— Researth Letter —

Geniposide Prevents PC12 Cells from Peroxynitrite via the Mitogen-Activated Protein Kinase Signaling Pathway

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Reactive oxygen species play an important role in the development of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and stroke. The present study investigated the neuroprotective action of geniposide and its signaling pathway in peroxynitrite induced PC12 cells. After coculture of PC12 cells with geniposide in the presence or absence of 3-morpholinosydnonmine hydrochloride (SIN-1), a donor of peroxynitrite, Bcl-2, Bcl-2 associated X protein (Bax) and the phosphorylation of mitogen-activated protein kinase (MAPK) were probed with Western blotting. The results showed that geniposide enhanced the expression of Bcl-2 to prevent the peroxynitrite damage induced by SIN-1 in PC12 cells. U0126, a selective inhibitor of MAPK/ERK, attenuated the protective effects of geniposide by inhibiting the phosphorylation of MAPK and expression of Bcl-2. All these data indicate that geniposide prevents PC12 cells from peroxynitrite damage induced by SIN-1 involved in the MAPK signaling pathway.

Key words —— geniposide, peroxynitrite, Bcl-2, mitogen-activated protein kinase

INTRODUCTION

Peroxynitrite formed by the reaction of superoxide and nitric oxide is a highly reactive species with a role in various pathologic processes, such as cancer, chronic inflammation, and cardiovascular and particularly neurologic diseases.[1] Since the endogenous antioxidant defenses are not always completely effective, a rapid rise in intracellular oxidant levels under oxidative stress could cause damage to biological molecules and result in various diseases.[2] It seems reasonable to propose that exogenous antioxidants could be very effective in diminishing the cumulative effects of oxidative damage.[3,4]

Geniposide, an iridoid glycoside isolated from the fruit of *Gardenia jasminoides* Ellis, is a popular Chinese herb used to treat febrile diseases including edema, hepatic disorders, acute conjunctivitis, and hematuria.[5] In our previous work, we found that geniposide activates the glucagon-like peptide 1 receptor (GLP-1R) to induce the neuronal differentiation of PC12 cells via the mitogen-activated protein kinase (MAPK) signaling pathway and prevents PC12 cells from oxidative damage induced by H_2O_2 via the phosphatidylinositol 3-kinase (PI3K) signaling pathway.[6] In the present study, using the model of peroxynitrite stress induced by 3-morpholinosydnonmine hydrochloride (SIN-1), a peroxynitrite generator, we wanted to explore the neuroprotective mechanism of geniposide in PC12 cells.

MATERIALS AND METHODS

Materials —— Geniposide was obtained from Sichuan Dicotyledonous Bio-tech Co., Ltd (Sichuan, China, purity greater than 99.5%, UR20060421). Other materials were obtained from the following sources: PC12 cells from the cell collection of the Shanghai Institute for Cell Research, Chinese Academy of Sciences; U0126, p-ERK1/2, ERK2, Bcl-2, Bcl-2 associated X protein (Bax), and horseradish peroxidase (HRP) -conjugated goat anti-rabbit (mouse) antibodies from Cell Signaling Technology (Danvers, MA, U.S.A.); HRP-conjugated Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Shanghai Kangcheng Bioengineering Co., Ltd. (Shanghai, China); fetal bovine serum, horse serum, penicillin/streptomycin, and Dulbecco's modified Eagle's medium (DMEM) from HyClone (Logan, UT, U.S.A.); Enhanced Chemiluminescence (ECL) Advance from Amersham (Arlington, Heightes, IL, U.S.A.); Polyvinyli-

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dene difluoride membrane from Millipore (Bedford, MA, U.S.A.); Lactate dehydrogenase (LDH) kit from Institute of Nanjing Jiancheng Biotech (Nanjing, China, A020). SIN-1 was from Calbiochem (San diego, CA, U.S.A.). All other reagents were purchased from Amersco (Solon, OH, U.S.A.) except as otherwise.

MTT Assay —— Cells were seeded in 6-well plates at a density of 5×10^5 cell/ml. To examine the cytotoxicity of geniposide, PC12 cells were treated with geniposide at the indicated concentrations for 24 hr at 37°C. For the cytoprotective assessment of geniposide, before SIN-1 was added, PC12 cells were treated with geniposide for 2 hr. Finally, cell viability was determined in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were incubated for 2 hr at 37°C with MTT (0.5 mg/ml final concentration) and dissolved in fresh complete medium, in which metabolically active cells reduced the dye to purple formazan. Formazan crystals were dissolved with dimethyl sulfoxide (DMSO), and the absorbance was measured on a BMG microplate reader (BMG LABTECH, Offenburg, Germany), using a reference wavelength of 630 nm and a test wavelength of 570 nm.

LDH Assay —— Cell death or cytotoxicity is classically evaluated by the quantification of plasma membrane damage. LDH is a stable enzyme present in all cell types and is rapidly released into the cell culture medium upon damage of the plasma membrane. LDH is therefore the most widely used marker in cytotoxicity studies. After treatment with the indicated doses of geniposide for 24 hr, LDH activities were detected using an LDH assay kit according to the manufacturer's instructions.

Western Blotting —— Western blot analysis was performed on 20 μg of protein from each cell lysate. Proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane after fractionation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were blocked with Tris 20 mM, NaCl 150 mM, pH 7.5, 5% nonfat dry milk at room temperature for 1 hr. Primary and second antibodies were diluted in blocking solution and incubated with the membranes for the indicated times as described previously. Excess antibody was washed off with Tris-buffered saline Tween-20 (TBST) 20 mM (Tris 20 mM, NaCl 150 mM, pH 7.5, and 0.1% Tween 20) before incubation in ECL advance. The membrane was subsequently exposed to photographic film. Western blotting results were quantified by the analysis of X-ray films using Quantity one software.

Statistical Analysis —— When necessary, data are expressed as mean ± S.D. In appropriate cases, significant differences between groups were determined using one-way analysis of variance (ANOVA) with Origin 7.5 software. The criterion for statistical significance was $p < 0.05$.

RESULTS

Neuroprotection of Geniposide in PC12 Cells

To determine whether geniposide itself affects cell viability, the LDH assay kit was used to detect the leakage of LDH from geniposide-treated PC12 cells. The results showed that geniposide had no influence on the normal growth of PC12 cells but, at dosages greater than 2 mM, it appeared to exert slight cytotoxicity on the cells (Fig. 1). Therefore, the experimental concentrations of geniposide in subsequent experiments were less than 50 μM.

We also investigated the influence of SIN-1 on PC12 cell viability; the data suggested that SIN-1 decreased the viability of PC12 cells in a dose-dependent manner (Fig. 2). Based on these results, to investigate the antioxidative action of geniposide, we determined the effects of geniposide on PC12 cell viability after challenge with SIN-1. The results from the MTT assay showed that preincubation with geniposide 50 μM for 2 hr increased the percentage of surviving PC12 cells by about 30% (Fig. 3).

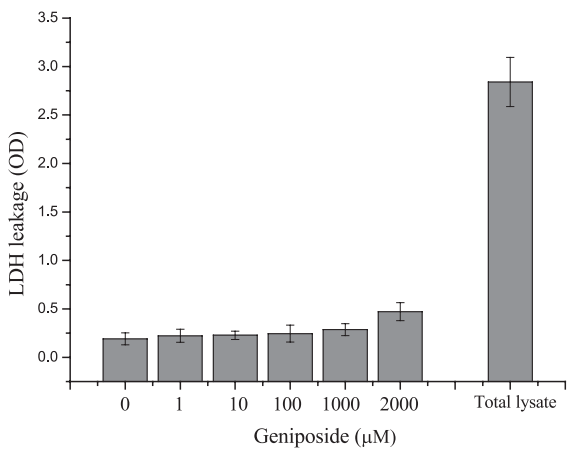

Fig. 1. Cytotoxicity of Geniposide in PC12 Cells

After PC12 cells were treated with the indicated concentrations of geniposide for 24 hr, the leakage of LDH in the cultured media was determined with an LDH assay kit. Data are expressed as mean ± S.D. of five separate experimental values performed in triplicate.

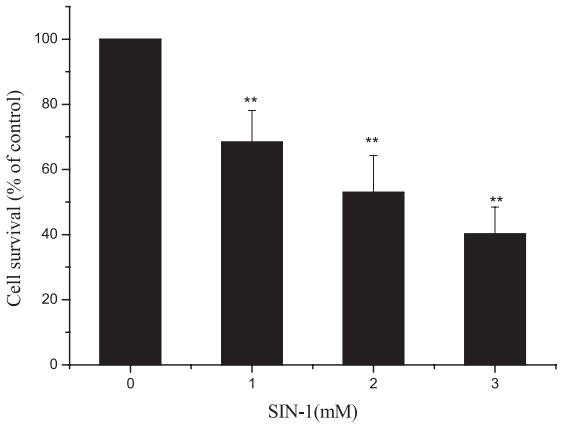

Fig. 2. SIN-1 Decreased PC12 Cells Activity in a Dose-Dependent Manner

After treatment with the indicated concentrations of SIN-1 for 24 hr, cell viability were determined in the MTT assay. Values are mean ± S.D. from four independent experiments. $^{**}p < 0.01$ *vs.* control (phosphate buffered solution (PBS) at equal volume).

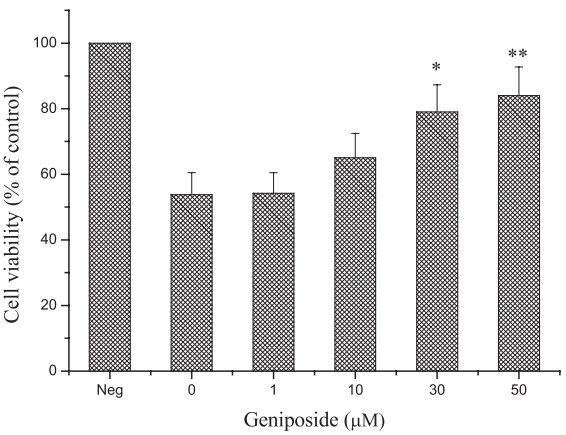

Fig. 3. Geniposide Protects PC12 Cells from Oxidative Damage Induced by SIN-1

Data are mean ± S.D. of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control.

Geniposide Regulates the Levels of Bcl-2 in PC12 Cells

To explore the molecular mechanism by which geniposide protects PC12 cells from peroxynitrite damage, we assessed the changes in Bcl-2 and Bax in geniposide-treated PC12 cells with Western blotting. The results demonstrated that preincubation with geniposide 50 μM for 6–8 hr increased the expression of Bcl-2 by about 2.2-fold but had no significant impact on the level of Bax (Fig. 4). These results suggest that geniposide regulates the expression of Bcl-2 and changes the ratio of Bcl-1/Bax to decrease the cytotoxicity of oxidative stress in PC12 cells.

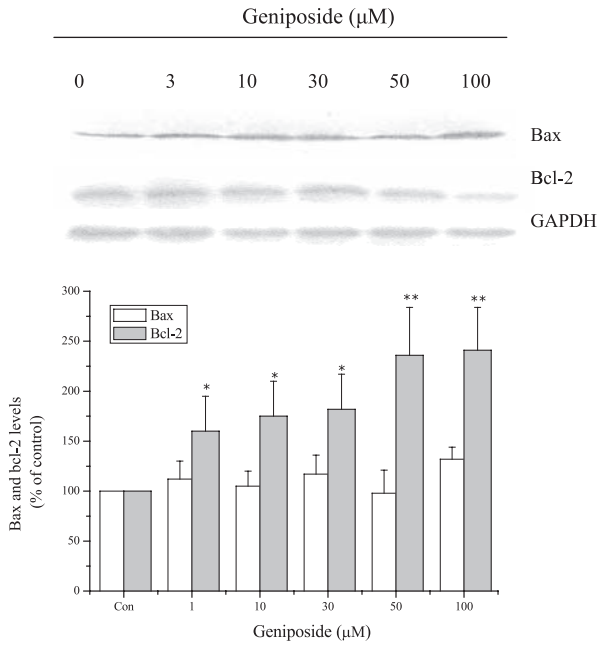

Fig. 4. Geniposide Regulates Levels of Bcl-2 and Bax in PC12 Cells

After PC12 cells were treated with the indicated concentrations of geniposide, equal lysates were separated with SDS-PAGE, and Bcl-2 and Bax were probed with antibodies as described in Methods. Experiments were replicated three times independently, and this figure shows representative results. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control.

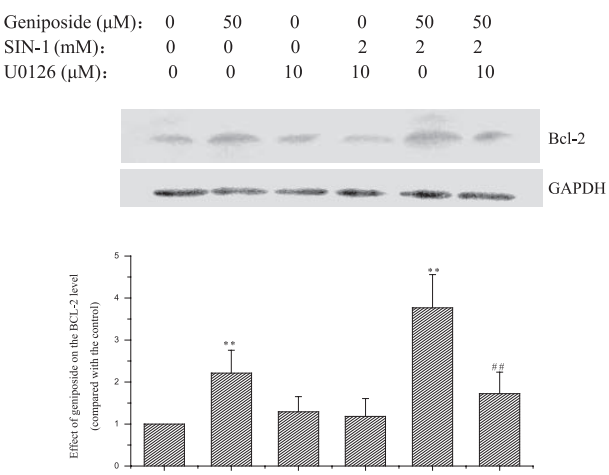

Fig. 5. Effects of U0126 on the Expression of Bcl-2 Induced by Geniposide in the Presence or Absence of SIN-1 in PC12 Cells

The experiments were repeated at least three times, and this figure shows representative results. Data are mean ± S.D. from three independent experiments, $^{**}p < 0.01$ *vs.* control; $^{\#\#}p < 0.01$ *vs.* the geniposide + SIN-1group.

MAPK Signaling Pathway Is Involved in the Neuroprotection of Geniposide in PC12 Cells

To investigate the possible signaling pathway for the neuroprotective activity of geniposide, U0126, a selective inhibitor of MEK, was used to

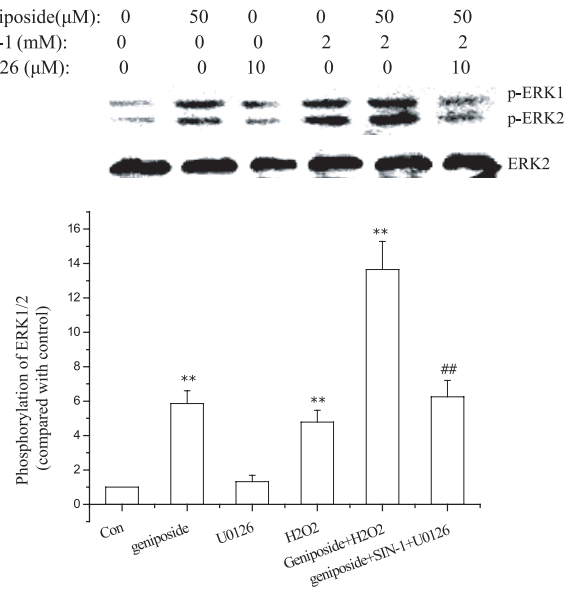

Fig. 6. Effects of Geniposide on the Phosphorylation and Expression of Mitogen-Activated Protein Kinase (MAPK, ERK) in PC12 Cells in the Presence or Absence of SIN-1

Data are from three independent experiments with mean ± S.D. $^{**}p < 0.01$ *vs.* control; $^{\#\#}p < 0.01$ *vs.* the geniposide + SIN-1 group.

determine the influence of MAPK phosphorylation on the expression of Bcl-2. The results demonstrated that preincubation with U012610 μM decreased the expression of Bcl-2 induced by geniposide from 3.77-fold to 1.77-fold in the presence of SIN-11 mM, and attenuated its neuroprotection (Fig. 5). This phenomenon suggests that MAPK signaling pathwayis involved in the neuroprotection by geniposide by enhancing the expression of Bcl-2 in PC12 cells. When we also examined the effects of geniposide on the expression and phosphorylation of ERK1/2, the results showed that geniposide increased the phosphorylation of ERK1/2 from 4.78-fold to 13.65-fold, but in the presence of U0126 10 μM, this value was decreased to 3.25-fold. Moreover, in this experiment, we also found that geniposide had no impact on the expression of ERK (Fig. 6).

DISCUSSION

Bcl family proteins, including Bcl-2, Bcl-XL, Bid, Bad, Bax etc., play a pivotal role in regulating cell apoptosis or survival.[7] In studying factors that could lead to the survival-promoting action of geniposide, we investigated its effects on some of the main regulators of cell viability. Our results showed that geniposide increased the expression of Bcl-2 to prevent the oxidative damage induced by free radicals in PC12 cells. Furthermore, this action of geniposide in PC12 cells was inhibited by U0126, a selective inhibitor of MEK. MAPK may therefore play an important role in the neuroprotection of geniposide in PC12 cells.

The MAPK pathways transmit a variety of external signals leading to a wide range of cellular responses, including growth, differentiation, inflammation, and apoptosis.[8] In this study, we observed that treatment with geniposide enhanced the phosphorylation of ERK1/2, and in the presence of oxidative stress, this effect was increased significantly, indicating that the MAPK signaling pathway was involved in the protection of geniposide in PC12 cells.

Geniposide, an iridoid glycoside from the fruit of *Gardenia jasminoides* Ellis, has been shown to have antitumor and anti-inflammatory activity and used to treat hepatic disorders.[9–11] It was reported that geniposide activated reduced glutathione (GSH) S-transferase by the induction of glutathione S-transferase (GST) M1 and GST M2 subunits involving the transcription and phosphorylation of MEK-1 signaling in rat hepatocytes.[5] In our previous papers, we reported that geniposide induced the neuronal differentiation of PC12 cells and protected PC12 cells from oxidative damage.[6,12] Lee reported that geniposide attenuated neuronal cell death in oxygen and glucose deprivation-exposed rat hippocampal slice cultures.[13] All these data show that geniposide is a promising antioxidative compound.

SIN-1 has been reported to be a peroxynitrite [$OONO^-$] donor because it produces both nitric oxide (NO) and superoxide upon decomposition in aqueous solution. However, SIN-1 can decompose primarily to NO in the presence of electron acceptors, including those found in biological tissues, making it necessary to determine the release product(s) formed in any given biological system.[14] Thus whether SIN-1 initiates neural cell death in an $OONO^-$-dependent or -independent manner is determined by the antioxidant status of the cells.

Accumulating data show that antioxidants may play a pivotal role in the treatment of neurodegenerative diseases.[15,16] Although more research is needed, the results of this study, at least in partly introduced the mechanism of action of geniposide in PC12 cells, which could also explain its frequent addition to the Chinese medicated diet (beverage and porridge) for promoting human health and for dis-

ease prevention.

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