ORIGINAL ARTICLE

# Enhancement of neutral endopeptidase activity in SK-N-SH cells by ginsenoside Rb1

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**Abstract**

**OBJECTIVES:** To investigate the effect of ginsenosides Rb1 and Rg1 on Neprilysin (NEP) activity in SK-N-SH cells, and probe the underlying mechanism.
**METHODS:** The effects of ginsenosides Rb1 and Rg1 on NEP activity were analyzed by NEP peptidase assay. Western blot was used to determine NEP gene expression at translational level, and RT-PCR was also performed to detect NEP gene expression at transcriptional level.
**RESULTS:** NEP peptidase assay indicated that ginsenoside Rb1 can improve the activity of NEP, and RT-PCR and western blot results showed that the enhancement of NEP activity by ginsenoside Rb1 was due to enhancing NEP gene expression, while Rg1 did not have this effect.
**CONCLUSION:** Our studies showed that ginsenoside Rb1 can enhance NEP activity by upregulating NEP gene expression. Our findings might offer a pharmacological explanation for the use of ginseng in traditional medicine.

**Abbreviations**

| | |
|---|---|
| NEP | – neprilysin |
| AD | – alzheimer's disease |
| SAAP-AMC | – Suc-L-Ala-L-Ala-Phe-7-amino-3-methyl-coumarin |
| APN | – aminopeptidase N |
| MEM | – minimum essential medium |
| FBS | – fetal bovine serum |
| ECL | – enhanced chemiluminescence |
| SDS-PAGE | – sodium dodecyl sulfate polyacrylamide gel electrophoresis |

## INTRODUCTION

Neprilysin (NEP; EC3.4.24.11) is a 97 kDa type II membrane metalloendopeptidase that functions as an ectoenzyme at the cell surface and acts mainly on peptides smaller than 5 kDa, such as enkaphalin, atrial natriuratic peptides, substance P, neurokinins, nociceptin, and corticotrophin-releasing factor (Barnes *et al.* 1995; Iwata *et al.* 2005; Johnson *et al.* 1999; Roques *et al.* 1995; Sakurada *et al.* 2002). Accumulating data have shown that NEP plays a key role in decreasing the levels of cerebral Aβ deposition, which is a pathological hallmark of Alzheimer's disease (AD) and is thought to play the key role in AD development (Hama *et al.* 2001; Iwata *et al.* 2001; Iwata *et al.* 2004; Marr *et al.* 2004). Therefore, it is speculated that upregulation of NEP activity in the brain may be an effective therapeutic approach for AD treatment.

Ginseng, the root of *Panax ginseng* (C.A. Mey.) is a traditional medicine extensively used to enhance stamina and to alleviate fatigue as well as the effects of physical stress for thousands of years (Attele *et al.* 1999). More than 40 different gin-

senosides have been identified and isolated from the root of *P. ginseng* (Cheng *et al*. 2005). Ginsenoside is the major pharmacologically active ingredient of ginseng.. The most abundant ginsenosides in ginseng are Rb1 and Rg1, and Rg1 and Rb1 are the representative constituents of panax ginseng and American ginseng, respectively. Many researchers believe that they share many beneficial effects of ginseng, including alleviating learning and memory impairment, reversing pathological and physiological changes induced by stress and aging, *etc* (Cheng *et al*. 2005; Wang *et al*. 2001). However, their effect on NEP was not studed. In the present study, we investigated the effects of ginsenosides Rb1 and Rg1 on NEP activity and discovered possible mechanisms involved in this process.

## MATERIALS AND METHODS

*Reagents and materials*

Suc-L-Ala-L-Ala-Phe-7-amino-3-methyl-coumarin (SAAP-AMC), Hip-L-His-L-Leu, MTT, and phosphoramidon were purchased from Sigma. Aminopeptidase N (APN) , mouse anti-human NEP antibody, and rabbit anti-human tubulin antibody were obtained from R&D. Ginsenosides Rb1 and Rg1, derived from cultivated *panax ginseng* (C.A. Mey) roots, were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of both Rg1 and Rb1 are ≥ 98%, analyzed by HPLC. Minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco. Goat anti-mouse and goat anti-rabbit secondary antibody were products of Zhongshan Company. The reverse transcription kit was purchased from Promega, rTaq from Takara, TRIzol reagent from Invitrogen, and enhanced chemiluminescence (ECL) system from Pierce.

*Cell culture and treatments*

SK-N-SH cells, human neuroblastoma cells, were purchased from Academia Sinica (China). 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 subcultured into 50-milliliter flasks when 80-90% confluence was reached. Twenty-four hours after plating, the cells were incubated with the indicated concentrations (see Tables 1, 2) of Rb1 or Rg1 in MEM with 8% FBS and cultivated for another seventy-two hours. For the enzymatic experiments, cells were seeded in 24-well plates.

*NEP activity*

The NEP activity was measured according to Bormann and Melzig (2000). Briefly, fifty microliters of SAAP-AMC (400 μM) and 400 microliters of Tris-HCL (pH 7.4) were added to the intact cell layer and incubated at 37°C. An hour later, the reaction was stopped by adding 50 microliters of phosphoramidon solution (50 μM). Subsequently, 400 microliters of the incubation mixture from each well was transferred to an eppendorf tube. Twenty microliters of APN solution (25 ng) was added, and the reaction mixture was incubated again for an hour at 56°C. The reaction was terminated by the addition of 800 microliters of acetone. Then the fluorescence of the released AMC was measured at λexcit = 367 nm and λemiss = 440 nm. A calibration curve with AMC was used to calculate the enzyme activity.

*Cell proliferation*

Cell proliferation was determined according to Bormann and Melzig (2000). Briefly, after enzyme assay determination, cells were washed with buffered saline, and then 400 μl of water were added. Plates were frozen twice, 100 μl from each well were transferred to an eppendorf tube, and 640 μl of phosphate-saline buffer and 60 μl Hoechst 33258 (10 μg/ml water) were added. Then, fluorescence was measured at λexcit = 356nm and λemiss = 458nm. A calibration curve with Herring Testes DNA (Sigma) was used to calculate the DNA amount per well.

*Western blot analysis*

Protein was extracted from treated cells with RIPA buffer and quantified by the BCA method. For western analysis, 50μg of protein were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were immediately blocked with 5% non-fat dry milk in PBS buffer for 1 hour, and then incubated for 2 hours at room temperature in primary antibody (1:1000). After washing, the membranes were incubated with peroxidase-labeled secondary antibody (1:1000) for 1 hour. Immunoreactive bands were visualized by ECL detection reagents. β-tubulin was used to normalize the quantity of the protein on the blot. At least 3 independent western blots were performed.

*RT-PCR*

Total RNA was isolated from the treated cells by TRIzol reagent according to the manufacturer's instruction. Then 1 μg total RNA was reverse transcribed with M-MuLV reverse transcriptase in the presence of a random primer (9-mer). The resulting cDNA preparation was subjected to PCR amplification in 20 μl total volume . The primers for NEP were: F: 5' AAGCCAAAGAAGAAAACAG 3' R: 5' CAGTGCCAACAAACAAAT 3' , and for housekeeping gene ß-actin: F: 5' ACCAACTGGGACGACAT 3', R: 5' CGCTCGGTGAGGATCTTCAT 3' (F: forward R: reverse). PCR was performed under a first initial denaturation of 94°C for 5 min, followed by 94°C denaturation for 30 sec, 53°C primer-annealing for 30 sec, 72°C extension for 45 sec, and 28 cycles later, 72°C extension for 10 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide and photographed under UV light. At least 3 independent RT-PCRs were performed.

**Table 1.** Influence of ginsenoside Rb1 on Neprilysin enzyme activity and cell proliferation

| Concentration (μmol/L) | NEP activity (%) | Cell proliferation (%) |
|---|---|---|
| Control | 100±4.2 | 100±2.4 |
| 20 | 120±7.9** | 99±5.1 |
| 50 | 127±3.5** | 95±4.1* |
| 100 | 135±2.3** | 94±2.4** |
| 200 | 129±5.8** | 91±5.2** |

Results are means ± SD, n=8, $^{*}p<0.05$, $^{**}p<0.01$. Asterisks indicate statistically-significant result in comparison to control value.

**Table 2.** Influence of ginsenoside Rg1 and Rb1 on Neprilysin enzyme activity and cell proliferation

| Concentration (μmol/L) | NEP activity (%) | Cell proliferation (%) |
|---|---|---|
| Control | 100±4.4 | 100±3.0 |
| 100 Rg1 | 106±3.4** | 95±6.1* |
| 100 Rb1 | 135±1.0** | 95±2.6* |
| 100 Rg1 + 100 Rb1 | 133±1.9** | 88±5.6** |

Results are means ± SD, n=8, $^{*}p<0.05$, $^{**}p<0.01$. Asterisks indicate statistically-significant result in comparison to control value.

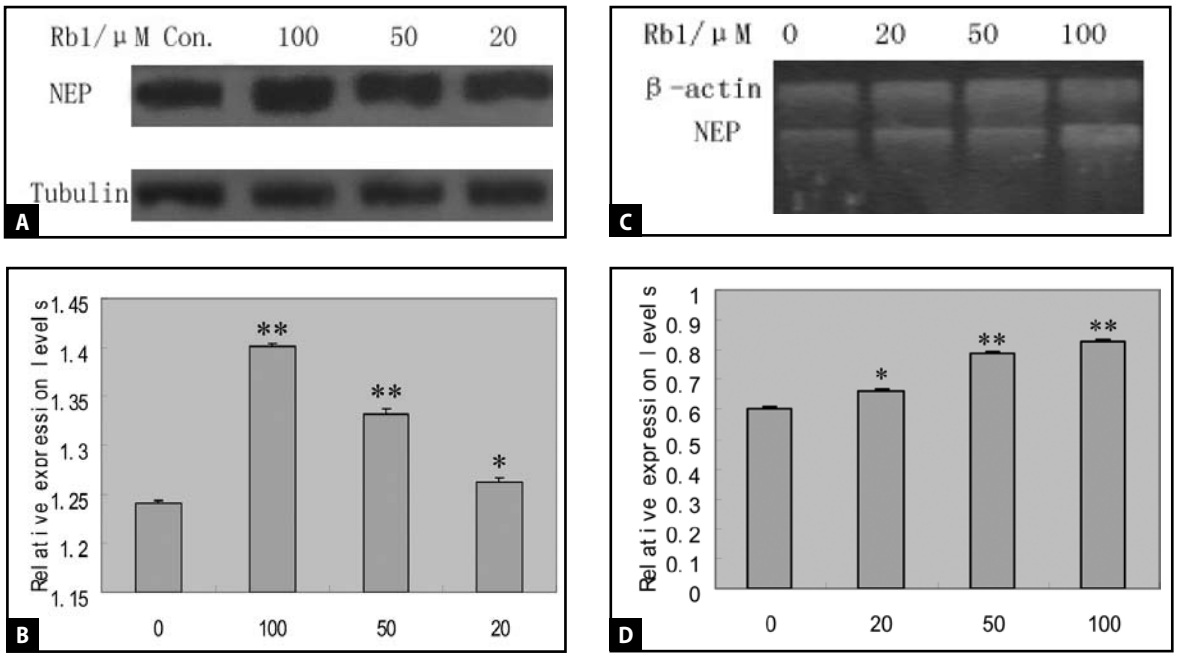


**Figure 1.** Ginsenoside Rb1 increased the expression of the neprilysin gene. (**A**) Western blot analysis for the neprilysin gene; β-tubulin was used as the internal control for protein loading and transfer efficiency. (**B**) Densitometric measurements for neprilysin expression levels normalized to internal control, respectively, and expressed as a relative number. (**C**) RT-PCR analysis for the neprilysin gene; β-actin was used as an internal control for loading. (**D**) Densitometric measurements for neprilysin mRNA expression levels normalized to internal control, respectively, and expressed as a relative number. This result was representative of three independent experiments. Each group contains 3 samples. * means $p<0.05$ when compared with control group, and ** means $p<0.01$ when compared with control group.

## STATISTICAL ANALYSIS

All data were expressed as the mean ± standard deviation (S.D.). Data were analysed by one-way analysis of variance (ANOVA) followed by Post Hoc Tests using SPSS for windows, differences with a value of $p<0.05$ were considered statistically significant.

## RESULTS

*Effects of Rb1, Rg1 on Neprilysin activity*

In the present study, we aimed to detect the effects of ginsenosides Rb1 and Rg1 on NEP activity. SK-N-SH cells, from a human neuroblastoma cell line with intact ecto-neuropeptidases, are widely used as a neuron model. So we detected the effects of ginsenosides on the NEP activity of SK-N-SH cells. As shown in Table 1, long-term (72 hours) treatment of SK-N-SH cells with Rb1 resulted in a dose-dependent enhancement of NEP activity, 50 μM ginsenoside Rb1 increased NEP activity significantly, 100 μM Rb1 increased NEP activity about 1.35 fold, but Rg1 did not show such an effect (Table 2). Moreover, simultaneous application of Rb1 and Rg1 did not show any increasing activity as compared to application of Rb1 alone. These results suggest that ginsenoside Rb1, a major pharmacologically active ingredient of ginseng, could up-regulate NEP activity.

*Effect of Rb1, Rg1 on Neprilysin gene expression*

To determine whether the effect of ginsenoside Rb1 on NEP activity might be due to the upregulation of NEP expression, Western blotting was performed. We treat-

ed SK-N-SH cells with different concentrations of Rb1 for 72 hours, then prepared the protein extracts. The results from Figure 1A show that the expression of NEP increased significantly. To further demonstrate the increased effect of ginsenoside Rb1 on the steady levels of NEP mRNA, RT-PCR was used. Figure 1C demonstrates that NEP mRNA expression was significantly increased by Rb1 in a dose-dependent manner, which was consistent with the effect of Rb1 on NEP protein expression. Therefore, we may conclude that the increased effect of Rb1 on NEP activity is related to the upregulation of NEP expression, which mainly occurs at the transcriptional level.

## DISCUSSION

NEP is believed to be the key enzyme in Aβ degradation in the human brain. Therefore, NEP activity could be a potential target for AD therapy. In this manuscript, we report a dose-dependent effect of ginsenoside Rb1 on NEP activity. Furthermore, our studies show that this compound increases NEP activity by upregulating NEP gene expression, especially on a transcriptional level.

Previous studies indicated that ginseng can enhance learning ability, memory, and decrease neurodegeneration in rodents (Bao *et al*. 2005; Kim *et al*. 1998; Koo *et al*. 2004; Lian *et al*. 2005; Nishijo *et al*. 2004; Petkov *et al*. 2003; Tohda *et al*. 2004; Tohda *et al*. 2005), as well as have a positive influence on memory in humans (Chen *et al*. 2004). However, few studies were performed to examine the influence of ginseng and its derivatives in AD, and fewer studies were performed to examine the effect of this natural product on NEP activity. In humans, the NEP gene is located on chromosome 3q21-27 and contains 24 exons (Barker *et al*. 1989; Roques *et al*. 1995). NEP is widely expressed in many normal tissues including the brush-border of intestinal and kidney epithelial cells, neutrophils, thymocytes, and lung, prostate, testes, and brain cells (Akiyama *et al*.2001; Connelly *et al*. 1985; Erdos & Skidgel, 1988; Erdos & Skidgel, 1989; Pierart *et al*. 1988). There have been many studies which show that neprilysin-deficient mice show a significant dose-dependent increase in cerebral Aβ levels (Iwata *et al*. 2001; Marr *et al*. 2004); over-expression of neprilysin leads to a reduction in Aβ levels in a dose-dependent manner (Hama *et al*. 2001; Iwata *et al*. 2004; Marr *et al*. 2004); infusion of neprilysin inhibitors into the brains of APP (Amyloid Precursor Protein) transgenic mice elevated Aβ levels in the brain (Iwata *et al*. 2001).

Up to now, compounds have been reported which are capable of increasing NEP activity (Kiss *et al*. 2006; Melzig & Ianka, 2003). However, ginseng and its isolated components represent a unique opportunity given that these agents have already been used on humans for thousands of years with little reported toxicity. In this study, ginsenosides were shown to have a slight inhibitive effect on SK-N-SH cell proliferation. This function has been detected in other studies (Kiss *et al*. 2006; Melzig & Ianka, 2003; Uehara *et al*. 2001). Uehara (2001) and Melzig etc (2003) thought the inhibition of cell proliferation was associated with the increase in cellular enzyme activity of NEP, which enhances cell differentiation, but not the result of a cytotoxic effect. So, our study, coupled with the positive results on learning and memory, strongly support the application of ginsenosides (especially Rb1) on AD patients in clinical trials. Moreover, our studies also offer a biochemical explanation of the effect of ginsenosides on NEP activity. We found that ginsenosides can upregulate NEP gene expression on a transcriptional level, but the underlying mechanism needs further study.

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