

Icariin and icaritin stimulate the proliferation of SKBr3 cells through the GPER1-mediated modulation of the EGFR-MAPK signaling pathway

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Abstract. Icariin (ICA) and icaritin (ICT), with a similar structure to genistein, are the important bioactive components of the genus *Epimedium*, and regulate many cellular processes. In the present study, using the estrogen receptor (ER)-negative breast cancer cell line, SKBr3, as a model, we examined the hypothesis that ICA and ICT at low concentrations stimulate SKBr3 cell proliferation *in vitro* through the functional membrane, G protein-coupled estrogen receptor 1 (GPER1), mediated by the epithelial growth factor receptor (EGFR)-mitogen-activated protein kinase (MAPK) signaling pathway. MTT assay revealed that ICA and ICT at doses of 1 nM to 1 μ M markedly stimulated SKBr3 cell proliferation in a dose-dependent manner. The ICA- and ICT-stimulated cell growth was completely suppressed by the GPER1 antagonist, G-15, indicating that the ICA- and ICT-stimulated cell proliferation was mediated by GPER1 activation. Semi-quantitative RT-PCR analysis revealed that treatment with ICA and ICT enhanced the transcription of c-fos, a proliferation-related early gene. The ICA- and ICT-stimulated mRNA expression was markedly attenuated by G-15, AG-1478 (an EGFR antagonist) or PD98059 (a MAPK inhibitor). Our data also demonstrated that ICA and ICT increased the phosphorylation of ERK1/2. The ICA- and ICT-stimulated ERK1/2 phosphorylation was blocked by pre-treatment of the cells with G-15 and AG-1478 or PD 98059. Flow cytometric analysis confirmed that the ICA- and ICT-stimulated SKBr3 cell

proliferation involved the GPER1-mediated modulation of the EGFR-MAPK signaling pathway. To the best of our knowledge, our current findings demonstrate for the first time that ICA and ICT promote the progression of ER-negative breast cancer through the activation of membrane GPER1.

Introduction

Over the past decade, a novel estrogen receptor (ER), GPR30, now known as G protein-coupled estrogen receptor 1 (GPER1), was identified as an orphan seven-transmembrane G protein-coupled receptor (GPCR) by multiple laboratories (1-3). As a functional membrane receptor, GPER1 is distinct from the classical nuclear ERs, including ER α and ER β). GPER1 mediates rapid non-genomic responses to estrogen stimulation which occur within seconds or minutes, such as cAMP production, calcium mobilization and the activation of intracellular kinases, including mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K), protein kinase A (PKA) and protein kinase C (PKC) (4-6). These responses usually cannot be explained by the classical nuclear ER α and ER β signaling pathways, which perform their functions through binding to estrogen response element (ERE) located within the regulatory region of target genes, and usually take hours to days to exert an effect on cells (7). Estrogen, tamoxifen (a selective ER modulator), 4-hydroxytamoxifen (OTH), ICI 172,780 (an antagonist of ER α and ER β) and some phytoestrogens are ligands of GPER1 (8).

Herba Epimedii (Yin Yang Huo) is a traditional Chinese herbal medicine that is widely used in the treatment of impotence, involuntary ejaculation, rheumatism, osteoporosis and bone fractures in China for thousands of years (9). Icariin (ICA) is a prenylated flavonol glycoside isolated from plants in the genus *Epimedium*, and icaritin (ICT) is a derivative hydroxylated at the 3,7-positions of ICA. Both ICA and ICT are important bioactive components of *Herba Epimedii*. They exert a number of beneficial cellular effects, including promoting apoptosis, stimulating

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angiogenesis, inducing osteogenic differentiation and upregulating extracellular matrix synthesis (10-12). Furthermore, ICA and ICT are also regarded to be active phytoestrogens, exerting estrogenic/anti-estrogenic effects due to their similar chemical structures to genistein, a well-known phytoestrogen (13). It has been reported that low concentrations of ICT (0.1 nM to 1 μ M) promote MCF-7 cell growth (14,15), while high concentrations of ICT (>1 μ M) have been shown to inhibit breast cancer cell proliferation, indicating a biphasic regulatory action of ICT (16). The proliferative effects of ICA and ICT on estrogen-dependent breast cancer cells are mainly due to the activation of nuclear ER α - and ER β -mediated signaling pathways (17). However, little is known about the interactions between GPER1 and ICA or ICT.

Epidermal growth factor receptor (EGFR) is the prototypical member of the family of transmembrane receptor tyrosine kinases. Increasing evidence suggests that the activation of EGFR is involved in GPER1-mediated multiple downstream events (18-20). Among these events, the GPER1-mediated activation of the EGFR-MAPK pathway plays a pivotal role in estrogen-dependent tumor cell proliferation, including breast, endometrial, ovarian and thyroid cancer (14,21-24).

In the present study, we used an ER α - and ER β -negative breast cancer cell line (SKBr3) as a model to identify the possible mechanisms that ICA and ICT promote breast cancer cell proliferation in a non-genomic GPER1-mediated pathway.

Materials and methods

Chemicals. ICA was purchased from the Chinese National Institute for Food and Drug Control (Beijing, China). ICT was purchased from Tauto Biotech (Shanghai, China). 17 β -estradiol (E2), DMSO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). G-1 (a GPER1 agonist), AG-1478 (an EGFR inhibitor) and PD98059 (a MAPK inhibitor) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). G-15 (a GPER1 antagonist) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against ERK2 or phospho-ERK1/2 were obtained from Boster Biotechnology (Wuhan, China).

Cell culture. SKBr3 breast carcinoma cells were obtained from the Chinese Type Culture Collection, Chinese Academy of Sciences (CAS; Shanghai, China) and were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose (high-glucose) and 0.37% sodium bicarbonate (Gibco, Rockville, MD, USA). In routine culture, the cells were supplemented with 10% fetal bovine serum (FBS) and 1X antibiotic mix (1x10⁴ U/l penicillin A and 100 mg/l of streptomycin) and grown at 37°C in a humidified atmosphere of 95% air/5% CO₂. Three days before the cells were treated, the medium was replaced with phenol-red-free DMEM supplemented with 4.5 g/l glucose and 0.37% sodium bicarbonate (Gibco), supplemented with 5% charcoal-dextran stripped FBS (CDT-FBS; Gibco) to eliminate endogenous estrogen throughout the whole experimental period.

MTT assay. Cell proliferation assay was determined by MTT colorimetric assay. Briefly, the SKBr3 cells were subcultured

on the logarithmic phase supplemented with phenol-red-free high-glucose DMEM with 5% FBS. The cells were counted and seeded in 96-well culture plates at an initial density of 3x10³ cells/well and allowed to attach to the plates. After 24 h of attachment, the culture medium was discarded, and the cells were treated with ICA (1 nM to 10 μ M), ICT (1 nM to 10 μ M) or ICA (1 nM to 10 μ M) with or without G-15 (20 μ M), and ICT (1 nM to 10 μ M) with or without G-15 (20 μ M) in 200 μ l of phenol red-free high-glucose DMEM with 5% FBS for 48 h. The media were then removed and replaced with 20 μ l of MTT (5 mg/ml) in PBS. The plates were incubated for 4 h at 37°C followed by the addition of 150 μ l DMSO to dissolve the purple crystals, which are products of the MTT substrates. The absorbance was read on a microplate reader at a wavelength of 570 nm. The proliferation rate was calculated as PR% = absorbance of experimental group/absorbance of control group x100%.

Semi-quantitative RT-PCR analysis. The measurement of c-fos mRNA expression was performed by semi-quantitative RT-PCR analysis. Briefly, the SKBr3 cells in phenol red-free DMEM containing 5% FBS were cultured in 6-well plates at an initial density of 1x10⁵ cells/plate. When the cells reached 70% confluency, they were cultured in phenol red-free DMEM containing 5% FBS for 24 h. The cells were then treated with E2 (10 nM), ICA (100 nM), or ICT (100 nM) for 15 to 60 min with or without pre-treatment with G-15 (20 μ M), AG-1478 (10 μ M), or PD98059 (10 μ M) for 1 h. Total cellular RNA was extracted using TRIzol reagent (CWbiotech, Beijing, China). First-strand cDNA was synthesized using a HiFi-MMLV cDNA kit (from CWbiotech). PCR was then performed using specific primer pairs. The level of 36B4 housekeeping mRNA was used as an internal control. The primers used were as follows: c-fos forward, 5'-AGAAAAGGAGAATCCGAA GGGAAA-3' and reverse, 5'-ATGATGCTGGGACAGGAA GTC-3'; and 36B4 forward, 5'-CTCAACATCTCCCCCTT CTC-3' and reverse, 5'-CAAATCCCATATCCTCGTCC-3'. The c-fos and 36B4 DNA products were 420 and 408 bp, respectively. Band intensity was quantified with ImageJ software (National Institutes of Health). The experiments were repeated at least 3 times.

Western blot analysis. For quantification of the ERK1/2 levels, the SKBr3 cells were serum-starved for 40 h, and were then treated with E2 (10 nM), ICA (100 nM), ICT (100 nM), or G-1 (10 nM) for 0 to 30 min with or without pre-treatment with G-15 (20 μ M) or AG-1478 (10 μ M) for 1 h. The cells were lysed in RIPA buffer and then lysates were separated by SDS-PAGE. Total ERK and phospho-ERK were detected using the anti-ERK2 or anti-phospho-ERK1/2 antibodies (Boster Biotechnology). The chemiluminescent signal was revealed by the enhanced chemiluminescence system (ECL; Pierce Biotechnology, Rockford, IL, USA) and the protein level was detected by exposure to x-ray film. Band intensity was quantified with ImageJ software. Experiments were repeated at least 3 times.

Cell cycle analysis. The SKBr3 cells were seeded in 6-cm culture plates at an initial density of 5x10⁵ cells/plate. Twenty-four hours after attachment, the cells were starved with phenol-red-free high-glucose DMEM with 0.5% FBS for 3 days.

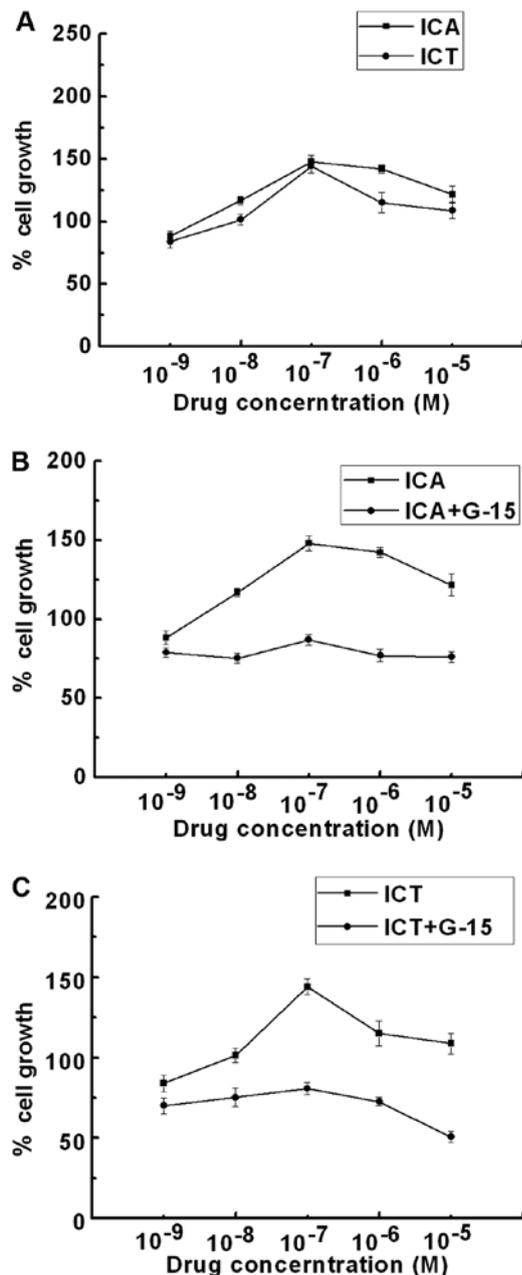


Figure 1. G protein-coupled estrogen receptor 1 (GPER1) mediates icariin (ICA)- and icaritin (ICT)-induced SKBr3 cell proliferation. Cells were treated with (A) ICA (1×10^{-9} to 1×10^{-5} M) or ICT (1×10^{-9} to 1×10^{-5} M), (B) ICA (1×10^{-9} to 1×10^{-5} M) with or without G-15 (20 μ M), and (C) ICT (1×10^{-9} to 1×10^{-5} M) with or without G-15 (20 μ M) in phenol red-free high-glucose DMEM medium with 5% fetal bovine serum (FBS) for 48 h before counting. The proliferation rate was determined by MTT assay. Results are the means \pm SD from 3 independent experiments.

The cells were then treated with E2 (10 nM), G-1 (10 nM), ICA (100 nM), or ICT (100 nM) with or without pre-treatment with G-15 (20 μ M), PD98095 (10 μ M), or AG-1478 (10 μ M). After 48 h, the cells were harvested and the cell cycle was analyzed as previously described (25). The cell proliferation index (PI) was calculated as $PI\% = (S + G2/M)/(G0/G1 + S + G2/M) \times 100\%$.

Statistical analysis. The statistical analysis of all data was performed using SPSS 19.0 software. Data are expressed as the means \pm SD, and the level of significance between 2 groups

was assessed using a Student's t-test. P values <0.05 were considered to indicate statistically significant differences.

Results

GPER1 mediates the ICA or ICT-induced proliferation of SKBr3 cells. Both ICA and ICT stimulated the proliferation of the SKBr3 ER-negative breast cancer cells in a dose-dependent manner (Fig. 1A). The maximal cell proliferative effects were 148 and 144% at a dose of 1×10^{-7} M ICA or ICT, respectively. To determine the involvement of GPER1 in the ICA- or ICT-stimulated proliferation, the SKBr3 cells were co-treated with G-15 (a high-affinity GPER1 antagonist, 20 μ M) and ICA (100 nM) or ICT (100 nM). The proliferation of the SKBr3 cells was completely suppressed by G-15 (Fig. 1B and C), suggesting that the ICA- or ICT-stimulated SKBr3 cell proliferation was mediated by the activation of GPER1.

ICA and ICT stimulate c-fos mRNA expression. The proto-gene, c-fos, is an immediate early gene and its expression is rapidly induced by various extracellular mitogens. In order to determine whether ICA or ICT induce c-fos expression, the SKBr3 cells were treated with ICA (100 nM) or ICT (100 nM) for 15, 30 and 60 min. Semi-quantitative RT-PCR analysis was performed to measure c-fos mRNA expression (Fig. 2). The maximal c-fos mRNA expression was detected at 30 min. c-fos mRNA expression was increased by 5.5- and 4.1-fold in response to ICA and ICT treatment, respectively (Fig. 2A and B).

GPER1 and EGFR mediate the ICA- and ICT-induced c-fos mRNA expression. To determine whether GPER1 and EGFR are required for the ICA- or ICT-induced c-fos expression, the SKBr3 cells were pre-treated with G-15 (20 μ M), AG-1478 (an EGFR inhibitor, 10 μ M) for 1 h, and then treated with E2 (10 nM), ICA (100 nM) or ICT (100 nM) for 30 min (Fig. 3). G-15 and AG-1478 markedly inhibited the E2-, ICA- or ICT-induced c-fos mRNA expression (Fig. 3A-C). These results indicate that ICA and ICT can mimic E2 that interacts with GPER1 and activates downstream EFGR-mediated c-fos gene expression.

Induction of c-fos mRNA expression by ICA and ICT requires ERK1/2 activation. It has been reported that GPER1/EGFR-mediated c-fos expression occurs through the activation of the MAPK signaling pathway (23). Thus, we investigated whether ICA or ICT increase the phosphorylation of ERK1/2. ICA or ICT increased ERK1/2 phosphorylation in the SKBr3 cells within 5 min (Fig. 4A and B). The effects of ICA and ICT were mimicked by G-1 (Fig. 4C). Furthermore, G-15 and AG-1478 significantly blocked the E2-, G-1-, ICA- or ICT-induced ERK1/2 phosphorylation (Fig. 4D and E). In addition, the MAPK inhibitor, PD98059, significantly decreased the E2-, ICA- and the ICT-induced c-fos mRNA expression (Fig. 4F).

ICA and ICT stimulate the proliferation of SKBr3 cells through the GPER1-mediated modulation of the EGFR-MAPK signaling pathway. To further confirm that ICA- or ICT-stimulated SKBr3 cell proliferation involves the GPER1-mediated activation of the ERFR-MAPK signaling pathway, the SKBr3 cells were starved for 3 days and were then treated with E2 (10 nM), ICA (100 nM) or ICT (100 nM) with or

Table I. Proliferation of SKBr3 cells at various phases of the cell cycle.

Drug treatment	G0/G1 (%)	S (%)	G2/M (%)	PI (%)
Negative control	64.1±3.8	2.4±0.5	33.6±0.6	35.9
E2 (10 nM)	26.5±4.2	35.3±0.5	38.3±0.9	73.5 ^a
ICA (100 nM)	25.9±2.7	61.7±0.3	12.3±0.8	74.1 ^a
ICT (100 nM)	13.8±2.6	50.1±0.6	36.1±1.1	86.2 ^a
E2 (10 nM) + G-15 (20 μM)	40.6±3.1	14.9±0.5	24.5±0.8	39.4
ICA (100 nM) + G-15 (20 μM)	42.3±1.8	19.8±0.2	18.0±1.7	37.7
ICT (100 nM) + G-15 (20 μM)	37.7±2.0	18.8±0.05	23.5±0.8	42.3
E2 (10 nM) + AG-1478 (10 μM)	34.5±5.2	15.1±0.6	20.4±0.6	35.5
ICA (100 nM) + AG-1478 (10 μM)	57.4±3.2	20.6±0.2	21.9±0.6	42.6
ICT (100 nM) + AG-1478 (10 μM)	66.2±4.0	9.5±0.2	24.4±0.8	33.8
E2 (10 nM) + PD 98059 (10 μM)	63.4±2.9	9.6±0.4	27.1±0.9	36.6
ICA (100 nM) + PD 98059 (10 μM)	76.0±3.5	13.1±0.2	20.9 ±0.5	34.0
ICT (100 nM) + PD 98059 (10 μM)	56.2±3.6	15.1±0.2	28.7±1.0	43.8

E2, 17β-estradiol; ICA, icariin; ICT, icaritin. Results are the means ± SD. ^ap<0.01 compared to the negative control (untreated) group.

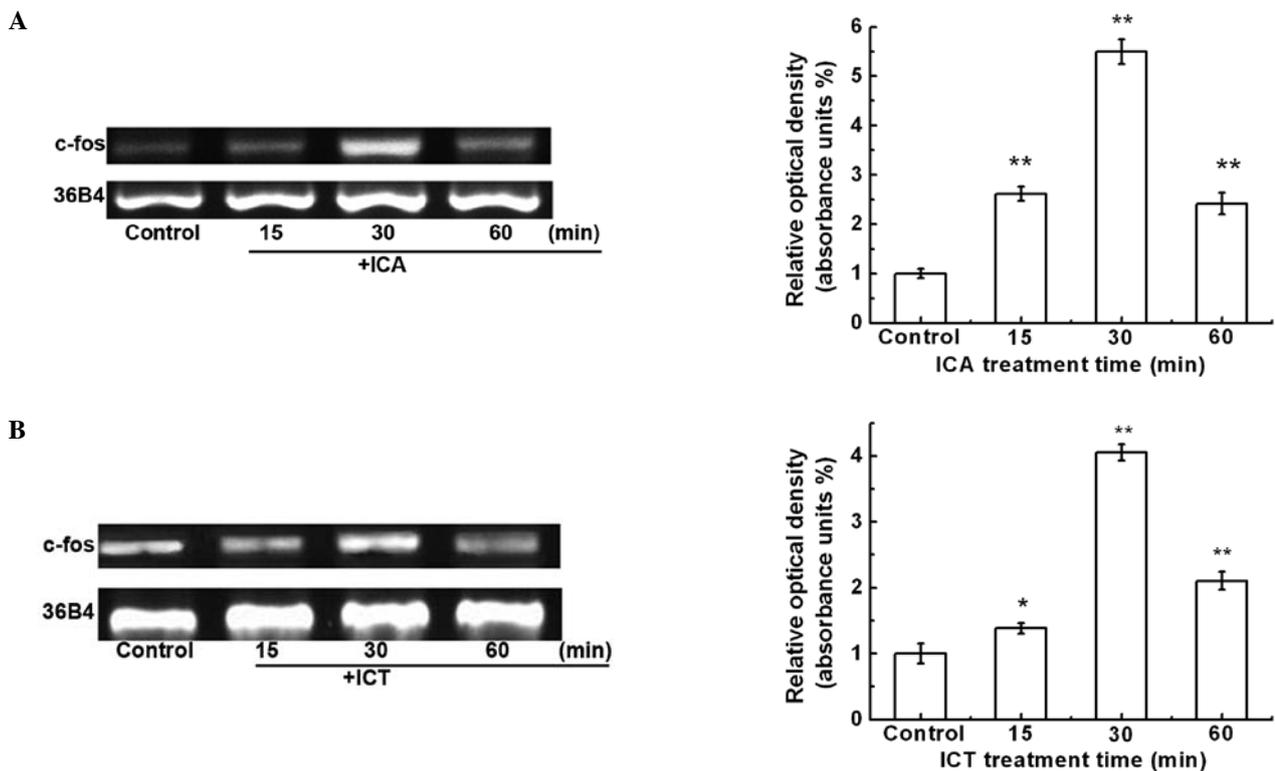


Figure 2. Icariin (ICA) and icaritin (ICT) induce c-fos mRNA expression in SKBr3 cells. Cells were treated with (A) ICA (100 nM), or (B) ICT (100 nM) for 15, 30 or 60 min. RT-PCR analysis was performed for the quantification of c-fos mRNA expression. The housekeeping gene, 36B4, was used as an internal control. The right side panels show the means ± SD from 3 independent experiments. *p<0.05, **p<0.01 compared to the control (untreated) group.

without pre-treatment with G-15 (20 μM), PD 98059 (10 μM) or AG-1478 (10 μM) for 2 days. The phase distribution of the cell cycle and proliferation rate was assessed by flow cytometry. We found that, similar to E2, ICA or ICT promoted SKBr3 cell proliferation that was blocked by pre-treatment with G-15, AG-1478 or PD98059. These data suggest that the ICA- and ICT-induced c-fos mRNA expression and cell proliferation

of SKBr3 cells requires the activation of the EGFR-MAPK signaling pathway, modulated by GPER1 (Table I).

Discussion

The present study demonstrates that ICA and ICT stimulate the proliferation of SKBr3 cells, an ERα- and ERβ-negative

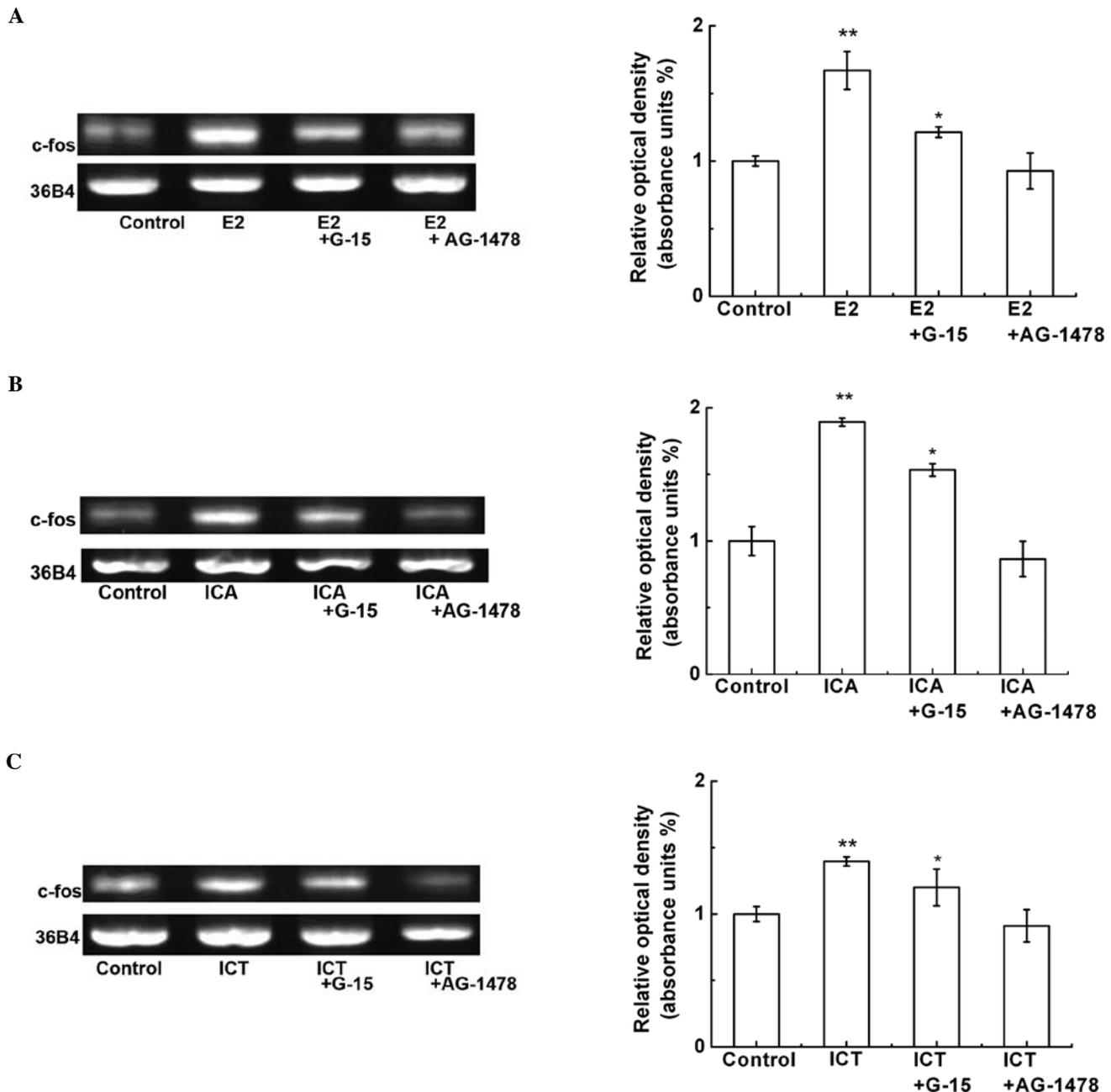


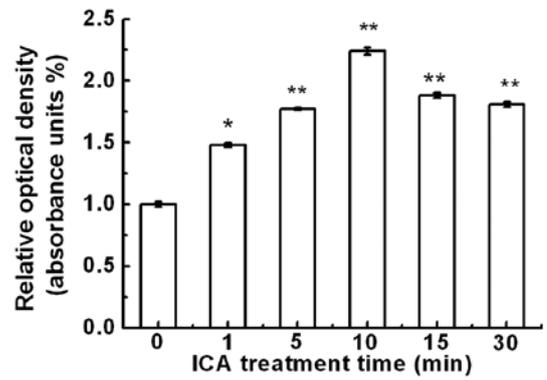
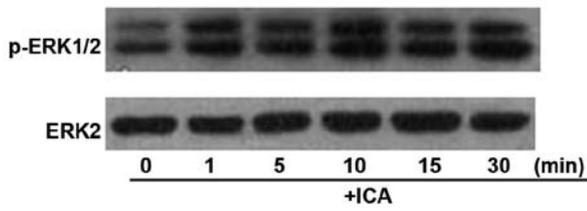
Figure 3. G-15 and AG-1478 inhibit icariin (ICA)- and icaritin (ICT)-induced c-fos mRNA expression in SKBr3 cells. Cells were pre-incubated with G-15 (a GPER1 antagonist, 20 μ M) or AG-1478 [epithelial growth factor receptor (EGFR) inhibitor; 10 μ M] for 1 h, then treated with (A) 17 β -estradiol (E2; 10 μ M), (B) ICA (100 nM), or (C) ICT (100 nM) ICT for 30 min. RT-PCR analysis was performed to quantify c-fos mRNA expression. The housekeeping gene, 36B4, was used as an internal control. The right side panels show the means \pm SD from 3 independent experiments. * p <0.05, ** p <0.01 compared to the control (untreated) group.

breast cancer cell line, *in vitro*. The effects of ICA and ICT on SKBr3 cell proliferation were mediated by rapid non-genomic GPER1 action, which was associated with the induction of EGFR, MAPK and c-fos expression. This ICA- and ICT-induced SKBr3 cell proliferation was inhibited by inhibitors of GPER1, EGFR and MAPK. These results suggest that ICA and ICT possess the ability to promote breast cancer cell growth through the activation of the EGFR-MAPK signaling pathway, mediated by GPER1.

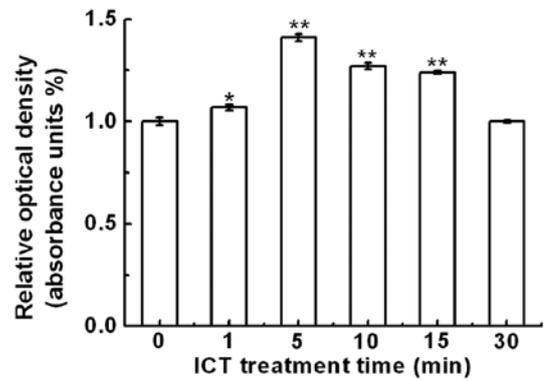
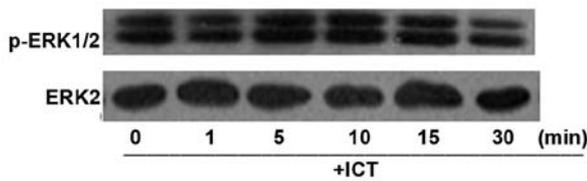
GPER1 is recognized as an important mediator for promoting breast cancer cell proliferation through rapid

non-genomic estrogenic actions. Some phytoestrogens have been proven to be the ligands of GPER1 (8). To the best of our knowledge, our study demonstrates for the first time that a low concentration of ICA and ICT (1 nM to 1 μ M) induces SKBr3 cell proliferation through the GPER1-mediated modulation of the MAPK pathway (Fig. 1). Consistent with our findings, genistein and quercetin have been shown to stimulate MCF-7 breast cancer cell growth through a rapid GPER1-mediated action (26). Another phytoestrogen, tectoridin, has also been reported to induce MCF-7 cell growth through the activation of GPER1-mediated MAPK signaling (27).

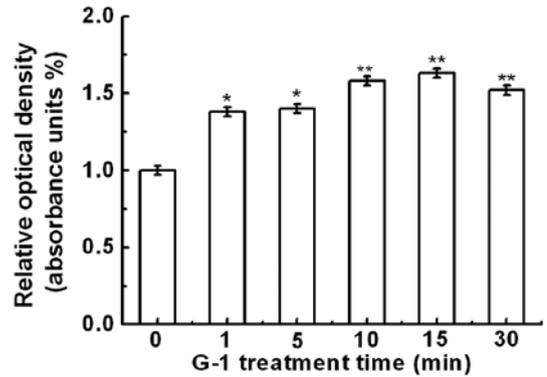
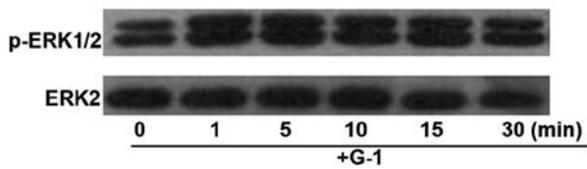
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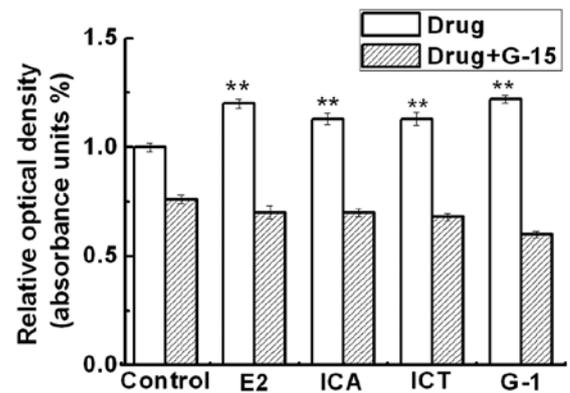
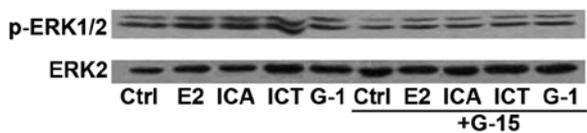
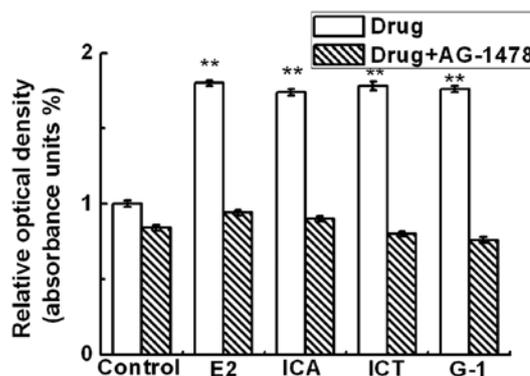
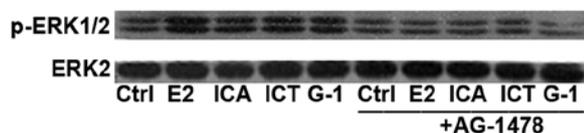


Figure 4. Icariin (ICA) or icaritin (ICT) induces ERK1/2 phosphorylation in SKBr3 cells. SKBr3 cells were serum-starved for 40 h before being treated for 0 to 30 min with: (A) ICA (100 nM), (B) ICT 100 nM and (C) G-1 (10 nM). (D) SKBr3 cells were pre-treated with G-15 (20 μ M) for 1 h, and then treated with E2 (10 nM), G-1 (10 nM), ICA (100 nM) or ICT (100 nM) for 30 min.

E



F

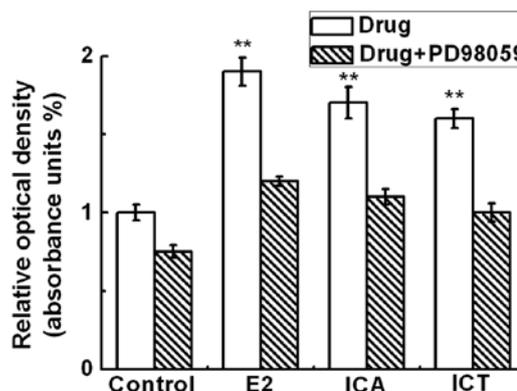
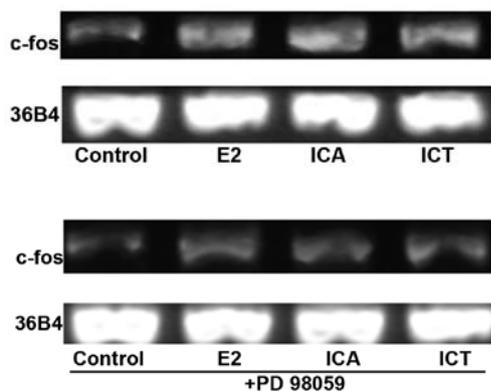


Figure 4. Continued. (E) SKBr3 cells were pre-treated with AG-1478 (10 μ M) for 1 h, and then treated with E2 (10 nM), G-1 (10 nM), ICA (100 nM) or ICT (100 nM) for 30 min. Cells lysates were used for western blot analysis for total ERK2 and phospho-ERK1/2. ERK2 protein levels were used to normalize ERK1/2 expression. (F) SKBr3 cells were pre-treated with PD98069 (10 μ M) for 1 h, and then treated with E2 (10 nM), ICA (100 nM) or ICT (100 nM) for 30 min. RT-PCR analysis was performed for quantification of c-fos mRNA expression. The housekeeping gene, 36B4, was used as an internal control. The right side panels show the means \pm SD from 3 independent experiments. ** p <0.01 compared to the control (untreated) group.

Several *in vitro* studies have demonstrated that EGFR-MAPK signaling is involved in GPER1-mediated cell proliferation. Filardo *et al* demonstrated that E2 activates ERK1/2 through the activation of EGFR by releasing heparin-bound epidermal growth factor (EGF) in SKBr3 breast cancer cells (21). Another study also demonstrated that the activation of GPER1 enhances EGF production, which triggers a rapid ERK phosphorylation and c-fos expression in ER-negative SKBr3 and BT20 cells (23). In our study, using SKBr3 cells as a model system, we demonstrated that the GPER1 antagonist, G-15, inhibited ICA- and ICT-induced SKBr3 cell growth, indicating that GPER1 mediated the ICA- and ICT-induced SKBr3 cell proliferation (Fig. 1A and B). Importantly, we found that the ICA- and ICT-induced c-fos mRNA expression was markedly attenuated by the GPER1 antagonist, G-15, and the EGFR antagonist, AG-1478, indicating that the ICA- and ICT-induced c-fos upregulation was mediated through the activation of GPER1 and EGFR (Figs. 2 and 3). Furthermore, we demonstrated that ICA and ICT rapidly stimulated the phosphorylation of ERK1/2 in serum-starved SKBr3 cells (Fig. 4A and B), which was blocked by pre-treatment with G-15 and AG-1478 (Fig. 4D and E). Consistent with the above results, the MAPK inhibitor, PD98059, blocked c-fos transcription which was induced by ICA and ICT treatment (Fig. 4F). Considering that the proliferation of the SKBr3 cells also

required the EGFR-dependent ERK1/2 activation, we ascertained their roles in the proliferation of SKBr3 cells by flow cytometry. Indeed, the EGFR antagonist, AG-1478, and the MAPK inhibitor, PD98059, abrogated the response to ICA and ICT (Table I). The above results demonstrated that the ICA- or ICT-stimulated SKBr3 cell proliferation occurred through the GPER1-mediated modulation of the EGFR-MAPK signaling pathway.

Of note, we demonstrated that both ICA and ICT promoted SKBr3 cell proliferation through an ER α - and ER β -independent, non-genomic pathway, and there was no difference in the proliferative activity between ICA and ICT. However, ICT has been demonstrated to have stronger estrogenic activity than ICA in MCF-7 cells through an ER-dependent pathway due to the steric hindrance produced by glycoside moieties of ICA, which prevents docking to the ER binding site (14,17). A possible explanation for the equal activity of ICA and ICT to promote SKBr3 cell proliferation is that ICA and ICT have a similar binding affinity to GPER1. The presence of 3,7-position glycoside moieties in ICA do not hamper binding to GPER1.

In conclusion, we demonstrate that both ICA and ICT activate the EGFR-MAPK signaling pathway in SKBr3 cells and that this pathway is mediated by the functional membrane, GPER1. GPER1 rapidly transduces a signal from EGFR to ERK1/2, which upregulates c-fos expression and results in

SKBr3 breast cancer cell proliferation. The precise mechanisms responsible for the ICA or ICT induction of c-fos expression in breast cancer cells require further investigation. To the best of our knowledge, our findings provide new insight into these mechanisms, demonstrating that ICA and ICT induce the progression of ER-negative breast cancer cell growth through the activation of GPER1.

Acknowledgements

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