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Ginsenoside-R_{b1} Acts as a Weak Phytoestrogen in MCF-7 Human Breast Cancer Cells

Young Joo Lee, Young Ran Jin[1], Won Chung Lim, Wan Kyu Park, Jung Yoon Cho, Siyoul Jang[2], and Seung Ki Lee[1]

College of Engineering, Institute of Biotechnology, Department of Bioscience and Biotechnology, Sejong University, Seoul, Korea, [1]College of Pharmacy, Seoul National University, Seoul, Korea, and [2]College of Engineering, Kookmin University, Seoul, Korea

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Ginseng has been recommended to alleviate the menopausal symptoms, which indicates that components of ginseng very likely contain estrogenic activity. We have examined the possibility that a component of *Panax ginseng*, ginsenoside-R_{b1}, acts by binding to estrogen receptor. We have investigated the estrogenic activity of ginsenoside-R_{b1} in a transient transfection system using estrogen-responsive luciferase plasmids in MCF-7 cells. Ginsenoside-R_{b1} activated the transcription of the estrogen-responsive luciferase reporter gene in MCF-7 breast cancer cells at a concentration of 50 μM. Activation was inhibited by the specific estrogen receptor antagonist ICI 182,780, indicating that the estrogenic effect of ginsenoside-R_{b1} is estrogen receptor dependent. Next, we evaluated the ability of ginsenoside-R_{b1} to induce the estrogen-responsive gene c-fos by semi-quantitative RT-PCR assays and Western analyses. Ginsenoside-R_{b1} increased c-fos both at mRNA and protein levels. However, ginsenoside-R_{b1} failed to activate the glucocorticoid receptor, the retinoic acid receptor, or the androgen receptor in CV-1 cells transiently transfected with the corresponding steroid hormone receptors and hormone responsive reporter plasmids. These data support our hypothesis that ginsenoside-R_{b1} acts a weak phytoestrogen, presumably by binding and activating the estrogen receptor.

Key words: Ginseng, Ginsenoside-R_{b1}, Estrogen receptor, Phytoestrogen

INTRODUCTION

Ginseng has been used over 2000 years in Oriental countries to enhance stamina and immune function. It has a wide range of pharmacological activities in cardiovascular, endocrine, immune, and central nervous systems (Bhattaacharya *et al.*, 1991). Among its various uses, ginseng is commonly used to alleviate menopausal symptoms as an alternative therapy even in Western countries (Gilis *et al.*, 1997). Various studies implicated that ginseng contains estrogenic activity supporting such uses. However, no conclusive reports showed that components of ginseng actually possess estrogenic ligands. Among many ingredients from ginseng, a group of saponin glycosides known as ginsenosides (Liu *et al.*, 2000), sugar conjugates of dihydroxyl or trihydroxyl dammarane triterpenes, most likely contains steroid hormone activity (Nah *et al.*, 1995). Among 26 identified ginsenosides, Ginsenoside-R_{b1}, -R_o, -R_{g1}, -R_c, and -R_e are highly abundant. Especially, ginsenosides-Rb1 is composed of 0.37-0.5% (http://www.netnam.vn/ic-asia/english/products/redkogin/redkogind/redkogind.htm) of ginseng and belongs to protopanaxdiol classes (Chan *et al.*, 2002; Scott *et al.*, 2001). It is possible that the hydrophobic properties of ginsenosides favor their binding to intracellular steroid hormone receptors, such as receptors for glucocorticoids, progesterone, androgens, mineralocorticoid, and estrogen. Like steroids, ginsenosides are lipophilic (Park *et al.*, 1982) that enters nucleus by simple diffusion to control gene transcription by binding to specific intracellular receptors. A few studies showed several biological effects resulting from steroid hormone receptor activation (Attele *et al.*, 1999). Lee *et al.* (1997) showed that ginsenoside-R_{g1} is a functional ligand of the glucocorticoid receptor

Correspondence to: Young Joo Lee, Ph. D., Department of Bioscience and Biotechnology, Sejong University, Kwang-Jin-Gu, Seoul 143-747, Korea
E-mail: yjlee@sejong.ac.kr

(GR). Ginsenoside-R_{g1} competitively inhibited the binding of the synthetic glucocorticoid dexamethasone (Dexa) to the GR, although the affinity of ginsenoside-R_{g1} for GR was lower than for Dexa (Chung *et al.*, 1998). Accumulating evidence indicates that some components of ginseng act as phytoestrogens (Amato *et al.*, 2002) and/or involve activation of the estrogen receptor (ER). Ginseng extracts are able to stimulate the growth of ER-positive cells (Duda *et al.* 1999). However, there has been no conclusive scientific data to show that ginseng contains phytoestrogens (Pinkerton *et al.*, 1999).

Our data show that ginsenoside-R_{b1} is an active estrogenic ligand as determined by measuring the transcription activation of estrogen-responsive reporter plasmids and an endogenous estrogen responsive gene. This is the first study showing that ginsenoside-Rb1, a major component of ginseng, elicits estrogen-induced responses *in vitro*.

MATERIALS AND METHODS

Reagents

Ginsenoside-Rb1 (Fig. 1) was provided by the Korea Ginseng and Tobacco Research Institute (Daejeon, Korea). 17β-estradiol (E2), Dexa, all-trans retinoic acid (ATRA), and testosterone (Tes) were purchased from Sigma (St. Louis MO). ICI 182,780 (ICI) was obtained from ZENECA Pharmaceuticals. Ginsenoside-R_{b1} was dissolved in 20% ethanol at a concentration of 3 mg/ml. Dexa, E2, and Tes were dissolved in 100% ethanol and ATRA in dimethylsulfoxide. All the compounds were added to the medium at a 1:10^3 to 10^4 dilution such that the total ethanol concentration was never higher than 0.1%. An untreated group served as a control.

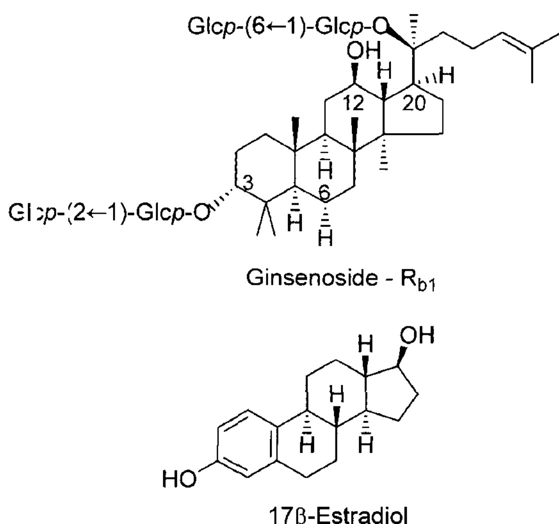

Fig. 1. Structures of ginsenoside-R_{b1} and E2

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), ($(\beta_2RARE)_2$-luc, and ARE4-luc, were kind gifts of Larry Jameson, Pierre Chambon, and Chawnshawn Chang, respectively. Expression vectors for GR, androgen receptor (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 Dulbecco's 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 estrogenic source before treatment. All treatments were done with DMEM/10% CD-FBS. 10 nM E2 was used to maximize the response unless otherwise noted.

Transient transfection and luciferase assays

Cells were seeded in 24-well plates at a density of 7×10^4 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 mean and standard errors of triplicate or quadruplicate samples are shown for representative experiments. All transfection experiments were repeated three or more times with similar results.

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 ginsenoside-Rb1 for 24 h. The wells were rinsed in PBS and total RNA was isolated by lysing the cells in guanidinium isothiocyanate

using the TRIzol reagent (Gibco, Grand Island, NY) according to the manufacturer's 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, Grand Island, NY) and 5 μl deionized water at 70°C for 5 min. Reverse transcription reactions were performed using 40 units of M-MuLV reverse transcriptase (Promega, Madison, WI) 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 KCl, 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'-TCC CAG AGG AGA TGT CTG TG-3' and 5'-GGC TCC AGC TCT GTG ACC AT-3' (Mercier *et al.*, 2001) and those of human β-actin were 5'-CCT GAC CCT GAA GTA CCC CA-3' and 5'-CGT CAT GCA GCT CAT AGC TC-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 and visualized by ethidium bromide staining, quantified using a bio-imaging analyzer (Bio-Rad), and band-intensity was normalized to the intensity of β-actin mRNA.

Western blotting

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, U.S.A.) 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). Fifty 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 rabit 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, U.S.A.).

RESULTS

ER-mediated transcription activation in MCF-7 cells

Proper ligand binding to the ER initiates transcriptional activation through the specific estrogen response element (ERE) in certain target genes (Gehm *et al.*, 2000). We have examined whether ginsenoside-R_{b1} activates the transcription of an ERE containing reporter plasmid in the human breast adenocarcinoma cell line, MCF-7.

Ginsenoside-R_{b1} induced luciferase activity to the same level as seen with 10 nM E2 in MCF-7 cells transiently transfected with a plasmid containing the estrogen responsive receptor gene at 50 μM concentration. Rough estimation from this luciferase data, ginsenoside-R_{b1} is 50,000 fold weaker as an ER ligand than E2. To confirm that the activities of ginsenosides-R_{b1} are ER-mediated, we co-treated the cells with the pure anti-estrogen, ICI, at a concentration of 1 μM, which is sufficient to saturate almost all the ERs on the cells. The transcriptional activation of the reporter plasmid by ginsenoside-R_{b1} was blocked by ICI (Fig. 2), indicating that luciferase gene activation by ginsenoside-R_{b1} is estrogen-specific.

Induction of an endogenous estrogen responsive c-fos mRNA levels in MCF-7 cells

To evaluate the potential of ginsenoside-R_{b1} as an activator of estrogen-responsive genes, we examined c-fos mRNA induction in MCF-7 cells after treatment with ginsenoside-R_{b1}. Steady-state c-fos mRNA levels were measured by carrying out RT-PCR assays on total RNA prepared from cells treated with the various compounds. As an internal control, constitutively expressed human β-actin mRNA was used. Ginsenoside-R_{b1} also activated transcription of the c-fos gene at 24 h of treatment as E2

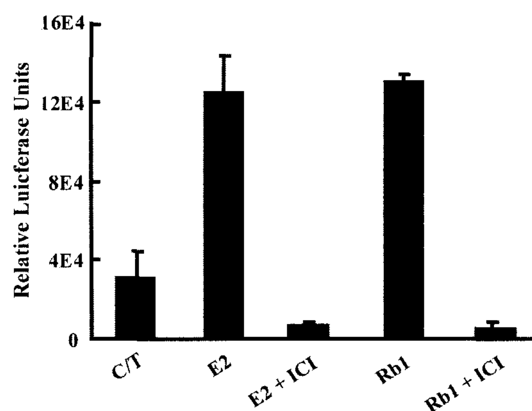

Fig. 2. Activation of estrogen-responsive reporter gene in MCF-7 cells. Cells were transiently transfected with ERE2-tk81-luc and treated with 10 nM E2 or 50 μM ginsenoside-Rb1 in phenol-red-free DMEM plus 10% CD-FBS, and assayed for luciferase activity after 48 h treatments. Data are representative of at least three independent experiments performed in triplicate. Mean ± SEM are shown.

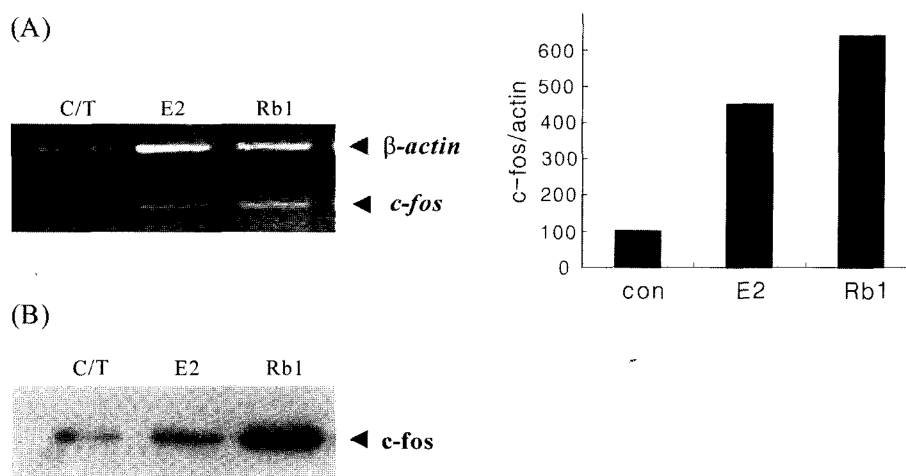

Fig. 3. Ginsenoside-Rb1 activates the expression of estrogen responsive genes in MCF-7 cells. MCF-7 cells were treated with ethanol vehicle, 10 nM E2, or 50 μM ginsenoside-Rb1 for 24 h as indicated. Total RNA prepared from each treatment was analyzed for steady-state (A) c-fos levels using RT-PCR assays. As an internal control, constitutively expressed human β-actin mRNA was used. 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. 3A). These data confirm that ginsenoside-R_{b1} can act as a weak estrogen agonist. 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-R_{b1} treatment as compared with untreated control (Fig. 3B).

Effects on glucocorticoid and androgen receptors

To examine whether estrogenic activity of ginsenoside-R_{b1} is specific, we have examined other steroid hormonal activity of ginsenoside-R_{b1}. We transiently transfected CV-1 cells with the corresponding steroid receptors and reporter plasmids containing the hormone responsive elements. Dexa at 10 nM, 1 μM ATRA or 100 nM Tes was used as a positive control to examine the glucocorticoid, retinoic acid, or androgen activity of ginsenoside-R_{b1}. Ginsenoside-R_{b1} failed to elicit any activity through the AR (Fig. 4A), GR (Fig. 4B).or RAR (Fig. 4C).

DISCUSSION

Estrogens are used in postmenopausal women to prevent hot flashes, depressed moods, and osteoporosis (Johnson *et al.*, 1998; Kenny *et al.*, 2000). However, such uses of estrogens are associated with life-threatening breast and endometrial cancers (Colditz *et al.*, 1999; Pickar *et al.*, 1998). Due to these problems, alternative medicine plays a significant role in hormone replacement therapy despite the lack of scientific information. For postmenopausal symptoms, ginseng is one of the commonly used herbs as with dong quai, black cohosh, and licorice roots (Tyler *et al.*, 1993). We have found a couple

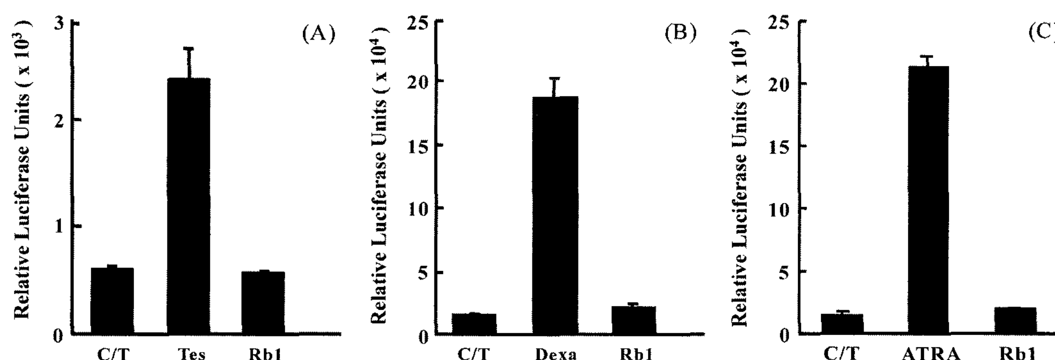

Fig. 4. Screening of androgen, glucocorticoid, and retinoic acid activity in CV-1 cells. (A) Cells were transiently co-transfected with (A) hAR and ARE4-luc (B) pGRE2-luc and hGR (C) hRAR and $(\beta_2RARE)_2$-luc and treated with 100 nM Tes, 10 nM Dexa, 1 μM ATRA, or 50 μM ginsenoside-Rb1 for 48 h as indicated and assayed for luciferase activity 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.

of ginsenoside with estrogenic activity from a panel of purified ginseng components (our unpublished results). In this study, we report a major component of ginseng, ginsenoisde-R_{b1}, of its estrogenic activity.

Accumulating evidence suggests that ginsenosides contain estrogen activity, raising the possibility that they may activate the ER directly or indirectly (Amato *et al.*, 2002). For example, ginseng extracts activates estrogen-responsive genes and regulates cellular growth of human breast cancer cells (Duda *et al.*, 1999). Although, these results suggest that ginsenosides in ginseng extracts, which shares structural similarity with estrogen, may activate ER, no direct evidence has been reported yet (Amato *et al.*, 2002; Duda *et al.*, 1999). American ginseng with high concentration of ginsenoside-R_{b1} implicated estrogenicity but no work was done using the purified components. Other studies using ginsenoside-R_{b1} from different point of view indirectly supports our data that two known estrogen responsive genes are regulated by ginsenoside-R_{b1} in different systems. Ginsenoside-R_{b1} decreased cardiac contraction in adult rat ventricular myocytes in part through increase in NO production (Morschl *et al.*, 2000; Scott *et al.*, 2001). It has been shown that ginsenoside-R_{b1} regulated adrenal tyrosine hydroxlase (Kim *et al.*, 1999). This is the first *in vitro* study to evaluate estrogenic efficacy of ginsenoside-R_{b1}. Further studies are required to determine the equilibrium binding constants, other cellular activities, and *in vivo* effects.

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