Endocrine, *vol. 11, no. 1, 69–74, August 1999* 0969–711X/99/8:69–74/$11.50

TGF-α Exerts Biphasic Effects on Estrogen- and Phytoestrogen-Mediated Gene Expression in Breast Cancer Cells

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Transforming growth factor-α (TGF-α) contributes to the progression of mammary carcinogenesis in part through synergistic augmentation of estradiol (E_2) action. To investigate this further, we sought to determine (1) whether the duration of TGF-α treatment might influence the nature of the TGF-α/E_2 interaction, and (2) whether TGF-α would behave in a similar manner when combined with phytoestrogens. To this end, we transfected T47-D breast cancer cells with an estrogen-responsive reporter and then treated the cells (for 4–48 h) with varying concentrations of TGF-α, E_2, the antiestrogen 4-hydroxy-tamoxifen (HOT), and/or one of three phytoestrogens. Our findings revealed that TGF-α has short-term synergistic and long-term inhibitory effects on E_2- and phytoestrogen-regulated gene expression. Furthermore, this secondary inhibition of E_2 action by TGF-α was similar in magnitude to that imposed by HOT. These findings demonstrate a novel role for TGF-α and invite reevaluation of current models regarding TGF-αs interactions with E_2 in breast cancer cells. Our results also raise the possibility that phytoestrogens, which interact with TGF-α in a manner conceptually identical to that of E_2, may subserve a regulatory function in breast cancer cells.

Key Words: Transforming growth factor-α; estrogen; phytoestrogen; gene expression; breast cancer.

Introduction

Transforming growth factor-α (TGF-α) is a polypeptide produced and secreted by certain breast cancer cell lines *(1)* and primary breast carcinomas *(2–4)*. This growth factor plays an autoregulatory role in breast cancer progression. It does so by binding to the epidermal growth factor (EGF) transmembrane receptor and initiating a cascade of events that leads to proliferation of neoplastic cells *(5–7)*. The autocrine feedback mechanisms controlling TGF-αs actions are regulated in part by estrogen mediation of TGF-α gene expression and secretion *(1,8–11)*. Moreover, TGF-α, as well as other growth factors including EGF and insulin-like growth factor-1 (IGF-1), can act synergistically with estrogen to accelerate cancer cell growth *(12)* and enhance estrogen-response element (ERE)–mediated transcriptional processes *(12–14)*. Additionally, TGF-α alone in the absence of estrogen can activate specifically ERE-regulated gene expression through mechanisms that have yet to be elucidated. Although overwhelming evidence exists that TGF-α plays an important role in breast cancer progression, the agonistic and synergistic effects of TGF-α on estrogenic pathways have been investigated largely over a very narrow time frame (18–24 h). Studies over a longer duration of treatment are required to appreciate more fully the overall role of TGF-α in mediating estrogen-regulated gene expression.

Although numerous studies support a role for TGF-α as an amplifier of estrogen action, they have not addressed whether the growth factor might interact similarly with other estrogenically active agents such as estrogenic endocrine disruptors (EEDs). The compounds that comprise this group are diverse and include both introduced (e.g., 2.3,7,8,-tetrachlorodibenzo-*p*-dioxin [TCDD], octylphenol) and naturally occurring (e.g., phytoestrogens) environmental agents with estrogenic activity *(15–22)*. These agents have been shown to display agonistic as well as antagonistic effects on estrogen-sensitive cell processes including estrogen receptor (ER)/ERE-mediated gene expression in breast cancer cells *(22–26)*. The physiological relevance of EEDs to breast cancer and their effects on other estrogen-sensitive tissues remains controversial primarily owing to the reduced potency of EEDs relative to endogenous estrogens *(27)*. However, what has not been considered is the potential for complementary mechanisms, such as the amplification of estrogen-mediated gene expression by TGF-α, to act in concert with EEDs. Potential synergistic effects between peptide growth factors and EEDs could have enormous implications to our understand-

Received March 4, 1999; Accepted April 14, 1999.

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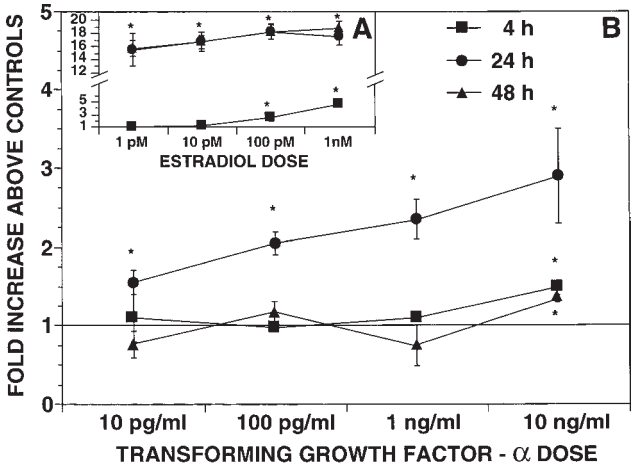

Fig. 1. Effects E_2; **(A)** and TGF-α **(B)** on MMTV-ERE-LUC expression. For data presented in this and Figs. 2–4, T47-D cells were transfected with a luciferase reporter plasmid driven by multiple copies of the vitellogenin ERE (MMTV-ERE-LUC). Cells were then treated for 4 (—■—), 24 (—●—), or 48 (—▲—) h with varying concentrations of E_2 (1 p*M* to 1 n*M*) or TGF-α (10 pg/mL to 10 ng/mL). All results presented here and elsewhere are expressed as fold-increases relative to serum-free control values. *, Treatments are different ($p < 0.05$) from serum-free control values.

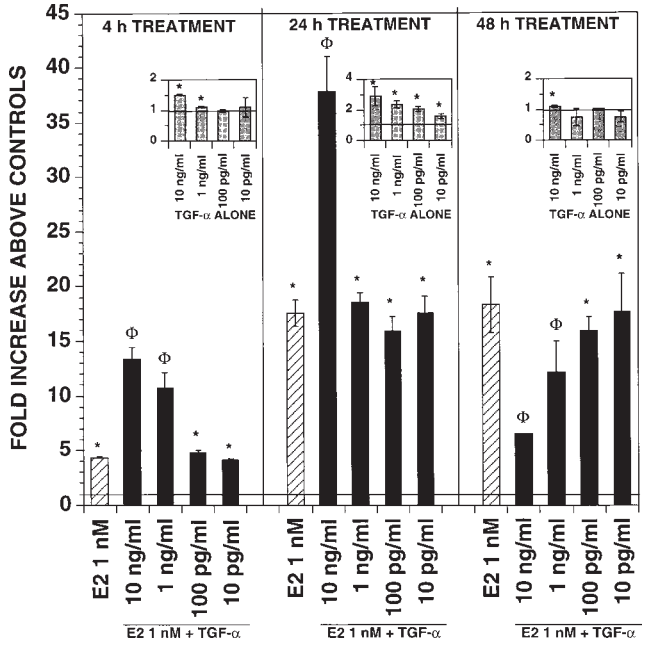

Fig. 2. Effects of combined E_2 and TGF-α treatment on MMTV-ERE-LUC expression. Transfected cells were treated for 4, 24, or 48 h with E_2 (1 n*M*) and/or TGF-α (10 pg/mL to 10 ng/mL). *, Treatments are different ($p < 0.05$) from serum-free control values; Φ, Treatments are different from E_2 treatment alone and serum-free control values.

ing of how EEDs operate and the physiological consequences of EED exposure.

In this article, we present findings to indicate that TGF-α can act as either an amplifier or inhibitor of estrogen-regulated gene expression, depending on the time course of treatment. Moreover, we also provide compelling evidence for both synergistic and inhibitory roles of TGF-α in mediating the estrogenic actions of naturally occurring phytoestrogens (EEDs).

Results

TGF-α in Combination with Estradiol Has Short-Term Synergistic and Long-Term Inhibitory Actions on ERE-Mediated Gene Expression

To investigate the short- and long-term actions (4–48 h) of TGF-α on E_2-regulated gene expression, we began by examining responsiveness of the ERE-driven luciferase reporter (MMTV-ERE-LUC) chemically transfected into T47-D breast cancer cells. This was accomplished by varying the concentrations of E_2 and/or TGF-α. Following 4 h of E_2 treatment, increases of luciferase activity ($p < 0.05$) above control values were observed only at the 100 p*M* and 1 n*M* E_2 doses (Fig. 1A). By contrast, treatment with E_2 for 24 or 48 h increased ($p < 0.05$) ERE-driven luciferase activity above control values at all concentrations tested (1 p*M* to 1 n*M*; Fig. 1A). However, no differences ($p > 0.10$) among concentrations of E_2 were observed during the 24- and 48-h treatment periods, presumably owing to the extended treatment of the cells with E_2 concentrations that approached the saturable range. Similarly, TGF-α alone increased ERE-driven luciferase activity but to a much lesser extent than E_2. As shown in Fig. 1B, TGF-α also augmented ($p < 0.05$) ERE-driven luciferase activity in a dose-dependent manner at 24 h, whereas at 4 and 48 h only the 10 ng/mL dose evoked an increase ($p < 0.05$) above that of controls.

Having established that E_2 or TGF-α alone could each increase ERE-driven reporter activity, we initiated further studies aimed at elucidating the combined actions of E_2 (1n*M*) in concert with varying concentrations of TGF-α on ERE-mediated gene expression. The data presented in Fig. 2 show that at 4 h, E_2 synergized with TGF-α (at doses of 1 and 10 ng/mL) to activate ERE-mediated gene expression. A similar magnitude of synergistic activation was also observed at 24 h, but only with the 10 ng/mL dose of TGF-α. In striking contrast, inhibitory effects of TGF-α (at 1 and 10 ng/mL) on E_2-stimulated luciferase activity were evident following 48 h of treatment. Taken together, these results demonstrate that TGF-α has biphasic effects on E_2-regulated gene expression that are time- and dose-dependent in breast cancer cells.

Inhibitory Effects of TGF-a on Estrogen-Regulated Gene Expression Are Quantitatively Similar to Those of the Antiestrogen 4-Hydroxy-Tamoxifen

Having established that TGF-α has short-term synergistic and long-term inhibitory effects on E_2-regulated

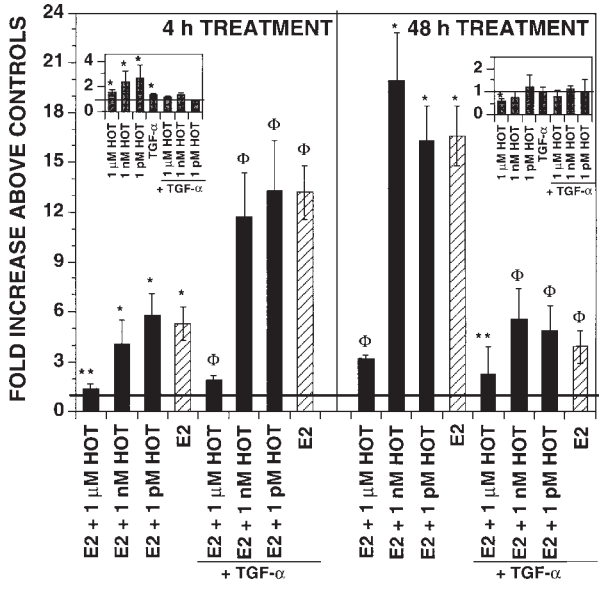

Fig. 3. Effects of HOT treatment on E_2- and TGF-α-mediated gene expression. Transfected cells were treated for 4 or 48 h with E_2 (1 n*M*), TGF-α (10 ng/mL) and/or HOT (1 p*M* to 1 μ*M*). *, Treatments are different ($p < 0.05$) from serum-free control values; **, treatments are different ($p < 0.05$) from E_2 treatment alone; Φ, treatments are different ($p < 0.05$) from E_2 treatment alone and serum-free control values.

gene expression, we proceeded to ask several ancillary questions:

1. What effect would TGF-α have on antiestrogen treatment alone (or vice versa) in the absence of E_2?
2. Could short-term TGF-α treatment reverse (partially or in full) the inhibitory effects of an antiestrogen on E_2-regulated gene expression?
3. Would long-term TGF-α treatment influence E_2-mediated gene expression in the presence of an antiestrogen?

To answer these questions, T47-D breast cancer cells were transfected with the MMTV-ERE-LUC reporter and cultured for 4 or 48 h in the presence of E_2 (1 n*M*), TGF-α (10 ng/mL), and/or varying concentrations of the antiestrogen 4-hydroxy-tamoxifen (HOT) (1 p*M* to 1 μ*M*). At 4 h, all concentrations of HOT alone exhibited some agonistic activity on ERE-mediated luciferase activity, which was not apparent at 48 h (Fig. 3, inset controls). However, in the presence of TGF-α, HOT treatment values at 4 and 48 h did not differ ($p > 0.10$) from controls (Fig. 3, inset controls). These findings demonstrate that TGF-α inhibited the short-term (4 h) partial agonist activity of HOT, whereas long-term (48 h) HOT treatment remained unaffected by the addition of TGF-α. As anticipated, 1 μ*M* HOT inhibited ($p < 0.05$) E_2-stimulated gene expression at 4 and 48 h whereas at lower doses (1 p*M* and 1 n*M*) HOT did not affect ($P > 0.10$) 1 n*M* E_2 action (Fig. 3). Moreover, reporter activity after combinatorial treatment of HOT with E_2 and TGF-α at 4 and 48 h did not differ ($P > 0.10$) from the depressed values of 1 μ*M* HOT + E_2 treatment alone.

These data show that although TGF-α has synergistic effects with E_2 at 4 h, this combined effect was not sufficient to reverse or even influence the antiestrogenic effects of 1 μ*M* HOT. Following long-term treatment (48 h), the combination of TGF-α with E_2 resulted in a level of inhibition comparable to E_2 + 1 μ*M* HOT treatment (Fig. 3). Moreover, while HOT treatment at lower doses (1 n*M* and 1 p*M*) in combination with E_2 did not differ ($p > 0.10$) from E_2 treatment alone, the addition of TGF-α to these treatments resulted in an inhibition of E_2-stimulated gene expression. Taken together these data show that TGF-α inhibited the short-term partial agonist activity of HOT treatment alone, does not reverse or affect the short-term inhibitory actions of HOT on E_2 stimulation, and has comparable long-term inhibitory effects similar to those of high-dose HOT treatment on E_2-mediated gene expression.

Synergistic and Inhibitory Actions of TGF-α on Estrogen-Regulated Gene Expression Are Also Observed with Phytoestrogens

To assess whether the synergistic and inhibitory actions of TGF-α were restricted to combination with estradiol-17β, other estrogenic agents, namely phytoestrogens, were examined by use of paradigms similar to those described previously. Briefly, T47-D cells transfected with the MMTV-ERE-LUC reporter were cultured in serum-free medium containing either E_2 (1 n*M*), genistein, daidzein, or equol (1 μ*M*, respectively) in the presence or absence of TGF-α (10 ng/mL) for 4 and 48 h. A dosage of 1 μ*M* for each phytoestrogen was used because studies conducted previously in our laboratory showed predictable elevations in the levels of MMTV-ERE-LUC gene expression for all phytoestrogens tested at this dose *(26)*, and were comparable to that elicited by 1 n*M* E_2. Moreover, 1 μ*M* concentrations of phytoestrogens are within the range (0.7–3 μ*M*) suggested by previous reports to be achieved in serum following heavy soy or phytoestrogen consumption *(28)*. Consistent with our earlier study *(26)*, we found here that each of the three phytoestrogens tested alone (at 1 μ*M*) exhibited agonistic actions on ERE-mediated gene expression comparable to that of E_2 alone (at 1 n*M*; $p > 0.10$) (Fig. 4). Maximal responses in this regard were approx 6- and 11-fold above control values at 4 and 48 h, respectively (Fig. 4). Interestingly, each of the three phytoestrogens, like E_2, was able to synergize with TGF-α to augment ERE-mediated reporter activity at 4 h (Fig. 4). In fact, their magnitudes of response were quite similar for all four treatments (>15-fold; $p < 0.05$). By contrast, the response was distinctly reversed at 48 h; E_2, daidzein, and equol in combination with TGF-α evoked a 50–60% reduction ($p < 0.05$) relative to respective individual treatments (Fig. 4). Although a reduction (~45%) in gene expression for cells treated with genistein in combination with TGF-α was also

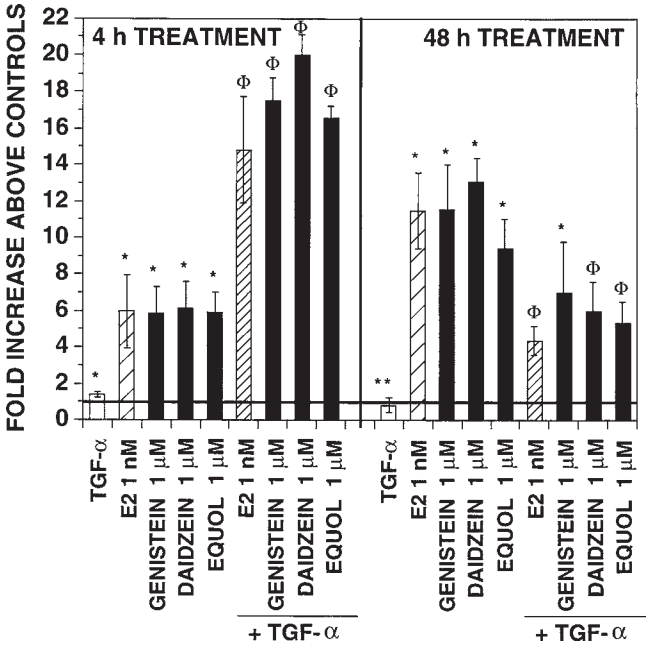

Fig. 4. Effects of TGF-α on phytoestrogen- and E_2-mediated MMTV-ERE-LUC expression. Transfected cells were treated for 4 or 48 h with E_2 (1 n*M*), one of three phytoestrogens (genistein, daidzein, or equol at 1 μ*M* doses) and/or TGF-α (10 ng/mL). *, Treatments are different ($p < 0.05$) from serum-free control values; **, treatments are different ($p < 0.05$) from E_2 treatment alone; Φ, treatments are different ($p < 0.05$) from E_2 treatment alone and serum-free control values.

observed at 48 h, this decrease did not achieve statistical significance ($p > 0.10$).

Collectively, these results support two important conclusions: First, TGF-α can act synergistically with phytoestrogens (under some circumstances) to enhance the agonistic actions of these environmental estrogens following initial or short-term exposure. Second, the synergistic and inhibitory effects of TGF-α on estrogen-regulated gene expression are not restricted to E_2; phytoestrogens can substitute for E_2, albeit at much higher concentrations.

Discussion

The results of the present study demonstrate clearly that TGF-α has synergistic as well as inhibitory effects on E_2-regulated gene expression, and that these effects are time- and dose-dependent. Although others have shown that growth factors (TGF-α, EGF, and IGF-1) can activate and/or enhance ER-mediated gene transcription in breast cancer and other E_2-sensitive cells *(12–14,29–31)*, inhibitory effects represent a significant departure from this classic stimulatory role for TGF-α. As further evidence for the biphasic effects (short-term synergistic and long-term inhibitory) of TGF-α on E_2-mediated gene expression, we showed that the dual actions of TGF-α are also manifested in the presence of other estrogenically active agents (i.e., phytoestrogens). Thus, these findings demonstrate not only a novel biphasic action of TGF-α on E_2-regulated gene expression, but also that phytoestrogens can substitute for E_2 in this regard.

Just as others found that growth factors such as TGF-α, EGF and IGF-1 can activate ERE-mediated gene expression in the absence of E_2 *(12–14,31)*, we too observed that TGF-α alone can evoke the same response. Interestingly, there is considerable evidence to suggest that both the ER and EGF receptor must be present for TGF-α (or EGF) to evoke an estrogenic response by itself *(14)*. Given the growth factor's dual receptor requirement, it is not surprising that coordinated responses between TGF-α and E_2 have already been investigated *(12–14,29–31)*. Consistent with results from these previous studies in breast cancer and other cell types, we observed short-term synergistic effects of TGF-α with E_2 on ERE-mediated gene expression, thereby adding another layer of support to the notion that TGF-α is an amplifier of E_2 action. Moreover, our data extend these findings to show that the synergistic activation of ERE-mediated gene transcription by TGF-α and E_2 can occur within a 4-h treatment period—a much shorter time frame than has been found previously (18–24 h) by others.

Although synergy between peptide growth factors and steroid hormones has been established for some time *(13,29–31)*, the mechanisms underlying these responses have yet to be elucidated. There are, however, a few hypotheses to explain this phenomenon. These include TGF-α induced phosphorylation and/or conformational changes in the ER to activate gene transcription *(29,32)*, modulation (i.e., up- or downregulation) of ER levels *(14)*, and regulation or coactivation of critical nuclear transcription or accessory factors necessary for formation of ER transcriptional complexes *(12)*. The precise mechanism of interaction notwithstanding, a synergy between TGF-α and E_2 on ERE-mediated gene expression is not inconsistent with most models of ER-positive breast cancer progression. In this scenario, E_2 is believed to stimulate, in an autocrine manner, the production and release of TGF-α, which indirectly mediates at least some of the effects of E_2. TGF-α, in turn, may also act to enhance the direct effects of E_2 on estrogen-responsive tissues.

In the present study, we also uncovered a seemingly paradoxical new role for TGF-α distinct from the growth factor's short-term synergistic effects: a long-term antagonism of estrogen action. Indeed, we found that long-term treatment (48 h) of breast cancer cells with E_2 and TGF-α resulted in a significant reduction (~60%) of E_2-stimulated gene expression. Such a response is not unprecedented. A similar inhibition of E_2-regulated gene expression has been reported for MCF-7 breast cancer cells treated for 48 h with the protein kinase C activator 12-*O*-tetradecanoylphorbol-13-acetate *(33)*. Furthermore, additional preliminary studies conducted in our laboratory using other ERE reporters (ERE-tk-LUC) also revealed a reduction (>30%) in tran-

scriptional activity following long-term TGF-α treatment (data not shown). Note that in the present study, TGF-α inhibition of E_2 action was not trivial. On the contrary, it was quantitatively similar to that imposed by a maximal dose (1 μ*M*) of the potent antiestrogen *HOT*. Although the mechanisms surrounding TGF-α's inhibitory interactions remain to be clarified (e.g., regulation of cellular EGF-R, and ER), the novel role for TGF-α as an inhibitor of E_2 action may prove useful for identifying additional mechanisms through which estrogenic pathways in breast cancer cells may be perturbed.

Having shown clearly that TGF-α can have biphasic (synergistic followed by inhibitory) effects on E_2-mediated gene expression, we next evaluated whether other estrogenically active compounds (e.g., phytoestrogens) might behave in a similar manner. Our findings demonstrate that the biphasic actions of TGF-α on E_2-regulated gene expression are not restricted to estradiol-17β. In fact, we observed that each of the three phytoestrogens (genistein, daidzein, and equol) tested in the short-term (4 h) synergized with TGF-α to produce a much greater increase of ERE-mediated gene expression than any of the phytoestrogens alone.

On the other hand, TGF-α acted in the long-term (48 h) to inhibit ERE-mediated gene expression when combined with two of the three phytoestrogens, just as it did when combined with E_2. To our knowledge, this is the first demonstration that any environmental estrogen (natural or otherwise) can substitute for E_2 in the induction of a synergistic (or inhibitory) mechanism within breast cancer cells. This finding is important because the diminished potency and lower ER binding affinities of phytoestrogens (and other EEDs) relative to endogenous E_2 has raised concerns about their physiological relevance *(27,34)*. Such concerns were diminished in part by our recent observation *(26)* that at least four phytoestrogens can exert combinatorial effects on E_2-regulated gene expression in breast cancer cells. When viewed together with our present findings that individual phytoestrogens can substitute for E_2 in evoking a synergistic interaction with TGF-α, it is reasonable to speculate that alternative mechanisms exist within breast cancer cells for magnification of EED action. Whether the net effect of these agents in vivo is carcinogenic or protective remains to be established. Clearly, the results of the present in vitro study suggest that the potential for either response in vivo is not modest.

In summary, we have shown that TGF-α can have short-term synergistic and long-term inhibitory effects on E_2-regulated gene expression in breast cancer cells. The long-term inhibitory actions of TGF-α are of considerable interest because the magnitude of the response approached that of the antiestrogen *HOT*. Future studies aimed at clearly delineating the mechanisms underlying this phenomenon (e.g., combinatorial TGF-α/E_2 regulation of cellular EGF-R, and ER) could open the door for identifying new ways to perturb estrogenic pathways involved with breast cancer progression. Finally, we have shown, for the first time, that secondary mechanisms exist in breast cancer cells for intensifying (short-term) or dampening (long-term) the estrogenic actions of at least one class of environmental endocrine disruptors—phytoestrogens. This finding invites further studies aimed at establishing the consequences of crosstalk between growth factor- and EED-responsive pathways in breast cancer cells.

Materials and Methods

T47-D Cell Culture

Cultures of T47-D human breast adenocarcinoma cells (American Type Culture Collection [ATCC], Rockville, MD) were maintained in phenol-free RPMI-1640 medium supplemented with 0.2 IU of bovine insulin, 2 m*M* L-glutamine, 1 mM sodium pyruvate, 10 m*M* HEPES, and 10% normal fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY) in a 95% air/5% CO_2 humidified 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-BRL) to yield a suspension of cells for plating in six-well culture plates (Falcon; Becton-Dickinson, Franklin Lakes, NJ). Monodispersed cells were plated at a concentration of 2.0×10^5 cells/well in phenol-free RPMI-1640 medium supplemented with 10% charcoal-stripped FBS (cs-FBS) for 24 h prior to chemical transfection. Following transfection, cells were maintained in serum-free RPMI-1640 for an additional 24 h until the administration of treatments.

Chemical Transfections and Treatments

T47-D cells cultured for 24 h in cs-FBS were chemically transfected with an estrogen-responsive reporter plasmid, MMTV-ERE-LUC (generously provided by Dr. D. McDonnell, Duke University, Durham, NC), according to the lipofectamine method (Gibco-BRL). The MMTV-ERE-LUC reporter plasmid has been described previously *(35)* and contains five tandem copies of a 33-bp vitellogenin ERE, which was inserted into the plasmid (MTV-LUC, for use as a "generic" estrogen-responsive reporter. Briefly, lipofectamine (10 μL/well) and the ΔMTV-ERE-LUC plasmid (2 μg/well) were incubated separately for 35 min, and then combined to allow the formation of liposome-DNA complexes during an additional 25-min incubation. Transfection components were then added to each well and the cells were transfected for 5 h. Next, transfection medium was removed and serum-free RPMI-1640 medium was added to each well. Twenty-four hours later, cells were treated with one, or a combination, of the following compounds: TGF-α (10 pg/mL to 10 ng/mL; R&D, Minneapolis, MN), estradiol-17β (E_2: 1 p*M* to 1 n*M*; Sigma, St. Louis, MO), HOT (1 p*M* to 1 μ*M*;

Sigma), or one of three phytoestrogens (1 μ*M* genistein, daidzein, or equol; Indofine, Belle Mead, NJ). Treatment dose and incubation times (4–48 h) varied depending on the experimental paradigm employed. Following the incubation of cells with respective treatments, cells were lysed and the extracts assayed for luciferase activity. Additionally, control experiments conducted in the same manner utilized a promoterless (i.e., "null") MMTV-LUC reporter. This was done in light of recent findings reporting the existence of a TGF-α-responsive enhancer in the upstream region of the MMTV long terminal repeat (LTR) *(36)*. These studies were conducted to determine whether any changes in gene expression seen after the addition of TGF-α alone or in combination with E_2 were the result of spurious activation of this region. Stated briefly, no transcriptional activation of the "null"-MMTV-LUC reporter was observed following short- or long-term treatment with TGF-α, E_2, or a combination thereof.

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). Results were expressed as actual luciferase activity, since preliminary experiments indicated no differences in well protein content (bicinchoninic acid protein assay; Pierce, Rockford, IL) or cell number (trypsinization of cells followed by trypan blue exclusion) between treated and control wells within the time-frames under study (data not shown). Data from three independent experiments with three replicates/treatment within each experiment were normalized as fold increases relative to serum-free controls. Statistical analysis was performed using single-factor analysis of variance, and significant differences between treated and control wells were made using the Student *t*-test (StatView; Abacus Concepts, Berkeley, CA).

Acknowledgments

We thank D. McDonnell (Duke University, Durham, NC) for providing the MMTV-ERE-LUC plasmid utilized in this study. This research was supported in part by a grant from the Department of Energy EHAP program: Cooperative Agreement: DE-FC02-98CH10902.

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