

Original Article

GLP-1 receptor plays a critical role in geniposide-induced expression of heme oxygenase-1 in PC12 cells

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Aim: To explore the role of activation of glucagon-like peptide 1 receptor (GLP-1R) and its relative cell signaling pathway in the cytoprotection of geniposide.

Methods: Cell viability was determined by MTT assay. Knockdown of the *Glp-1r* gene was carried out with shRNA. The levels of HO-1 protein and cAMP response element binding protein (CREB) phosphorylation were measured by Western blotting.

Results: Geniposide protected PC12 cells from oxidative damage induced by 3-morpholinopyridone hydrochloride (SIN-1) by enhancing the expression of heme oxygenase 1 (HO-1) via the cAMP-PKA-CREB signal pathway. After transfecting PC12 cells with the AB1 enhancer from the HO-1 gene, luciferase activity induced by geniposide increased in a dose-dependent manner, but not in the PC12 cells whose *Glp-1r* gene was disrupted. Additionally, inhibition of HO-1 activity by Sn-protoporphyrin IX (SnPP) or shRNA-mediated knockdown of *Glp-1r* decreased the neuroprotection of geniposide in PC12 cells.

Conclusion: GLP-1R plays a critical role in geniposide-induced HO-1 expression to attenuate oxidative insults in PC12 cells.

Keywords: geniposide; glucagon-like peptide 1 receptor; heme oxygenase 1; oxidative stress; PC12 cells

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Introduction

Glucagon-like peptide 1 (GLP-1), secreted from intestinal L-cells after food intake, is an endogenous insulinotropic peptide that controls serum glucose level via its action on the pancreas, specifically, via the G-protein-coupled GLP-1 receptor (GLP-1R)^[1]. Upon GLP-1 binding, adenylate cyclase is activated and intracellular cAMP is generated. The main effect of GLP-1 is to exert potent effects on glucose-dependent insulin secretion and insulin gene expression^[2]. Although predominantly localized to pancreatic islets, numerous reports have documented GLP-1R expression within the nervous system of rodents and humans^[3,4].

In addition to the described stimulatory action of GLP-1R on pancreatic β cells, it is coupled to the cAMP second messenger pathway, the enhancement of which is well documented to be associated with neuroprotection. In rat pheochromocytoma PC12 cells and rat primary hippocampal neurons, GLP-1R activation, stimulating adenylate cyclase, leads to an increase in intracellular cAMP in a manner similar to that in pancreatic

β cells^[5]. GLP-1 induces the neuronal differentiation of PC12 cells similarly to nerve growth factor (NGF), which is reversed by co-incubation with a selective GLP-1R antagonist^[5]. In addition, GLP-1 analogs provide complete protection against apoptotic cell death induced by glutamate neurotoxicity in cultured hippocampal neurons^[6]. Likewise, hippocampal neurons are protected against cell death induced by $A\beta_{1-42}$ and membrane lipid peroxidation caused by iron^[7]. Unfortunately, *in vivo*, GLP-1 is rapidly degraded by dipeptidylpeptidase IV (DPPIV), resulting in a half-life of only about 2 min^[8].

In previous work, we found that geniposide was a novel selective agonist for GLP-1R that accelerated the generation of cAMP and induced the neuronal differentiation of PC12 cells via the mitogen-activated protein kinase (MAPK) pathway^[9]. Furthermore, activation of GLP-1R by geniposide showed neuroprotection in PC12 cells challenged by oxidative stress^[10-12]. However, direct evidence of geniposide activating GLP-1R to prevent cells from oxidative damage remains to be established. In this study, we explore the effect of knocking down *Glp-1r* by RNA interference on the neuroprotectin of geniposide in PC12 cells.

Heme oxygenase 1 (HO-1), a stress-inducible protein, is the rate-limiting enzyme of heme degradation, and it is associated

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with protection against cellular injury and oxidative stress. HO-1 thus provides a relevant and sensitive index by which to assess alterations in cellular redox state^[13]. Additionally, the protein kinase A (PKA)/cAMP response element binding protein (CREB) pathway also plays an important role in neuronal survival in the central and peripheral nervous system. PKA phosphorylates CREB at Ser-133 to recruit the CREB binding protein to activate the expression of target genes, including HO-1 and Bcl-2^[14]. Therefore, in addition to determine the influence of GLP-1R activation by geniposide on the phosphorylation of CREB (Ser-133), we also analyzed the expression of HO-1 in the geniposide-treated PC12 cells as an index of the neuroprotective properties of geniposide.

Materials and methods

Materials

Materials were obtained from the following sources: PC12 cells from the cell collection of the Shanghai Institute for Cell Research, Chinese Academy of Sciences; HO-1, p-CREB, and CREB antibodies and horseradish peroxidase-conjugated goat anti-rabbit antibody from Cell Signaling Technology (Lake Placid, NY); HRP-labeled GAPDH primary antibody from Shanghai Kangcheng Bioengineering Co, Ltd; fetal bovine serum, horse serum, penicillin/streptomycin, and Dulbecco's modified Eagle's medium (DMEM) from HyClone (UT, USA); ECL advance from Amersham; polyvinylidene difluoride membranes from Millipore (Billerica, MA); 3-morpholinopyridone hydrochloride (SIN-1), hemin, Sn-protoporphyrin IX (SnPP), and G418 from Calbiochem; GLP-1 (7-36) from Anaspec (CA, USA); and geniposide from the National Institute for the Control of Pharmaceutical and Biological Products. All other reagents were purchased from Amersco (Solon, OH).

Cell culture and treatment

PC12 cells were grown at 37 °C in DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum in a humidified atmosphere of 5% CO₂. To assay the effect of geniposide on the expression of HO-1, PC12 cells were seeded in 6-well plates at a density of 1×10⁶ cells/mL. After PC12 cells were treated with geniposide for the indicated time, the cells were washed with cold PBS and scraped in the presence of lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% NP40, 1 mmol/L Na₃VO₄) containing a mixture of protease inhibitors (25 mmol/L PMSF, 10 μg/mL leupeptin).

Preparation of GLP-1 receptor shRNA and plasmid

Based on the rat *Glp-1r* (NM_012728) gene, an oligonucleotide sequence for *Glp-1r* shRNA was selected to knock down *Glp-1r* expression in PC12 cells. The rat *Glp-1r*-specific shRNA [5'-GTATCTCTACGAGGACGAG-3' (#1) and 5'-GACCGA-CATCAATGCAGA-3' (#2)] plasmids and the control vector [Scrambled shRNA 5'-TTCTCCGAACGIGTCACGT-3'] were provided by Shanghai Kangcheng Bioengineering Co, Ltd. The single-stranded sense and antisense DNA sequences of each shRNA were linked by a loop ring, which included a

stop site for RNA polymerase III. Then, the template DNA was inserted into the pRNAT-shRNA-U6 plasmid. According to the protocol provided by the supplier, transfection of *Glp-1r* shRNAs was carried out in a 6-well plate with Lipofectamine™ 2000 transfection reagent (Invitrogen). Twenty-four hours after transfection, total RNA and protein were extracted from untransfected, scrambled shRNA-transfected, and *Glp-1r* shRNA-transfected PC12 cells, the efficiency of transfection was determined by RT-PCR and Western blotting. Based on this, stable cell clones (dnGLP-1R) were selected in the presence of G418 (100 μg/mL).

RT-PCR

Total RNA was extracted from wild-type PC12 cells, scrambled shRNA- and *Glp-1r* shRNA-transfected PC12 cells using the Catrimox-14TM RNA isolation Kit Ver 2.11 (TaKaRa, Japan). The total RNA (2 μg) was reverse-transcribed and amplified using a TaKaLa RNA PCR kit Ver 3.0 (Japan). Expression of *Glp-1r* (79 bp) was normalized to *Gapdh* (443 bp). The primers were 5'-CTGCATCGTGATAGCCAAGCT-3' (*Glp-1r* sense), 5'-GGACTTCGCGAGTCTGCATT-3' (*Glp-1r* antisense), 5'-CATCACCATCTTCCAGG AGCG-3' (*Gapdh* sense) and 5'-TGACCTTGCCCACAGCCTTG-3' (*Gapdh* antisense). The elongation was done at 42 °C for 30 min and 94 °C for 2 min, and amplification consisted of 24 cycles of denaturing at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The levels of gene expression were semi-quantified using Quantity One software (Bio-Rad, Hercules, CA).

Western blotting

Western blotting was performed on 20 μg of protein from each cell lysate. Proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane after fractionation 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 secondary antibodies were diluted in blocking solution and incubated with the membranes for 2 h and 1 h, respectively. Excess antibody was washed off with 20 mmol/L TBST (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, and 0.1% Tween-20) before incubation in enhanced chemiluminescence (ECL) solution. The membrane was subsequently exposed to photographic film. Western blotting results were quantified by the analysis of X-ray films using Quantity One.

Effect of geniposide on the activity of the HO-1 promoter in PC12 cells

The core promoter sequence of human heme oxygenase-1 (*ho-1*) (-56 to -206) was cloned from the human genome and inserted into the pGL3-Basic vector (Promega). Further, the AB1 enhancer-like sequence of human *ho-1* was amplified using the primers Fw-Ab1: 5'-GCTAGCTAATCCTTTC-CCGAGCCA-3' and Rv-Ab1: 5'-GCTGGATATCTGAGGAA-AAC-3'^[15]. The amplified AB1-like sequence was inserted into the upstream site of the constructs described above.

Before the luciferase reporter assay was performed, scram-

bled shRNA PC12 cells (shCon) and *Glp-1r* shRNA PC12 cells (shGlp-1r) were seeded at a density of 1×10^5 cells per well in 6-well plates. For each sample, 2.0 μg of the luciferase reporter plasmid construct harboring the ho-1 enhancer or a negative control was transfected using Lipofectamine™ 2000 according to the manufacturer's protocol (Invitrogen). After 12 h, the medium was changed, and the cells were transferred into 96-well plates and treated with geniposide for 8 h. The luciferase activities in the geniposide treated shCon and shGlp-1r PC12 cells were measured using Bright-Glo (Promega).

Determination of cell viability

To explore the neuroprotection of geniposide in shCon and shGlp-1r PC12 cells, cells were plated in 6-well culture dishes at 2×10^5 cells/mL. After cells adhered, they were pre-incubated with SnPP for 15 min before adding geniposide or hemin (a heme oxygenase-1 inducer). After treatment with geniposide for 2 h, the cells were treated with SIN-1 (1 mmol/L) for 12 h. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were incubated for 2 h 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 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.

Statistical analysis

When necessary, data are expressed as mean \pm SD. In appropriate cases, significant differences between groups were determined by one-way analysis of variance (ANOVA) using Origin 8.0 software. The criterion for statistical significance was $P < 0.05$.

Results

Knockdown of *Glp-1r* with shRNA in PC12 cells

To evaluate the function of *Glp-1r* in the PC12 cells, we designed and constructed two shRNA plasmids for GLP-1R and transfected them into PC12 cells. The results from RT-PCR and Western blotting showed that both of the shRNA sequences had a noticeable effect on the expression of *Glp-1r* in PC12 cells, but plasmid #1 had better knockdown efficiency than #2 (Figure 1A and B). Therefore, to obtain stable cell clones (shGlp-1r PC12 cells), we treated the plasmid #1-transfected PC12 cells with 100 $\mu\text{g}/\text{mL}$ G418 to isolate individual clones.

Effect of GLP-1R on geniposide-induced HO-1 expression

To determine the effect of geniposide on the expression of HO-1, we determined the level of HO-1 protein after treatment with geniposide. Geniposide induced the expression of HO-1 in a dose-dependent manner (Figure 2). Furthermore, we found that pre-treatment with 10 $\mu\text{mol}/\text{L}$ H89 (a selective

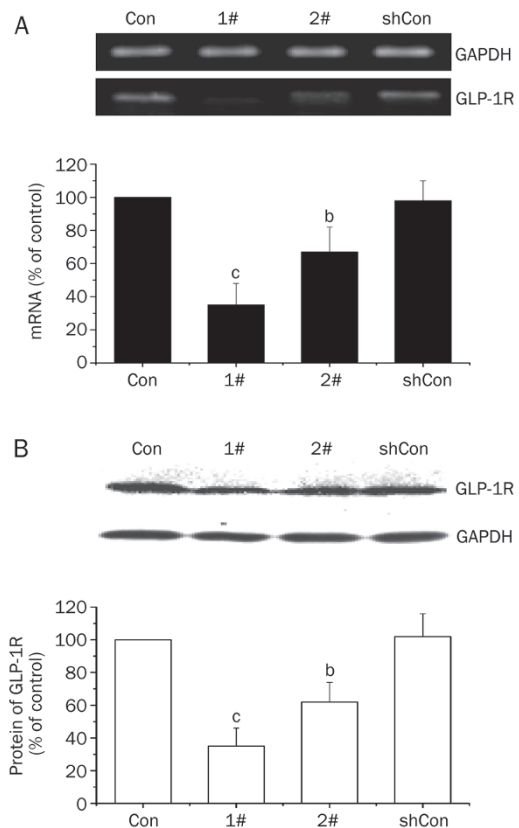


Figure 1. PC12 cells were transfected with *glp-1r* shRNA (1# and 2#) or its scrambled shRNA using lipofectamine reagent. After transfected 24 h, the cells were collected, RT-PCR (A) and Western blotting (B) were used to assay the change of GLP-1R mRNA and protein in PC12 cells. β -actin and GAPDH were taken as internal references respectively. Experiments were repeated three times, band intensities were quantified, and SD values were calculated. ^b $P < 0.05$, ^c $P < 0.01$ vs scrambled control (transfected with scrambled shRNA, shCon).

inhibitor of PKA) for 30 min decreased geniposide-induced HO-1 expression. However, in shGlp-1r PC12 cells, pre-treatment with geniposide had no apparent impact on the expression of HO-1 (Figure 3). These results suggest that GLP-1R is involved in geniposide-induced expression of HO-1 in PC12 cells and that PKA might have an important role in this process.

Geniposide regulates the enhancer activity of HO-1 by activating GLP-1R

To explore the specific mechanism by which geniposide induces the expression of HO-1 through activation of GLP-1R, we constructed a reporter gene plasmid including an enhancer (AB1) of *ho-1* gene and transfected it into control and shGlp-1r PC12 cells. The results from reporter gene assay demonstrate that geniposide increased the expression of the reporter gene in a dose-dependent manner in control PC12 cells but not in the shGlp-1r PC12 cells (Figure 4).

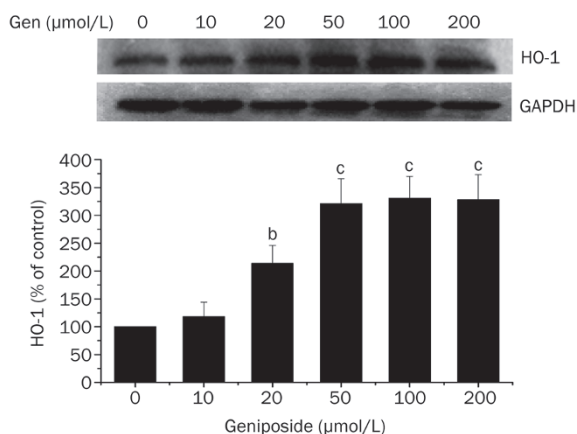


Figure 2. Geniposide induced the expression of HO-1 in a dose-dependent manner. After PC12 cells were treated with indicated doses of geniposide for 8 h, HO-1 protein was determined with Western blotting. Anti-GAPDH serves as the protein load control. Representative results are shown. $n=3$. ^b $P<0.05$, ^c $P<0.01$ vs Control groups.

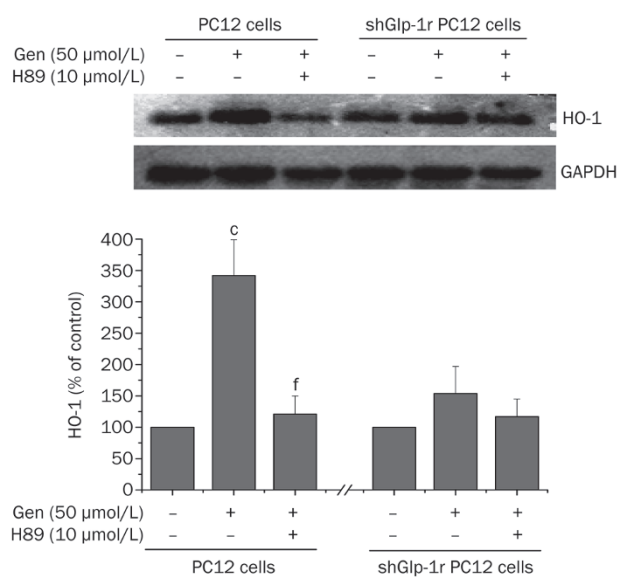


Figure 3. Effect of H89, a PKA inhibitor, on the expression of HO-1 induced by geniposide in PC12 and glp-1r shRNA transfected PC12 cells (shGlp-1r). Anti-GAPDH serves as the protein load control. Representative results are shown. $n=3$. ^c $P<0.01$ vs Control. ^f $P<0.01$ vs the group of geniposide treatment.

Effect of Glp-1r RNAi on the neuroprotective effect of geniposide in PC12 cells

To explore the role of activation of GLP-1R by geniposide in PC12 cells, we measured cell viability after an oxidative stress challenge. Similar to hemin (a strong inducer of HO-1), geniposide prevented PC12 cells from SIN-1-induced oxidative damage, and the cytoprotection of PC12 cells by geniposide was also inhibited by pretreatment with SnPP (an inhibitor of HO-1 activity). By contrast, in shGlp-1r PC12 cells, neither of these phenomena was observed (Figure 5), suggesting that

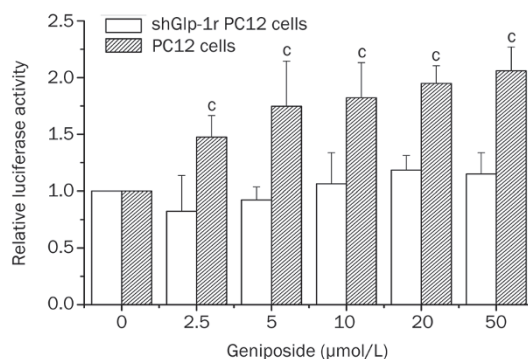


Figure 4. Geniposide increased the enhancer activity of HO-1 gene. PC12 cells were plated and transfected with plasmid including the AB1 enhancer of *ho-1* gene or the control plasmid as described in *Materials and Methods*. Transfected cells were treated with different doses of geniposide for 8 h, and luciferase activity was assayed for reporter gene activity. Data are shown as mean±SD. $n=5$ in three different experiments. ^c $P<0.01$ vs Control.

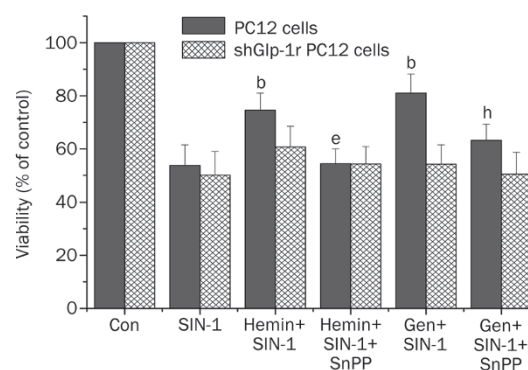


Figure 5. SnPP, an inhibitor of HO-1 activity, attenuated the neuroprotection of geniposide in PC12 cells and glp-1r shRNA transfected PC12 cells (shGlp-1r) in the presence of SIN-1. Hemin, an inducer of HO-1, was taken as positive control in this experiment. Values are expressed as mean±SD from three different experiments. $n=5$. ^b $P<0.05$ vs the SIN-1 treated group; ^e $P<0.05$ vs the group of SIN-1 plus hemin; ^h $P<0.05$ vs the group of SIN-1 plus geniposide.

GLP-1R-induced up-regulation of HO-1 plays a critical role in the neuroprotection of geniposide in PC12 cells.

Geniposide induces the phosphorylation of CREB in a PKA-dependent manner in PC12 cells

Western blotting showed that treatment with geniposide increased the level of phosphorylation of CREB ~2.1-fold over untreated PC12 cells. In the presence of SIN-1, the level of geniposide-induced CREB phosphorylation was increased to 3.5-fold over the untreated cells. Moreover, this effect of geniposide on CREB phosphorylation was inhibited by preincubation with 10 μmol/L H89 (a PKA inhibitor) for 30 min (Figure 6). Together with the results from Figure 3, these data indicate that phosphorylation of CREB by PKA is involved in the cytoprotection of geniposide in PC12 cells.

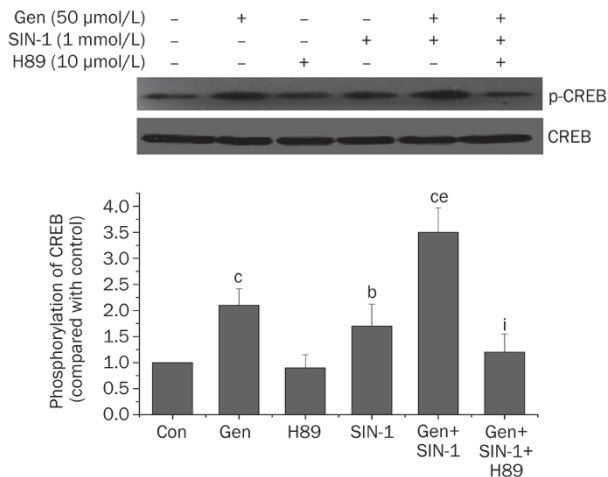


Figure 6. Geniposide enhances the phosphorylation of CREB induced by SIN-1 in PC12 cells. After PC12 cells were pretreated with 10 µmol/L H89 for 30 min, and the treated with 50 µmol/L geniposide for 2 h followed by incubation with 1 mmol/L SIN-1 for 1 h, the cells lysates were subjected to SDS-PAGE, phosphorylation of CREB and CREB were detected by Western blotting. Experiments were repeated three times, and representative results are shown. ^b $P < 0.05$, ^c $P < 0.01$ vs Control; ^e $P < 0.05$ vs SIN-1 treated group; ⁱ $P < 0.01$ vs the group of SIN-1 plus geniposide.

Discussion

Heme oxygenase (HO) is the rate-limiting enzyme in the oxidative degradation of free heme, which prevents the heme-catalyzed production of highly reactive hydroxyl radicals from hydrogen peroxide. Up-regulation of oxidative stress-inducible HO-1 expression might confer an adaptive survival response to oxidative insults and delay cell death^[14,16]. Several putative mechanisms have been proposed to explain the cytoprotective roles of HO-1 induction. Up-regulation of HO-1 is associated with an enhancement in the catabolism of the pro-oxidant heme into bile pigments, which are potent endogenous antioxidants^[17]. In addition, induction of HO-1 expression is accompanied by increased ferritin activity, which exerts an anti-apoptotic effect by chelating the excess free iron in the oxidatively stressed cells^[18]. In previous work, we found that geniposide, a novel agonist for GLP-1R, shows neurotropic and neuroprotective functions in PC12 cells^[9-12]. It was not clear whether GLP-1R activation by geniposide is involved in these actions directly. In this study, treatment with geniposide induced the expression of HO-1 in a dose-dependent manner in PC12 cells, but when GLP-1R in PC12 cells was knocked down with shRNA, accompanying the decline of HO-1 expression, the neuroprotective effect of geniposide was inhibited significantly. Furthermore, neuroprotection by geniposide was also greatly reduced by SnPP (an inhibitor of HO-1 activity) in PC12 cells. These results demonstrate that HO-1 expression induced by GLP-1R activation plays a pivotal role in the neuroprotection of geniposide in PC12 cells.

Geniposide, an iridoid glycoside from the fruit of *Gardenia jasminoides* Ellis, has the interesting property of activating the

glucagon-like peptide-1 receptor (GLP-1R), and it is a popular Chinese herb used to treat febrile diseases including edema, hepatic disorders, acute conjunctivitis and hematuria^[19,20]. In rat hepatocytes, geniposide activates the expression of GSH S-transferase by inducing the GST M1 and GST M2 subunits involved in the transcription and phosphorylation of MEK-1 signaling^[21]. Lee and colleagues have reported that geniposide attenuates neuronal cell death in oxygen- and glucose-deprived rat hippocampal slice cultures^[22], suggesting that geniposide is a promising anti-oxidative compound.

Similar to GLP-1, geniposide activating GLP-1R could induce the release of cAMP in PC12 cells^[10]. As a downstream target of cAMP, PKA might be involved in the neuroprotection of geniposide. Accumulating evidence shows that cAMP induces gene transcription through the activation of cAMP-dependent protein kinase A (PKA) and subsequent phosphorylation of the transcription factor cAMP response element binding protein (CREB) at Ser-133^[23,24]. Based on the strong correlation between HO-1 stimulation and resistance to apoptosis, we tested whether the PKA signal pathway was directly related to the neuroprotection of geniposide in PC12 cells. Geniposide enhanced the SIN-1-induced phosphorylation of CREB, and this effect was inhibited by H89, a selective inhibitor of PKA. Moreover, pre-incubation with H89 decreased the neuroprotective effect of geniposide in PC12 cells, suggesting that up-regulation of HO-1 expression by geniposide is a neuroprotective response mediated by the PKA pathway.

The anti-apoptotic effect of GLP-1R activation in oxidative stress models and the ability of GLP-1 to modify the synthesis/processing of APP and A β have also been evaluated^[25]. It has been suggested that GLP-1 analogs lower A β in normal mouse brain by 20%, and in cell culture studies, both secreted and cellular APP levels are also lowered by GLP-1 analogs^[5]. All of these results suggest that GLP-1R activation may prove a useful therapeutic strategy in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Unfortunately, the short half-life in GLP-1 and its analogs limit their application. Geniposide not only activates GLP-1R to accelerate the release of cAMP, but it also improves SIN-1-induced phosphorylation of CREB to enhance the expression of HO-1 in PC12 cells, suggesting that geniposide might be a promising compound in the treatment of neurodegenerative diseases.

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Author contribution

Jian-hui LIU and Fei YIN designed the research; Fei YIN, Jian-hui LIU, Xu-xu ZHENG, and Li-xia GUO performed the research; Jian-hui LIU and Fei YIN analyzed the data and wrote the paper.

Abbreviations

CREB, cAMP response element binding protein; DPP IV, dipeptidyl peptidase IV; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP-1R, glucagon-like peptide 1 receptor; HO-1, heme oxygenase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, NF-E2-related factor 2; PBS, phosphate-buffered saline; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered solution.

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