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Research Article

Ginsenoside Rg1 activates ligand-independent estrogenic effects via rapid estrogen receptor signaling pathway

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ABSTRACT

Background: Ginsenoside Rg1 was shown to exert ligand-independent activation of estrogen receptor (ER) via mitogen-activated protein kinase–mediated pathway. Our study aimed to delineate the mechanisms by which Rg1 activates the rapid ER signaling pathways.

Methods: ER-positive human breast cancer MCF-7 cells and ER-negative human embryonic kidney HEK293 cells were treated with Rg1 (10^{-12}M, 10^{-8}M), 17ß-estradiol (10^{-8}M), or vehicle. Immunoprecipitation was conducted to investigate the interactions between signaling protein and ER in MCF-7 cells. To determine the roles of these signaling proteins in the actions of Rg1, small interfering RNA or their inhibitors were applied.

Results: Rg1 rapidly induced ERα translocation to plasma membrane via caveolin-1 and the formation of signaling complex involving linker protein (Shc), insulin-like growth factor-I receptor, modulator of nongenomic activity of ER (MNAR), ERα, and cellular nonreceptor tyrosine kinase (c-Src) in MCF-7 cells. The induction of extracellular signal-regulated protein kinase and mitogen-activated protein kinase kinase (MEK) phosphorylation in MCF-7 cells by Rg1 was suppressed by cotreatment with small interfering RNA against these signaling proteins. The stimulatory effects of Rg1 on MEK phosphorylation in these cells were suppressed by both PP2 (Src kinase inhibitor) and AG1478 [epidermal growth factor receptor (EGFR) inhibitor]. In addition, Rg1-induced estrogenic activities, EGFR and MEK phosphorylation in MCF-7 cells were abolished by cotreatment with G15 (G protein-coupled estrogen receptor-1 antagonist). The increase in intracellular cyclic AMP accumulation, but not Ca mobilization, in MCF-7 cells by Rg1 could be abolished by G15.

Conclusion: Ginsenoside Rg1 exerted estrogenic actions by rapidly inducing the formation of ER containing signalosome in MCF-7 cells. Additionally, Rg1 could activate EGFR and c-Src ER-independently and exert estrogenic effects via rapid activation of membrane-associated ER and G protein-coupled estrogen receptor.

1. Introduction

Phytoestrogens are increasingly popular as alternatives to hormone replacement therapy whose clinical application is limited because it is associated with an increased risk of reproductive cancers. However, as many of the physiological effects of phytoestrogen are similar to those of estrogen and their effects are mediated through similar receptors and pathways, there is concern over the safe use of phytoestrogen [1]. Recent studies indicated that estrogen receptors (ERs) can be directly or indirectly activated by xenoestrogens and phytoestrogens in different tissues and cell types through differential mechanisms of actions, resulting in either

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undesirable or beneficial effects [2]. Thus, it is of the utmost importance to delineate how individual phytoestrogen activates ER to determine if the phytoestrogen of interest can have safer tissue- or pathway-selective estrogenic effects over the use of estrogen.

Ginsenoside Rg1, an abundant active ingredient in *Panax ginseng*, is previously reported to be a unique class of phytoestrogens which act like estrogen but without direct binding to ERs [3]. It has been shown to exert estrogen-like effects in neuronal [4,5], endometrial [6], and breast cancer [3,7,8] cells. Our earlier studies demonstrated that Rg1 could activate ER-dependent insulin-like growth factor-I receptor (IGF-IR) signaling by enhancing tyrosine phosphorylation of insulin receptor substrate-1 in MCF-7 cells [9,10]. Our study also showed that the neuroprotective actions of Rg1 were mediated by ER and IGF-IR pathways as demonstrated by its ability to attenuate the neurotoxicity of 6-hydroxydopamine (6-OHDA) and β-amyloid peptide fragment 25-35 ($A\beta_{25-35}$) in SK-N-SH and PC12 cells [10]. Subsequent studies showed that Rg1 induced ERα via mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated protein kinase (ERK)–mediated phosphorylation of ERα at Ser118 [7,8]. However, the mechanism by which Rg1 activates MAPK signaling pathways and exerts rapid estrogenic effects is far from clear.

Recent studies have indicated that rapid nongenomic responses that involve either membrane ERα or G protein-coupled estrogen receptor 1 (GPER1, formerly known as GPR30) may play an essential role in mediating the actions of phytoestrogens in different cell types [11–13]. Membrane-associated ERs play an important role in mediating the rapid signaling of E2. Hall et al [14] reported that E2 exerted rapid biological effects in bones, breasts, the vasculature, and the nervous system through the cell-surface ER forms. Estrogen is reported to initiate membrane signaling through growth factor receptors or membrane ERs, thereby modulating cellular functions via both transcriptional and nontranscriptional events [15]. Binding of estrogen to membrane ERα resulted in the assembly of a large protein signalosome at the plasma membrane which consisted of scaffold proteins such as caveolin and modulator of nongenomic activity of ER (MNAR, also known as PELP1), linker protein (Shc), cellular nonreceptor tyrosine kinase (c-Src), and in some situations, growth factor receptor tyrosine kinases (IGF-IR). The exact proteins involved in conveying specificity to the signaling from membrane ERα are cell- and context-dependent [16]. This indicates that estrogenic actions in different cell types and contexts are mediated by a complex interplay between classical ER pathways and rapid signaling pathways. GPER, a 7-transmembrane G protein-coupled receptor, mRNA, and protein are expressed in the lung, liver, prostate, ovary, and placenta [17]. The binding affinity of E2 for GPER was 10-fold lower than that determined for ERα [18]. Upon activation of GPER, E2 activated the MEK1/2/ERK1/2 pathway and exerted its effects via the transactivation of the epidermal growth factor receptor (EGFR) [19] and increased intracellular cyclic adenosine monophosphate (cAMP) in human breast cancer MCF-7 cells [20,21].

The present study was designed to determine the mechanisms by which Rg1 activates the rapid ER signaling pathways. As Rg1 is a potent activator of ER but not a ligand of nuclear ER, characterization of its estrogenic actions will provide new insights into understanding the role of rapid signaling pathways in mediating the actions of phytoestrogens.

2. Methods

2.1. Cell culture and treatment

Ginsenoside Rg1 was purified as described previously [3]. MCF-7 cells (HTB-22) and HEK293 cells (CRL-1573) were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS), 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. Two days after plating, the medium was replaced by phenol red–free DMEM containing 1% charcoal-stripped FBS (csFBS) and cultured for another 2 days. Cells were treated with different concentration of Rg1 or its vehicle for various periods of time in the presence or absence of different blockers, including AG1478 (EGFR inhibitor), PP2 (Src kinase inhibitor), and G15 (GPER1 blocker).

2.2. ERα translocation by wide-field fluorescence microscopy

MCF-7 cells were cotransfected with green fluorescent protein (GFP)-tagged ERα and mCherry-tagged caveolin-1. The transfected cells were treated with ginsenoside Rg1 (10^{-8}M) for 5 mins. Images were acquired using a wide-field fluorescence microscopy (Zeiss Observer.Z1). Captured images were analyzed using the MetaMorph software (MDS Analytical Technologies). Autofluorescence was negligible.

2.3. Small interfering RNA

Small interfering RNA (siRNA) targeting IGF-IR, Shc, MNAR, c-Src, and scramble RNA oligo were purchased from Dharmacon (Chicago, IL). Cells were seeded on a 6-well plate at a confluency of 50% 1 day before transfection. siRNA was transfected into the cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

2.4. Immunoblotting

Cells were lysed with Nonidet P-40 buffer containing protease inhibitors (aprotinin 2 μg/ml, leupeptin 2 μg/ml, and 1mM PMSF) and phosphatase inhibitors (1mM sodium orthovanadate and 10mM NaF). 50 μg of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilin-P, Millipore Corp.) at constant voltage (22V) overnight [22]. The blots were probed with primary antibodies, including polyclonal rabbit anti-human GPER1 (1:1000; Santa Cruz Biotechnology), rabbit anti-ERK (1:1000; Cell Signaling), mouse anti-phospho-ERK (1:1000; Santa Cruz Biotechnology), rabbit anti-MEK1/2, rabbit anti-phospho-MEK (Ser218/Ser222) (1:2000; Santa Cruz Biotechnology), Shc (1:1000; anta Cruz Biotechnology, Inc., Santa Cruz, CA), IGF-IR (1:1000; Santa Cruz Biotechnology), rabbit anti-MNAR (1:1000; Cell Signaling, Inc., MA, USA), rabbit anti-Src (1:1000; Millipore), phospho-EGFR (Tyr1148), EGFR (1:2000, Cell Signaling Technology, Inc., MA, USA), monoclonal rabbit anti-human phospho- ERα (Ser118, 1:2000, Upstate), ERα (1:2000; Santa Cruz Biotechnology), or monoclonal mouse anti-human B-actin (1:2000; Abcam). After washing, the PVDF membrane was subsequently incubated with goat anti-rabbit (1:2000; Santa Cruz Biotechnology) or anti-mouse IgG (1:2000; Cell Signaling) conjugated with horseradish peroxidase as the secondary antibody.

2.5. Immunoprecipitation

Cells were lysed with Nonidet P-40 buffer as described above. Protein lysate was precipitated with the corresponding antibodies overnight. And then the antibody/antigen complex was pulled out by addition of protein A Sepharose slurry. Precipitated proteins were resuspended and subjected to immunoblotting as described above. The antigen–antibody complexes were then detected with enhanced chemiluminescence reagent and visualized by the Lumi-Imager using Lumi Analyst, version 3.10, software (Roche, Mannheim, Germany).

Table 1
Primers used for real-time RT-PCR

Primer	Accession No.	Sequence (5′–3′)	Product	
			Orientation	Tm (°C)
GAPDH	NM_002046	GAAGGACTCATGACCACAGT	Sense	
		GTTGAAGCTAGAGGAGACCA	Antisense	57
GPER1	NM_001098201	AGTCGGATGTGAGGTTCAG	Sense	
		TCTGTGTGAGGAGTGCAAG	Antisense	53
pS2	NM_003225	ATGGCCACCATGGAGAACAAGG	Sense	
		CATAAATTCACACTCCTCTTCTGG	Antisense	55
IGF-IR	NM_000875	ACTATGCCGGTGTCTGTGTG	Sense	
		TGCAAGTTCTGGTTGTCGAG	Antisense	55

GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GPER1, G protein-coupled estrogen receptor 1; IGF-IR, insulin-like growth factor-I receptor; RT-PCR, reverse transcription polymerase chain reaction.

2.6. Cell proliferation assay

MCF-7 cells were treated with 10^{-8}M E2 and 10^{-12} M and 10^{-8}M Rg1 or its vehicle with or without G15 (10^{-6}M) (Tocris Bioscience, Bristol, United Kingdom) for 48 h. Upon incubation, cell viability was quantified by performing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

2.7. Transient transfection and luciferase assay

MCF-7 cells were seeded into 24-well plates (37,500cells/well) and cultured in phenol red–free DMEM containing 1% sFBS. 0.8 μg of the estrogen response element (ERE)-containing luciferase reporter plasmid vERETkluc, a gift from Dr. Vincent Giguere of McGill University, together with 0.01 μg of an inactive control plasmid pRL-TK, a *Renilla* luciferase control vector, were cotransfected into the cells using the Lipofectamine™ 2000 reagent following the manufacturer's instructions. 4–6 h after transfection, cells were subjected to E2 (10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) in the presence or absence of G15 (10^{-6}M) for another 24h. Upon treatment, luciferase activity of estrogen promoter was measured by a Dual Luciferase Reporter Assay System (Promega), and signals were detected by a TD-20/20 Luminometer (Turner Design).

2.8. Real-time RT-PCR

Total RNA was extracted from cells by using Trizol reagent according to the standard protocol. 1μg of RNA (1μg) was reverse-transcribed in 20μL of a reaction mixture (high-capacity cDNA reverse transcription kit, Applied Biosystem) at 25°C for 10 min, 37°C for 2 h, and 85°C for 5 s. The sequences of the PCR primers for pS2, IGF-IR, GPER1, and the house keeping gene glyceraldehydes-3-phosphate dehydrogenase were described in Table 1. PCR was carried out in 20μL reaction mixture containing 10μL SsoFast™ Eva Green supermix (BIO-RAD) and 0.5μL of cDNA template 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. Expression levels of the target genes were normalized by glyceraldehydes-3-phosphate dehydrogenase [8].

2.9. Intracellular cAMP accumulation

MCF-7 cells were treated with E2 (10^{-8}M), Rg1 (10^{-12}M, 10^{-8}M), or its vehicle in the presence or absence of G15 for 10 min. Treated cells were lysed with 0.1M HCL. cAMP levels were measured using the Cyclic AMP EIA Kit (Cayman, USA) according to the manufacturer's instructions and normalized by total protein concentration determined by Bio-Rad Bradford.

2.10. Calcium imaging

MCF-7 cells were incubated with 10μM Ca^{2+} green (Invitrogen) and subjected to treatment with E2 (10^{-8}M), Rg1 (10^{-12}M, 10^{-8}M), or its vehicle for 5 min in the presence or absence of ICI 182,780 or G15. Images were acquired using a wide-field fluorescence microscopy (Zeiss Observer.Z1). Captured images were analyzed using the MetaMorph software (MDS Analytical Technologies). Autofluorescence was negligible.

2.11. Statistical analysis

Data are reported as the mean ± standard error of mean. Differences between the means of groups were determined by *t* test. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Rg1 induced translocation of ERα to plasma membrane in MCF-7 cells

ERα translocation to plasma membrane requires the interaction with the caveolin-1 protein [23]. To determine if Rg1 increases ER translocation to plasma membrane via the interaction with caveolin-1 protein, the colocalization of ER and caveolin-1 in MCF-7 cells were studied by time-lapse microscopy. MCF-7 cells cotransfected GFP-tagged ER and mCherry-tagged caveolin-1 were treated

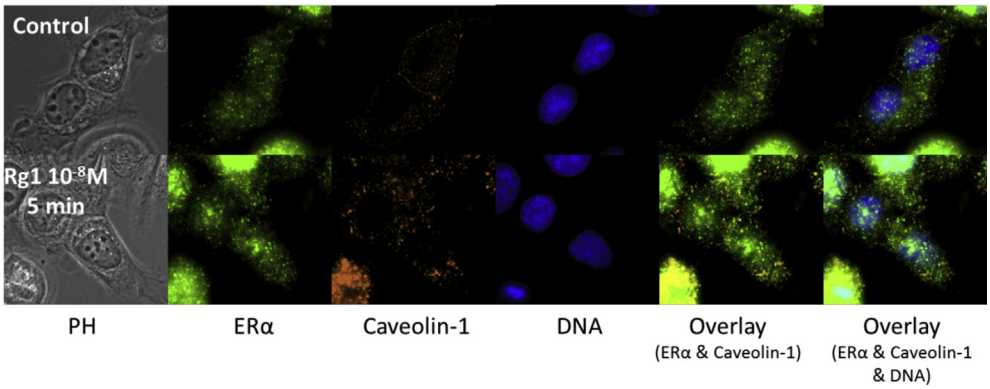

Fig. 1. Rg1 rapidly induced ERα translocation to plasma membrane via caveolin-1 within 5 min in MCF-7 cells. MCF-7 cells were cotransfected with GFP-tagged ERα and mCherry-tagged caveolin-1. The transfected cells were treated with Rg1 (10^{-8}M) for 5 mins. Images were acquired using a wide-field fluorescence microscopy (Zeiss Observer.Z1). Captured images were analyzed using the MetaMorph software (MDS Analytical Technologies). Autofluorescence was negligible.
ERα, estrogen receptor alpha.

with Rg1 and its vehicle. As shown in Fig. 1, Rg1 at 10^{-8}M rapidly increased ERα as well as caveolin-1 protein expression in MCF-7 cells within 5 minutes of incubation. Moreover, both ERα and caveolin-1 appeared to be colocalized near the plasma membrane of MCF-7 cells in response to treatment with Rg1. The results indicated that Rg1 rapidly induced ERα translocation to plasma membrane via caveolin-1 within 5 minutes of incubation in MCF-7 cells.

3.2. Rg1 induced the formation of ternary complex involving IGF-IR, Shc, and ERα in MCF-7 cells

Our previous study indicated that in addition to the increase in IGF-IR expression, Rg1 also enhances IGF-IR signaling pathways in MCF-7 cells [9]. Shc is an adapter protein that binds to many growth factor receptors in the membrane, including IGF-IR [24]. Its recruitment to the intracellular domains of the activated membrane receptors would eventually lead to the activation of MAPK pathway. It is reported that the recruitment of Shc by estrogen can facilitate the cross talk between IGF-IR and ERα [25]. To determine if Shc facilitates cross talk between IGF-IR and ER pathway in response to Rg1 treatment, the ability of the MCF-7 cells to form ternary complex involving Shc, ERα, and IGF-IR was studied by immunoprecipitation. As shown in Fig. 2A (Input), Rg1 at 10^{-12}M treatment increased IGF-IR, Shc and ERα protein expression in MCF-7 cells. In addition, Rg1 increased the binding between Shc and ERα, IGF-IR and ERα, and Shc and IGF-IR within 10 min of incubation in MCF-7 cells (Fig. 2A IP). The results suggest that Rg1 increased the physical interaction amongst Shc, IGF-IR, and ERα in MCF-7 cells.

3.3. Rg1 induced interactions amongst MNAR, c-Src, and ERα in MCF-7 cells

MNAR/PELP1 is a scaffold protein that contains multiple protein–protein interaction domains and links conventional steroid receptors with extranuclear signal transduction pathways [26]. It is shown to be required for estrogen-induced ERα activation of Src and downstream MAPK pathway [27]. To determine whether MNAR is involved in mediating the effects of Rg1 in MCF-7 cells, interactions among MNAR, ERα, and Src were studied. As shown in Fig. 2B (Input), Rg1 at 10^{-12}M increased MNAR, c-Src, and ERα protein expression in MCF-7 cells. In addition, coimmunoprecipitation studies showed that Rg1 increased the binding between MNAR and ERα (as shown by coimmunoprecipitation with anti-MNAR antibody), MNAR and c-Src (as shown by coimmunoprecipitation with anti-MNAR antibody), and ERα and c-Src. The inability for ERα and c-Src to coimmunoprecipitate with MNAR from the total protein lysate might be due to the large molecular size of MNAR that hindered its stable association with these proteins during immunoprecipiation. Neverttheless, the results suggest that MNAR might help mediate the actions of Rg1 in the activation of c-Src and ERα in MCF-7 cells.

3.4. Knockdown of IGF-IR, Shc, MNAR, and c-Src expression decreased Rg1-induced MAPK signaling in MCF-7 cells

A previous study of our group found that Rg1 activate MAPK within 5 min of incubation in MCF-7 cells [8]. As indicated in Fig. 2C, Rg1 at 10^{-12}M increased the phosphorylation of MEK and

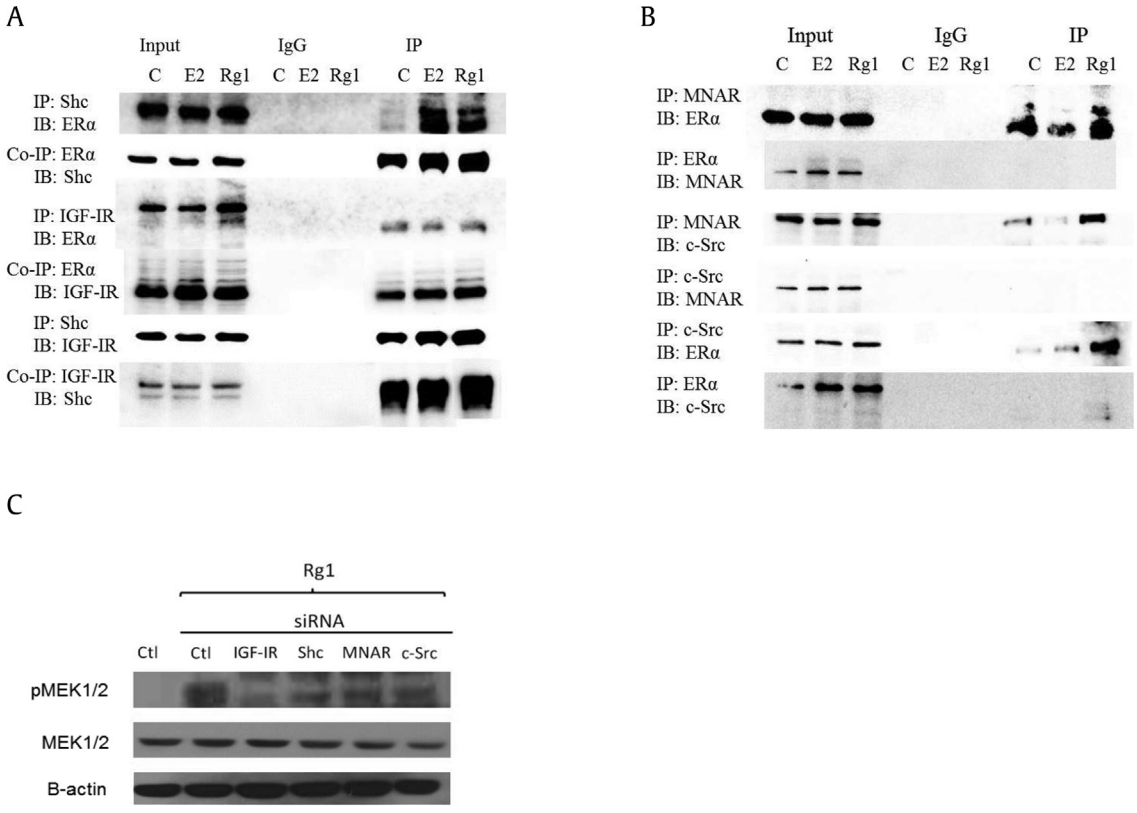

Fig. 2. Rg1 induced the formation of signaling complex containing Shc, ERα, IGF-IR MNAR and c-Src and subsequent MEK and ERK phosphorylation in MCF-7 cells. (A) Formation of ternary complex involving Shc, ERα, and IGF-IR: MCF-7 cells were treated with 17β-estradiol (E2, 10^{-8}M) or Rg1 (10^{-12}M) for 10 min. (B) Formation of ternary complex involving MNAR, c-Src, and ERα: MCF-7 cells were treated with 17β-estradiol (E2, 10^{-8}M) or Rg1 (10^{-12}M) for 10 min. After protein extraction, immunoprecipitation and immunoblotting were performed. The experiment was repeated independently three times. (C) MEK phosphorylation: MCF-7 cells were transfected with siRNA directly against IGF-IR, Shc, MNAR, and c-Src. After treatment with Rg1 (10^{-12}M) for 10 min, protein was extracted for immunoblotting detection. The experiment was repeated independently three times.
ERα, estrogen receptor alpha; ERK, extracellular signal-regulated protein kinase; IGF-IR, insulin-like growth factor-I receptor; MEK, mitogen-activated protein kinase kinase; siRNA, small interfering.

MEK in MCF-7 cells within 10 minutes of incubation (lane 2, blots 1 & 3). Knockdown of IGF-IR, Shc, MNAR, and c-Src by transfecting with 200pM siRNA against corresponding proteins successfully suppressed Rg1-induced phospho-MEK expression (lane 3–6). This indicated that the potential function of these signaling proteins in the actions of Rg1 on mitogen-activated protein (MAP) activation.

3.5. *Rg1-induced MEK phosphorylation was suppressed by Src kinase inhibitor PP2 in MCF-7 and HEK293 cells*

Our previous study suggested that the most effective dosages of Rg1 to exert estrogenic effects in MCF-7 cells were 10^{-12}M and 10^{-8}M [28]. To characterize the function of Src in mediating the effects of Rg1 on MAPK signaling, MCF-7 cells were preincubated with Src kinase inhibitor PP2 (10^{-8}M) prior to treatment with Rg1. E2 (10^{-8}M) and Rg1 at 10^{-12} and 10^{-8}M significantly induced MEK phosphorylation in MCF-7 cells, and PP2 abolished the stimulatory effects of Rg1 on MEK phosphorylation at both dosages in MCF-7 (Fig. 3A). To determine if Rg1 stimulates MEK phosphorylation in HEK293 cells, the time- and dose-response of MEK phosphorylation to treatment with Rg1 were determined. Rg1 significantly increased the expression of phosphorylated MEK as early as at 5 min and did not alter MEK expression in HEK293 cells throughout the course of incubation (Fig. 3B). Rg1 significantly increased phosphorylated MEK/MEK expression at all concentrations tested (10^{-14} to 10^{-6}M) in HEK293 cells (Fig. 3C). PP2 abolished the stimulatory effects of Rg1 on MEK phosphorylation at 10^{-12}M and 10^{-8}M in HEK293 cells (Fig. 3D). The results indicate that c-Src mediates the actions of Rg1 on MAPK signaling in both ER-positive

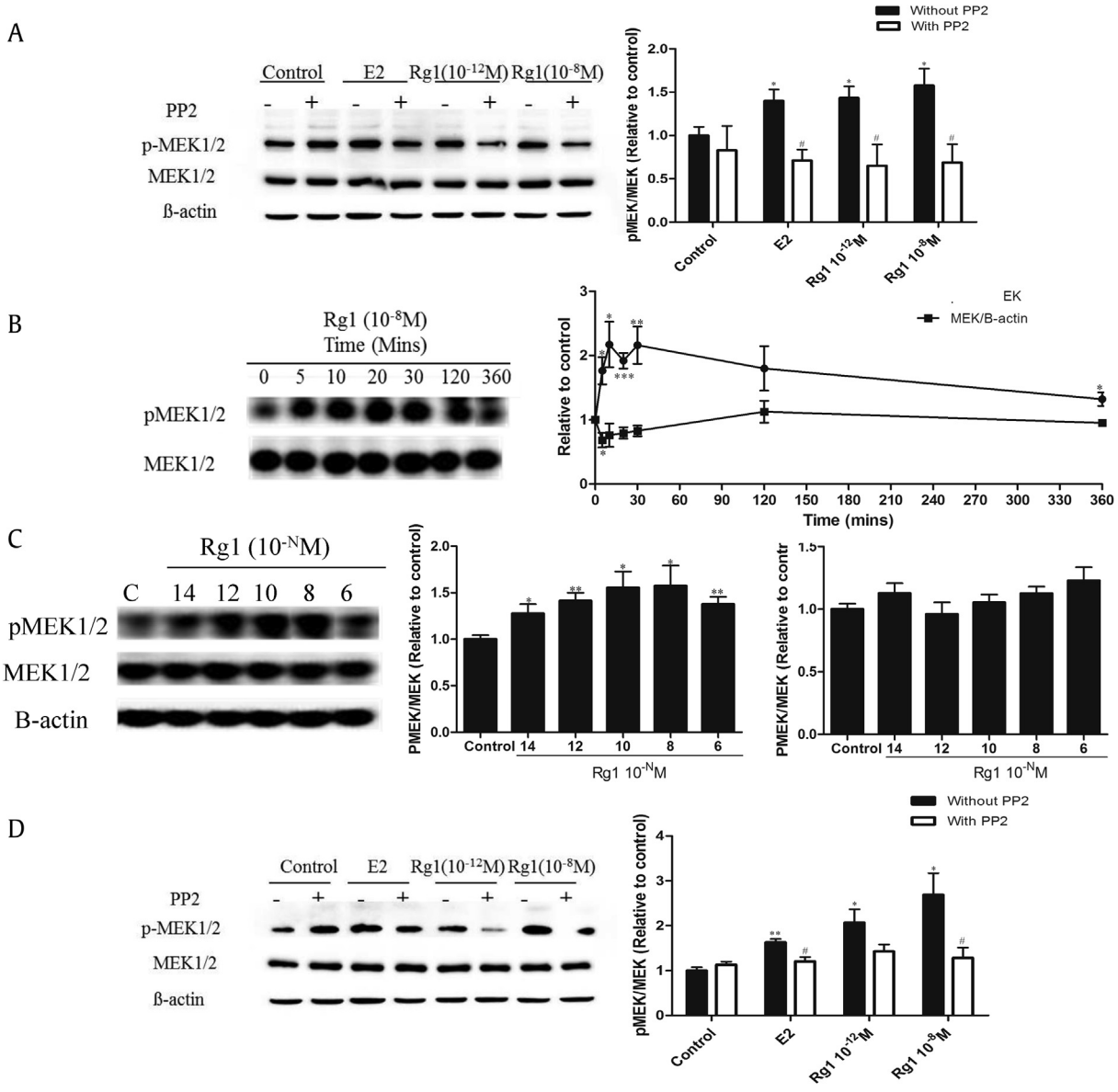

Fig. 3. Effect of Src kinase inhibitor PP2 [1] on MEK phosphorylation induced by Rg1 in ER-positive MCF-7 cells and ER-negative HEK 293 cells. (A) MCF-7 cells were pretreated with Src kinase inhibitor PP2 (10nM) for 30 mins and then co-treated with 17β-estradiol (E2, 10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) for 30 mins. (B) HEK293 cells were treated with Rg1 at 10^{-8}M for 5 min, 10 min, 20 min, 30 min, 2 hr, and 6 hr. (C) HEK293 cells were treated with different dosages of Rg1 (10^{-14} M–10^{-6}M) for 30 mins. (D) HEK293 cells were pretreated with Src kinase inhibitor PP2 (10nM) for 30 mins and then cotreated with 17β-estradiol (E2, 10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) for 30 mins. After protein extraction, western blotting was performed. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group; $^{\#}p < 0.05$ versus without G15 group, n = 4.
ER, estrogen receptor; MEK, mitogen-activated protein kinase kinase; SEM, standard error of mean.

MCF-7 and ER-negative HEK293 cells and that its involvement is ER-independent.

3.6. The induction of MEK phosphorylation by Rg1 involved EGFR phosphorylation in both MCF-7 and HEK293 cells

EGFR is a type I receptor tyrosine kinase that has been shown to be the convergence point for many membrane tyrosine cytokine receptors on MAPK activation [29]. EGFR was also shown to mediate the activation of MAPK in MCF-7 cells [30]. As shown in Fig. 4A, E2 at 10^{-8}M rapidly induced EGFR phosphorylation within 10 min of incubation in MCF-7 cells, which peaked at 2 hr (2.7-fold) and subsequently fell at 6 hr (1.61-fold). For Rg1, it produced rapid EGFR phosphorylation as early as at 5 min, followed by a fall at 20 min, then increased again, and peaked at 2 hr (2.2-fold). To determine if EGFR plays a role in Rg1 activation of MAPK, MCF-7 and HEK293 cells were pretreated with 1μM AG1478 (a selective EGFR inhibitor) prior to treatment with Rg1. As shown in Fig. 4B, AG1478 significantly abolished the stimulatory effect of Rg1 at 10^{-12} and 10^{-8}M on MEK phosphorylation in MCF-7 cells. Similarly, the stimulatory effects of Rg1 at both dosages on MEK phosphorylation in HEK293 cells were completely blocked by AG1478. The results show the involvement of EGFR in mediating the stimulation of Rg1 on MAPK signaling in both MCF-7 cells and HEK293 cells.

3.7. The estrogenic effects of Rg1 in MCF-7 cells were abolished by GPER1 antagonist, G15

GPER is also implicated in the mediating of both rapid and transcriptional events in response to estrogen [31,32]. To determine its potential function in mediating the actions of Rg1, the effects of Rg1 on GPER1 mRNA and protein expression in MCF-7 cells were first determined. As shown in Fig. 5A, Rg1 at 10^{-12}M and 10^{-8}M and E2 at 10^{-8}M significantly decreased GPER1 mRNA expression in MCF-7 cells time-dependently (2–72 hr) (Fig. 5A). Similarly, the significant inhibitory effect of Rg1 could be detected in MCF-7 cells

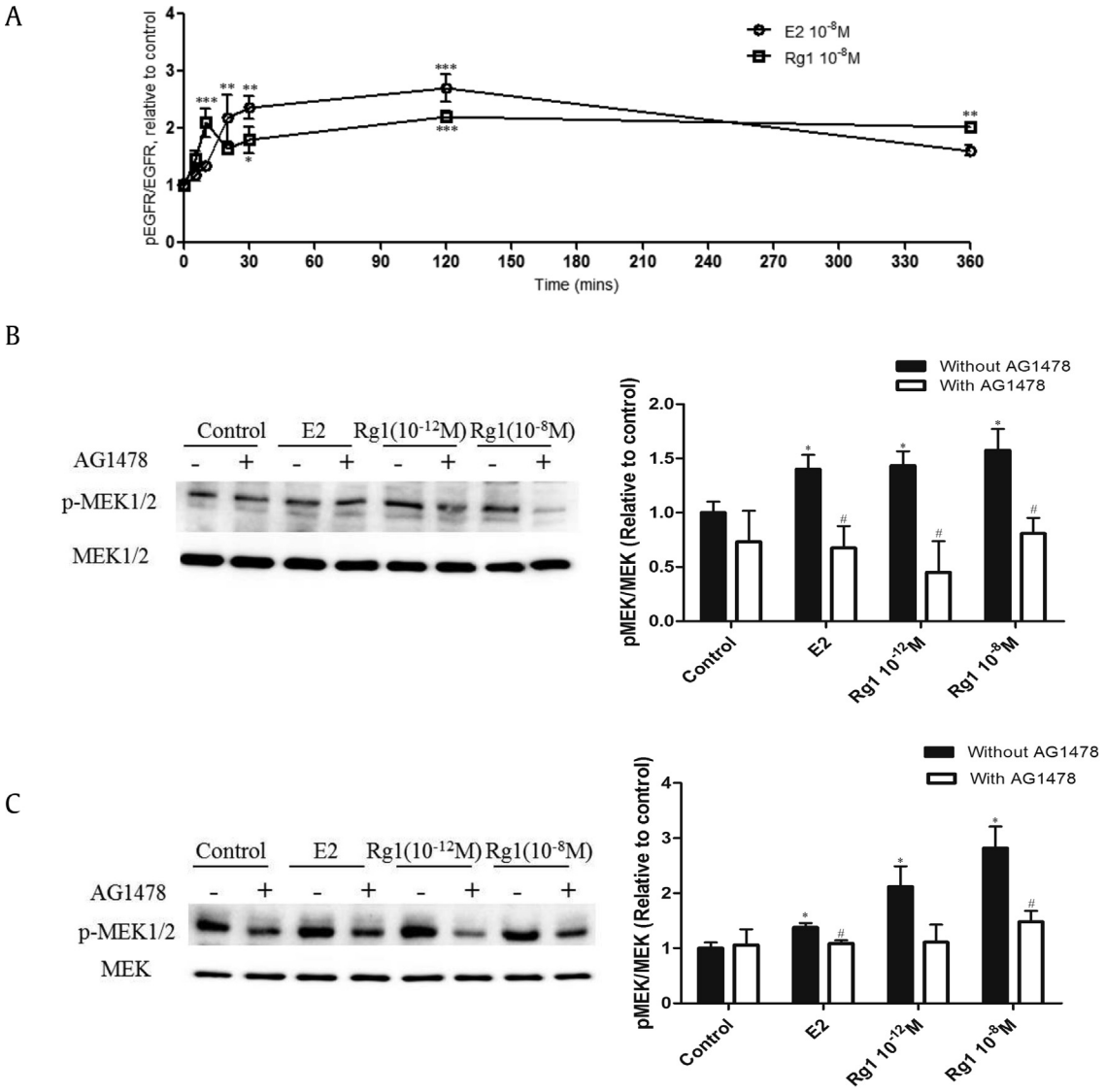

Fig. 4. EGFR is involved in mediating Rg1-induced MEK phosphorylation in both ER-positive MCF-7 and ER-negative HEK 293 cells. (A) MCF-7 cells were treated with E2 (10^{-8}M) or Rg1 (10^{-8}M) for 5 min, 10 min, 20 min, 30 min, 2 hr, 6 hr. (B) and (C) MCF-7 cells and HEK293 cells were pre-treated with EGFR inhibitor AG1478 (1μM) for 30 mins, and then co-treated with 17β-estradiol (E2, 10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) for 30 mins. After protein extraction, western blotting was performed. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group; $^{\#}p < 0.05$ versus without G15 group, n = 4.
EGFR, epidermal growth factor receptor; ER, estrogen receptor; MEK, mitogen-activated protein kinase kinase; SEM, standard error of mean.

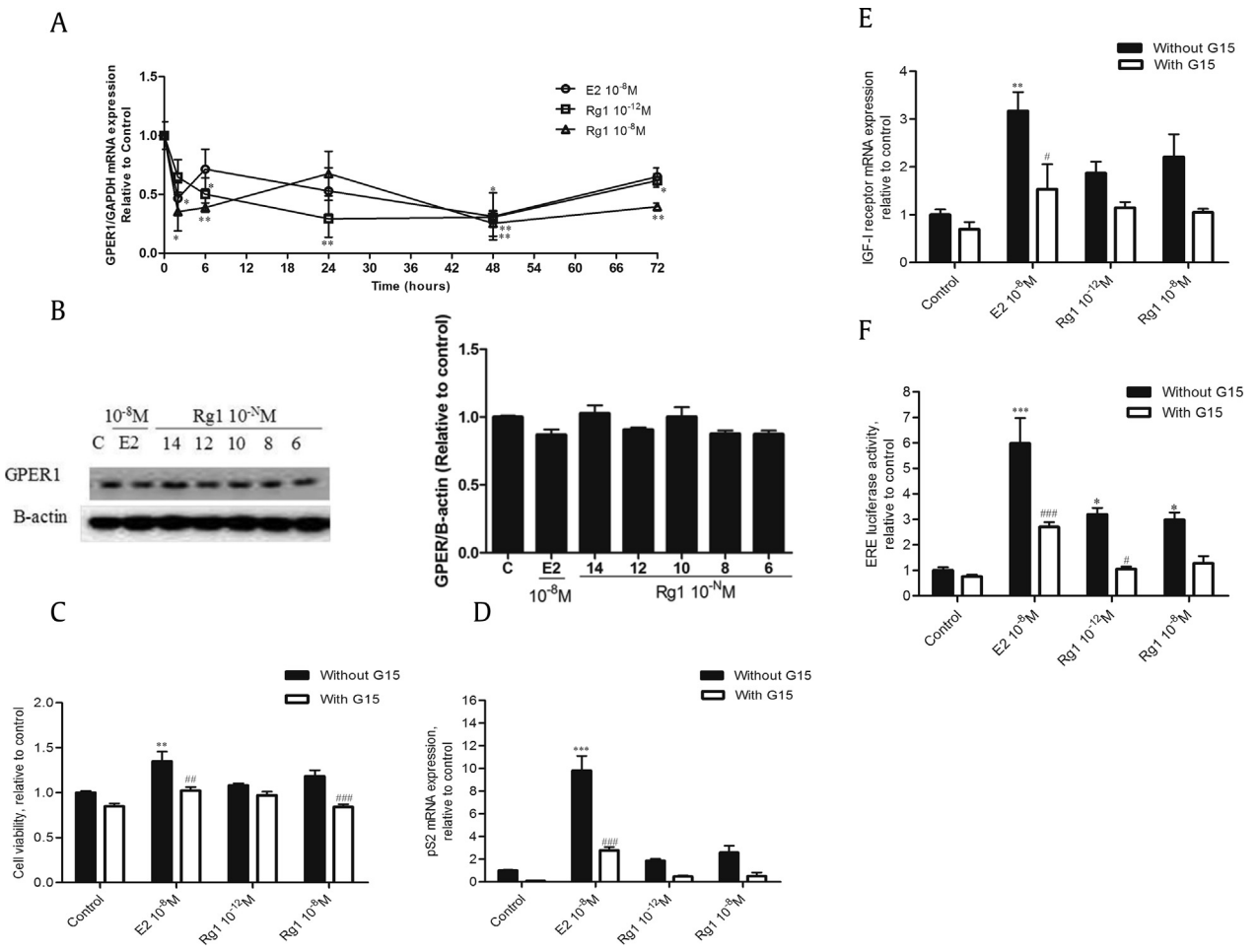

Fig. 5. GPER plays a role in mediating the estrogenic effects of Rg1 in MCF-7 cells. (A) GPER1 mRNA expression: MCF-7 cells were treated with E2 (10^{-8}M), Rg1 (10^{-12}M, 10^{-8}M) or its vehicle for 2 hr, 6 hr, 24 hr, 48 hr, 72 hr. After total RNA extraction, real-time PCR was performed. (B) GPER protein expression: MCF-7 cells were treated with E2 (10^{-8}M) or different dosages of Rg1 (10^{-14} M–10^{-6}M) for 48 hr. After protein extraction, western blotting was performed. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group, n = 4–5. (C) MCF-7 cells were pre-treated with G15 (10^{-6}M) for 20 min, and then co-treated with E2 (10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) for 48h. The growth of MCF-7 cells was quantified using the MTS proliferation assay according to the manufacturer's instructions. n = 6–8. (D) and (E) MCF-7 cells were pre-treated with G15 (10^{-6}M) for 20 min, and then co-treated with E2 (10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) for 48h. After total RNA extraction, real-time RT-PCR was performed. n = 4. (F) Transfected MCF-7 cells were pre-treated with G15 (10^{-6}M) for 20 min, then co-treated with E2 (10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) for 24h; dual-luciferase assay was performed. Activities of luciferase encoded by experimental and internal control plasmid were measured sequentially with dual luciferase reporter (DLR) assay reagents. The ERE *firefly* luciferase activities were normalized for pRL-TK *Renilla* luciferase values. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus without G15 group, n = 4.

DLR, dual luciferase reporter; GPER, G protein-coupled estrogen receptor; RT-PCR, Reverse transcription polymerase chain reaction; SEM, standard error of mean.

as early as after 2 hr and 24 hr of treatment in the concentrations of 10^{-8}M and 10^{-12}M, respectively (Fig. 5A). However, E2 and Rg1 did not alter GPER protein expression in MCF-7 cells (Fig. 5B). Our previous study suggested that Rg1 stimulated cell proliferation and increased IGF-I receptor mRNA expression as well as pS2 mRNA expression in MCF-7 cells [9]. In the present study, MCF-7 cells were pretreated with G15 (a GPER1 selective antagonist, 10^{-6}M) for 20 min followed by cotreatment with Rg1 (10^{-12}M, 10^{-8}M) or E2 (10^{-8}M) for 48h. G15 significantly abolished the stimulatory effects of 10^{-8}M of E2 and Rg1 on cell proliferation (Fig. 5C). Similarly, G15 completely abolished the stimulatory effects of Rg1, but only partially blocked the stimulatory effects of E2 on pS2 mRNA expression in MCF-7 cells (Fig. 5D). G15 could only partially block the stimulatory effects of E2 and Rg1 on IGF-I receptor mRNA expression (Fig. 5E). The results suggest that GPER is at least in part involved in mediating the estrogenic effects of Rg1 in MCF-7 cells. At the transcriptional level, G15 cotreatment could abolish the stimulatory effects of Rg1 and partially block the effects of E2 on ERE-dependent luciferase activities in MCF-7 cells transiently transfected with ERE-luciferase construct (Fig. 5F), suggesting that the GPER pathway might be in part responsible for the transcriptional activation of ERE-dependent gene expression by E2 and Rg1 in MCF-7 cells.

3.8. Rg1-induced EGFR and MEK phosphorylation was abolished by cotreatment with G15 in MCF-7 cells

It is reported that EGFR could be transactivated by estrogen via GPER1 [19]. In addition, GPER was also reported to mediate the actions of estrogen in inducing MAPK signaling pathways in breast cancer cells [20]. To determine if the activation of EGFR and MEK by Rg1 is mediated by GPER, MCF-7 cells were cotreated with G15 (10^{-6}M) and Rg1 (10^{-12}, 10^{-8}M), E2 (10^{-8}M), or vehicle. For EGFR, estrogen and Rg1 at 10^{-8}M significantly induced EGFR phosphorylation in MCF-7 upon treatment for 10 minutes (Fig. 6A) and cotreatment with G15 partially blocked E2- and Rg1-induced phosphorylation of EGFR (Fig. 6A). The results suggest that both E2 and Rg1 might in part activate EGFR via GPER1 in MCF-7 cells.

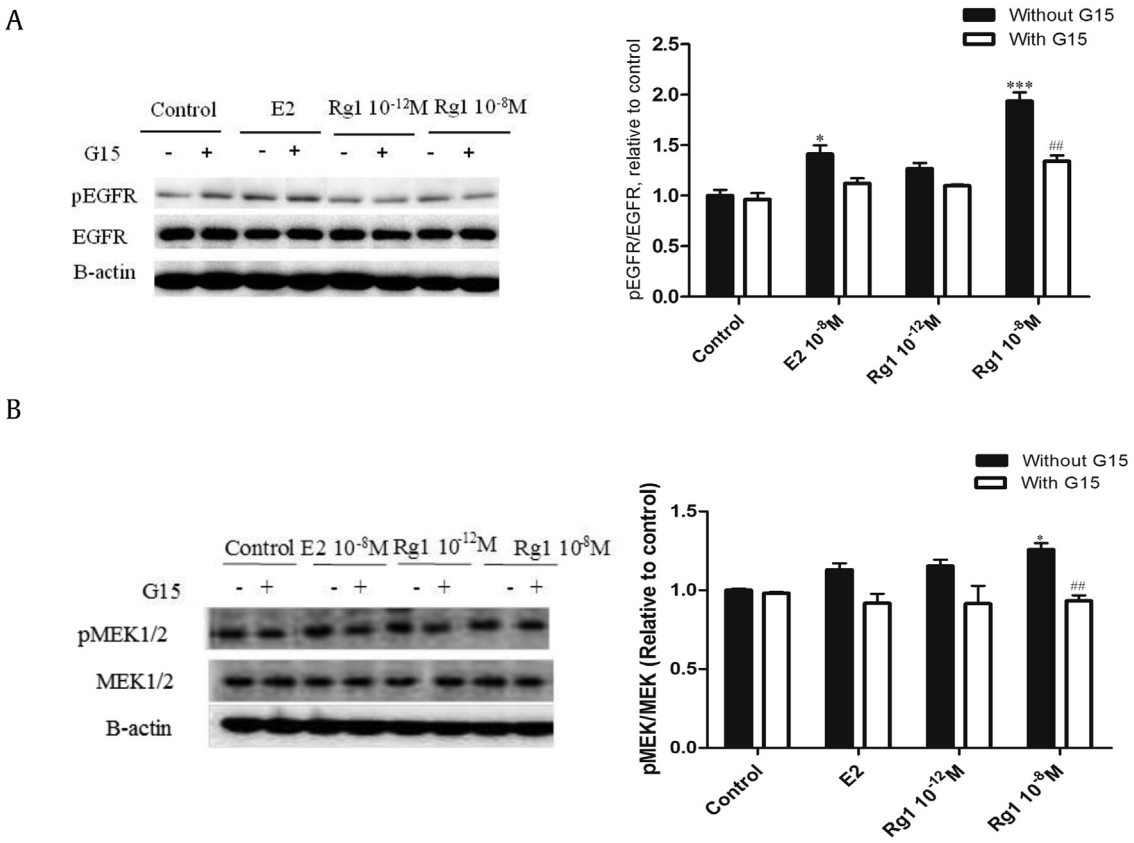

Fig. 6. Rg1-induced EGFR and MEK phosphorylation in MCF-7 cells was GPER1-dependent. (A) MCF-7 cells were pre-treated with G15 (10^{-6}M) for 20 min, then co-treated with E2 (10^{-8}M) or Rg1 (10^{-12}M, 10^{-8}M) for 10 min. (B) MCF-7 cells were pre-treated with G15 (10^{-6}M) for 20 min and then co-treated with E2 (10^{-8}M), Rg1 (10^{-12}M, 10^{-8}M) for 30 min. After protein extraction, western blotting was performed. Signals were detected and quantified by the lumi-Imager. The degree of phosphorylation was calculated as a ratio of pMEK to MEK and finally expressed as a ratio to the control [1]. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ versus control group; $^{\#\#}p < 0.01$ versus without G15 group, n = 3–4.
EGFR, epidermal growth factor receptor; GPER1, G protein-coupled estrogen receptor 1; MEK, mitogen-activated protein kinase kinase; SEM, standard error of mean.

For MEK, E2 at 10^{-8}M and Rg1 at 10^{-12}M and 10^{-8}M significantly induced MEK phosphorylation in MCF-7 cells upon treatment for 30 min (Fig. 6B). Cotreatment with G15 (10^{-6}M) completely abolished the stimulatory effects of E2 and Rg1 on MEK1/2 phosphorylation without alteration of MEK phosphorylation in MCF-7 cells (Fig. 6B). The results indicate the potential role of GPER in activation of EGFR and MAPK signaling pathway in MCF-7 cells.

3.9. Rg1-induced intracellular cAMP accumulation, but not Ca mobilization, was abolished by cotreatment with G15 in MCF-7 cells

cAMP plays an important role in GPER1-mediated estrogen signaling [19]. It is of interest to determine if Rg1 acts like E2 in alteration of intracellular cAMP accumulation in MCF-7 cells. Both E2 (10^{-8}M) and Rg1 at 10^{-12}M and 10^{-8}M significantly stimulated intracellular cAMP accumulation in MCF-7 cells upon treatment for 10 min (Fig. 7A). Cotreatment with G15 abolished E2- and Rg1-induced intracellular cAMP accumulation in MCF-7 cells. The results suggest that the induction of intracellular cAMP accumulation by Rg1 in MCF-7 cells is mediated by the GPER1 pathway.

Estrogen is reported to induce extremely rapid increase in the concentrations of intracellular calcium in MCF-7 cells [33]. To delineate whether Rg1 can induce Ca mobilization in MCF-7 cells, changes in fluorescence intensity were measured using Ca^{2+} green by wide-field fluorescence microscopy. E2 and Rg1 at 10^{-8}M significantly increased fluorescence intensity upon treatment for 5 min in MCF-7 cells (Fig. 7B), indicating that they could rapidly induce Ca mobilization in MCF-7 cells. Cotreatment of MCF-7 cells with a specific ER antagonist, ICI 182780, significantly reduced Rg1-induced, but not E2-induced, Ca mobilization in MCF-7 cells (Fig. 7B). The results indicated the involvement of ER in Rg1-induced Ca mobilization in MCF-7 cells. In contrast, G15 cotreatment significantly reduced the induction of Ca mobilization by E2, but not by Rg1, in MCF-7 cells (Fig. 7B). The results suggest that GPER1 is involved in mediating E2-induced Ca mobilization in MCF-7 cells and that the molecular actions of Rg1 in inducing Ca mobilization are different from those of E2.

4. Discussion

It is now well established that the actions of estrogen are mediated by genomic, classical, or nuclear pathways and by rapid nongenomic, extranuclear effects that do not involve any nuclear localization of the ERs [34]. Our previous study demonstrated that Rg1 induced ERα nuclear translocation and ERα phosphorylation at Ser118 primarily in the cytoplasm in MCF-7 cells [28]. Specifically, Rg1 rapidly induced ERα Ser118 phosphorylation by 2.8 fold in the cytoplasm within 5 min of incubation, but it only significantly induced ERα Ser118 phosphorylation in the nucleus upon treatment for 2 h (1.8 fold) and 6 h (2.74 fold). As Rg1 did not bind to ERs, these results suggest that the delayed nuclear genomic events induced by Rg1 might be initiated by the rapid extranuclear pathways at the plasma membrane. Rapid extranuclear signaling pathway is known to be mediated by cytoplasmic signaling proteins, growth factor receptors, and G-protein coupled receptor (GPCR) signaling pathways [34]. The present study attempted to

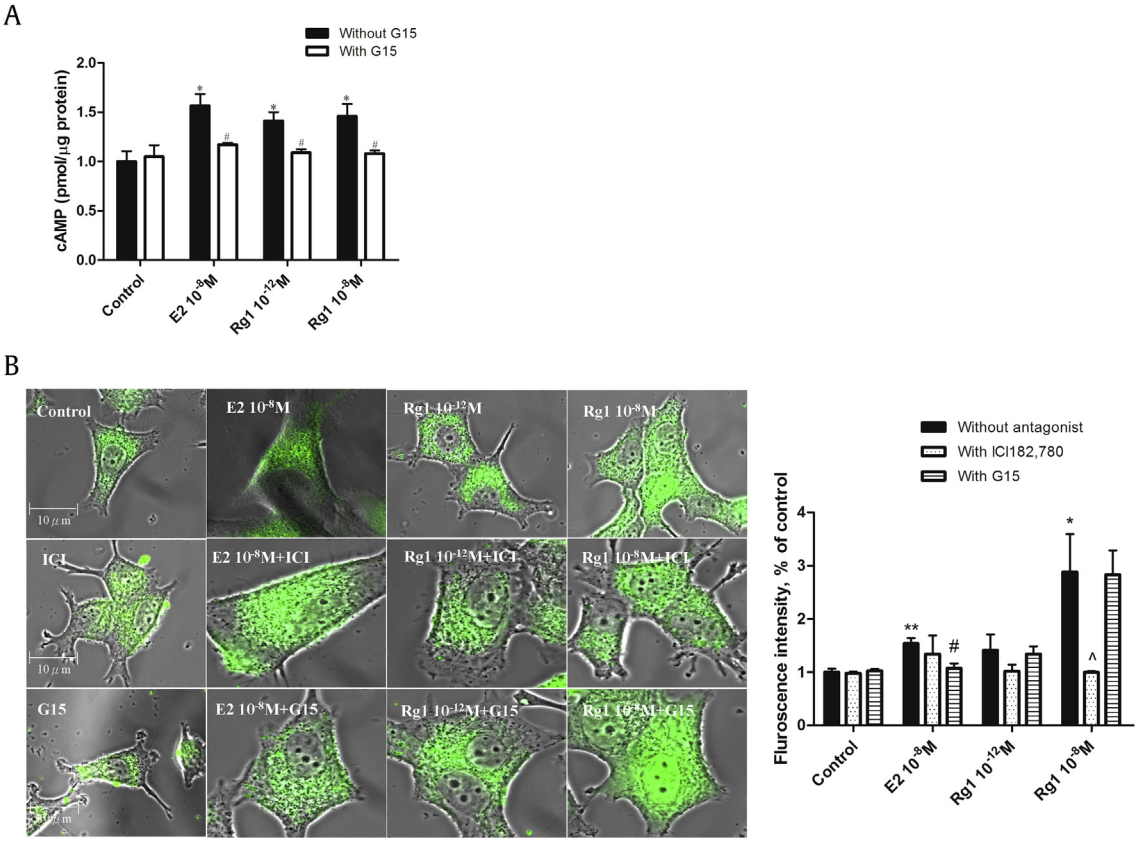

Fig. 7. GPER mediates the effects of E2 and Rg1 on cAMP accumulation, but not calcium mobilization in MCF-7 cells. (A) MCF-7 cells were treated with E2 (10^{-8}M), Rg1 (10^{-12}M, 10^{-8}M), or its vehicle for 10 min. Cell lysate (50μL) was directly used for cAMP measurement using the Cyclic AMP EIA Kit according to the manufacturer's instructions. Protein concentration was determined using the Bio-Rad Bradford methods and used to normalize the cAMP levels. Data are expressed as mean ± SEM. $p < 0.05$ versus control, n = 3. (B) MCF-7 cells were incubated with 10 μM Ca^{2+} green (Invitrogen) at room temperature for 20 min. Cells were treated with E2 (10^{-8}M), Rg1 (10^{-12}M, 10^{-8}M) or its vehicle for 5 min. Images were acquired using a wide-field fluorescence microscopy. Captured images were analyzed using the MetaMorph software. Autofluorescence was negligible. Data are expressed as mean ± SEM. p versus $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group; $^{\#}p < 0.05$ versus without G15 group; $\hat{}p < 0.05$ versus without ICI182,780 group, n = 5. GPER, G protein-coupled estrogen receptor; SEM, standard error of mean.

determine the role of several scaffolding proteins (caveolins, Shc, MNAR/PELP1), Src kinase, growth factor receptors (EGFR and IGF-IR), and estrogen receptors (ERα and GPER1) in mediating the rapid estrogenic actions of Rg1 via the activation of MEK. The results clearly demonstrated that these signaling proteins and receptors were all involved in mediating the rapid estrogenic actions of Rg1 in MCF-7 cells and that ER-independent activation of MEK by Rg1 in ERα-negative HEK293 cells involved both c-Src and EGFR.

Our studies demonstrated that Rg1 rapidly induced ERα translocation to plasma membrane in MCF-7 cells in which the expression of ERα appeared to colocalize with expression of caveolin-1 protein, a structural component of caveolae. Caveolae are known to be involved in the regulation of signal transduction and form 50–100 nm plasma membrane invaginations which support functional protein–protein interactions and clusters of several discrete signaling pathways [35]. In fact, it is well known that ERα localization to the plasma membrane require its binding to caveolin-1 [36]. Therefore, our results suggest that the Rg1-induced recruitment of ERα to cell membrane is in part by interacting with caveolin-1 located in the caveolae of MCF-7 cells. This observation is also in agreement with our findings that Rg1 rapidly induced ERα phosphorylation of Ser118 in the cytoplasm within 5 min of incubation in MCF-7 cells [28].

Another important scaffolding protein that may be involved in mediating the estrogenic actions of Rg1 in MCF-7 cells is Shc, a cytoplasmic adaptor protein that is known to interact with IGF-IR to lead to the activation of Ras/Raf/MAPK pathway [24,37]. Estrogen is known to induce phosphorylation of the Shc binding sites located at the intracellular domain of IGF-IR, thereby facilitating the ERα/Shc complexes to bind to IGF-IR and mediating ERα membrane translocation [25]. Thus, it is possible that Rg1 could mimic estrogen and induce Shc binding to ERα as well as to IGF-IR. Indeed, the results of the immunoprecipitation studies clearly showed that Rg1 enhanced the formation of ternary complex involving Shc, ERα, and IGF-IR, suggesting that the translocation of ERα by Rg1 to plasma membrane might also involve Shc in MCF-7 cells. Similarly, our results demonstrated that Rg1 also rapidly induced the formation of ERα-MNAR-c-Src complex in MCF-7 cells. Indeed, MNAR is another scaffolding protein that contains multiple protein–protein interaction domains, and the formation of ER-MNAR-c-Src complex is reported to lead to activation of Src and downstream Ras/Raf/MAPK pathway [38]. Thus, our results suggest that MNAR is also involved in mediating the rapid actions of Rg1 by facilitating the interactions between ERα and c-Src. Furthermore, our results indicated that Rg1 failed to induce MEK phosphorylation when the expression of any of one of these proteins (IGF-IR, shc, MNAR, and c-Src) was suppressed in MCF-7 cells, thus confirming their role in mediating the actions of Rg1 in activating of MAPK signaling pathways.

Our earlier study showed that Rg1 could activate MEK phosphorylation in both ER-positive MCF-7 cells and ER-negative HEK293 cells in a time- and dose-dependent manner, indicating that Rg1 activation of MAPK is ER-independent [8]. The current study further demonstrated that Rg1 activation of MEK phosphorylation in both MCF-7 and HEK293 cells could be abolished by cotreatment with either c-Src inhibitor (PP2) or EGFR inhibitor

(AG1478). These results indicated that Rg1 could activate MEK phosphorylation via c-Src and EGFR and that the activation is ER-independent. This observation further confirms our previous hypothesis that Rg1 exerts estrogen-like activities via cross talk between receptor tyrosine kinases (IGF-IR and EGFR) and ER signaling pathways.

Other than membrane-associated ERα, GPER was reported to mediate the rapid, nongenomic estrogen signaling that could activate MAPK/ERK phosphorylation and phosphatidylinositide 3 kinase /AKT signaling pathway via transactivation of the EGFR pathway in ER-negative but GPER-positive breast cancer cells [20,21]. Thus, it is possible that GPER might also be involved in mediating the rapid estrogenic effects of Rg1. Our results indicated both E2 and Rg1 downregulated GPER mRNA expression in MCF-7 cells. The result is in line with the report by Ariazi et al [39] in which E2 was shown to repress GPER mRNA expression in MCF-7 cells. Such observation also agrees with those observed for other GPCRs in which activated GPCRs undergo a series of adaptive cytosolic changes to effectively prevent excessive receptor signaling [40]. In addition, our results showed that the estrogen-like activities of Rg1, including stimulation of cell viability, pS2, and IGF-I receptor mRNA expression as well as ERE-dependent transcriptional activities, were abolished when MCF-7 cells were cotreated with GPER1 antagonist G15, suggesting that GPER1 is indeed involved in mediating the actions of Rg1 in MCF-7 cells.

Our study showed that Rg1 mimicked E2 in rapidly inducing EGFR phosphorylation in MCF-7 cells time-dependently. Most importantly, the activation of EGFR by E2 and Rg1 was shown to be GPER-dependent as cotreatment with G15 partially blocked their stimulatory effects on EGFR in MCF-7 cells. Furthermore, the stimulatory effect of E2 and Rg1 on the phosphorylation of MEK in MCF-7 cells could also be blocked by cotreatment with G15. These results suggest that GPER is the upstream signaling molecule of EGFR and MEK which mediates the rapid actions of E2 and Rg1. Indeed, E2-stimulated activation of MAPKs was shown to require GPER and occur via Gβγ-dependent transactivation of EGFR [20,41]. The stimulation of GPER induces the activation of matrix-metalloproteinases and the release of heparin-bound epidermal growth factor from cell surface, resulting in EGFR-mediated signaling. This transactivation also involves the Src family and leads to downstream activation of MAPKs [20,41]. Thus, as the present study showed that MEK phosphorylation induced by Rg1 in MCF-7 cells requires GPER, c-Src, and EGFR, and our results strongly support that GPER–EGFR cross talk is involved in mediating the estrogenic actions of Rg1 in ER-positive cells.

Our results indicated that Rg1 appeared to mimic E2 in increasing intracellular cAMP accumulation in MCF-7 cells within 10 min of incubation. Similar actions have been reported for genistein and tectoridin [42] through which they could rapidly increase the intracellular cAMP content in MCF-7 cells. As estrogen was reported to stimulate GPER-dependent adenylyl cyclase activity and intracellular cAMP production in MCF-7 and SKBR3 cells [19], it is also possible that Rg1 stimulation of cAMP production is GPER-dependent. Indeed, our results indicated that Rg1 stimulation of intracellular cAMP in MCF-7 cells could be abolished by cotreatment with G15, suggesting that Rg1 might activate GPER-dependent adenyl cyclase in MCF-7 cells. Estrogen has been reported to induce extremely rapid increase in the concentrations of the intracellular Ca via membrane ERs. mERα-dependent activation of phospholipase C catalyzes phosphatidylinositol into diacglycerol and IP3, which activates the Ca channel, leading to the release of Ca from endoplasmic reticulum [43]. Another mER is GPER which increases cAMP level via activation of adenyl cyclase and activates IP3 receptors upon their phosphorylation by protein kinase A (PKA) [19]. Activation of IP3 receptors either via binding to IP3 or phosphorylation by PKA triggers the release of Ca from endoplasmic reticulum. Our results showed that 10nM of E2 and Rg1 significantly induce intracellular Ca mobilization within 5 min of incubation in MCF-7 cells. The induction of intracellular Ca mobilization by E2 in MCF-7 cells could be abolished by cotreatment with G15, but not ICI 182,780. The results suggest that GPER, but not ER, is involved in mediating the effects of E2 on intracellular Ca mobilization. Such observation agrees with studies by others that E2-activation of GPER result in intracellular Ca mobilization in MCF-7 cells [39]. In contrast, our results showed that Rg1-induced intracellular Ca mobilization in MCF-7 cells was ER-dependent, but not GPER-dependent. The results suggest that the mechanisms by which Rg1 activates second messengers in MCF-7 cells are different from that of E2 and that the activation of GPER by Rg1 might not lead to increased intracellular Ca mobilization. Based on our results, we speculate that the Rg1-induced formation of signalosome helps to recruit more ERα to membrane surface to facilitate the rapid activation of IP3 receptor and Ca release from endoplasmic reticulum, thereby increase intracellular Ca in MCF-7 cells.

The present study also increases our understanding of the actions by which phytoestrogens exert rapid ligand-independent estrogenic actions in estrogen-sensitive tissues and cells. Our previous study suggested that Rg1 exerted potent estrogenic effects in MCF-7 cells and activated ER via MEK/ERK in a ligand-independent manner [7,8]. Together with the results of this study, the mechanism of actions of Rg1 in ER-positive cells could be summarized as follows (Fig. 8): Rg1 might (1) activate the recruitment of ERα to plasma membrane via the formation of signalsome containing caveolin, Shc, MNAR, insulin-like growth factor receptor (IGFR), and c-Src and induce an increase in intracellular Ca mobilization; (2) activate GPER–EGFR cross talk and induce an increase in intracellular cAMP; and (3) lead to an increase in MEK1/2 phosphorylation and ERα phosphorylation at Ser118, followed by an induction of ERE-dependent transcription and finally the exertion of estrogenic effects. Based on our results, we speculated that cell-surface receptors, like membrane ERα and GPER–EGFR cross talk, might be involved in mediating the rapid actions of Rg1 in MCF-7 cells.

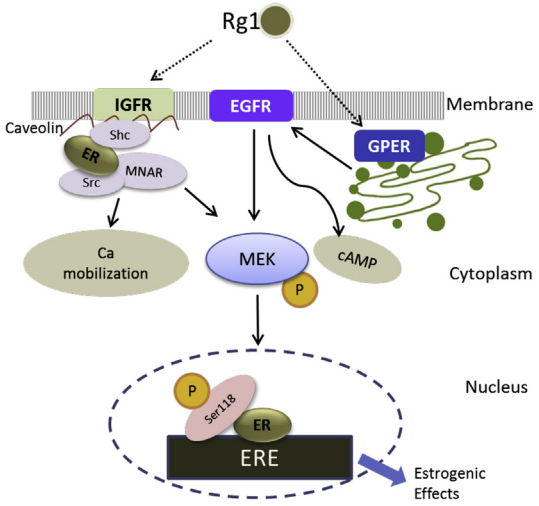

Fig. 8. Possible mechanisms of actions of Rg1 in ER positive cells. Rg1 (1) activates the recruitment of ERα to plasma membrane via the formation of signalsome containing caveolin, Shc, MNAR, IGFR, and c-Src and induces an increase in intracellular Ca mobilization; (2) activates GPER–EGFR cross talk and induces an increase in intracellular cAMP; and (3) leads to an increase in MEK1/2 phosphorylation and ERα phosphorylation at Ser118, followed by an induction of ERE-dependent transcription and finally the exertion of estrogenic effects.
EGFR, epidermal growth factor receptor; ER, estrogen receptor; GPER, G protein-coupled estrogen receptor; MEK, mitogen-activated protein kinase kinase.

However, further study will be needed to identify the direct targets of Rg1 in estrogen receptor−positive cells.

Our study is the first to report the possible involvement of both membrane ERα and GPER pathways in mediating the estrogenic actions of ginsenoside Rg1. The discovery that rapid ER signaling pathway is involved in the estrogen-like action of ginsenosides provides new insight into the understanding of the pharmacological actions of ginseng. Recent studies demonstrated that both membrane ERα and GPER-mediated pathways play important physiological roles in the reproductive, skeletal, nervous, endocrine, immune, and cardiovascular systems, and they are believed to be an important target for developing tissue-selective treatment against various diseases including cancer, bone disorders, cardiovascular diseases, and neurological disorders [37,44−46]. It is very likely that the previously reported cardioprotective [47] and the neuroprotective effects [48,49] previously reported for ginseng might be mediated through the rapid actions of Rg1 on membrane ERα and GPER-mediated pathways. Indeed, our previous studies [4,10,50] clearly indicated that ER and IGFR are involved in mediating the neuroprotective effects of Rg1. In addition, membrane ERα was recently reported to mediate estrogenic response in bone in a tissue-selective manner [37]. It should be noted that the effective dosages of Rg1 for activating rapid estrogenic responses via membrane ERα and GPER as shown in the present study are 1 pM and 10nM. Since these concentrations are easily achieved in the circulation through oral consumption of ginseng, the ability of Rg1 to activate rapid ER signaling pathways might account for some of the reported beneficial effects of ginseng on human health. Further study will be needed to determine the role of the membrane ERα and GPER1 pathway in mediating the molecular actions of Rg1 in other tissues, such as bone, brain, and vascular tissues.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2018.03.004.

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