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Original Article

Neuroprotection of geniposide against hydrogen peroxide induced PC12 cells injury: involvement of PI3 kinase signal pathway

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Aim: Oxidative stress plays a critical role in the pathogenic cascade leading to neuronal degeneration in AD. Consequently, the induction of endogenous antioxidative proteins by antioxidants seems to be a very reasonable strategy for delaying the disease's progression. In previous work, we identified the neurotrophic and neuroprotective effects of geniposide, which result from the activation of glucagon-like peptide 1 receptor (GLP-1R). In this study, we explore the role of PI3 kinase signaling pathway in the neuroprotection of geniposide in PC12 cells.
Methods: Cell viability was determined by MTT assay. Apoptosis was detected by Hoechst and PI double staining. The protein expression of Bcl-2 and phosphorylation of Akt308, Akt473, GSK-3β, and PDK1 was measured by Western blot.
Results: Geniposide induced the expression of the antiapoptotic protein Bcl-2, which inhibited apoptosis in PC12 cells induced by H_2O_2, and this effect could be inhibited by preincubation with LY294002, a selective inhibitor of PI3K. Furthermore, geniposide enhanced the phosphorylation of Akt308, Akt473, GSK-3β and PDK1 under conditions of oxidative stress.
Conclusion: These results demonstrate that the PI3K signaling pathway is involved in the neuroprotection of geniposide in PC12 cells against the oxidative damage induced by H_2O_2 in PC12 cells.

Keywords: geniposide; glucagon-like peptide 1 receptor; PI3K; GSK-3β; PDK1
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Introduction

Glucagon-like peptide-1 (GLP-1) is an endogenous peptide that is secreted from the gut in response to the ingestion of food[1]. It has potent effects on glucose-dependent insulin secretion and insulin gene expression *via* its action on the pancreas following its binding at the G-protein-coupled GLP-1 receptor (GLP-1R)[2]. There is also evidence to indicate that type II diabetes, or at least impaired glucose tolerance, is associated with impaired recognition of GLP-1, independent of age[3]. In activating its receptor, GLP-1 shows diverse physiological actions that include insulinotropic, cardiovascular and neurotrophic effects. The ability to modulate these physiological parameters may be proved beneficial in a variety of diseases prevalent in aging, including stroke and Alzheimer's disease (AD)[4–7]. Unfortunately, the biological activity of GLP-1 is inhibited through N-terminal degradation by the common endogenous aminopeptidase enzyme dipeptidyl peptidase IV (DPP IV), which not only inactivates GLP-1 but may turn it into an antagonist for the GLP-1R receptor[8]. This represents a potential challenge to therapeutic efforts directed at enhancing GLP-1 activity *in vivo*. Because numerous reports document GLP-1R expression in both the rodent and human brains, the development of natural products as lead compounds to activate GLP-1R in the brain may be useful in central nervous system degenerative disorders such as stroke and Alzheimer's disease[3, 6, 9].

In our previous work, we demonstrated that geniposide, isolated from *Gardenia jasminoides* Ellis, activated the glucagon-like peptide 1 receptor (GLP-1R) in a manner similar to GLP-1, and showed neurotrophic properties that induced the neuronal differentiation of PC12 cells by stimulating cAMP production. Furthermore, we found that geniposide could prevent oxidative damage induced by H_2O_2 in PC12 cells via the MAP kinase signaling pathway[10, 11]. Activation of GLP-1R on the cell membrane of pancreatic β cells has been shown to modulate mitosis, growth and differentiation, and much is known about the cellular signaling pathway

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triggered by GLP-1 binding to its pancreatic receptor[12–14]. However, little has been confirmed in neuronal cells. In this study, we explore the signaling pathway of geniposide in activating GLP-1R in PC12 cells.

Bcl-2 is a gene family involved in the suppression of apoptosis in response to a wide range of cellular insults[15]. Many papers have suggested a link between Bcl-2 and oxidative damage/antioxidant protection[16, 17]. Here we report that geniposide, a selective agonist for GLP-1R, induces the expression of Bcl-2, which in turn decreases oxidative damage in PC12 cells. This effect is mediated by the phosphorylation of Akt, PDK1 and GSK. Furthermore, this effect is inhibited partly by LY294002, a selective inhibitor of phosphatidylinositol 3-kinase (PI3K). Together, these data suggests that PI3K is involved in the neuroprotection of geniposide in PC12 cells challenged by H_2O_2.

Materials and methods

Materials

Materials were obtained from the following sources: PC12 cells were obtained from the cell bank of type culture collection of Chinese Academy of Sciences; LY294002, anti-phospho-Akt308, -Akt473, -PDK1, -GSK-3β, anti-Bcl-2, anti-Akt, anti-HO-1, anti-β-actin, anti-GADPH and horseradish peroxidase-conjugated goat anti-rabbit (mouse) antibodies were purchased from Cell Signaling Technology; fetal bovine serum, horse serum, penicillin/streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from HyClone; ECL advance was purchased from Amersham; polyvinylidene difluoride membranes were purchased from Millipore; and geniposide was purchased from Sichuan Dicotyledonous Bio-tech Co, Ltd (purity is over 99.5%, UR20060421). All other reagents were purchased from Amersco, except where indicated.

Cell culture and drug treatment

PC12 cells were maintained in DMEM containing 5% fetal bovine serum, 10% horse serum, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate at 37 °C with 5% CO_2. For experiments involving geniposide and PC12 cells, the cells were plated in 6-well culture dishes at 5×10^5 cells/mL. The cells were preincubated for 30 min with or without LY294002 before the addition of H_2O_2. After treatment with 50 μmol/L geniposide for 2 h, cells were incubated with 100 μmol/L H_2O_2 for the indicated time. LY294002, a specific inhibitor of PI3K, was diluted in dimethyl sulfoxide to a final concentration in the medium of 10 μmol/L.

Determination of cell viability

Cells were seeded in 6-well plates at a density of 5×10^5 cells/mL. In the cytotoxic studies, PC12 cells were treated with geniposide and H_2O_2 at the indicated concentration for 24 h at 37 °C. To assess the cytoprotection of geniposide, PC12 cells were treated with geniposide for 2 h before H_2O_2 was added. Finally, the cells' viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The cells were incubated for 2 h at 37 °C with MTT (0.5 mg/mL final concentration) and then dissolved in fresh complete medium, in which the metabolically active cells reduced the dye to purple formazan. The formazan crystals were dissolved in DMSO, and the absorbance was measured on a BMG microplate reader (BMG Technologies), using a reference wavelength of 630 nm and a test wavelength of 570 nm.

Hoechst and PI double staining

In the morphological studies, the cells were grown on slides and treated with geniposide and H_2O_2 as described above. After a wash with PBS, the cells were stained with Hoechst 33258 (1 μg/mL) for 7–10 min at room temperature, and then stained with 5 μg/mL PI (propidium iodide) for 1–2 min. After a final wash in PBS, the cells were photographed under UV light with a microscope (Nikon, Japan).

SDS-PAGE and Western blot analysis

Western blot analysis was performed on 20 μg of protein from each cell lysate. The proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane after being fractionated by SDS-PAGE. The membranes were blocked with 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, and 5% nonfat dry milk at room temperature for 1 h. Primary and second antibodies were diluted in blocking solution and incubated with the membranes for the indicated times as described previously[18]. Excess antibody was washed off with a 20 mmol/L TBST (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, and 0.1% Tween 20) solution before incubation in ECL advance. The membrane was subsequently exposed to photographic film. Western blot results were quantified by analyzing the X-ray films using software from Quantity One.

Statistical analysis

When necessary, data are expressed as mean±SD, and variance analysis was carried out using Origin 7.5. Values of $P<0.05$ were considered statistically significant.

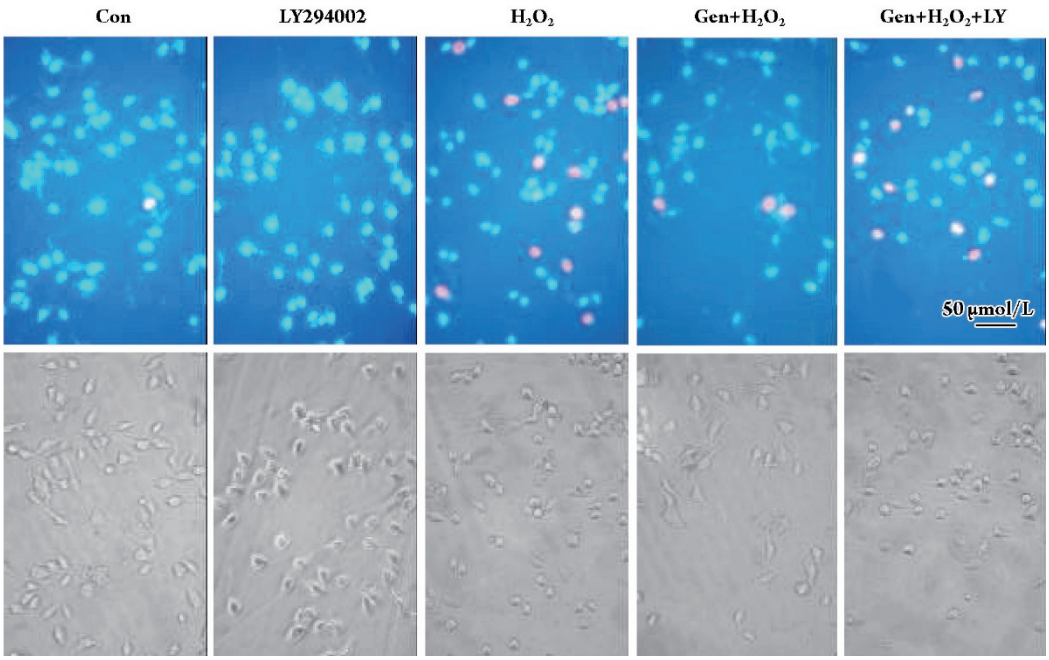

Figure 1. Geniposide (Gen) inhibits the apoptosis of PC12 cells induced by H_2O_2. To analyze the role of geniposide in preventing the apoptosis of PC12 cells induced by H_2O_2, PC12 cells were plated in 6-well culture dishes at 5×10^5 cells/mL and preincubated for 30 min in the presence or absence of LY294002 (10 μmol/L) before the addition of geniposide and H_2O_2. After treatment with 50 μmol/L geniposide for 2 h, cells were treated with 100 μmol/L H_2O_2 for 2–4 h, and the treated cells were double stained with PI/Hoechst 33258 and the positive cells were counted. The results shown are from a representative experiment, which was repeated at least three times. (Scale bar is 50 μmol/L)

Results

Geniposide decreases apoptosis in PC12 cells induced by H_2O_2

In previous work, we identified that geniposide activated the GLP-1 receptor and increased PC12 cells' viability in a dose-dependent manner[11]. With PI/Hoechst 33258 double staining, we found that preincubation with geniposide could inhibit apoptosis in PC12 cells induced by H_2O_2, with the percentage of apoptotic cells decreasing from 42.7%±7.6% to 15.9%±6.3% (Figure 1). We also observed that the neuroprotective effect of geniposide was attenuated by LY294002, a selective inhibitor of PI3K. Using the MTT method, we determined the influence of geniposide on the viability of PC12 cells treated with H_2O_2 in the presence or absence of LY294002. The results showed that geniposide increased the viability of the cells from 51.7%±11.3% to 77.9%±9.3%. Furthermore, this effect could be inhibited by preincubation with LY294002 (Figure 2). In total, these results suggest that the PI3K pathway is involved in the neuroprotection of geniposide in PC12 cells.

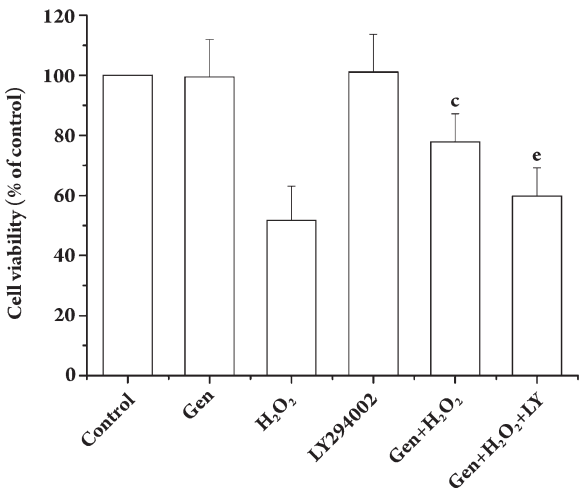

Figure 2. Effect of LY294002 on the neuroprotection of geniposide(Gen) against oxidative damaged PC12 cells. PC12 cells were maintained for 12–16 h in normal media supplied with 10% HS and 5% FBS and then stimulated with vehicle (Me_2SO) or Gen 50 μmol/L for 2–4 h. After that, the cells were pretreated with 10 μmol/L LY294002 for 15–30 min and submitted to 100 μmol/L H_2O_2 as indicated for 2–4 h, and then analyzed by the MTT method. All data are shown as mean±SD from three independent repeats. $^cP<0.01$ *vs* H_2O_2 treated group. $^eP<0.05$ *vs* the group treated with Gen+H_2O_2.

Geniposide increases the expression of antiapoptotic Bcl-2 proteins

The bcl-2 proteins are a family of proteins involved in responding to apoptotic signals[19]. A lot of evidences have demonstrated that Bcl-2 plays a critical role in preventing oxidative damage to cells induced by highly reactive hydroxyl radicals (·OH) from H_2O_2[20]. An excess of Bcl-2 in cells will tend to make them more resistant to oxidative stress. In studying the factors that could lead to the prosurvival effect of geniposide, we investigated its influence on the expression of Bcl-2, a main regulator of cell apoptosis. The results demonstrated that, treatment of the PC12 cells with 50 μmol/L geniposide increased the expression of bcl-2 by about 1.57-fold relative to the control. Furthermore, in the presence of H_2O_2, the over-expression of bcl-2 in the PC12 cells exposed to geniposide increased to 2.21-fold that of the control. However, preincubation with LY294002 attenuated the expression of Bcl-2 induced by geniposide (Figure 3).

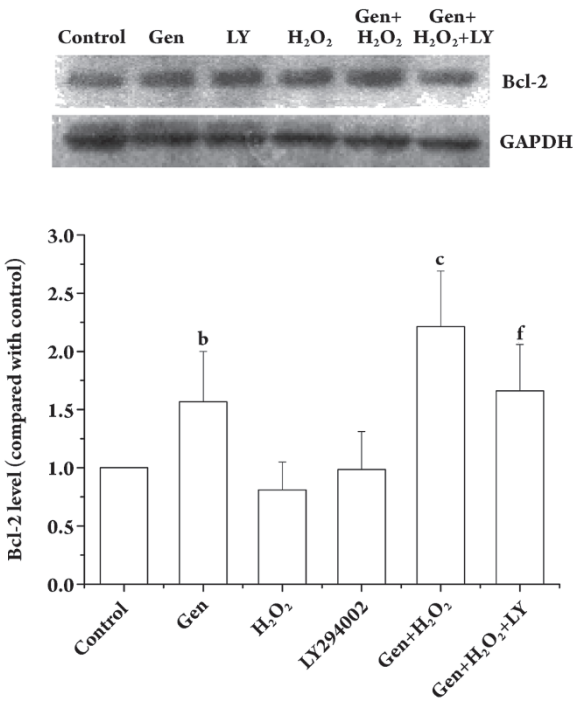

Figure 3. Geniposide regulates the expression of Bcl-2 to antagonize the oxidative damage induced by H_2O_2 in PC12 cells. After the PC12 cells were treated with the agents listed in the Materials and methods section, equal proteins were separated with SDS-PAGE, and the Bcl-2 proteins were probed by Western blot. The experiments were repeated at least three times, and the results are shown as mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* the control group. $^fP<0.01$ *vs* the group treated with Gen+H_2O_2. LY: LY294002.

Neuroprotective action of geniposide is mediated by the activation of a PI3K-dependent signaling pathway in PC12 cells

Activation of the PI3K pathway is known to elicit a survival signal against multiple proapoptotic insults[21]. To determine whether the activation of the PI3K pathway is involved in the downstream signaling pathway responsible for the up-regulation of Bcl-2 expression and for protection from H_2O_2 induced apoptosis, we examined the effects of LY294002, a selective inhibitor of PI3K, on the phosphorylation of Akt, a downstream substrate of PI3K activity. The results from Western blot experiments showed that treatment with 50 μmol/L geniposide for 1 h increased the phosphorylation of Akt308 by about 3.72-fold in PC12 cells, and that after the addition of H_2O_2 and incubation for one additional hour, the level of Akt308 phosphorylation increased to 4.71-fold that of the control cells. We also found that treatment with geniposide alone had no significant influence on the phosphorylation of Akt473, another kinase downstream of PI3K, but in the presence of H_2O_2, geniposide did increase phosphorylation by about 1.92-fold. This activity was also inhibited by preincubation with LY294002. However, the level of unphosphorylated Akt enzyme remained unchanged in PC12 cells in the presence of H_2O_2 (Figure 4).

Moreover, we also explored the effects of geniposide on the activity of upstream and downstream kinases, such as PDK1 and GSK-3β. We found that oxidative stress decreased the level of phosphorylated GSK-3β in PC12 cells, but that geniposide enhanced the phosphorylation of PDK1 and GSK-3β in the presence or absence of H_2O_2. These effects were attenuated by the PI3K inhibitor, LY294002. The levels of phosphorylated PDK1 and GSK-3β decreased from 4.59-fold to 2.91-fold and from 2.93-fold to 1.45-fold, respectively, after a 30-min preincubation with 10 μmol/L LY294002 (Figure 5).

Discussion

Oxidative stress contributes, at least in part, to the pathogenic cascade leading to neuronal degeneration in AD[22, 23]. The induction of endogenous antioxidative proteins by antioxidants seems to be a very reasonable strategy for delaying the disease's progression[24]. In previous work, we reported that geniposide, a selective agonist for the GLP-1 receptor, showed neurotrophic properties, such as inducing neuronal differentiation of PC12 cells and preventing oxidative damage *via* the MAP kinase pathway in PC12 cells[10, 11]. However, the mechanisms by which geniposide activates GLP-1R and imparts its neuroprotective effects are not fully under-

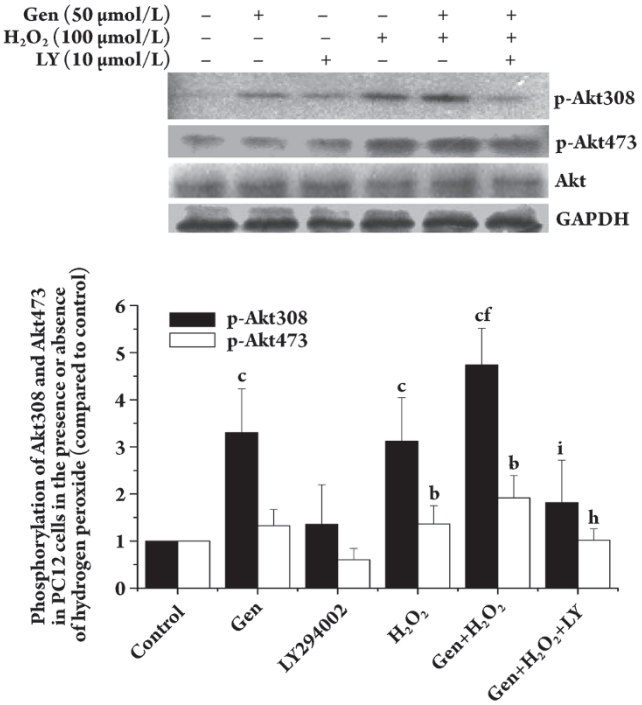

Figure 4. The effect of LY294002, a PI3K inhibitor, on the phosphorylation of Akt308 and Akt473 in PC12 cells treated with H_2O_2. PC12 cells were plated into 6-well culture dishes at 1×10^6 cells/mL and maintained for 12–16 h in normal media supplied with 10% HS and 5% FBS, and then treated with 50 μmol/L geniposide for 2–4 h. After that, the cells were pretreated with 10 μmol/L LY294002 for 15–30 min and submitted to 100 μmol/L H_2O_2 for about 1 h. After the cells were washed three times with PBS, the cell lysate was collected to assay the phosphorylated Akt308 and Akt473. The results shown are from a representative experiment, which was repeated at least three times. The data are shown as mean±SD from three independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* the control; $^fP<0.01$ *vs* the group of treatment with H_2O_2 alone; $^hP<0.05$, $^iP<0.01$ *vs* the group treated with Gen+H_2O_2. Gen: geniposide; LY: LY294002.

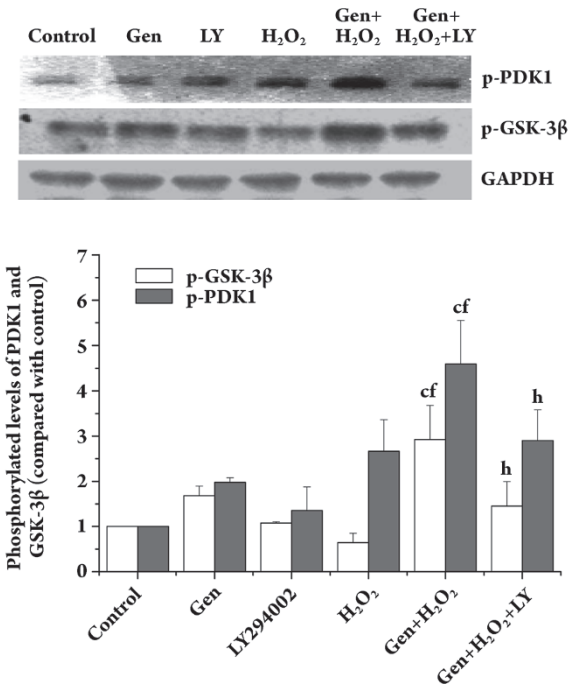

Figure 5. Geniposide regulates the phosphorylation of PDK1 and GSK-3β in PC12 cells treated with H_2O_2. PC12 cells were plated into 6-well culture dishes at 1×10^6 cells/mL and maintained for 12–16 h in normal media supplied with 10% HS and 5% FBS, and then treated with 50 μmol/L geniposide for 2–4 h. Then the cells were pretreated with 10 μmol/L LY294002 for 15–30 min and submitted to 100 μmol/L H_2O_2 for about 1 h The cells were then washed with PBS three times, and the cell lysate was collected to assay the phosphorylated PDK1 and GSK-3β. The results shown are one from a representative one from at least three repeating experiments. The data are shown as mean±SD from three independent experiments. $^cP<0.01$ *vs* the control group. $^fP<0.01$ *vs* the group treated with H_2O_2 alone; $^hP<0.05$ *vs* the group treated with Gen+H_2O_2. Gen: geniposide; LY: LY294002.

stood. In this study, we proposed that the neuroprotection associated with geniposide was caused by an antiapoptotic effect related to the drug-induced over-expression of Bcl-2. The over-expression of Bcl-2 resulting from the activation of PI3K, a major signaling pathway implicated in supporting neuronal survival. We first investigated the effect of geniposide on cellular apoptosis and viability. Preincubation with geniposide significantly decreased apoptosis and increased the viability of PC12 cells challenged with H_2O_2. Additionally, we also identified that this effect involved the modulation of geniposide on the phosphorylation of Akt, PDK1, and GSK-3β.

The data increasingly support the notion that the GLP-1R signal is mediated by the phosphatidyl-inositol-3-kinase, cAMP/protein kinase A, and Ca^{2+}-CamKIIα pathways[21, 25, 26]. To explore the signal pathway by which geniposide prevents PC12 cells from oxidative damage, we used LY294002 to investigate whether geniposide activates the PI3K pathway. It has been shown that PI3K is regulated primarily by one of its downstream targets-Akt[27]. Akt has a direct effect on the apoptosis pathway, by regulating the expression of anti-apoptotic proteins such as Bcl-2[28]. Our results demonstrate that geniposide could induce the expression of the endogenous antiapoptotic protein Bcl-2, which protects against oxidative damage in PC12 cells, and that this neuroprotective effect of geniposide is attenuated by preincubation with the PI3K inhibitor, LY294002. Additionally, we investigated the effect of geniposide on the phosphorylation of Akt and the kinases upstream and downstream of Akt (for example, PDK1, and GSK-3β). We showed that

geniposide induced the phosphorylation of Akt308, PDK1, and GSK-3β, and enhanced the phosphorylation of Akt308, Akt473, GSK-3β, and PDK1 under oxidative stress. These results strongly suggest that the PI3K pathway is involved in neuroprotection of geniposide in PC12 cells.

There is growing evidence that GSK-3β is involved in the pathogenesis of central nervous system diseases. GSK-3β is an enzyme that was initially identified as a regulator of glycogen metabolism, but it also has broader functions[29]. Following the PI3K/Akt pathway, we investigated whether geniposide had an influence on GSK-3β phospho-regulation and whether this effect was involved with PI3K by using LY294002. We are the first to demonstrate that geniposide strongly increases the phosphorylation of GSK-3β in the presence of H_2O_2, and that this increase is partly abolished by LY294002. Together these data suggest a probable mechanism by which geniposide protects against cell death.

3-Phosphoinositide-dependent protein kinase 1 (PDK1) was originally identified as the activator of the cell survival kinase Akt/PKB[30, 31]. Subsequently, PDK1 has been shown to be a more versatile kinase. It is involved in a variety of cell functions, including protein synthesis, cell survival, glucose metabolism, and cell adhesion and migration[32, 33]. In this study, we found that geniposide enhanced the phosphorylation of PDK1 in the presence of H_2O_2, and that this effect was inhibited by LY294002, suggesting that PDK1 was also involved in neuroprotection of geniposide in PC12 cells.

At present, the increase in the phosphorylation of Akt, PDK1 and GSK-3β induced by geniposide in H_2O_2 challenged cells and inhibition of PI3K, by LY294002, leading to a decrease in the neuroprotection mediated by geniposide, suggest that the PI3K/Akt pathway and GSK-3β could be involved in the mechanism by which geniposide protects PC12 cells from H_2O_2-induced cell death. Strikingly, geniposide did not influence Akt phosphorylation in untreated cells but did increase the phosphorylation of PDK1 and GSK-3β in H_2O_2-treated PC12 cells, indicating that geniposide is not a direct activator of these pathways but acts as a facilitator of their activation. Although further work is needed to understand the precise mechanism of the neuroprotection provided by geniposide, the present study provides evidence that the PI3K cascade may play an important role in geniposide-induced neuroprotection from oxidative stress, and we are the first to delineate the signaling pathway for the GLP-1 receptor in PC12 cells.

Abbreviations

GLP-1R, glucagon-like peptide 1 receptor; DPP IV, dipeptidyl peptidase IV ; PI3K, phosphoinositide 3-kinase; GSK-3β, glycogen synthase kinase-3beta; PDK1, phosphoinositide-dependent protein kinase 1; MAPK, mitogen-activated protein kinase; AD, Alzheimer's disease; PD, Parkinson's disease; PI, propidium iodide; PBS, phosphate buffer solution; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

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Author contribution

Jian-hui LIU and Fei YIN designed research; Jian-hui LIU, Fei YIN, Li-xia GUO, and Xiao-hong DENG performed research; Jian-hui LIU and Fei YIN analyzed data; Jian-hui LIU and Yin-he HU wrote the paper.

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