Original Paper

Biochanin A Promotes Proliferation that Involves a Feedback Loop of MicroRNA-375 and Estrogen Receptor Alpha in Breast Cancer Cells

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Key Words

Biochanin A • miR-375 • Estrogen receptor α • OVX

Abstract

Background: Biochanin A and formononetin are O-methylated isoflavones that are isolated from the root of *Astragalus membranaceus*, and have antitumorigenic effects. Our previous studies found that formononetin triggered growth-inhibitory and apoptotic activities in MCF-7 breast cancer cells. We performed *in vivo* and *in vitro* studies to further investigate the potential effect of biochanin A in promoting cell proliferation in estrogen receptor (ER)-positive cells, and to elucidate underlying mechanisms. **Methods:** ER α -positive breast cancer cells (T47D, MCF-7) were treated with biochanin A. The MTT assay and flow cytometry were used to assess cell proliferation and apoptosis. mRNA levels of ER α , Bcl-2, and miR-375 were quantified using real-time polymerase chain reaction. Compared with the control, low biochanin A concentrations (2–6 μ M) stimulated ER α -positive cell proliferation (T47D, MCF-7). The more sensitive T47D cells were used to study the relevant signaling pathway. **Results:** After treatment with biochanin A, ER α , miR-375, and Bcl-2 expression was significantly upregulated. Additionally, in the *in vivo* studies, uterine weight in ovariectomized mice treated with biochanin A increased significantly. **Conclusion:** This study demonstrated that biochanin A promoted ER α -positive cell proliferation through miR-375 activation and this mechanism is possibly involving in a miR-375 and ER α feedback loop.

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Introduction

Phytoestrogens, a class of plant-derived phenolic compounds, have either estrogenic or/and antiestrogenic effects owing to their structural similarity to estrogens [1]. The isoflavone biochanin A ($C_{16}H_{12}O_5$) is a phytoestrogen and a main active component of *Astragalus membranaceus*, a traditional Chinese herbal medicine which has been widely used for centuries. The effects of biochanin A include regulating steroid receptor (AR and ER) expression, inhibition of cell proliferation and DNA topoisomerase II, promotion of cancer cell apoptosis [2]. Our previous studies have shown that another isoflavone (formononetin) had an inhibitory effect on ER α -positive human breast cancer cells (MCF-7) but not on ER-negative cells at concentrations higher than 30 μ M. These results confirmed the underlying mechanism [3-5].

The ERs are activated by estrogen and, together with the type of ligand, are important factors in estrogen target gene transcription. Existing evidence has shown that ER ligands such as 17 β -estradiol (E_2), estradiol, and raloxifene play central roles in the proliferation and migration of ER α -positive cells [6-8]. Therefore, E_2 was used as a positive control in the present study.

MicroRNAs (miRNAs) are a class of endogenous, non-coding single-stranded RNA molecules with 21–25 nucleotides that bind by imperfect pairing to their target mRNA 3' untranslated region and suppress mRNA translation [9, 10]. miRNAs have been shown to act as potential mediators of multiple biologic processes and are involved in providing feedback loops for various signal transduction pathways [11, 12]. In addition, miR-375 was identified as the first miRNA with the capacity to enhance ER α signaling in breast cells to promote cell proliferation. Previous studies showed that high expression levels of the microRNA, miR-375, stimulated proliferation in ER α -positive breast cell lines [13].

Bcl-2 (B-cell CLL/lymphoma 2), a potent inhibitor of apoptosis cell death, is activated as the target in the downstream signaling pathway and it contributed to cell proliferation in our previous studies [14]. We demonstrated that the Bcl-2 signaling pathway was involved in formononetin-induced anti-proliferative effects on ER α -positive MCF-7 cells [15, 16], so we expect the same mechanism to stimulate cell proliferation on ER α -positive breast cancer cells treated with biochanin A.

Therefore, we aim to further investigate the effects of the isoflavone, biochanin A, on ER α -positive cell proliferation. In addition to MCF-7 cells, another human ER α -positive breast cancer cell line, T47D, was also examined to provide additional support and was used to further confirm the downstream targets. We hypothesized that miRNA-375 was involved in the ER α -mediated pathway and thus forms a positive feedback loop induced by low biochanin A concentrations.

Materials and Methods

Drugs and animals

Kaighn's modification of Ham's F12 medium (F-12K), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and charcoal-stripped fetal bovine serum (CS-FBS) were all used for cell culture. Biochanin A ($C_{16}H_{12}O_5$, purity >98%, verified using high performance liquid chromatography) was purchased from Sigma-Aldrich Ltd (St. Louis, USA). Biochanin A was dissolved in dimethyl sulfoxide (DMSO) to form a 200 mM solution. 17 β -Estradiol (E_2 , Sigma), the positive control, was dissolved in DMSO to a final concentration of DMSO < 0.1% (v/v). The solution was stored at 4°C until further use.

Fifty mice (female) were kept under sterile conditions and in isolated pathogen-free ventilation chambers under an ambient temperature of 22–25°C and 55–65% relative humidity. Besides, all chambers conditioned with alternating 12 h periods of light and darkness. The experimental procedures were approved by our institutional animal research ethics committee with reference to the Experimental Research Institution of Guilin Medical University for the use of experimental animals (NO 20140213).

Cell culture

ER α -positive T47D cells and MCF-7 cells, all of which were obtained from the Shanghai Institute of Cell Biology (The Chinese Academy of Sciences, Shanghai, China). These were placed in Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). At 37°C in a humidified atmosphere of 5% CO₂, the two cell lines were exposed to phenol red-free RPMI-1640 with 10% charcoal-stripped (CS) FBS instead of normal FBS for at least 4 days before the experiments. Cells were then starved using low-serum medium (containing 0.5% CS-FBS) for 24 h for the cell proliferation assay before stimulation with biochanin A. The 96-well plates and 6-well plates were pre-coated with 0.1% gelatin.

MTT assay

ER α -positive T47D and MCF-7 cells were trypsinized and then seeded at 4×10^3 cells each well in 96-well plates. After incubating for 24 h, various doses (0.5, 1, 2, 3, 6, 12, 25, 50 μ M) of biochanin A were added and the cells were incubated for another 48 h. Then, 20 μ L MTT (Sigma) solution (5 g/L) in phosphate-buffered saline (PBS) was added. After 3 h incubation, the optical density (OD) for each well was measured at a wavelength of 490 nm under a microculture plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Flow cytometry assay

ER α -positive T47D and MCF-7 cells were separately seeded on 6-well plates with 4×10^3 cells per well. After 24 h, cells were treated with biochanin A for 48 h, collected, and then washed three times using ice-cold PBS. Cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 40 min in the dark at room temperature in 400 μ L of binding buffer. Under a FACS Aria flow cytometer (Becton Dickinson), unstained cells were viable, and cells stained with Annexin V were judged at the early stage of apoptosis. Surface exposure of phosphatidylserine in apoptotic cells was measured using an Annexin V-FITC apoptosis detection kit (Boster, China), according to the manufacturer's instructions. Additional exposure to PI made it possible to differentiate the early apoptosis cells (Annexin V+/PI-) from the late apoptotic cells (Annexin V+/PI+).

Real-time PCR assay

Biochanin A may be potentially used in angiogenesis, and thus, T47D cells were used to further investigate the downstream signaling pathway and thereby confirm the relevant mechanism. ER α -positive T47D cells were treated with biochanin A for 48 h. The cells were then lysed in TRIzol reagent (Gibco-BRL, USA) to extract total RNA. RNA was reverse transcribed to single-strand cDNA with the Revert Aid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, USA), according to the manufacturer's instructions. Bcl-2, ER α , and miR-375 levels were determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with specific primers for Bcl-2, ER α , miR-375, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and miRNA U6 using SYBR Green qPCR Master Mix (Fermentas Life Sciences). GAPDH and miRNA U6 were used as internal controls to calculate the relative expression level of Bcl-2, ER α , and miR-375.

Animals study design

The mice underwent either dorsal ovariectomy (OVX) or were sham-operated (sham) with 10% chloral anesthesia. The mice were adequately anesthetized, fixed on an operating table, and masked with light ether for the duration of the surgery. Six days later, they were divided into five groups: sham (n=10); OVX (n=10); OVX mice that received intraperitoneal (i.p.) biochanin A (2, 4 mg/kg/day, n=10); OVX mice that received i.p. E₂ (20 μ g/kg/day, n=10). After 20 days' treatment, each uterus was weighted to confirm the success of the ovariectomy and to determine the effects of biochanin A, ER α , and miR-375 on the uterine index in the OVX mice.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation. The Statistical Package for Social Sciences (SPSS) 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analyses including one-way analysis of variance and the Student's t-test. A p-value <0.05 was considered statistically significant.

Results

Biochanin A regulates proliferation of ER α -positive cells

To explore the proliferative effect of biochanin A, ER α -positive breast cancer cells (T47D, MCF-7) were treated with different concentrations of biochanin A for 48 h. MTT assay results showed that, compared with the vehicle control, biochanin A at low concentrations (2, 3, and 6 μ M) significantly induced dose-dependent proliferation in ER α -positive T47D and MCF-7 cells ($p < 0.05$; Fig. 1); this was shown as increasing optical density (OD) values. However, as the concentration increased, the proliferation effect of biochanin A decreased, and even inhibited T47D and MCF-7 cell proliferation at 50 μ M ($p < 0.05$). E $_2$, the positive control, induced a dramatic increase in proliferation ($p < 0.05$). We suggest that biochanin A induces cell proliferation through regulation of the ER α -mediated signaling pathway.

Fig. 1. Effects of biochanin A on ER α -positive MCF-7 and T47D cell proliferation. MCF-7 and T47D cells were treated with different concentrations of biochanin A, E $_2$ for 48 h. Final concentrations of the compounds are shown. Cell viability was determined using the MTT assay. Ten independent experiments were repeated. ** $p < 0.05$ vs. control; $n = 10$.

Fig. 2. Effects of biochanin A on early apoptosis of MCF-7 and T47D cells. MCF-7 and T47D cells were cultured in RPMI-1640 containing 10% FBS. Cellular apoptosis was examined using flow cytometry. MCF-7 and T47D cells were then cultured in phenol red-free RPMI-1640 with 0.5% CS-FBS. Apoptosis was assessed in the two cell lines after treatment with control (0 μ M), 3 μ M biochanin A, 6 μ M biochanin A, 10 nM E $_2$. Means at 3 μ M and 6 μ M biochanin A and E $_2$ are different from the control. Three independent experiments were repeated. ** $p < 0.05$ vs. control; $n = 3$.

Low concentrations of biochanin A inhibit apoptosis in ER α -positive cells

To understand whether the proliferation effect of biochanin A was related to apoptosis, we conducted a flow cytometry. Conversely, Annexin V binding to phosphatidylserine exposed on the cell membrane is generally recognized as an early indicator of apoptosis. As shown in Fig. 2, only 1.68% of T47D cells in the normal group and 1.62% of MCF-7 cells were positive for Annexin V staining; conversely, 17.12% and 16.88% of T47D and MCF-7 cells in the vehicle group (in phenol red-free RPMI with 0.5% CS-FBS) were positively stained by Annexin V. However, 11.21% and 4.89% of T47D cells treated with 3 and 6 μ M biochanin A, respectively, for 48 h were positive for Annexin V staining. This suggests that low concentrations of biochanin A had an inhibitory effect on apoptosis in T47D cells, which was in agreement with the MTT assay results. Decreased apoptosis in the cells further confirmed the involvement of ER α activation in isoflavone-mediated growth regulation of ER α -positive cells.

Low concentrations of biochanin A regulate ER α and miR-375

We investigated further applications of biochanin A for angiogenesis using T47D cells to study the possible mechanism. The results showed that low concentrations of biochanin A gradually upregulated ER α , miR-375, and Bcl-2 expression levels (Fig. 3). Given the high miR-375 expression level in T47D cells, we evaluated the potential contribution of miR-375 to the proliferation of ER α -positive cells. These results suggested a reciprocal regulatory connection between miR-375 and ER α . Collectively, these data indicate the existence of positive feedback regulation between ER α and miR-375.

Low concentrations of biochanin A regulate the uterine index of the OVX mice

Compared with sham-operated mice, the uterine weight of the uterine tissues of ovariectomized (OVX) mice was lower, which suggests that the bilateral oophorectomies in these mice were successful. OVX mice that received biochanin A at concentrations of 2

Fig. 3. Biochanin A increased miR-375, ER α , and Bcl-2 mRNA expression in T47D cells. After treatment with different concentrations (0, 3, 6 μ M) of biochanin A for 48 h, miR-375 (A), ER α (B) and Bcl-2 (C) expression levels were determined using real-time PCR. Means for 3 μ M and 6 μ M are different from the control. Three independent experiments were repeated. ** $p < 0.05$ vs. control; $n = 3$.

Fig. 4. Effects of biochanin A on the uterine index in OVX mice. The OVX mice were treated with different concentrations of biochanin A (2, 4 mg/kg) and E_2 (20 μ g/kg) for 20 days and the uterine index of the OVX mice was then determined. Means at OVX plus 2 mg/kg, 4 mg/kg biochanin A, and OVX plus E_2 are different from the OVX group. ** $p < 0.05$ vs. OVX group; $n = 10$.

and 4 mg/kg showed a significant increase in uterine weight relative to that of the controls ($p < 0.05$; Fig. 4). Moreover, in accordance with the *in vitro* results, the uterine weight of the OVX mice that received E_2 showed a significant increase ($p < 0.05$).

Discussion

Biochanin A is the main component of isoflavones that possesses antitumorigenic properties. Previously, we studied its inhibitory effect in human breast cancer MCF-7 cells at relative high concentrations and found that it might exhibit anticancer properties through an ER-dependent mechanism. In addition, calycosin, another novel component of isoflavones, was suggested to exhibit stimulatory effects on the proliferation of ER-positive cells *in vitro* and *in vivo* [15]. Based on these findings, we further observed low concentrations of biochanin A acted on ER-positive cell proliferation, to provide more valuable information for evaluating the feasibility of its clinical application. Our results demonstrate that low concentrations of biochanin A not only activated MCF-7 cell proliferation, but also increased ER α -positive T47D cells, suggesting that biochanin A may be important for *in vitro* angiogenesis. This may be because angiogenesis is involved in the establishment of a mature blood vessel network and it plays a key role in the adult organism by the induction of endothelial proliferation from pre-existing capillaries [17, 18].

Estrogens are steroidal hormones that play important roles in the growth, development and function of the female reproductive system. By binding to the ERs, ER α and ER β , estrogen regulates target gene expression. E_2 , a ligand of ER α , was found to downregulate the levels of ER α in breast cancer cell lines. In this study, the results *in vivo* showed that biochanin A stimulated a noticeable increase in uterine weight in OVX mice compared to controls, and this increase was associated with its estrogenic activity. Phenol red, which mimics estrogen, also stimulates cell proliferation in estrogen-responsive cells [19]. In this study, the ER-positive MCF-7 and T47D cells were cultured in phenol red-free RPMI with 10% CS-FBS instead of normal FBS for at least 4 days to induce cell apoptosis. As a result, biochanin A at low concentrations inhibited cell apoptosis, and this was mainly owing to its proliferative effect. Thus, we concluded that low concentrations of biochanin A inhibited cell apoptosis via its estrogenic effect.

However, previous studies have shown that miRNAs, a class of small non-coding RNAs, play a major role in cell growth and differentiation by regulating gene expression at the post-transcriptional level [20, 21]. miR-375 was highly expressed in ER α -positive MCF-7 cells, and there was a positive loop between ER α and miR-375 [9]. Our findings supported our hypothesis that a positive loop between ER α and miR-375 might exist in ER α -positive cells that were induced by biochanin A.

Notably, the role of Bcl-2 is commonly recognized as a suppressor of apoptosis cellular effects and it may prolong the length of a cell's life by regulating programmed cell death (PCD) [22, 23]. Thus, Bcl-2 expression was determined at the mRNA levels in this study. As expected, their expression was dose-dependently enhanced by biochanin A treatment. These results further confirm the important role of Bcl-2 in the proliferation of ER α -positive cells and also in biochanin A-mediated activation of ER α -positive cells.

Taken together, our study shows that biochanin A at low concentrations shows the stimulatory action in ER α -positive MCF-7 and T47D cells. However, this proliferation effect possibly occurred by activating a positive feedback loop of miR-375 and ER α modulation. Thus, biochanin A may be a promising therapeutic agent for treating patients with estrogen-related conditions.

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