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Phytoestrogens Have Agonistic and Combinatorial Effects on Estrogen-Responsive Gene Expression in MCF-7 Human Breast Cancer Cells

Scott T. Willard and L. Stephen Frawley

Laboratory for Molecular Dynamics, Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC

Phytoestrogens can exhibit agonistic actions on estrogen-dependent gene expression in breast cancer cells. Since several different phytoestrogens may be found within a single dietary plant source, we sought to investigate whether estrogen-dependent gene expression may be further influenced by the collective treatment of breast cancer cells with multiple phytoestrogens. Accordingly, we transfected MCF-7 breast cancer cells with estrogen-responsive reporters followed by treatment with one of four phytoestrogens (genistein, daidzein, formononetin, and equol) or a combination of these in the absence of estradiol. Our results demonstrated clear-cut agonistic effects of phytoestrogens on estrogen-dependent gene expression. Moreover, combinatorial treatment consistently stimulated reporter activity above that observed for individual phytoestrogens. Inasmuch as the phytoestrogens tested are frequently found together in food sources, these combinatorial responses may more accurately reflect the consequences of in vivo exposure.

Key Words: Phytoestrogens; breast cancer; estrogen; gene expression.

Introduction

Phytoestrogens, naturally occurring estrogenic compounds found in a variety of edible plants, exhibit both agonistic and antagonistic effects on estrogen-sensitive pathways in mammalian systems *(1–5)*. Additionally, these naturally occurring agents appear to display anticarcinogenic effects on breast cancer cells *(3,6,7)* and, thus, have been suggested to account, at least in part, for epidemiological evidence which suggests that phytoestrogens have cancer-protective properties *(8–11)*. Although phytoestrogen-induced anticarcinogenesis has been reported both in vivo and in vitro to varying degrees, a consensus on whether the ingestion of phytoestrogens in the diet is in fact physiologically relevant, beneficial, harmful, or a mixture of these has yet to be reached. Moreover, the dual actions of phytoestrogens as agonists and antagonists have resulted in a general confusion in the literature regarding the role these compounds may play in cancer progression and/or protection.

Clearly, phytoestrogens can act through the estrogen receptor *(3,12,13)* and subsequently affect the expression of estrogen-responsive breast cancer genes *(14–16)*. However, many studies have focused on the actions of only a single phytoestrogen at one time and have largely ignored the effect that these compounds may have in combination with one another on specific breast cancer genes. In order to establish firmly the physiological relevance of phytoestrogens, it is necessary to examine these compounds in relevant combinations associated with their origin in the environment, metabolism, and presence in vivo. It has been suggested that when environmental estrogens in general are found in combination with one another, they may either:

1. "Cancel each other out," resulting in no physiological effect *(3)*.
2. Have a combinatorial estrogenic effect.
3. Have a combinatorial antiestrogenic effect *(13)* on estrogen-sensitive pathways.

Definitive answers regarding the possible outcomes of combinatorial phytoestrogenic treatment on breast cancer cells are critical to our understanding of the positive or negative actions these compounds may have on the incidence and progression of breast cancer.

In this article, we show that four common phytoestrogens have both agonistic and combinatorial actions on estrogen-regulated gene expression in breast cancer cells. The implications of these results relative to the observed estrogenic actions are discussed.

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Author to whom all correspondence and reprint requests should be addressed: Dr. L. Stephen Frawley, Laboratory for Molecular Dynamics, Department of Cell Biology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. E-mail: frawleys@musc.edu

Results

Phytoestrogens Alone and in Combination Stimulate Estrogen-Responsive Element- (ERE) Driven Luciferase Activity

To investigate the actions of phytoestrogens on estrogen-regulated gene expression, we began by using MCF-7 cells chemically transfected with a "generic" estrogen-responsive reporter construct (MMTV-ERE-LUC), which harbors five EREs. These cells were then exposed to treatment with varying concentrations of one of four phytoestrogens. All phytoestrogens tested along with estrogen (E_2, the positive control) exhibited varying degrees of dose-dependent effects on ERE-driven luciferase activity (as is evident from top to bottom in Fig. 1). Specifically, daidzein and equol increased ($p < 0.05$) ERE-driven luciferase activity above control values at all concentrations tested, whereas genistein and formononetin were effective at all but the lowest doses tested. These response data demonstrate clearly that phytoestrogens can exert agonistic effects on ERE-dependent gene expression, and in some cases (daidzein and equol), they can do so within a physiologically relevant range. It is interesting to note that the magnitude of responses for individual phytoestrogens varied as much as seven- to eightfold across the range of doses tested. By contrast, the dose-related increment evoked by E_2 was only twofold owing to the fact that even the lower doses employed approached the saturable range. Also shown in Fig. 1 are the combinatorial effects of phytoestrogens on MMTV-ERE-LUC expression. Here, the combination of four individual phytoestrogens at equimolar doses significantly ($p < 0.05$) increased ERE-driven luciferase activity above that for controls or for any of the individual phytoestrogen treatments at all doses tested. Taken together, these results demonstrate that the four phytoestrogens studied have combinatorial as well as individual agonistic effects on ERE-mediated gene expression in breast cancer cells.

Phytoestrogens Alone and in Combination Stimulate pS2-Driven Luciferase Activity

Having established phytoestrogen action on a "generic" ERE-driven reporter construct, we then asked whether similar responses might be evoked from a more physiologically relevant breast cancer gene. To this end, we employed the same experimental paradigm to study the effects of phytoestrogens on the expression of a luciferase reporter under the control of regulatory sequences from the estrogen-responsive pS2 gene, characteristic of the MCF-7 cell line and some primary breast cancers. Consistent with the results obtained for the MMTV-ERE-LUC studies, we found that E_2 and three of the four phytoestrogens (excluding daidzein) exhibited varying degrees of dose-dependent effects on pS2-driven luciferase activity (as is evident from top to bottom in Fig. 2). However, significant increases occurred only at higher concentrations (1 μ*M* and 100 n*M*); genistein was remarkable in that it increased ($p < 0.05$) pS2-driven

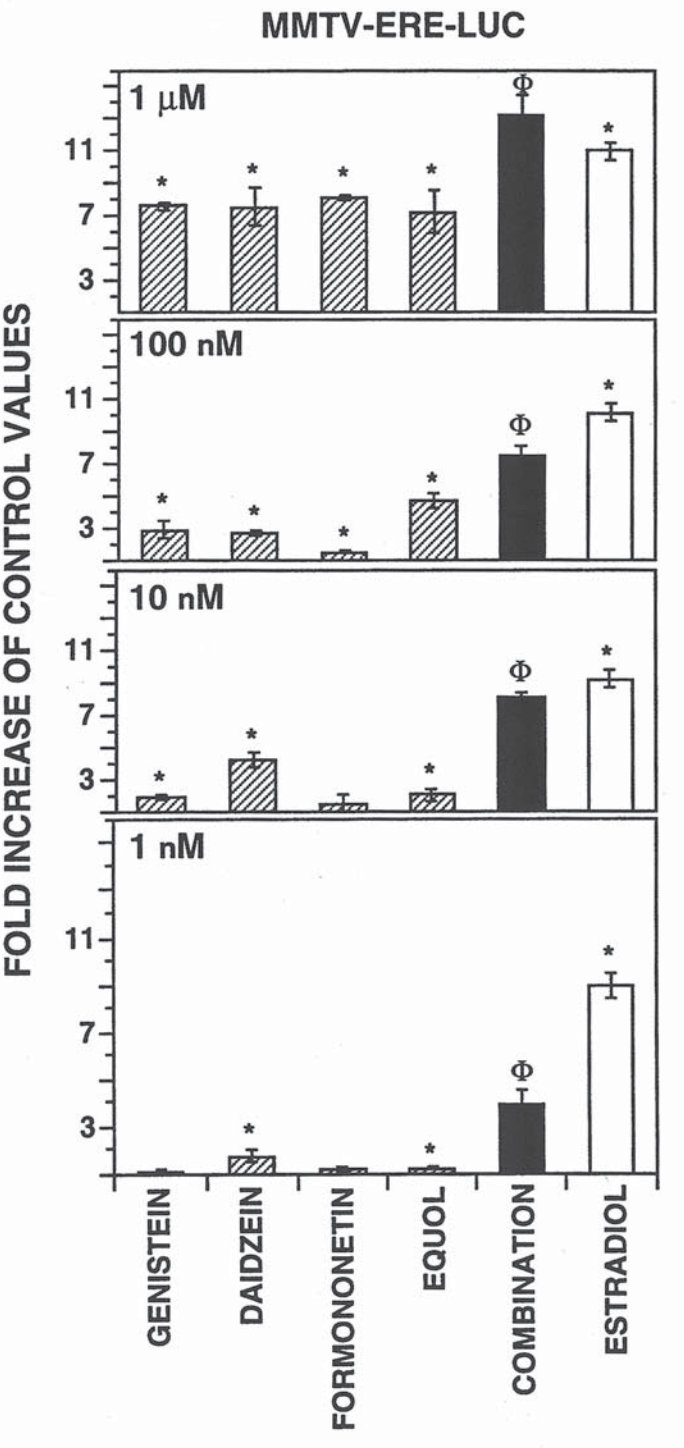

Fig. 1. Effects of phytoestrogens on MMTV-ERE-LUC expression. MCF-7 cells were transfected with a luciferase reporter plasmid driven by multiple copies of the vitellogenin ERE (MMTV-ERE-LUC). Cells were then treated for 42 h with one of four phytoestrogens, a combination of the four phytoestrogens at equimolar doses (i.e., 4× final dose), or estradiol. Results are expressed as fold increases of control values. *Treatments are different ($p < 0.05$) from control values; ΦCombinatorial treatment is different ($p < 0.05$) from individual phytoestrogen treatments.

luciferase activity above control values at all concentrations tested. The combination of the four individual phytoestrogens at equimolar doses significantly ($p < 0.05$)

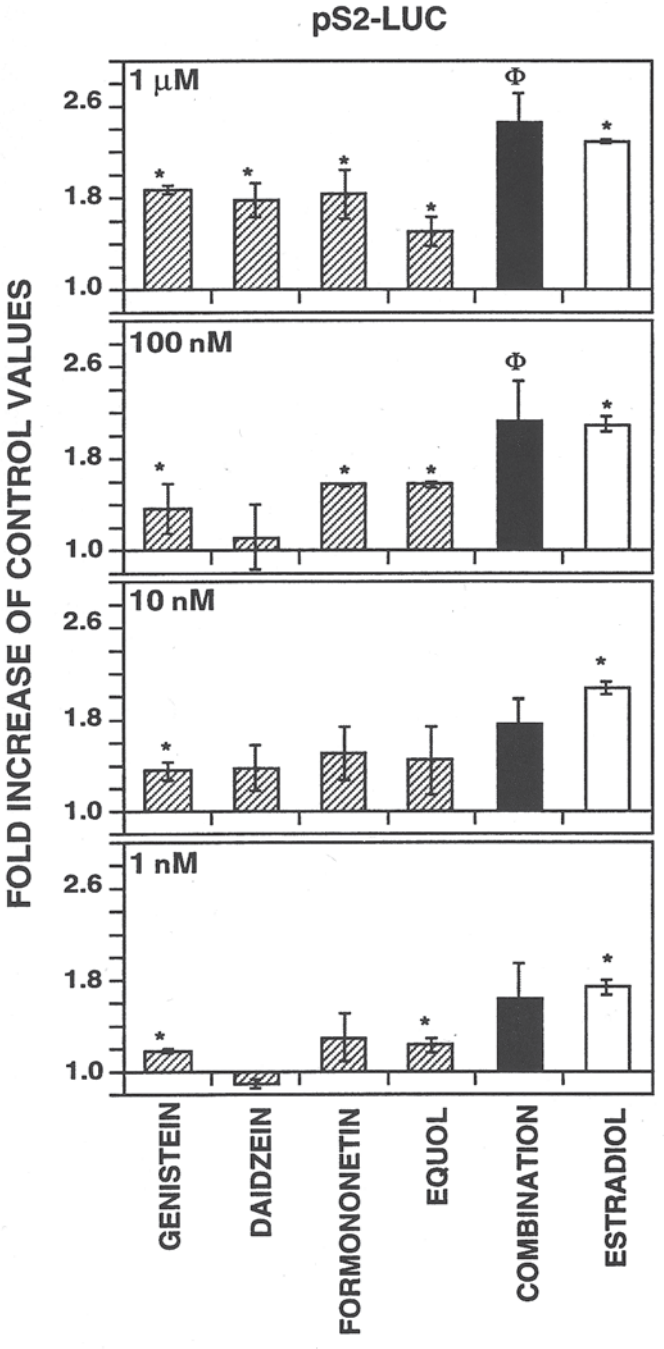

Fig. 2. Effects of phytoestrogens on pS2-LUC expression. MCF-7 cells were transfected with a luciferase reporter plasmid driven by regulatory sequences of the pS2 gene (pS2-LUC). Cells were then treated for 42 h with one of four phytoestrogens, a combination of the four phytoestrogens at equimolar doses (i.e., 4× final dose), or estradiol. Results are expressed as fold increases of control values. *Treatments are different ($p < 0.05$) from control values; ΦCombinatorial treatment is different ($p < 0.05$) from individual phytoestrogen treatments.

increased pS2-driven luciferase activity only at 1 μ*M* and 100 n*M* concentrations (Fig. 2). Thus, our findings with a more physiologically relevant breast cancer gene parallel those obtained with the "generic" construct under most circumstances.

Discussion

Results of this study show agonistic effects of phytoestrogens on estrogen-regulated gene expression in breast cancer cells. Others have shown this as well for a number of phytoestrogens *(4,14,15)* and our data agree with respect to genistein *(4,16,17)*, daidzein and equol *(14)*. We now extend this knowledge to include formononetin. The ability of each of these four compounds to stimulate both a "generic" estrogen-responsive reporter and one containing the regulatory sequences of a relevant breast cancer gene (pS2) supports the notion that phytoestrogens may be important effectors of estrogen-regulated pathways in breast cancer cells. However, the importance of re-demonstrating the agonistic actions of phytoestrogens in general on gene expression does not lie in their individual, separate effects, but rather in their collective actions.

Genistein, daidzein, and formononetin are found together in abundant quantities within various plant sources, specifically dietary soya protein *(18–20)*. It has been established that these compounds (once ingested) are detectable in the human body *(8,20–23)* and act as precursors for equol formation in vivo *(23)*. Given these data, it would stand to reason that in order to examine fully the actions of these four phytoestrogens on estrogen-sensitive pathways, both individual as well as combinatorial effects must be addressed. To this end, we showed that genistein, daidzein, formononetin, and equol clearly have combinatorial agonistic actions on estrogen-responsive gene expression in breast cancer cells. Although others have reported additive antiestrogenic activity by phytoestrogens in the presence of E_2 (biochanin A, chrysin, and flavone in a yeast hER/ERE expression system; ref. *13*), this is the first report in breast cancer cells demonstrating a relevant combinatorial effect. Our results show that combinatorial effects may occur within a physiological range (as was the case when multiple EREs were present) and can stimulate the expression of a physiologically relevant breast cancer gene, such as pS2. Although the interaction between a phytoestrogenic cocktail and endogenous E_2 remains to be investigated in breast cancer cells, our findings suggest that the collective responses of phytoestrogens alone may have important biological ramifications.

Most of the focus on human exposure to environmental estrogens has been related to the influence of environmental contaminants, such as pesticides and plastic derivatives *(24,25)*. Of significance, however, is the fact that the average daily exposure to naturally occurring phytoestrogens may far exceed that of estrogenically active agents introduced into the environment *(26)*. Even with these data, the significance of phytoestrogens as estrogenic effectors in humans have been suggested to be insignificant, given that they may differ from E_2 in estrogenic potency by a factor of 0.001–0.0001 *(4,26)*. Alternatively, the potential combinatorial actions of these compounds would suggest that the collective effects of

multiple phytoestrogens may result in an overall increase in estrogenic potency and possibly clinical significance. This may be particularly relevant to members of Eastern cultures and other persons choosing vegetarian or semi-vegetarian lifestyles. In support of this are numerous reports about growth of estrogen-responsive tissues in animals *(5,27,28)* and humans *(29)* fed diets rich in phytoestrogens. Therefore, it follows logically that steroid-dependent tumors, particularly estrogen-dependent breast cancers, might be affected similarly.

Our present results are not entirely consistent with epidemiological findings that suggest that phytoestrogen consumption may have a cancer-protective effect or with in vitro studies that report antiestrogenic and antiproliferative effects of phytoestrogens on breast cancer cells. Unfortunately, studies of such relationships have not incorporated combinatorial paradigms, which may more accurately reflect the in vivo condition, where the presence of numerous phytoestrogens may collectively augment the total dose of estrogen present. Consistent with this idea is the report by Miksicek *(4)*, who suggested that dietary phytoestrogens may actually stimulate cancer progression under certain conditions, specifically with regard to estrogen-dependent tumors in postmenopausal women where endogenous E_2 may be limiting. This hypothesis is supported by data that confirm that some phytoestrogens (apigenin and 4,4-Dihydroxy chalcone) can actually stimulate the growth of MCF-7 cells under estrogen-deprived conditions, thus satisfying the E_2 requirement for cancer cell growth *(4)*. Such a relationship may be further enhanced when one considers potential combinatorial phytoestrogenic actions on gene expression in breast cancer cells.

In summary, we have shown that phytoestrogens exert agonistic and combinatorial effects on estrogen-regulated gene expression in breast cancer cells, and that they do so in the absence of E_2. Because of their estrogenic activity when tested alone or in combination, they have the potential to subserve a stimulatory role in breast cancer progression. Of importance is the need for additional investigation to establish firmly the interrelationships among similar phytoestrogens, endogenous E_2, and relevant combinatorial effects on the growth of breast cancer cells.

Materials and Methods

MCF-7 Cell Culture

Cultures of MCF-7 breast adenocarcinoma cells (ATCC; Rockville, MD) were maintained in phenol-free minimum essential medium (MEM) supplemented with 0.2 IU bovine insulin, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 10 m*M* HEPES, and 10% normal FBS (n-FBS; Gibco BRL, Grand Island, NY) in a 95% air/5% CO_2 environment at 37°C. Cells were grown as a monolayer under these conditions in accordance with routine cell-culture procedures. Cells were harvested as needed for use in experimental trials by trypsinization (0.05% trypsin + 0.53 m*M* EDTA-4Na; Gibco) to yield a suspension of cells for plating in six-well tissue-culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ). Cells were plated at a concentration of 1.5×10^5 cells/well in phenol-free MEM supplemented with 10% heat-inactivated dextran/charcoal-stripped FBS (cs-FBS) for 48 h prior to chemical transfection.

Chemical Transfections and Treatments

MCF-7 cells cultured for 48 h in cs-FBS were chemically transfected with one of two E_2-responsive luciferase (LUC) reporter plasmids according to the lipofectamine method (Gibco). Reporter plasmids used in these experiments consisted of the MMTV-ERE-LUC (generously provided by D. McDonnell, Duke University, Durham, NC) and the pS2-LUC (generously provided by T. Zacharewski, University of Western Ontario, Ontario, Canada) reporter constructs. The MMTV-ERE-LUC plasmid has been previously described *(30)* and consists of five tandem copies of a 33-bp vitellogenin ERE, which were inserted into the plasmid ΔMTV-LUC for use as a "generic" E_2-responsive reporter. The pS2-LUC plasmid contains regulatory sequences of the pS2 gene *(31)*, an estrogen-inducible gene characteristic of the MCF-7 human breast cancer cell line *(32,33)*, placed in tandem with the luciferase gene. Briefly, lipofectamine (10 μL/well) and the appropriate plasmid (2 μg plasmid/well) were incubated separately in phenol/serum-free MEM for 35 min and then combined to allow the formation of liposome–DNA complexes during an additional 25-min incubation. Cells were then washed with phenol/serum-free MEM and 1 mL of the transfection components added to each well. Immediately following a 5-h incubation with the transfection components, transfection medium was removed, the cells washed with phenol/serum-free MEM, and then treated with differing concentrations of the appropriate compound in the presence of phenol-free MEM supplemented with 10% cs-FBS. Treatments were given at 1-n*M* to 1-μ*M* concentrations, and consisted of one of the following phytoestrogens: genistein, daidzein, formononetin, and equol (Indofine, Belle Mead, NJ) or estradiol-17β (E_2; Sigma, St. Louis, MO). Cells designated as controls were treated with 0.01% ethanol (vehicle) in medium supplemented with cs-FBS. In addition to treatment with a single phytoestrogen, phytoestrogenic combinatorial effects were investigated by treating cells with equimolar concentrations (1 n*M* to 1 μ*M*) of each of the four phytoestrogens (i.e., 4-n*M* to 4-μ*M* final combined molar concentration). Cells were incubated with treatments for 42 h, then lysed, and cell extracts assayed for luciferase activity. The 42-h treatment period was determined from preliminary time-course studies and is a similar paradigm to that reported previously for estrogenic stimulation of the pS2-LUC plasmid in MCF-7 cells *(15)*.

Luciferase Assay and Data Analysis

Cell lysate extracts were measured (Luciferase Assay System, Promega, Madison, WI) in a luminometer for 30 s (Monolight 2010, Analytical Luminescence Laboratory, Ann Arbor, MI) to quantify luciferase activity. Results were expressed as actual luciferase activity, since preliminary experiments indicated no differences among treatments and control wells in cell number (trypsinization of cells followed by trypan blue exclusion) or protein content (BCA protein assay; Pierce, Rockford, IL) within the time frame with which experiments were conducted in the presence of treatment (i.e., 42 h; data not shown). Data from three independent experiments for each plasmid (MMTV-ERE-LUC and pS2-LUC) with 3 replicates/treatment within each experiment were normalized by the expression of treatment values as fold-increases relative to controls. Statistical analysis was then carried out using single-factor ANOVA, and significant differences between individual treatment groups made using the Student's *t*-test. The individual contrasts of dose–response within treatment, treatment vs control, and single estrogen or individual phytoestrogen treatment vs combinatorial phytoestrogen treatment were examined.

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