British Journal of Pharmacology (2006) 147, 542–551
www.nature.com/bjp

Activation of insulin-like growth factor I receptor-mediated pathway by ginsenoside Rg1

[1,2]Wen-Fang Chen, [1,3]Wai-Sum Lau, [1]Pik-Yuen Cheung, [4]De-An Guo & *,[1,3]Man-Sau Wong

[1]Central Laboratory of the Institute of Molecular Technology for Drug Discovery and Synthesis, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, People's Republic of China; [2]Department of Physiology, Medical College of Qingdao University, Qingdao 266021, People's Republic of China; [3]State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen 518057, People's Republic of China and [4]Shanghai Research Center for TCM Modernization, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, People's Republic of China

1 Ginsenoside Rg1, an active ingredient in ginseng, was previously shown to be a novel class of potent phytoestrogen. The present study aims at investigating the molecular mechanisms involved in mediating its actions in human breast cancer (MCF-7) cells.

2 Rg1 (1 pM) stimulates cell proliferation ($P<0.01$) and estrogen-responsive pS2 mRNA expression ($P<0.05$) without alteration of estrogen receptor alpha (ERα) protein or mRNA expression in MCF-7 cells. In addition, 10^{-14}–10^{-4} M of Rg1 does not demonstrate specific binding to ERα.

3 We hypothesize that Rg1 may exert its actions in MCF-7 cell *via* the activation of crosstalk between ER- and insulin growth factor I receptor (IGF-IR)-dependent pathways.

4 The results indicate that Rg1 significantly increases IGF-IR expression and IGF-IR promoter activity in MCF-7 cells ($P<0.05$). Cotreatment of MCF-7 cells with 1 μM of estrogen antagonist ICI 182,780 completely abolishes the effects of Rg1 on IGF-IR expression.

5 Furthermore, Rg1 enhances tyrosine phosphorylation of IRS-1 in MCF-7 cells upon IGF-I stimulation and the activation of IRS-1 phosphorylation is also ER-dependent.

6 Taken together, our results suggest that Rg1 not only increases IGF-IR expression but also enhances IGF-IR-mediated signaling pathways in MCF-7 cells. The stimulation of IGF-IR expression by Rg1 in MCF-7 cells appears to require ER, and its actions might involve ligand-independent activation of ER.
British Journal of Pharmacology (2006) **147,** 542–551. doi:10.1038/sj.bjp.0706640;
published online 16 January 2006

Keywords: Ginsenoside Rg1; estrogen; insulin-like growth factor I receptor; human breast cancer; phytoestrogen

Abbreviations: CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; E_2, 17β-estradiol; ER, estrogen receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF, insulin-like growth factor; IGF-IR, insulin-like growth factor I receptor; IRS-1, insulin receptor substrate 1; PBS, phosphate-buffered saline; RT–PCR, reverse transcriptase–polymerase chain reaction; Shc, Src homology/collagen; sFBS, charcoal-stripped fetal bovine serum

Introduction

Ginsenosides, the principal active components of ginseng (Liu & Xiao, 1992), have been shown to possess a variety of beneficial effects on human health, including anti-inflammatory, antioxidant and anticancer effects (Mochizuki *et al*., 1995; Kim *et al*., 1998; Wakabayashi *et al*., 1998). There are two major classes of ginsenosides, namely protopanaxatriol (Rg1, Rg2, Re, Rf, and Rh1) and protopanaxadiol (Rb1, Rb2, Rc, Rd, Rg3, Rh2, and Rh3). Numerous studies have demonstrated that ginseng extracts (Punnonen & Lukola, 1980; Duda *et al*., 1996; Liu *et al*., 2001; Amato *et al*., 2002) and its constituents (Cui *et al*., 2001; Chan *et al*., 2002; Lee *et al*., 2003a, c) possess estrogen-like activities in both *in vitro* and *in vivo* models. Ginseng extracts can stimulate the growth of estrogen receptor (ER)-positive cells and the induction of estrogen-dependent *pS2* gene expression (Duda *et al*., 1996; Liu *et al*., 2001; Amato *et al*., 2002).

Our previous study demonstrated that ginsenoside Rg1, a steroidal saponin of high abundance in ginseng, could mediate its action *via* the ER in human breast cancer cells (Chan *et al*., 2002). Subsequent studies by others have reported that ginsenoside Rb1 (Lee *et al*., 2003c; Cho *et al*., 2004) and Rh1 (Lee *et al*., 2003a) could activate ER in human breast cancer cells, whereas ginsenoside Rc and Re can stimulate MCF-7 cell growth as well as induce c-Fos expression independent of ER activation (Lee *et al*., 2003b). Among these ginsenosides, Rg1 appears to be of the highest potency as its EC50 for activation of human breast cancer cell proliferation is 0.05 pM (Chan *et al*., 2002), while the reported activities of Rb1 and Rh1 are in micro-molar range. Similarly, it is by far the most potent phytoestrogens being reported as most of

*Author for correspondence at: Central Laboratory of the Institute of Molecular Technology for Drug Discovery and Synthesis, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, People's Republic of China; E-mail: bcmswong@polyu.edu.hk

the other naturally occurring compounds (nonsteriodal agents like flavonoids, coumestan derivatives and lignans; polyphenolic compounds) demonstrated weak estrogenic activity at micro-molar range (Murkies *et al*., 1998; Tham *et al*., 1998; Anter *et al*., 2005; Klinge *et al*., 2005). Most importantly, the binding affinities of different ginsenosides for ER are found to be different. Both Rg1 (Chan *et al*., 2002) and Rb1 (Cho *et al*., 2004) did not show specific binding to ER, while Rh1 could displace ^{3}H-labeled 17β-estradiol (E_2) binding to ER (Cho *et al*., 2004). The demonstration of estrogenic activities of ginsenosides in the absence of direct interaction with ER suggests that ginsenosides may activate ER *via* a ligand-independent pathway.

Recent studies indicated that ERα can be activated in a ligand-independent manner by a variety of stimuli, including insulin-like growth factor I (IGF-I) (Lee *et al*., 1997a, b), epidermal growth factor (EGF) (Kato *et al*., 1995), serum (Karas *et al*., 1998), and, recently, leptin (Catalano *et al*., 2004). Moreover, crosstalk between ER and growth factor has been observed in ER-positive breast cancer cells (Kato *et al*., 2000). E_2 can induce the expression of several members of the IGF family in human breast cancer cells, including IGF-I receptor (IGF-IR), IGF-II receptor (IGF-IIR), IGF binding proteins (IGFBPs), insulin receptor substrate 1 (IRS-1), and insulin receptor substrate 2 (IRS-2). The upregulation of these proteins by E_2 provides the potential mechanisms for its autocrine and paracrine control of breast cancer cell mitogenesis (Stewart *et al*., 1992; Lee *et al*., 1999). Reciprocally, IGF-I was found to modulate E_2 action by acting *via* the tyrosine kinase of IGF-IR. Ligands for both the EGF receptor and IGF-IR can regulate ER gene expression and ER-mediated transcription in an estrogen-independent manner (Kato *et al*., 1995; Lee *et al*., 1997a, b).

In the present study, we systematically evaluated the molecular actions of ginsenoside Rg1 in ERα-positive breast cancer (MCF-7) cell line. We hypothesize that Rg1 might exert its action *via* the crosstalk between ER- and IGF-IR-dependent pathways. We have studied the effect of Rg1 on IGF-IR expression and IGF-IR promoter activity, as well as on tyrosine phosphorylation of IRS-1, a downstream signaling molecule in IGF-IR-mediated pathways, in MCF-7 cells. The results indicate that IGF-I signaling pathway is indeed involved in mediating the action of Rg1 in human breast cancer cells, and provide new evidence to support our hypothesis for the activation of ER and IGF-IR crosstalk by Rg1 in MCF-7 cells.

Methods

Purification of ginsenoside Rg1 from Panax notoginseng

The method of ginsenoside Rg1 purification used was as described previously (Chan *et al*., 2002). Briefly, the powder of roots of *P. notoginseng* was extracted using 70% ethanol three times. The extract was evaporated to dryness at less than 50°C under reduced pressure, and the residue was dissolved in water and extracted by aqueous butanol three times. The butanol extract was evaporated to dryness at less than 50°C under reduced pressure and was then subjected to chromatography on D101 resin, which was eluted by using a 0–100% gradient of water–ethanol mixture. After removal of solvent, the 50% ethanol extract was subjected to chromatography on silica gel. Rg1 was eluted from the column using $CHCl_3$–MeOH–H_2O (50 : 10 : 1). The purity of Rg1 was determined by HPLC and was found to be more than 99% pure. The structure of Rg1 is shown in Figure 1.

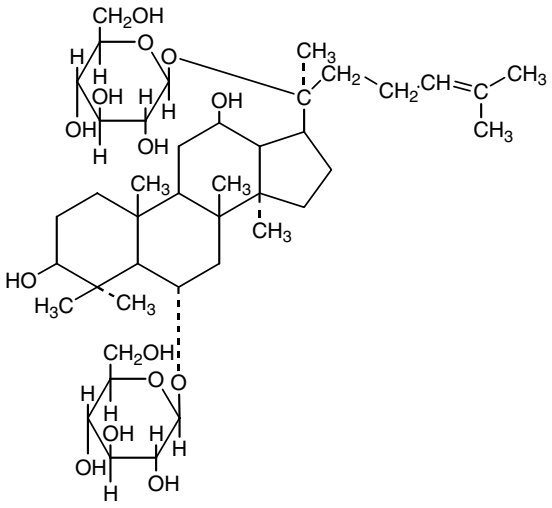

Figure 1 Chemical structure of ginsenoside Rg1.

Culture of human breast cancer cell line (MCF-7) and human embryonic kidney cell line (HEK293)

MCF-7 cells (ATCC No. HTB-22) and HEK293 cells (ATCC No. CRL-1573, a human embryonic kidney cell line) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin 100 IU ml^{-1}, and streptomycin 100 μg ml^{-1} (Invitrogen, Carlsbad, CA, U.S.A.) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were transferred to phenol-red free DMEM supplemented with 1% charcoal-stripped fetal bovine serum (sFBS), penicillin 100 IU ml^{-1}, and streptomycin 100 μg ml^{-1} by standard methods of trypsinization, plated in six-well dishes for 5 days and allowed to replicate to 80% confluence. Cells were then treated either with Rg1 (1 pM) or E_2 (10 nM) (Sigma, St Louis, MO, U.S.A.) for 6, 24, 48, and 72 h. For dose–response treatment, MCF-7 cells were exposed to 0.01 pM–1 μM of Rg1 for 48 h. The medium and test compounds were replenished at 24 h. For antiestrogen or cycloheximide (CHX) treatment, MCF-7 cells were exposed to Rg1 or E_2 in the presence or absence of estrogen antagonist ICI 182,780 (1 μM) (Tocris, Bristol, U.K.) or CHX (5 μg ml^{-1}) (Sigma, St Louis, MO, U.S.A.) for 48 h. For immunoprecipitation experiment, cells were then stimulated with either IGF-1 (5 min) or Rg1 (48 h) separately, or sequentially (Rg1 for 48 h followed by IGF-1 for 5 min). The cell lysates were used for immunoprecipitation.

Cell proliferative assays

For growth study, MCF-7 cells were seeded in 96-well plates (3×10^3 cells per well) in phenol-red free DMEM supplemented with 1% sFBS for 4 days and then treated with various concentrations (10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}, and 10^{-14} M) of Rg1 or E_2 for 48 h. As an indirect measure of growth, the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT)

assay was used as described previously (Denizot & Land, 1986). Briefly, the medium was removed and replaced with 100 μl of tetrazolium (MTT, 5 mg ml^{-1}, Sigma, St Louis, MO, U.S.A.) in PBS. The plates were incubated for 4 h at 37°C and followed by addition of 100 μl lysis buffer (0.04 N HCl in propan-2-ol). The multiwell plates are shaken for 1 h and read on a microplate reader at a wavelength of 595 nm.

ERα competition assay

The ERα competition assay was performed using the human recombinant Estrogen Receptor α Competitor Assay Beacon Kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, U.S.A.). In this assay kit, an ER/fluorescent estrogen ligand (ES_2) complex with a high polarization value is added to the test compound. If the compound competes with estrogen for ER binding, it will displace ES_2 from the ER/ES_2 complex and cause a reduction in the polarization value. Non-ER-binding competitors will not displace ES_2 from the ER/ES_2 complex, and the polarization value remains high. The shift in polarization in the presence of a test compound is used to determine the relative affinity of the compound for ERα that was calculated from the resulting curve using Prism 3.03 software (GraphPad Software, San Diego, CA, U.S.A.).

Reverse transcriptase–polymerase chain reaction (RT–PCR) for pS2, ERα and IGF-IR expression

Total RNA was isolated from cells by using Trizol reagent according to the standard protocol. Total RNA (2 μg) was used to generate cDNA in each sample using SuperScript II reverse transcriptase with oligo(dT) 12–18 primers (Invitrogen, Carlsbad, CA, U.S.A.). The evaluation of pS2, ERα, and IGF-IR expression was performed by semiquantitative RT–PCR as described previously (Chen & Wong, 2004). For pS2, ERα, IGF-IR, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers used were 5′-ATGGCCACCATGGAGAACAAGG-3′ (pS2 forward) and 5′-CATAAATTCACACTCCTCTTCTGG-3′ (pS2 reverse), 5′-AAGTTCAGGCACAATTGGATG-3′ (ERα forward) and 5′-CCCTGCATGACACTGATTACA-3′ (ERα reverse), 5′-ACTATGCCGGTGTCTGTGTG-3′ (IGF-IR forward) and 5′-TGCAAGTTCTGGTTGTCGAG-3′ (IGF-IR reverse), and 5′-ACCACAGTCCATGCCTACAC-3′ (GAPDH forward) and 5′-TTCACCACCCTGTTGCTGTA-3′ (GAPDH reverse) to yield products of 252, 502, 522, and 422 bp, with 23, 23, 30, and 20 PCR cycles, respectively. PCR amplification was performed on a GeneAmp 9600 PCR system (Perkin-Elmer, Foster City, CA, U.S.A.). The PCR products were analyzed using agarose gel electrophoresis. Optical densities of ethidium bromide-stained DNA bands were quantified using a Lumi-Imager (Roche Mannheim, Germany) and the mRNA expression levels were normalized to the expression of GAPDH.

IGF-IR promoter luciferase assay

The effect of Rg1 on IGF-IR promoter activity was studied by transient transfection assays using a genomic DNA fragment extending from nucleotides −2350 to +640 (nucleotide corresponds to the transcription site of rat *IGF-IR* gene) or promoterless luciferase construct (pOLuc) that was kindly provided by Dr Derek LeRoith (Diabetes Branch, Bethesda, MD, U.S.A.). MCF-7 cells were seeded in 12-well plates for 1 day in DMEM medium supplement with 5% FBS; cells were then transferred to phenol-red free DMEM supplement with 1% sFBS for another 2 days. MCF-7 cells were transfected with 0.8 μg of reporter plasmid along with 0.4 μg of the control reporter plasmids pRL-TK using the Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). At 5 h after transfection, cells were treated with Rg1 or E_2 for another 24 h and then harvested. Luciferase activity encoded by experimental and internal control plasmid was measured sequentially using TD-20/20 Luminometer (Turner Design, Sunnyvale, CA, U.S.A.) and the DLR assay reagents according to the supplier's recommendation (Promega, Madison, WI, U.S.A.). The IGF-IR promoter activity was expressed as firefly luciferase values normalized by pRL-TK renilla luciferase values.

Immunoblotting and immunoprecipitation

For Western blotting, protein was isolated from cells using Trizol reagent according to the standard protocol. Protein concentrations were analyzed by the method of Bradford (Bio-Rad, Hercules, CA, U.S.A.) (Bradford, 1976). Equal amount of proteins (5 μg) were separated by SDS–PAGE on 10% reducing gels at a constant voltage (150 V) for 1 h as described previously (Sriussadaporn *et al*., 1995), and transblotted onto PVDF membranes (Immobilin-P, Millipore Corp., MA, U.S.A.). Immunodetection was performed after blocking nonspecific binding sites on the membrane with 5% skimmed milk. The blots were probed with polyclonal rabbit anti-human IGF-IRβ, IRS-1 (1 : 2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) or ERα (1 : 3000; Sigma, St Louis, MO, U.S.A.) as the primary antibody, which was followed by incubation with goat anti–rabbit antibody conjugated with horseradish peroxidase (1 : 2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) as the secondary antibody for 1 h. The antigen–antibody complexes were then detected with enhanced chemiluminescence (ECL) reagent (Mattson & Bellehumeur, 1996) and visualized by the Lumi-Imager using Lumi Analyst version 3.10 software (Roche, Mannheim, Germany).

For immunoprecipitation, cells were lysed with Nonidet P-40 buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40) containing protease inhibitors (aprotinin 2 μg ml^{-1}, leupeptin 2 μg ml^{-1}, 1 mM PMSF) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM NaF). Lysates were centrifuged at 14,000 × *g* for 30 min at 4°C and supernatant protein concentrations were analyzed by the method of Bradford. Phosphorylation of IRS-1 was determined as follows: 500 μg of protein lysate was precipitated with 6.5 μg of the corresponding antibodies at 4°C on a rocker platform for 2 h, followed by the addition of 100 μl protein A Sepharose slurry, and incubated for 1.5 h at 4°C. After three sequential washes using Nonidet P-40 buffer, the resulted pellets were resuspended in electrophoresis sample buffer and boiled for 5 min and subsequently detected by immunodetection with antiphosphotyrosine monoclonal antibody (P-Tyr-100, 1 : 1000; Cell Signaling Technology, Hitchin, Herts, U.K.) as the primary antibody and goat anti-mouse-conjugated horseradish peroxidase as the secondary antibody (1 : 1000). The sample was

then detected by ECL kit (Pierce, IL, U.S.A.), and analyzed by Lumi-Analyst 3.1 software (Roche, Mannheim, Germany).

Statistical analysis

Data are reported as the mean ± s.e.m. Significance of difference between group means was determined by one-way analysis of variance (ANOVA). The independent Student's *t* test was used to calculate statistical significance between the control group and each treatment group in MTT assay and tyrosine Phosphorylation of IRS-1. A *P*-value of <0.05 was considered statistically significant.

Results

Ginsenoside Rg1 stimulates cell proliferation and pS2 mRNA expression in human breast cancer (MCF-7) cells

As shown in Figure 2a, Rg1 increased cell proliferation of MCF-7 cells in a dose-dependent manner. The stimulation of cell proliferation by different concentrations of Rg1 appeared to be biphasic. The maximal stimulation of MCF-7 at low concentrations of Rg1 (from 10^{-14} to 10^{-8} M) occurred at 1 pM, and 1 μM of Rg1 appeared to increase MCF-7 cell proliferation to an extent higher than that achieved by 1 pM of Rg1. Treatment of MCF-7 cells with 1 pM and 1 μM of Rg1 for 48 h resulted in 1.33- and 1.55-fold increase in cell number, respectively ($P<0.05$ vs vehicle-treated cells). E_2 also increased cell proliferation of MCF-7 cells in a dose-dependent manner; the cell number increased as the concentration of E_2 increased.

To determine if Rg1 mimics E_2 and induces ER-dependent gene transcription, the ability of Rg1 to induce estrogen-regulated gene pS2 was determined. The *pS2* gene was originally identified as an estrogen-inducible transcript in MCF-7 cells and encoded for a secretary protein from MCF-7 cells (Masiakowski *et al*., 1982). *pS2*-gene expression is frequently used as a marker for assessing the estrogenicity of various compounds (Soto *et al*., 1997). Figure 2b clearly shows that 1 pM Rg1 increased *pS2* gene expression in a time-dependent fashion. The significant increase was found at 72 h treatment. In the case of E_2 (10 nM), the induction of *pS2* gene expression occurred as early as 24 h and throughout the 72 h of treatment. These results indicate that Rg1 indeed behaves as a potent phytoestrogen at a concentration as low as 1 pM and its activation pattern in MCF-7 cells is not completely similar to estrogen.

Role of ERα in mediating the action of ginsenoside Rg1

The binding of Rg1 or E_2 to human recombinant ERα was determined by ERα competitive binding assay. In the case of E_2, specific binding of ES_2 with recombinant ERα was displaced by increasing concentration of unlabeled E_2 and the polarization value decreased in a dose-dependent manner (Figure 3). In contrast, Rg1 (0.01 pM–100 μM) failed to displace the specific binding of ES_2 from its receptor (Figure 3), confirming our previous finding (Chan *et al*., 2002) that no direct interaction exists between ERα and Rg1 at concentration as high as 100 μM.

ER is previously demonstrated to be an essential mediator of Rg1 action; we therefore determined if Rg1 exerts E_2-like activity by regulating the level of ER expression in MCF-7 cells. As ERα, but not ERβ, is the major isoform expressed in MCF-7 cell line, its expressions in response to Rg1 were being studied in the present study. As shown in Figure 4, the dose–response effects of Rg1 on ERα protein and mRNA expressions were determined. Our results showed that Rg1 (10^{-14}–10^{-6} M) did not significantly alter ERα protein expression, although an upward trend of stimulation was observed when treated with 10^{-12}–10^{-8} M of Rg1 (Figure 4a and c). Similarly, the expressions of ERα mRNA expression in MCF-7 cells were not altered in response to treatment with 10^{-14}–10^{-6} M of Rg1 (Figure 4b and d).

The time-course responses of ERα expression to treatment with E_2 and Rg1 in MCF-7 cells were also determined. As shown in Figure 4e and f, 10 nM of E_2 significantly reduced ER protein and mRNA expressions in MCF-7 cells throughout the duration of treatment ($P<0.05$). In contrast, Rg1 did not

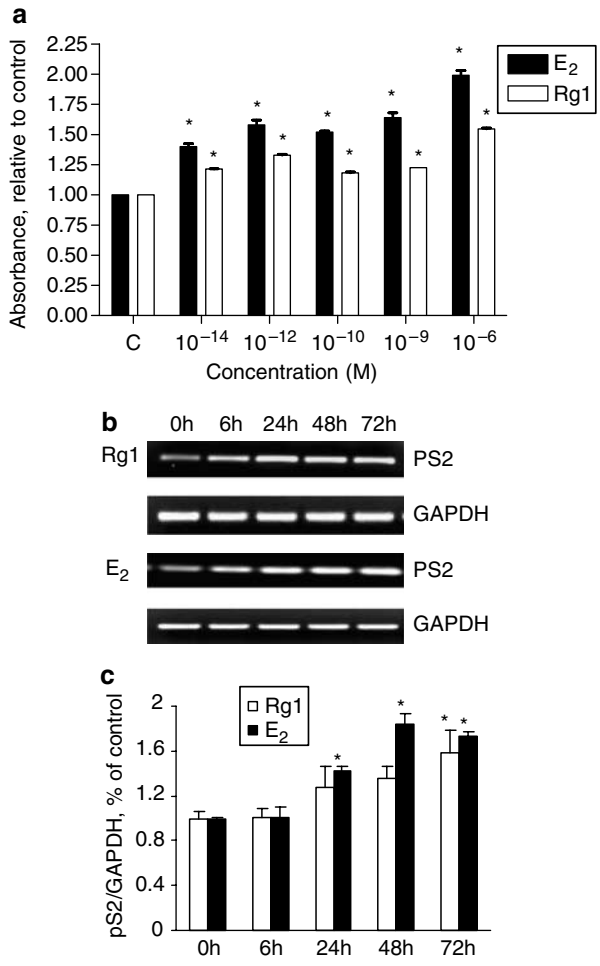

Figure 2 Ginsenoside Rg1 stimulation of cell proliferation and pS2 mRNA expression in human breast cancer (MCF-7) cells. (a) MCF-7 cells were treated with increasing doses of Rg1 and 17-β estradiol (E_2) for 48 h. Cell number was then determined by MTT assay. The result is representative of three independent experiments expressed as mean ± s.e.m. $^*P<0.05$ vs control (c), $n=6$. (b) MCF-7 cells were treated with either 1 pM Rg1 or 10 nM E_2 with increasing time (6, 24, 48 and 72 h). Total RNA was isolated and pS2 and GAPDH mRNA expression were subjected to semiquantitative RT–PCR analysis. The mRNA expression level was expressed as a ratio to the expression of GAPDH. Graphic results shown are representative of three independent experiments and expressed as mean ± s.e.m. $^*P<0.05$ vs control, $n=3$.

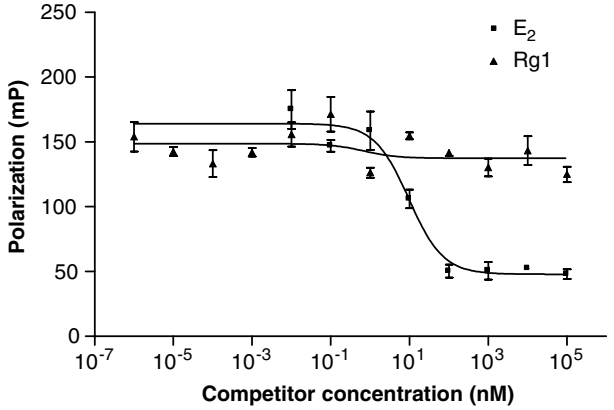

Figure 3 ERα competition assay. Serial dilutions of Rg1 or E_2 mixed with 50 μl of 2 × complex were incubated in the dark at room temperature for 2 h and the polarization values were determined. A sample containing 50 μl of ES_2 screening buffer and 50 μl of 2 × complex was used as a negative control, which represents 0% competition. Results were obtained from three independent experiments and expressed as mean ± s.e.m.

suppress both ER protein and mRNA expressions in MCF-7 cells, and it, though insignificantly, appeared to raise ER protein expression in a time-dependent manner. Thus, it appears that Rg1 behaves differently from E_2 and does not suppress the expression of ERα in MCF-7 cells.

Ginsenoside Rg1 stimulates insulin-like growth factor I receptor (IGF-IR) expression in MCF-7 cells

The fact that Rg1 exerts its E_2-like activity without direct interaction with ERα suggests that other signaling pathways might be involved in mediating its actions. A recent report (Kato *et al*., 2000) indicated that crosstalk exists between ERα and IGF-IR signaling in human breast cancer cells. We, therefore, studied if Rg1 could alter IGF-IR protein expressions in MCF-7 cells in response to Rg1 for a time course ranging from 6 to 72 h. As shown in Figure 5a and c, the expression of IGF-IR protein increased significantly in a time-dependent manner and reached 1.8-fold by 72 h in response to treatment with 1 pM Rg1 ($P<0.05$). Such change mimicked the effect of 10 nM E_2 on IGF-IR protein expression in MCF-7 cells (Figure 5a and c). We then determined if Rg1 would also mimic the effects of E_2 on IGF-IR mRNA expression. As shown in Figure 5b and d, both Rg1 (1 pM) and E_2 (10 nM) induced mRNA expression of IGF-IR mRNA in MCF-7 cells in a time-dependent manner, and reached 1.8- and 2.1-fold, respectively, by 48 h of treatment. Thus, our results suggest that the E_2-like actions of Rg1 might be mediated through the crosstalk between ERα and IGF-IR-dependent signaling pathways *via* the upregulation of IGF-IR expression in MCF-7 cells.

Mechanisms involved in ginsenoside Rg1 induction of IGF-IR expression in MCF-7 cells

Our previous study (Chan *et al*., 2002) reported that the proliferative actions of Rg1 in MCF-7 cells were ER-dependent. To determine if the observed effects of Rg1 on IGF-IR expression are also ER-dependent, ICI 182,780, an estrogen antagonist that abolishes the activation functions (both AF-1 domain and AF-2 domain) of ER, was used. Coincubation of MCF-7 cells with ICI 182,780 (1 μM) completely prevented the upregulation of IGF-IR protein (Figure 6a and c) and mRNA (Figure 6b and d) expression by Rg1 (1 pM) and E_2 (10 nM). These results suggest that the induction of IGF-IR expression by Rg1 requires the functional activity of ER.

To further investigate the potential mechanism involved in upregulation of IGF-IR expression by Rg1, MCF-7 cells treated with either Rg1 or E_2 were coincubated with or without CHX (5 μg ml^{-1}), a *de novo* protein synthesis inhibitor. The expressions of IGF-IR induced by Rg1 and E_2 were completely blocked by CHX at both protein (Figure 7a and c) and mRNA level (Figure 7b and d), indicating that *de novo* protein synthesis is indeed required for the action of Rg1 and E_2 on IGF-IR regulation.

Effect of ginsenoside Rg1 on IGF-IR promoter activity

To determine if the increase in IGF-IR expression by ginsenoside Rg1 was mediated by the upregulation of IGF-IR promoter activities, transient transfection assay was performed. MCF-7 cells were transiently transfected with IGF-IR luciferase promoter p(2350/ + 640)Luc or promoterless luciferase construct (pOLuc) for 5 h, followed by treatment with Rg1 (1 pM) or E_2 (10 nM) for another 24 h. As shown in Figure 8, Rg1 and E_2 significantly increased the IGF-IR promoter luciferase activity and their induction could be abolished by cotreatment with antiestrogen ICI 182,780, while no luciferase activity was determined in the cells which were transfected with promoterless luciferase construct. These results suggest that Rg1 and E_2 could upregulate IGF-IR gene expression by their stimulatory effects on IGF-IR promoter activities, and these effects are also ER dependent.

Ginsenoside Rg1 potentiates the effect of IGF-I on IGF-IR signaling cascade in MCF-7 cells

To investigate if Rg1 can alter IGF-IR signaling cascade, we assessed the responses of tyrosine phosphorylation of IRS-1 in MCF-7 cells to IGF-I (50 ng ml^{-1}) in the presence or absence of 1 pM of Rg1. MCF-7 cells were stimulated with either IGF-1 (5 min) or Rg1 (48 h) separately, or sequentially (Rg1 for 48 h followed by IGF-1 for 5 min). Protein expression level as well as tyrosine phosphorylation of IRS-1 were then determined (Figure 9). The results indicate that the basal tyrosine phosphorylation levels of IRS-1 were low and undetectable in MCF-7 cells (Figure 9b, lane 1). Rg1 alone did not cause an induction of tyrosine phosphorylation of IRS-1 (Figure 9b, lane 2). Treatment with IGF-I for 5 min alone resulted in an increase in tyrosine phosphorylation level of IRS-1 (Figure 9b, lane 3). Most importantly, IGF-I stimulation of MCF-7 cells pretreated with Rg1 resulted in enhanced tyrosine phosphorylation of the IRS-1 (Figure 9b, lane 4). Figure 9a indicates that the observed effects on tyrosine phosphorylation of IRS-1 were not due to unequal loading of proteins for immunoprecipitation as well as Western blotting. These results indicate that Rg1 not only stimulates IGF-IR expression but also enhances tyrosine phosphorylation of downstream signaling proteins, such as IRS-1, in MCF-7 cells.

In order to investigate if the Rg1-mediated enhancement of IRS-1 phosphorylation by IGF-I is ER-dependent, an

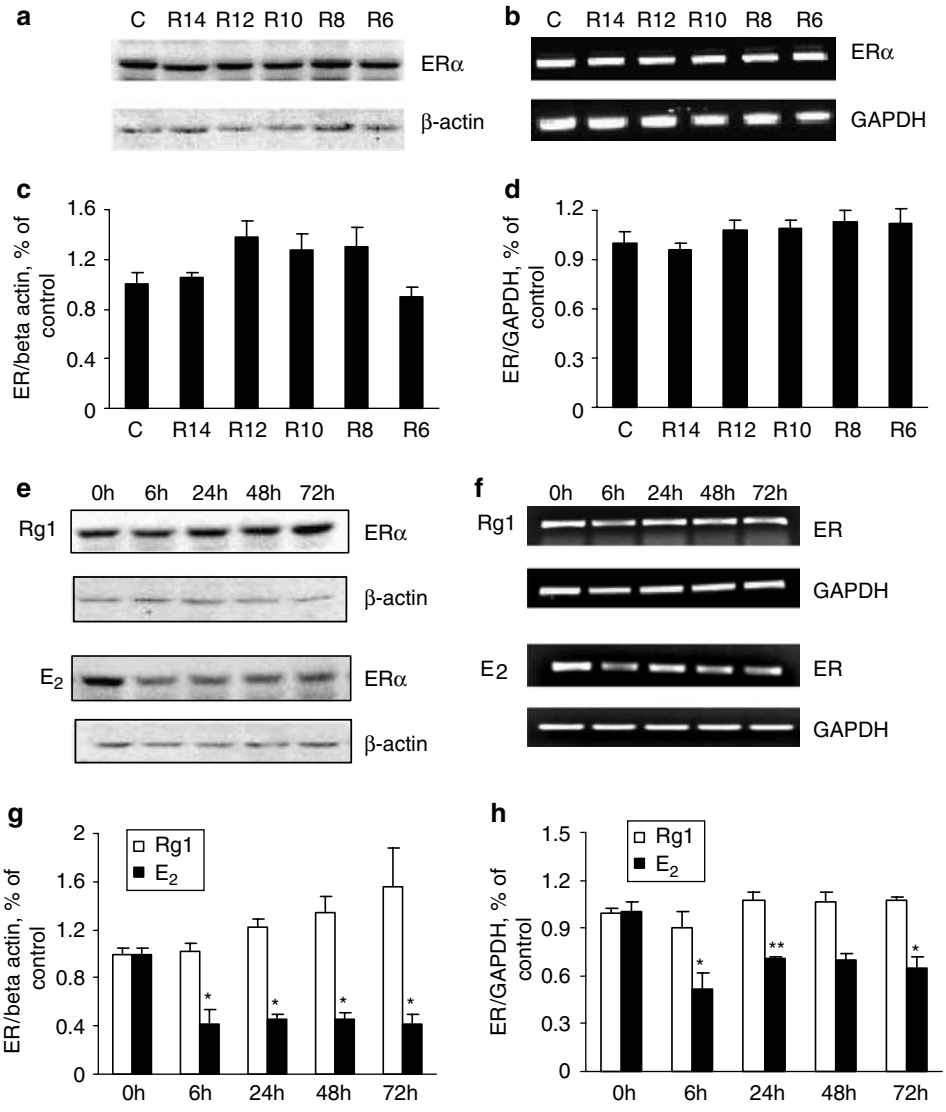

Figure 4 Effect of Rg1 on the ERα protein and ERα mRNA expression. MCF-7 cells were treated with 0.01 pM (R14), 1 pM (R12), 100 pM (R10), 100 nM (R8), 1 μM (R6) Rg1 for 48 h (a, b) and treated with 10 nM E_2 and 1 pM (R12) with increasing time (6, 24, 48 and 72 h) (e, f). Protein and total RNA were isolated and subjected to Western blotting and RT–PCR analysis. (a) The dose response of Rg1 on ERα and β-actin protein expression. (b) The dose response of Rg1 on ERα and GAPDH mRNA expression. (c, d) The graphic representation of the ERα protein and mRNA expression levels, which were expressed as a ratio to the expression of β-actin and GAPDH, respectively. (e) ERα protein and mRNA expression treated with E_2 and Rg1. (f) Semiquantitative RT–PCR analysis of ERα and GAPDH mRNA expression treated with E_2 and Rg1. (g, h) Graphic representation of the ERα protein and mRNA expression levels, which were expressed as a ratio to the expression of β-actin and GAPDH, respectively. Results were obtained from three independent experiments and expressed as mean $\pm$ s.e.m. $^*P<0.05$ vs control, $n=3$.

ER-negative human embryonic kidney (HEK293) cell was used instead of MCF-7 cells. As shown in Figure 9b, treatment with IGF-I for 5 min alone resulted in an increase in tyrosine phosphorylation level of IRS-1 (Figure 9b, lane 7). However, pretreatment of HEK293 cells with Rg1 did not enhance IGF-I-induced tyrosine phosphorylation of the IRS-1 (Figure 9b, lane 8). Thus, the result strongly suggests that the enhancement of IGF-I-induced phosphorylation of IRS-1 by Rg1 requires the presence of ER.

Discussion

Ginsenoside Rg1 is a steroid saponin that shares similar structural features and target organs with steroid hormone, including the brain and cardiovascular system (Attele *et al*., 1999). Ginsenosides are amphiphilic in nature and have the ability to intercalate into the plasma membrane (Banthorpe, 1994). There is evidence suggesting that ginsenosides interact directly with specific membrane proteins (Kimura *et al*., 1994; Brann *et al*., 1995). Moreover, like steroid hormones, Rg1 has previously been shown to interact with glucocorticoid receptor and initiate genomic effects (Lee *et al*., 1997a, b). Our previous study has shown that Rg1 has estrogen-like properties and exerts its action *via* the activation of, but without direct binding to, ERα in human breast cancer MCF-7 cells (Chan *et al*., 2002). The present study further demonstrates that in MCF-7 cells ginsenoside Rg1: (1) mimics the effects of E_2 and induces cell proliferation, ER-dependent transcription of pS2, IGF-IR protein, and mRNA expression, as well as IGF-IR

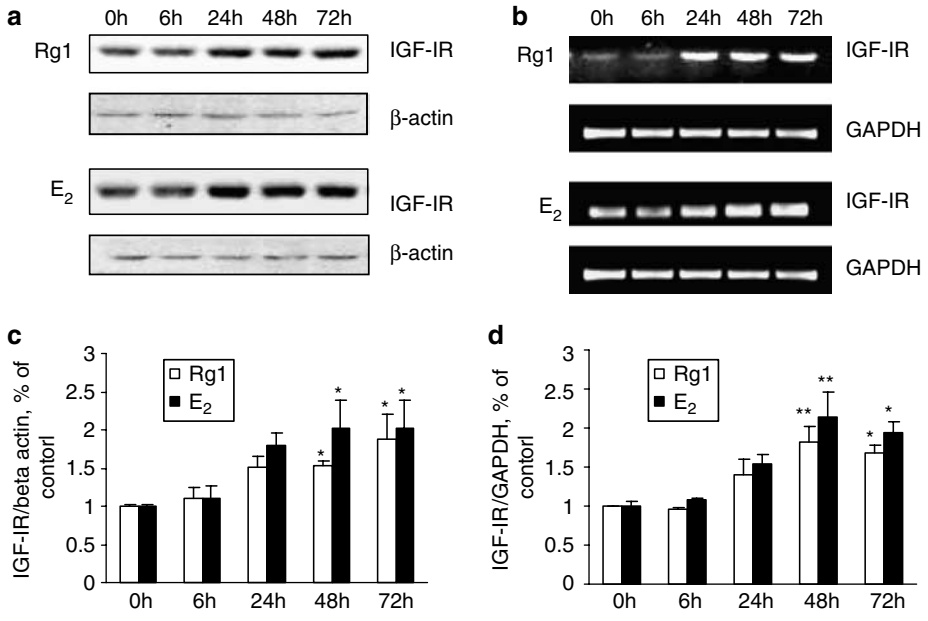

Figure 5 Western blotting and RT–PCR analysis of the expression of IGF-IR in MCF-7 cells treated with Rg1. MCF-7 cells were cultured and treated with 1 pM Rg1 or 10 nM E_2 for 6, 24, 48 and 72 h. (a) IGF-IR and β-actin protein expression. (b) Semiquantitative RT–PCR analysis of IGF-IR and GAPDH mRNA expression. (c, d) Graphical representation of IGF-IR protein and mRNA expression, respectively. Results were obtained from three independent experiments and expressed as mean $\pm$ s.e.m. $^{*}P<0.05$ and $^{**}P<0.01$ vs control, $n=3$.

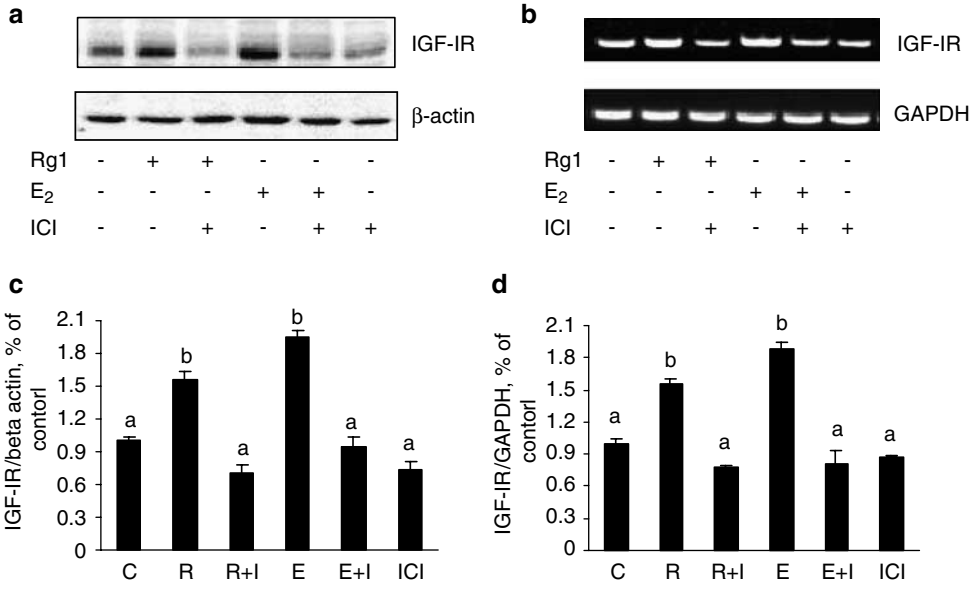

Figure 6 Effect of ICI 182,780 on the regulation of IGF-IR expression by Rg1 or E_2. MCF-7 cells were cultured and treated with 1 pM Rg1 (R) or 10 nM E_2 (E) in the presence and absence of 1 μM ICI 182,780 (ICI) for 48 h. (a) IGF-IR and β-actin protein expression. (b) Semiquantitative RT–PCR analysis of IGF-IR and GAPDH mRNA expression. (c, d) Graphical representation of IGF-IR protein and mRNA expression, respectively. Results shown were obtained from three independent experiments. Columns with different letters are significantly different from one other ($P<0.05$, $n=3$).

promoter activity; (2) does not significantly alter ERα expression; and (3) enhances IGF-I-induced tyrosine phosphorylation of IRS-1, but not IGF-IR.

It is apparent that ER is essential for mediating the actions of Rg1 in MCF-7 cells. In our previous study, Rg1 stimulation of MCF-7 cell proliferation and estrogen response element (ERE)-dependent activation of luciferase reporter gene activity could be completely abolished by cotreatment with a pure ER antagonist, ICI 182,780 (Chan *et al*., 2002). In the present study, our results clearly demonstrate that upregulation of IGF-IR expression and induction of IGF-IR promoter activity by Rg1 could also be completely blocked by cotreatment with ICI 182,780. In addition, our results provide further support that Rg1 behaves as a phytoestrogen and stimulates estrogen-regulated *pS2* gene expression in a time-dependent manner. However, unlike E_2 and other phytoestrogen such as genistein

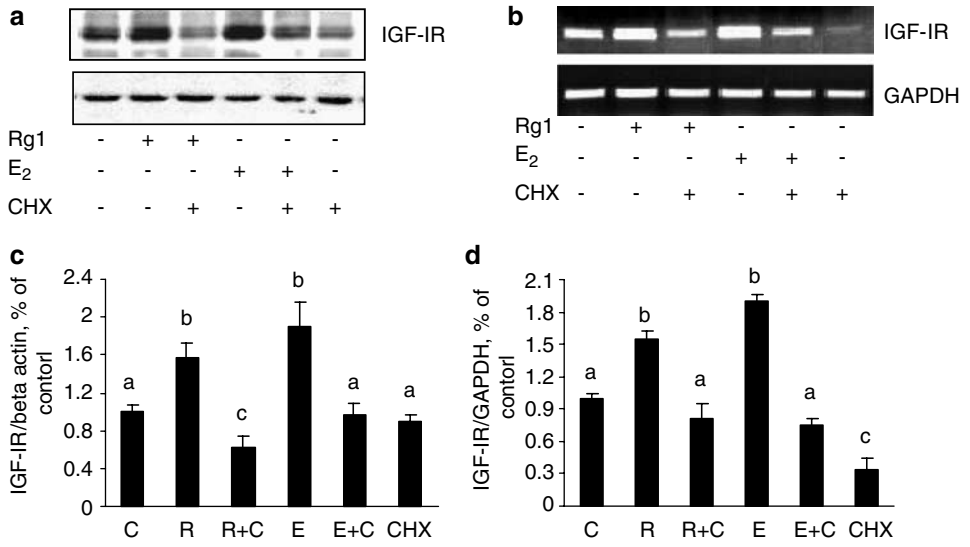

Figure 7 Effect of CHX on the regulation of IGF-IR expression by Rg1 or E_2. MCF-7 cells were cultured and treated with 1 pM Rg1 (R) or 10 nM E_2 (E) in the presence and absence of 1 μM CHX for 48 h. (a) IGF-IR and β-actin protein expression. (b) Semiquantitative RT–PCR analysis of IGF-IR and GAPDH mRNA expression. (c, d) Graphical representation of IGF-IR protein and mRNA expression, respectively. Results shown were obtained from three independent experiments. Columns with different letters are significantly different from one other ($P<0.05$, $n=3$).

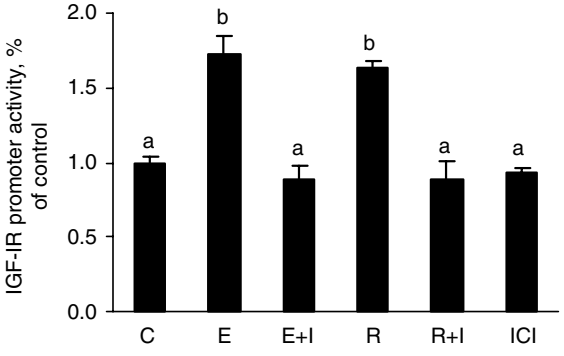

Figure 8 IGF-IR promoter luciferase assay. Activities of luciferases encoded by experimental and internal control plasmid were measured sequentially with the DLR assay reagents. The IGF-IR promoter firefly luciferase activities were normalized with pRL-TK renilla luciferase values. The IGF-IR promter luciferase activity of control was defined as 100%. Results were obtained from three independent experiments and expressed as mean $\pm$ s.e.m. Columns with different letters are significantly different from one other ($P<0.05$, $n=3$).

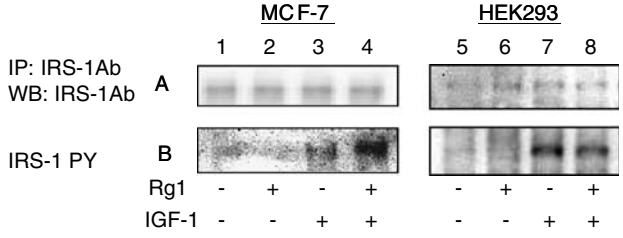

Figure 9 Effects of Rg1 on tyrosine phosphorylation of IRS-1 in MCF-7 and HEK293 cells. MCF-7 and HEK293 cells were stimulated with IGF-1 (50 ng ml^{-1}) for 5 min, Rg1 (1 pM) for 48 h, or with both agents. Cell lysates were immunoprecipitated with anti-IRS-1 (A, B) antibodies. After SDS–PAGE, blots were incubated with anti-IRS-1 (A) or antiphosphotyrosine of IRS-1 (B). IP stands for immunoprecipitation; WB stands for Western blotting and PY stands for tyrosine phosphorylation. Results shown were obtained from three independent experiments; $n=3$.

(Chen & Wong, 2004), Rg1 did not bind to ERα nor suppress its expression level in MCF-7 cells. Thus, the increase in E_2-like activity by Rg1 in MCF-7 cells appears to activate ERα through distinct molecular mechanisms.

The present study clearly demonstrates the involvement of the crosstalk between ER-IGF-IR-dependent signaling pathways in mediating the actions of Rg1 in MCF-7 cells. Rg1 can stimulate IGF-IR expression in MCF-7 cells in a manner similar to E_2. Such mechanism was originally proposed to serve as a means for E_2 to exert autocrine and paracrine control of breast cancer cells mitogenesis (Stewart *et al*., 1992; Lee *et al*., 1997a, b; Kato *et al*., 2000). In addition, Rg1 can enhance IGF-I-induced tyrosine phosphorylation of IRS-1 in MCF-7 cells, suggesting that Rg1 can enhance IGF-IR-mediated signaling pathways in this cell type. Our results using ER-negative HEK293 cells showed that Rg1 failed to increase tyrosine phosphorylation of IRS-1 in the absence of ER, suggesting that ER is required for Rg1 enhancement of IGF-IR-mediated signaling pathways. Thus, our results suggest that the molecular mechanism of Rg1 in MCF-7 cells involves: (1) the ER-dependent enhancement of IGF-IR signaling pathway; (2) the ER-dependent increase in IGF-IR protein and mRNA expression.

Though E_2-like activities of ginseng or its constituents have been reported repeatedly, this study is the first to report that another signaling pathway, namely IGF-IR-mediated pathway, is involved in the E_2-like action of ginsenosides in human breast cancer MCF-7 cells. This newly discovered mechanism of action of Rg1 provides a new insight to understand the pharmacological effects of ginseng in other physiological systems. Accumulating evidence support the fact that crosstalk between ER and IGF-IR pathways plays an important role in

regulating neural functions, including the promotion of neuronal differentiation, survival, and neuroprotection (Duenas *et al*., 1996; Garcia-Segura *et al*., 2000; Gardona-Gomez *et al*., 2002). Treatment of adult ovariectomized rats with E_2 resulted in dose-dependent increase in the phosphorylation of ERK in brain tissues (Gardona-Gomez *et al*., 2002), suggesting that E_2 can activate growth factor receptor-mediated pathways in brain tissues. Previous studies have reported-that Rg1 play a major role in modulating neurotransmission and prevent scopolamine-induced memory deficits by increasing cholinergic activity (Yamaguchi *et al*., 1996). Recent *in vivo* and *in vitro* experiments also showed that ginsenoside Rg1 has neurotropic and neuroprotective effects (Chen *et al*., 2003; Radad *et al*., 2004). Thus, it is possible that the effect of Rg1 in brain tissue might be mediated through the activation of crosstalk between ERα- and IGF-IR-mediated pathways. Further study will be needed to determine the role of IGF-IR-mediated pathway in mediating the molecular actions of Rg1 in brain tissue.

In summary, the present study provides a new paradigm for characterization of the molecular actions of newly identified phytoestrogen. In contrast to other well-characterized phytoestrogens such as genistein (Chen & Wong, 2004), the E_2-like activities of ginsenoside Rg1 are not mediated by direct binding interaction with ERα. Most importantly, our results showed that ER is essential for mediating the actions of Rg1 in stimulation of cell proliferation and IGF-IR expression, as well as in enhancement of growth factor receptor-mediated signaling pathways in human breast cancer cells. However, it should be noted that there are many unresolved issues relating to the mechanism of action of ginsenoside Rg1. For example, it is not clear whether ERβ, a low-abundance isoform of ER in MCF-7 cells, plays a role in mediating the actions of Rg1 and whether Rg1 indeed activates ER in a ligand-independently manner in MCF-7 cells. It is also intriguing to determine the role of these signaling pathways in mediating the action of Rg1 or other ginsenosides in different ER-positive cell types such as those found in brain and bone tissues. Future study will be needed to delineate the detailed mechanism of action of ginsenosides in both MCF-7 cells and other ER-positive cell types, as well as to provide insights for understanding the complex actions of ginseng extract in human body.

We thank Dr LeRoith D. for the IGF-IR promoter construct and the support provided by the State Key Laboratory of Chinese Medicine and Molecular Pharmacology. This work was supported by the Areas of Excellence Scheme Established under the University Grants Committee of the Hong Kong Special Administrative Region, China (AOE/P-10/01), the Area of Strategic Development Grant of the Hong Kong Polytechnic University (A014), the Central allocation grant from the Research Committee of the Hong Kong Polytechnic University (G-W105, G-YC81), and the National Natural Science Foundation of China (30570573).

References

AMATO, P., CHRISTOPHE, S. & MELLON, P.L. (2002). Estrogenic activity of herbs commonly used as remedies for menopausal symptoms. *Menopause,* **9,** 145–150.

ANTER, E., CHEN, K., SHAPIRA, O.M., KARAS, R.H. & KEANEY JR, J.F. (2005). p38 mitogen-activated protein kinase activates eNOS in endothelial cells by an estrogen receptor alpha-dependent pathway in response to black tea polyphenols. *Circ. Res.,* **96,** 1072–1078.

ATTELE, A.S., WU, J.A. & YUAN, C.S. (1999). Ginseng pharmacology multiple constituents and multiple actions. *Biochem. Pharmacol.,* **58,** 1685–1693.

BANTHORPE, D.V. (1994). In: *Terpenoids: Natural Products,* ed. Mann J. pp. 331–339. Essex: Longman Scientific and Technical.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.,* **72,** 248–254.

BRANN, D.W., HENDRY, L.B. & MAHESH, V.B. (1995). Emerging diversities in the mechanism of action of steroid hormones. *J. Steroid Biochem. Mol. Biol.,* **52,** 113–133.

CATALANO, S., MAURO, L., MARSICO, S., GIORDANO, C., RIZZA, P., RAGO, V., MONTANARO, D., MAGGIOLINI, M., PANNO, M.L. & ANDO, S. (2004). Leptin induces, *via* ERK1/ERK2 signal, functional activation of estrogen receptor alpha in MCF-7 cells. *J. Biol. Chem.,* **279,** 19908–19915.

CHAN, R.Y., CHEN, W.F., DONG, A., GUO, D. & WONG, M.S. (2002). Estrogen-like activity of ginsenoside Rg1 derived from Panax notoginseng. *J. Clin. Endocrinol. Metab.,* **87,** 3691–3695.

CHEN, W.F. & WONG, M.S. (2004). Genistein enhances insulin-like growth factor signaling pathway in human breast cancer (MCF-7) cells. *J. Clin. Endocrinol. Metab.,* **89,** 2351–2359.

CHEN, X.C., FANG, F., ZHU, Y.G., CHEN, L.M., ZHOU, Y.C. & CHEN, Y. (2003). Protective effect of ginsenoside Rg1 on MPP+-induced apotosis in SHSY5Y cells. *J. Neural. Transm.,* **110,** 835–845.

CHO, J., PARK, W., LEE, S., AHN, W. & LEE, Y. (2004). Ginsenoside-Rb1 from Panax ginseng C.A. Meyer activates estrogen receptor-α and β, independent of ligand binding. *J. Clin. Endocrinol. Metab.,* **89,** 3510–3515.

CUI, L., WU, T., LIU, X.Q., LI, Q.N., LIN, L.S. & LIANG, N.C. (2001). Preventive effects of ginsenosides on osteopenia of rats induced by ovariectomy. *Acta Pharmacol. Sin.,* **22,** 428–434.

DENIZOT, F. & LAND, R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods.,* **89,** 271–277.

DUDA, R.B., TABACK, B., KESSEL, B., DOOLEY, D.D., YANG, H., MARCHIORI, J., SLOMOVIC, B.M. & ALVAREZ, J.G. (1996). pS2 expression induced by American ginseng in MCF-7 breast cancer cells. *Ann. Surg. Oncol.,* **3,** 515–520.

DUENAS, M., TORRES-ALEMAN, I., NAFTOLIN, F. & GARCIA-SEGURA, L.M. (1996). Interaction of insulin-like growth factor I and estradiol signaling pathways on hypothalamic neuronal differentiation. *Neuroscience,* **74,** 531–539.

GARCIA-SEGURA, L.M., CARDONA-GOMEZ, G.P., CHOWEN, J.A. & AZCOITIA, I. (2000). Insulin-like growth factor-I receptors and estrogen receptors interact in the promotion of neuronal survival and neuroprotection. *J. Neurocytol.,* **29,** 425–437.

GARDONA-GOMEZ, G.P., MENDEZ, P., DONCARLOS, L.L., AZCOITIA, I. & GARCIA-SEGURA, L.M. (2002). Interaction of estrogen and insulin-like growth factor-I in the brain: molecular mechanisms and functional implications. *J. Steroid. Biochem. Mol. Biol.,* **83,** 211–217.

KARAS, R.H., GAUER, E.A., BIEBER, H.E., BAUR, W.E. & MENDELSOHN, M.E. (1998). Growth factor activation of the estrogen receptor in vascular cells occurs *via* a mitogen-activated protein kinase-independent pathway. *J. Clin. Invest.,* **101,** 2851–2861.

KATO, S., ENDOH, H., MASUHIRO, Y., KITAMOTO, T., UCHIYAMA, S., SASAKI, H., MASUSHIGE, S., GOTOH, Y., NISHIDA, E. & KAWASHIMA, H. (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science,* **270,** 1491–1494.

KATO, S., MASUHIRO, Y., WATANABE, M., KOBAYASHI, Y., TAKEYAMA, K.I., ENDOH, H. & YANAGISAWA, J. (2000). Molecular mechanism of a cross-talk between oestrogen and growth factor signaling pathways. *Genes Cells,* **5,** 593–601.

KIM, Y.S., KIM, D.S. & KIM, S.I. (1998). Ginsenoside Rh2 and Rh3 induce differentiation of HL-60 cells into granulocytes: modulation of protein kinase C isoforms during differentiation by ginsenoside Rh2. *Int. J. Biochem. Cell Biol.,* **30,** 327–338.

KIMURA, T., SAUNDERS, P.A., KIM, H.S., RHEU, H.M., OH, K.W. & HO, I.K. (1994). Interactions of ginsenosides with ligand-bindings of GABA(A) and GABA(B) receptors. *Gen. Pharmacol.,* **25,** 193–199.

KLINGE, C.M., BLANKENSHIP, K.A., RISINGER, K.E., BHATNAGAR, S., NOISIN, E.L., SUMANASEKERA, W.K., ZHAO, L., BREY, D.M. & KEYNTON, R.S. (2005). Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors alpha and beta in endothelial cells. *J. Biol. Chem.,* **280,** 7460–7468.

LEE, A.V., JACKSON, J.G., GOOCH, J.L., HILSENBECK, S.G., CORONADO-HEINSOHN, E., OSBORNE, C.K. & YEE, D. (1999). Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression *in vitro* and *in vivo*. *Mol. Endocrinol.,* **13,** 787–796.

LEE, A.V., WENG, C.N., JACKSON, J.G. & YEE, D. (1997a). Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *J. Endocrinol.,* **152,** 39–47.

LEE, Y., JIN, Y., LIM, W., JI, S., CHOI, S., JANG, S. & LEE, S. (2003a). A ginsenoside-Rh1, a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells. *J. Steroid Biochem. Mol. Biol.,* **84,** 463–468.

LEE, Y.J., CHUNG, E., LEE, K.Y., LEE, Y.H., HUH, B. & LEE, S.K. (1997b). Ginsenoside-Rg1, one of the major active molecules from *Panax ginseng*, is a functional ligand of glucocorticoid receptor. *Mol. Cell. Endocrinol.,* **133,** 135–140.

LEE, Y.J., JIN, Y.R., LIM, W.C., JI, S.M., CHO, J.Y., BAN, J.J. & LEE, S.K. (2003b). Ginsenoside Rc and Re stimulate c-fos expression in MCF-7 human breast carcinoma cells. *Arch. Pharm. Res.,* **26,** 53–57.

LEE, Y.J., JIN, Y.R., LIM, W.C., PARK, W.K., CHO, J.Y., JANG, S. & LEE, S.K. (2003c). Ginsenoside-Rb1 acts as a weak phytoestrogen in MCF-7 human breast cancer cells. *Arch. Pharm. Res.,* **26,** 58–63.

LIU, C.X. & XIAO, P.G. (1992). Recent advances on ginseng research in China. *J. Ethnopharmacol.,* **36,** 27–38.

LIU, J., BURDETTE, J.E., XU, H., GU, C., VAN BREEMEN, R.B., BHAT, K.P., BOOTH, N., CONSTANTINOU, A.I., PEZZUTO, J.M., FONG, H.H., FARNSWORTH, N.R. & BOLTON, J.L. (2001). Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J. Agric. Food Chem.,* **49,** 2472–2479.

MASIAKOWSKI, P., BREATHNACH, R., BLOCH, J., GANNON, F., KRUST, A. & CHAMBON, P. (1982). Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Res.,* **10,** 7895–7903.

MATTSON, D.L. & BELLEHUMEUR, T.G. (1996). Comparison of three chemiluminescent horseradish peroxidase substrates for immunoblotting. *Anal. Biochem.,* **240,** 306–308.

MOCHIZUKI, M., YOO, Y.C., MATSUZAWA, K., SATO, K., SAIKI, I., TONO-OKA, S., SAMUKAWA, K. & AZUMA, I. (1995). Inhibitory effect of tumor metastasis in mice by saponins, ginsenoside-Rb2, 20(R)- and 20(S)-ginsenoside-Rg3, of red ginseng. *Biol. Pharm. Bull.,* **18,** 1197–1202.

MURKIES, A.L., WILCOX, G. & DAVIS, S.R. (1998). Clinical review 92: phytoestrogens. *J. Clin. Endocrinol. Metab.,* **83,** 297–303.

PUNNONEN, R. & LUKOLA, A. (1980). Oestrogen-like effect of ginseng. *Br. Med. J.,* **281,** 1110.

RADAD, K., GILLE, G., MOLDZIO, R., SAITO, H. & RAUSCH, W.D. (2004). Ginsenoside Rb1 and Rg1 effects on mesencephalic dopaminergic cells stressed with glutamate. *Brain Res.,* **1021,** 42–53.

SOTO, A.M., FERNANDEZ, M.F., LUIZZI, M.F., OLES KARASKO, A.S. & SONNENSCHEIN, C. (1997). Developing a marker of exposure to xenoestrogen mixtures in human serum. *Environ. Health Perspect.,* **105** (Suppl 3), 647–654.

SRIUSSADAPORN, S., WONG, M.S., PIKE, J.W. & FAVUS, M.J. (1995). Tissue specificity and mechanism of vitamin D receptor up-regulation during dietary phosphorus restriction in the rat. *J. Bone Miner. Res.,* **10,** 271–280.

STEWART, A.J., WESTLEY, B.R. & MAY, F.E. (1992). Modulation of the proliferative response of breast cancer cells to growth factors by oestrogen. *Br. J. Cancer,* **66,** 640–648.

THAM, D.M., GARDNER, C.D. & HASKELL, W.L. (1998). Clinical review 97: potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J. Clin. Endocrinol. Metab.,* **83,** 2223–2235.

WAKABAYASHI, C., MURAKAMI, K., HASEGAWA, H., MURATA, J. & SAIKI, I. (1998). An intestinal bacterial metabolite of ginseng protopanaxafiol saponins has the ability to induce apoptosis in tumor cells. *Biochem. Biophys. Res. Commun.,* **246,** 725–730.

YAMAGUCHI, Y., HIGASHI, M. & KOBAYASHI, H. (1996). Effects of oral and intraventricular administration of ginsenosides Rg1 on the performance impaired by scopolamine in the rats. *Biomed. Res.,* **17,** 487–490.

(Received November 1, 2005
Accepted November 25, 2005
Published online 16 January 2006)