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ScienceDirect
Food Chemistry 108 (2008) 719–726
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Determination of the differential estrogenicity of isoflavonoids by E_2–ER–ERE-dependent gene expression in recombinant yeast and MCF-7 human breast cancer cells

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Received 17 July 2007; received in revised form 5 November 2007; accepted 8 November 2007

Abstract

The use of phytoestrogens-containing natural sources as alternative hormone replacement therapy (HRT) has been a subject of debate for decades. Development of assays to characterize these phytoestrogens is required. In this study, differential estrogenicities of five isoflavonoids found in red clover and soy, including biochanin A, daidzein, formononetin, genistein and glycitein were examined in a yeast-based screen system with a classical palindromic estrogen response element (ERE)-*ADE2* reporter and in a MCF-7 cell culture system with mRNA levels of ER-dependent genes compared. In a yeast-based assay, five isoflavonoids showed various extents of estrogenic potencies. A collection of primary estrogen receptor (ER)-regulated genes by estradiol (E_2), including *hTERT*, *c-MYC*, *BCL2* and *Ha-ras* (oncogenic) and quinone reductase (*QR*), human complement 3 (*C3*) and *COX7RP* (non-oncogenic) were selected as marker genes for a MCF-7 cell-based endogenous gene expression assay. The results indicated that the mRNA levels of these E_2–ER–ERE-dependent marker genes were regulated differentially by five isoflavonoids, leading to distinct expression patterns, which are also significantly different from that of E_2. Moreover, the anti-estrogenic effects of biochanin A and formononetin on E_2-induced transcriptions of marker genes in MCF-7 cells were also displayed. Taken together, these results are significant for these naturally occurring isoflavonoids regarding the issues of safety and efficacy.

Keywords: Estrogen receptor; Estrogen response element; Estrogenicity; Isoflavaonoid; Oncogene

1. Introduction

The epidemiological data and experimental animal studies both suggested that diets rich in phytoestrogens may prevent the estrogen-related cardiovascular, menopausal symptoms, osteoporosis and reduce the risk of breast cancer (Adlercreutz, 1995; Albertazzi & Purdie, 2002; Cornwell, Cohick, & Raskin, 2004; Magee & Rowland, 2004). On the contrary, involuntary exposure to hormonally-active phytoestrogens might also cause adverse effects on endocrine system (Setchell, Zimmer-Nechemias, Cai, & Heubi, 1997; Sheehan, 1998). In animal models, though the majority of studies have shown that phytoestrogens can protect against chemically induced mammary cancer, the estrogenic or anti-estrogenic roles of phytoestrogens are still conflicting depending on the experimental conditions employed (Barnes, 1997; Hsieh, Santell, Haslam, & Helferich, 1998; Lamartiniere et al., 2002). In clinical studies, a statistical analysis revealed the ratio of the positive results/total clinical studies was 64/105 in all aspects (Cornwell et al., 2004). Several in vitro studies have indicated

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doi:10.1016/j.foodchem.2007.11.020

that phytoestrogens, especially isoflavonoids, exhibit biphasic effects on the proliferation of ER-positive cell lines, by stimulating the growth at low concentration and inhibiting cell proliferation at higher concentrations (Hsieh et al., 1998; Zava & Duwe, 1997). Nevertheless, whether post-menopausal women should take alternative HRT is still debated regarding mainly the above mentioned issues of safety and efficacy. It is therefore important to investigate the impact of different phytoestrogens on estradiol (E_2)-associated oncogenic and non-oncogenic pathways.

Based on the chemical structures, phytoestrogens are divided into four main groups, isoflavonoids, flavonoids, coumestans and mammalian lignans with isoflavonoids being the most studied ones (Albertazzi & Purdie, 2002; Cornwell et al., 2004; Magee & Rowland, 2004). In natural sources, red clover contains genistein, daidzein, biochanin A and formononetin, whereas soy only contains two of those compounds (genistein and daidzein) and an additional glycitein (Beck, Rohr, & Jungbauer, 2005). In a mechanistic study, the estrogenic and/or anti-estrogenic effects of isoflavonoids were usually explained by their unique structural characteristics resembling that of E_2, which is mediated by binding to a ligand-dependent estrogen receptor (ER), thus inducing its conformational change and subsequently interacting with estrogen response elements (EREs) together with multiprotein complexes of transcription factors and co-activators to activate transcription (Klinge, 2001). The consensus ERE sequence consists of a 13-bp palindrome that is presented as vit-ERE (GGTCAcagTGACC). In mammalian cells, most of the identified EREs show one or more different nucleotides compared to the consensus palindrome, and several genes contain multiple half-sites (Klinge, 2001).

Several test systems have been developed to evaluate the estrogenic activities of herbal extracts and phytoestrogens used in menopausal symptoms (Dornstauder et al., 2001; Kostelac, Rechkemmer, & Briviba, 2003; Matsumura, Ghosh, Pope, & Darbre, 2005; Overk et al., 2005; Zava & Duwe, 1997). These systems were often discussed and compared with their advantages and limitations (Diel, Schmidt, & Vollmer, 2002; Mueller, 2002). The competitive estrogen receptor binding assay, rodent uterotrophic assay and the E-screen proliferation assay are the most commonly used procedures used to measure estrogenicity. However, the mechanistic information of regulation cannot be distinguished by these assays. The gel-shift assay for testing of the ER–ERE binding and the transactivation assays in yeast or mammalian cells, on the other hand, are either in vitro or artificial systems that would have difficulty to predict the in situ effect of the test compounds in the native cellular environment. Although the microarray technique has also been employed for the screening of the estrogen responsive genes in response to phytoestrogens, the cost, accuracy and required reconfirmation preclude its wide application (Ise et al., 2005).

In this paper, a rapid and less-expensive yeast-based assay with a classical palindromic ERE-*ADE2* reporter system and a MCF-7 cell-based endogenous gene expression assay to dissect the estrogenic activity of phytochemicals was employed. The differences of these isoflavonoids and E_2 on influencing the expression levels of primary ER-regulated genes were further determined and compared, especially their effects on the estrogen-dependent expression of oncogense and non-oncogenes in MCF-7 cancerous cells.

2. Materials and methods

2.1. Chemicals and reagents

Yeast nitrogen base was purchased from DIFCO (Sparks, MD). Test compounds (17β-estradiol and isoflavonoids) and all other chemicals for yeast and mammalian culture medium preparation were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Plasmids, yeast strain and growth

The yeast reporter assay was conducted as previously described (Balmelli-Gallacchi et al., 1999). Briefly, the multicopy plasmid Yep90 is a 2μ-derived plasmid carrying a *HIS3* selectable marker and a yeast *PGK1* promoter. Plasmid Yep90HEGO contains the complete coding sequence of *hER* gene cloned into the unique *Eco*RI site of Yep90. The *pU/ERE-ADE2* was originally linearized with *Bst*BI within the *URA3* gene and integrated into the *ura3* locus of YPH250 to yield strain DP160. Strain CLY3 was derived from DP160 by transformation of the plasmid YepHEGO. General yeast operation was performed as described by Guthrie and Fink (1991). CLY3 was routinely cultured on synthetic complete medium supplemented with adenine (25 g/ml) to avoid selection of suppressors of the endogenous mutant ade2 locus. The media for red/white screening consists of synthetic complete medium without uracil and histidine, plus limiting amounts of adenine (5 μg/ml) (Guthrie & Fink, 1991).

2.3. MCF-7 cell culture

MCF-7 were cultured in 10-cm cell-culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, MEM nonessential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin in the presence of 5% of CO_2 at 37 °C. Prior to conducting E_2 or test compound treatments, the cells were washed twice with phosphate-buffered saline (PBS). The cells were then cultured in phenol red-free medium containing 10% dextran-coated charcoal-treated fetal calf serum (DCC medium) for 48 h. For real-time RT-PCR analysis, each experiment was performed in triplicate. Cells were seeded in microtiter plates and treated with 50 nM E_2 or various concentrations of test compounds for various periods of time.

2.4. Real-time quantitative reverse transcriptase (RT)-PCR analysis

Cells were harvested by digestion with trypsin and collected after centrifugation. Total cellular RNAs were extracted using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) as described by the manufacturer's protocol and cDNA was synthesized according to the standard procedure. The expression levels of *hTERT*, *c-MYC*, *BCL2*, *Ha-ras*, *C3*, *QR*, *COX7RP* and *GAPDH* genes were determined quantitatively by real-time qRT-PCR using the ABI PRISM 7700 Sequence Detector and the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). *GAPDH* was used as a reference gene for an internal control and the untreated control sample was used as a calibrator. In the quantification method, the fold change is indicated as the target/reference of each sample divided by the target/reference ratio of the calibrator. The RT-PCR was performed three times for each gene. All primer sequences for PCR are available upon request.

2.5. Statistical methods

All values are expressed as means ± standard error of the mean. Differences between groups were tested using Student's *t*-test and Fisher Exact test. *P* values <0.05 were considered significant.

3. Results

3.1. Screening of a series of phytochemicals for estrogenicity by a recombinant yeast system with a classical consensus ERE-ADE2 reporter

A recombinant yeast-based assay, which was constructed with a classical consensus palindromic ERE incorporated and an *ADE2* reporter system, to test the estrogenicity of a collection of phytochemicals were employed (see Fig. 1 for structural representation of five isoflavonoids). Activation of the reporter gene depends on the amounts of estrogenic components in the medium. The *ADE2* gene encodes an enzyme of the adenine biosynthesis pathway. When the reporter gene is off, colonies turn red (the colour of metabolic intermediate from adenine metabolism). Estrogenic compounds can activate the expression of the reporter gene through the binding of estrogen receptor to ERE, resulting in white colonies. The quantitative of estrogenic activity of test sample was converted to the relative potency compared to E_2. In the study, two dozens of phytochemicals in different structural categories, including flavonoid, isoflavonoid, terpenoid and their glycoside, coumarin, alkaloid and others, have been tested by our yeast-based assay. Surprisingly, five of the eight isoflavonoids in our phytochemical pool were positive (Fig. 2). Among eight isoflavonoids tested, prominent

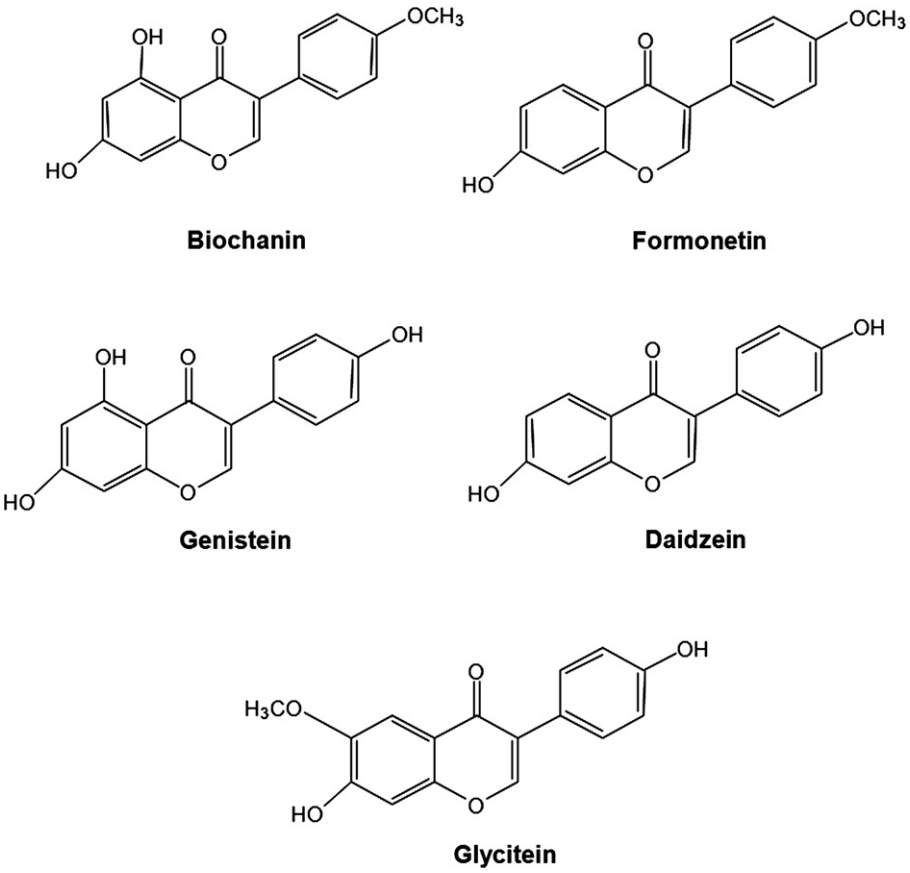

Fig. 1. Chemical structures of the isoflavonoids used in this study.

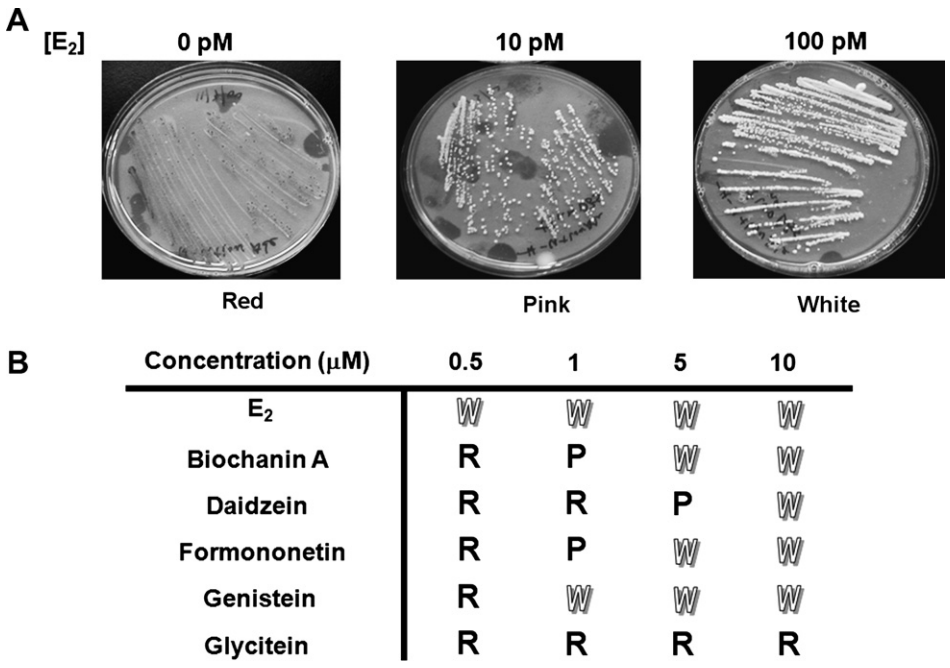

Fig. 2. Comparison of the estrogenicity of isoflavonoids in the recombinant yeast-based assay. Yeast strain CLY3 was grown on synthetic complete medium without uracil and histidine, plus limiting amounts of adenine. E_2 (A) or various concentrations of isoflavonoids (A and B) were added to the media and each culture was grown for 3 days. R – red colonies (negative); P – pink colonies (equivalent to 10 pM of estradiol); W – white colonies (equivalent to 100 pM of E_2). Each experiment was performed in triplicate.

estrogenic activity was observed for five aglucons with their potencies following the hierarchy – genistein (1 μM) > biochanin A, formononetin (5 μM) > daidzein (10 μM) > glycitein (>10 μM), as indicated by the concentrations required to active the *ADE2* reporter equivalent to 100 pM of E_2. These data demonstrate that our yeast reporter system is sensitive enough to evaluate the estrogenicity of structurally diversified phytochemicals.

3.2. Time-course of E_2 on the mRNA expression levels of selected E_2–ER–ERE-dependent genes in MCF-7 cells

Based on literature search, a collection of ER-dependent genes regulated by E_2 were selected as marker genes to evaluate the estrogenicity of these isoflavonoids in MCF-7 cells (Table 1). Previous studies demonstrated that E_2 activates these genes and EREs were identified in the flanking or coding sequences of these genes (Klinge, 2001). Among these, *hTERT*, *c-MYC*, *BCL2* and *Ha-ras* are recognized as oncogenes, whose gene expressions represent the increment of potential risk for breast cancer. The gene products of non-oncogenic *QR*, *C3* and *COX7RP*, on the other hand, represent the beneficial factors in the maintenance of physiological regulation. In order to optimize the mRNA expression levels of E_2-induced genes, a time-course study was performed on these seven genes by real-time RT-PCR. The results showed that exposure of MCF-7 cells to 50 nM of E_2 for various periods of time, five of seven genes, *c-MYC*, *BCL2*, *C3*, *QR* and *COX7RP*, reached their maximum induction after 2 h of treatments

Table 1
Sequences of the EREs from selected E_2-responsive marker genes

	Accession number	Sequence
Oncogene		
TERT	AF015950	5′-GGTCAGGCTGATC-3′
C-MYC	NM_002467	5′-GGAAGCAGACCACGTGGTCTGCTTCC-3′
BCL-2	NM_000633	ERE-E-3 + 195: 5′-GGTCGCCAGGACC-3′ERE-E-4 + 276: 5′-GGTCCCCATGACC-3′
RAS	NM_176795	+1713: 5′-GCGCTGACCATCCAGCTGATCCAGAACC-3′
Non-oncogene		
C3	NM_000064	−236: 5′-GTGTTCACCAGGTGGCCCTGACCCTGGGAGAGTCCA-3′ +25: 5′-TGTCCCTCTGTCCCTCTGACCCTGCACTGTCCCAGCAACCATG-3′
QR	NM_000903	−476: 5′-AATTAAATCGCAGTCACAGTGACTCAGCAGAATCTGAGCCTAGG-3′
COX7RP	AB007618	+433: 5′-TCACTGCAGGGGTCAAGGTGACCCCCGGGGTCA-3′
Consensus palindromic sequence		
Xenopus vitellogenin A2 (vit-ERE)		5′-GTCAGGTCACAGTGACCTGA-3′

(Fig. 3). However, for *hTERT* and *Ha-ras*, the maximum peak was achieved after 8 h of induction.

3.3. Dose–response study of the five isoflavonoids on the regulation of the gene expression profile

Next, the dose–response studies of the five isoflavonoids on the expression profile of these primary ER-regulated genes were conducted individually (Fig. 4). Interestingly, five isoflavonoids induced distinct expression patterns in these seven marker genes examined, which were also different from that of E_2. In the case of oncogenes, E_2 up-regulated the mRNA expression levels of *hTERT*, *c-MYC*, *Ha-ras* and *BCL2* by folds of 4.6, 4.1, 2.5, and 5.1, respectively (Fig. 4). None of these isoflavonoids enhanced the transcriptions of *hTERT* and *Ha-ras* in all tested doses. Instead, all five isoflavonoids showed significant down-regulation of *hTERT* transcripts ($P < 0.05$) at certain concentrations. For example, 1 μM of formononetin and glycitein reduced 50% of the *hTERT* expression (Fig. 4).

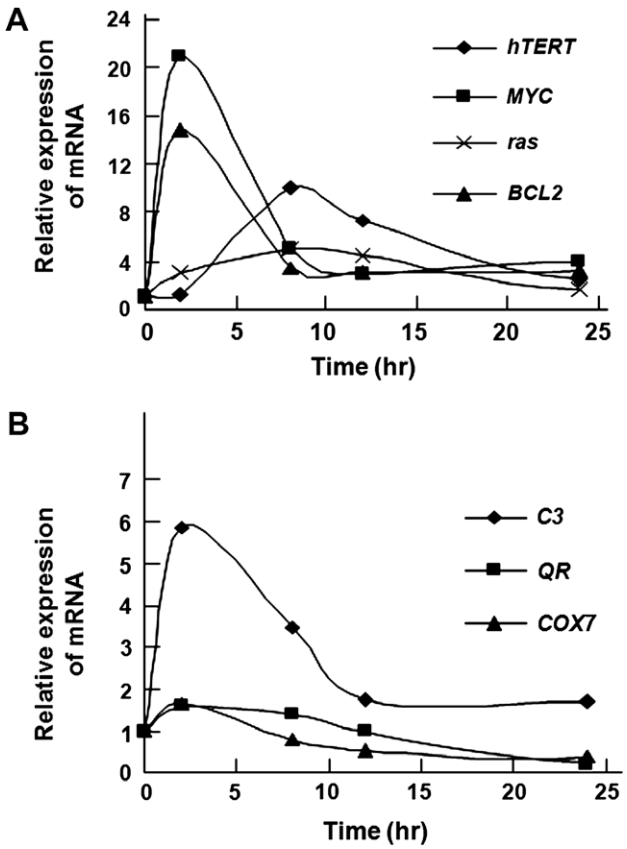

Fig. 3. Time-course study of E_2 on the expression levels of seven selected E_2–ER–ERE-dependent genes in MCF-7 cells. MCF-7 cells were treated with 100 nM of E_2 for 2, 8, 12, or 24 h. RNA was extracted and cDNA was then synthesized. Relative expression folds of (A) *hTERT*, *c-MYC*, *Ha-ras* and *BCL2*; (B) *C3*, *QR* and *COX7RP* were measured by quantitative RT real-time PCR analysis. Graphs represent expression levels upon normalization to *GAPDH*. The relative expression of mRNA was indicated as ratio for the fold change at each time indicated divided by the fold change at time 0.

Both formononetin at 1 μM and daidzein at all doses attenuated *c-MYC* transcriptions, while genistein exhibited significant enhancement at dosage over 1 μM. Formononetin, biochanin A and glycitein down-regulated the *Ha-ras* mRNA expression levels significantly. It is notable that the structural features of these three compounds are distinguishable from genistein and daidzein by bearing a methoxy group on the benzene ring of the isoflavonoid (Fig. 1). In *BCL2*, it is noteworthy that four of the five isoflavonoids up-regulated its mRNA expression level, except for genistein, where no significant regulation was observed. However, as mentioned previously, in contrast to the other four isoflavonoids, genistein specifically up-regulated the *c-MYC* mRNA, which makes it differentiated from the others.

In the expression of non-oncogenes, it is interesting to observe that all tested compounds significantly up-regulated the mRNA expression levels of C3 by two to three folds at all doses (Fig. 4). Similar to the minimal effect of E_2 on the gene expression of *QR*, the five isoflavonoids only slightly down-regulated its mRNA expression at certain concentrations. In *COX7RP*, E_2 up-regulated its expression by 1.6-fold, while the five isoflavonoids did not affect the mRNA expression levels of *COX7RP*. All together, the expressions of these E_2–ER–ERE-dependent marker genes were regulated differentially by five isoflavonoids determined. These results also suggested differential oncogenic and non-oncogenic expression patterns of structurally distinct isoflavonoids, which are also significantly different from that of E_2. Most significantly, the response of the treatment of these isoflavonoids on most tested genes, except for *BCL2* and *C3*, were very mild.

3.4. Formononetin and biochanin A inhibited the E_2-induced ER-dependent response

In order to further investigate the effects of the isoflavonoids on the regulation of expression profiles of E_2-inducible genes, two most abundant isoflavonoids found in red clover, biochanin A and formononetin, were selected for further investigations. In a dose–response study, after co-treatment of 50 nM of E_2 with biochanin A or formononetin, both isoflavnoids significantly inhibited the E_2-induced expression of four oncogenes at all the test doses (1, 10 and 50 μM) ($P < 0.05$) (Fig. 5). Among non-oncogenes, formononetin also attenuated the expression of *C3* and *COX7 RP* transcriptions that were induced by E_2, while biochanin A only reduced the *C3* mRNA expression level with statistical significance at 50 μM ($P < 0.05$). These studies demonstrate that these isoflavonoids inhibit the E_2-induced expression of these ER-dependent genes.

4. Discussion

In this study, we first used an established yeast-based assay for high-throughput screening of estrogenic compounds. The E_2-dependent transcriptional activation in

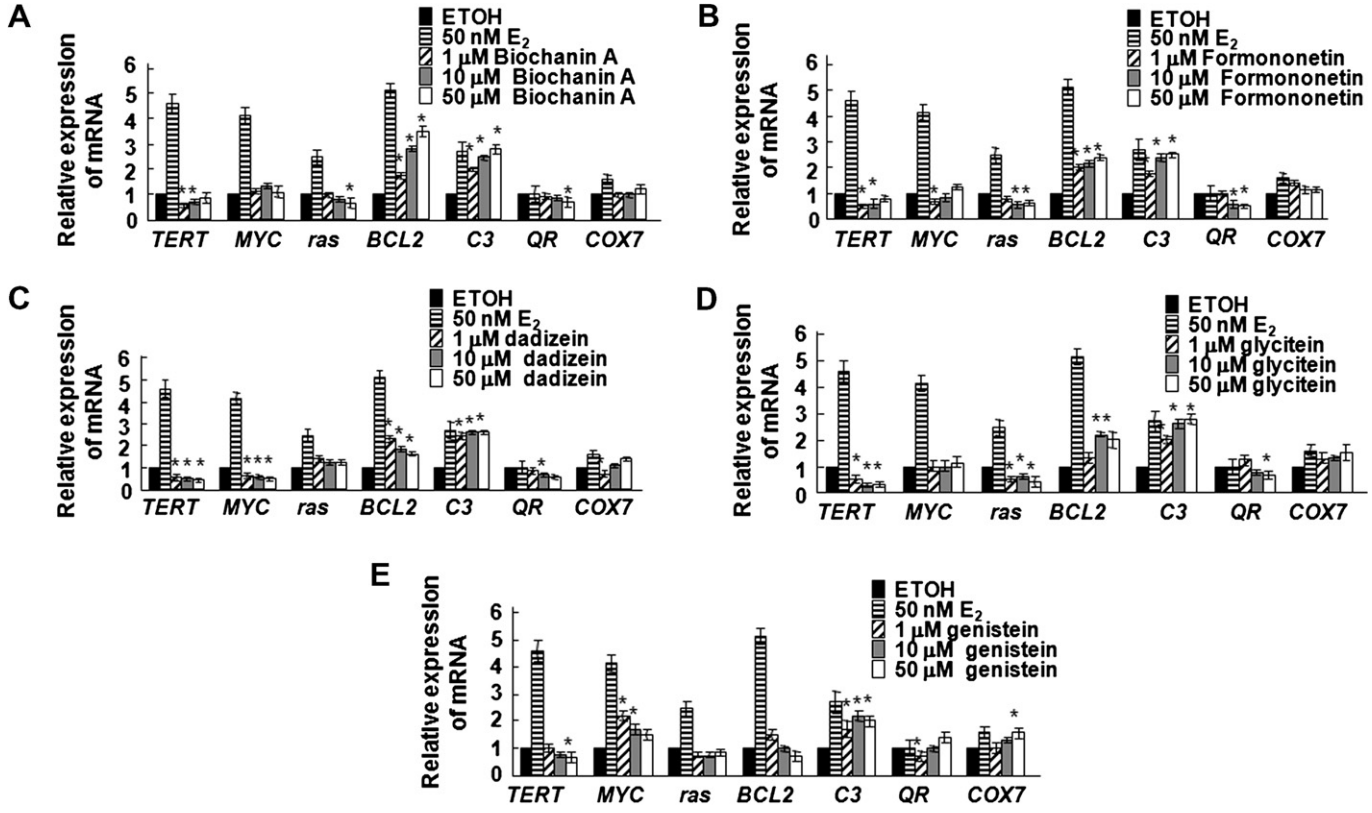

Fig. 4. Dose–response effect of isoflavonoids on the expression levels of primary ER-regulated gene profile in MCF-7 cells. MCF-7 cells were treated with ethanol (EtOH, solvent), 50 nM of E_2 or various concentrations (as inidcated in the figures) of biochanin A (A), formononetin (B), daidzein (C), glycitein (D) and genistein (E) for 8 h to detect the mRNA expression levels of *hTERT* and *Ha-ras* or 2 h to detect others. Relative expression levels of these seven transcripts were measured by qRT-PCR analysis. The fold change of each gene is indicated as the target/reference of each sample divided by the target/reference ratio of the untreated control. Data are presented as the mean ± S.D. from triplicate determinations. Significant differences ($P < 0.05$) from EtOH-treated groups are indicated by asterisks.

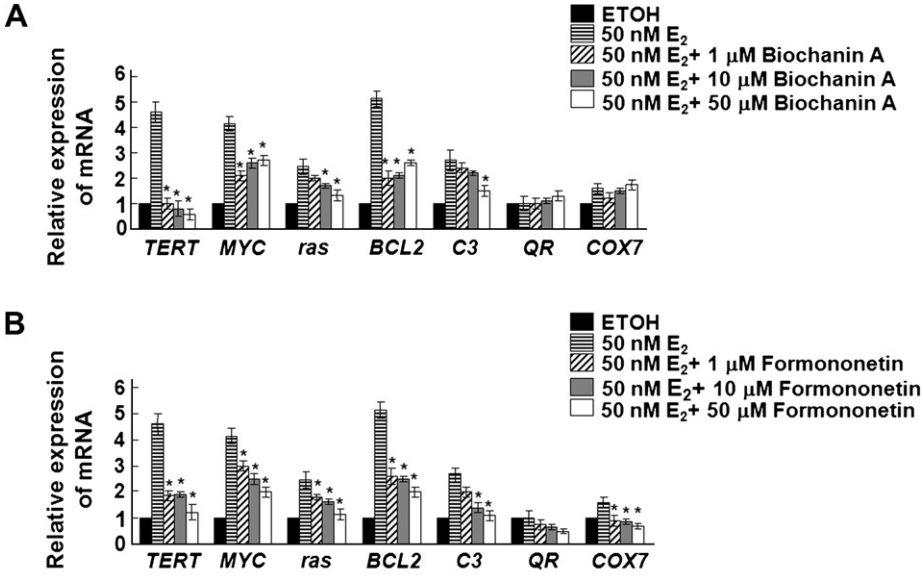

Fig. 5. Dose–response effect of formononetin and biochanin A on the E_2-induced expression of the primary ER-regulated genes. MCF-7 cells were treated with EtOH, 50 nM E_2, or 50 nM E_2 plus various concentrations of biochanin A (A) or formononetin (B) for 8 h to detect the mRNA expression levels of *hTERT* and *Ha-ras* or 2 h to detect others. Relative expression levels of transcripts were measured by qRT-PCR analysis. Data are presented as in Fig. 4 Significant differences ($P < 0.05$) from E_2-treated groups are indicated by asterisks.

mammalian cells has previously been proven to operate in yeast (Metzger, White, & Chambon, 1988). From economical consideration, yeast growth is cost-effective and easy to achieve efficient screening work. Our results in the order of potency among the five isoflavonoids are consistent with the previous report using yeast with a β-galactosidase reporter (Beck et al., 2005). In previous comparative studies of estrogenic properties of phytoestrogens using the competitive binding assay, transfected ERE-CAT reporter assay and cell proliferation within the same receptor and cellular context of MCF-7 have been reported (Matsumura et al., 2005). The results showed that the rank orders of potency were similar in all three assays, where E_2 exhibited the strongest, genistein was 1000-fold less, and daidzein was 10^4-fold less. These data are also consistent with those of ours from the yeast-based assay (Fig. 2). Furthermore, in the MCF-7 cell-based endogenous gene expression assay, we developed a sensitive way to differentiate the estrogenicity of a variety of phytoestrogens with gene-profiling fingerprints. Our results also presented evidence that these phytoestrogens, especially isoflavonoids, affect mRNA expression levels of E_2-induced ER-dependent genes. Seven selected ER-dependent marker genes were regulated differentially by isoflavonoids when compared to that of E_2. Most of the identified EREs show one or more different nucleotides compared to the consensus palindrome (Table 1). These variations might therefore contribute to the discrepancy in the E_2-ERE regulated gene expression.

The relationship of estrogenic activities and the structural features of flavonoids has been studied by several groups (Miksicek, 1995; Vaya & Tamir, 2004). It was concluded that hydroxyl groups of 4′ and 7′ on the two benzene rings of flavonoid are mainly responsible for the E_2-like activity. However, the interplay between the diversified ERE sequences and the ability of liganded ER to bind to each specific promoter containing different ERE has received much less attention. A study using in vivo chromatin immunoprecipitation assay to analyze the interaction of the ligand-bound ER with an imperfect ERE indicated that ER ligand and ERE sequence are two important determinants for the regulation of the binding of ER to estrogen responsive promoters in vivo (Krieg, Krieg, Ahn, & Shapiro, 2004). Here, we applied an assay to predict the in situ effect of the test compounds in native cellular environment, which may provide a better assessment of profile of individual gene expression.

The quantitative analyses of estrogenicity in vivo and in vitro by determining the expression levels of endogenous estrogen-regulated genes have been reported. These results indicated that E_2 and genistein alter the expression levels of the same 179 genes in immature mouse model (Moggs et al., 2004). In another study performed in MCF-7 cells, genistein exhibited a weaker effect on the regulation of four estrogen-regulated marker genes by over 10^3-fold lower than that of E_2 (Jorgensen, Vendelbo, Skakkebaek, & Leffers, 2000). Our study first demonstrated the differentiated expressions among the treatments of five isoflavonoids and indicated that the expression patterns of the selected ER-dependent markers from the naturally occurring isoflavonoids were distinct from that of E_2. In addition, in comparison of the effect of five isoflavonoids on the mRNA expression levels of oncogenes, genistein up-regulated *c-MYC*, while others showed up-regulation of *BCL2*, which might be explained by the structural uniqueness of genistein, which possesses three hydroxyl group at 5, 7, and 4′ in the skeleton of isoflavonoid.

Two in vivo studies using primate model and xenogragted mice showed the anti-estrogenic effect of isoflavonoid-containing products when co-treatment with E_2 (Gallo, Ferlini, Fabrizi, Prislei, & Scambia, 2006; Wood, Register, Franke, Anthony, & Cline, 2006). Our results demonstrated the anti-estrogenic effects of isoflavonids on E_2-induced expression of the proliferation-related genes in MCF-7 cells, which might provide a mechanistic evidence for these two in vivo data. Moreover, it is notable that the abrupt decrease of *hTERT* transcripts as observed by isoflavonoid treatments indicated potential mechanisms other than the E_2–ER–ERE-dependent pathway was involved in the regulation of *hTERT* expression.

In summary, these results demonstrated (1) the distinct effects of the naturally occurring isoflavonoids from E_2 in vivo on the expression levels of primary ER-regulated oncogenes vs. non-oncogenes; (2) the anti-estrogenic effects of isoflavonids on E_2-induced expressions of seven marker genes in MCF-7 cells. Taken together, these studies support a positive angle of using isoflavonoid-containing natural products as chemopreventing agents. It provides the beneficial as well as hazardous effects of these isoflavonoids through understanding their biological effects at the molecular level, especially their effects on the estrogen-dependent expression of oncogense vs. non-oncogenes in MCF-7 cancerous cells, which would represent, in part, the risk factor and efficacy of E_2 replacement therapy.

Acknowledgements

We thank Drs. P. Chambon and D. Picard for providing plasmids and strains and Drs. J.-H. Guh and T.-K. Li for their critical comments on the manuscript. This work was supported by a National Science Council Grant (95-2313-B-157-002) to C.-C. Lin and grants from National Research Program for Genomic Medicine (NRPGM-95-3112-B-002-023) and National Health Research Institute (NHRI-EX95-9328SI) to S.-C. Teng.

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