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Inhibitory Effect of Luteolin on Estrogen Biosynthesis in Human Ovarian Granulosa Cells by Suppression of Aromatase (CYP19)

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Supporting Information

ABSTRACT: Inhibition of aromatase, the key enzyme in estrogen biosynthesis, is an important strategy in the treatment of breast cancer. Several dietary flavonoids show aromatase inhibitory activity, but their tissue specificity and mechanism remain unclear. This study found that the dietary flavonoid luteolin potently inhibited estrogen biosynthesis in a dose- and time-dependent manner in KGN cells derived from human ovarian granulosa cells, the major source of estrogens in premenopausal women. Luteolin decreased aromatase mRNA and protein expression in KGN cells. Luteolin also promoted aromatase protein degradation and inhibited estrogen biosynthesis in aromatase-expressing HEK293A cells, but had no effect on recombinant expressed aromatase. Estrogen biosynthesis in KGN cells was inhibited with differing potencies by extracts of onion and bird chili and by four other dietary flavonoids: kaempferol, quercetin, myricetin, and isorhamnetin. The present study suggests that luteolin inhibits estrogen biosynthesis by decreasing aromatase expression and destabilizing aromatase protein, and it warrants further investigation as a potential treatment for estrogen-dependent cancers.

KEYWORDS: *luteolin, aromatase, estrogen, granulosa cell*

INTRODUCTION

Estrogen is a sex steroid hormone and plays a pivotal role in the regulation of many biological processes. Estrogen is known to induce physiological responses in the reproductive tract, mammary tissue, and the pituitary gland and to affect nonreproductive processes such as bone formation and cardiovascular health.[1] The biological actions of estrogen are mediated through nuclear and membrane estrogen receptors (ERs), which are expressed in a variety of cell types and mediate the genomic and nongenomic effects of estrogen.[1] Numerous factors have been implicated in the increased incidence of breast cancer in humans, including the Western-style diet and environmental endocrine-disrupting chemicals, which are thought to alter the production, metabolism, and action of estrogen.[2,3] Some ER antagonists such as tamoxifen and fulvestrant have been widely used to treat hormone-responsive breast cancer, but their clinical use is often limited by side effects including vaginal bleeding, thromboembolism, and increased risk of endometrial cancer.[4] Fruits and vegetables are rich in flavonoids such as genistein. These compounds are structurally similar to estrogen and are well-known for their cancer-preventive effects, which are partly mediated through competitive inhibition of the ER. However, flavonoids also exhibit estrogenic activity that can enhance cell proliferation in the uterus and the mammary gland.[5,6] Thus, reduction of estrogen levels by means of inhibition of endogenous estrogen biosynthesis becomes another option in the prevention and treatment of estrogen-mediated carcinogenesis.[7]

In humans, estrogen biosynthesis occurs at a number of different sites, with the major sites being the granulosa cells of the ovary in premenopausal women and stromal cells of the adipose tissue in postmenopausal women.[8] In women, estrogen is also produced locally at other sites, including the brain, bone, and breast. Estrogen biosynthesis is catalyzed by aromatase (cytochrome P450 19; CYP19), which binds the C_{19} androgenic steroid substrate and catalyzes successive reactions to form the phenolic A ring characteristic of estrogens.[9] In humans, aromatase expression at various sites is regulated by tissue-specific promoters by alternative splicing mechanisms.[10] In the ovary and testes, aromatase expression is controlled by promoter II, which binds the transcription factors cAMP-response element binding protein (CREB) and steroidogenic factor-1. Aromatase expression in the gonads is thus regulated by gonadotropins through stimulation of cAMP generation.[11] Aromatase inhibitors such as anastrozole, letrozole, and exemestane have been developed for the treatment of hormone-dependent breast cancer in postmenopausal women, and they show superior efficacy to conventional antiestrogen receptor drugs such as tamoxifen.[12] However, the estrogen deprivation that results from inhibition of aromatase activity leads to side effects such as increased risk for osteoporosis and cardiovascular disease.[13] Thus, there remains a need to discover and develop novel aromatase inhibitors that offer greater clinical efficacy and fewer side effects than the currently available drugs.

Several natural flavonoids are known to be potent aromatase inhibitors; this activity partly contributes to the importance of certain vegetables and fruits in the daily diet to decrease the risk of chronic diseases.[14−16] Luteolin (3′,4′,5,7-tetrahydroxyflavone) is one of the most common flavonoids present in edible and medicinal plants. Preclinical studies have shown that this

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compound possesses a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer activities.[17] Accumulating evidence suggests that luteolin could be developed as an anticancer agent to sensitize some tumor cells, such as tumor necrosis factor-related apoptosis-inducing ligand-sensitive or -resistant cancer cells and hepatoma cells, for apoptosis.[18,19] Luteolin has also been proposed to be a promising adjuvant for multiple sclerosis therapy due to its additive effects with interferon-β in modulating the immune responses of peripheral blood mononuclear cells isolated from multiple sclerosis patients.[20] Dietary sources of luteolin include celery, green peppers, bird chili, onion, parsley, cabbages, and apple skin. Although luteolin is found to inhibit estrogen biosynthesis in breast cancer MCF-7 cells, preadipocyte cells and placental choriocarcinoma JEG-3 cells, its tissue specificity and mechanism of action remain poorly understood.[21−23]

In this study, we investigated the effect and mechanism of luteolin on estrogen biosynthesis in human ovarian granulosa-like KGN cells. We also compared the effects of luteolin on estrogen biosynthesis with those of other commonly consumed flavonoids and vegetables.

MATERIALS AND METHODS

Materials. Luteolin, quercetin, kaempferol, myricetin, and isorhamnetin were purchased from Must Biotechnology Co., Ltd. (Chengdu, China). Testosterone, formestane, forskolin, and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bird chili (*Capsicum frutescens*) and onion (*Allium fistulosum*) were purchased from a market near the institute and were identified by one of the authors (Prof. Guolin Zhang). The pCMV6-aromatase plasmid was purchased from OriGene (Rockville, MD, USA), and the pSV-β-galactosidase plasmid was purchased from Promega (Beijing, China).

Cell Culture and Transfection. Human ovarian granulosa-like KGN cells (kindly supplied by Prof. Yiming Mu, Chinese PLA General Hospital, Beijing, China) were maintained in Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F-12) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% (v/v) fetal bovine serum (Gibco-Invitrogen), penicillin (100 units/mL), and streptomycin (0.1 mg/mL) at 37 °C in a humidified 5% CO_2 atmosphere. Human embryonic kidney 293A (HEK293A) cells (Qbiogene, Carlsbad, CA, USA) were cultured at 37 °C in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/mL), and streptomycin (0.1 mg/mL). For the transient transfections, subconfluent HEK293A cells grown in 24-well plates were cotransfected with 0.5 μg of pCMV6-aromatase plasmid and 0.3 μg of pSV-β-galactosidase plasmid using Trans-EZ transfection reagent according to the the manufacturer's protocol (Sunbio Medical Biotechnology, Shanghai, China). Aromatase activity was normalized to β-galactosidase activity to allow for differences in transfection efficiency.

Cell Proliferation Assay. KGN cells were seeded into 96-well plates (1.0×10^3 cells/100 μL) DMEM/F-12 medium at 37 °C in a 5% CO_2 humidified atmosphere. Compounds were then added to the cells, and the plates were incubated for a further 24 h. Alamar Blue reagent (10 μL/well) was added, and the fluorescence intensities were measured using a Verioskan Flash Multimode Reader with excitation at 544 nm and emission at 590 nm. Cytotoxicity was defined as the ratio of the fluorescence intensity in test wells to that in solvent control wells (0.1% DMSO). The assay was conducted three times in triplicate. The IC_{50} value was obtained by fitting dose−response data to a four-parametric logistic nonlinear regression model using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA).

Cell-Based Estrogen Biosynthesis Assay. KGN cells or transiently transfected HEK293A cells were seeded in 24-well plates and cultured overnight. The next day, the medium was replaced with serum-free medium, and the cells were pretreated for 24 h with the test chemicals. Testosterone (10 nM) was then added to each well, and the cells were incubated for a further 24 h. At the end of this incubation, the culture supernatants were collected and stored at −20 °C. Levels of 17β-estradiol in the supernatants were quantified using a magnetic particle-based enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Bio-Ekon Biotechnology, Beijing, China). Optical densities (OD) were measured at 550 nm using a Verioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). Results were normalized to the total cellular protein content and were expressed as percentage 17β-estradiol production compared with the control samples. Protein determination was carried out with a bicinchoninic acid (BCA) protein assay kit (Bestbio, Shanghai, China). For the calculation of the IC_{50} value of luteolin in inhibiting estrogen biosynthesis, various concentrations of luteolin (1 nM, 5 nM, 10 nM, 50 nM, 1 μM, 5 μM, 10 μM, 35 μM) were used to treat KGN cells for 24 h. Testosterone (10 nM) was then added to each well, and the cells were incubated for a further 24 h. The IC_{50} value was obtained by fitting dose−response data to a four-parametric logistic nonlinear regression model using GraphPad Prism 5.0 software.

Recombinant Expressed Aromatase Activity Assay. An in vitro recombinant expressed aromatase activity assay was conducted as described previously with minor modifications.[24,25] In brief, the test compounds (5 μL) were preincubated with an NADPH regenerating system (45 μL of 2.6 mM $NADP^+$, 7.6 mM glucose-6-phosphate, 0.8 U/mL glucose-6-phosphate dehydrogenase, and 1 mg/mL albumin, in 50 mM potassium phosphate, pH 7.4) for 10 min at 37 °C before 50 μL of the enzyme and substrate mixture (40 pM recombinant aromatase and 0.4 μM dibenzylfluorescein in 50 mM potassium phosphate, pH 7.4) was added. The reaction mixture was then incubated for 2 h at 37 °C to allow the aromatase to generate the product and then quenched with 37.5 μL of 2 N NaOH. The mixture was then shaken for 5 min and incubated for 2 h at 37 °C to enhance the noise/background ratio. The fluorescence intensity was measured at 485 nm (excitation) and 530 nm (emission). Three independent experiments were performed in duplicate.

Western Blotting. Cells cultured in 60 mm dishes were lysed with RIPA lysis buffer (Beyotime, Haimen, China) supplemented with protease inhibitor cocktail (Sigma). The protein concentration was determined by using a BCA protein assay kit (Bestbio). Aliquots of total cell lysates (40 μg protein) were mixed in loading buffer, boiled for 5 min, and subjected to SDS-PAGE (10%). Proteins were blotted onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin and then incubated at 4 °C overnight with rabbit anti-human aromatase monoclonal antibody (1:500 dilution) or rabbit anti-human GAPDH monoclonal antibody (1:5000 dilution) (Epitomics, Burlingame, CA, USA). Membranes were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, USA). The intensity of each signal was determined by a computer image analysis system (Quantity One, Bio-Rad, Hercules, CA, USA).

Quantitative Real-Time RT-PCR. Total cellular RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The purity of extracted RNA was determined by examining the ratio of the absorbance at 260 and 280 nm using a Verioskan Flash Multimode Reader (Thermo Scientific). The value of 1.8−2.0 for A260/A280 indicates that the RNA is pure. The concentration of extracted RNA was determined by examining the absorbance at 260 nm. An A260 reading of 1.0 is univalent to 40 μg/mL of RNA. Total RNA (2 μg) was reverse-transcribed using SuperScript III rReverse Transcriptase (Invitrogen) with oligo$(dT)_{18}$ primers. Equal amounts of cDNA were subjected to real-time quantitative PCR with the fluorescent dye SYBR Green I using a Chromo4 detection system (Bio-Rad). Reaction mixtures contained 12.5 μL of 2× TransStart Top Green qPCR SuperMix (TransGen Biotech), 0.5 μL of each primer (0.2 μM), and 1 μL of template cDNA. Sterile distilled water was added to a final volume of 25 μL.

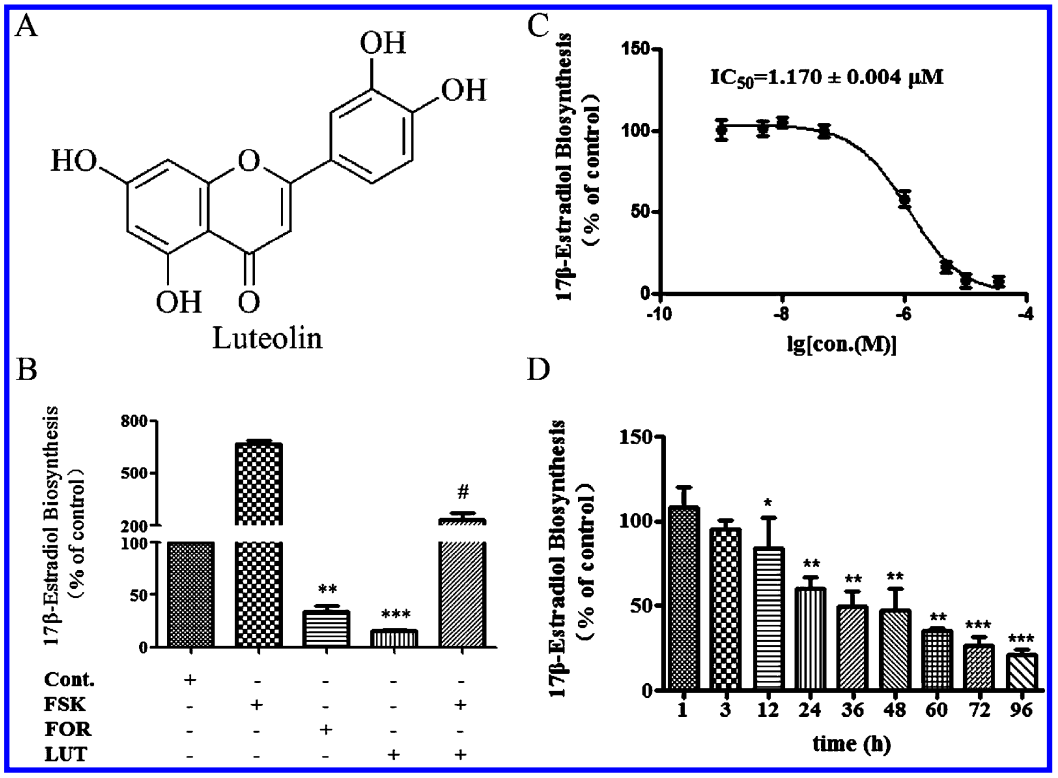

Figure 1. Effect of luteolin on estrogen biosynthesis in KGN cells: (A) presents the chemical structure of luteolin. (B) KGN cells seeded in 24-well plates overnight were pretreated with the test compounds for 24 h. Testosterone (10 nM) was added for a further 24 h of incubation. Concentrations of 17β-estradiol in the culture supernatants were quantified by ELISA. (C) presents the concentration−response curve of luteolin (1 nM, 5 nM, 10 nM, 50 nM, 1 μM, 5 μM, 10 μM, 35 μM) for inhibition of estrogen biosynthesis in KGN cells. (D) presents the time course of luteolin (10 μM) for the inhibition of estrogen biosynthesis in KGN cells. Cont., DMSO control; FSK, 10 μM forskolin; FOR, 50 μM formestane; LUT, 10 μM luteolin. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared with the control; (#) $p < 0.05$ compared with FSK-treated cells ($n = 3$).

The primer pair for aromatase was 5′-ACCCTTCTGCGTCGTGTC-3′ (sense) and 5′-TCTGTGGAAATCCTGCGTCTT-3′ (antisense), and that for GAPDH was 5′-CCACCCATGGCAAATTCCATGGCA-3′ (sense) and 5′-GGTGGACCTGACCTGCCGTCTAGA-3′ (antisense). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 54 °C for 15 s, and 72 °C for 15 s. Standard curves were established for each primer set, and both reference and target gene reactions were performed for each sample. The relative quantity (*n*-fold) of aromatase mRNA was calculated by the Δ (ΔC_t) method by using GAPDH amplified from the same sample as a reference.

Plant Extraction and HPLC Analysis. Samples of bird chili and onion were extracted and hydrolyzed to aglycons using a method modified from that of Miean et al.[26] Briefly, the fresh plants (500 g) were cleaned, cut into pieces, and oven-dried at 50 °C. After the addition of 100 mL of 90% methanol containing 2 mg/mL *tert*-butylhydroquinoneTBHQ) and 10 mL of 6 M HCl, the extracts were mixed and refluxed at 100 °C for 2 h. The extracts were then filtered and concentrated to dryness by evaporation. The resultant flavonoid aglycons were quantified by reversed-phase HPLC (Scientific Systems, Inc., State College, PA, USA) on an Agilent C_{18} column (4.6 × 150 mm, 5 μm) using methanol/0.4% aqueous phosphoric acid (55:45, v/v) as mobile phase (isocratic) and UV detection (360 nm) at the flow rate of 1 mL/min. The contents of luteolin and quercetin were calculated in triplicate for each sample on the basis of the external standard technique, from a standard curve of peak area versus concentration.

Statistical Analysis. Statistical analyses were performed with GraphPad Prism 5.0 software. Results are expressed as the mean ± standard deviation of individual values from three independent experiments. Data were compared by one-way ANOVA followed by Duncan's multiple-range tests. *P* values of <0.05 were considered to be statistically significant.

RESULTS

Effect of Luteolin on Estrogen Biosynthesis in Human Ovarian Granulosa-like KGN Cells. We screened a chemical library consisting of 1431 natural products for small molecule modulators of estrogen biosynthesis[27] and identified luteolin, a natural flavone, as a potent inhibitor of estrogen biosynthesis in human granulosa-like KGN cells. The chemical structure of luteolin is given in Figure 1A. To examine the effect of luteolin on 17β-estradiol synthesis, KGN cells were incubated for 24 h with various concentrations of the test compounds followed by a further 24 h incubation with testosterone. As shown in Figure 1B, forskolin (FSK), which is an adenylate cyclase agonist and up-regulates aromatase expression by increasing intracellular cAMP levels and activating the protein kinase A (PKA)/CREB pathway,[28] significantly increased the production of 17β-estradiol, whereas formestane (FOR), an aromatase inhibitor in clinical use for the treatment of breast cancer,[29] significantly inhibited 17β-estradiol biosynthesis. These results indicated that the established assay is suitable for examining the effect of bioactive compounds on estrogen biosynthesis. At 10 μM, luteolin (LUT) significantly inhibited 17β-estradiol biosynthesis and had a more potent effect than 50 μM formestane. Luteolin at 10 μM also potently inhibited forskolin-induced 17β-estradiol biosynthesis.

To examine the effect of luteolin on estrogen biosynthesis in more detail, KGN cells were treated with luteolin at

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concentrations between 1 nM and 50 μM. As shown in Figure 1C, luteolin inhibited 17β-estradiol biosynthesis in a concentration-dependent manner, with a calculated IC_{50} value of 1.17 μM. Inhibition of 17β-estradiol biosynthesis by luteolin also exhibited a time-dependence manner (Figure 1D). Inhibition of estrogen secretion was first evident at 12 h and was sustained until 96 h, when 17β-estradiol biosynthesis was inhibited by approximately 80% compared with the control (Figure 1D). The tested concentrations of luteolin were not cytotoxic to KGN cells (see Figure S1 in the Supporting Information). These results indicate that luteolin could potently inhibit the biosynthesis of estrogen in human ovarian granulosa cells.

Luteolin Inhibits Aromatase Expression in KGN Cells. KGN cells lack endogenous 17α-hydroxylase and cannot synthesize androgens or estrogens de novo.[30] Therefore, the 17β-estradiol measured in the culture supernatants was likely synthesized from the exogenously added testosterone through the action of aromatase—the only enzyme able to convert testosterone to 17β-estradiol. To determine whether luteolin inhibits 17β-estradiol biosynthesis through an effect on aromatase, we examined aromatase mRNA and protein levels in KGN cells treated with luteolin. As previously reported,[28,31] we found that aromatase mRNA levels were significantly increased in cells treated by forskolin and significantly inhibited by formestane (Figure 2A). Luteolin inhibited aromatase

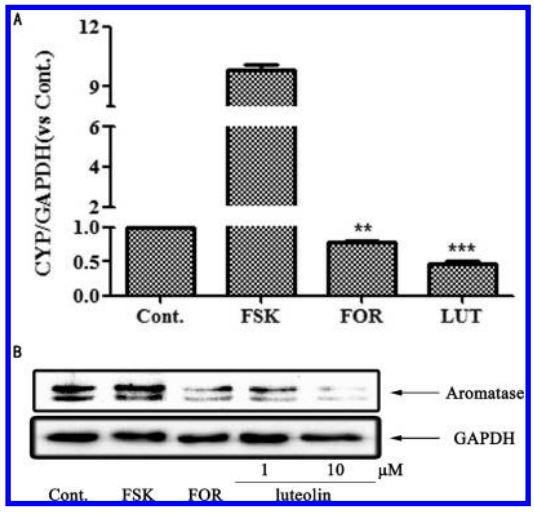

Figure 2. Luteolin inhibits aromatase expression in KGN cells. KGN cells were pretreated for 24 h with 10 μM luteolin, 10 μM forskolin, or 50 μM formestane. (A) Aromatase mRNA was measured in total cellular RNA by using real-time qPCR. The results are expressed as fold increase relative to levels in untreated cells. GAPDH was used as an internal control. (B) Cell lysates were immunoblotted with anti-aromatase or anti-GAPDH antibodies. Cont., DMSO control; FSK, 10 μM forskolin; FOR, 50 μM formestane; LUT, 10 μM luteolin. (**) $p < 0.01$ and (***) $p < 0.001$ compared with the control ($n = 3$).

transcript levels by about 50% compared with the DMSO-treated control cells. Aromatase protein expression was also examined by Western blotting of KGN cell lysates following treatment with luteolin. Consistent with their effects on aromatase transcription, luteolin and formestane treatmenta of KGN cells reduced the expression of aromatase protein, whereas forskolin treatment increased the expression (Figure 2B). These results indicate that luteolin inhibits estrogen biosynthesis in KGN cells by decreasing the expression of aromatase.

Effect of Luteolin on Estrogen Biosynthesis in Aromatase-Expressing HEK293A Cells. To further confirm that luteolin exerts it inhibitory effect on estrogen biosynthesis by suppression of aromatase, HEK293A cells, which do not express aromatase, were transiently transfected with an aromatase expression vector and then treated with luteolin before the addition of testosterone. As shown in Figure 3A,

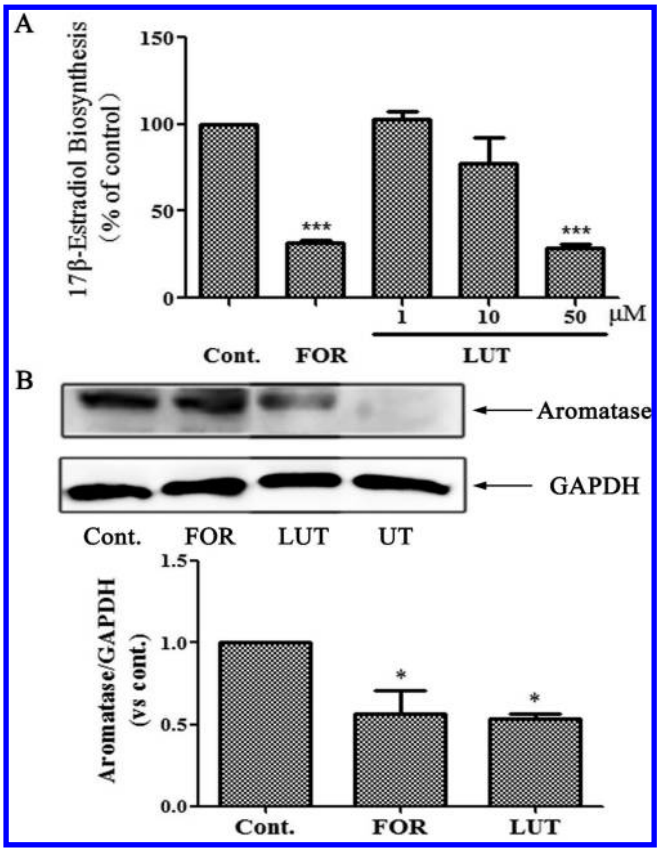

Figure 3. Effect of luteolin on estrogen biosynthesis in aromatase-expressing HEK293A cells. HEK293A cells were cotransfected with pCMV6-aromatase and pSV-β-galactosidase expression plasmids. After 24 h, the cells were incubated with the indicated compounds for an additional 24 h, and then testosterone was added for a further 24 h. (A) 17β-Estradiol concentrations in the culture supernatants were quantified by ELISA. (B) Cell lysates were immunoblotted with anti-aromatase or anti-GAPDH antibodies. The quantitative results are depicted. Cont., DMSO control; FOR, 50 μM formestane; LUT, 50 μM luteolin; UT, untransfected HEK293A cells. (*) $p < 0.05$ and (***) $p < 0.001$ compared with the control ($n = 3$).

17β-estradiol biosynthesis by HEK293A cells was decreased by formestane and by luteolin in a concentration-dependent manner, consistent with the results in KGN cells. Furthermore, luteolin at 50 μM was found to significantly decrease aromatase protein expression in HEK293A cells compared with the DMSO- or formestane-treated cells (Figure 3B). These results indicate that luteolin exerts it inhibitory effect on estrogen biosynthesis by promoting aromatase protein degradation.

Effect of Luteolin on Aromatase Activity in Vitro. Flavonoids have long been found to act directly on aromatase to inhibit its catalytic activity.[14] To examine whether luteolin can directly inhibit aromatase activity, we conducted an in vitro assay by using recombinant expressed aromatase. As shown in Figure 4, formestane inhibited aromatase activity in a concentration-dependent manner, as reported.[24] Compared

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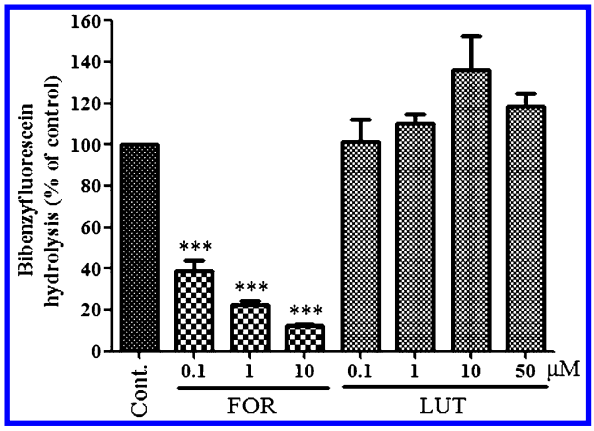

Figure 4. Effect of luteolin on aromatase activity in vitro. An in vitro aromatase assay using recombinant expressed aromatase and dibenzylfluorescein as a substrate was conducted as described under Materials and Methods. Various concentrations of formestane and luteolin were added into the mixtures containing recombinant expressed aromatase and NADPH regenerating system, and fluorescence intensity induced by the aromatase-catalyzed dibenzylfluorescein hydrolysis was detected. Cont., DMSO control; FOR, formestane; LUT, luteolin. (***) $p < 0.001$ compared with the control ($n = 3$).

with that, luteolin had no effect on aromatase activity. These results indicate that luteolin does not directly inhibit aromatase activity as other active flavonoids generally do.

Effects of Dietary Flavonoids on Estrogen Biosynthesis. We next examined the effects of several flavonoids found in commonly consumed vegetables and fruits on estrogen biosynthesis. First, KGN cells were treated with various concentrations of kaempferol, quercetin, myricetin, or isorhamnetin to test their effects on cell viability and to determine the optimal concentrations for estrogen biosynthesis assays (see Figure S2 in the Supporting Information). The chemical structures of these flavonoids are given in Figure 5A. As shown in Figure 5B, kaempferol inhibited 17β-estradiol biosynthesis by KGN cells in a concentration-dependent manner, with approximately 30 and 50% inhibition following treatment with 10 and 50 μM kaempferol, respectively. Quercetin inhibited 17β-estradiol biosynthesis by up to 75% at a high concentration (50 μM) but increased 17β-estradiol biosynthesis slightly when added at low concentrations (1−10 μM). Myricetin inhibited 17β-estradiol biosynthesis at a low concentration (0.1 μM) but had no effect at higher concentrations (1−10 μM). In contrast, isorhamnetin had no effect on 17β-estradiol biosynthesis at any concentration examined (1−50 μM). These results indicate that kaempferol and quercetin potently inhibit estrogen biosynthesis in KGN cells, whereas myricetin and isorhamnetin show only weak or no effects.

Effects of Onion and Bird Chili Extracts on Estrogen Biosynthesis. Onion and bird chili are commonly consumed vegetables that are natural sources of luteolin; therefore, we next investigated whether extracts of onion or bird chili could affect estrogen biosynthesis. Quantification of quercetin and luteolin in the extracts indicated that onion contained high amounts of both quercetin and luteolin, whereas bird chili contained relatively low levels of both compounds (Table 1). KGN cells were treated with various noncytotoxic concentrations of the extracts (see Figure S3 in the Supporting Information). As shown in Figure 6A, a high concentration

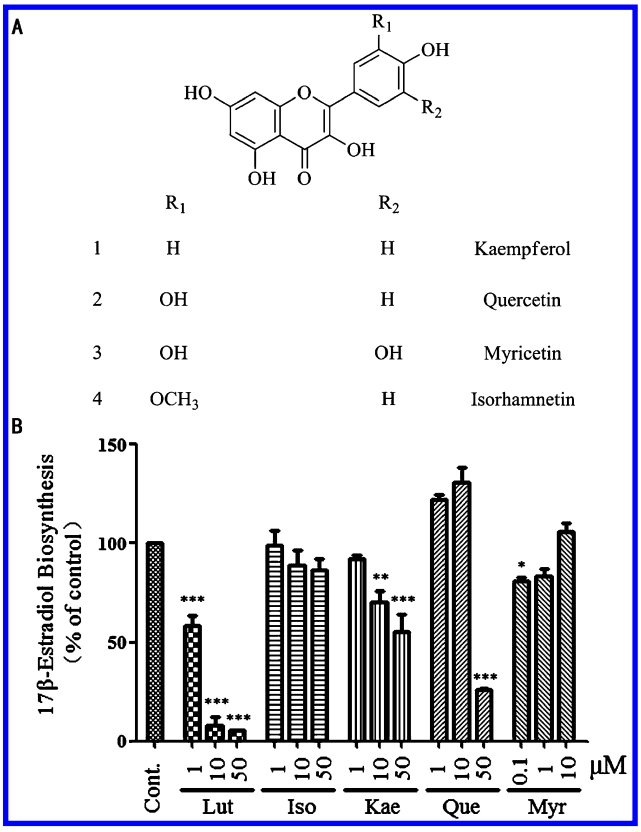

Figure 5. Effects of dietary flavonoids on estrogen biosynthesis. (A) presents the chemical structures of isorhamnetin, kaempferol, quercetin, and myricetin. (B) KGN cells were seeded in 24-well plates overnight and pretreated with the test compounds for 24 h. Testosterone (10 nM) was then added for a further 24 h, and 17β-estradiol concentrations in the culture supernatants were quantified using ELISA. Cont., DMSO control; Lut, luteolin; Iso, isorhamnetin; Kae, kaempferol; Que, quercetin; Myr, myricetin. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared with the control ($n = 3$).

Table 1. Quantification of Quercetin and Luteolin in Onion and Bird Chili Extracts

		content, mg/kg of dry weight[a]	
sample	scientific name	quercetin	luteolin
bird chili	*Capsicum frutescens*	2.57 ± 0.05	6.40 ± 0.39
onion (interior)	*Allium fistulosum*	52.0 ± 0.19	46.1 ± 0.08

[a]Each value is expressed as mg/kg of dry weight of three replications ± SE.

(500 μg/mL) of onion extract potently inhibited 17β-estradiol biosynthesis, whereas the bird chili extract had no such effect. We then examined the effect of onion extracts on aromatase expression in KGN cells. As shown in Figure 6B, onion extracts (100−500 μg/mL) could significantly decrease aromatase protein expression. We also examined the effect of the vegetable extracts on estrogen biosynthesis in aromatase-expressing HEK293A cells. In contrast to the effects on KGN cells, both onion and bird chili extracts significantly inhibited 17β-estradiol biosynthesis in HEK293A cells in a concentration-dependent manner (1−100 μg/mL) (Figure 6C). These results indicate the onion and bird chili extracts can inhibit estrogen biosynthesis, and the inhibitory potency is concentration- and cell type-dependent.

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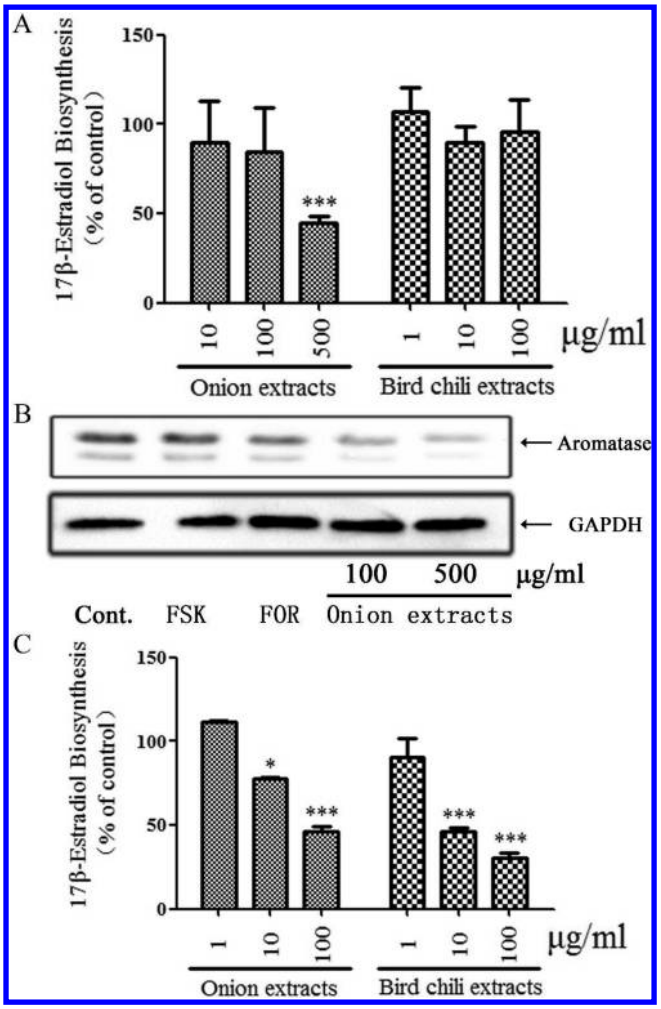

Figure 6. Effects of onion and bird chili extracts on estrogen biosynthesis. (A) KGN cells were seeded in 24-well plates overnight and pretreated with the plant extracts for 24 h. Testosterone (10 nM) was then added for a further 24 h, and 17β-estradiol concentrations in the culture supernatants were quantified by ELISA. (B) KGN cell lysates were immunoblotted with anti-aromatase or anti-GAPDH antibodies. (C) HEK293A cells were cotransfected with pCMV6-aromatase and pSV-β-galactosidase expression plasmids. After 24 h, the cells were incubated with the plant extracts for an additional 24 h. Testosterone (10 nM) was then added for a further 24 h, and 17β-estradiol concentrations in the culture supernatants were quantified by ELISA. Cont., DMSO control; FSK, 10 μM forskolin; FOR, 50 μM formestane. (*) $p < 0.05$ and (***) $p < 0.001$ compared with the control ($n = 3$).

DISCUSSION

In the ovary, a major tissue for estrogen biosynthesis in premenopausal women, aromatase expression is regulated through a follicle-stimulating hormone-mediated cAMP pathway.[28] In the present study, we found luteolin could significantly decrease aromatase mRNA expression in KGN cells derived from the ovary. This is consistent with an observation in MCF-7 breast cancer cells that luteolin inhibits AP-1 binding in the I.3/II promoter region of aromatase and reduces aromatase mRNA expression.[21] In KGN cells, aromatase expression is mainly controlled by promoter II; thus, it is possible that luteolin inhibits aromatase expression in these cells by similar mechanism a to that in MCF-7 cells. Luteolin has been shown to decrease intracellular cAMP levels and inhibit melanin production in B16 melanoma cells treated with α-melanocyte-stimulating hormone,[32] further indicating that luteolin may modulate the cAMP pathway to decrease aromatase transcription in KGN cells. Exemestane, an aromatase inhibitor used clinically, has been shown to promote aromatase degradation through the ubiquitin/26S proteasome pathway in aromatase-overexpressing MCF-7 cells without effect on the aromatase mRNA expression.[33] Similar to this, we found that luteolin significantly promoted aromatase degradation in aromatase-transfected HEK293A cells, in which the expression of aromatase was driven by a constitutive CMV promoter. Luteolin is not a general gene expression inhibitor and has no effect on CMV promoter-driven gene transcription,[19,34] so it may directly change the aromatase stability at the protein level. Luteolin promotes the X-linked inhibitor of apopotosis protein ubiquitination and proteasomal degradation by inhibition of protein kinase C (PKC).[18] Thus, it will be of great interest to investigate whether luteolin and exemestane promote aromatase degradation by the same mechanism and whether PKC is a novel regulator for aromatase ubiquitination and proteasomal degradation. No direct inhibitory effect of luteolin on recombinant expressed aromatase was observed, which was inconsistent with the observation that luteolin can inhibit aromatase activity by using partially purified aromatase prepared from human placenta.[35] This discrepancy is possible due to the different in vitro assay methods used. However, we found that luteolin exhibited the aromatase inhibitory effect only beginning at 12 h, indicating it may not directly inhibit the enzyme catalytic activity. Thus, whether luteolin can directly modulate the catalytic activity of aromatase needs to be further studied. Taken together with previous observations that some flavonoids such as apigenin and kaempferol can directly inhibit the catalytic activity of aromatase in placental microsomes or purified aromatase,[14] our results indicate that inhibition of aromatase by flavonoids may result from inhibition of aromatase expression, aromatase catalytic activity, and/or destabilization of aromatase protein.

Aromatase inhibitors derived from food sources or traditional medicines are preferred for more tissue specificity and less toxicity than those currently in clinical use.[14] We compared five widely consumed food-derived flavonoids—luteolin, isorhamnetin, quercetin, kaempferol, and myricetin—for their ability to inhibit aromatase activity. All except isorhamnetin could inhibit aromatase activity, albeit with differing potencies. Luteolin, one of the potent naturally occurring aromatase inhibitors, is present in many vegetables and fruits known to have multiple health benefits.[17] One of the prominent side effects of clinically used aromatase inhibitors is the increased risk for osteoporosis due to whole-body depletion of estrogen. However, luteolin has been found to have antiosteoporotic effects in vitro and in vivo through stimulation of osteoblast function and inhibition of osteoclast function.[36,37] Luteolin appears to be a safe natural flavonoid because long-term administration to rats (30 mg/kg orally for 20 days) did not reveal any obvious toxicity.[38] Thus, it will be important to examine whether luteolin can inhibit estrogen biosynthesis in vivo. A maximal plasma concentration of 3 μM luteolin was measured following oral dosing of rats with 15 mg/kg luteolin.[39] In the present study, we found that luteolin inhibited estrogen biosynthesis at a concentration as low as 1 μM. Thus, oral dosing with luteolin could achieve physiologically relevant plasma concentrations to inhibit aromatase enzyme activity and expression, making it one of the most promising flavonoids for therapeutic targeting of

aromatase. Flavonoids with potent aromatase inhibitory activities, such as chrysin, naringenin, and apigenin, failed to inhibit uterine growth via aromatase in vivo.[40] The contradiction was possibly due to the negative feedback effect of estrogen depletion by increased secretion of follicle-stimulating hormone from the hypothalamus in rats with functional ovaries.[41] Thus, the ovariectomized rats with the aromatase-overexpressing MCF-7 breast tumor should be used to evaluate the efficacy of these flavonoids, including luteolin, in the inhibition of aromatase-mediated breast tumorigenesis in vivo to obviate such negative feedback effect.

Overexposure to environmental estrogenic compounds and the Western-style diet have contributed to the increased incidence of breast cancer.[2,3] In contrast, consumption of large quantities of fruits and vegetables conveys protection against a variety of cancers, including breast cancer.[16] It has been found that red wine, grape seed, white button mushroom, and beer can inhibit aromatase activity and modulate estrogen biosynthesis.[42−46] Despite this, the role of fruits and vegetables in endogenous estrogen biosynthesis is still largely unknown. In this study, we compared two luteolin-containing vegetables—onion and bird chili—for their effects on estrogen biosynthesis. Interestingly, onion extracts significantly inhibited estrogen biosynthesis in both KGN cells and aromatase-expressing HEK293A cells, whereas bird chili extracts inhibited estrogen biosynthesis only in the HEK293A cells. The difference is possibly due to the cell permeability of the compounds, their composition, or concentration range of flavonoids contained in the food source. Different combinations of flavonoids may also change the solubility and/or accessibility of the compounds to intracellular aromatase or may act on aromatase through different mechanisms. As an example, some dietary flavonoids such as hesperetin can increase aromatase expression.[21] Thus, the composition and concentrations of flavonoids and the cell type being tested should be considered when fruits and vegetables are compared for their effects on estrogen biosynthesis.

In conclusion, we identified luteolin, distributed widely in consumed fruits and vegetables, as a potent inhibitor of estrogen biosynthesis in human ovarian granulosa-like KGN cells by decreasing aromatase expression and promoting its degradation. We also showed that other daily consumed flavonoids, including isorhamnetin, quercetin, kaempferol, and myricetin, inhibited estrogen biosynthesis with differing potencies. Two widely consumed vegetables, onion and bird chili, also differed in their ability to inhibit estrogen biosynthesis in KGN cells and in aromatase-expressing HEK293A cells. The present study suggests that luteolin may exert its estrogen biosynthesis inhibitory effect through suppression of aromatase expression and promotion of aromatase degradation, and it warrants further investigation as a potential agent for the treatment of breast cancer. Consumption of fruits and vegetables rich in flavonoids will be helpful for reducing endogenous estrogen levels in the prevention of estrogen-dependent disease, such as breast cancer.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Cytotoxic effects of luteolin, kaempferol, quercetin, myricetin, isorhamnetin, and plant extracts on KGN or HEK293A cells (Figures S1−S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

ER, estrogen receptor; CREB, cAMP-response element binding protein; HEK293A, human embryonic kidney 293A; ELISA, enzyme-linked immunosorbent assay; BCA, bicinchoninic acid; PKA, protein kinase A; PKC, protein kinase C.

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