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Neuroprotective Effects of Glycyrrhizic Acid and 18β-Glycyrrhetinic Acid in PC12 Cells via Modulation of the PI3K/Akt Pathway

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Glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (18βGA) are the bioactive compounds of licorice. The neuroprotective effects of GA and 18βGA against serum/glucose deprivation and 6-hydroxydopamine (6-OHDA)-induced cytotoxicity in PC12 cells were investigated. The intracellular reactive oxygen species (ROS) content, the activity of the antioxidant enzymes of glutathione peroxidase (GPx) and catalase, the mitochondrial membrane potential (MMP), and the mitochondrial Bax/Bcl-2 ratio were determined. PI3K/Akt pathway signaling was also evaluated to study the possible protective mechanism. The results showed that GA treatment decreased the ROS content by elevating the activities of GPx and catalase, leading to a decreased MMP. GA and 18βGA also lowered the mitochondrial Bax/Bcl-2 ratio and activated PI3K/Akt signal. The results suggest that GA may protect PC12 cells from ischemic injury via modulation of the intracellular antioxidant system and mitochondria-induced apoptosis. Moreover, GA and 18βGA may modulate the ratio of the mitochondrial Bcl-2 family and influence PI3K/Akt signaling. These results demonstrate the neuroprotective ability of GA and 18βGA and suggest that the cytotoxicity of 6-OHDA may influence the mitochondrial Bax/Bcl-2 ratio without altering the expression of Bax. This study also suggests a possible compound for treating neural disease and general neuronal health.

KEYWORDS: Glycyrrhizic acid; 18β-glycyrrhetinic acid; mitochondria; PI3K/Akt pathway; neuroprotection

INTRODUCTION

There are many situations that cause neuronal damage. One example is ischemic injury, which occurs as a result of the temporary suspension of circulation. This causes mitochondria dysfunction and intracellular reactive oxygen species (ROS) content to become elevated (*1*). Parkinson's disease is a common neurodegenerative disease in which neuronal damage occurs. Many studies have suggested that compounds that inhibit the apoptosis pathway may be helpful in reducing neuronal injury (*2*−*4*). It has been shown that antioxidants such as edaravone may reduce the ROS content both in vitro and in vivo (*5*, *6*). There is, however, evidence showing that antioxidants are not always safe to use. For example, edaravone, a potent antioxidant, causes acute renal failure and fulminant hepatitis (*7*). It would, therefore, be beneficial to find a compound that is less toxic and that protects neurons from damage.

Licorice, a well-known herb plant with pharmaceutical effects, has been used for thousands of years as a treatment for liver disease in traditional Chinese medicine. Studies on licorice have shown that glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (18βGA) are the biologically active compounds (*8*). In a previous study, roasted licorice has been shown to protect against oxygen and glucose deprivation (OGD)-induced PC12 cell damage and to reduce the damaged area in gerbil brains after transient forebrain ischemia (*9*). Recently, Yim et al. (*10*) also indicated that GA and 18βGA protect PC12 from 1-methyl-4-phenylpyridinium-induced cytotoxicity. The detail of the protective pathway, however, remains to be elucidated. It has also been shown that GA can block the activation of NF-κB in primary neurons (*11*). Activation of NF-κB is essential to dopamine-induced apoptosis in PC12 cells, and it may be involved in nigral neurodegeneration in patients with Parkinson's disease (*12*). This suggests that GA and 18βGA may protect against 6-hydroxydopamine (6-OHDA)-induced neuronal damage. Therefore, we hypothesized that GA and 18βGA may be promising new compounds for neuronal protection. The purpose of this study was to investigate the neuroprotective effects of GA and 18βGA against serum/glucose deprivation and 6-hydroxydopamine (6-OHDA)-induced cytotoxicity in PC12 cells.

MATERIALS AND METHODS

Chemicals. GA, 18βGA, 2′,7′-dichlorofluorescein (DCF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 4′,6-diamidino-2-phenylindole (DAPI), 6-hydroxydopamine, ascorbic acid, LY294002, sodium bicarbonate, and tris(hydroxymethyl)aminomethane (Tris) were

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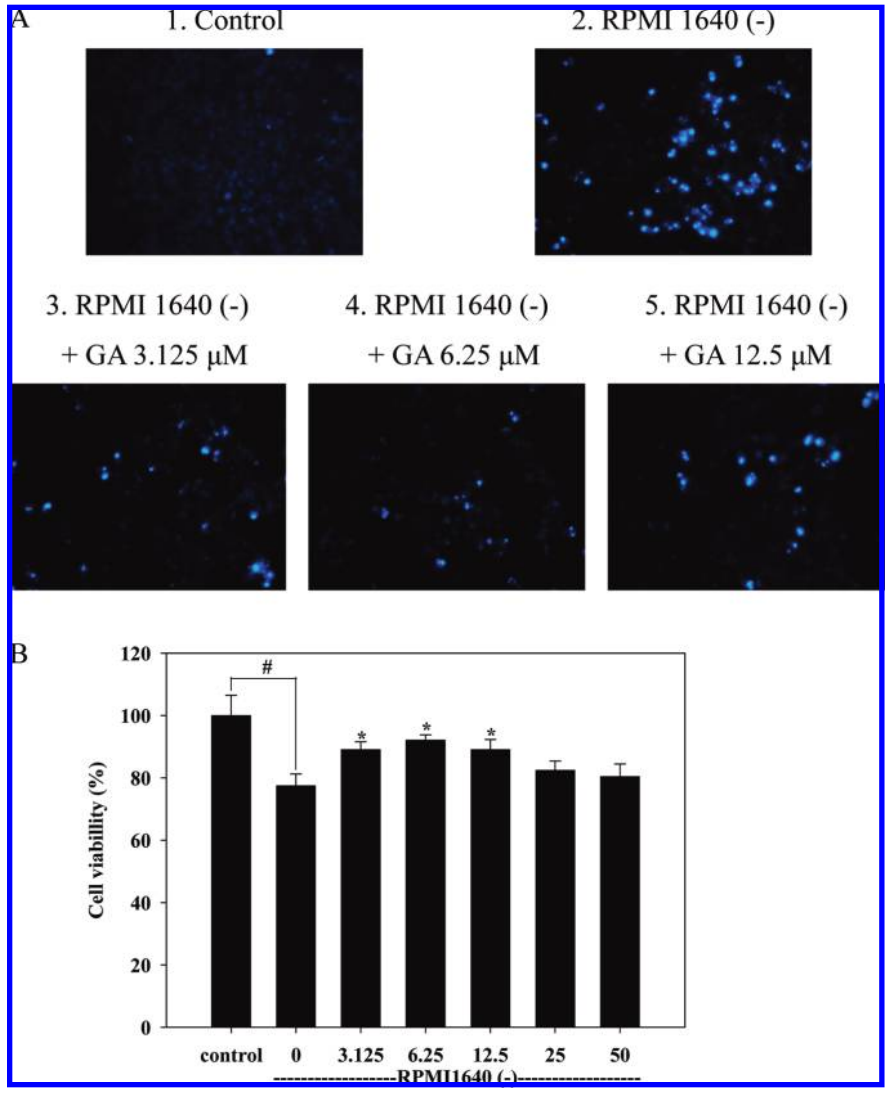

Figure 1. (**A**) Effect of glycyrrhizic acid (GA) on cell viability in serum/glucose-deprived (RPMI 1640 (−)) PC12 cells. PC12 cells were co-incubated in normal condition (A1), serum/glucose-deprived condition only (A2), and serum/glucose-deprived condition with co-incubated condition with a 3.125 μM (A3), 6.25 μM (A4), and 12.5 μM concentration (A5) of GA for 16 h. After treatment, cells were washed three times and stained by DAPI. Intracellular chromatin condensation was observed in a light-inverted microscope (Olympus, magnification = 200×). (**B**) Quantification of cell death induced by the serum/glucose-deprived condition and the protective ability of GA. Following treatment, cell viability was measured by MTT assay. Data are presented as means ± SD (n = 3). #, $p < 0.05$; ∗, $p < 0.05$, compared to RPMI 1640 (−) alone.

obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum and RPMI 1640 were purchased from Invitrogen (Carlsbad, CA). Anti-Bax, anti-Bcl-2, anti-phosphorylated Akt, and HRP conjugated second antibody were purchased from Cell Signaling (Boston, MA). All fine chemicals were obtained from Sigma-Aldrich and Showa Chemical (Tokyo, Japan).

Cell Culture. The rat pheochromocytoma cell (PC12) line (BCRC 60048) was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 10% horse serum, 5 mg/L penicillin, 5 mg/L streptomycin, and 10 mg/L neomycin at 37 °C and under 5% CO_2. All treatments were performed when cells seeded at 80% confluence.

Cell Viability Assay. The protective ability of GA and 18βGA against ischemic or 6-OHDA-induced cell death was measured by MTT assay. Briefly, PC12 cells (5×10^4 cells/mL) were seeded in 96-well microtiter plates. After co-incubation with serum/glucose deprivation or 6-OHDA treatments, the medium was removed and replaced with fresh medium containing 0.5 mg/mL MTT and incubated for 3 h at 37 °C. The violet crystal was dissolved by DMSO, and the optical density was measured at 570 nm by using BMG LABTECH FLUOstar (Jena, Germany).

Cell Morphology. Intracellular chromatin condensation was observed by DAPI stain. At the end of the incubation period, the cells were incubated with PBS containing 0.5 mg/mL DAPI at 37 °C for 30 min. The results were observed by IX71 microscope (Olympus, Osaka, Japan) and recorded by ImagePro (Media Cybernetics, Bethesda, MD).

Intracellular ROS Content Measurement. Intracellular ROS was estimated using DCF. At the end of the incubation period, cells were collected and washed three times with PBS and resuspended with PBS containing 2 μM DCF. Cells were analyzed using a Becton Dickinson FACScan flow cytometer (Franklin Lakes, NJ).

Assay for Antioxidant Enzymes. The glutathione peroxidase (GPx) activity was determined using a spectrophotometer. The following solutions were pipetted into a cuvette: 0.1 mL of cell lysate and 0.8 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GSH reductase, and 1 mM GSH. The overall reaction was initiated by adding 0.1 mL of 2.5 mM H_2O_2. Enzyme activity was calculated by the change of absorbance at 340 nm for 5 min. The GPx activity was expressed as a ratio of control.

The catalase activity was determined using a spectrophotometer. A mixture of 50 mM phosphate buffer (pH 7.0), 20 mM H_2O_2, and cell

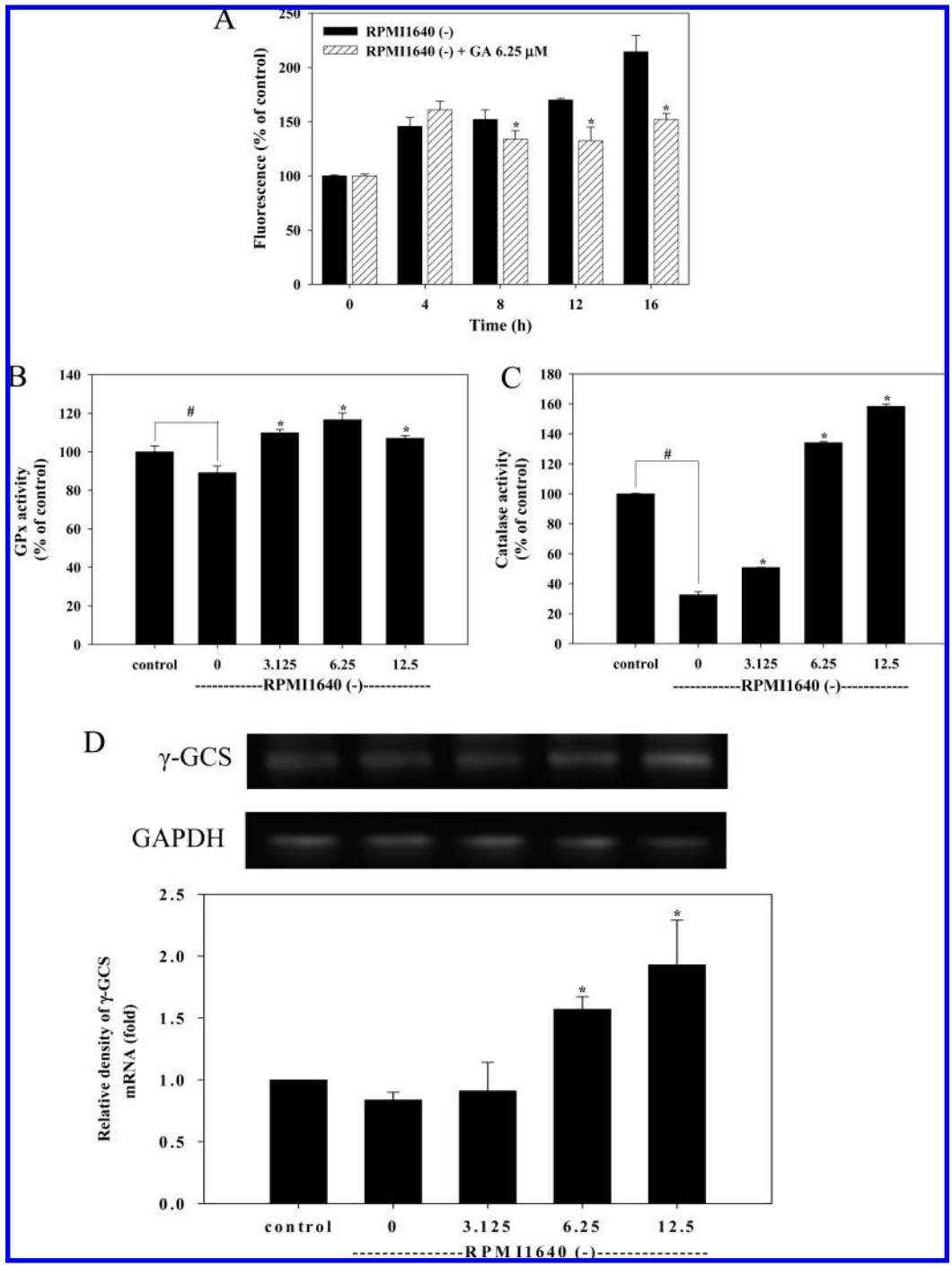

Figure 2. Effect of glycyrrhizic acid on ROS production and intracellular antioxidant enzyme system in serum/glucose-deprived (RPMI 1640 (−)) PC12 cells. The intercellular ROS content (**A**) was measured by fluorescence intensity of DCF-DA and recorded by flow cytometry. Intracellular glutathione peroxidase (GPx) (**B**) and catalase (**C**) activities were also measured after incubation for 12 h with GA in the serum/glucose-deprived condition. The mRNA expression of γ-GCS (**D**) was also measured at 12 h. Data are presented as means ± SD ($n = 3$). #, $p < 0.05$; ∗, $p < 0.05$, compared to RPMI 1640 (−) alone.

lysate in a final volume of 3 mL was incubated at room temperature for 2 min. The change in absorbance at 240 nm for 2 min was calculated. The catalase activity was expressed as a ratio of control.

RNA Extraction and RT-PCR. After treatment, cells were washed three times with PBS, and Trizol (Invitrogen) was used to extract the intracellular RNA. To analyze the target mRNA, a SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) was used to amplify the specific mRNA sequence. The primers used to amplify γ-GCS and GAPDH are described as follows: γ-GCS, forward = 5′-CCTTCTGGCACAGCACGTTG-3′, reverse = 5′-TAA-GACGGCATCTCGCTCCT-3′; GAPDH, forward = 5′-TCCCTCAA-GATTGTCAGCAA-3′, reverse = 5′-AGATCCACAACGGATACATT-3′. The RT-PCR product electrophoresed on a 1.8% agarose gel, and the signal was detected using ethidium bromide staining. The gel was then photographed under UV transillumination. The results were expressed as a ratio after normalization of the gene signal relative to the corresponding GAPDH signal from each sample.

Mitochondrial Membrane Potential (MMP) in Cells. The PC12 cells were seeded in six-well plates for MMP detection. After incubation, cells were washed three times with PBS, and MitoPT (Immunochemistry Technologies, Bloomington, MN) was used to evaluate the intracellular MMP level. Data were recorded at Ex = 485 nm and Em = 590 nm by a Flowstar microplate reader (BMG Labtech, Jena, Germany).

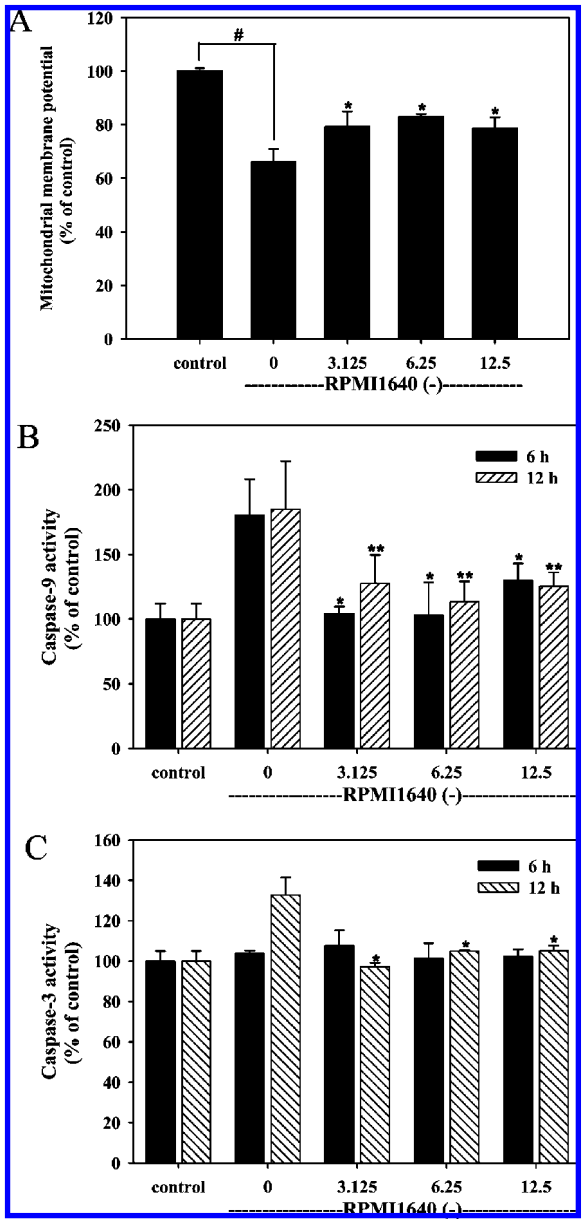

Figure 3. Effect of glycyrrhizic acid on mitochondrial membrane potential ($\Delta\psi_m$) and caspase activity in serum/glucose-deprived (RPMI 1640 (−)) PC12 cells. Cells were treated with GA and RPMI 1640 (−) for 12 h. The mitochondrial membrane potential was measured by MitoPT, and all procedures were performed according to the instruction manual. After treatment for 6 and 12 h, intracellular caspase-9 and caspase-3 activities were measured by commercial kits. Data are presented as means ± SD ($n = 3$). ∗, $p < 0.05$, compared to RPMI 1640 (−) alone.

Assay for Caspase Activity. Following treatment, PC12 cells were dissolved by RIPA lysis buffer. To measure the intracellular caspase-9 (Caspase-9 Fluorometric Assay Kit, purchased from Invitrogen) and caspase-3 (Caspase-3 Activity Detection Kit, purchased from Millipore) activity, the commercial kits were used according to the manufacturers' instructions. The protein concentration was determined by using bovine serum albumin as a standard (*13*). All results were normalized by the protein concentration of each sample.

Isolation of Mitochondria and Cytosol. The mitochondria and cytosol isolation procedure was performed as described in ref *14*. Briefly, PC12 cells were harvested by centrifugation at 600*g* for 10 min at 4 °C. The cell pellets were washed once with ice-cold PBS and resuspended in 5 volumes of buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA-2NA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride) containing 250 mM sucrose. The cell homogenates were centrifuged twice at 750*g* for 10 min at 4 °C. The supernatants were centrifuged at 10000*g* for 15 min at 4 °C, and the resulting mitochondrial pellets were resuspended in buffer A containing 250 mM sucrose and frozen as multiple aliquots at −20 °C. The supernatants of the 10000*g* spin were further centrifuged at 14000*g* for 20 min at 4 °C. After processing, a Western blot analysis was performed to determine the intracellular content of Bax and Bcl-2.

Western Blot Analysis. After incubation, cells were dissolved in RIPA lysis buffer (Millipore, Billerica, MA) and boiled at 100 °C for 10 min with 4× protein loading dye (8% SDS, 0.04% Coomassie Blue R-250, 40% glycerol, 200 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol). The samples were then subjected to SDS−polyacrylamide gel electrophoresis. Proteins in the gel were transferred onto an Immobilon-P PVDF membrane (Millipore) and incubated with the primary antibody (Bax, Bcl-2, phospho-Akt, α-tubulin) overnight (1:1000 dilution). Finally, the samples were incubated with horseradish peroxide-linked secondary antibody (1:8000 dilution) and analyzed using the Chemilucent ECL Detection System (Millipore). The protein expression was normalized by α-tubulin. The intensity of the chemiluminescence signal was quantified using LabWorks 4.5 (UVP, Cambridge, U.K.).

Neutral Sphingomyelinase (N-SMase) Activity Analysis. This analysis was modified from the method of ref *15*. Briefly, after treatment, PC12 cells were harvested and washed three times with PBS. The cells were then lysed by RIPA lysis buffer and centrifuged at 2000*g* for 10 min. The pellets were collected and homogenized by an ultrasonic processor (MISONIX, Farmingdale, NY) as the sample for measurement. After mixing with 2 mL of reagent (1.5 mM HNP, 100 mM Tris-HCl buffer (pH 7.4), 6 mM $MgCl_2$), the mixture was incubated for 1 h at 37 °C. To terminate the reaction, a mixture of 400 μL of 100 mM glycine buffer (pH 10.5) and 700 μL of ethanol was used. The absorbance of the mature solution was measured at 410 nm.

Statistical Analysis. All data are expressed as means ± standard deviation (SD). An ANOVA was used to evaluate the difference between multiple groups. If significance was observed between the groups, Duncan's test was used to compare the means of two specific groups. A p value of <0.05 was considered to be significant.

RESULTS

GA and 18βGA Inhibit the Death Induced by the Deprivation of Serum and Glucose in PC12 Cells. DAPI stain and an MTT assay were used to evaluate the protective activity of GA and 18βGA against serum/glucose deprivation. The result indicated that 18βGA did not restore cell viability after co-incubation with serum/glucose deprivation for 16 h (data not shown). **Figure 1A** shows the cell morphology after 16 h of co-incubation treatment with different concentrations of GA in serum/glucose deprivation. Serum/glucose deprivation caused intracellular chromatin condensation, and, after the 3.125−12.5 μM GA treatment, the chromatin condensation of PC12 cells was attenuated. **Figure 1B** shows that 3.125−12.5 μM GA treatment increased the PC12 cells' viability against the serum/glucose deprivation. This demonstrates that, after 16 h of treatment with 6.25 μM GA, cell viability was elevated from 77.4 to 92.1% ($p < 0.05$).

GA Treatment Decreases the ROS Content by Elevating the Intracellular Antioxidant System. The intracellular ROS content induced by serum/glucose deprivation is illustrated in **Figure 2**. Intracellular ROS content decreased ($p < 0.05$) after co-incubation with 6.25 μM GA over 8 h (**Figure 2A**). Previous study showed that catalase and GPx play an important role in the prevention of ROS-induced neuron damage (*16*). The activities of GPx and catalase and the mRNA expression of γ-GCS were also evaluated. After 3.125−12.5 μM GA treatment for 12 h, the GPx and catalase activities were higher than after treatment only by serum/glucose deprivation (**Figure 2B**,**C**). These data suggest that serum/glucose deprivation induces ROS

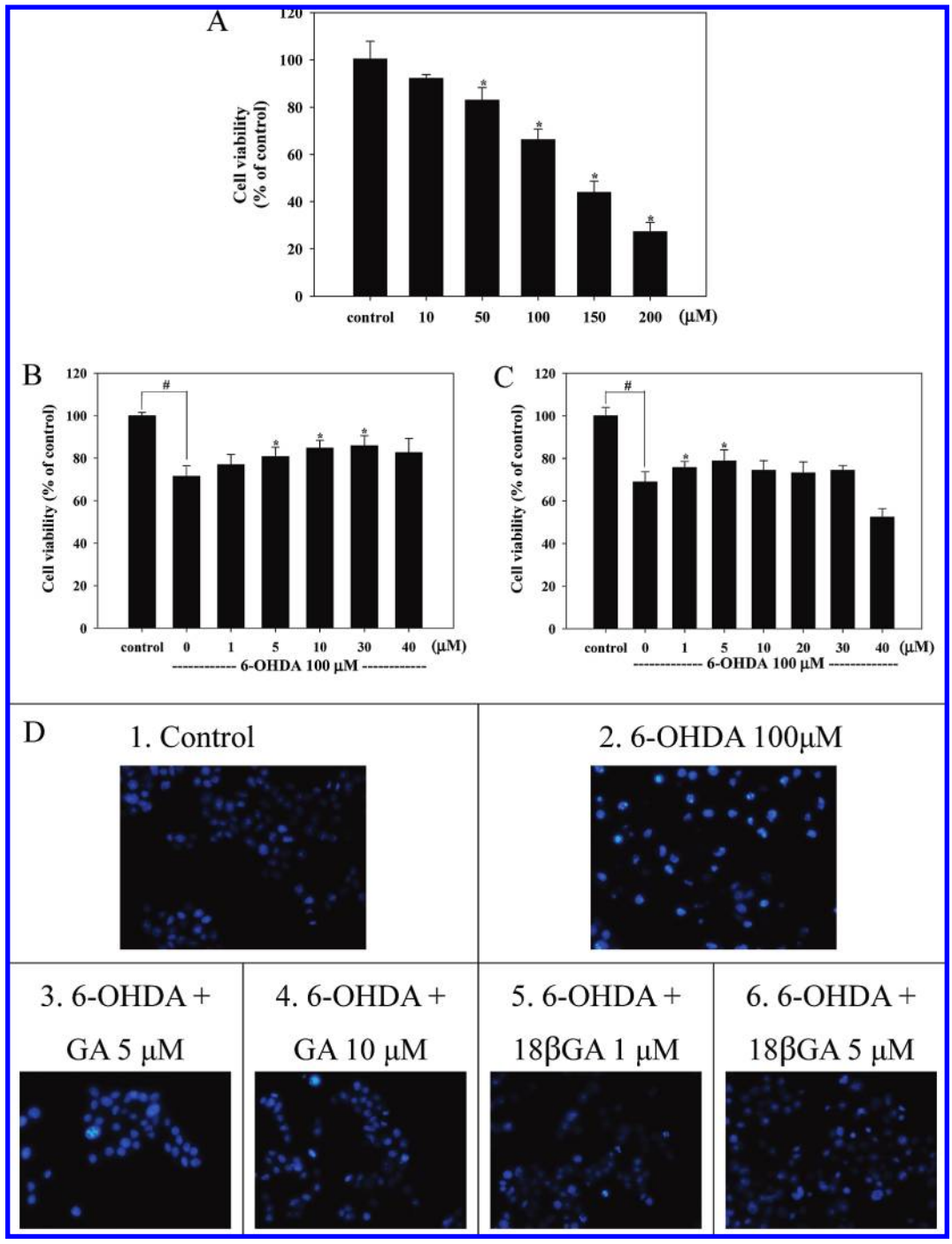

Figure 4. Effect of glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (18βGA) on cell viability of 6-OHDA-treated PC12 cells. Cells were treated with 6-OHDA only (**A**) or along with GA (**B**) or 18βGA (**C**) for 24 h. Following treatment, cell viability was measured by MTT assay (Sigma). DAPI stain was used to observe the intracellular chromatin condensation. (**D**) Cells were treated in the normal condition (D1), 100 μM 6-OHDA only (D2), and co-incubated in 100 μM 6-OHDA with GA (5 μM), GA (10 μM), 18βGA (1 μM), and 18βGA (5 μM). Intracellular chromatin condensation was observed by light-inverted microscope (Olympus, magnification = 200×). Data are presented as means ± SD (n = 3). #, $p < 0.05$; ∗, $p < 0.05$, compared to RPMI 1640 (−) alone.

overproduction in PC12 cells. Glutathione peroxidase and catalase activities also increased following GA treatment. This was also the case for the mRNA expression of γ-GCS (**Figure 2D**).

GA Restores Serum/Glucose Deprivation-Induced Mitochondrial Dysfunction. It has previously been reported that mitochondria are involved in ROS-induced apoptosis. Therefore, the effects GA treatment on serum/glucose deprivation-induced MMP were examined. As shown in **Figure 3A**, after 12 h of serum/glucose deprivation, the MMP of PC12 cells was decreased. GA increased the MMP from 66.1 to 83.1%. This effect was also associated with caspase-9 and caspase-3 activities. Caspase-9 activity was raised at 6 and 12 h, but caspase-3 activity increased only at 12 h (**Figure 3B**,**C**, respectively). This result suggests not only that the GA may protect the PC12 cells from hypoxic injury but also that the protective effect of GA is not limited to increasing the intracellular antioxidant system.

GA and 18βGA Inhibit the Death of PC12 Cells Induced by 6-Hydroxydopamine (6-OHDA)-Induced Cytotoxicity. **Figure 4A** illustrates the cytotoxicity of 6-OHDA in PC12 cells.

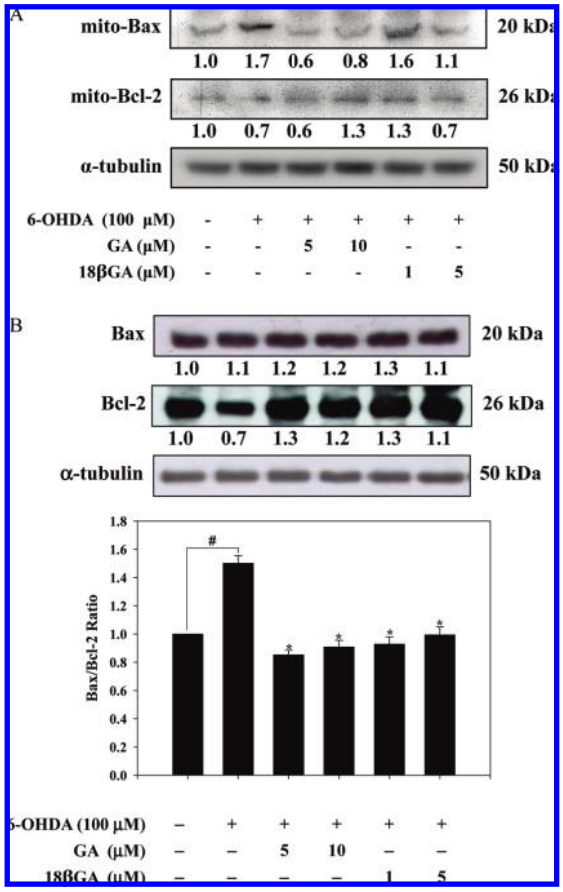

Figure 5. Effects of glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (18βGA) on the protein expression of Bax and Bcl-2 in 6-OHDA-induced PC12 cells. Cells were co-incubated with 100 μM 6-OHDA and different concentrations of GA and 18βGA. The mitochondrial (**A**) and total (**B**) Bax and Bcl-2 expressions in PC12 cells were measured by Western blot analysis. The α-tubulin antibody was used as an internal control in this experiment. The bands of Bax and Bcl-2 were visualized at the molecular masses of 23 and 26 kDa, respectively. Data are presented as means $\pm$ SD ($n = 3$). #, $p < 0.05$; $*$, $p < 0.05$, compared with 6-OHDA 100 μM alone.

After 24 h of 6-OHDA treatment, cell viability showed a decrease in a dose-dependent manner from 10 to 200 μM. We therefore chose a 100 μM concentration of 6-OHDA for further study. The cytoprotection ability of GA (**Figure 4B**) and 18βGA (**Figure 4C**) against 6-OHDA was quantified by MTT assay. Data showed that 5−10 μM GA and 1−5 μM 18βGA could protect against 6-OHDA-induced PC12 cytotoxicity. The DAPI stain (**Figure 4D**) also confirmed this result. Furthermore, the level of chromatin condensation induced by 6-OHDA was decreased after 24 h of GA and 18βGA treatment (**Figure 4D**).

GA and 18βGA Modulates Bax and Bcl-2 Expression in a Model of 6-OHDA-Induced Cytotoxicity. Because of the MMP-recovering ability of GA in serum/glucose deprivation-induced injury, we evaluated the Bax and Bcl-2 expression in mitochondria (**Figure 5A**) and in whole cell (**Figure 5B**). After 4 h of 6-OHDA treatment, the data suggested that GA and 18βGA might modulate the mitochondrial Bax and Bcl-2 expression. The regulation ability of GA and 18βGA, however, was limited by the total Bax and expression in whole cell lysate, both of them resulting in attenuation of the Bax/Bcl-2 ratio. The ratio of Bax/Bcl-2 is very important in the mitochondrial apoptosis pathway, and decreasing the Bax/Bcl-2 ratio results in the inhibition of apoptosis.

Protective Ability of GA and 18βGA via Modulation of the PI3K/Akt Pathway. The PI3K/Akt pathway plays an important role in the regulation of many important cell functions, such as maintaining the cell's viability and the antioxidant system. We evaluated intracellular PI3K/Akt pathway activation by detecting pAkt expression (**Figure 6A**), PI3K inhibitor treatment (**Figure 6B**,**C**), and N-SMase activity (**Figure 6D**). **Figure 6A** shows evidence that GA and 18βGA up-regulate pAkt expression, both in normal conditions and following 6-OHDA treatment. We then used a specific PI3K inhibitor (LY294002) for further confirmation. After pretreatment of 5 μM LY294002 for 1 h, the cytotoxicity of 6-OHDA was enhanced, and the protective abilities of GA (**Figure 6B**) and 18βGA (**Figure 6C**) were reduced. This suggests that the PI3K/Akt pathway plays an important role in the protective ability of GA and 18βGA. Finally, the activity of N-SMase in PC12 cells (**Figure 6D**) was evaluated. Data showed that GA and 18βGA also decreased the N-SMase activity that was induced by 6-OHDA.

DISCUSSION

Licorice is a very popular herb for treating most kinds of liver disease, and GA and 18βGA have been shown to be its primary bioactive compounds. Its neuroprotective mechanisms, however, are still unknown. The aim of our study was to examine the neuroprotective activity of licorice's main bioactive compounds and their possible mechanisms. We selected PC12 cells, a neuronal cell line isolated from a rat adrenal gland pheochromocytoma. These cells are widely used as a model to study apoptosis in many other fields (*17*) and to study protective properties against hypoxic damage and neurodegenerative disease.

Damage brought about by hypoxia is fatal for all kinds of living tissue, especially tissue in the brain. Today, studies simulate in vitro hypoxic damage by directly removing glucose and oxygen from the culture environment. This, however, causes unrecoverable damage to cells (*18*). Because of this, we used serum/glucose deprivation as an in vitro model of hypoxia (*15*) to evaluate the possible protective mechanisms of GA and 18βGA on ischemic injury. It is known that ROS are related to ischemic injury, lower the MMP, and lead to mitochondrial apoptosis (*1*). In the present study, we observed that, after GA treatment, the intracellular ROS content was decreased and the cell viability recovered (**Figure 2A**). Because GA itself is not a potent antioxidant (*19*), the decrease in ROS activity may not be due to GA but may be modulated by other intracellular regulatory pathways. Activities of GPx and catalase were also up-regulated following GA treatment, working against the serum/glucose-deprived injury. The mRNA expression of γ-GCS, the key enzyme for GSH synthesis, was also up-regulated (**Figure 2D**). It has been shown that the activity of γ-GCS can be induced by nerve growth factor and may be useful for the intracellular oxidative stress balance (*20*). Our study also found that GA decreased the activities of caspase-9 and caspase-3 (**Figure 3B**,**C**), which suggests that GA may influence the mitochondrial function. Mitochondria are an important organelle in cells, as they modulate many antioxidant mechanisms (*21*). These results suggest that GA modulates mitochondrial function and the intracellular antioxidant system to protect PC12 cells from ischemic injury.

The cause of Parkinson's disease remains to be elucidated. Treatment with 6-OHDA is one of the most common inducers for studying Parkinson's disease (*22*). It is unstable and easily autoxidizes into *p*-quinone and H_2O_2 (*23*). According to previous

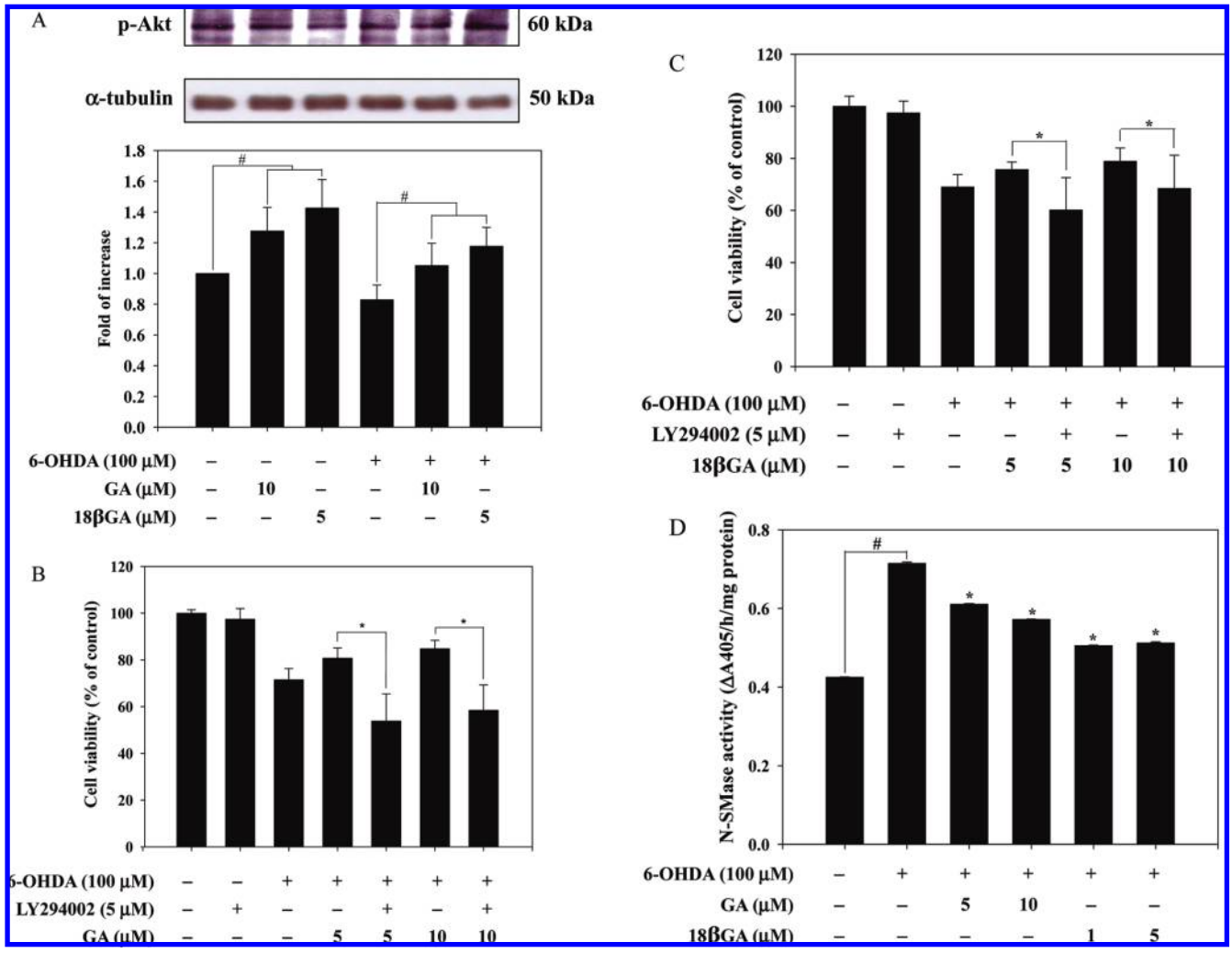

Figure 6. Effect of glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (18βGA) of Akt pathway in PC12 cells toward 6-OHDA cytotoxicity. The phospho-Akt (p-Akt) expression in PC12 cells (**A**) was measured by Western blot assay after 4 h of GA and 18βGA treatment. Cells were pretreated with 5 μM LY294002 (PI3K inhibitor) for 1 h and then co-incubated with 100 μM 6-OHDA and different concentrations of GA (**B**) and 18βGA (**C**). N-SMase activity (**D**) was measured after cells were co-incubated for 4 h with 100 μM 6-OHDA and different concentrations of GA or 18βGA. Data are presented as means $\pm$ SD ($n = 3$). #, $p < 0.05$; $*$, $p < 0.05$, compared with 100 μM 6-OHDA alone.

research, ascorbic acid might be suitable for preventing 6-OHDA autoxidation, and it has also been shown to enhance cytotoxicity due to 6-OHDA (*24*). We used 10 mM ascorbic acid to stabilize 10 mM 6-OHDA stock solution in our experiments. In the pre-experiment, we applied 6-OHDA without ascorbic acid and found that the cytotoxicity of 6-OHDA at the same concentration is significantly decreased (data not shown). After dilution, the concentration of ascorbic acid in our experiment was far below its protective concentration (*25*).

In this study, we also found that GA may protect PC12 from ischemic damage by modulating the mitochondrial apoptosis pathway. It has already been shown that 6-OHDA treatment decreases Bcl-2 expression in PC12 cells (*26*), but Bax expression is not known. In this study, the results show that 6-OHDA lowers Bcl-2 content and elevates Bax content in mitochondria, but only Bcl-2 content changed in whole cytosol lysate (**Figure 5**). This result may provide a new aspect of the mechanism for 6-OHDA-induced cytotoxicity. We discovered that 6-OHDA may also influence the protein expression of Bax and Bcl-2 in mitochondria but that it does not directly regulate their gene expression. This hypothesis, however, needs to be evaluated in future research.

PI3K/Akt signaling is very important to cell viability. By activating PI3K/Akt signaling, the mRNA expression of HO-1 (*27*), GPx activity (*28*), and catalase activity (*29*) are increased. These are important mechanisms for promoting cell viability. For further confirmation, we performed the measurement for N-SMase activity. N-SMase can hydrolyze sphingomyelin into ceramide, an apoptosis-induced factor in neurons. For example, after hypoxia or ischemia injury, the ceramide content increases and results in neuronal apoptosis (*30*). Inhibition of N-SMase activity has been shown to be helpful for preventing apoptosis caused by ischemic stress in PC12 cells (*15*). In this study, the result showed that the activity of N-SMase was increased after 6-OHDA treatment (**Figure 6D**). The possible cause of this phenomenon may be the autoxidation of 6-OHDA. Considering that GA and 18βGA are not potent antioxidants, the inhibition of N-SMase activity may be due to the modulation of the pathway. It is known that N-SMase activity can be inhibited by Akt activation (*30*). Our data suggest that GA and 18βGA may modulate the PI3K/Akt signaling to up-regulate the antioxidant system.

In conclusion, the results of this study have demonstrated that GA can protect PC12 cells from serum/glucose deprivation and that both GA and 18βGA can protect against 6-OHDA-induced damage. The protective ability may be via PI3K/Akt signaling and mitochondrial Bcl-2 family modulation. This research implicates a new promising medicine for neuronal

ischemia and degenerative disease. Furthermore, on the basis of this experiment, GA and 18βGA may be used in the diet to promote neuronal health.

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