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Ginsenoside Rd enhances glutathione levels in H4IIE cells via NF-κB-dependent γ-glutamylcysteine ligase induction

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Panax ginseng is widely used as herbal medicine in East Asia and the pharmacological effects of *P. ginseng* against certain chronic diseases might be explained by its antioxidative effects. Here, we show that ginsenoside Rd significantly increases both cellular glutathione (GSH) contents and the protein level of γ-glutamylcysteine ligase (γ-GCL) heavy chain in H4IIE cells (a rat hepatocyte cell line). Subcellular fractionation and Western blot analysis revealed that ginsenoside Rd increased the nuclear level of p65, but not of Nrf2. Moreover, ginsenoside Rd increased luciferase reporter gene activity in cells transfected with nuclear factor-κB (NF-κB) binding site-containing −1088 bp γ-GCL promoter. However, ginsenoside Rd-inducible reporter activity was abolished when cells were transfected with NF-κB deletion mutant. These effects of ginsenoside Rd are suggested to underlie the putative anti-oxidative effect of *Panax ginseng*.

1. Introduction

Panax ginseng has been used as an herbal medicine to enhance stamina and the ability to deal with fatigue and physical stress in Asia for thousands of years. A large number of studies have described the pharmacological effects of *P. ginseng* on certain chronic disease states and on aging (Cheng et al. 2005). Moreover, these effects have been suggested to be due to enhanced immune function and antioxidant capacity (Lee et al. 2005; Cheng et al. 2005). Ginsenosides, which are considered to be the biologically active components of *P. ginseng*, are a mixture of triterpene glycosides, and the major components can be classified based on the presence of protopanaxadiol or protopanaxatriol moieties (Shibata et al. 1966), i.e., ginsenosides Rb1, Rc, Rd and Rg3 are protopanaxadiols, whereas ginsenoside Rg1 and Re are protopanaxatriols. In a series of previous studies, we found that ginsenosides from *P. ginseng* reduce blood pressure by inducing the production of endothelium-derived nitric oxide (Kim et al., 1999) and by increasing the expressions of inducible nitric oxide synthase in aortic smooth muscle and macrophages (Kim et al. 2003).

Although no unequivocal evidence demonstrates the anticancer effect of ginsenosides, various research groups have suggested that ginsenosides have tumor-inhibitory actions, especially during the carcinogenesis and cancer progression phases (Helms, 2004). Moreover, the prolonged administration of red ginseng extract was found to significantly inhibit the prevalences of hepatoma and pulmonary tumor induced by diethylnitrosamine or urethane (Wu et al. 2001; Yun et al. 1987), and in case-controlled clinical studies, the odds ratios of a number of tumor types, including those of the lung, stomach and liver, were found to be significantly reduced by ginseng administration (Yun 2003). Moreover, the relative risks of gastric and lung cancer were found to be reduced in ginseng users (Yun 2003).

Cancer chemoprevention may be defined as the use of naturally occurring compounds to prevent or reverse the carcinogenic process, and a recent report concluded that standardized *P. ginseng* extracts have chemopreventive and anti-mutagenic effects on papilloma formation in Swiss albino mice (Panwar et al. 2005). Many plant derived compounds can scavenge reactive oxygen species (ROS), and thereby directly reduce oxidative stress *in vivo*, and in addition to this, a direct scavenging effect many are also enhance endogenous defense systems. Several chemopreventives derived from plants exert their effects by inducing cellular detoxifying enzymes, such as, γ-glutamylcysteine ligase (γ-GCL) and glutathione S-transferases (GST). Glutathione (GSH) is a non-protein sulfhydryl containing species, which is present in all cell types. It can react with a variety of ROS types, and thereby control cellular redox status (Hayes and Strange 1995). Moreover, GSH-conjugation with xenobiotics can be accelerated by the phase II detoxifying enzyme, GST, and both γ-GCL and GST can be induced by phytochemicals like dithiolethione (Kang et al. 2003) and diallylsulfide (Wu et al. 2001).

In the present study, we found that ginsenoside Rd increases cellular GSH levels by inducing the γ-GCL gene in H4IIE cells. Furthermore, we monitored the activations of the NF-E2 related factor2 (Nrf2)/antioxidant response element (ARE) and NF-κB pathways in order to investigate the mechanistic basis of γ-GCL induction by ginsenoside Rd.

2. Investigations, results and discussion

GSH serves as an effective oxygen radical scavenger via its recycling oxidized antioxidants (Anderson 1998). Moreover, the conjugation of carcinogens to GSH reduces their toxicities and carcinogenic abilities. In order to estimate whether ginsenosides affect intracellular GSH levels, H4IIE cells (a rat hepatocyte cell line) were treated with ginsenoside Rd, Re, or Rg3 (10 μg/ml) and cellular GSH levels were then determined. H4IIE cells exposed to ginsenoside Rd for 24 h showed significant GSH upregulation versus DMSO-treated controls (Fig. 1A). In contrast, ginsenoside Re or Rg3 did not affect GSH levels (Fig. 1A).

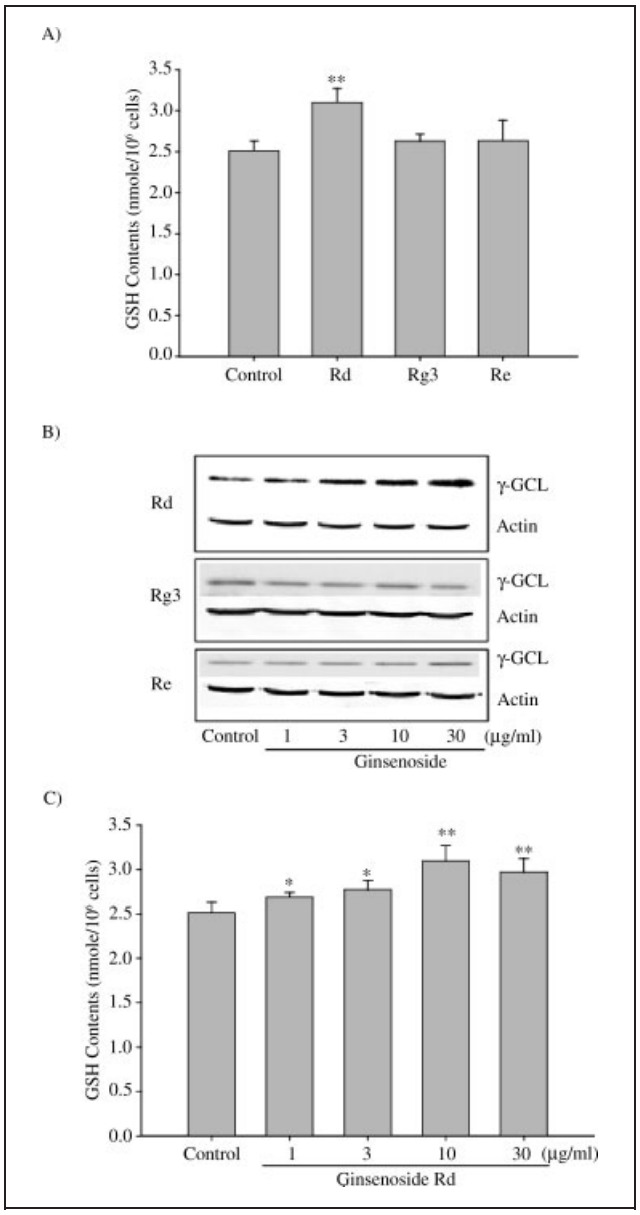

Fig. 1: A) Effects of ginsenoside Rd, Rg3 and Re on the cellular GSH contents in H4IIE cells. Cells were serum-starved and incubated in the presence or absence of each ginsenoside (10 μg/ml) for 24 h, and cell lysates were used to determine GSH contents. Data represent the means ± SD of 4 different samples (significant as compared to the untreated control, ** $p < 0.01$). B) Induction of γ-glutamylcysteine ligase (γ-GCL) heavy chain by ginsenoside Rd. Cells were serum-starved and incubated in the presence or absence of each ginsenoside (1–30 μg/ml) for 18 h, and cell lysates were used to perform Western blot analyses. C) Concentration-dependent effects of ginsenoside Rd on the cellular GSH contents in H4IIE cells. Cells were serum-starved and incubated with or without ginsenoside Rd (1–30 μg/ml) for 24 h, and cell lysates were used to determine GSH contents. Data represent the means ± SD of 4 different samples (significant as compared to the untreated control, * $p < 0.05$; ** $p < 0.01$)

The two-step biosynthesis of intracellular GSH is catalyzed by γ-GCL and GSH synthetase, and the efficiency of GSH production is mainly dependent on the activity of γ-GCL (Anderson 1998). To determine whether ginsenoside Rd exerts its GSH upregulating effect via γ-GCL gene induction, heavy chain γ-GCL levels were monitored by Western blotting using a specific antibody. Treatment of H4IIE cells with ginsenoside Rd (1–30 μg/ml) for 18 h upregulated γ-GCL protein, although this induction ratio is marginal compared to other chemical inducers (i.e., *tert*-butylhydroquinone) and appeared saturated at >10 μg/ml; actin levels were comparable in all the samples (Fig. 1B). However, ginsenoside Re or Rg3 did not increase γ-GCL protein levels (Fig. 1B). Thus, the selective upregulation of GSH by ginsenoside Rd appeared to be due to γ-GCL induction. Cellular GSH levels were also measured after exposing H4IIE cells to ginsenoside Rd at 1–30 μg/ml, and the pattern of GSH increase induced by ginsenoside Rd was found to closely resemble that observed by Western blotting (Fig. 1C).

The expression of *γ-GCL* gene by cancer chemopreventive agents depends on ARE sequences in the distal and proximal parts of γ-GCL promoter (Myhrstad et al. 2001). Given the roles of ARE in γ-GCL expression, we performed reporter gene analyses using a pGL-797 reporter plasmid, which contained the GSTA2-ARE but not the XRE/CEBP binding region (Kang et al. 2003). However, ginsenoside Rd at 1 or 10 μg/ml was not found to evoke a significant increase in luciferase activity (Fig. 2A).

Nrf2 is a key transcription factor that binds to ARE sequences, and is implicated in the regulation of γ-GCL ex-

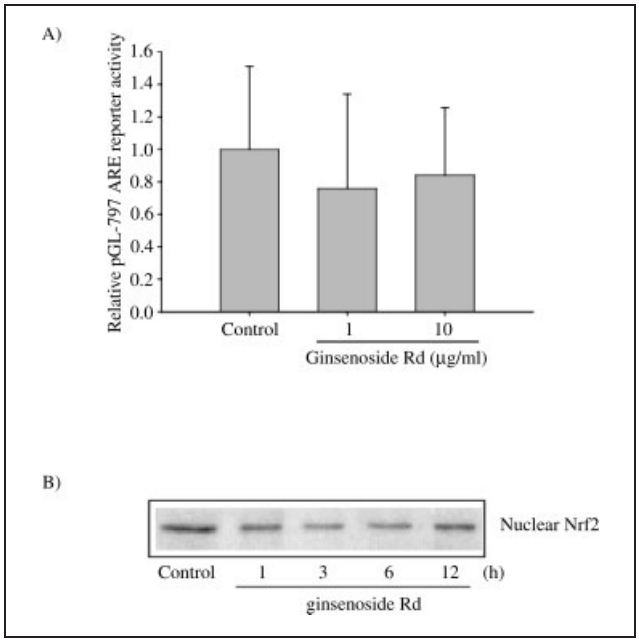

Fig. 2: A) Effect of ginsenoside Rd on the ARE-reporter activity. H4IIE cells were transiently transfected with the GSTA2 chimeric gene construct pGL-797 containing the ARE element. Dual luciferase reporter assays were performed on the lysed cells co-transfected with pGL-797 (firefly luciferase) and pRL-SV (*Renilla* luciferase)(in a ratio of 100 : 1) after exposure to ginsenoside Rd (1 or 10 μg/ml) for 18 h. Reporter gene activation was calculated relative to *Renilla* luciferase activity. Data represented the means ± SD of 4 different samples. B) Effect of ginsenoside Rd on the nuclear translocation of Nrf2. The subcellular localization of Nrf2 was immunochemically assessed in cells treated with ginsenoside Rd (10 μg/ml) for 0–12 h

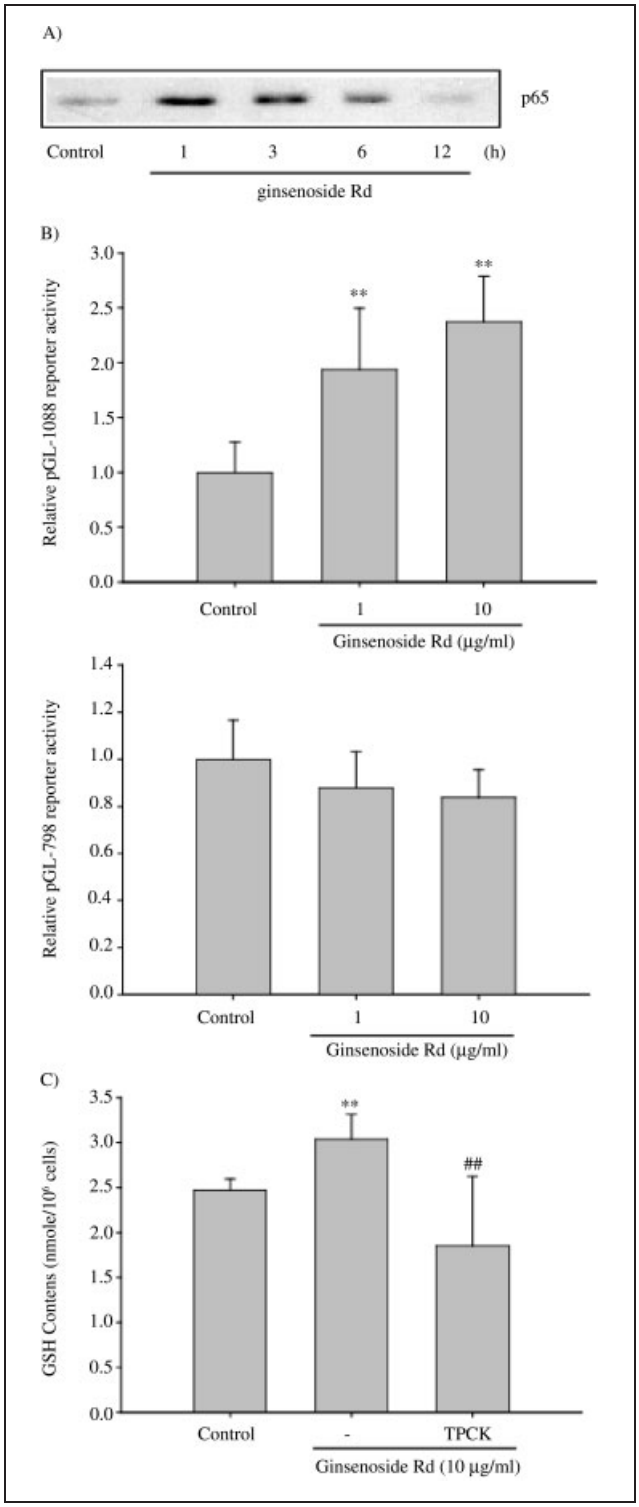

Fig. 3: A) Effect of ginsenoside Rd on the nuclear translocation of p65. The nuclear localization of Nrf2 was immunochemically assessed in cells treated with ginsenoside Rd (10 μg/ml) for 0–12 h. B) Induction of luciferase activity ginsenoside Rd in H4IIE cells transiently transfected with the heavy chain of γ-GCL chimeric gene constructs; pGL-1088 (first panel) containing a functionally active NF-κB element and pGL-798 NF-κB deletion mutant (Right panel). Dual luciferase reporter assays were performed as described in the panel (A) of Fig. 2. Data represented the means ± SD of 4 or 5 different samples. C) Effect of N-alpha-tosyl-L-phenylalanine chloromethyl ketone (TPCK) on the ginsenoside Rd-inducible GSH increases in H4IIE cells. Cells were serum-starved and preincubated in the presence or absence of TPCK (50 μM) for 30 min, and incubated with or without ginsenoside Rd (10 μg/ml) for 24 h. The cell lysates were used to determine GSH contents. Data represent the means ± SD of 6 different samples (significant as compared to the untreated control, ** $p < 0.01$)

pression (Chan and Kwong 2000). Thus, we examined whether ginsenoside Rd stimulates the translocation of Nrf2 into the nucleus, since the nuclear translocation of Nrf2 is essentially required for ARE activation (Huang et al. 2000; Kang et al. 2002). Subcellular fractionation and Western blot analyses showed that ginsenoside Rd did not affect the level of nuclear Nrf2 (Fig. 2A), which suggests that γ-GCL induction by ginsenoside Rd is not associated with Nrf2-mediated ARE activation.

We then tried to clarify the potential role of NF-κB activation in γ-GCL induction by ginsenoside Rd, since human and rat heavy chain γ-GCL promoter also possess a putative NF-κB binding site, which is also known to be required for the transactivation of the *γ-GCL* gene in response proinflammatory cytokine or ionizing radiation challenge (Kondo et al. 1999; Iwanaga et al. 1998; Yang et al. 2005). Because the nuclear translocation of p65 is a key event during NF-κB activation, we examined nuclear p65 levels to assess ginsenoside Rd-mediated NF-κB activation. It was found that nuclear p65 levels were increased by ginsenoside Rd at 10 μg/ml after 1 h to 6 h (Fig. 2B). A promoter deletion study was then performed to clarify the role of NF-κB activation during *γ-GCL* heavy chain transactivation by ginsenoside Rd. Functional NF-κB and AP-1 sites are present in p1088-GCL-Luc reporter plasmid, which also contains the luciferase structural gene and a −1.1 kb human heavy chain of γ-GCL promoter (Morales et al. 1997). Exposure of H4IIE cells, transiently transfected with p1088-GCL-Luc, to ginsenoside Rd (10 μg/ml) resulted in a 2.3-fold increase in luciferase activity (Fig. 3B, first panel). In p798-GCL-Luc plasmid, the NF-κB binding site (from −1052 bp), but not the AP-1 site (from −275 bp), is deleted from the promoter sequence (Morales et al., 1997), and p798-GCL-Luc reporter activity was not altered by ginsenoside Rd (10 μg/ml) treatment (Fig. 3B, second panel), which strongly suggests that NF-κB activation plays a key role in the GSH upregulation observed in ginsenoside Rd-treated H4IIE cells.

Given the roles of NF-κB in the induction of γ-GCL by ginsenoside Rd, we further examined the effect of *N*-alpha-tosyl-L-phenylalanine chloromethyl ketone (TPCK; a specific NF-κB inhibitor) on GSH production. As shown in Fig. 3C, ginsenoside Rd-inducible GSH production was completely blocked in H4IIE cells pretreated with TPCK (50 μM) (Fig. 3C). These findings provide evidence that NF-κB plays an essential role in the induction of γ-GCL and in subsequent GSH synthesis in ginsenoside Rd-treated hepatocytes.

3. Experimental

Ginsenoside Rd, Re, and Rg3 were obtained from Dr. JH Park at the College of Pharmacy, Seoul National University. Anti-heavy chain of γ-GCL antibody was supplied by Neomarkers (Westinghouse, CA), and anti-Nrf2 and anti-p65 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). H4IIE cells were from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cellular GSH levels were quantified using a commercially available GSH determination kit (Oxis International, Portland, OR). Total cell lysates and nuclear fraction isolations, immunoblot analysis, and reporter gene assays were performed as described in our previous report (Kang et al. 2003). One way analysis of variance (ANOVA) was used to assess significant differences between the different treatment groups. Statistical significance was set at either the $p < 0.05$ or < 0.01 level as indicated.

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