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# Ginsenoside Rc and Re Stimulate c-Fos Expression in MCF-7 Human Breast Carcinoma Cells

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We have found that ginsenoside Rc and Re induce c-fos in MCF-7 human breast carcinoma cells at both the mRNA and protein levels. However, neither ginsenoside activated the expression of reporter gene under the control of AP-1/TPA response elements. We have also examined the possibility that ginsenoside Rc and Re act by binding to intracellular steroid hormone receptors that act as transcriptional factors in the nucleus in inducing c-fos mRNA in MCF7 human breast carcinoma cells. However, ginsenoside Rc and Re did not bind to glucocorticoid, androgen, estrogen, or retinoic acid receptors as examined by the transcription activation of the luciferase reporter genes in CV-1 cells that were transiently transfected with the corresponding steroid hormone receptors and hormone responsive luciferase reporter plasmids. These data demonstrate that ginsenoside Rc and Re act via other transcription factors and not via estrogen receptor in c-Fos expression.

**Key words:** Ginseng, Ginsenoside Rc, Ginsenoside Re, c-Fos, Estrogen receptor

## INTRODUCTION

Ginseng is a popular herbal medicine that has been used for over 2,000 years in oriental countries. It has been reported that ginseng has a wide range of pharmacological activities in cardiovascular, endocrine, immune, and central nervous systems (Bhattaacharya *et al.*, 1991). Ginseng and its constituents have been used for their tonic, immunomodulatory adaptogenic, anticancer, and anti-aging activities (Gillis *et al.*, 1997). These beneficial effects of ginseng have prompted tremendous effort to discover the pharmacology of its action through biochemical and molecular biology techniques.

A number of components have been isolated and characterized from ginseng including ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids (Liu *et al.*, 2000). However, how each of these components contributes to the pharmacological properties of ginseng is not very well known. Among these ingredients, a group of saponin glycosides known as ginsenosides, comprising a four trans-ring rigid steroid skeleton with a modified side chain at C-20, have received considerable attention because of their biological activity (Nah *et al.*, 1995).

Ginsenosides could exercise their activities at either the cellular membrane, inside the cell or even in the nucleus (Attele *et al.*, 1999). Ginsenosides may initiate their mode of action by binding to transmembrane membrane receptors and subsequently activating the associated downstream signaling pathways. It is also possible that the hydrophobic properties of ginsenosides favor their binding to intracellular steroid hormone receptors, such as glucocorticoid receptor (GR), progesterone receptor, androgen receptor (AR), mineralocorticoid receptor, and estrogen receptor (ER) to control gene transcription by binding to specific intracellular receptors. Ginseng has been recommended for the alleviation of the symptoms of menopause, indicating that some components of ginseng act as phytoestrogens (Amato *et al.*, 2002) and/or involve activation of the ER. Ginseng extracts are able to stimulate the growth of ER-positive cells (Duda *et al.*, 1999). In estrogen-positive cells, estrogen is known to stimulate cell proliferation through binding to the estrogen receptor followed by activation of protooncogene expression such as c-fos, c-jun, and c-myc. c-fos is rapidly

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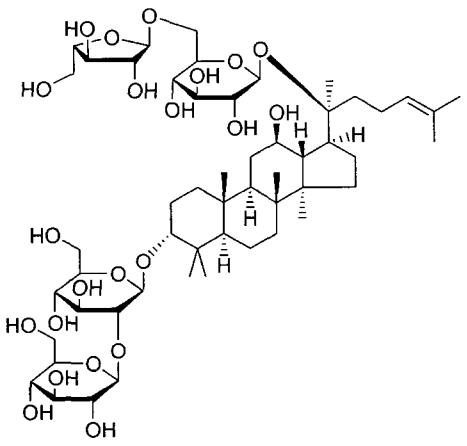
induced in response to diverse extracellular stimuli including various mitogens and the steroid hormones at the transcription level, which is modulated by several nuclear proteins through binding to the corresponding cis-elements located in the gene (Duan *et al.*, 2001; Lee *et al.*, 2001). Among them, estrogen receptor is one of the known regulators of expression of c-fos. We have found that ginsenoside Rc and Re increased the expression of c-fos both at mRNA and protein levels in MCF-7 cells at 24 h of treatment. However, this expression was not mediated by ER or sufficient to activate AP-1 pathway.

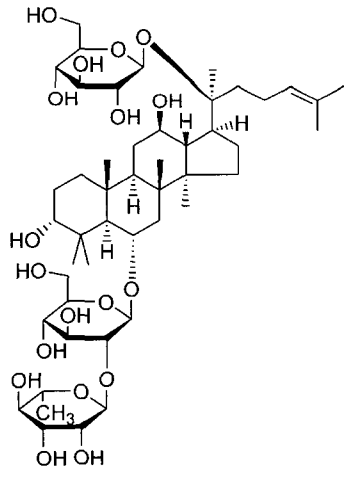
## MATERIALS AND METHODS

### Reagents

Ginsenoside Rc and Re (Fig. 1) were provided by Korea Ginseng and Tobacco Research Institute (Daejeon, Korea). 17β-estradiol (E2), dexamethasone (Dexa), all-trans retinoic acid (ATRA), testosterone (Tes), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma.



Ginsenoside - $R_c$



Ginsenoside - $R_e$

**Fig. 1.** Chemical structures of ginsenoside Rc and Re

Ginsenoside Rc and Re were dissolved in 80% ethanol at a concentration of 15 mg/ml. Dexa, E2, and Tes were dissolved in 100% ethanol, and ATRA in dimethylsulfoxide.

### Plasmids

ERE2-tk81-luc constructed by inserting the fragment of the herpes simplex thymidine kinase promoter and two copies of the vitellogenin ERE into pA3luc (Gehm *et al.*, 1997), ARE4-luc, and $(\beta_2RARE)_2$-luc were kind gifts of Larry Jameson, Chawnshawn Chang, and Pierre Chambon, respectively. Expression vectors for GR, AR, and retinoic acid receptor (RAR) were from Ron Evans, Chawnshawn Chang, and Pierre Chambon, respectively.

### Cell cultures

ER-positive human breast adenocarcinoma, MCF-7 cells were purchased from the Korea Cell Line Bank. MCF-7 cells were maintained in phenol red-free Dulbeccos modified Eagles medium (DMEM) containing 1×antibiotic/antimycotic mix, 5 mM *N*-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulfonic acid, and 0.37% sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere of 95% air/5% $CO_2$ and fed every 3-4 days. Before hormone induction, the cells were washed with phosphate-buffered saline (PBS) and cultured in DMEM/10% charcoal-dextran stripped FBS (CD-FBS) for 2 days to eliminate any hormonal source before treatment. All treatments were done with DMEM/10%/CD-FBS. Untreated samples served as controls.

### Transient transfection and reporter gene assays

Cells were seeded in 24-well plates at a density of $7\times10^4$ cells/well. After 24 h, plasmids were transiently transfected with calcium phosphate-DNA coprecipitation method. A total of 0.5 μg of DNA in 25 μl of $CaCl_2 \cdot H_2O$ (250 mM $CaCl_2$) was mixed with 25 μl of 2×HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, 50 mM HEPES) with constant bubbling and within 5 to 10 min this solution was added to each well. The next day, transfected cells were washed with PBS, and treated with compounds. Luciferase activity was determined 24 or 48 h after transfection by using an AutoLumaat LB953 luminometer and expressed as relative light units. The levels of CAT were measured by commercially available ELISA kit (Boehringer Mannheim, Switzerland). Compared to the classical radioisotope-based CAT assay, the CAT ELISA provides more accurate measurements for the actual amount of CAT protein synthesized. The mean and standard errors of triplicate or quadruplicate samples are shown for representative experiments.

### RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

MCF-7 cells were grown in 6-well plates in phenol red-

free DMEM containing 10% CD-FBS. Near confluent monolayers were treated with or without compounds for 24 h. The cells were rinsed in PBS and total RNA was isolated by lysing the cells in guanidinium isothiocyanate using the TRIzol reagent (Gibco BRL) according to the manufacturers instructions. After extraction, RNA was precipitated by recommended procedures and dissolved in diethylpyrocarbonate-treated water. To synthesize first strand cDNA, 5 μl total RNA was incubated in 0.5 μg of oligo(dT)$_{18}$ primer (Gibco BRL) and 5 μl deionized water at 70°C for 5 min. And reverse transcription reactions were performed using 40 units of M-MuLV reverse transcriptase (Promega) in 5× reaction buffer (250 mM Tris-HCl; pH 8.3 at 25°C, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) and 1 mM dNTP mixtures at 37°C for 60 min. The reaction was terminated by heating at 70°C for 10 min, followed by cooling at 4°C. The resulting cDNA was added to the PCR reaction mixture containing 10× PCR buffer (100 mM Tris-HCl; pH 8.3, 500 mM KC, 15 mM $MgCl_2$), 25 units of rTaq polymerase (TaKaRa, Japan) 4 μl of 2.5 mM dNTP mixtures, and 100 pmole of primers each. The final volume was 50 μl. The sequences of c-fos primers were 5'-TCCCAGAGGAGATGTCTGTG-3' and 5'-GGCTCCAGCTCTGTGACCAT-3' (Mercier *et al.*, 200) and those of human b-actin were 5'-CCTGACCCT-GAAGTACCCCA-3' and 5'-CGTCATGCAGCTCATAGCTC-3' (Ren *et al.*, 1997). The PCR-product for c-fos is 330 bp and 550 bp for β-actin. The reactions were initiated by 3 min of denaturation at 94°C followed by amplification at 94°C for 45 s, and 55°C for 45 s, and 72°C for 45 s; 30 cycles for c-fos, and 20 cycles for β-actin. The PCR reaction was ended by elongation at 72°C for 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified using a bio-imaging analyzer (Bio-Rad), and band-intensity was normalized to the intensity of β-actin mRNA.

### Immunoblotting

Protein was isolated by using radioimmune precipitation buffer (containing 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS) with protease inhibitor cocktail (Sigma) on ice for 1 h and then centrifuged for 20 min at 13,000 g. Supernatant was collected and protein concentrations were measured using the Bradford method (Bio-Rad). Twenty microgram of protein was dissolved in sample buffer and boiled for 5 min prior to loading onto an 8% acrylamide gel. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in Tris-buffered saline (TBS), and incubated with with rabbit anti-monoclonal antibody to c-fos (Santa Cruz, U.S.A.) for 1 h at 1:1000. After washing with TBS/0.1% Tween, blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence ECL kits (Amersham).

## RESULTS

### Effect of ginsenoside Rc and Re on c-fos mRNA expression in MCF-7 cells

To evaluate the potential of ginsenoside Rc and Re activating the expression of a protooncogene, we examined c-fos mRNA induction in MCF-7 cells. Total RNA prepared from cells treated with the compounds was analyzed for steady-state c-fos mRNA levels using RT-PCR assays.

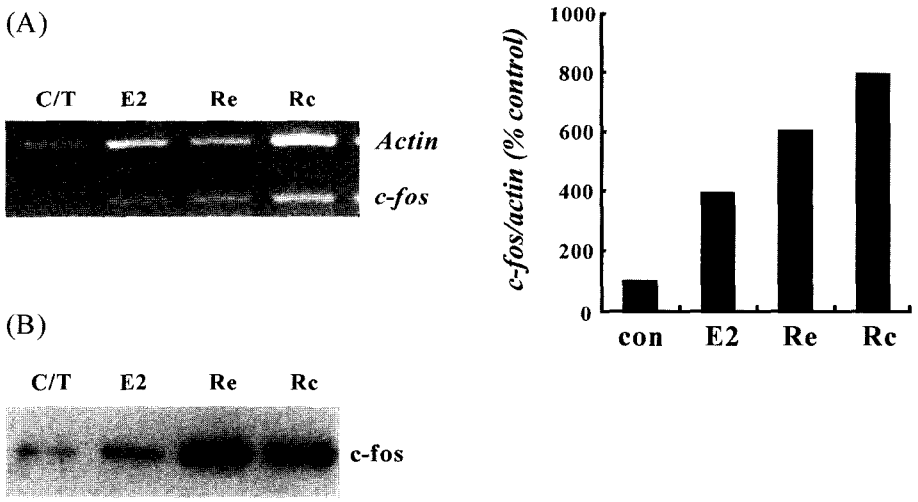



**Fig. 2** Ginsenoside-Rc activates the expression of c-fos on the mRNA level in MCF-7 cells. (A) Total RNA prepared from each treatment was analyzed for steady-state c-fos mRNA levels using RT-PCR assays. As an internal control, constitutively expressed human β-actin mRNA was used. MCF-7 cells were treated with 45 μg/ml ginsenoside-Rc, Re, or 10 nM E2. A representative pattern is shown from two independent experiments. The quantified results expressed as percentage-changes to the control value is shown left to the PCR results. (B) Western blotting of c-fos protein from protein samples treated as above. A representative pattern is shown from two independent experiments.

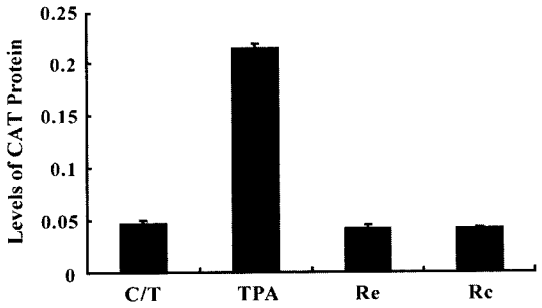



**Fig. 3.** Activation of reporter constructs containing AP-1 binding sites. MCF-7 cells were transfected in 24-well plates with TRE-CAT and treated with 50 nM TPA, 45 μg/ml ginsenoside Rc, or 45 μg/ml ginsenoside Re in phenol-red-free DMEM plus 10% CD-FBS, and assayed for CAT activity after 48 h treatments as described in Materials and Methods. Transfections were performed in triplicate twice. One representative result is shown in this figure. Data are expressed as the mean ± SEM.

As an internal control, constitutively expressed human β-actin mRNA was used. The primers for c-fos and β-actin were designed to span more than one intron. Therefore, these primers can discriminate any signal from contaminating genomic DNA in RNA samples since they span intron junctions. Amplification from genomic DNA would result in large and different size fragments. MCF-7 cells were treated with 45 mg/ml ginsenoside Rc and Re for 24 h resulted in activation of c-fos gene (Fig. 2A). To further verify the RT-PCR results, we have examined c-fos protein levels by Western analysis. Protein levels of c-fos were increased after 24 h of either E2 or ginsenoside-Rb1 treatment as compared with untreated control (Fig. 2B).

## Activation of gene expression from a TPA-response element containing reporter plasmids

Next, we have examined whether ginsenoside Rc and Re treatment of cells could stimulate the activity of AP-1 pathway. AP-1 is composed of dimmers form the Jun and Fos protein families (Duan *et al.*, 2001). MCF-7 cells were transfected with a TRE-CAT reporter plasmid and treated with 50 nM TPA, ginsenoside Rc, or Re. TPA strongly induced TRE-dependent gene expression (Fig. 3). In contrast, ginsenoside Rc and Re failed to increase transcription from the reporter plasmids indicating that c-fos induction was not sufficient to activate AP-1 mediated transactivation.

## Screening of steroid hormone-like activity of ginsenoside Rc and Re

In the presence of a ligand, the ER initiates transcriptional activation by binding to specific estrogen responsive element (ERE) in target genes such as c-fos (Klein-Hitpass *et al.*, 1986). Based on this theory, we studied whether ginsenoside Rc and Re could lead to transcriptional activation of ERE containing reporter plasmid to examine the possibility of ginsenoside Rc and Re acting through ER in c-fos induction. After transient transfection with the reporter plasmid, ERE2-tk81-luc, cells were treated for 48 h with the ligands as indicated in Fig. 4 at the concentration depicted in the figure legends. As shown in Fig. 4A,

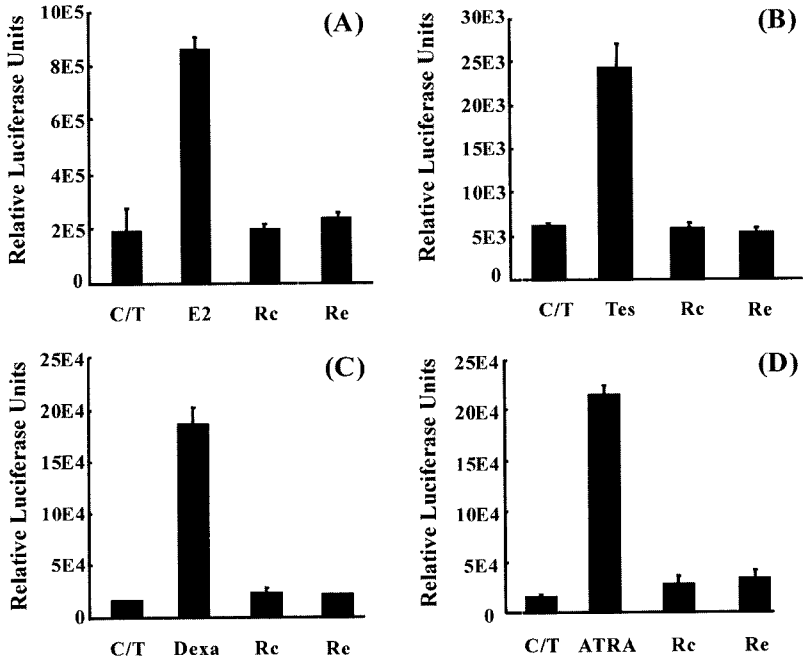



**Fig. 4.** Screening of estrogen, androgen, glucocorticoid, and retinoic acid activity. Cells were transfected in 24-well plates with (A) ERE-luc (B) ARE4-Luc and AR (C) pGRE2-luc and hGR (D) hRAR and $(\beta_2RARE)_2$-luc and treated with 10 nM 17β-estradiol, 100 nM Tes, 10 nM Dexa, 1 μM ATRA, 45 μg/ml ginsenoside Rc, or 45 μg/ml ginsenoside Re in phenol-red-free DMEM plus 10% CD-FBS, and assayed for luciferase activity after 48 h treatments as described in Materials and Methods. Transfections were performed in triplicate more than three times. One representative result is shown in this figure. Data are expressed as the mean ± SEM.

ginsenoside Rc nor Re activate transcription of estrogen-responsive luciferase gene. To examine whether ginsenoside Rc or Re have the activities of glucocorticoid, androgen, and retinoid, we transiently transfected CV-1 cells with steroid receptors and reporter plasmids containing hormone responsive elements. Ten nM Dexa, 1 μM ATRA, or 100 nM Tes were used as a positive control to examine the glucocorticoic, retinoid, or androgen activities of ginsenoside Rc and Re. Ginsenoside Rc nor Re showed activities of androgen (Fig. 4B), glucocorticoid (Fig. 4C), or retinoid (Fig. 4D).

## DISCUSSION

The ginseng root has been used for over 2,000 years, as a treatment for various illnesses and also as a daily supplement (Goldstein, 1975; Liu and Xiao, 1992). It is an herb with many active components, and there is evidences from numerous studies that ginseng have beneficial effects. Recently, there has been a renewed interest in investigating mechanisms of ginseng action. We have observed ginsenoside Rc and Re increased an immediate early gene c-fos at 24 h of treatment. We have examined a possibility that ginsenoside acts by binding to nuclear steroid receptor. As a tool for monitoring the hormone-like activities of ginsenosides, we used the transient transfection with steroid hormone receptors and hormone responsive element containing reporter plasmids, followed by luciferase expression assay. However, ginsenoside Rc nor Re showed androgen, estrogen, glucocorticoid, or retinoid activities. Further study will reveal the molecular mechanism of ginsenoside Rc and Re activating c-fos transcription and will aid the understanding of the cellular effect and pharmacological profiles of ginsenoside Rc and Re.

## ACKNOWLEDGEMENT

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