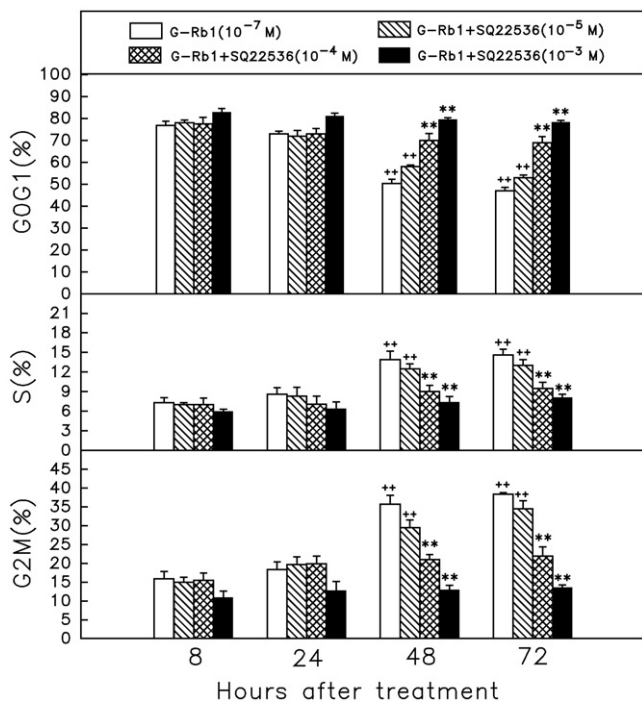


Fig. 3. Quantitative analysis of SQ22536 (10^{-5} – 10^{-3} mol/L) on G-Rb1 (10^{-7} mol/L)-stimulated PC12 cell cycle kinetics (G0G1, S, and G2M phase) by flow cytometry for 8, 24, 48, and 72 hours. Each point represents the mean of 3 independent experiments performed in duplicate ($n = 6$), and error bars indicate the SEM. The cell numbers in different phases (G0G1, S, and G2M) of the cell cycle were expressed as relative percentage. $**P < .01$ as compared with the G-Rb1 (10^{-7} mol/L)-treated group. $^{++}P < .01$ as compared with the 8-hour-treated group.

by a decrease in the percentage of cells in the resting G0G1 phase at 48 and 72 hours ($P < .01$; Fig. 4). Compared with 8 hours, there were no time difference in the percentage of G-Rb1 plus H89 (10^{-4} and 10^{-3} mol/L)-treated PC12 cells in the S and G2M phases at 24, 48, and 72 hours (Fig. 4).

4. Discussion

Parkinson disease is characterized by selective loss of dopaminergic neurons in the substantia nigra of the brain [10,21]. PC12 cells produce dopamine and have been used extensively as model “dopaminergic” cell lines for the study of Parkinson disease [10–14]. Here, we used PC12 cells to investigate mechanisms for dopaminergic cell cycle kinetics induced by G-Rb1. The ginsenosides (Rb1 and Rg1), a key family of components of Radix ginseng, are neuroprotective, are anti-amnesic, and cause improvement in learning and memory [8,22–25]. Glutamate is a major excitatory neurotransmitter in the mammalian nervous system and plays an important role in many physiologic functions including learning, memory, and brain development [26,27]. Ginsenoside Rb1 promotes glutamate release in PC12 cells by modulating phosphorylation of synapsins through a cAMP-dependent PKA pathway [3,4]. We hypothesized that G-Rb1

stimulates the proliferation and mitosis of PC12 cells by promoting the cell cycle at all regulatory points. Moreover, part of this mechanism is mediated via the adenylate cyclase-dependent PKA signaling pathway.

The cell cycle in its simplest form involves the cell transiting from resting phase or differentiated function (G0) through proliferative protein synthesis (G1), DNA synthesis (S), preparatory phase for mitosis (G2), and mitosis (M) phases [15]. In the present study, we used multiparameter flow cytometric analysis as a quantitative and qualitative method to examine the changes in relative cell number and specific changes within the cell cycle.

In this study, we found that G-Rb1 produced the most powerful enhancement of the proliferation rate of PC12 cells (data not shown). Based on this finding, we suspected a direct effect of G-Rb1 on the cell cycle. In the next step, we analyzed cell cycle distribution of G-Rb1-treated and untreated cells. Maximal DNA synthesis was seen at 48 and 72 hours after both vehicle and G-Rb1 (10^{-9} – 10^{-6} mol/L) treatment (Fig. 2, middle panel). The higher dose of G-Rb1 (10^{-7} and 10^{-6} mol/L) induced an increase in the percentage of S and G2M cells and a corresponding decrease in the G0G1 phase in PC12 cells at 48 and 72 hours. Flow cytometric assay confirms that G-Rb1 increases PC12 cell cycle kinetics in a dose-dependent manner.

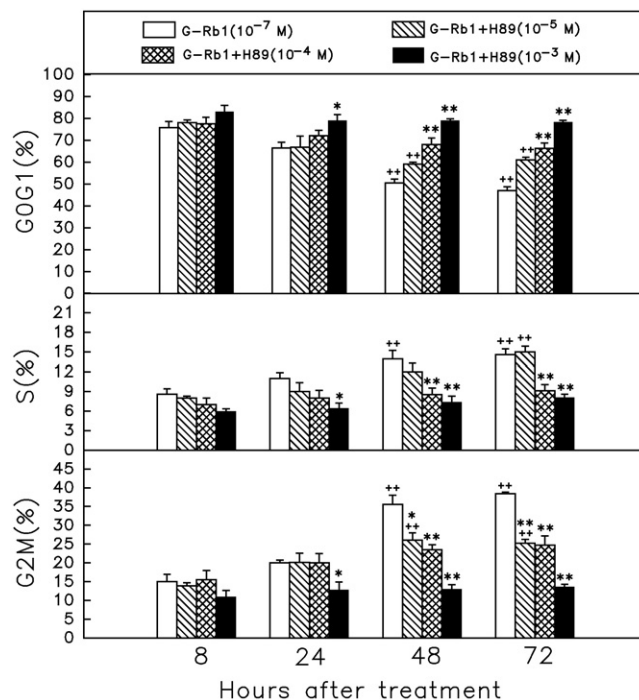


Fig. 4. Quantitative analysis of H89 (10^{-5} – 10^{-3} mol/L) on G-Rb1 (10^{-7} mol/L)-stimulated PC12 cell cycle kinetics (G0G1, S, and G2M phase) by flow cytometry for 8, 24, 48, and 72 hours. Each point represents the mean of 3 independent experiments performed in duplicate ($n = 6$), and error bars indicate the SEM. The cell numbers in different phases (G0G1, S, and G2M) of the cell cycle were expressed as relative percentage. $*P < .05$ or $**P < .01$ as compared with the G-Rb1 (10^{-7} mol/L)-treated group, respectively. $^{++}P < .01$ as compared with the 8-hour-treated group.

It has been indicated that PKA and extracellular signal-regulated kinase play a key role in KCl- and forskolin (an adenylate cyclase activator)-induced neuronal differentiation of PC12 cells by integration of signals from both pathways [28]. The inflammatory mediator interleukin-1 α stimulates dopamine release from PC12 cells by activating PKA [29]. We found that the stimulatory effects of G-Rb1 on cell cycle kinetics in PC12 cells were attenuated by the administration of adenylate cyclase inhibitor SQ22536. The PKA inhibitor H89 produced a marked decrease in the G-Rb1-stimulated cell cycle kinetics by inhibiting the G0G1 phase from transiting to the S phase. It has been shown that G-Rb1 may bind to its receptors to enhance the cAMP activity and then increase neurotransmitter release by up-regulating the phosphorylation of synapsins via the PKA pathway [3,4,30]. These results suggest that G-Rb1 stimulates PC12 cell proliferation and mitosis by promoting the cell cycle at all regulatory points. Moreover, part of this mechanism is mediated via the adenylate cyclase-dependent PKA signaling pathway.

Ginsenoside Rb1 plays a major role in exerting these effects on the central nervous system [31], such as modulation of neurotransmitters, learning, and memory [4,23,25]. It is believed that the medicinal herbs of ginseng have antiaging properties [32]. Recent studies have shown that ginseng is effective in the prevention or intervention of aging-associated neurologic disorders, such as Parkinson disease or Alzheimer disease [6-8,32]. These findings suggest that G-Rb1 may effect all phases of cell cycle and stimulate PC12 cell proliferation by stimulating transiting of the G0G1 phase to the S and G2M phases. The results demonstrate that G-Rb1 exerts a proliferative effect in the PC12 cell. This is in part due to the increase of adenylate cyclase and PKA enzyme activities and the stimulation of cell division rates.

Our results support our hypothesis. Ginsenoside Rb1 may provide a nutritional benefit for humans with prevention of aging-related disorders such as Parkinson disease or memory decline; however, specific studies must be conducted. The limitation of this study the inability to define whether the observed cell cycle changes are due to the effects of G-Rb1 on signal transduction pathways (eg, calcium) or on specific cell cycle-related proteins.

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References

- [1] Kim HS, Lee JH, Goo YS, Nah SY. Effects of ginsenosides on Ca²⁺ channels and membrane capacitance in rat adrenal chromaffin cells. *Brain Res Bull* 1998;46:245-51.
- [2] Radad K, Gille G, Moldzio R, Saito H, Rausch WD. Ginsenosides Rb1 and Rg1 effects on mesencephalic dopaminergic cells stressed with glutamate. *Brain Res* 2004;1021:41-53.
- [3] Xue JF, Hu JF, Liu ZJ, Chen H, Zhang JT, Chen NH. The mechanism of ginsenosides Rg1 and Rb1 promoting glutamic acid release from PC12 cells. *Yao Xue Xue Bao* 2006;41:1141-5.
- [4] Xue JF, Liu ZJ, Hu JF, Chen H, Zhang JT, Chen NH. Ginsenoside Rb1 promotes neurotransmitter release by modulating phosphorylation of synapsins through a cAMP-dependent protein kinase pathway. *Brain Res* 2006;1106:91-8.
- [5] Zhao R, McDaniel WF. Ginseng improves strategic learning by normal and brain-damaged rats. *Neuroreport* 1998;9:1619-24.
- [6] Zhong YM, Nishijo H, Uwano T, Tamura R, Kawanishi K, Ono T. Red ginseng ameliorated place navigation deficits in young rats with hippocampal lesions and aged rats. *Physiol Behav* 2000;69:511-25.
- [7] Mook-Jung I, Hong HS, Boo JH, Lee KH, Yun SH, Cheong MY, et al. Ginsenoside Rb1 and Rg1 improve spatial learning and increase hippocampal synaptophysin level in mice. *J Neurosci Res* 2001;63:509-15.
- [8] Chen F, Eckman EA, Eckman CB. Reductions in levels of the Alzheimer's amyloid beta peptide after oral administration of ginsenosides. *FASEB J* 2006;20:1269-71.
- [9] Shafer TJ, Atchison WD. Transmitter, ion channel and receptor properties of pheochromocytoma (PC-12) cells: a model for neurotoxicological studies. *Neurotoxicology* 1991;12:473-92.
- [10] Kitazawa M, Wagner JR, Kirby ML, Anantharam V, Kanthasamy AG. Oxidative stress and mitochondrial-mediated apoptosis in dopaminergic cells exposed to methylcyclopentadienyl manganese tricarbonyl. *J Pharmacol Exp Ther* 2002;302(1):26-35.
- [11] Hartley A, Stone JM, Heron C, Cooper JM, Schapira AH. Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. *J Neurochem* 1994;63:1987-90.
- [12] Kang D, Miyako K, Kuribayashi F, Hasegawa E, Mitumoto A, Nagano T, et al. Changes of energy metabolism induced by 1-methyl-4-phenylpyridinium (MPPt)-related compounds in rat pheochromocytoma PC12 cells. *Arch Biochem Biophys* 1997;337:75-80.
- [13] Kitazawa M, Anantharam V, Kanthasamy AG. Diethylindole induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. *Neuroscience* 2003;119:945-64.
- [14] Viswanath V, Wu Y, Boonplueang R, Chen S, Stevenson FF, Yantiri F, et al. Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. *J Neurosci* 2001;21:9519-28.
- [15] Marx J. The cell cycle: spinning farther afield. *Science* 1991;252:1490-2.
- [16] Bergstrom CS, Emdin GR, Stenling R. DNA content in colorectal carcinoma: a flow cytometric study of the epithelial fraction. *Anal Cell Pathol* 1995;8:287-95.
- [17] Begg AC, Hofland I. Cell kinetic analysis of mixed populations using three-color fluorescence flow cytometry. *Cytometry* 1991;12(5):445-54.
- [18] Chen XC, Zhu YG, Zhu LA, Huang C, Chen Y, Chen LM, et al. Ginsenoside Rg1 attenuates dopamine-induced apoptosis in PC12 cells by suppressing oxidative stress. *Eur J Pharmacol* 2003;473(1):1-7.
- [19] Suzuki T, Fujikura K, Higashiyama T, Takata K. DNA staining for fluorescence and laser confocal microscopy. *J Histochem Cytochem* 1997;45(1):49-53.
- [20] Steel RD, Torrie H. Principles and procedures of statistics. New York: McGraw-Hill; 1981.
- [21] Klintworth H, Newhouse K, Li T, Choi WS, Faigle R, Xia Z. Activation of c-Jun N-terminal protein kinase is a common mechanism underlying paraquat- and rotenone-induced dopaminergic cell apoptosis. *Toxicol Sci* 2007;97(1):149-62.
- [22] Tohda C, Matsumoto N, Zou K, Meselhy MR, Komatsu K. Axonal and dendritic extension by protopanaxadiol-type saponins from ginseng drugs in SK-N-SH cells. *Jpn J Pharmacol* 2002;90:254-62.
- [23] Kim EH, Jang MH, Shin MC, Shin MS, Kim CJ. Protective effect of aqueous extract of Ginseng radix against 1-methyl-4-

- phenylpyridinium–induced apoptosis in PC12 cells. *Biol Pharm Bul* 2003;26:1668-73.
- [24] Kim S, Nah SY, Rhim H. Neuroprotective effects of ginseng saponins against L-type Ca^{2+} channel-mediated cell death in rat cortical neurons. *Biochem Biophys Res Commun* 2008;365:399-405.
- [25] Chen LW, Wang YQ, Wei LC, Shi M, Chan YS. Chinese herbs and herbal extracts for neuroprotection of dopaminergic neurons and potential therapeutic treatment of Parkinson's disease. *CNS Neuro Disord Drug Targets* 2007;6:273-81.
- [26] Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 1993;361:31-9.
- [27] Reis HJ, Guatimosim C, Paquet M, Santos M, Ribeiro FM, Kummer A, et al. Neuro-transmitters in the central nervous system & their implication in learning and memory processes. *Curr Med Chem* 2009; 16:796-840.
- [28] Hansen TO, Rehfeld JF, Nielsen FC. KCl potentiates forskolin-induced PC12 cell neurite outgrowth via protein kinase A and extracellular signal–regulated kinase signaling pathways. *Neurosci Lett* 2003;347:57-61.
- [29] Joseph A, Kumar A, O'Connell NA, Agarwal RK, Gwosdow AR. Interleukin-1 alpha stimulates dopamine release by activating type II protein kinase A in PC-12 cells. *Am J Physiol* 1995;269:E1083-8.
- [30] Wang ZJ, Nie BM, Chen HZ, Lu Y. Panaxynol induces neurite outgrowth in PC12D cells via cAMP- and MAP kinase–dependent mechanisms. *Chem Biol Interact* 2006;159(1):58-64.
- [31] Benishin CG. Actions of ginsenoside Rb1 on choline uptake in central cholinergic nerve endings. *Neurochem Int* 1992;21(1):1-5.
- [32] Ho YS, So KF, Chang RC. Anti-aging herbal medicine—how and why can they be used in aging-associated neurodegenerative diseases? *Ageing Res Rev* 2010;9(3):354-62.