

RESEARCH ARTICLE

Emodin Inhibits Breast Cancer Cell Proliferation through the ER α -MAPK/Akt-Cyclin D1/Bcl-2 Signaling Pathway

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Abstract

Background: The aim of the present study was to investigate the involvement of emodin on the growth of human breast cancer MCF-7 and MDA-MB-231 cells and the estrogen (E2) signal pathway *in vitro*. **Materials and Methods:** MTT assays were used to detect the effects of emodin on E2 induced proliferation of MCF-7 and MDA-MB-231 cells. Flow cytometry (FCM) was applied to determine the effect of emodin on E2-induced apoptosis of MCF-7 cells. Western blotting allowed detection of the effects of emodin on the expression of estrogen receptor α , cyclin D1 and B-cell lymphoma-2 (Bcl-2), mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinases (PI3K). Luciferase assays were employed to assess transcriptional activity of ER α . **Results:** Emodin could inhibit E2-induced MCF-7 cell proliferation and anti-apoptosis effects, and arrest the cell cycle in G0/G1 phase, further blocking the effect of E2 on expression and transcriptional activity of ER α . Moreover, Emodin influenced the ER α genomic pathway via downregulation of cyclin D1 and Bcl-2 protein expression, and influenced the non-genomic pathway via decreased PI3K/Akt protein expression. **Conclusions:** These findings indicate that emodin exerts inhibitory effects on MCF-7 cell proliferation via inhibiting both non-genomic and genomic pathways.

Keywords: Emodin - breast cancer - proliferation - estrogen receptor α - signalling pathways

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Introduction

Breast cancer is one of the most common malignant tumor. the mortality rate of breast cancer has increased yearly in China since 1991. In addition, the trend will continue to increase (Shi et al., 2014). Breast cancer is a kind of estrogen-dependent malignant tumors. Evidence indicates that estrogen is one of most important pathogenic factors to the formation and development of breast cancer, and its biological activity is dependent on the estrogen receptor- α (ER α) (Kong et al., 2003). ESR- α polymorphisms are associated with susceptibility to breast cancer (Lu et al., 2013). ER α plays an important role in breast cancer initiation and progression and represents a major target in cancer therapy. Izadi's study show that ER α methylation is correlated with poor prognosis subtypes of breast tumors in Iranian patients and may play an important role in pathogenesis of the more aggressive breast tumors (Izadi et al., 2012). The expression and activity of ER α is regulated by multiple mechanisms at the transcriptional and post-translational levels (Coombes et al., 2007). Estrogen interaction of ER α regulates the transcription gene through activating estrogen reactive element (ERE), and regulates the proliferation of breast cancer through MAPK/Erk1/2 and PI3K/Akt signaling

pathways (Kaufmann et al., 2007). Selective estrogen receptor modulators (SERMs), such as tamoxifene and raloxifene, can bind to estrogen receptor and exert estrogen-like actions and are widely used in treatment and prevention of postmenopausal osteoporosis, breast cancer and other gynecological diseases. However, SERMs may also be responsible for some potentially very serious adverse effects, such as thromboembolic disorders and immunity suppression. Therefore, finding an effective, safe and less side effect antitumor drug is necessary to overcome this current and urgent gap in therapy. Emodin is isolated and extracted from traditional Chinese drugs rhubarb, polygonum cuspidate and aloe that exhibits high antitumor activity. However, there is no report about emodin in treatment of breast cancer and its involvement in ER α signaling pathway *in vitro*. The objectives of present study are to investigate if emodin has anti-breast cancer effect through ER α signaling pathway, which will provide a theoretical foundation for development of emodin as antitumor drugs.

Materials and Methods

Reagents and chemicals

MCF-7 cells and MDA-MB-231 cells were provided

by cell center of Institute of Basic Medical Science (CAMS). Emodin, purity $\geq 98\%$, was purchased from National Institute of Food and Drug Control. RPMI-1640 and Charcoal stripped fetal calf serum (FCS) were purchased from GIBCO. Trypsin, methyl thiazolyl tetrazolium (MTT), PI3K inhibitor LY-294-002, MAPK inhibitor PD-98095 and 17- β estradiol were purchased from Sigma. Antibodies of ER α , p-Akt, Akt, p-MAPK and MAPK were purchased from Santa Cruz Biotechnology. Lipofectam ine™ 2000 transfection reagent kit was purchased from Invitrogen. pcDNA3.1/ER α 66 expression vector and 2 \times ERE/TATA-Luciferase reporter were a kind of gift from Dr Wang Zhaoyi, School of Medicine, Creighton University. Cell Cycle and Apoptosis Kit were purchased from Beyotime Institute of Biotechnology. All of the other reagents were analytical grade.

Cell culture and treatment

MCF-7 cells and MDA-MB-231 cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100U/mL) and streptomycin (100U/mL) at 37°C in a 5% CO₂ incubator, cells in log phase were used. For E2 treatment, cells were maintained in phenol red-free media with 5% charcoal-stripped FCS for 48h and then in serum-free medium for 24h. After that, cells were treated with different emodin (the final concentrations were 20, 40, 60 μ mol/L) or/and E2 for 48h before cell harvest. The cells lysates were prepared for Western blot. To test the effects of different inhibitors, all inhibitors and emodin (40 μ mol/L) were added 24h before the E2 addition. The cells lysates were prepared for Western blot.

MTT cell viability assay

MCF-7 cells at log phase were seeded in 96-well plates at 5×10^3 cells/well. When cells attached to the plate, MCF-7 cells were treated either with different concentrations of emodin (the final concentrations were 10, 20, 40, 60 μ mol/L) for 48h, or with above concentrations of emodin and E2 for 24/48h. In control experiment, the cells treated with 1% DMSO. Before incubated with E2 and emodin, cells were pretreated with 5% charcoal stripped FCS for 48h, then in instead of phenol red-free serum-free medium for 24h. Then, MTT (20 μ l at 5 mg/ml) was added to each well and culture was continued for 4h. Medium was then replaced with 150 μ l of DMSO. Absorption was measured at 490nm with reference wavelength at 570nm. Similar experiment was repeated three times.

Cell cycle and apoptosis analysis

MCF-7 cells were incubated with E2 and different concentrations of emodin for 24h. The Cell suspension was washed with PBS. Then, cells were fixed with 70% ethanol over 24h at 4°C. Finally, cells were treated with 20 μ g/ml RNase A and 50 μ g/ml of propidium iodide for 30 min. Stained cells were monitored by flow cytometry.

Transient transfection experiments

MCF-7 cells were plated in 24-well plates at 1×10^5 cells/well with RPMI-1640 (phenol red free) supplemented with 5% charcoal stripped FCS. The cells were transfected with phenol red-free RPMI-1640 at 95% confluence. The

transient transfection was performed using Lipofectam ine™ 2000 according to the manufacturer's directions. Cells were treated in triplicate, 24h later with emodin and E2. The cells were harvested 24h later, and the luciferase activities were assayed.

Western blot assay

For western blot assays, samples containing 30-50 μ g of protein were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. Blots were incubated overnight at 4°C with (a) anti-Cyclin D1 antibody (1:1000), (b) anti-Bcl-2 antibody (1:1000), (c) anti-MAPK antibody (1:1000), (d) anti-pMAPK antibody (e), anti-Akt antibody (1:1000), (f) anti-pAkt antibody (1:1000) and anti- β -actin antibody (1:2000), then incubated at 37°C in shaker with HRP-conjugated secondary antibody for 1h. Upon color development with ECL, X-ray film was used to detect light. The film was developed at room temperature, fixed, and scanned with scanner. Transillumination light density was measured with gel image system.

Statistical analysis

Data were expressed as mean \pm SD. SPSS13.0 and Excel XP with t-text were used for statistic analysis. A *p* value of <0.05 was considered significant.

Results

Effects of emodin on E2 induced MCF-7 cell and MDA-MB-231 cell proliferation

We evaluated the effect of emodin on the proliferation of breast cancer cells *in vitro*, determined by MTT assay. As shown in Figure 1A, emodin inhibits the proliferation of ER α -positive MCF-7 cell and ER α -negative MDA-MB-231 cell in dose-dependent manner. At 48h, the inhibition of 40 μ mol/L emodin on MCF-7 cell and MDA-MB-231 cell were $38.15 \pm 2.93\%$, $24.15 \pm 2.43\%$, respectively (*p*<0.05). The effect of emodin on MCF-7 cell is more significant.

To understand molecular mechanisms underlying the growth inhibition induced by emodin, we examined the proliferation of E2-induced MCF-7 cell *in vitro* using MTT assay. It turned out that 10nM E2 significantly increases the proliferation of MCF-7 cell (*p*<0.05) and MDA-MB-231 cell (*p*<0.05) which is inhibited by emodin in a dose-dependent manner. Treatment of MCF-7 cell

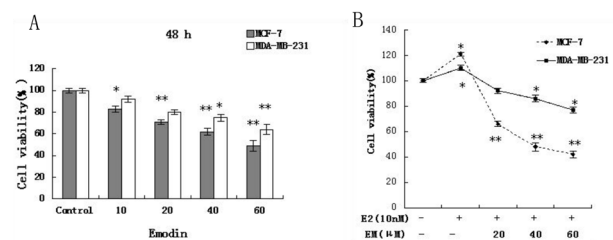


Figure 1. The Effect of Emodin on E2-induced Proliferation of MCF-7 Cells and MDA-MB-231 cells. A) The effect of emodin on proliferation of MCF-7 cells and MDA-MB-231 cells; B) The effect of emodin on E2-induced proliferation of MCF-7 cells and MDA-MB-231 cells. **p*<0.05, ***p*<0.01, versus control

and MDA-MB-231 cell with emodin at 40 μ mol/L for 48h and with E2, cell viability is reduced by 58.90 \pm 2.72%, 21.58 \pm 3.07% (p <0.05, Figure 1B), respectively. This result suggests emodin could inhibit E2-induced cells proliferation effect which is interaction with E2. Based on above results, we selected MCF-7 cells for the subsequent experiments.

The effect of emodin on E2-induced MCF-7 cells cycle progression and apoptosis

As shown in Figure 2, Flow cytometric analysis showed that E2 slightly decreases the number of cells in G0/G1-phase while slightly increases the number of cells in S- and G2/M-phase, there is no significant difference. Combined treatment with 40 μ mol/L of emodin and 10 nmol/L E2 significantly increases the number of cells in the G0/G1-phase by 28.4 \pm 1.82% (p <0.05). In addition, the cell apoptosis rates in control group and E2 group are 13.24 \pm 0.67% and 7.93 \pm 0.53%, respectively, there is statistical significance (p <0.01, Figure 2B), suggesting that E2 inhibits the apoptosis of MCF-7 cells. This result indicated that emodin inhibits E2-induced cell against apoptosis. Furthermore, combined treatment with emodin and E2 significantly increases the apoptosis rate of MCF-7 cells (Figure 2B, p <0.05).

The effect of emodin on ER α expression and transactivation

As shown in Figure 3, the Western blot experiment indicated that the expression of ER α in MCF-7 cells is reduced by emodin in a dose-independent manner. Treatment of MCF-7 cells with 20 μ mol/L, 40 μ mol/L, 60 μ mol/L emodin reduced ER α expression by 43.09 \pm 3.07%,

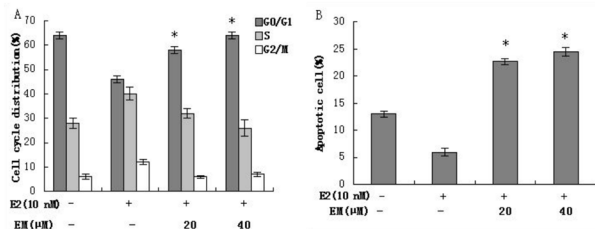


Figure 2. The Effect of Emodin on E2-induced Cell Cycle Distribution and Apoptosis in MCF-7 Cells. A) The effect of emodin on E2-induced cell cycle distribution and B) apoptosis in MCF-7 cells. # p <0.05 vs control; * p <0.05 vs E2 group. Note: EM, emodin

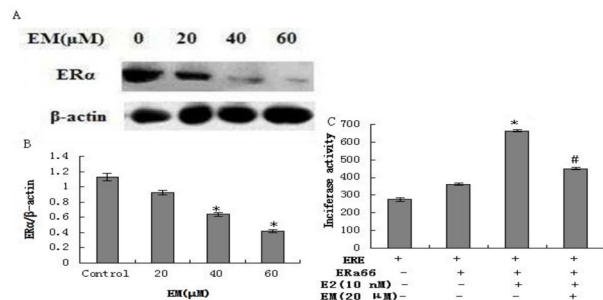


Figure 3. The Effect of Emodin on Expression and Transcription Activity of ER α in MCF-7 Cells. A, B) The effect of emodin on the expression of ER α protein in MCF-7 cells; C) The effects of emodin on E2 induced transcription activity of ER α . * p <0.05 vs control, ** p <0.01 vs control, ## p <0.01 vs E2 group. Note: EM

48.81 \pm 2.93%, 71.52 \pm 3.86% (p <0.01, Figure 3A, B), respectively, when compared with control.

ER α -specific siRNA as shown in Figure 3C, transient transfection of ER α -specific siRNA slightly increases the luciferase activity but without significant difference when compared with control group (p >0.05). E2 (10nm) treatment further increases luciferase activity of MCF-7 cells, with significance (51.01 \pm 3.28%, p <0.01). However, combined treatment of the cells with emodin (20 μ mol/L) and E2 (10nmol/L) reduces this increase of luciferase activity with significance (44.31 \pm 2.23%, p <0.01). These results suggest that emodin significantly inhibited E2-activated ER α expression.

The effect of emodin on E2-induced ER α target genes Cyclin D1 and Bcl-2 expression

To investigate the signaling pathway for the involvement of ER α in the inhibitory effect of emodin on E2-induced cell growth, we tested the effect of emodin on E2-induced ER α target genes Cyclin D1 and Bcl-2 expression. In E2-treated cells, the expression of ER α target genes Cyclin D1 and Bcl-2 are obviously increased compared with control (p <0.01; Figure 4). However, the increases of Cyclin D1 and Bcl-2 are significantly inhibited by 20 μ mol/L emodin (p <0.01) and further reversed by 40 μ mol/L emodin (87.34 \pm 0.83%, 85.48 \pm 0.86%, p <0.01).

The effect of emodin on AKT and MAPK activation induced by E2

To investigate the possible involvement of emodin in E2-

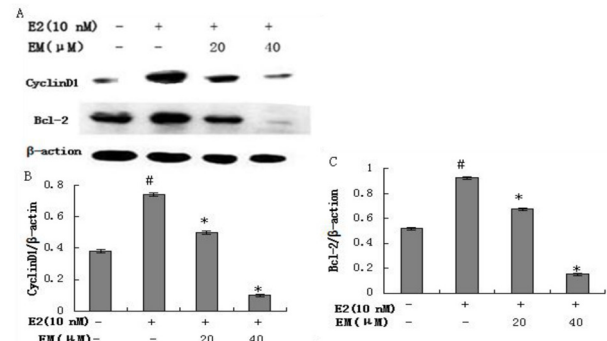


Figure 4. Effects of Emodin on the Expression of E2-Induced Cyclin D1 and Bcl-2 Proteins. A, B) Effects of emodin on the expression of E2-induced Cyclin D1 and A, C) Bcl-2 proteins. ## p <0.01 vs control; ** p <0.01 vs E2 group

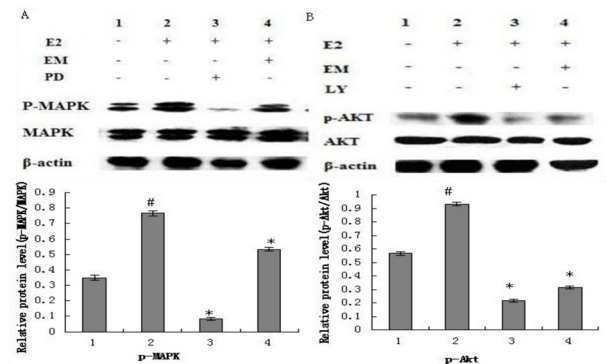


Figure 5. The Effects of Emodin on the Expression of p-MAPK and p-AKT Proteins. A) The effects of emodin on the expression of p-MAPK and B) p-AKT proteins. # p <0.01 vs control, * p <0.05, ** p <0.01 vs E2 group

dependent rapid activated pathways, we studied the AKT and MAPK activation by examine their phosphorylation status. E2 increases the phosphorylation of AKT and MAPK ($p < 0.01$, Figure 5). The phosphorylation of AKT and MAPK is significantly reduced by specific inhibitor PD-98095 and LY-294-002, respectively ($p < 0.01$; Figure 5). Furthermore, the expression of *p*-AKT and *p*-MAPK is reduced of $29.89 \pm 2.49\%$ ($p < 0.05$), $63.42 \pm 3.04\%$ ($p < 0.01$) by $40 \mu\text{mol/L}$ emodin.

Discussion

As the nature anthraquinone compounds found in rhubarb, polygonum, cuspidate and aloe, emodin was found to have effect of anti-cancer for different stages of cancer development (Liu et al., 2006), He et al. (2012) have showed that emodin extracted from Chinese herbs may inhibited cell proliferation in NSCLC cells by downregulation ERCC1 and Rad51 (He et al., 2012). All of this indicating that emodin could be potentially developed for clinical applications. Recently, emodin has been a focal point in the development of breast cancer therapy. Our study indicates that emodin inhibits the proliferation of ER-positive MCF-7 cell and ER-negative MDA-MB-231 cell in a dose-dependent manner, in which the inhibition of MCF-7 cell is more significant. It has been indicated that ER α regulates genomic and non-genomic mechanism in breast cancer initiation and progression. Mechanistically, dysregulation of ER α signaling pathway contributes to the onset and progression of breast cancer (Yamaguchi, 2007; Ma et al., 2012). Here we show that emodin inhibits the effects of ER α on MCF-7 cells. Emodin may exert its effect through associating with ER α . Our results indicate emodin could inhibit E2-activated ER α transactivation activation, suggesting that ER α is a key target for drugs.

The genomic mechanism of ER signaling is direct action of E2 bound to ER α . This is followed by ER α dimerization, binding to estrogen-responsive genes (ERE). Increased transcription of these genes enhances mammary epithelial cell proliferation and tumorigenesis, thereby regulates the transcription of target gene (Yao et al., 2010). In breast cancer tissues, several estrogen response proteins containing ERE directly promote breast cancer cells proliferation and survival, we main talk about Cyclin D1 and Bcl-2. CyclinD1 is a kind of cell cycle regulator protein which is closely related to tumorigenesis (Takahashi-Yanaga et al., 2008). Bcl-2 is the most important regulator protein of apoptosis protein family, playing the main role in tumor cells to prevent apoptosis and promote cell survival, Expression of Bcl-2 in tumor cells was strongly associated with expression of estrogen and progesterone receptors (Borner et al., 2004; Jaafar et al., 2012). Our results indicate that emodin significantly inhibit the E2-induced expression of ER α target genes Cyclin D1 and Bcl-2, and arrested MCF-7 cells at G0/G1 phase, suggesting that emodin may inhibit tumor play antitumor effect through the down-regulation of Cyclin D1 and Bcl-2.

The non-genomic mechanism of E2 plays an important role in the progression and apoptosis of tumor cells. E2 activates transcriptional activity via a number of pathways

including PI3K/Akt, MAPK/Erk1/2 pathway which exists in cell membrane. Furthermore, it has been suggested that E2 could rapidly bind to receptors related to the development and progression of tumor and result in its phosphorylation, the effect is very rapid and not related to gene expression (Chakraborty et al., 2010; Mendoza et al., 2011). Our data are supported by previous studies showing that emodin exerts its anticancer effects via inhibiting PI3K/Akt and MAPK/Erk1/2 pathways. Therefore, ER α is directly involved in the inhibition of PI3K/AKT by emodin through regulating non-genomic pathway, this hypothesis is consistent with Zhang's study (2005), which showing that E2 mediates rapid non-genomic pathway through stimulating IGF-IR in breast cancer cells, thereby activating PI3K/Akt signaling pathway, promoting breast cancer cells proliferation and differentiation to exert its biological effect.

Taken together, our results indicate that emodin inhibits E2-induced MCF-7 cells proliferation and anti-apoptosis effect through ER α -MAPK/Akt- Cyclin D1 and Bcl-2 signaling pathway.

Acknowledgements

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