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Ginsenoside-Rb1 from *Panax ginseng C.A. Meyer* Activates Estrogen Receptor-α and -β, Independent of Ligand Binding

JUNGYOON CHO, WANKYU PARK, SEUNGKI LEE, WOONGSHICK AHN, AND YOUNGJOO LEE

College of Life Science (JY.C., W.P., YJ.L.), Institute of Biotechnology, Department of Bioscience and Biotechnology, Sejong University, Seoul 143-747, Korea; College of Pharmacy (SK.L.), Seoul National University, Seoul 151-742, Korea; and Department of Obstetrics and Gynecology (W.A.), Catholic Research Institutes of Medical Science, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

We studied the estrogenic activity of a component of *Panax* ginseng, ginsenoside-Rb1. The activity of ginsenoside-Rb1 was characterized in a transient transfection system, using estrogen receptor isoforms and estrogen-responsive luciferase plasmids, in COS monkey kidney cells. Ginsenoside-Rb1 activated both α and β estrogen receptors in a dose-dependent manner with maximal activity observed at 100 μM, the highest concentration examined. Activation was inhibited by the estrogen receptor antagonist ICI 182,780, indicating that the effects were mediated through the estrogen receptor. Treatment with 17β-estradiol or ginsenoside-Rb1 increased expression of the progesterone receptor, pS2, and estrogen receptor in MCF-7 cells and of AP-1-driven luciferase genes in COS cells. Although these data suggest that it is functionally very similar to 17β-estradiol, ginsenoside-Rb1 failed to displace specific binding of [^{3}H]17β-estradiol from estrogen receptors in MCF-7 whole-cell ligand binding assays. Our results indicate that the estrogen-like activity of ginsenoside-Rb1 is independent of direct estrogen receptor association. (*J Clin Endocrinol Metab* 89: 3510–3515, 2004)

GINSENG HAS BEEN used for over 2000 yr in oriental countries to enhance stamina and immune function, where it has been suggested to have pharmacological activities in the cardiovascular, endocrine, immune, and central nervous systems (1). Its use has expanded to Western countries and continues to rise with the increasing popularity of complementary and alternative medicine. It is one of the best-selling herbs in the United States (2). Although many studies have examined the pharmacological action of ginseng extracts, a detailed mechanism has yet to be determined. The major pharmacologically active components of ginseng are ginsenosides, which are steroidal saponins comprising 3–6% of ginseng (3). It has been shown that ginsenosides decrease the levels of total cholesterol and triglyceride via cAMP production and inhibit the accumulation of calcium ions in liver cells (4). Ginsenosides potentiate analgesia and inhibit analgesic tolerance (5). The cardioprotective action of ginsenosides is because of effects on vasodilation via nitric oxide (NO) release (6, 7). Other activities, such as anticarcinogenic and neurological effects, have also been reported for ginsenosides (8, 9).

In the United States, ginseng is used to alleviate menopausal symptoms, as are black cohosh (*Cimicifuga racemosa*), chaste tree berry (*Vitex agnus-castus*), dong quai (*Angelica sinensis*), evening primrose oil (*Oenethera biennis*), motherwort (*Leonurus cardiaca*), red clover (*Trifolium pratense*), and licorice (*Glycyrrhiza glabra*) (10). One recent randomized controlled clinical trial showed that only black cohosh had a beneficial effect on postmenopausal hot flashes (10). Other *in vitro* studies have measured estrogenic activity in red clover and licorice, which was not demonstrated in black cohosh (11). Various studies have indicated that ginseng has estrogenic activity, although no clinical trials have demonstrated real efficacy as an estrogen-replacement therapy (10). Ginseng extracts are able to stimulate the growth of estrogen receptor (ER)-positive cells (12). Ginsenoside-Rg1 and -Rh1 have been shown to contain estrogen-like activity (13, 14), but more comprehensive data are needed to adequately evaluate this activity.

Among 26 identified ginsenosides, ginsenoside-Rb1, -Ro, -Rg1, -Rc, and -Re are highly abundant. In particular, ginsenoside-Rb1 makes up 0.37–0.5% of ginseng extracts, depending on manufacturing and processing methods, and belongs to the protopanaxadiol class of ginsenosides (http://www.netnam.vn/icasia/english/products/redkogin/redkogind/redkogind.htm). We have previously reported that ginsenoside-Rb1 has estrogenic activity (15). In the present study, we aimed to characterize the differential activity of ginsenoside-Rb1 toward ER isoforms α and β. We examined its ability to induce endogenous ER-responsive genes and to act as an ER ligand. Our data show that ginsenoside-Rb1 activates both ERs α and β in the absence of receptor binding.

Materials and Methods

Reagents

Ginsenoside-Rb1 was provided by the Korea Ginseng and Tobacco Research Institute (Daejeon, Korea). 17β-Estradiol (E2) was purchased from Sigma Chemical Co. (St. Louis, MO). ICI 182,780 (ICI) was obtained from ZENECA Pharmaceuticals (Tocris, UK). Ginsenoside-Rb1 was dis-

Abbreviations: CD-FBS, Charcoal-dextran stripped fetal bovine serum; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; PR, progesterone receptor.

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solved in 20% ethanol at a concentration of 15 mg/ml. E2 was dissolved in 100% ethanol. All the compounds were added to the medium such that the total ethanol concentration was never higher than 0.15%. An untreated group served as a control.

Plasmids

ERE2-tk81-luc, constructed by inserting the fragment of the herpes simplex thymidine kinase promoter and two copies of the vitellogenin estrogen-responsive element (ERE) into pA3luc (16), was a kind gift of Dr. Larry Jameson. Expression vector for ERs α and β were from Dr. Pierre Chambon and Dr. Vincent Giguere, respectively. pAP-1-LUC plasmid was purchased from Stratagene (La Jolla, CA).

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 DMEM containing 1× antibiotic/antimycotic mix (Invitrogen, Gaithersburg, MD), 5 mM HEPES, and 0.37% sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT). COS cells were maintained under identical conditions except that 10% calf serum was used instead of FBS. Cells were grown at 37 C in a humidified atmosphere of 95% air/5% CO_2 and fed every 3–4 d. Before hormone induction, the cells were washed with PBS and cultured in DMEM/10% charcoal-dextran stripped FBS (CD-FBS) for 2 d to eliminate any estrogenic source before treatment. All treatments were done with DMEM/10% CD-FBS, and 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 per well. After 24 h, plasmids were transiently transfected into the cell by a 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, and 50 mM HEPES) with constant bubbling, and within 5–10 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 drug treatments with an AutoLumaat LB953 luminometer using the luciferase assay system (Promega Corp., Madison, WI) and expressed as relative light units. The mean and SES 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 RT-PCR

MCF-7 cells were grown in six-well plates in phenol red-free DMEM containing 10% CD-FBS. Near-confluent monolayers were treated with the compounds 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 (Invitrogen) 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 (Invitrogen) and 5 μl deionized water at 70 C for 5 min. RT reactions were performed using 40 U of Moloney murine leukemia virus reverse transcriptase (Promega Corp.) in 5× reaction buffer [250 mM Tris-HCl (pH 8.3) at 25 C, 375 mM KCl, 15 mM $MgCl_2$, and 50 mM dithiothreitol] and 20 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 mixture containing 10× PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$], 25 U of rTaq polymerase (TaKaRa, Japan), 4 μl of 2.5 mM dNTP mixtures, and 10 pmol of primers each. The final volume was 50 μl. The sequences for the human progesterone receptor (PR) and pS2 primers were 5′-CCA TGT GGC AGA TCC CAC AGG AGT T-3′ and 5′-TGG AAA TTC AAC ACT CAG TGC CCG G-3′ for PR (17) and 5′-CAT GGA GAA CAA GGT GAT CTG -3′ and 5′-CAG AAG CGT GTC TGA GGT GTC-3′ for pS2 (18), 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′ (19). The PCR product for PR is 271 bp and 365 bp for pS2 and 550

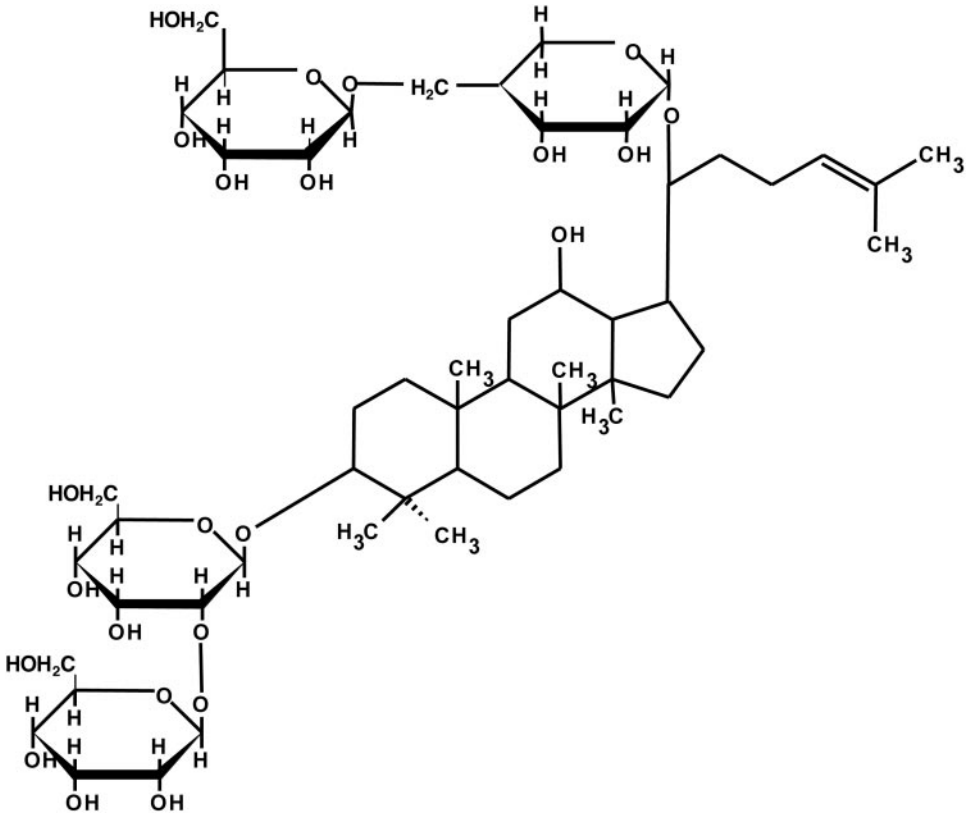

FIG. 1. Chemical structure of ginsenoside-Rb1.

bp for β-actin. The reactions were initiated by 3 min of denaturation at 94 C followed by amplification at 94 C for 45 sec, 55 C for 45 sec, and 72 C for 45 sec; 24 cycles for PR or pS2 and 20 cycles for β-actin. The PCR 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 Laboratories, Inc., Hercules, CA), 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)] 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 micrograms of protein were dissolved in sample buffer and boiled for 5 min before 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/0.05% Tween, and incubated with rabbit anti-polyclonal antibody to ER (0.4 mg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h at 1:500. After washing with Tris-buffered saline/0.05% Tween, blots were incubated with goat antirabbit horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence ECL kits (Amersham Bioscience, Little Chalfont, UK).

ER binding assay

MCF-7 cells were stripped of any estrogen by keeping them in phenol red-free DMEM supplemented with 10% CD-FBS for 2 d. Cells were incubated with 1 nM [2,4,6,7-^{3}H]E2 (89 Ci/mmol; Amersham Bioscience) and a 100-fold excess of nonlabeled E2 (100 nM) or 25–50 μM ginsenoside-Rb1 for 1 h at 37 C in a humidified atmosphere of 95% air/5% CO_2. Aliquots of the medium were measured before and after the incubation with the cells to determine the total uptake of E2 into the cells. After removal of the medium, cells were washed with ice-cold PBS/0.1% methylcellulose twice, harvested by scraping and centrifugation, and lysed with 100% ethanol, 500 μl per 60 mm dish, for 10 min at room temperature (20). The radioactivity of extracts was measured by liquid scintillation counting.

Statistical analysis

Values shown represent mean ± SD. Statistical analysis was performed by Student's *t* test with a *P* value of less than 0.05 being considered statistically significant.

Results

Ginsenoside-Rb1 activates estrogen-responsive luciferase genes in the presence of either ERα or ERβ

We have previously demonstrated that ginsenoside-Rb1 (Fig. 1) activates ER in MCF-7 cells (15). It has been shown that some phytoestrogens such as genistein and coumestrol

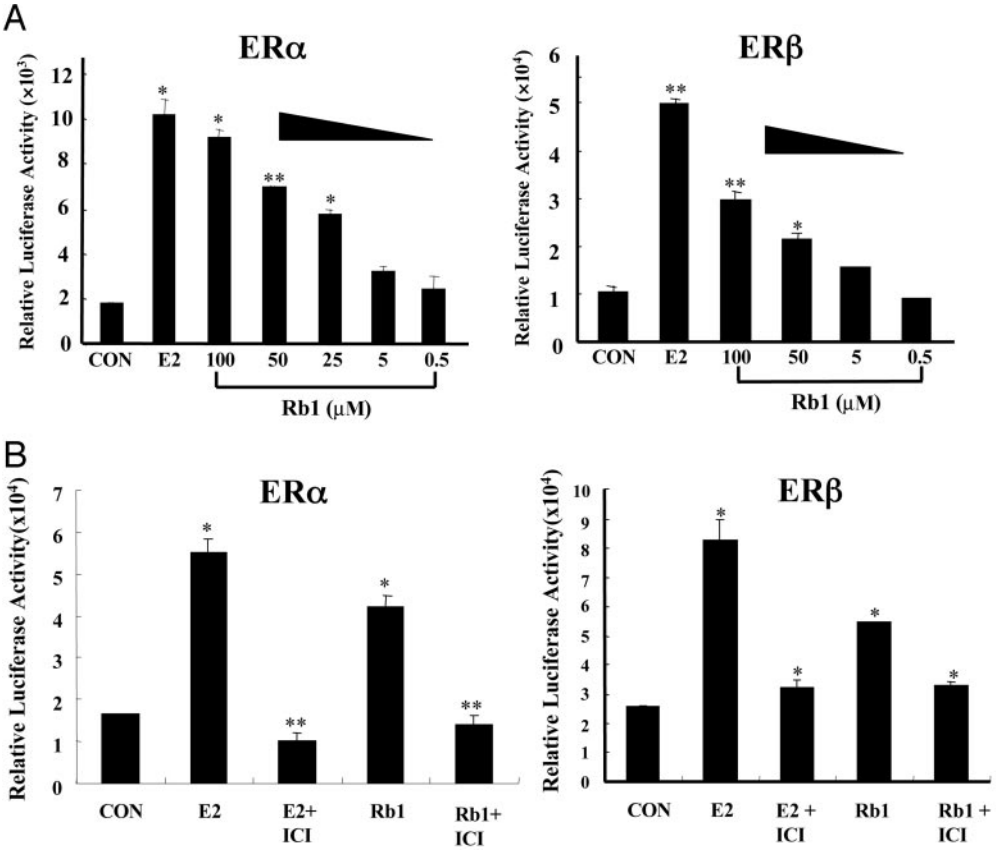

FIG. 2. Ginsenoside-Rb1-induced transactivation was assessed by cotransfection of the (ERE)-luciferase with either ERα or ERβ expression plasmids in COS cells. Cell extracts were prepared and analyzed by a luciferase assay. A, Ginsenoside-Rb1 activated both ERα and ERβ in a dose-dependent manner. Cells were exposed to E2 (10 nM), ginsenoside-Rb1 (100–0.5 μM) for 24 h. *, $P < 0.05$; **, $P < 0.005$. B, Ginsenoside-Rb1 activation was inhibited by ICI. Cells were exposed to E2 (10 nM) or ginsenoside-Rb1 (100 μM) or in combination with ICI (1 μM) for 24 h. The data are representative of at least three independent experiments performed in triplicate with similar results, expressed as relative luciferase units and the SEM of triplicate samples. *, $P < 0.05$; **, $P < 0.005$.

differ in their activity on ERα and ERβ (21). In the present study, we investigated whether there is differential activation of these two receptor isoforms in response to ginsenoside-Rb1 by examining the transcription of an ERE-containing reporter plasmid in the presence of ERα or ERβ in ER-negative COS cells. Ginsenoside-Rb1 activated both ERα and ERβ in a dose-dependent manner (Fig. 2A). Luciferase gene activation was observed over two orders of ginsenoside-Rb1 concentration up to 100 μM, the highest concentration tested. Higher concentrations would have resulted in unacceptably high concentrations of ethanol in the cell media. In the presence of the ERα isoform, ginsenoside-Rb1 and E2 activated ERE-driven luciferase expression to a similar extent (91% of E2 activity); approximately 60% of the E2 response was seen with the ERβ isoform (Fig. 2A). These data indicate that ginsenoside-Rb1 acts as a dose-dependent agonist, stimulating transcription through both receptors, but that it has slightly higher affinity for ERα than for ERβ.

To confirm that the activities of ginsenoside-Rb1 were ER mediated, we coincubated the cells with the pure antiestrogen ICI, at a concentration sufficient to saturate almost all the ERs in the cells (1 μM). Transcriptional activation of the reporter plasmid by ginsenoside-Rb1 was blocked by ICI (Fig. 2B) with either ERα or ERβ, indicating that the gene activation was estrogen-specific.

Ginsenoside-Rb1 activates AP-1 luciferase reporter plasmids, PR and pS2 mRNA, and ER protein in an ER-dependent manner

To further characterize the effects of ginsenoside-Rb1, the effects on ER-mediated AP-1 responses, endogenous estrogen-responsive PR and pS2 mRNAs, and ER protein were examined. Ligand-occupied ERs are known to mediate gene transcription from AP-1 enhancer elements (22). AP-1 is implicated in the inducible expression of a variety of genes involved in the regulation of proliferation and apoptosis, as well as in cellular stress responses and inflammation (23). With transient ERα expression in COS cells, both ginsenoside-Rb1 and E2 stimulated AP-1-driven luciferase plasmid transactivation (Fig. 3A). Ginsenoside-Rb1 did not affect luciferase production in the absence of ER (data not shown). After treatment of MCF-7 cells with the compounds for 24 h, steady-state mRNA levels were measured by RT-PCR of total RNA, as indicated in Fig. 3B. Constitutively expressed human β-actin mRNA was used as an internal control. Ginsenoside-Rb1 increased steady-state mRNA levels of the PR and pS2 genes after 24 h of treatment, as did E2 (Fig. 3B). Coincubation with 1 μM ICI efficiently blocked the activation of PR and pS2 mRNA expression, indicating activation through ER. We also have examined the ER protein levels in MCF-7 cells by Western analysis. ER protein levels were down-regulated after 24 h of either E2 or ginsenoside-Rb1 treatments as compared with control (Fig. 3C). These data indicate that ginsenoside-Rb1 is capable of activating an ER-mediated pathway.

Ginsenoside-Rb1 fails to compete with estrogen binding to ER

To determine whether ginsenoside activation occurs via direct binding to ER, we examined the ability of ginsenoside-Rb1 to compete with ^{3}H-labeled E2 for ER binding in MCF-7 cells (Fig. 4). Specific binding was calculated as total binding

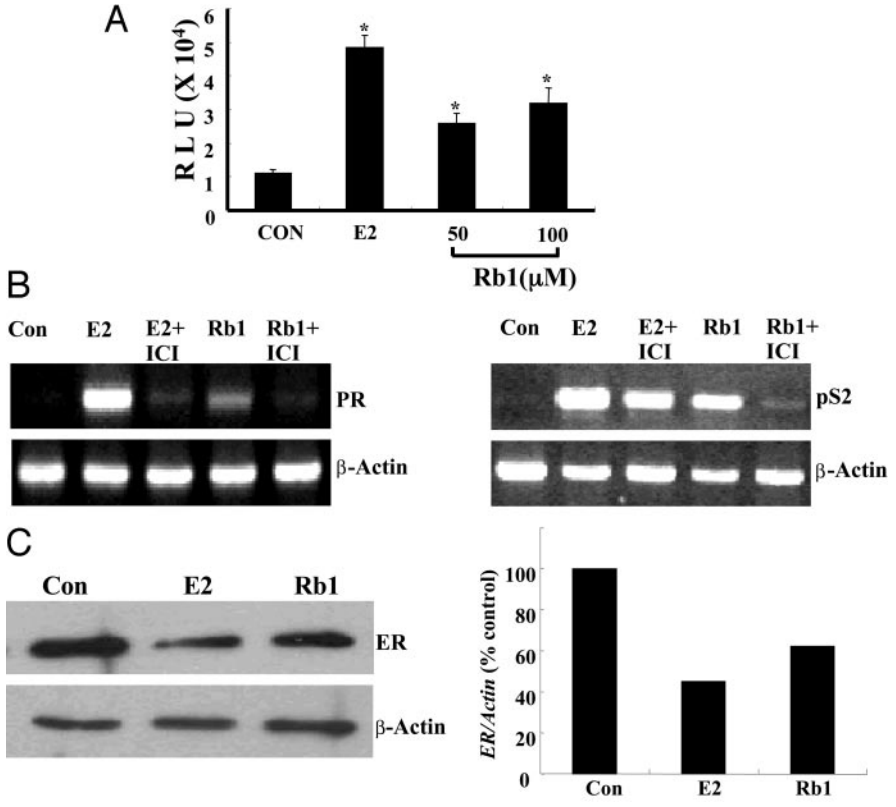

FIG. 3. Effects of ginsenoside-Rb1 on AP1-mediated activation, PR and pS2 mRNA, and ER protein levels. A, Ginsenoside-Rb1-induced expression of AP1-driven reporter plasmids. COS cells were transiently transfected with an AP1-containing reporter and ERα expression plasmids and treated with E2 (10 nM) or ginsenoside-Rb1 (50 μM) at the indicated concentrations for 24 h. Cell extracts were prepared and analyzed by a luciferase assay. The data are representative of at least three independent experiments performed in triplicate with similar results, expressed as relative luciferase units and the SEM of triplicate samples. B, The semiquantitative RT-PCR for PR and pS2 in the MCF-7 cells shown is a representative of three independent experiments. Cells were exposed to ginsenoside-Rb1 (50 μM) with and without ICI (1 μM) for 24 h. PR amplification product was detected at 271 bp (*left*) and 365 bp for pS2 (*right*). C, The Western blot analysis shown is a representative of two independent experiments. Cells were treated with E2 (10 nM) or ginsenoside-Rb1 (50 μM) alone for 24 h. ER protein was detected at 62 kDa (*right*). Equal loading of protein in each lane was confirmed by β-actin protein (43 kDa). ER densitometry values are expressed as a percentage of the control (*left*).

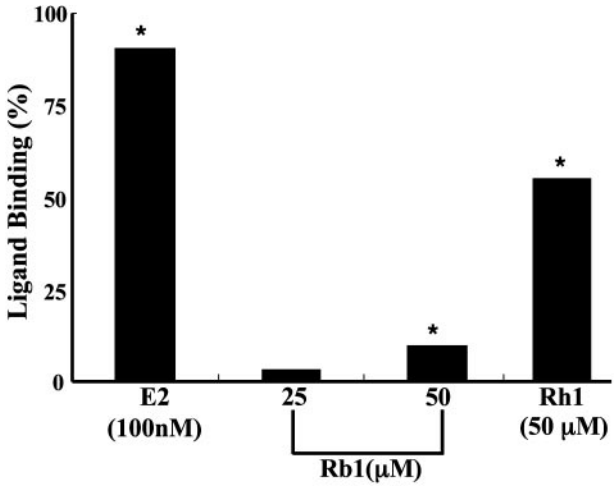

FIG. 4. Competitive binding of ginsenside-Rb1 to ER in MCF-7 cells. Ginsenoside-Rb1 failed to compete E2 occupancy of ER. Cells were incubated with 1 nM ^{3}H-labeled E2 (89 Ci/mmol) plus vehicle, nonlabeled E2 (100 nM), ginsenoside-Rb1 (25–50 μM), or ginsenoside-Rh1 (50 μM) for 1 h. Radioactivity of ethanol extracts of cell lysates was measured by scintillation counting. Specific binding was calculated as total binding minus nonspecific binding. Data were expressed as percentage of ligand binding, which is (total binding – nonspecific binding)/total binding $\times$ 100. *, $P < 0.05$.

minus nonspecific binding (determined in the presence of unlabeled E2 or ginsenoside-Rb1 at the concentration as indicated in the figure). At 50 μM, ginsenoside-Rb1 did not inhibit ^{3}H-labeled E2 binding to ER (Fig. 4). We also failed to observe any binding in a cell line that was stably transfected with ER (data not shown). In contrast, ginsenoside-Rh1, a weak phytoestrogen, displaced approximately 44% of ^{3}H-labeled E2 binding to ER at a concentration of 50 μM in MCF-7 cells.

Discussion

Hormone replacement therapy is used to prevent or combat heart disease, stroke, osteoporosis, Alzheimer's disease, and postmenopausal symptoms such as hot flashes and depression (24). However, such uses of estrogens are associated with side effects, including increased risks of breast and endometrial cancers (24). Owing to these problems, public interest in alternative medicines for hormone replacement therapy has increased. The most commonly used alternative herbal medicines for estrogen replacement are soy, black cohosh, dong quai, chastetree berry, and ginseng (10). Phytoestrogens that include isoflavones, lignans, and coumestans are found in some of these herbs (20, 25). This is likely to be one reason for the lower prevalence of menopausal symptoms in countries like Korea, Japan, and China, where consumption of soy is high (26). Although accumulating studies suggest important potential health benefits, both the clinical efficacies and mechanisms of action of these herbs are still not fully known. We evaluated the estrogenic activity of ginseng, using purified ginseng components from *Panax* ginseng. Two ginsenosides with estrogenic activity, ginsenoside-Rb1 and -Rh1, had been identified previously by screening a panel of ginsenosides. In this study, we characterized the *in vitro* estrogenic activity of ginsenoside-Rb1, providing a scientific foundation for potential clinical development. However, it should be noted that, as with other phytoestrogens, ginsenoside-Rb1 is likely to contain biological activities that are independent of ER, such as antioxidant, antiproliferative, and antiangiogenic effects (27, 28).

In Asia, ginseng has been used for thousands of years as a tonic, to fight various aspects of stress, and to restore homeostasis (1). In Western countries, it is being used as an alternative herb for postmenopausal women. Accumulating evidence suggests that ginseng contains either direct or indirect estrogenic activity (29). Ginseng extracts activate estrogen-responsive genes and regulate the growth of human breast cancer cells (12). Recent studies by Chan *et al.* (13) showed that picomolar ginsenoside-Rg1 from *Panax notoginseng* activated ER-mediated transcription without direct receptor interaction. Our group has reported estrogenic activity of ginsenoside-Rh1 in the micromolar range (14). Although ginsenosides share structural similarities with estrogen and may activate ERs, more detailed studies are needed to fully characterize their activities (12, 29).

The results from other studies in different systems indirectly suggest the regulation of estrogen-responsive genes by ginsenoside-Rb1. It was shown to decrease cardiac contraction in adult rat ventricular myocytes, in part through an increase in NO production (6). Although a correlation between the increase in NO and ER activation was not evaluated, estrogen is known to enhance NO production (30). Ginsenoside-Rb1 also regulates adrenal tyrosine hydroxylase (31), which is known to be under estrogen regulation (32).

The data presented here show that ginsenoside-Rb1 activated both ERα and ERβ, leading to the transactivation of estrogen-responsive genes. However, this activation occurred in the absence of direct receptor binding, as examined using receptor competition assays. This indicates that ginsenoside-Rb1 activates ER via a mechanism or mechanisms other than that of classical, hormone-mediated activation. A variety of agents, including growth factors, neurotransmitters, and cAMP, activate ER in ligand-independent manners (33). Recent data on the pharmacokinetics of ginsenoside-Rb1 show that its absolute oral bioavailability is as low as 4.35% in rats (34). Based on a calculation using the figures in the report by Xu *et al.* (34), approximately 180 mg/kg ginsenoside-Rb1 should be taken orally to obtain a serum concentration of 50 μM, the concentration that activated estrogen receptors in our assays. The ginsenoside persists for 3 d, but because of its low absorption, initial administration or formulation changes are necessary before clinical application (34). It has been shown recently that ginsenoside-Rb1 is teratogenic in the rat embryo at concentrations over 30 μM (35). Potential toxicity should be kept in mind during the clinical development of this compound. The exact cause of the estrogenic effects of ginseng may not be ginsenside-Rb1, because of its low serum concentration despite its *in vitro* estrogenic activity. However, these approaches are essential to provide a scientific rationale for using ginseng for estrogen-related symptoms. In this report, we have addressed the *in vitro* mechanism of one of the main components of ginseng. Studies investigating the upstream targets of ginsenoside-Rb1 that lead to ER activation and

in vivo studies will improve our understanding of the clinical application of ginseng.

Acknowledgments

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Address all correspondence and requests for reprints to: YoungJoo Lee, Ph.D., Department of Bioscience and Biotechnology, Sejong University, Kwang-Jin-Gu, Seoul 143-747, Korea. E-mail: yjlee@sejong.ac.kr.

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JY.C. and W.P. contributed equally to this work.

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