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Research Paper

# Ginsenoside Rg3 promotes beta-amyloid peptide degradation by enhancing gene expression of neprilysin

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## Abstract

**Objectives** It has been hypothesized that the accumulation of beta-amyloid peptide (A$\beta$) in the brain is a triggering event leading to the pathological cascade of Alzheimer's disease. The steady-state levels of A$\beta$ are determined by the metabolic balance between anabolic and catabolic activity and the dysregulation of this activity leads to Alzheimer's disease. Recent evidence has shown that neprilysin (NEP) is the rate-limiting enzyme in the A$\beta$ degradation in the brain. Ginseng, the root of *Panax ginseng* C.A. Meyer, is widely used as a tonic for the prevention and treatment of age-related disorders in China. We aimed to investigate the basis of this use.

**Methods** In this study, we investigated the effect of ginsenoside Rg3, one of the major active components of ginseng, on the metabolism of A$\beta$40 and A$\beta$42 in SK-N-SH cells transfected with Swedish mutant $\beta$-amyloid precursor protein (SweAPP).

**Results** The ELISA result showed that Rg3 significantly reduced the levels of A$\beta$40 and A$\beta$42, 19.65 ± 6.05%, 23.61 ± 6.74%, respectively ($P < 0.01$). The Western blot analysis showed that Rg3 reduced the levels of A$\beta$40 and A$\beta$42 through enhancing NEP gene expression, and real-time PCR assay showed that 50 $\mu$M Rg3 could significantly enhance NEP gene expression (2.9 fold at 48 h).

**Conclusions** Our findings suggest that the Rg3 compound of ginseng may be useful for treating patients suffering with Alzheimer's disease.

**Keywords** Alzheimer's disease; beta-amyloid peptide; ginsenoside; neprilysin; Swedish mutant $\beta$-amyloid precursor protein

## Introduction

Alzheimer's disease is the most common form of senile dementia and is characterized by a variety of pathological features, such as amyloid plaques, neurofibrillary tangles, synaptic loss and brain atrophy.[1–3] Up to now, the pathogenesis of Alzheimer's disease is still unclear. It is widely accepted that the abnormal accumulation of beta-amyloid peptide (A$\beta$) plays a key role in the pathogenesis and development of Alzheimer's disease. A$\beta$, a physiological peptide, is constantly anabolized and catabolized in the brain,[4] and the steady-state A$\beta$ levels are determined by the metabolic balance between anabolic and catabolic activity. Much more attention has been paid to abnormal A$\beta$ production but recently the role of A$\beta$ degradation in A$\beta$ homoeostasis has been increasingly recognized, as several enzymes that degrade A$\beta$ have been identified, such as neprilysin (also called neutral endopeptidase, NEP),[5] endothelin-converting enzyme (ECE),[6,7] insulin-degrading enzyme (IDE)[8–12] and angiotensin-converting enzyme (ACE).[13] Among these enzymes, NEP is believed to be the key enzyme in degrading A$\beta$ in the brain.[14–17]

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a traditional Chinese medicine extensively used throughout the Far East Asian countries to enhance stamina and to alleviate fatigue, as well as treat a variety of disorders for thousands of years.[18] Many components of ginseng, such as ginsenosides, polysaccharides, peptides, polyacetylenic alcohols and fatty acids, have been found to be biologically active.[18] However, it was found that much of the pharmacological activity of ginseng is attributed to the ginsenosides, which are structurally similar to steroid hormones.[19] More than 40 different ginsenosides have been identified and isolated from the root of *P. ginseng*. Ginsenoside Rg3 is one of the major pharmacologically

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active ingredients of ginseng and consists of three different parts: a steroid-like backbone structure, a carbohydrate portion and an aliphatic side chain (-$CH_2CH_2CH=C(CH_3)_2$), which is coupled to carbon-20 of the backbone structure. It contains two isoforms, the 20(R)- and the 20(S)- form. Recently, some studies have shown that Rg3 exerts anti-tumorigenic or anti-mutagenic activity in experimental animals and cultured cells.[20–22] However, the effect of Rg3 on nervous system diseases has seldom been reported. Chen *et al.*[23] have reported that Rg3 significantly decreased A$\beta$40 and A$\beta$42 in cultured cells and transgenic mice, Tg2576, which express the Swedish mutant $\beta$-amyloid precursor protein (APP K670N, M671L) at high levels, but the underlying mechanism was not elucidated. In this study, we investigated the effect of ginsenoside Rg3 on A$\beta$ metabolism and found that it decreased A$\beta$40 and A$\beta$42 in cultured SK-N-SH cells transfected with Swedish mutant $\beta$-amyloid precursor protein (SweAPP) gene by enhancing the NEP expression.

## Materials and Methods

### Materials

Suc-L-Ala-L-Ala-Phe-7-amino-3-methyl-coumarin (SAAP-AMC), Hip-L-His-L-Leu, 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2*H*-tetrazolium (MTT), and phosphoramidon were purchased from Sigma (MO, US). Aminopeptidase N, mouse anti-human NEP antibody and rabbit anti-human tubulin antibody were obtained from R&D (MN, US), and rabbit anti-human APP was purchased from CST (Cell Signaling Technology, MA, US); ECL system was from Santa Cruz (CA, US). Goat anti-mouse and goat anti-rabbit secondary antibody were products of Zhongshan Company (Zhongshan, China). Ginsenoside Rg3 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity was ≥ 98% as analysed by HPLC, and it contains one single form, 20(R)-Rg3. Rg3 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. Minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco (NY, US). SuperScrip III First-strand synthesis kit, TRIzol reagent, Lipofectamine$^{2000}$ and SyBr greenER qPCR supermix universal were purchased from Invitrogen (CA, US), rTaq from Takara (Dalian, China). Human amyloid $\beta$ (1-40) and (1-42) assay kits were products of IBL (Japan).

### Cell culture

SK-N-SH cells, human neuroblastoma cells, were purchased from the Chinese Academy of Science. The cells were cultured in MEM medium supplemented with 10% FBS and kept at 37°C in a humidified atmosphere containing 5% $CO_2$. Cells were passaged when 80–90% confluence was reached.

### Cell viability assay

The effect of ginsenoside Rg3 on cell viability was assessed with a 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2*H*-tetrazolium (MTT) assay. Briefly, SK-N-SH cells were cultured in 96-well plates and treated with different concentrations of Rg3 for 12, 24, 48, 72 and 96 h, respectively. Then, 10 $\mu$l of MTT solution (5 mg/ml) was added into each well and cells were incubated for another 4 h at 37°C to allow MTT oxidation by mitochondrial dehydrogenase in viable cells. After 4 h, 100 $\mu$l DMSO per well was added to dissolve the resulting blue formazan crystals. The absorbance was measured at 570 nm on a microplate reader (Bio-Rad Model 680). All readings were compared with the control (treated with vehicle), which represented 100% viability.

### Stable transfection

SK-N-SH cells were cultured in 24-well plates. When cells reached 90–95% confluence, we performed transfection with Lipofectamine$^{2000}$ according to the manufacturer's instruction. Briefly, culture medium was discarded and cells were washed with Opti-MEM twice, 0.8 $\mu$g plasmid was added (pcDNA3.1-SweAPP, kindly provided by Dr Weihong Song) into 50 $\mu$l Opti-MEM and 2 $\mu$l Lipofectamine$^{2000}$ was added into 50 $\mu$l Opti-MEM, respectively, then mixed together gently for 5 min. After incubating for 20 min at room temperature, we added the mixture to cells and continued culturing the cells. Twenty-four hours later, cells were subcultured from 24-well plates into 50-ml flasks and G418 was added to screen positive cell clones. After about 3–4 weeks, 50-cell clones were picked out and identified. Cells highly expressing mutant APP protein were designated SweAPP-SK.

### Quantification of beta-amyloid

We quantified the protein levels of A$\beta$40 and A$\beta$42 in SweAPP-SK cells by means of enzyme-linked immunosorbent assays (ELISA). Briefly, cells were treated with 50 $\mu$M Rg3 for 72 h, then the culture medium was collected and added into 96-well ELISA plates (100 $\mu$l/well), incubated at 4°C overnight, the wells were washed nine times with washing buffer, 100 $\mu$l/well second antibody was added and incubated for an hour at 4°C. The wells were washed 10 times with washing buffer, 100 $\mu$l/well chromogen solution was added and incubated for half an hour at room temperature, and finally, 100 $\mu$l/well stop solution was added and the $OD_{450}$ was measured on a Bio-Rad microplate reader within 30 min. The A$\beta$ concentration was determined according to the manufacturer's instruction. Data was shown as percent reduction relative to vehicle control (0.5 $\mu$l DMSO/ml culture medium).

### Neprilysin activity

The NEP activity assay was performed according to the method of Bormann and Melzig.[24] Briefly, after treating cells with drugs, the culture medium was discarded, 50 $\mu$l of SAAP-AMC (400 $\mu$M) and 400 $\mu$l of Tris-HCl (pH 7.4) were added to the intact cell layer and incubated at 37°C. One hour later, the reaction was stopped by adding 50 $\mu$l of phosphoramidon solution (50 $\mu$M). Subsequently, 400 $\mu$l of the incubation mixture from each well was transferred to an Eppendorf tube. Twenty microlitres of APN (2-aminopropionitrile) solution (25 ng) was added, and the reaction mixture was incubated again for 1 h at 56°C. The reaction was terminated by the addition of 800 $\mu$l acetone. Then the fluorescence of the released AMC was measured at $\lambda$excit = 367 nm and $\lambda$emiss = 440 nm. A calibration curve with AMC was used to calculate the enzyme activity. All results were compared with the vehicle control, which represented 100% activity.

### Western blot analysis

Protein was extracted from treated cells with RIPA buffer and quantified by the BCA method. For Western analysis, 50 $\mu$g of protein were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was immediately blocked with 5% non-fat dry milk in PBS buffer for 1 h, and then incubated for 2 h at room temperature in primary antibody (1 : 1000). After washing, the membrane was incubated with peroxidase-labelled secondary antibody (1 : 1000) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection reagents. $\beta$-Tubulin was used to normalize the quantity of the protein on the blot. At least three independent Western blots were performed.

### RNA isolation and real-time PCR

Total RNA was isolated from cultured SK-N-SH cells using TRIzol according to the manufacturer's instructions. Reverse transcription reactions were carried out in a 20-$\mu$l reaction mixture containing total RNA (1 $\mu$g), 1 $\mu$l primer (50 $\mu$M), 1 $\mu$l 10 mM dNTP mix, 2 $\mu$l 10× reaction buffer, 4 $\mu$l 25 mM $Mg^{2+}$, 2 $\mu$l 0.1 M dTT, 1 $\mu$l SuperScrip III RT (200 U/$\mu$l) and 1 $\mu$l RNaseout (40 U/$\mu$l), under conditions described by the supplier (Invitrogen). Following cDNA synthesis, the resultant mixture was heated at 95°C for 5 min to inactivate RT. Expression of NEP was analyzed by real-time PCR using the SyBr green method (Invitrogen). The reactions were performed in a 50-$\mu$l system containing cDNA template 1.5 $\mu$l, 10× PCR buffer 5 $\mu$l, 25 mM $Mg^{2+}$ 3 $\mu$l, 25 mM dNTP 0.4 $\mu$l, 1 $\mu$l each of forward and reverse primers (10 $\mu$M), 50× sybr 1 $\mu$l, PlatinumTaq 0.3 $\mu$l, $ddH_2O$ 36.8 $\mu$l, and the reactions were run in a Corbett RG6000 thermal cycler. The cycling conditions included an initial phase at 95°C for 2 min, 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. Three independent PCRs from the same reverse transcription sample were performed. The presence of a single specific PCR product was verified by melting curve analysis and confirmed by agarose gel electrophoresis. The expression level of genes was calculated according the following formula: $F = 2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct$ = (average Ct (threshold cycle) of neprilysin gene in treated sample – average Ct of actin gene in treated sample) – (average Ct of neprilysin gene in control – average Ct of actin gene in control). Sequences for the PCR primers were human-actin: F 5′-TCACCCACACTGTGCCCATCTACGA-3′, R 5′-CAGCGGAACCGCTCATTGCCAATGG-3′, human NEP: F 5′-TGACCACTCGACCAGGTTCT-3′, R 5′-CGATCACTGTCGCTATGACAAC-3′ (F: forward, R: reverse).

### Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Data were analysed by one-way analysis of variance followed by post-hoc tests using SPSS for windows; differences with a value of $P < 0.05$ were considered statistically significant.

## Results

### Establishment of SweAPP-SK cell strain

In our experiment, we first constructed a cell strain continuously expressing Swedish mutant APP. We transfected pcDNA3.1-SweAPP with Lipofectamine$^{2000}$ into neuroblastoma cells (SK-N-SH cells), and screened with G418; 3–4 weeks later, we picked out monoclones and identified them with Western blot (Figure 1b). We kept the cell clone that highly expressed APP, and further measured A$\beta$40 and A$\beta$42 in culture medium with ELISA. The ELISA results showed that cells highly expressing APP protein did highly produce A$\beta$40 and A$\beta$42 in culture medium, 8.6-fold and 4.3-fold higher than controls, respectively ($P < 0.05$) (Figure 1a). These results indicated that we had successfully established SK-N-SH cells that highly produced both A$\beta$40 and A$\beta$42, and we designated them SweAPP-SK cells.

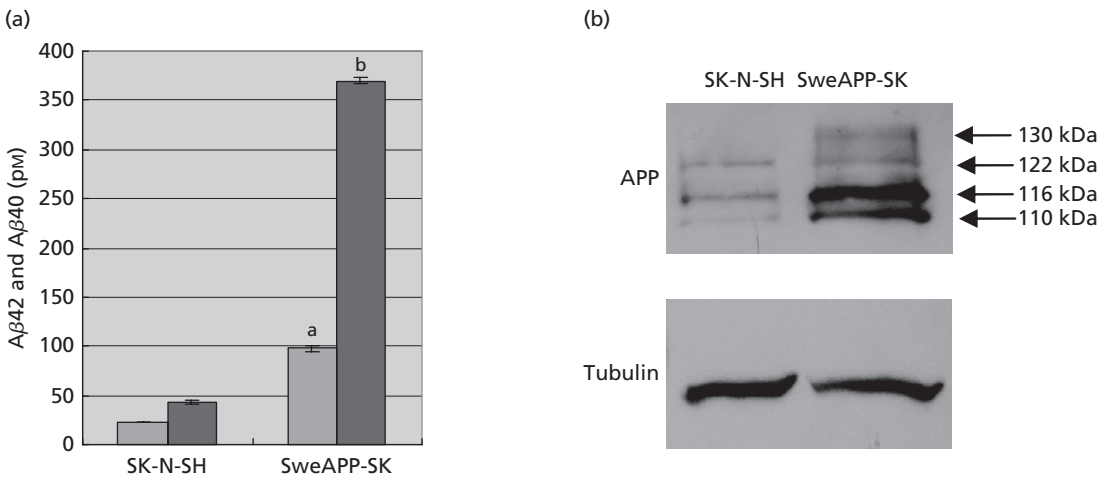


**Figure 1** Effect of SweAPP gene transfection on $\beta$-amyloid precursor protein (APP) protein production and extracellular A$\beta$40 and A$\beta$42 levels. (a) SK-N-SH cells were transfected with pcDNA3.1-SweAPP, followed by G418 screening for 3–4 weeks, then the positive clones were picked out and cultured, named as SweAPP-SK. A$\beta$40 and A$\beta$42 in the culture medium of SweAPP-SK cells were detected by ELISA. First column of each pair, A$\beta$42; second column of each pair, A$\beta$40. Values shown are representative of three independent experiments, each done in triplicate. [a]$P < 0.01$ vs SK-N-SH; [b]$P < 0.01$ vs SK-N-SH. (b) Western blot analysis for APP protein level. There are several isoforms of APP with molecular weights ranging from 100 kDa to 140 kDa. $\beta$-Tubulin was used as the internal control for protein loading and transfer efficiency.

### Ginsenoside Rg3 significantly reduces A$\beta$40 and A$\beta$42 in culture medium of SweAPP-SK cells

To investigate whether Rg3 affects A$\beta$ metabolism, we detected A$\beta$40 and A$\beta$42 levels in culture medium with ELISA after treating SweAPP-SK cells. Before examination of the effect of Rg3 on A$\beta$40 and A$\beta$42, we first performed an MTT assay to identify optimal Rg3 concentration. The results showed that 50 $\mu$M Rg3 did not affect the cell viability significantly ($P > 0.05$) (Table 1). We thus treated SweAPP-SK cells with 50 $\mu$M Rg3 for 72 h, and measured the A$\beta$40 and A$\beta$42 levels in cell culture medium. We found that 50 $\mu$M Rg3 significantly reduced both A$\beta$40 and A$\beta$42 levels in cell culture medium, 19.65 ± 6.05% and 23.61 ± 6.74%, respectively ($P < 0.05$). Our finding was consistent with previous results reported by Chen *et al.*[23]

### Ginsenoside Rg3 reduces A$\beta$40 and A$\beta$42 by enhancing neprilysin activity

As shown in Table 1, after treating with Rg3, the NEP activity increased significantly in a dose-dependent way: 117 ± 4.5% at 25 $\mu$M ($P < 0.05$), 127 ± 2.8% at 50 $\mu$M ($P < 0.05$), 140 ± 8.5% at 100 $\mu$M ($P < 0.05$), respectively. Moreover, we also examined the effect of Rg3 on NEP activity at different times. As shown in Table 2, the NEP activity was 107 ± 10.4%, 123 ± 3.1% ($P < 0.05$) and 130 ± 1.3% ($P < 0.05$) after treating the cells with 50 $\mu$M Rg3 for 24, 48 and 72 h, respectively.

### Ginsenoside Rg3 enhances NEP activity by promoting neprilysin gene expression

As shown in Table 3, after treating cells with Rg3, NEP gene transcription was enhanced: by 1.9 fold at 25 $\mu$M (72 h), 2.3 fold at 50 $\mu$M (72 h), 2.9 fold at 50 $\mu$M (48 h) and 2.1 fold at 50 $\mu$M (24 h). These results indicated that Rg3 enhanced NEP activity by promoting NEP gene transcription.

## Discussion

It is widely accepted that increased A$\beta$ production and deposition plays a key role in triggering neuronal dysfunction and death in Alzheimer's disease.[3] For improving the treatment of Alzheimer's disease, there is a need to find compounds that are capable of reducing A$\beta$ levels. However, normally, both intracellular and extracellular A$\beta$40 and A$\beta$42 levels of cultured cells are very low and not easily measured, so it is necessary to construct a cell strain that highly produces A$\beta$ for screening of therapeutic compounds. Therefore, in our experiment, we first constructed a cell strain continuously expressing Swedish mutant APP, because it was reported that this kind of mutant APP caused up to a 3- to 4-fold increase in A$\beta$ generation in its human carrier.[25] ELISA showed that cells highly expressing APP protein did indeed highly produce A$\beta$40 and A$\beta$42 in culture medium, 8.6-fold and 4.3-fold higher than controls, respectively (Figure 1a). These results indicated that we had successfully established SK-N-SH cells that highly produced both A$\beta$40 and A$\beta$42, and we designated them SweAPP-SK cells.

Ginseng has already been used for a long time to prevent and treat age-related disorders in Chinese medicine. It has been reported that ginseng enhances spatial memory and protects neurodegeneration in rodents.[26–33] However, the effect of ginsenoside Rg3 on A$\beta$ metabolism and the underlying mechanism has been little studied. To investigate whether Rg3 affects A$\beta$ metabolism, we detected A$\beta$40 and A$\beta$42 levels in culture medium with ELISA after treating SweAPP-SK cells. We found that 50 $\mu$M Rg3 significantly reduced both A$\beta$40 and A$\beta$42 levels in cell culture medium, 19.65 ± 6.05% and 23.61 ± 6.74%, respectively ($P < 0.05$). Our finding was consistent with previous results reported by Chen *et al.*[23]

A$\beta$ levels are determined by the metabolic balance between anabolic and catabolic activity, and NEP is thought to be the key enzyme degrading A$\beta$ *in vivo*. We thus performed a NEP peptidase assay to detect whether Rg3 could affect NEP activity. Our results (Tables 1 and 2) showed that Rg3 increases

**Table 1** Effect of ginsenoside Rg3 on SK-N-SH cell viability and neprilysin activity

| Concentration ($\mu$M) | Cell viability (%) | NEP activity (%) |
|---|---|---|
| 0 | 100 ± 3.06 | 100 ± 5.5 |
| 25 | 102 ± 4.73 | 117 ± 4.5$^{\#}$ |
| 50 | 97 ± 6.58 | 127 ± 2.8$^{\#}$ |
| 100 | 93 ± 6.09* | 140 ± 8.5$^{\#}$ |

SK-N-SH cells were treated with vehicle (0.5 $\mu$l DMSO/ml culture medium), 25, 50 or 100 $\mu$M Rg3, respectively. Cell viability was determined 72 h later. *$P < 0.05$, vs vehicle control ($n = 6$). Neprilysin (NEP) activity was also determined 72 h after treating with Rg3. $^{\#}P < 0.05$, vs vehicle control. Values shown are representative of three independent experiments, each done in quadruplicate.

**Table 2** Time-dependent influence of ginsenoside Rg3 on neprilysin activity of SK-N-SH cells

| Time (h) | NEP activity (%) |
|---|---|
| 0 | 100 ± 5.5 |
| 24 | 107 ± 10.4 |
| 48 | 123 ± 3.1* |
| 72 | 130 ± 1.3* |

SK-N-SH cells were treated with 50 $\mu$M Rg3, and neprilysin (NEP) activity was determined after 24, 48 and 72 h, respectively. *$P < 0.05$ vs vehicle control. Values shown are representative of three independent experiments, each done in quadruplicate.

**Table 3** Influence of ginsenoside Rg3 on neprilysin gene transcription

| Treatment | $F = 2^{-\Delta\Delta Ct}$ |
|---|---|
| Control | 1.00 ± 0.00 |
| Rg3 (25 $\mu$M, 72 h) | 1.88 ± 0.26* |
| Rg3 (50 $\mu$M, 24 h) | 2.14 ± 0.00* |
| Rg3 (50 $\mu$M, 48 h) | 2.93 ± 0.55* |
| Rg3 (50 $\mu$M, 72 h) | 2.25 ± 0.18* |

After treatment, RNA was isolated and reverse transcription was performed. Three independent PCRs from the same reverse transcription sample were performed. The expression level of genes was calculated according the fomula $F = 2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct$ = (average Ct (threshold cycle) of neprilysin gene in treated sample – average Ct of actin gene in treated sample) – (average Ct of neprilysin in control – average Ct of actin gene in control). *$P < 0.05$ vs control, $n = 3$.

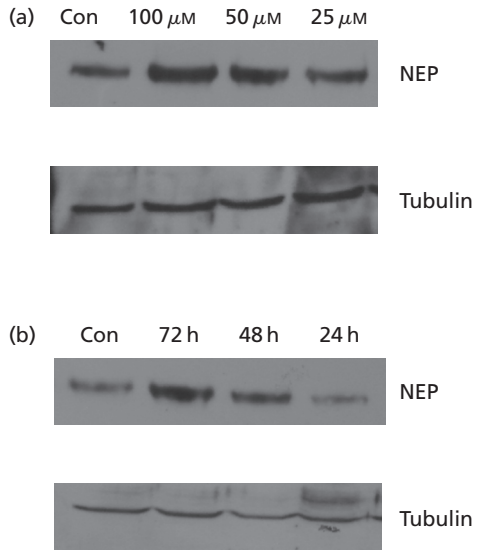


**Figure 2** Effect of ginsenoside Rg3 on the expression of the neprilysin gene by concentration gradient and time gradient. (a) Cells were treated with 25, 50 or 100 $\mu$M Rg3, and 72 h later, Western blot analysis was performed. (b) Cells were treated with 50 $\mu$M Rg3 for 24, 48 or 72 h, then Western blot analysis was performed. $\beta$-Tubulin was used as the internal control for protein loading and transfer efficiency. Con, control.

the NEP activity in SweAPP-SK cells in a dose- and time-dependent manner.

To study the possible mechanism underlying the effect of Rg3 on NEP activity, we firstly performed Western blot analysis to detect the expression of NEP protein. The results showed that Rg3 improved NEP gene expression in a dose-dependent and time-dependent manner (Figure 2). It is well known that gene expression regulation can take place in multiple stages, such as the transcription and translation levels. To determine whether the increase of NEP protein is caused by enhancement of NEP gene transcription, we performed real-time PCR. Our results indicated that Rg3 enhanced NEP activity by promoting NEP gene transcription.

At least three cell type-specific ligands capable of up-regulating NEP activity have been identified so far: opioid for neutrophils,[34] calcitonin for osteoblast-like cells,[35] and substance P for fibroblasts[36] and bone marrow cells.[37] These ligands are known to act via specific G-protein-coupled receptors (GPCRs). These studies suggested that Rg3 may enhance NEP expression via the G-protein-coupled receptor (GPCR)-mediated signal pathway, but the exact molecular mechanism needs further studying.

## Conclusions

In our experiment, we first successfully established a cell strain that highly secreted A$\beta$42 and A$\beta$40 (SweAPP-SK cells). Then we identified the optimal Rg3 concentration to act on cells. Consistent with a previous report,[23] we found that 50 $\mu$M Rg3 could reduce extracellular A$\beta$42 and A$\beta$40 levels significantly without affecting the cell viability significantly. To study the underlying mechanism of the effect of ginsenoside Rg3 on A$\beta$ metabolism, we performed a peptide assay to examine if it produced its effect by enhancing NEP activity, and we found that Rg3 significantly enhanced NEP activity in a dose- and time-dependent way. Furthermore, we provided evidence showing that Rg3 significantly enhanced expression of NEP protein via an increase in NEP gene transcription. In summary, our data show that Rg3 decreases A$\beta$42 and A$\beta$40 in SK-N-SH cells expressing Swedish mutant APP by promoting NEP gene transcription. These findings suggest the possibility of using ginsenoside Rg3 for the treatment of patients suffering with Alzheimer's disease.

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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### Note

Lingling Yang and Jianrong Hao contributed equally to this paper.

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