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Ginsenoside Rg1 attenuates dopamine-induced apoptosis in PC12 cells by suppressing oxidative stress

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Abstract

In Parkinson's disease, neuroprotective therapy to rescue dopamine neurons has been proposed. Ginsenoside Rg1, one of the biologically active ingredients of ginseng, may be a candidate neuroprotective drug. In the present study, the mechanism underlying the neuroprotection provided by ginsenosde Rg1 was studied against apoptosis induced by exogenous dopamine in PC12 cells. Pretreatment with ginsenoside Rg1 markedly reduced the generation of dopamine-induced reactive oxygen species and the release of mitochondrial cytochrome *c* into the cytosol, and subsequently inhibited the activation of caspase-3. In addition, Rg1 pretreatment also reduced inducible nitric oxide (NO) synthase protein level and NO production. These results suggested that ginsenoside Rg1 may attenuate dopamine-induced apoptotic cell death through suppression of intracellular oxidative stress, and that it may rescue or protect dopamine neurons in Parkinson's disease.

Keywords: Dopamine; Parkinson's disease; Ginseng; Saponin; Oxidative stress; Apoptosis

1. Introduction

Parkinson's disease is a progressive motor disorder of the central nervous system. Extensive postmortem studies have provided evidence to support the notion that oxidative stress is involved in the pathogenesis of Parkinson's disease. Dopamine can be oxidized to generate semiquinones, quinones, oxygen radicals and other reactive oxygen species, which are toxic and ultimately contribute to inhibition of mitochondrial respiration and to lipid peroxidation, and may play an important role in neuronal cell death (Jenner and Olanow, 1996; Barzilai et al., 2001; Zeevalk et al., 1998). Indeed, dopamine has been shown to be toxic to cell cultures in vitro and administration of dopamine directly into the brain results in cell death (Michel and Hefti, 1990; Luo et al., 1999). Furthermore, some antioxidants such as glutathione and *N*-acetylcysteine protect against dopamine-induced apoptosis (Offen et al., 1996).

Therefore, interest has focused on antioxidants as candidates for the current and future treatment of Parkinson's disease. Ginseng has long been used for the alleviation of many ailments, particularly those associated with aging. Recently, studies have shown that ginsenoside Rg1, one of the active ingredients of ginseng, has potential neurotrophic and neuroprotective effects (Rudakewich et al., 2001). Studies also indicated that Rg1 could attenuate dopamine-induced apoptosis in PC12 cells (Chen et al., 2001). However, the molecular events involved in the processes are still unclear. In this study, we investigated the effects of ginsenoside Rg1 on dopamine-induced apoptosis in PC12 cells in terms of suppression of intracellular oxidative stress.

2. Materials and methods

2.1. Materials

Ginsenoside Rg1 (purity>98%) was obtained from the Department of Organic Chemistry of Norman Bethune Medical University (Changchun, Jilin, China). It is a dammrane-type tetracyclic triterpenoid saponin and its aglycone is 20-*S*-protopanaxatriol (Fig. 1).

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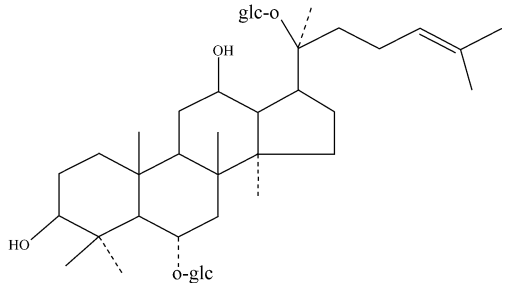

Fig. 1. Structure of ginsenoside Rg1. Molecular formula: $C_{42}H_{72}O_{14}$. Molecular weight: 800.

Dulbecco's modified Eagel's medium (DMEM), fetal bovine serum and horse serum were obtained from Gibco BRL (Grand Island, NY, USA). Dopamine, cyclosporin A, nerve growth factor (NGF), aminoguanidine and propidium iodide were from Sigma (St. Louis, MO, USA). Primary antibodies included rabbit monoclonal antibodies to rat cytochrome *c*, inducible nitric oxide synthase (iNOS), and cleaved caspase-3 were from BD PharMingen (San Diego, CA, USA), Santa Cruz (Santa Cruz, CA, USA) and Cell Signaling (Beverly, MA, USA), respectively. Caspase-3 Assay Kit and *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were from BD PharMingen. Western Blot Chemiluminescent Detection System (LumiGLO system) was obtained from KPL (Gaithersburg, MD, USA).

2.2. Cell culture and treatment

Pheochromocytoma-derived PC12 cells, which acquire a neuronal phenotype in the presence of NGF, were induced to differentiate by treating them with NGF (50 ng/ml) in DMEM containing 1% horse serum for 7 days. The medium, including NGF, was subsequently replaced every 2 days. Then the differentiated cells were cultured in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin and were kept at 37 °C in humidified 5% CO_2/95% air. PC12 cells were treated with reagents as follows: (1) For treatment, PC12 cells were treated for 24 h with various dopamine concentrations. (2) For pretreatment, PC12 cells were pretreated for 24 h with aminoguanidine, cyclosporin A, Ac-DEVD-CHO or ginsenoside Rg1, respectively, and then the cells were further treated with 0.45 mM dopamine for 24 h.

2.3. Assay of DNA fragmentation

Approximately 5×10^6 cells from each experimental condition were harvested, and the procedure for extraction of DNA was followed as described (Herrman et al., 1994). In brief, the cells were scraped from the dishes using a rubber, then washed with 100 mM phosphate-buffered saline (PBS) and centrifuged at $1000 \times g$ for 5 min. The cell pellets were then treated for 10 s with 50 μl lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5). After centrifugation at $1600 \times g$ for 5 min, the supernatants were incubated with 1% sodium dodecyl sulfate (SDS) and 5 mg/ml RNase A at 56 °C for 2 h, and then digested with 2.5 mg/ml proteinase K at 37 °C for at least 2 h. After incubation and addition of 1 M ammonium acetate, the DNA was precipitated with 2.5 volume of ethanol, dissolved in gel loading buffer, and then separated by electrophoresis on 1.0% agarose gels containing 0.1 μg/ml ethidium bromide. DNA was visualized under ultraviolet light.

2.4. Flow cytometric analysis of DNA content

To quantify the apoptotic cells, the treated PC12 cells were harvested and washed twice with 100 mM cold PBS and fixed with 70% ethanol. Then the cells were centrifuged at $200 \times g$ for 10 min and resuspended in 100 μl PBS containing 200 μg/ml propidium iodide and 50 μg/ml RNase A at room temperature for 30 min. The fluorescence of cells was measured with a FACScan flow cytometer (Becton Dickinson, FACScan). Data were analyzed with CellQuest™ software.

2.5. Nitrite content measurement

Nitrite was quantified by the Griess reaction. In brief, 4 M HCl was added to the supernatant for 10 min, and then 2 mg/ml sulfanilic acid and 1 mg/ml *N*-(1-naphthyl)-ethylenediamine were added. After incubation for 30 min, absorbance was measured with a spectrophotometer at a wavelength of 550 nm. The absorbance of a sample was compared with that of standard sodium nitrite solution.

2.6. Measurement of intracellular reactive oxygen species

2,7-Dichlorofluorescein diacetate (DCF-DA) is a nonfluorescent compound that, in the presence of oxidants, is intracellularly oxidized to the fluorescent 2,7-dichlorofluorescein (DCF), which can be quantified by flow cytometry (Cassarino et al., 1997). To monitor intracellular reactive oxygen species generation, cultured cells were incubated with 10 mM DCF-DA for 15 min at 37 °C and rinsed three times with 100 mM PBS, then the cell fluorescence was measured with a FACScan flow cytometer.

2.7. Assay for caspase-3 activity

Caspase-3 activity was measured with the Colorimetric Caspase-3 Assay Kit in accordance with the protocol supplied by the manufacturer. The colorimetric substrate *N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin) was added to 100 μg of cell lysate. After incubation for 1 h, the activity of caspase-3 was analyzed by spectrofluorometry with an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Enzyme activity is

expressed as fluorescence units per minute per milligram of protein. The protein concentration was determined by the method of Bradford.

2.8. Western blotting for determination of iNOS and cleaved caspase-3

Proteins were extracted by washing with ice-cold PBS and incubating them for 30 min in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% solution deoxycholate, 1 mM phenylmethysulfonyl fluoride and 100 μg/ml leupeptin. Cells lysates were centrifuged at 12,000 × *g* for 10 min, and the protein concentrations were determined by the Bradford method. For Western blots, 50 μg of total cell protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were incubated with the appropriate antibody using Western Blot Chemiluminescence Detection System.

2.9. Detection of cytochrome c release

Approximately 5×10^6 cells were collected by centrifugation and the pellets were washed with ice-cold PBS and resuspended in 100 μl buffer containing 250 mM sucrose, 20 mM HEPES–KOH, pH 7.5, 10 mM KOH, 1.5 mM $MgCl_2$, 1 mM ethylenediaminetetra-acetic acid (EDTA), 1 mM dithiothreitol and 1 mM phenylmethysulfonyl fluoride. Cells were then homogenized. The mitochondria-containing fraction was pelleted by centrifugation at 18,000 × *g* for 25 min. The supernatant was used for Western blot analysis with a monoclonal antibody (7H8.2C12) to cytochrome *c*.

2.10. Statistical analysis

Statistical analysis of the data for multiple comparisons was performed by analysis of variance (ANOVA) followed by Dunnett's test. For single comparisons, the significance of differences between means was determined by *t*-test.

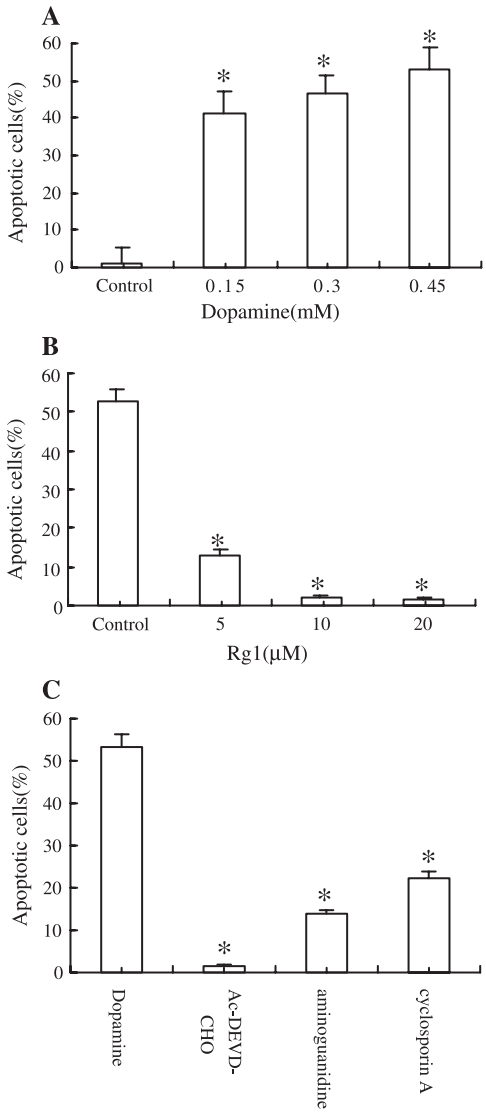

Fig. 2. Quantitative analysis of apoptotic PC12 cells by flow cytometry. PC12 cells were treated with 0.15–0.45 mM dopamine for 24 h (A) or were pretreated with 5–20 μM ginsenoside Rg1 (B) or with 0.1 mM Ac-DEVD-CHO or aminoguanidine, or 10 μM cyclosporin A (C) for 24 h, and then were cultured with 0.45 mM dopamine for 24 h. Each value represents the mean ± S.D. of three independent experiments. *$P < 0.01$ versus control or dopamine group.

3. Results

3.1. Effect of ginsenoside Rg1 on the neurotoxicity of dopamine in PC12 cells

Caspase-3 is a prototypical caspase that becomes activated during apoptosis and can be used as an apoptotic marker. Exposure of PC12 cells to 0.15–0.45 mM dopamine for 24 h resulted in a concentration-dependent increase in apoptotic cell death (Fig. 2A), and DNA fragmentation was observed, too (Fig. 3A). In addition, dopamine induced caspase-3 activation, with increased caspase-3 cleavage and caspase-3 activity (Figs. 4 and 5A). These biochemical changes are characteristic of apoptosis. However, pretreatment of PC12 cells with 5–20 μM ginsenoside Rg1 for 24 h resulted in significant protection against DNA fragmentation and caspase-3 activation compared with treatment with 0.45 mM dopamine alone (Figs. 2B, 3B, 4 and 5B). In addition, pretreatment with 0.1 mM Ac-DEVD-CHO, an inhibitor of caspase-3, resulted in a decrease of dopamine-induced cell apoptosis and caspase-3 activation (Figs. 2C, 4 and 5C). These results showed that dopamine was capable of promoting PC12 cell apoptosis by activation of caspase-3 and that ginsenoside Rg1 might attenuate dopamine-induced apoptotic death in PC12 cells.

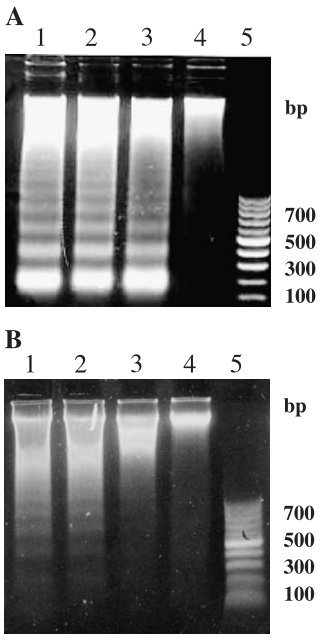

Fig. 3. DNA gel electrophoresis after treatment of PC12 cells with dopamine or pretreatment with ginsenoside Rg1. Cells were cultured with 0.15–0.45 mM dopamine for 24 h (A) or pretreated with 5–20 μM ginsenoside Rg1 for 24 h, and then cultured with 0.45 mM dopamine for 24 h (B). (A) Lines 1–3, treatment with 0.15, 0.30 and 0.45 mM dopamine, respectively; line 4, control; line 5, DNA marker (1031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp). (B) Lines 1–3, pretreatment with 5–20 μM ginsenoside Rg1, and then with 0.45 mM dopamine; line 4, control; line 5, DNA marker.

3.2. Involvement of oxidative species in the effects of dopamine and Rg1

Reactive oxygen species and nitric oxide (NO) were used to evaluate the role of oxidative species in dopamine-induced apoptosis. Treatment of PC12 cells with 0.15–0.45 mM dopamine for 24 h produced a significant increase in the intracellular generation of reactive oxygen species and

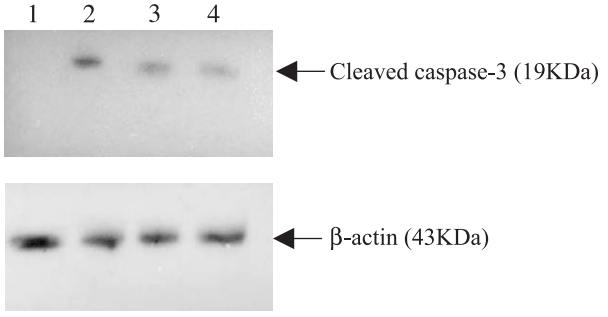

Fig. 4. Dopamine treatment induced caspase-3 cleavage and the effects of ginsenoside Rg1. PC12 cells were treated with 0.45 mM dopamine for 24 h or pretreated with 10 μM ginsenoside Rg1 or pretreated with 0.1 mM Ac-DEVD-CHO for 24 h. Next, they were cultured with 0.45 mM dopamine for 24 h. 1: Normal; 2: dopamine; 3: Rg1 + dopamine; 4: Ac-DEVD-CHO + dopamine.

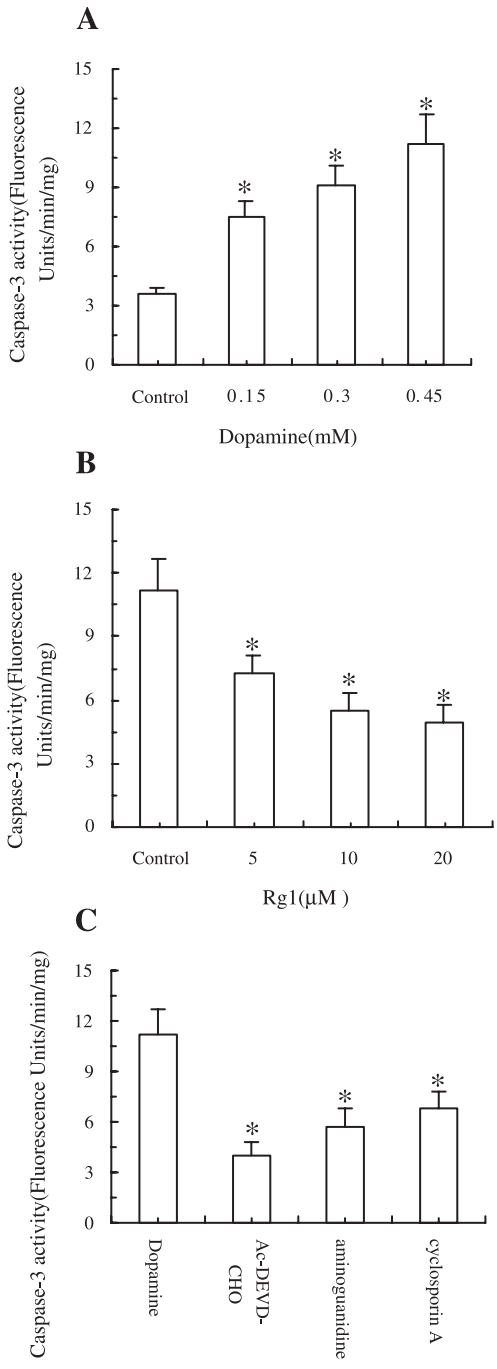

Fig. 5. Caspase-3 activity in PC12 cells after treatment with dopamine or ginsenoside Rg1. PC12 cells were treated with 0.15–0.45 mM dopamine for 24 h (A) or were pretreated with 5–20 μM ginsenoside Rg1 (B) or with 0.1 mM Ac-DEVD-CHO or aminoguanidine, or 10 μM cyclosporin A (C) for 24 h. Next, they cultured with 0.45 mM dopamine for 24 h. Each value represents the mean ± S.D. of three independent experiments. *$P < 0.01$ versus control or dopamine group.

NO in a concentration-dependent manner (Figs. 6A and 7A). The level of iNOS protein was also increased (Fig. 8). However, pretreatment with aminoguanidine, an iNOS inhibitor, resulted in a marked decrease in iNOS protein

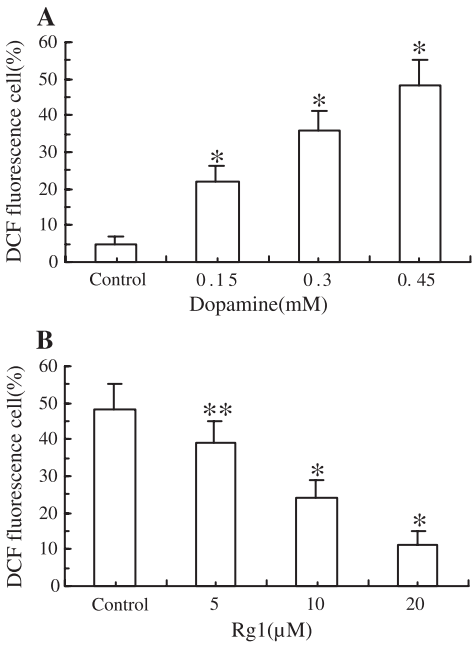

Fig. 6. Flow cytometric analysis of reactive oxygen species production in PC12 cells. Cells were cultured with 0.15–0.45 mM dopamine for 24 h (A) or were pretreated with 5–20 μM ginsenoside Rg1 for 24 h, and then they were cultured with 0.45 mM dopamine for 24 h (B). Each value represents the mean ± S.D. of three independent experiments. *$P<0.01$ versus control or dopamine group; **$P<0.05$ versus dopamine group.

expression and NO generation (Figs. 7C and 8). Pretreatment of the cells with ginsenoside Rg1 decreased the generation of reactive oxygen species and NO, as well as the iNOS protein level (Figs. 6B, 7B and 8). These results indicated that both reactive oxygen species and NO were generated during dopamine-induced apoptotic cell death, and that Rg1 could act as a potential candidate to attenuate the dopamine-induced increase in reactive oxygen species and NO generation.

3.3. Involvement of cytochrome c release in the effects of dopamine and Rg1

To address whether or not cytochrome *c* release contributes to dopamine-induced apoptosis, cytosolic fractions were isolated from lysates of cultured PC12 cells treated with 0.15–0.45 mM dopamine for 24 h. Western blot analysis revealed the accumulation of cytosolic cytochrome *c* (Fig. 9A). However, pretreatment of the cells with ginsenoside Rg1 decreased the release of cytochrome *c* into the cytosol (Fig. 9B). Importantly, preincubation with 10 μM cyclosporin A, a mitochondrial permeability transition pore inhibitor, decreased cytochrome *c* release from the mitochondria. Pretreatment with Ac-DEVD-CHO or aminoguanidine at 0.1 mM did not affect cytochrome *c* release (Fig. 9C). These results suggested that the dopamine-induced generation of reactive oxygen species or NO was a trigger

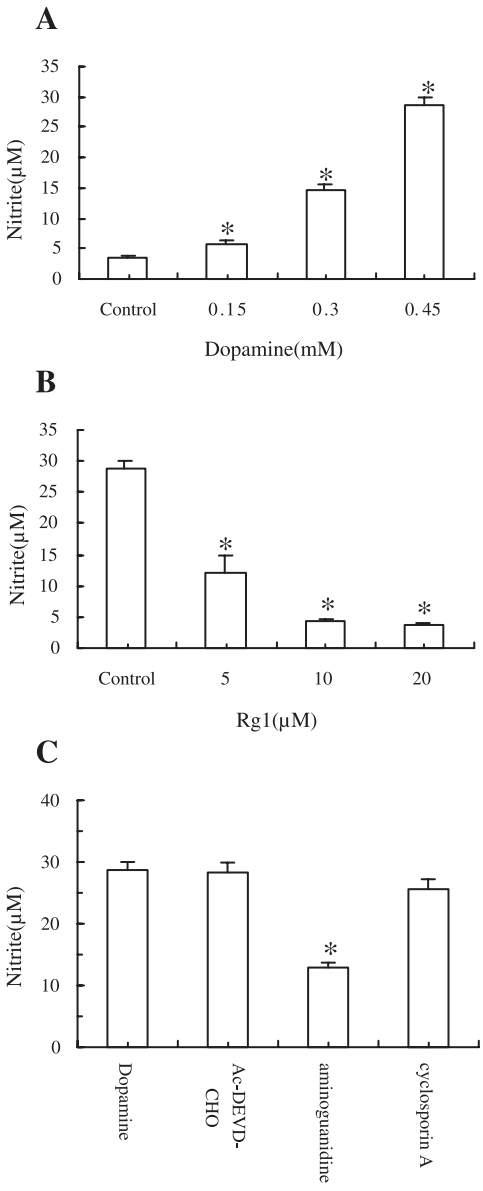

Fig. 7. Cellular nitric oxide production was analyzed by evaluation of nitrite in PC12 cells. PC12 cells were treated with 0.15–0.45 mM dopamine for 24 h (A) or were pretreated with 5–20 μM ginsenoside Rg1 (B) or with 0.1 mM Ac-DEVD-CHO or aminoguanidine, or 10 μM cyclosporin A (C) for 24 h. Then they were cultured with 0.45 mM dopamine for 24 h. Each value represents the mean ± S.D. of three independent experiments. *$P<0.01$ versus control or dopamine group.

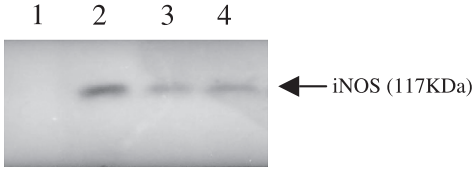

Fig. 8. Dopamine treatment caused an increase in iNOS protein and the effect of ginsenoside Rg1. PC12 cells were treated with 0.45 mM dopamine for 24 h or were pretreated with 10 μM ginsenoside Rg1 or with 0.1 mM aminoguanidine for 24 h. Then they were cultured with 0.45 mM dopamine for 24 h. 1: Normal; 2: dopamine; 3: aminoguanidine + dopamine; 4: Rg1 + dopamine.

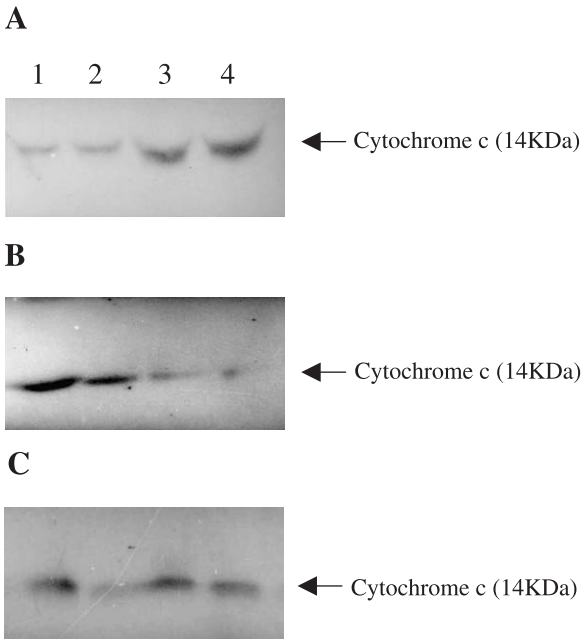

Fig. 9. Dopamine treatment induced cytochrome *c* release and the effects of ginsenoside Rg1. PC12 cells were treated with 0.15–0.45 mM dopamine for 24 h (A) or were pretreated with 5–20 μM ginsenoside Rg1 (B) or with 10 μM cyclosporin A or 0.1 mM Ac-DEVD-CHO or aminoguanidine (C) for 24 h. Next, they were cultured with 0.45 mM dopamine for 24 h. (A) 1: normal; 2, 3, 4: dopamine 0.15, 0.30 and 0.45 mM, respectively. (B) 1: dopamine, 0.45 mM; 2, 3, 4: Rg1 5, 10, 20 μM and dopamine 0.45 mM, respectively. (C) 1: dopamine, 0.45 mM; 2, 3, 4: cyclosporin A, Ac-DEVD-CHO, or aminoguanidine and dopamine 0.45 mM, respectively.

rather than a consequence of cytochrome *c* release and caspase-3 activation in dopamine-induced apoptosis.

4. Discussion

Increasing evidence indicates that dopamine might act as a neurotoxin and thereby participate in neurodegenerative processes (Barzilai et al., 2001). Dopamine has been shown to induce apoptosis in PC12 cells, and direct administration of dopamine into rat striatum resulted in neuronal loss and apoptosis (Offen et al., 1997; Hattori et al., 1998). In agreement with previous studies, the present study also demonstrated that dopamine was capable of promoting apoptotic cell death in PC12 cells. The characteristic features of apoptosis, including DNA fragmentation and caspase-3 activation, were seen in this study. The present study also provided evidence that dopamine-induced apoptosis is mediated by increased oxidative stress.

Oxidative stress is a leading mechanism for dopaminergic neuronal degeneration in Parkinson's disease (Seaton et al., 1997). The present results also indicate that dopamine-induced apoptosis in PC12 cells is associated with an increase in reactive oxygen species generation. Thus, excess reactive oxygen species resulting from dopamine autoxidation or metabolism may inhibit mitochondrial metabolism, while inhibition of energy metabolism may increase reactive oxygen species generation, thereby generating a vicious cycle of toxic events, eventually leading to cellular apoptosis.

In addition to reactive oxygen species, the current results also suggested a role for NO in dopamine-induced apoptosis. However, the contribution of NO to dopamine toxicity is unclear. Previous studies have demonstrated a direct association between dopaminergic loss and NO release in the striatum (Fujiyama and Masuko, 1996). Yoshie and Ohshima (1997) have shown that DNA fragmentation is mediated synergistically by NO and dopamine. It was suggested that NO may react with superoxides generated from dopamine oxidation to form $ONOO^-$, a strong nitrating agent capable of inducing DNA strand breakage and apoptosis (Lin et al., 1995). NO is endogenously produced by nitric oxide synthase (NOS), and a previous study showed that of the three NOS isoforms, only the activation of iNOS is regulated at the transcriptional level, mediated by the activation of nuclear factor-κβ (Xie et al., 1994). The present study showed that dopamine treatment of PC12 cells led to an increase in NO generation and iNOS protein level. Pretreatment with aminoguanidine almost completely inhibited iNOS expression and attenuated NO generation and subsequently cell apoptosis. These data strongly indicated that iNOS expression contributed to NO generation and the subsequent apoptotic cell death in PC12 cells after dopamine treatment.

Activation of caspase-3 is an important step in the execution of apoptosis and its inhibition blocks apoptotic cell death (Chen et al., 1998). Cytosolic cytochrome *c* has previously been demonstrated to bind apoptosis protease activating factor-1 and to subsequently trigger the sequential activation of caspase-9 and caspase-3 (Li et al., 1997). In this study, we showed that dopamine treatment also resulted in cytosolic accumulation of cytochrome *c* and in caspase-3 activation. Importantly, cyclosporin A, an inhibitor of mitochondrial permeability transition pore, reduced cytochrome *c* release and inhibited caspase-3 activation. However, inhibitor of caspase-3 Ac-DEVD-CHO only blocked the activation of caspase-3 and did not affect the release of cytochrome *c*. Furthermore, aminoguanidine also resulted in a decrease in cytochrome *c* release and caspase-3 activity. However, cyclosporin A and Ac-DEVD-CHO did not affect the generation of reactive oxygen species and NO. Taken together, these results indicate that dopamine is capable of promoting PC12 cell apoptosis through an oxidative mechanism. Oxidative species produced from dopamine might be a trigger and could in turn affect the release of cytochrome *c* from the mitochondria and the subsequent activation of caspase-3. Furthermore, in agreement with a previous report (Suzuki et al., 1999), cytochrome *c* release was a central event in mitochondria-triggered apoptosis, leading to caspase activation, and the generation of reactive oxygen species or NO might be an upstream event in the process of dopamine-induced apoptosis.

Although the exact mechanism of dopamine-induced apoptosis is unclear, antioxidants have proved to be effective in preventing dopamine-induced apoptosis (Offen et al., 1996). Antioxidant molecules such as *N*-acetyl-L-cysteine, vitamin E and catalase appear to protect cells from oxidative insults and the resultant induction of apoptotic cell death (Ferrari et al., 1995; Behl et al., 1994). Long-term *N*-acetyl-L-cysteine pretreatment has been shown to protect PC12 cells and sympathetic neurons from trophic factor deprivation-induced cell death and to influence cellular glutathione levels (Ferrari et al., 1995). In addition, Zhou and Zhu (2000) reported that Egb 761, an extract of Ginkgo biloba, was effective in preventing reactive oxygen species-induced apoptosis in PC12 cells, which indicates that some plant extracts may have potential protective effects against some oxidants. Ginsenoside, such as Rg1, represents the major active ingredient of ginseng, which has been shown to exert a variety of biomedical effects, such as anti-apoptotic activity (Chen et al., 2001). In this study, Rg1 dose-dependently attenuated dopamine-induced apoptosis. These results provide the first direct evidence that ginsenoside Rg1 could protect neuronal cells from dopamine neurotoxicity through an antioxidative mechanism, and that ginsenoside Rg1 might be act as a free radical scavenger.

In this study, the mechanism by which ginsenoside Rg1 protected PC12 cells from dopamine-induced apoptosis was further investigated. Results showed that ginsenoside Rg1 might attenuate the dopamine-induced elevation of reactive oxygen species or NO generation and subsequently protect mitochondria from reactive oxygen species-induced injury and reduced cytochrome *c* release, and eventually inhibit caspase-3 activation. Thus, the target of ginsenoside Rg1 for protecting PC12 cells from dopamine-induced apoptosis in PC12 cells may be the mitochondria.

In summary, the present study indicate that dopamine is capable of promoting neuronal cell death through an oxidative mechanism. Ginsenoside Rg1, an extract of ginseng, could interrupt the dopamine-induced elevation of reactive oxygen species or NO generation and attenuate the neurotoxicity of dopamine, which might offer a potential means to rescue or protect dopamine neurons in Parkinson's disease.

Acknowledgements

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