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Differential ERα-mediated rapid estrogenic actions of ginsenoside Rg1 and estren in human breast cancer MCF-7 cells

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Abbreviations

MAPK, mitogen-activated protein kinase; ER, estrogen receptor; ERE, estrogen response element; SRC-1, steroid receptor co-activator-1; MEK, mitogen-activated protein kianse kianse; ERK, extracellular signal-regulated protein kinase; AR, androgen receptor; E2, 17β-estradiol; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; sFBS, charcoal-stripped fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; ChIP assay, Chromatin immunoprecipitation assay; AFs, activation functions

Abstract

Recent studies indicated that both estren and Rg1 appear to be able to activate mitogen-activated protein kinase (MAPK) pathway in estrogen responsive cells. Rg1 could lead to MAPK activation through ligand-independent activation of estrogen receptor (ER), while estren could activate the Src-MAPK pathway in an ERE-independent manner. Thus, it is important to understand the mechanistic insights on the difference in transcriptional activation between estren and Rg1. The present study also addressed the differential abilities of Rg1 and estren in terms of the ability to activate ER and the ability to induce ER translocation in MCF-7 cells. Our data indicated that Rg1 could increase pS2 gene expression, and could recruit the co-activator steroid receptor co-activator-1 (SRC-1) to the pS2 promoter. Rg1 could also induce ERα nuclear translocation as well as ERα phosphorylation at Ser18 principally in the cytoplasm in MCF-7 cells. We deduced that estren induced ERE-dependent transcriptional activity and activated ERα at Ser18 occurred in the nucleus of MCF-7 cells. However, it was found to decrease pS2 gene expression and failed to induce the recruitment of SRC-1 to the pS2 promoter in MCF-7 cells. Our results suggest that the abilities of Rg1 and estren to regulate pS2 gene expression, to recruit co-activators as well as to induce sub-cellular distribution of ERα are dramatically different.

Key words: Ginsenoside Rg1, estren, 17β-estradiol, estrogen receptor, MCF-7 cells

1. Introduction

The actions of estrogen are traditionally conceived to be mediated through the classical steroid receptor pathway, in which estrogen receptor (ER) acts as a ligand-dependent transcription factor that binds to estrogen response elements (EREs) in promoters of target genes [1]. Recent research indicated that the actions of estrogen can also be mediated by rapid signaling that occurs within seconds to minutes of cell stimulation [2], in which the rapid actions of estrogen are shown to be mediated through membrane associated receptors [3-4], thereby modulating cellular functions via both transcriptional and non-transcriptional events. In particular, ERs have been shown to be activated by mitogen-activated protein kinase (MAPK) via phosphorylation upon activation of growth factor receptors in a ligand-independent manner [5-6].

Ginsenoside Rg1 is one of the most active and abundant compounds found in ginseng. The pharmacological effects of Rg1 in the skeletal system, central nervous system and cardiovascular system as well as its involvement in the pathogenesis of endometrial, breast and ovarian cancers have been studied [7]. Our previous study identified Rg1 as a potent phytoestrogen [8] and that exert estrogenic activities at picomolar concentration. Its actions are more potent than other naturally occurring compounds such as flavonoids, coumestan derivatives and ligands which exert estrogenic activities at the micromolar range [9-12]. We have also shown previously that Rg1 can stimulate the growth of human breast cancer MCF-7 cells, as well as activate the ERE-dependent luciferase activity without directly binding to the

ERα [8, 13]. Furthermore, Rg1 was found to preferentially activate ERα and rapidly induce ERα phosphorylation at the AF-1 domain via mitogen-activated protein kianse kianse (MEK)/ extracellular signal-regulated protein kinase (ERK) pathway in MCF-7 cells [12, 14]. This reveals that Rg1 exerts rapid oestrogenic effects by activating ERα via MAPK pathway in a ligand-independent manner in MCF-7 cells.

Estren (4-estren-3α, 17β-diol) is a synthetic ligand of ER or androgen receptor (AR) with very low affinities to ER (about 0.15% of that of 17β-estradiol (E2)) and AR (about 2% of that of the potent androgen R1881) [15-16]. It was initially reported to selectively activate the rapid membrane-mediated pathway rather than through the classical nuclear ER-mediated pathway to increase bone mass and strength in gonadectomized Swiss Webster mice, but it had no effects on uterine weight [16-17]. The anti-apoptotic action of estren on osteoblasts, osteocytes, embryonic fibroblasts and HeLa cells was shown to be mediated by ligand binding domain of ER localized exclusively to the cell membrane and involve the activation of Src/Shc/ERK signaling without inducing transcriptional activity of the ER *in vitro* [15]. In addition, its ability to induce osteoblast differentiation is also shown to be mediated by rapid non-genomic ER actions in which the membrane-localized ERα is selectively activated in bone cells [16]. However, others have shown that estren is able to induce transcriptional activity of an ERE-driven reporter gene via ERα and ERβ in human 293 kidney epithelial ERα (293/h ERα)- and ERβ(293/h ERβ)-expressing reporter cell lines [18]. Despite

discrepancies in reports regarding the estrogenic actions exerted by estren, these studies demonstrated that the bone-protective effects of estren involve the activation of ER as well as the induction of rapid cell membrane-mediated Src-MAPK pathways without inducing transcriptional activity. However, it is unclear if estren can induce ERE-dependent trasncription via ER-mediated pathway in cancer cells such as MCF-7 cells.

As both Rg1 and estren appear to be able to activate MAPK pathway in estrogen responsive cells, it is of importance to determine why activation of MEK (an enzyme upstream of MAPK) by Rg1 results in ligand-independent activation of ER and induce ERE-dependent transcription while activation of Src-MAPK pathway by estren does not result in activation of ERE-dependent transcription. Thus, the present study was designed to compare the actions of Rg1 and estren in inducing transcriptional events, in activating ER and inducing ER translocation in MCF-7 cells. It is hope that this study can provide insights for understanding non-classical ER signaling pathways activated by phytoestrogens as well as synthetic ER analogs.

2. Material and Methods

2.1 Culture and treatment of human breast cancer MCF-7 cells

MCF-7 cells (HTB-22) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin 100 U/ml and streptomycin 100 μg/ml (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Twenty-four hours after plating, the medium was changed to 1% charcoal-stripped fetal bovine serum (sFBS) in phenol red-free DMEM and cultured for 48h before treatment.

2.2 Cell proliferation assay

MCF-7 cells were plated in 96-well plates at a density of 5000 cells/well; 24h after being plated, the cells were cultured in phenol red-free DMEM medium containing 1% sFBS for 48h and subsequently treated with various concentrations of 17β-estradiol (E2, 10^{-14}M-10^{-6}M), estren (10^{-14}M-10^{-6}M), Rg1 (10^{-14}M-10^{-6}M) or its vehicle for 48h. Upon incubation, the growth of the cells was quantified using the MTS proliferation assay according to the manufacturer's instructions. The 96-well plate was incubated at 37^{o}C for 2h and then read on a microplate reader at a wavelength of 490nm.

2.3 Transient transfection of MCF-7 cells and luciferase assay

MCF-7 cells were seeded into 24-well plates and transient transfected by Lipofectamine 2000 reagent (Invitrogen). The ERE-containing luciferase reporter plasmid vERETkluc was kindly provided by Dr Vincent Giguere (McGill University, Montreal, Quebec, Canada). 0.8μg of

this reporter construct, together with 0.01μg of an inactive control plasmid pRL-TK, a Renilla luciferase control vector, was co-transfected into the cells in triplicate. At 6 hours after transfection, cells were treated with various concentrations of 17β-estradiol (10^{-14}M-10^{-6}M), estren (10^{-14}M-10^{-6}M), Rg1 (10^{-14}M-10^{-6}M) or its vehicle for another 24h. Luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega), and signals were detected by a TD-20/20 Luminometer (Turner Desing, Sunnyvale, CA). Estrogen promoter activity was expressed as firefly luciferase values normalized by pRL-TK Renilla luciferase values.

2.4 Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells by using Trizol reagent according to the standard protocol. Total RNA (1μg) was reverse-transcribed in 20μL of a reaction mixture containing reverse transcription buffer, deoxynucleotide triphosphate mixture, random primers and MultiScribe reverse transcriptase, using a high-capacity cDNA reverse transcription kit at 25^{o}C for 10 min, 37 ^{o}C for 2h and 85 ^{o}C for 5s. For pS2 and the house keeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH), the primers used were 5'-ATGGCCACCATGGAGAACAAGG-3' (pS2 forward) and 5'-CATAAATTCACACTCCTCTTCTGG-3' (pS2 reverse), 5'-GAAGGACTCATGACCACAGT-3' (GAPDH forward) and 5'-GTTGAAGCTAGAGGAGACCA-3' (GAPDH reverse). PCR was carried out in 20μL

reaction mixture containing 10μL SsoFast™ Eva Green supermix (BIO-RAD) and 0.5μL of cDNA template. The PCR was performed in an iO5 Multicolor Real-time PCR Detection System (BIO-RAD) using the following cycle parameters: 1 cycle of 95°C for 30s, and 40 cycles of 95°C for 5s, annealing temperature for 30s and followed by melting curve. Standard curves were generated using serially diluted solutions of cDNA derived from untreated MCF-7 cells. The target gene transcripts in each sample were normalized on the basis of its GAPDH.

2.5 Chromatin immunoprecipitation assay (ChIP assay)

ChIP assays were performed by using the ChIP kit according to the manufacturer's instruction. MCF-7 cells were cross-linked with 1% formaldehyde. Cells were harvested by centrifugation and resuspended in the lysis buffer (1% SDS, 10mM EDTA, 50mM Tris PH 8.1, 1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml Aprotinin and 1.5μg/ml Pepstatin A). After sonication in Sonifier 450 (Branson, Danbury, CT), the samples were centrifuged and cell supernatant was diluted for 10-fold in ChIP dilution buffer containing protease inhibitors. Protein A Agarose/Salmon Sperm DNA was added to reduce non-specific background. After centrifugation, the supernatant fraction was collected and incubated overnight at 4°C with rotation by adding the SRC-1 antibody (Mouse IgG). For a negative control, no-antibody immunoprecipitation and anti-Mouse IgG antibody immunoprecipitation were performed. After that, the samples were incubated with protein A Agarose/Salmon Sperm DNA for one

hour at 4°C with rotation to precipitate the DNA-protein complex and then eluted in the elution buffer (1% SDS and 0.1M $NaHCO_3$). The mixture was incubated at 65°C for 4h to reverse the formaldehyde cross-linking. Protein was removed by proteinase K digestion (10mg/mL) and phenol/$CHCL_3$ extraction. The extracted DNA was used for performing RT-PCR using the pS2 promoter primers: upstream 5'-GATTACAGCGTGAGCCACTG-3' and downstream 5'-TGGTCAAGCTACATGGAAGC-3' [19]. The PCR conditions were as follows: 95°C 1min, 58°C 1min, 72°C 50s. The amplification products, obtained in 38 cycles, were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

2.6 Immunoblotting

After treatment, MCF-7 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, leupeptin 2 μg/ml, 1 mM PMSF) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM NaF). Lysates were centrifuged at 14,000g at 4°C for 30 min and protein concentrations were analyzed by the method of Bradford (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (50 μg) were separated by SDS-PAGE, and transferred onto PVDF membranes (Immobilin-P, Millipore Corp., MA, USA) as previously described [20]. The blots were probed with monoclonal rabbit anti-human phosphor-ERα at Ser 118 (1:2000; Millipore), monoclonal mouse anti-human B-actin (1:2000; Abcam) and polyclonal rabbit anti-human ERα (1:2000; Santa Cruz Biotechnology, Inc.,

Santa Cruz, CA). This was followed by incubation with goat anti-rabbit (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-mouse antibody (1:2000; Cell Signaling) conjugated with horseradish peroxidase. The antigen-antibody complexes were then detected with enhanced chemiluminescence (ECL) reagent and visualized by the Lumi-Imager using Lumi Analyst version 3.10 software (Roche, Mannheim, Germany).

2.7 Wide-field fluorescence microscopy

After treatment, cells were fixed with PBSAT (2% BSA, 0.5% Triton-X 100 in PBS) which contained the Triton X-100. Followed by incubation with the ERα antibody (1:100) or phosphorylated-ERα (Ser 118) antibody (1:100) for 2h at room temperature, the cells were incubated with Alexa Fluor 488 anti-rabbit antibody (1:100, Molecular Probes) for 1h at room temperature. After washing with PBSAT 8 times, images were acquired using the wide-field fluorescence microscopy (Zeiss Observer.Z1). Before image acquisition, the samples were incubated with the DNA-binding vital stain Hoechst 33342 for 10 min to stain the DNA.

2.8 Statistical analysis

Data are reported as the mean ± SEM. Significance of differences between the means of various was determined by t-test. A p-value <0.05 was considered statistically significant.

3. Results

3.1 Rg1 and estren increased MCF-7 cell proliferation

As shown in Fig.1, 17β-estradiol significantly increased cell proliferation at all tested dosages from 10^{-14} M to 10^{-6} M with the most effective dosage of 10^{-6} M (1.23-fold) in MCF-7 cells. However, estren significantly stimulated cell proliferation only at 10^{-14} M (1.15-fold) and 10^{-10} M (1.27-fold) (Fig. 1). For Rg1, it significantly increased cell proliferation from 10^{-12} M-10^{-6} M with the most effective concentrations of 10^{-6} M (1.27-fold) (Fig. 1). These results indicate that Rg1 and estren exert estrogen-like effects on increasing cell proliferation in MCF-7 cells, although their effective dosages are different.

3.2 Both Rg1 and estren induced ERE-dependent transcription in MCF-7 cells

To determine if Rg1 or estren stimulated ERE-dependent transcription, MCF-7 cells were transfected with ERE-luciferase reporter and subjected to treatment with different concentrations of 17β-estradiol, Rg1 and estren. The results showed that 17β-estradiol significantly induced ERE-dependent transcription at all tested dosages (10^{-14} M- 10^{-6} M) in MCF-7 cells, and the most effective dosage was 10^{-8} M (6.4-fold) (Fig. 2). Rg1 significantly induced ERE-dependent transcription from 10^{-14} M to 10^{-8} M, the most effective dosage were 10^{-12} M (3.46-fold) and 10^{-8} M (3.33-fold) (Fig. 2). Estren significantly induced ERE-dependent transcription from 10^{-10} M to 10^{-6} M, but not at 10^{-14} and 10^{-12} M, and the most effective concentration was 10^{-10} M (4.4-fold) (Fig.2). This suggests that both Rg1 and estren

exert estrogen-like effects in activating ERE-dependent transcription in MCF-7 cells.

3.3 Rg1, but not estren, increased pS2 gene expression and induced recruitment of co-activator SRC-1 to ERE-containing pS2 promoter in MCF-7 cells

The pS2 gene was the human breast cancer prognostic marker. It was originally identified as an estrogen-inducible transcript in MCF-7 cells [21]. pS2 gene expression was frequently used as a marker for assessing the estrogenicity of various compounds [22]. Our previous study suggested that 1 pM Rg1 significantly increased pS2 gene expression [13]. To determine if estren mimicked Rg1 at inducing the expression of estrogen responsive gene in MCF-7 cells, the ability of estren to induce estrogen-regulated pS2 mRNA expression was determined. As expected, 17β-estradiol significantly increased pS2 gene expression in MCF-7 cells at 10^{-10} M, 10^{-8} M and 10^{-6} M upon treatment for 48 h (Fig. 3A). Similarly, Rg1 significantly increased pS2 gene expression from 10^{-12} M to 10^{-8} M, and the most effective concentration was 10^{-8} M (2.58-fold) (Fig. 3A). However, estren significantly decreased pS2 gene expression at the concentrations of 10^{-8} M and 10^{-6} M in MCF-7 cells upon treatment for 48 h (Fig. 3A).

ERE element in the pS2 gene promoter (region -405 to -394) was a classic site of direct ER regulation [19, 23]. p160 co-activator SRC-1 was known to interact with the ERE-containing pS2 promoter to modulate its transcriptional activities [24]. To determine if the differential regulation of pS2 gene expression by Rg1 and estren was due to differences in co-activator

recruitment, ChIP assay was used to study the co-activator recruitment of SRC-1 to pS2 promoter in MCF-7 cells treated with 17β-estradiol, Rg1 or estren. As shown in Fig.3B, 17β-estradiol significantly increased the binding of SRC-1 to the pS2 promoter by 4.2-fold after immnunoprecipitation by SRC-1 antibody. 10^{-12} M and 10^{-8} M Rg1 increased the binding of SRC-1 to the pS2 promoter by 1.66-fold and 5.18-fold, respectively (Fig. 3B). In contrast, estren failed to induce any recruitment of SRC-1 to pS2 promoter (Fig. 3B). The result suggested that the inability of estren to recruit SRC-1 to pS2 promoter might contribute to its negative regulation of pS2 mRNA expression in MCF-7 cells. This reveals that different co-activators and promoters are involved in the estrogenic actions of Rg1 and estren in MCF-7 cells.

3.4 Both Rg1 and estren rapidly induced ERα translocation from the cytoplasm to the nucleus in MCF-7 cells

ER is previously demonstrated to be an essential mediator of actions of 17β-estradiol and Rg1 [13]; we therefore determined if estren exerts similar 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 [13], its expressions in response to 17β-estradiol, Rg1 and estren were being studied in the present study. As expected, 17β-estradiol decreased ERα protein expression as early as 10 mins treatment and the suppression was significant at 30 min and 2 hr, with the maximal decrease at 2 hr (Fig.4A). In contrast, both Rg1 and estren did not alter ERα protein

250 expression at all test points (Fig.4A). Thus, it appears that Rg1 and estren behave differently
251 from E2 and do not suppress the expression of ERα in MCF-7 cells.
252 To determine if Rg1 and estren activated ERα in a way similar to 17β-estradiol in MCF-7
253 cells, their abilities to induce ERα translocation from the cytoplasm to the nucleus were
254 determined. 17β-estradiol induced rapid ERα translocation within 5mins and the maximal
255 point was 30 min (3.2-fold), followed by a fall at 2 hr in MCF-7 cells (Fig.4B). Rg1 at 10^{-8} M
256 induced significant and maximal ERα translocation from the cytoplasm to the nucleus as early
257 as 5 min (1.89-fold) (Fig. 4B). For estren, it induced rapid ERα translocation from the
258 cytoplasm to nucleus within 5mins by approximately 1.3-fold (Fig. 4B). The increase was
259 sustained and appeared to reach the peak by 6hrs although the changes were not statistically
260 significant (Fig. 4B). In addition, immunofluorescent staining was performed to confirm the
261 effect of 17β-estradiol, Rg1 and estren on ERα translocation in MCF-7 cells treated for 30
262 min. As shown in Fig.4C, ERα was diffusely expressed in the whole cell and predominately in
263 the nucleus in the control group (untreated group). Upon treatment with 17β-estradiol for 30
264 min, ERα could be clearly shown to translocate to nucleus, especially to the nucleolus, from
265 the cytoplasm in MCF-7 cells (Fig.4C). Similarly, 10^{-8} M Rg1 and estren also increased ERα
266 translocation from the cytoplasm to nucleus in MCF-7 cells (Fig.4C). These results were
267 consistent with the results of our western blotting data. Additionally, picomole of Rg1 also
268 increased ERα translocation from the cytoplasm to nucleus in MCF-7 cells upon treatment for

30 min (Fig.4C). This suggests that both Rg1 and estren induced ERα translocation from the cytoplasm to nucleus in MCF-7 cells in a way similar to that of 17β-estradiol.

3.5 Rg1 rapidly induced ERα phosphorylation at Ser118 principally in the cytoplasm in MCF-7 cells, while estren rapidly induced Ser118 phosphorylation principally in the nucleus

ERs contain two regions called activation functions (AFs) that are important for ligand-dependent ERs transcriptional activity [6]. The Ser118 residue in the AF-1 region of ERα is the phosphorylation sites essential for the ligand-independent activation of ERs through the Ras-MAPK signaling cascade [6]. In the present study, the phosphorylation level was calculated as a ratio of p-ERα to ERα and finally expressed as a ratio to the basal reading where time 0 (untreated as basal) equals 1. We evaluated the effects of Rg1 on ERα phosphorylation at Ser118 in MCF-7 cells in comparison to 17β-estradiol and estren. As shown in Fig.5A, a steady activation of ERα phosphorylation at Ser 118 in MCF-7 cells in response to 10^{-8}M 17β-estradiol occurred within the first 20 mins of incubation and peaked at 20 min (3.15-fold), the activation persisted throughout the incubation. Similarly, Rg1 induced Ser118 phosphorylation as early as 10 min, followed by a steady increase and peaked at 30 min (1.27-fold) (Fig. 5A). Treatment of MCF-7 cells with estren produced rapid and maximal phosphorylation of Ser 118 (2.6-fold) within 20 min, followed by a decrease in phosphorylation at 30 min and the activation persisted for the whole treatment (Fig.5A).

Fig. 5B shows the time-course of the protein expression of phosphorylated ERα and ERα in

288 the cytoplasm and nucleus in MCF-7 cells in response to treatment with 10^{-8} M 17β-estradiol,
289 10^{-8} M estren or 10^{-8} M Rg1. 17β-estradiol induced a steady activation of ERα within 30 min
290 of incubation both in the cytoplasm and the nucleus, the activation continued to slightly
291 increase and then peaked at 6 hr (4.5-fold and 10.4-fold, respectively) (Fig.5B). It should be
292 noted that the upregulation of phosphorylated ERα expression by 17β-estradiol was more
293 pronounced in the nucleus than in the cytoplasm (Fig.5B). Rg1 induced Ser118
294 phosphorylation within 30 min in the cytoplasm and peaked at 5 min (2.76-fold) (Fig. 5B),
295 but only significantly induced ERα phosphorylation at Ser 118 in the nucleus upon treatment
296 for 2 h (1.78-fold) and 6 h (2.74-fold) (Fig. 5B). In contrast, estren produced a slight increase
297 in ERα phosphorylation at Ser 118 in the cytoplasm where significant activation could only
298 be observed in MCF-7 cells upon 6 h of incubation, while induced a steady and significant
299 increase in Ser 118 phosphorylation in the nucleus within 30 min of incubation and peaked at
300 6 hr (8.8-fold) (Fig.5B). Wide-field fluorescence microscopy confirmed that 17β-estradiol
301 induced ERα phosphorylation both in the cytoplasm and nucleus but principally in the
302 nucleus, Rg1 at 10^{-12} M and 10^{-8} M activated ERα principally in the cytoplasm, while estren
303 activated ERα principally in the nucleus (Fig.5C). Our results indicate that Rg1 and estren
304 rapidly induced ligand-independent activation of ER with different activation pattern in
305 MCF-7 cells.
306

4. Discussion

Our previous study suggests that Rg1 exerts estrogenic effects and induces ERE-dependent transcription by activating ERα via MAPK pathway in a ligand-independent manner in MCF-7 cells [8, 12, 14]. Estren was known to exert bone-protective effects via the activation of ER as well as the induction of rapid cell membrane-mediated Src-MAPK pathways without inducing transcriptional activity [15]. Thus, it is intriguing to investigate why activation of MEK (an enzyme upstream of MAPK) by Rg1 results in ligand-independent activation of ER and induces ERE-dependent transcription while activation of Src-MAPK pathway by estren does not result in activation of ERE-dependent transcription. The present study clearly demonstrated that the effects of Rg1 are different from those of estren on estrogen-related pS2 gene expression, recuritment of co-activator SRC-1 to ERE-containing pS2 promoter and subcellular activation of ERα in MCF-7 cells. In addition, estren indeed induced ERE-dependent transcription in human breast cancer MCF-7 cells via rapid ER signaling pathway.

As expected, Rg1 increased cell proliferation at 10^{-12} M to 10^{-6} M in MCF-7 cells in a way similar to that of 17β-estradiol. However, estren could only increase cell proliferation at 10^{-14} M and 10^{-10} M. Discrepancies exist amongst reported studies regarding whether estren could stimulate MCF-7 cell proliferation. Windahl et al. [25] previously reported that estren increased MCF-7 cell proliferation at the concentrations of 10^{-10}M-10^{-6}M while Kousteni et al.

326 [17] and Almeida et al. [26] reported that estren (10^{-12} M-10^{-7} M) failed to induce any
327 proliferation of MCF-7 cells. Our results demonstrated that 17β-estradiol (10^{-14} M-10^{-6} M),
328 Rg1 (10^{-14} M-10^{-8} M) and estren (10^{-10} M-10^{-6} M) were found to alter ERE-dependent gene
329 transcription in MCF-7 cells. 17β-estradiol was more potent than Rg1 and estren in inducing
330 ERE-dependent reporter activities in MCF-7 cells.
331 Our ChIP assay studies indicated that the difference in regulation of pS2 expression by estren
332 and that by 17β-estradiol and Rg1 was due to the difference in their abilities to induce
333 recruitment of SRC-1 to the ERE-containing pS2 promoter. Our result was in agreement with
334 the report of Cascio et al. [19] that 17β-estradiol increased SRC-1 recruitment to pS2
335 promoter upon treatment for 1h. Similarly, Rg1 at 10^{-12} M and 10^{-8} M also significantly
336 induced recruitment of SRC-1 to pS2 promoter in MCF-7 cells. However, estren failed to
337 induce SRC-1 recruitment to pS2 promoter. The difference in the abilities to induce SRC-1
338 recruitment to pS2 promoter by 17β-estradiol, Rg1 and estren appeared to result in the
339 difference in pS2 mRNA expression induced by treatment of MCF-7 cells with these
340 compounds. These results indicated that the recruitment of co-activators to estrogen-sensitive
341 promoters in MCF-7 cells was differentially regulated by Rg1 and estren.
342 The reports of others [27-28] and the results of our wide-field fluorescence microscopy
343 experiment suggested that ERα was diffusely expressed in the whole cell, and predominately
344 expressed in the nucleus, especially in the nucleoli in MCF-7 cells. Results of our western

345 blotting indicated that 17β-estradiol rapidly induced ERα translocation from the cytoplasm to
346 the nucleus within 30 minutes of incubation in MCF-7 cells. Similarly, Rg1 and estren
347 significantly induced ERα translocation from the cytoplasm to the nucleus as early as 5 min in
348 MCF-7 cells. Gutierrez et al. (2012) reported that ER was classically shown to be constantly
349 shuttling between the nucleus and cytoplasm [27] and the localization of ER affected both
350 rapid and genomic signaling pathways [29]. This suggests that the nuclear translocation of
351 ERα is involved in the rapid signaling pathways of both Rg1 and estren in MCF-7 cells. In
352 addition, the levels of nuclear localization induced by Rg1 and estren were much lower that
353 induced by 17β-estradiol. This indicates that different mechanisms might be involved in
354 inducing nuclear translocation by 17β-estradiol, Rg1 and estren as both the rapid and genomic
355 signaling pathways are known to be activated by 17β-estradiol but not by Rg1 and estren.
356 For the activation of ERα, results of western blotting suggest that both Rg1 and estren
357 increased total ERα phosphorylation at Ser118 in MCF-7 cell within 10 min of incubation in a
358 way similar to that of 17β-estradiol. As Rg1 and estren are known to have very limited
359 binding affinity to ER, our results suggest that both of them might activate ERE-dependent
360 transcriptional activities in MCF-7 cells rapidly via ER phosphorylation. Indeed, the induction
361 levels of ERα phosphorylation in response to 17β-estradiol and Rg1 as well as estren in
362 MCF-7 cells were in agreement with the level of induction of ERE-dependent transcriptional
363 activities. This indicates that both Rg1 and estren can induce ERE-dependent transcription by

ligand-independent activation of ER via rapid signaling in MCF-7 cells. Most importantly, the sub-cellular distributions of phosphorylated ERα were differentially altered in MCF-7 cells in response to treatment with Rg1, estren and 17β-estradiol. Our results clearly indicated that Rg1 rapidly induced ERα phosphorylation at Ser118 principally in the cytoplasm by 5 min while estren could induce the expression of phosphorylated ERα at Ser 118 principally in the nucleus of MCF-7 cells. The induction patterns of Rg1 and estren were different from that of 17β-estradiol as the latter could induce the expression of phosphorylated ERα in both the cytoplasm and the nucleus. This suggests that different mechanisms are involved in determining the sub-cellular distribution of ERα by 17β-estradiol, Rg1 and estren. Both the rapid and genomic signaling pathways are involved in the effects of 17β-estradiol. The ability of estren and Rg1 to trigger kinase-mediated events would eventually lead to genomic events in MCF-7 cells which expressed high level of growth factor receptors, despite the low or negligible affinities towards ERα. However, it is unclear why Rg1 can induce more ERα phosphorylation in the cytoplasm than estren in MCF-7 cells in a ligand-independent manner.

5. Conclusion

In conclusion, the abilities of Rg1 and estren to regulate pS2 gene expression and recruit co-activators as well as induce sub-cellular distribution of ERα in MCF-7 cells are dramatically different, although both estren and Rg1 activate ERα via phosphorylation at Serine 118 in ligand-independent manner. Future studies will be needed to delineate how different phytoestrogens or estrogen analogs activate ER differently in different cell types.

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Figure legends

Figure 1 Effects of ginsenoside Rg1 and estren on cell proliferation in human breast cancer MCF-7 cells

MCF-7 cells were treated with various concentrations of 17β-estradiol (10^{-14} M-10^{-6} M), estren (10^{-14} M-10^{-6} M), Rg1 (10^{-14} M-10^{-6} M) or its vehicle for 48h. The growth of MCF-7 cells was quantified using the MTS proliferation assay according to the manufacturer's instructions. The 96-well plate was incubated at 37^{o}C for 2h and the absorbance at 490nm was measured. n=6-8. Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs control group.

Figure 2 Effects of ginsenoside Rg1 and estren on ERE-dependent transcription in human breast cancer MCF-7 cells

MCF-7 cells were transfected with 0.8μg the ERE-containing luciferase reporter plasmid vERETkluc and 0.01μg the inactive control plasmid pRL-TK. 5h after transfection, the cells were treated with 17β-estradiol (10^{-14} M-10^{-6} M), estren (10^{-14} M-10^{-6} M), Rg1 (10^{-14} M-10^{-6} M) or its vehicle for 24h. Activities of luciferase encoded by experimental and internal control plasmid were measured sequentially with DLR assay reagents. The ERE *firefly* luciferase activities were normalized for pRL-TK *Renilla* luciferase values. n=4. Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs control group

Figure 3 Effects of ginsenoside Rg1 and estren on pS2 gene expression and recruitment of co-activator SRC-1 to ERE-containing pS2 promoter in human breast cancer MCF-7

cells

A, MCF-7 cells were treated with various concentrations of 17β-estrodiol (10^{-14}M-10^{-6}M), estren (10^{-14}M-10^{-6}M), Rg1 (10^{-14}M-10^{-6}M) or its vehicle for 48h. After total RNA extraction, real-time RT-PCR was performed. Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs control group. n=3. B, MCF-7 cells were treated with 17β-estradiol (10^{-8} M), estren (10^{-8} M), Rg1 (10^{-12} M, 10^{-8} M) or its vehicle for 1h. ChIP assay was performed to study their effects on the recruitment of SRC-1 to pS2 promoter. pS2 promoter sequences containing ERE were detected by PCR amplification with specific primers. To determine input DNA, the pS2 promoter fragment was amplified from the purified soluble chromatin before immunoprecipitation. For negative control, the specific SRC-1 antibody was replaced by nonimmune mouse IgG. PCR products obtained at 38 cycles as shown. This experiment was repeated four times with similar results.

Figure 4 Effects of ginsenoside Rg1 and estren on ERα protein expression and ERα translocation in human breast cancer MCF-7 cells

A, MCF-7 cells were treated with 17β-estradiol (10^{-8} M), estren (10^{-8} M) or Rg1 (10^{-8} M) for 10 min, 20 min, 30 min and 2 h. After protein extraction, western blotting was performed. n=3. B, MCF-7 cells were treated with 17β-estradiol (10^{-8} M), estren (10^{-8} M) or Rg1 (10^{-8} M) for 5 min, 10 min, 20 min 30 min, 2 h and 6 h. After protein extraction from the cytoplasm and nucleus using the nuclear/cytosol fractionation kit, western blotting was performed to

555 investigate ERα, B-action and Histone H3 protein expressions. B-actin was used to normalize
556 ERα protein expression in the cytoplasm. Histone H3 was used to normalize ERα protein
557 expression in the nucleus. n=3. Data was expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$,
558 $^{***}P<0.001$ vs control group (0 point). C, MCF-7 cells were treated with 17β-estradiol (E2,
559 10^{-8}M), estren (10^{-8}M) or Rg1 (10^{-12} M, 10^{-8} M) for 30 min. After fixing with PBSAT
560 containing the Triton X-100, the cells were incubated with the ERα antibody (1:100) **(Green)**
561 at room temperature for 2h. After washing with PBSAT for 5 times, the cells were incubated
562 with Alexa Fluor 488 anti-rabbit antibody for 1h at RT. Before scanning with the Wide-field
563 Fluorescence Microscopy, the cells were incubated with the DNA-binding reagent Hoechst
564 33342 for 10 mins to stain the nucleolus **(Blue)**.

565 **Figure 5 Effects of ginsenoside Rg1 and estren on sub-cellular ERα phosphorylation at**
566 **Ser 118 in human breast cancer MCF-7 cells**

567 A, MCF-7 cells were treated with 17β-estradiol (E2, 10^{-8} M), estren (10^{-8} M) or Rg1 (10^{-8} M)
568 for 10 min, 20 min, 30 min and 2 h. After protein extraction, western blotting was performed.
569 n=3. B, MCF-7 cells were treated with 17β-estradiol (E2, 10^{-8} M), estren (10^{-8} M) or Rg1 (10^{-8}
570 M) for 5 min, 10 min, 20 min 30 min, 2 h and 6 h. After protein extraction from the cytoplasm
571 and nucleus using the nuclear/cytosol fractionation kit, western blotting was performed to
572 study the phospho-ERα and ERα protein expressions. The phosphorylation level was
573 calculated first as a ratio of pERα to ERα and finally expressed as a ratio to the basal reading

where time 0 (untreated as basal) equals 1. n=4. Data are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group (0 point). C, MCF-7 cells were treated with 17β-estradiol (E2, 10^{-8} M), estren (10^{-8} M) or Rg1 (10^{-12} M, 10^{-8} M) for 30 mins. After fixing with PBSAT containing the Triton X-100, the cells were incubated with the phosphor-ERα (Ser 118) antibody (1:100) **(Green)** at room temperature for 2h. After washing with PBSAT for 5 times, the cells were incubated with Alexa Fluor 488 anti-rabbit antibody for 1h at RT. Before scanning with the Wide-field Fluorescence Microscopy, the cells were incubated with the DNA-binding reagent Hoechst 33342 for 10 mins to stain the nucleolus **(Blue)**.

Figure 1

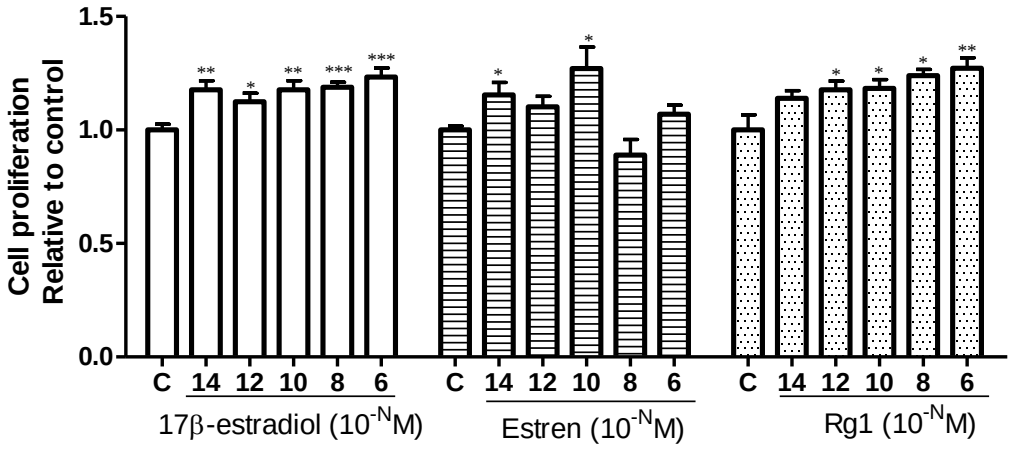

Cell proliferation
Relative to control
1.5
1.0
0.5
0.0
C 14 12 10 8 6
17β-estradiol (10-NM)
C 14 12 10 8 6
Estren (10-NM)
C 14 12 10 8 6
Rg1 (10-NM)

Figure 2

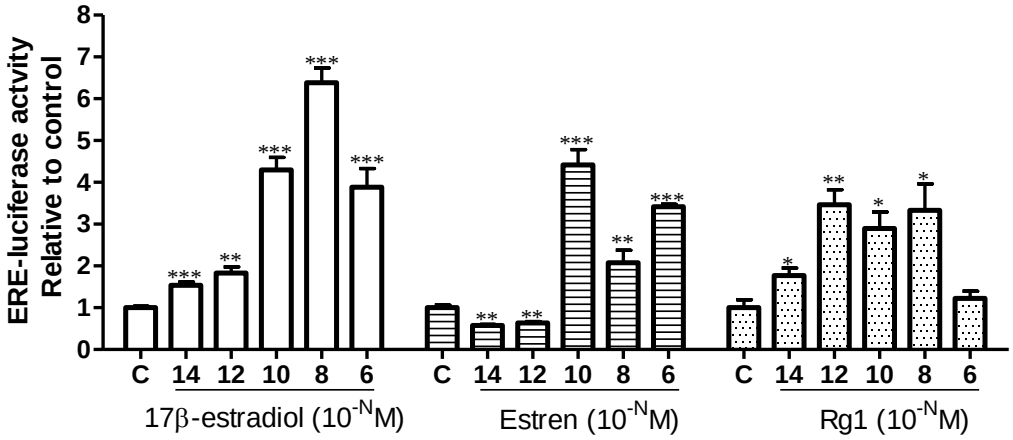
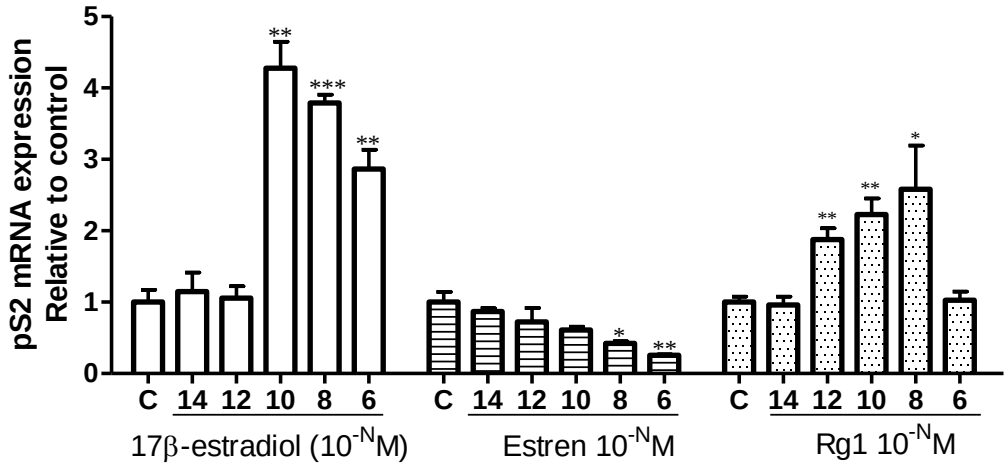
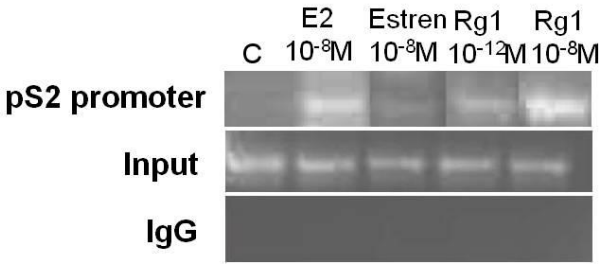

ERE-luciferase actvity
Relative to control
0
1
2
3
4
5
6
7
8
C 14 12 10 8 6
17β-estradiol (10-NM)
C 14 12 10 8 6
Estren (10-NM)
C 14 12 10 8 6
Rg1 (10-NM)

Figure 3

A.


pS2 mRNA expression
Relative to control
0
1
2
3
4
5
**
***
**
**
*
**
**
*
C 14 12 10 8 6
C 14 12 10 8 6
C 14 12 10 8 6
17β-estradiol (10-NM)
Estren 10-NM
Rg1 10-NM

B.


C
E2 10-8M
Estren 10-8M
Rg1 10-12M
Rg1 10-8M
pS2 promoter
Input
IgG

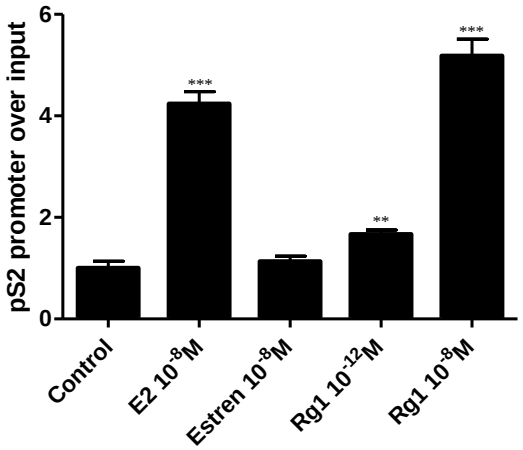
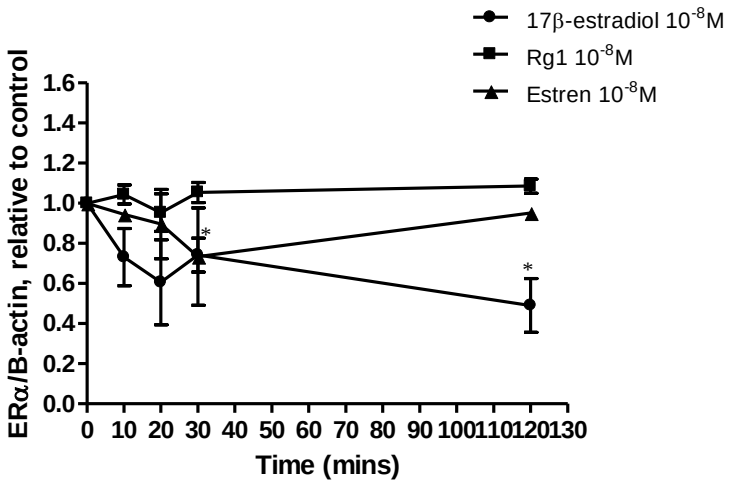
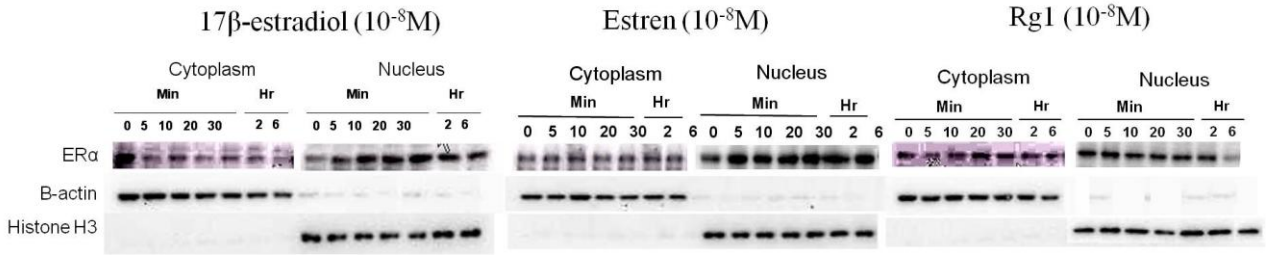

pS2 promoter over input
0
2
4
6
***
***
**
Control
E2 10-8M
Estren 10-8M
Rg1 10-12M
Rg1 10-8M

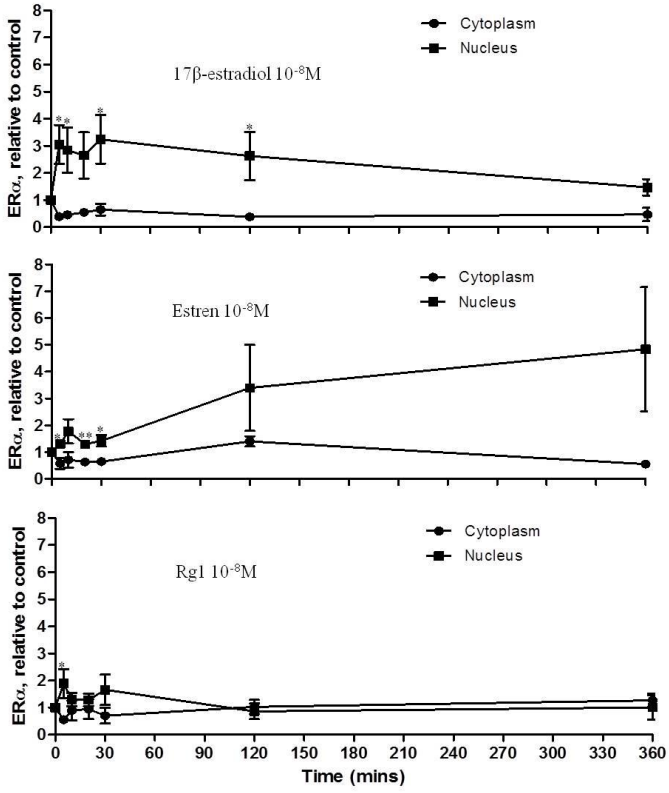
Figure 4

A.


17β-estradiol 10-8M
Rg1 10-8M
Estren 10-8M
ERα/B-actin, relative to control
Time (mins)

B.


17β-estradiol (10-8M)
Estren (10-8M)
Rg1 (10-8M)
Cytoplasm
Nucleus
Min
Hr
ERα
B-actin
Histone H3


Cytoplasm
Nucleus
17β-estradiol 10-8M
Estren 10-8M
Rg1 10-8M
ERα, relative to control
Time (mins)

C.

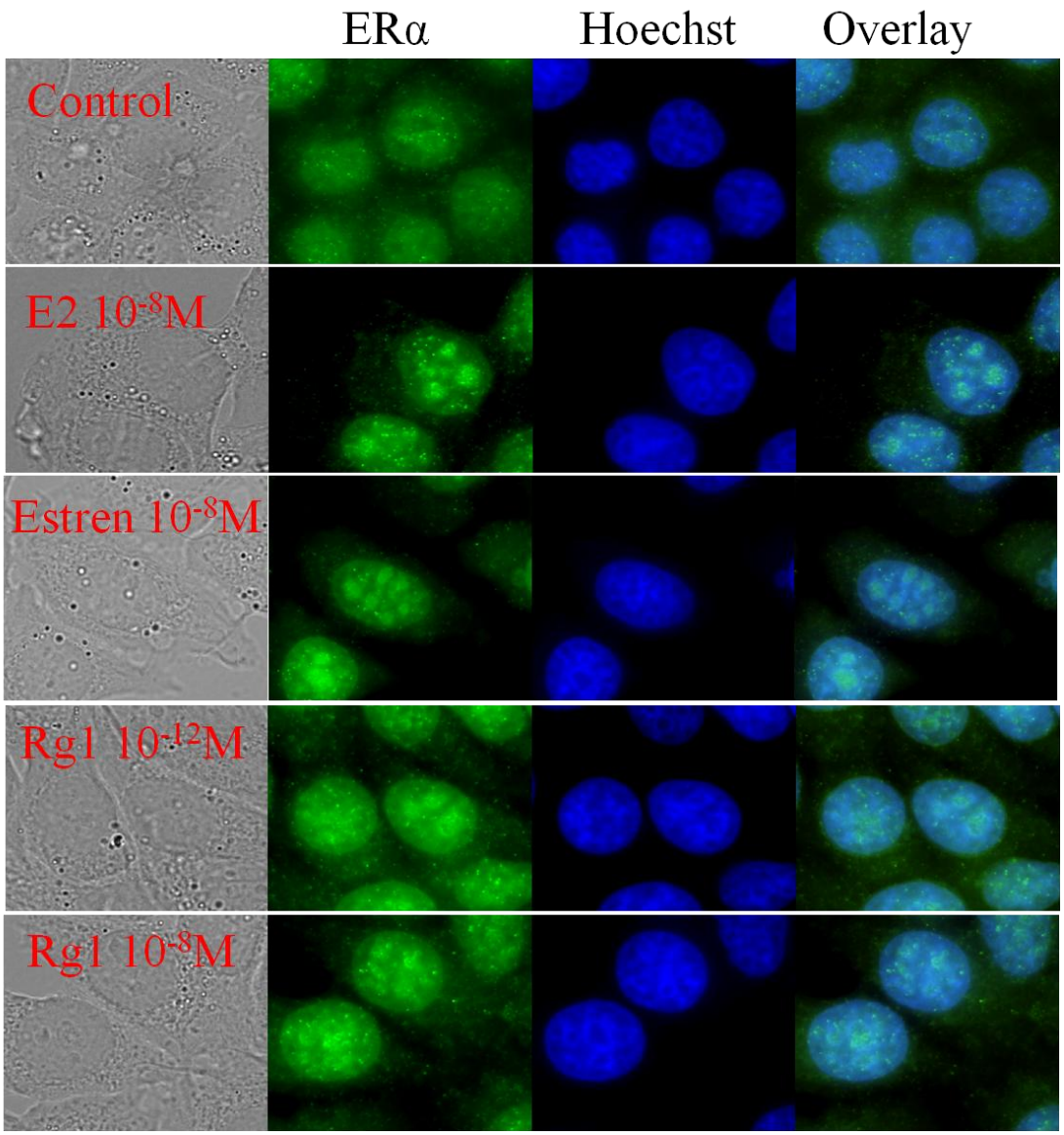
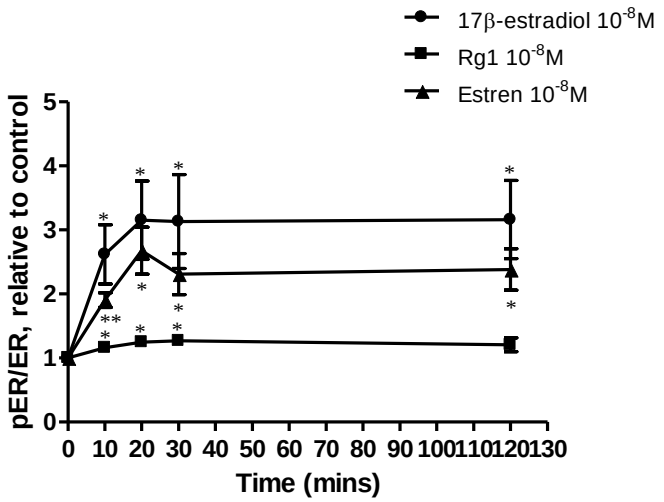
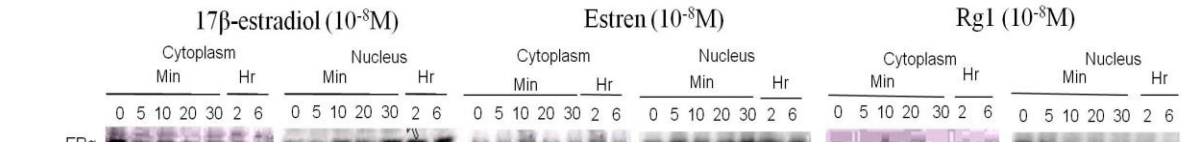

ERα
Hoechst
Overlay
Control
E2 10-8M
Estren 10-8M
Rg1 10-12M
Rg1 10-8M

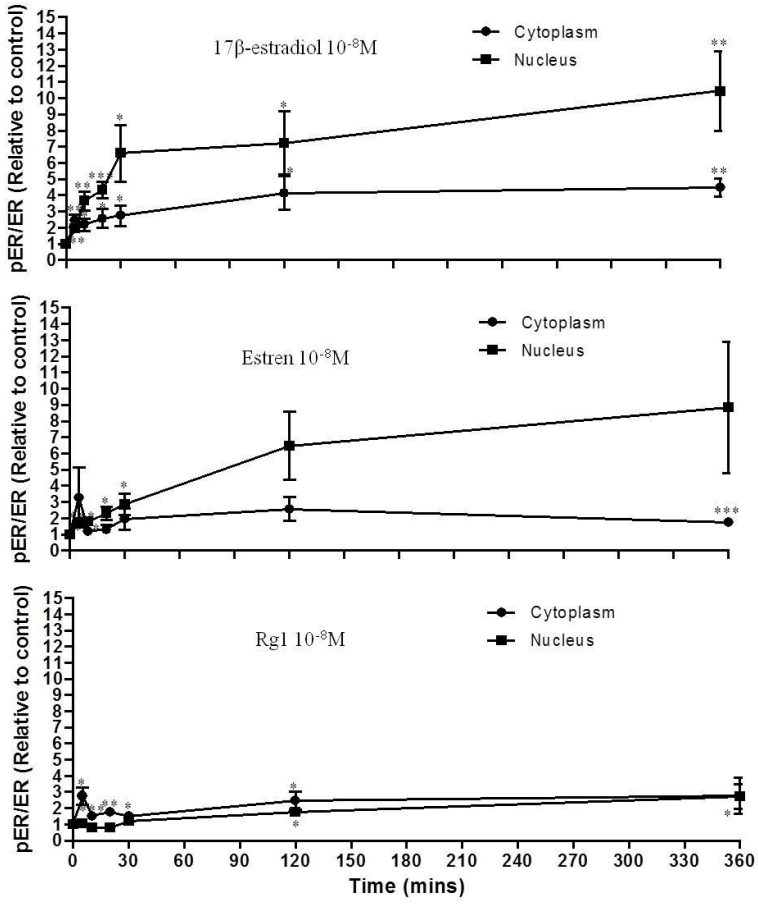
Figure 5

A.

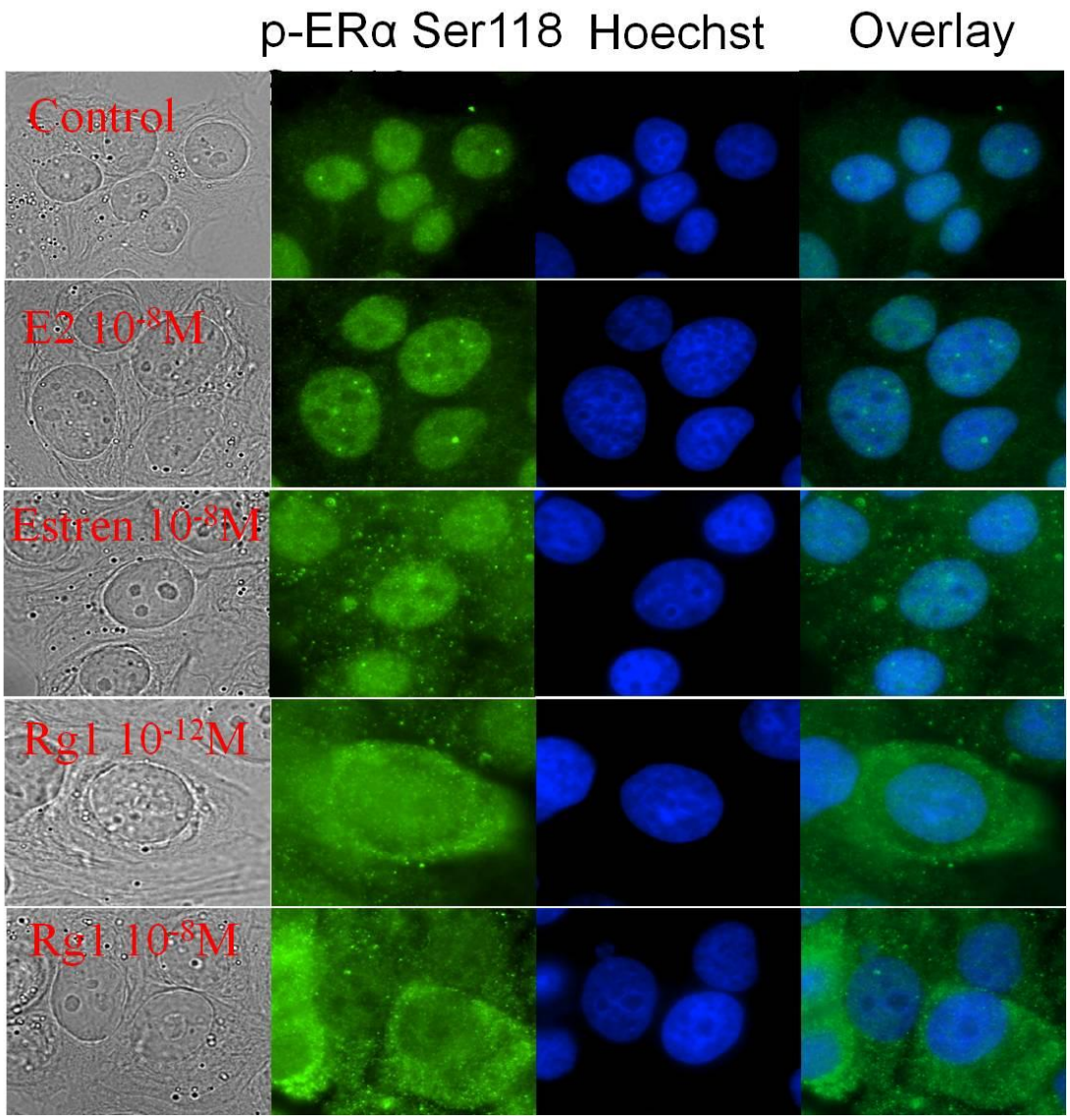

17β-estradiol 10-8M
Rg1 10-8M
Estren 10-8M
pER/ER, relative to control
Time (mins)

B.


17β-estradiol (10-8M)
Estren (10-8M)
Rg1 (10-8M)
Cytoplasm
Nucleus
Min
Hr
ERα
p-ER Ser 118


17β-estradiol 10-8M
Estren 10-8M
Rg1 10-8M
Cytoplasm
Nucleus
pER/ER (Relative to control)
Time (mins)

C.


p-ERα Ser118
Hoechst
Overlay
Control
E2 10-8M
Estren 10-8M
Rg1 10-12M
Rg1 10-8M