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Ginsenoside Re, a Main Phytosterol of *Panax ginseng*, Activates Cardiac Potassium Channels via a Nongenomic Pathway of Sex Hormones

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ABSTRACT

Ginseng root is one of the most popular herbs throughout the world and is believed to be a panacea and to promote longevity. It has been used as a medicine to protect against cardiac ischemia, a major cause of death in the West. We have previously demonstrated that ginsenoside Re, a main phytosterol of *Panax ginseng*, inhibits Ca^{2+} accumulation in mitochondria during cardiac ischemia/reperfusion, which is attributable to nitric oxide (NO)-induced Ca^{2+} channel inhibition and K^+ channel activation in cardiac myocytes. In this study, we provide compelling evidence that ginsenoside Re activates endothelial NO synthase (eNOS) to release NO, resulting in activation of the slowly activating delayed rectifier K^+ current. The eNOS activation occurs via a nongenomic pathway of each of androgen receptor, estrogen receptor-α, and progesterone receptor, in which c-Src, phosphoinositide 3-kinase, Akt, and eNOS are sequentially activated. However, ginsenoside Re does not stimulate proliferation of androgen-responsive LNCaP cells and estrogen-responsive MCF-7 cells, implying that ginsenoside Re does not activate a genomic pathway of sex hormone receptors. Fluorescence resonance energy transfer experiments with a probe, *SCCoR* (single cell coactivator recruitment), indicate that the lack of genomic action is attributable to failure of coactivator recruitment. Thus, ginsenoside Re acts as a specific agonist for the nongenomic pathway of sex steroid receptors, and NO released from activated eNOS underlies cardiac K^+ channel activation and protection against ischemia-reperfusion injury.

The earliest evidence of humans' use of herbs for healing dates back to the Neanderthal period (Winslow and Kroll, 1998; Goldman, 2001). In the late 20th century, concerns over the iatrogenic effects of conventional medicine and desire for more self-reliance led to increased interest in natural health, and use of herbal medicines again became popular (Winslow and Kroll, 1998; Goldman, 2001). Among the >20,000 herbal products that are currently on the market, ginseng root is one of the most popular herbs (Attele et al., 1999). Ginseng is known as a panacea (cure-all), and it exhibits a variety of actions, including modulation of immune responses and antineoplastic effects (Attele et al., 1999). Although estrogenic activities (Kim et al., 2004) and nitric oxide (NO) action (Gillis, 1997) have been suggested as a mechanism of ginseng's actions, the precise mechanism remains unknown, which is major hindrance for use of ginseng in modern medicine.

Ginseng root exhibits protection against cardiac ischemia-

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ABBREVIATIONS: NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; E_2, 17β-estradiol; DHT, 5α-dihydrotestosterone; AR, androgen receptor; ERα, estrogen receptor-α; PR, progesterone receptor; FRET, fluorescence resonance energy transfer; *SCCoR*, single cell coactivator recruitment; LBD, ligand binding domain; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; P_4, progesterone; SMTC, *S*-methyl-L-thiocitrulline; L-NIO, L-N_5-(l-iminoethyl)ornithine; SH-6, D-2,3-dideoxy-myo-inositol 1-[(*R*)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; ICI182,780, fulvestrant; DMSO, dimethyl sulfoxide; PI3, phosphoinositide 3; pyrazole, 1,2,5-tris(4-hydroxyphenyl)-4-propylpyrazole; estren, 4-estren-3α,17β-diol; DMEM, Dulbecco's modified Eagle medium; $I_{Ca,L}$, L-type Ca^{2+} current; I_{Ks}, the slowly activating delayed rectifier K^+ current.

reperfusion injury (Gillis, 1997), a major cause of death in the West. We have demonstrated previously, in an in vivo rat model, that ginsenoside Re, one of the main constituents of *Panax ginseng*, prevents accumulation of mitochondrial Ca^{2+} in the heart during ischemia-reperfusion injury (Bai, 1993). We have also reported in isolated single cardiomyocytes that ginsenoside Re inhibits L-type Ca^{2+} current ($I_{Ca,L}$) and enhances the slowly activating delayed rectifier K^+ current (I_{Ks}), which we consider a possible mechanism underlying prevention of mitochondrial Ca^{2+} overload (Bai et al., 2003, 2004). Both inhibition of $I_{Ca,L}$ and activation of I_{Ks} by ginsenoside Re are attributable to NO actions, because NO trappers and NO synthase (NOS) inhibitors prevented ginsenoside Re-induced $I_{Ca,L}$ inhibition and I_{Ks} enhancement (Bai et al., 2004). However, the way in which ginsenoside releases NO is still an enigma. In the present study, therefore, we use NO-dependent I_{Ks} activation to unveil the mechanism by which ginsenoside releases NO. Results indicate that ginsenoside acts as a specific agonist for the nongenomic pathway of sex hormone receptors; it activates endothelial NOS (eNOS) and releases NO without activation of the genomic pathway.

Materials and Methods

The investigation was conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Patch-Clamp Experiments. Single ventricular myocytes were harvested from adult female guinea pig hearts, and I_{Ks} was recorded with a perforated configuration of patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) as described previously (Bai et al., 2005b). I_{Ks} was elicited by a 3.5-s depolarizing pulse from a holding potential of −40 mV to various test potentials between −30 and +50 mV in 10-mV increments at 0.1 Hz. All experiments were performed at 36 ± 1°C.

External solution was K^+-free solution containing 135 mM NaCl, 0.33 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5.0 mM 2-HEPES (pH adjusted to 7.4 with NaOH) that is known to suppress I_{Kr} and enhance I_{Ks} (Sanguinetti and Jurkiewicz, 1992). Nisoldipine (3 μM) and E-4031 (10 μM), drugs that selectively block $I_{Ca,L}$ and I_{Kr} channels, were added to bath solution. The standard pipette solution contained 110 mM aspartic acid, 30 mM KCl, 5.0 mM magnesium-ATP, 5.0 mM creatine phosphate dipotassium salt, and 5.0 mM HEPES (pH adjusted to 7.25 with KOH). Amphotericin B (Sigma-Aldrich, St. Louis, MO) was used in pipette solution to achieve patch perforation. Amphotericin B was prepared as a 600 mg/ml stock solution in dimethyl sulfoxide (DMSO) and diluted to 600 μg/ml in the pipette solution. We front-filled patch pipettes by dipping them into pipette solution and then back-filled with pipette solution containing amphotericin B (600 μg/ml). The averaged membrane capacitance in 119 cells was 150 ± 13 pF.

Immunoblot Analysis. Immunoblot analysis was performed as described previously (Zheng et al., 2002). In brief, cardiomyocytes isolated from adult guinea pig ventricles were maintained in culture medium without serum or growth factors for 1 h and were incubated with culture medium to which ginsenoside Re (10 μM) with or without various blockers was added for 15 min. Cell lysates were prepared from approximately 1×10^8 cardiomyocytes; those with 20 μg of total proteins were electrophoresed on SDS/acrylamide gels and subjected to immunoblot analysis by incubation with a 1:1000-diluted anti-phosphoAkt (^{473}Ser) antibody (Cell Signaling, Danvers, MA) or a 1:1000-diluted anti-Akt antibody (Cell Signaling), followed by incubation with a 1:40,000-diluted horseradish peroxidase-conjugated anti-rabbit IgG (Dako Japan Co. Ltd., Kyoto, Japan). Proteins were detected using an advanced enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

Proliferation Assay of MCF-7 and LNCaP. MCF-7 cells were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan), and LNCaP cells from American Type Culture Collection (Manassas, VA). They were maintained in 1:1 Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (F12) medium (DMEM/F12) with 10% fetal bovine serum at 37°C in a moist environment. Cells were seeded in triplicate at a density of 1.6×10^5 cells/ml in phenol-red-free DMEM/F12 with 10% charcoal-treated fetal bovine serum. Five days after cells had been incubated in the presence of 17β-estradiol (E_2; 10 nM), 5α-dihydrotestosterone (DHT; 10 nM), or ginsenoside Re (10 μM), they were collected and cell numbers were counted.

Receptor-Binding Assay. Binding of ginsenoside Re to the androgen receptor (AR), estrogen receptor-α (ERα), and progesterone receptor (PR) was analyzed with the receptor competitor assay (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, fluorescently tagged receptor ligands that are bound to the ligand-binding domain (LBD) of the human AR, ERα, and PR show high fluorescent polarization; displacement of fluorescently tagged ligands by unlabeled ligands decrease fluorescent polarization (Boyer et al., 2000). In this system, the change in polarization reflects displacement of fluorescently tagged ligands (Boyer et al., 2000); therefore, we measured the change in fluorescent polarization with a polarizer-attached fluorescent spectrometer (FP-6500; JASCO Corporation, Tokyo, Japan).

A Fluorescence Resonance Energy Transfer-Based Coactivator Recruitment Assay. Recruitment of coactivator upon agonist binding to ERα receptor was assayed using a FRET indicator, *SCCoR* (single cell coactivator recruitment), as described previously (Awais et al., 2004, 2006). In brief, an intramolecular FRET-based indicator was constructed to visualize the ligand-dependent recruitment of a coactivator peptide containing a LXXLL motif to the ERα-LBD connected via a short flexible linker. This fusion protein was sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) in such a way that excitation and emission spectra of these fluorescent proteins are suitable for FRET in single living cells. The indicator was designated as ER-*SCCoR*. An agonist promotes interaction between a receptor and a coactivator within *SCCoR*, which results in an increase in FRET from CFP to YFP. In contrast, an antagonist inhibits receptor/coactivator interaction. To construct AR-*SCCoR* and PR-*SCCoR*, the LBD of ER in the ER-SCCoR was replaced with the LBD of AR and PR, respectively (Awais et al., 2004, 2006). CHO-K1 cells (ATCC) were transfected with indicators for AR-*SCCoR*, ERα-*SCCoR*, or PR-*SCCoR* in the presence of LipofectAMINE 2000 reagent (Invitrogen) in glass-bottomed dishes. Twelve to 24 h after transfection, cells were imaged at room temperature using a microscope (Axiovert 135; Carl Zeiss, Jena, Germany) with a cooled charged-coupled device camera MicroMAX (Roper Scientific Inc., Tucson, AZ), controlled by MetaFluor (Molecular Devices). Cells were excited at 440 ± 10 nm for 100 ms, and fluorescence images were obtained using filters at 480 ± 15 and 535 ± 12.5 nm in a microscope with a 40× oil immersion objective.

Reagents. E-4031 was purchased from Eisai Co. Ltd. (Tokyo, Japan); ginsenoside Re, DHT, mifepristone, and progesterone (P_4) were purchased from Wako (Osaka, Japan); nisoldipine, *S*-methyl-L-thiocitrulline (SMTC), L-N_5-(l-iminoethyl)ornithine (L-NIO), wortmannin, and E_2 were purchased from Sigma-Aldrich; SH-6 and PP2 were purchased from Merck (Darmstadt, Germany), and ICI182,780 and nilutamide were purchased from Tocris (Ellisville, MO). Stock solutions of E_2 (5 mM), DHT (5 mM), and mifepristone (10 mM) were prepared in ethanol; those for E-4031 (5 mM), SMTC (5 mM), and L-NIO (1 mM) in distilled water; and those for nisoldipine (10 mM), SH-6 (20 mM), PP2 (20 mM), wortmannin (5 mM), ICI182,780 (5 mM), and nilutamide (5 mM) in DMSO. They were diluted in the

bath solution to achieve the desired concentrations. The final concentrations of ethanol [<0.01% (v/v)] and DMSO [<0.05% (v/v)] did not affect K^+ channel activity, shape and proliferation of cells, or a FRET signal.

Data Analysis. All values are presented as mean ± S.E. Statistical significance was examined by repeated-measures nonparametric Friedman test for experiments of time course of I_{Ks}, multiple comparison with Kruskal-Wallis test followed by Dunn's multiple comparison test for immunoblot analysis and cell proliferation assay, and analysis of variance followed by paired Student's *t* test for FRET experiments. A *p* value less than 0.05 was considered to be significant.

Results

Both Panaxadiols and Panaxatriols Activate I_{Ks}. Ginseng root contains more than 30 types of ginsenosides divided into two major groups based on their chemical structure; panaxadiols with sugar moieties at the C-3 and C-21 positions of the sterol structure, and panaxatriols with sugar moieties at positions C-6 and C-21 (Kaku et al., 1975). We have previously reported that ginsenoside Re enhanced I_{Ks} in cardiac myocytes via a NO-dependent manner (Bai et al., 2004). To examine whether activation of I_{Ks} was specific to ginsenoside Re, we tested five ginsenosides that are commercially available: three panaxadiols (Rb1, Rc, Rd) and two panaxatriols (Re, Rg1) (Fig. 1A). Both panaxatriols (Re and Rg1) and panaxadiols (Rb1 and Rc) activated I_{Ks} (Fig. 1B). EC_{50} values were similar among Rb1, Rc, Re, and Rg1, but their maximum responses differed. The maximum extent of I_{Ks} activation was greatest for Re followed by Rc, Rg1, and Rb1, whereas Rd did not activate I_{Ks} (Fig. 1B). Because ginsenoside Re is the most potent among the five ginsenosides, in the following experiments, we used ginsenoside Re-induced I_{Ks} enhancement to examine the mechanism by which ginsenoside Re produces NO.

Ginsenoside Re Releases NO via eNOS Activation. The current-voltage curves showed that enhancement of I_{Ks} by ginsenoside Re (3 μM) was voltage-independent (Fig. 2A), which agrees with our previous report (Bai et al., 2003); thus, test potential at a single voltage (+50 mV) was used to analyze statistical significant changes. Ginsenoside Re-induced I_{Ks} enhancement started to occur within approximately 5 min and reached a pseudo-steady state between 10 and 15 min after its application, implying a role of constitutive NOS, neuronal NOS (nNOS), or eNOS, rather than inducible NOS. Application of SMTC at 3 μM, a concentration that inhibits nNOS but not eNOS (Narayanan and Griffith, 1994), did not alter enhancement of I_{Ks} amplitude by ginsenoside Re (Fig. 2B), whereas L-NIO at 1 μM, a concentration that inhibits eNOS but not nNOS (McCall et al., 1991), decreased I_{Ks} to the initial levels observed before ginsenoside Re application (Fig. 2C), indicating that ginsenoside Re produces NO via eNOS activation.

Ginsenoside Re Activates eNOS via a Phosphoinositide 3-Kinase/Akt-Dependent Pathway. eNOS is activated at least through two mechanisms: a Ca^{2+}-dependent mechanism involving the Ca^{2+}-binding protein calmodulin (Kone, 2000; Goligorsky et al., 2002) and a phosphorylation-dependent mechanism involving the serine/threonine kinase Akt (Kone, 2000; Goligorsky et al., 2002). SH-6 (10 μM), an Akt inhibitor, completely reversed enhancement of I_{Ks} by ginsenoside Re (Fig. 3A). c-Src and PI3-kinase are key upstream signaling molecules of Akt. Ginsenoside Re-induced I_{Ks} activation was inhibited by the c-Src inhibitor PP2 (Fig. 3B) and the PI3-kinase inhibitor wortmannin (Fig. 3C), indicating that ginsenoside Re activates eNOS via a c-Src/PI3-kinase/Akt-dependent mechanism.

As a complementary experiment, we examined effects of preincubation of various blockers on ginsenoside-induced I_{Ks} enhancement. The fractional enhancement of I_{Ks} was obtained as I_{Ks} tail currents averaged from five consecutive traces in the steady state after drug application divided by control I_{Ks} tail currents averaged from five consecutive traces just before drug application. We first examined effects of SMTC, L-NIO, SH-6, PP2, and wortmannin on I_{Ks} in the control condition; I_{Ks} density was slightly decreased (Fig. 4, B–G). However, the magnitude of I_{Ks} reduction was not significantly different from the value at the corresponding time (30 min) in the time-control experiments without addition of any reagents (Fig. 4, A and G), suggesting that the observed I_{Ks} reduction is time-dependent run-down of I_{Ks}, rather than specific effects of blockers. Then, we found that preincubation with L-NIO, SH-6, PP2, and wortmannin abolished enhancement of I_{Ks} by ginsenoside Re, whereas preincubation with SMTC did not affect enhancement of I_{Ks} by ginsenoside Re

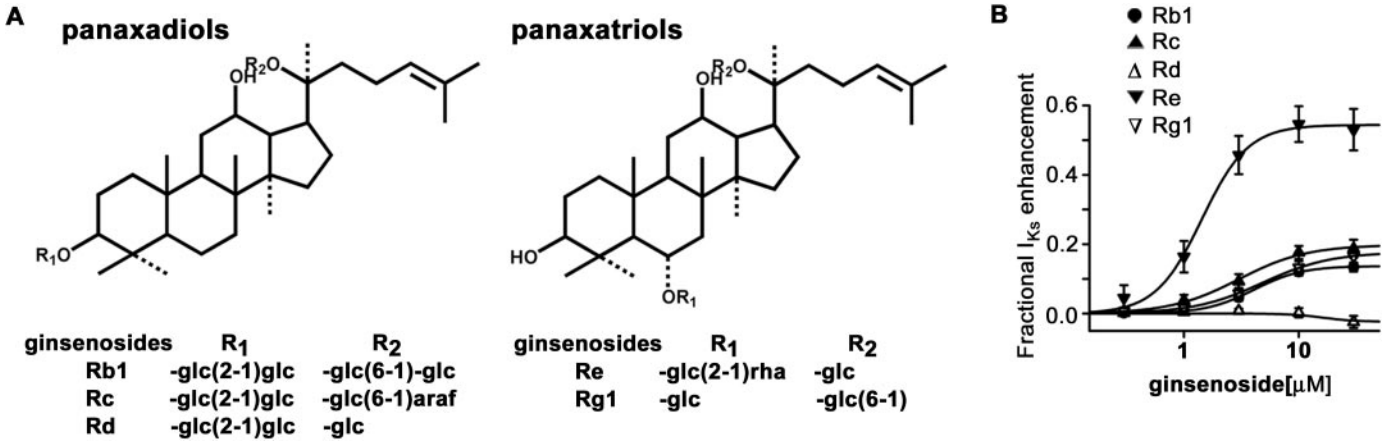

Fig. 1. Both panaxadiols and panaxatriols activate I_{Ks}. A, chemical structures of panaxadiols and panaxatriols used. B, dose-response curves for I_{Ks} activation by five ginsenosides. Continuous lines are results of fitting to the Hill equation. The EC_{50} values were 4.1 ± 0.5 μM for Rb1, 3.0 ± 0.5 μM for Rc, 1.4 ± 0.4 μM for Re, and 4.8 ± 0.7 μM for Rg1; the maximum responses were 13.7 ± 2.3, 19.8 ± 3.1, 54.4 ± 7.1, and 17.7 ± 4.1%, respectively. Ginsenoside Rd did not significantly enhance I_{Ks}.

(Fig. 4, B–G), further supporting that ginsenoside Re activates eNOS via a c-Src/PI-3kinase/Akt-dependent mechanism.

Ginsenoside Re Activates I_{Ks} via Sex-Hormone Receptors. Receptor-type tyrosine kinases and receptors that link to nonreceptor type tyrosine kinases, such as c-Src, activate PI3-kinase (Porter and Vaillancourt, 1998; Wymann and Pirola, 1998). Those include growth factor receptors, an insulin receptor, and gonadal steroid receptors (Porter and Vaillancourt, 1998; Wymann and Pirola, 1998). Gonadal steroids such as testosterone and E_2 exert some biological effects that are too rapid (seconds to minutes) to be compatible with the conventional transcriptional mechanism ("nontranscriptional mechanism") (Weiss and Gurpide, 1998; Baron et al., 2004). We have recently demonstrated that testosterone and E_2 enhance I_{Ks} via the nontranscriptional pathway involving Akt-dependent eNOS activation in cardiomyocytes (Bai et al., 2005a). Because ginsenoside has a four-ring steroid-like structure (Fig. 1A) (Kaku et al., 1975) and exhibits estrogenic activities (Kim et al., 2004), we tested the hypothesis of whether ginsenoside Re exhibited its action via activation of gonadal steroid receptors using inhibitors of sex steroid receptors. Nilutamide (1 μM), ICI-182,780 (5 μM), and mifepristone (1 μM), inhibitors of AR, ERα, and PR, respectively, partially inhibited ginsenoside Re-induced I_{Ks} enhancement in cardiac myocytes (Fig. 5, A–C). A simultaneous application of three inhibitors completely inhibited I_{Ks} enhancement by ginsenoside Re (Fig. 5D).

Ginsenoside Re Induces Akt Phosphorylation. Phosphorylation of Akt at ^{473}Ser occurs when Akt is activated via a PI3-kinase-dependent pathway (Kohn et al., 1996). We confirmed that ginsenoside Re induced phosphorylation of Akt in cardiomyocytes in a concentration-dependent manner (Fig. 6, A and B). Phosphorylation of Akt in cardiac myocytes was inhibited by PP2, wortmannin, and SH-6 (Fig. 6, C and D). It was partially inhibited by ICI182,780, nilutamide, or mifepristone and was completely inhibited by a combination of ICI182,780, nilutamide, and mifepristone (Fig. 6, C and D). These biochemical data further confirm that ginsenoside Re activates Akt via the nongenomic pathway of gonadal steroid receptors.

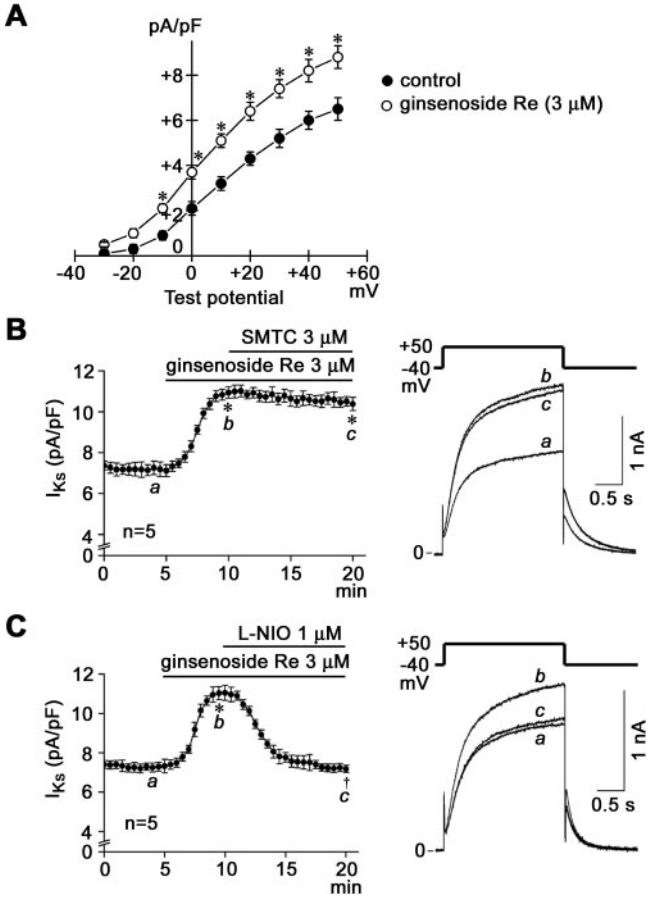

Fig. 2. NO produced by eNOS, but not nNOS, is responsible for I_{Ks} enhancement by ginsenoside Re. A, the current-voltage curves in the absence (control) and presence of ginsenoside Re (3 μM). *, $p < 0.05$ versus control. B and C, effects of SMTC (B), a nNOS inhibitor, and L-NIO (C), an eNOS inhibitor, on I_{Ks} enhancement by ginsenoside Re. Left, time course of experiments in 5 cells. *x*-Axis is time after start of experiments, and *y*-axis is averaged current density of I_{Ks}. I_{Ks} were continuously elicited by depolarizing pulses to +50 mV at 0.1 Hz. *, $p < 0.05$ versus control, †, $p < 0.05$ versus in the presence of ginsenoside Re. Right, representative superimposed current traces recorded at the timing indicated by italic lower-case alphabets.

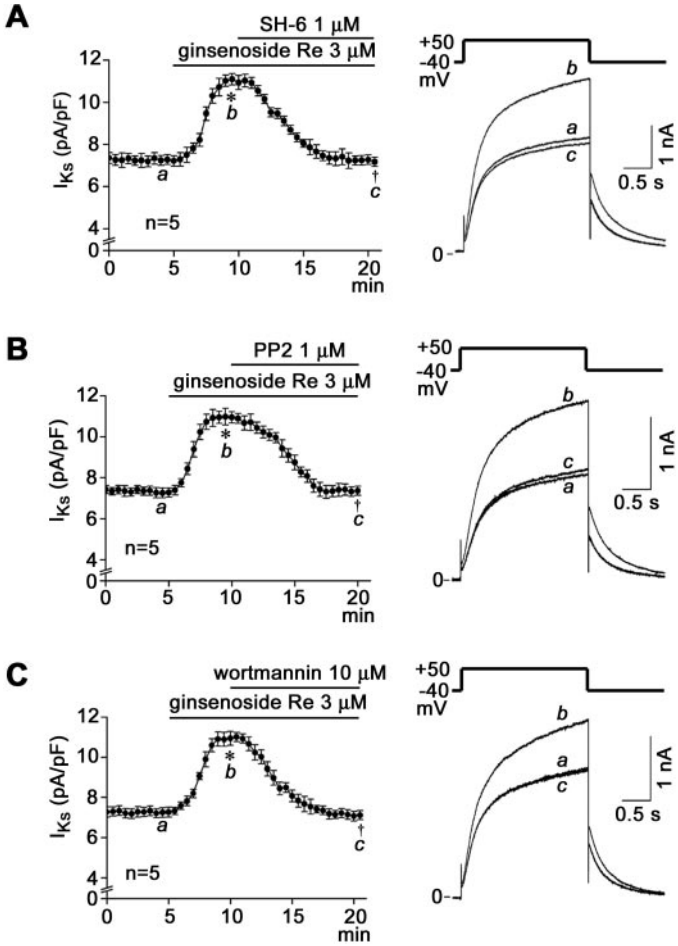

Fig. 3. Signaling cascade of I_{Ks} enhancement by ginsenoside Re. A–C, effects of SH-6 (A), an Akt inhibitor, PP2 (B), a c-Src inhibitor, and wortmannin (C), a PI-3 kinase inhibitor, on ginsenoside Re-induced I_{Ks} enhancement. Left, time course of experiments in five cells. *x*-Axis is time after start of experiments, and *y*-axis is averaged current density of I_{Ks}. I_{Ks} were continuously elicited by depolarizing pulses to +50 mV at 0.1 Hz. *, $p < 0.05$ versus control; †, $p < 0.05$ versus in the presence of ginsenoside Re. Right, representative superimposed current traces recorded at the timing indicated by italic lower-case alphabets.

Ginsenoside Re Binds to AR, ERα, and PR. Receptor competitor assay was performed to test whether ginsenoside Re could bind to the LBD of human AR, ERα, and PR. DHT, E_2, and P_4 showed dose-dependent displacement of fluorescently tagged receptor ligands; IC_{50} was 2.8 nM for DHT, 33.8 nM for E_2, and 50.0 nM for P_4 (Fig. 7). Ginsenoside Re also showed dose-dependent displacement of fluorescently tagged receptor ligands; the IC_{50} values were 56.2 μM for the AR, 59.0 μM for the ERα, and 80.6 μM for the PR (Fig. 7). The binding of ginsenoside Re to the AR, ERα, and PR was not saturable up to the concentration of 1 mM, suggesting that ginsenoside Re is a partial agonist of the AR, ERα, and PR.

Ginsenoside Re Does Not Activate Genotropic Action of AR and ERα. Genotropic action of E_2 is generally assessed by its effects on proliferation of the estrogen-responsive human breast cancer cell line MCF-7 (Lippman et al., 1976) and that of DHT via proliferation of the testosterone-responsive human prostate cancer cell line LNCaP (Hasen-

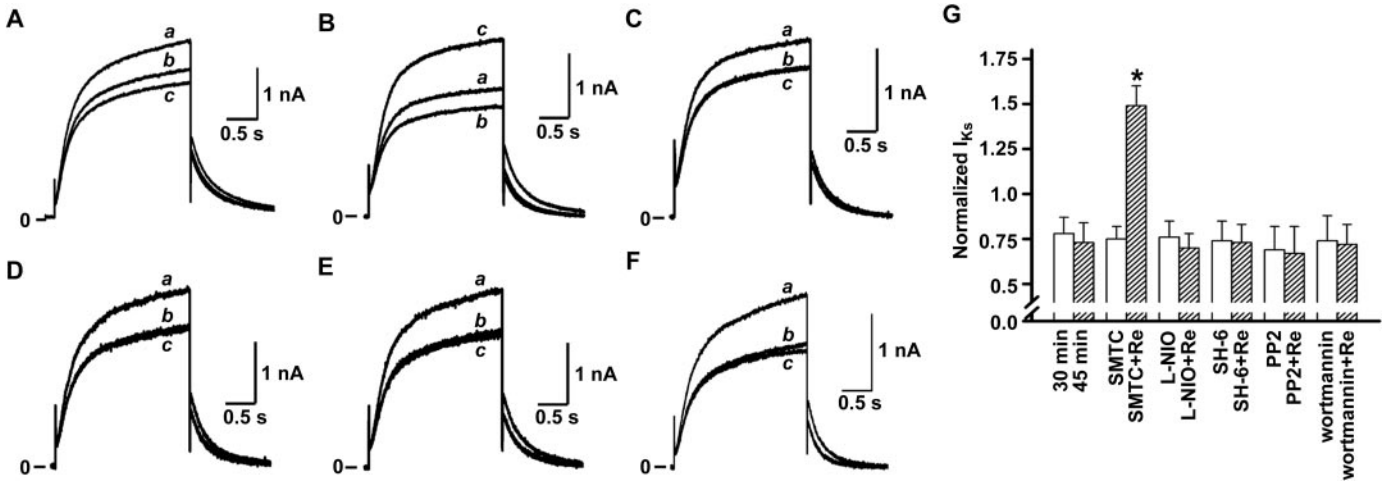

Fig. 4. Effects of preincubation with various blockers on ginsenoside Re (3 μM)-induced I_{Ks} enhancement. A, representative superimposed current traces in the time control experiment in the control state (trace a), at 30 min after start of experiment (b), and at 45 min after start of experiment (c). B–F, representative superimposed current traces in the control state (trace a), after incubation with various blockers (trace b), and after addition of ginsenoside Re (3 μM) in the continued presence of various blockers (trace c). B, SMTC (3 μM); C, L-NIO (1 μM); D, SH-6 (1 μM); E, PP2 (1 μM); and F, wortmannin (10 μM). G, averaged I_{Ks} after incubation with various blockers for 30 min, and 15 min after addition of ginsenoside Re in the continued presence of various blockers (45 min after start of experiment). I_{Ks} was normalized to the control value. *, $p < 0.05$ between before and after ginsenoside Re application.

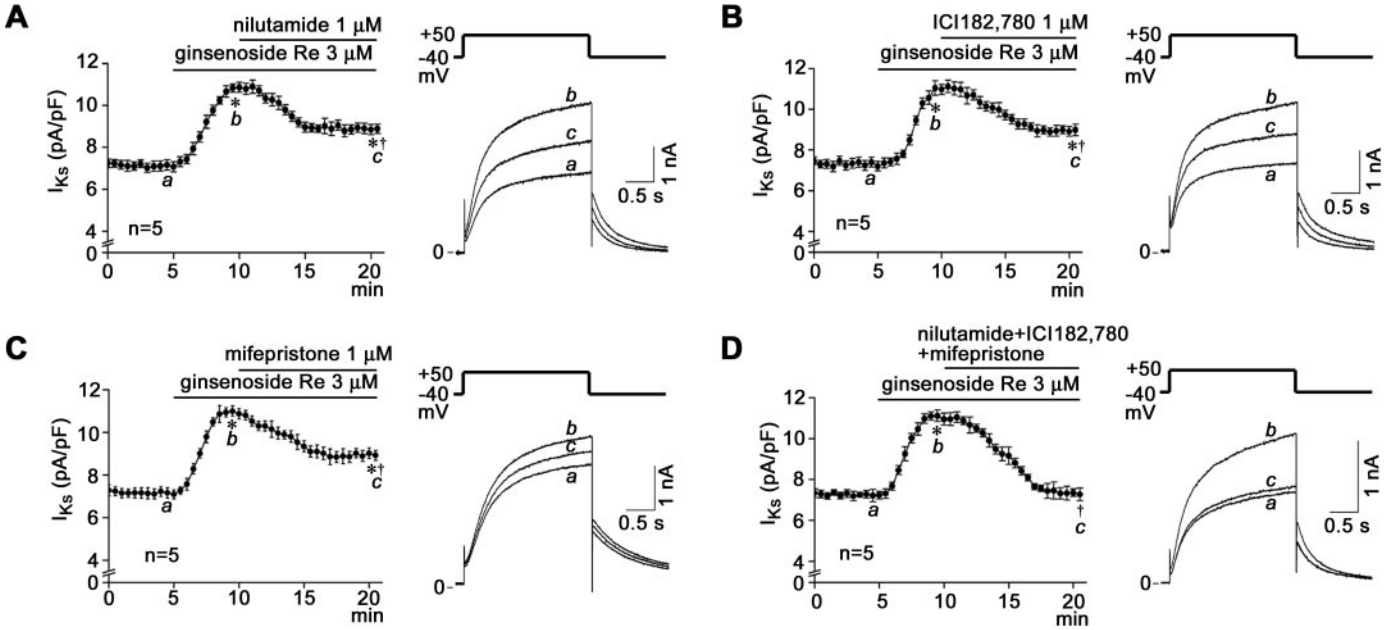

Fig. 5. Involvement of sex hormone receptors on I_{Ks} enhancement by ginsenosides. A–D, effects of nilutamide (A), an AR inhibitor, ICI182,780 (B), an ER inhibitor, mifepristone (C), a PR inhibitor, and a combination of nilutamide, ICI182,780, and mifepristone (D) on ginsenoside Re-induced I_{Ks} enhancement. Left, time course of experiments in 5 cells. *x*-Axis is time after start of experiments, and *y*-axis is averaged current density of I_{Ks}. I_{Ks} were continuously elicited by depolarizing pulses to +50 mV at 0.1 Hz. *, $p < 0.05$ versus control, †, $p < 0.05$ versus in the presence of ginsenoside Re. Right, representative superimposed current traces recorded at the timing indicated by italic lower-case alphabets.

son et al., 1985). We found that, unlike DHT or E_2, ginsenoside Re did not stimulate proliferation of LNCaP or MCF-7; rather, it partially inhibited DHP-induced LNCaP proliferation and E_2-induced MCF-7 proliferation (Fig. 8). Thus, ginsenoside Re is a partial antagonist, but not an agonist, of the genomic pathway of AR or ERα.

Ginsenoside Re Fails to Recruit CoActivator of AR, ERα, and PR. To further seek for the mechanism underlying lack of genomic action by ginsenoside Re, we examined whether ginsenoside Re triggered binding of a coactivator peptide containing a canonical LXXLL-motif (L = leucine, X = any amino acid) to the LBD of ERα, AR, and PR. We used a FRET indicator, *SCCoR*, in which agonist-induced recruitment of coactivator to the LBD of receptors was designed to induce FRET signals between enhanced CFP and enhanced YFP (Awais et al., 2004, 2006). We first confirmed that ginsenoside Re did not change FRET signals for AR-*SCCoR*, ERα-*SCCoR*, or PR-*SCCoR* (Fig. 9, A and D). Then, we found that ginsenoside Re significantly inhibited E_2-, DHT-, or P_4-induced FRET signals (Fig. 9, B–D), indicating that ginsenoside did not induce coactivator recruitment to AR, ERα, and PR, and rather inhibited agonist-induced coactivator recruitment.

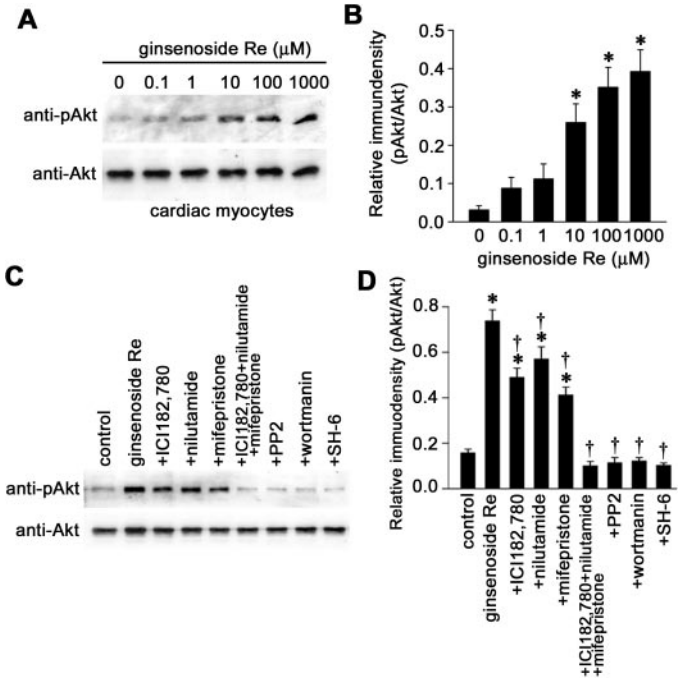

Fig. 6. Effects of ginsenoside Re on Akt phosphorylation. A, representative immunoblots showing dose-dependent effects of ginsenoside Re on Akt phosphorylation. B, densitometric analysis of dose-dependent phosphorylation of Akt by ginsenoside Re in three experiments. *, $p < 0.05$ versus without ginsenoside Re. C, representative immunoblotting showing effects of various blockers on Akt phosphorylation by ginsenoside Re. D, densitometric analysis of effects of various blockers on Akt phosphorylation by ginsenoside Re in three experiments. *, $p < 0.05$ versus in the control state; †, $p < 0.05$ versus in the presence of ginsenoside Re without any blockers.

Discussion

The present study provides convincing evidence to clarify a mechanism underlying the bioactivity of ginseng in cardiovascular system. We have previously reported that ginsenoside Re enhances I_{Ks} via a NO-dependent manner in isolated cardiac myocytes (Bai et al., 2003, 2004). In the present study, we found that ginsenoside Re releases NO via a nongenomic pathway of sex steroid receptors, resulting in I_{Ks} activation in cardiac myocytes. Ginsenoside Re does not activate the genomic pathway of sex steroid hormones, because it fails to recruit coactivators upon binding of ginsenoside to the LBD of sex hormone receptors. Thus, ginsenoside is a specific agonist for the nongenomic pathway of sex steroid receptors.

Our pharmacological experiments indicate that ginsenoside Re activates the nongenomic pathway of sex steroid receptors to activate eNOS and release NO. Because reliable methods to measure NO at nanomolar range have not been available until very recently (Sato et al., 2005), we did not directly assess NO release from cardiac myocytes induced by ginsenoside Re. However, our previous report that two different types of NO-trapper, carboxy-2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide and LNAC, abolished ginsenoside Re-induced I_{Ks} activation (Bai et al., 2004) supports the idea that I_{Ks} enhancement by ginsenoside Re is caused by NO. Ginsenoside Re-induced I_{Ks} activation was reversed by inhibitors of c-Src, PI3-kinase, Akt, and eNOS that are key signal molecules of the nongenomic pathway of sex steroid receptors (Figs. 2 and 3). In the preincubation with these inhibitors, ginsenoside Re did not activate I_{Ks} (Fig. 4). Akt phosphorylation by ginsenoside Re was suppressed by inhibitors of c-Src, PI3-kinase, and Akt (Fig. 6).

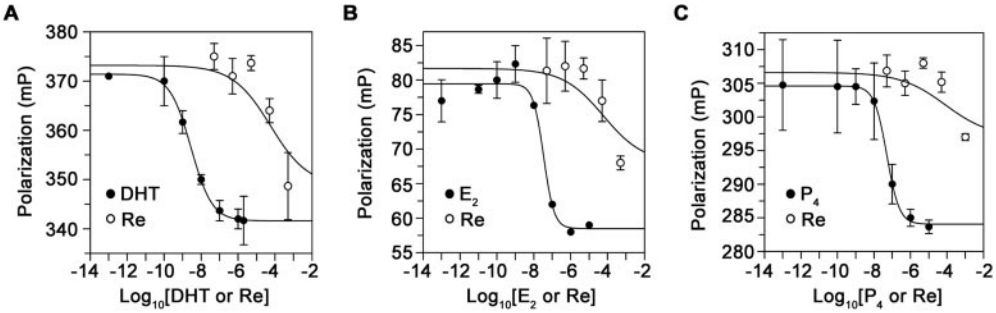

Fig. 7. Ginsenoside Re binds to the human AR, ERα, and PR. Fluorescently tagged receptor ligands bound to the LBD of the human AR were displaced by DHT and partially by ginsenoside Re (A); those to the LBD of the human ERα were displaced by E_2 and partially by ginsenoside Re (B); and those to the LBD of the human PR were displaced by P_4 and partially by ginsenoside Re (C). *x*-Axes are logarithm of concentration of DHT, E_2, P_4, and ginsenoside Re; *y*-axes are intensity of fluorescent polarization. Continuous lines are results of fitting of data to the Hill equation in the following formula using the least-squares method: $mP = mP_{0\%} - (mP_{0\%} - mP_{100\%})/[1 + (IC_{50}/[\text{ginsenoside Re}])^{n_H}]$, where mP is intensity of fluorescent polarization, $mP_{0\%}$ is mP without radioactive competitor, $mP_{100\%}$ is mP with the highest concentration of competitor (0.1 mM DHT, E_2, or P_4), and n_H is the Hill coefficient.

Finally, inhibitors of AR, ERα, and PR inhibited I_{Ks} enhancement and Akt phosphorylation by ginsenoside Re (Figs. 5 and 6).

In this study, each inhibitor of AR, ERα, and PR only partially suppressed ginsenoside Re-induced I_{Ks} enhancement, whereas the combination of all three inhibitors completely abolished ginsenoside Re actions. Competitive binding assays revealed that ginsenoside Re bound to AR, ERα, and PR. FRET experiments showed that ginsenosides competitively inhibited DHT-, E_2-, and P_4-induced coactivator recruitment further imply that ginsenoside Re somehow interacts with the LBD of AR, ERα, and PR. Taken together, we speculate that sex hormone receptors might be primary targets of ginsenoside Re. However, we would not completely eliminate the possibility that ginsenoside activates some common signaling molecules downstream of AR, ERα, and PR rather than binding to each of three receptors. Ginsenoside Re required relatively higher concentration to competitively displace fluorescently tagged receptor ligands compared with the concentration to enhance I_{Ks}. Although it is possible that the concentration to interact with the LBD is different between receptors present in the cytosol (receptor binding assays) and those localized in the plasma membrane (electrophysiological experiments), ginsenoside could also act primarily on molecules other than sex hormone receptors. Because a phytosterol genistein is a well established nonspecific inhibitor of tyrosine kinases (Akiyama et al., 1987), c-Src, a tyrosine kinase that is a common downstream signal of the AR, ERα, and PR, may be a potential candidate for target of ginsenoside Re. Therefore, these points are not settled yet, and further experiments are certainly needed

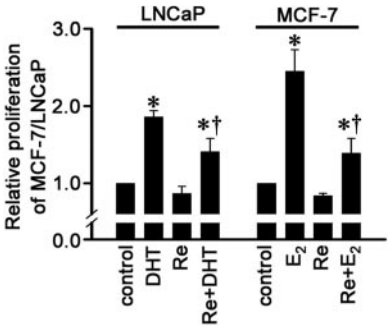

Fig. 8. Effects of ginsenosides on proliferation of MCF-7 and LNCaP. Relative proliferation of cells was calculated as (cell counts in the presence of drugs)/(cell counts in the control state). *, $p < 0.05$ versus control; †, $p < 0.05$ versus in the presence of DHT alone or E_2 alone.

Although our data indicate that ginsenoside Re does not activate the genomic pathway of sex hormone receptors, reported effects of ginsenoside on MCF-7 breast cancer cell growth are controversial. Ginsenoside Re induces expression of genes with estrogen-responsive element and proliferation of MCF-7 (Lee et al., 2003), whereas American ginseng inhibits MCF-7 breast cancer cell growth (Duda et al., 1999). Our data are consistent with the latter; ginsenoside Re does not enhance proliferation of MCF-7 cells or LNCaP cells. Experiments with FRET probes, *SCCoRs*, further provide supporting evidence that ginsenoside Re fails to activate the genomic pathway; ginsenoside Re does not induce coactivator recruitment upon binding to the LBD and inhibits coactivator recruitment induced by E_2, DHT, or P_4. A structural basis analysis seems to provide further supporting evidence. A structural basis of ERα/coactivator recognition is well documented from the analysis of the crystal structure of ERα-LBD bound to both an agonist diethylstilbestrol and a coactivator GRIP1 (Brzozowski et al., 1997; Shiau et al., 1998). The LBD pocket bound by an agonist is covered by helix 12 of

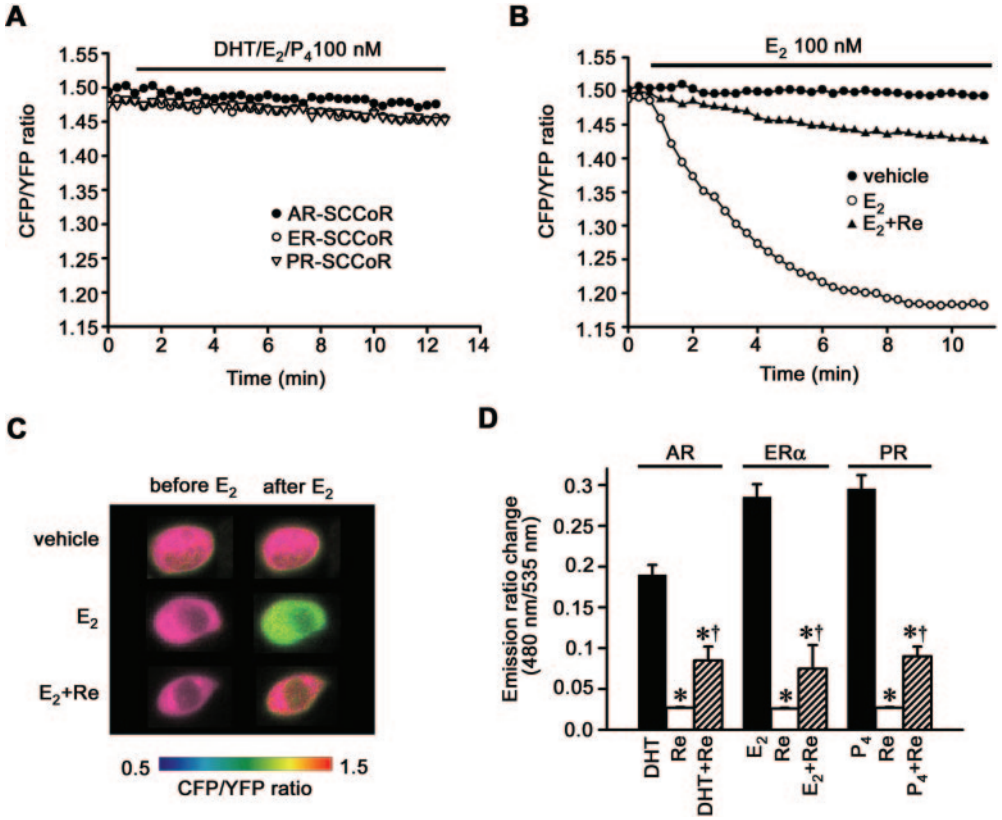

Fig. 9. Effects of ginsenoside on recruitment of coactivator examined by FRET experiments. A, representative time course of FRET responses of AR-*SCCoR*, ER-*SCCoR*, and PR-*SCCoR* upon addition of ginsenoside Re (10 μM). B, representative time course of FRET responses of ER-*SCCoR* upon E_2 (100 nM) in addition in the presence and absence of ginsenoside Re (10 μ). C, representative pseudocolor images of CFP/YFP emission ratios before (left) and after E_2 (100 nM) addition (right). D, averaged emission ratio change. *, $p < 0.05$ versus ligand alone.

LBD, creating a hydrophobic groove on the surface of LBD, where a coactivator can bind (Brzozowski et al., 1997; Shiau et al., 1998). The LBD pocket bound by a selective antagonist 4-hydroxytamoxifen disturbed motion of helix 12 and creation of a coactivator recognition groove, because of the presence of a bulky side chain in 4-hydroxytamoxifen. Because, like 4-hydroxytamoxifen, every ginsenoside has a bulky side chain (Fig. 1A), ginsenoside is unlikely to promote coactivator binding.

Hormone replacement therapy has been used for rapidly developing cardiovascular events, osteoporosis, disturbed cognition, and other symptoms in postmenopausal women; however, there are accompanying serious adverse events including high risk of estrogen-sensitive cancers (breast cancer, ovarian cancer, and certain types of lung cancer) (Barrett-Connor et al., 2005). Likewise, testosterone replacement therapy has recently been used for various symptoms in male menopause (andropause) with a risk of testosterone-sensitive prostate cancer (Hijazi and Cunningham, 2005). 4-Estren-3α,17β-diol (estren) is a synthetic compound that selectively induces nongenomic actions of estrogens and androgens without classic transcriptional activity (Kousteni et al., 2002). Conversely, 1,2,5-tris(4-hydroxyphenyl)-4-propylpyrazole (pyrazole) has potent transcriptional activity with minimal effects on nongenomic-induced events: estren, but not pyrazole, reversed bone loss in mice (Kousteni et al., 2002). From these findings, they propose that mechanism-specific ligands of steroid nuclear receptors represent a novel class of pharmacotherapeutics (Kousteni et al., 2002). Our data imply that ginsenoside is a naturally harvested, mechanism-specific agonist of sex steroid receptors. In the Eastern world, *P. ginseng* has been successfully prescribed for health problems associated with the post- and perimenopausal periods, which includes not only cardiac events, but also hot flashes, loss of bone matrix, and cognition disturbance (Punnonen and Lukola, 1984; Kropotov et al., 2002; Hartley et al., 2004; Low Dog, 2005). In the present study, we used ginsenoside Re at a concentration of 3 μ, because this is the concentration prescribed to patients in China (Bai et al., 2003). It does not necessarily reflect the plasma concentration in humans. Nevertheless, we expect that effects of ginsenoside described in the present article may provide a potential of ginsenoside as a medicinal seed for treatment of cardiac events, and potentially other symptoms, in postmenopausal women and postandropausal men.

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