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Growth Factors Differentially Augment the Effects of HPTE on Estrogen Response Element-mediated Gene Transcription in a Dose- and Time-dependent Manner Among Human Breast Cancer Cell Lines

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Abstract: Methoxychlor's active metabolite (HPTE; 2,2-bis(p-hydroxyphenyl)- 1,1,1-trichloroethane) acts via the estrogen receptor (ER) to stimulate estrogen response element (ERE)-mediated gene transcription in estrogen-sensitive tissues. Moreover, growth factors can synergize with estrogenic agents enhancing transcription from estrogen-responsive genes. The objective of this study was to examine whether growth factors can augment HPTE-induced ERE-mediated responses in two breast cancer cell lines (MCF-7 and T47-D). Cells were transfected with an estrogen-responsive reporter (MMTV-ERE-Luc) and treated for 6 h or 24 h with HPTE (1 nM to 1 μM) in the presence or absence of single growth factor treatment (10 ng/ml; transforming growth factor [TGF], epidermal growth factor [EGF] or insulin-like growth factor-1 [IGF-1]), media alone (control) or estradiol-17β (1 nM; positive control). HPTE alone increased ($P < 0.05$) ERE-mediated gene expression at >1 nM concentrations in both cell lines. IGF-1 did not alter HPTE-induced ERE-mediated gene expression in MCF-7 cells ($P > 0.10$); whereas in T-47D cells 24 h treatment with IGF-1 increased ($P < 0.05$) gene expression, but only with 1 mM HPTE. EGF with HPTE (>1 nM) increased ($P < 0.05$) ERE-mediated gene expression at 6 h in both cell lines, but not at 24 h. TGF with HPTE (>1 nM) produced a differential response between cell lines. Specifically, T47-D cells exhibited a greater response ($P < 0.05$) at 6 h than MCF-7 cells, whereas MCF-7 cells exhibited a greater response ($P < 0.05$) at 24 h than T-47D cells. To address the effects of TGF further we chose to investigate "low dose" HPTE treatment (1 pM to 1 nM) in the presence of TGF on ERE-mediated gene transcription. For MCF-7 cells, only at the 1 nM HPTE treatment did TGF increase ($P < 0.05$) ERE-mediated gene transcription at 6 and 24 h. For T47-D cells, treatment with 1 nM HPTE with TGF for 6 h resulted in a increase in gene transcription ($P < 0.05$). However no effect ($P > 0.10$) was observed for TGF with pM HPTE treatment for ether cell line. These data demonstrate synergistic combinatorial effects of growth factors on HPTE-induced ERE-mediated gene expression. Moreover, differential effects between MCF-7 and T-47D cells were noted in time-course and extent of ERE activation; suggesting heterogeneity in responsiveness among cancer cells.

Key Words: Breast Cancer, HPTE, Growth Factors, Estrogen

INTRODUCTION

Some pesticides have been shown to have estrogenic activity in mammalian systems, including neoplasias of the breast [1]. While pesticide-induced carcinogenesis has been suggested based on both *in vitro* and *in vivo* evidence, whether exposure to relatively weak estrogenic pesticides is relevant or even harmful has yet to be definitively determined. While estrogen receptor (ER)-mediated pathways are the most obvious means through which pesticides may influence endocrine-responsive cancer cells, alternate or convergent pathways may be of equal or greater significance in the etiology of chemical-induced cancer progression (e.g., via synergism with growth factors; [2]). While synergism for a number of growth factors with estradiol to enhance estrogen response element (ERE)-mediated gene transcription in human breast cancer cell lines have been reported [2], few have evaluated these relationships in combination with xenoestrogens (e.g., estrogenic pesticide mimics). Moreover, studies have not addressed the heterogeneity in responsiveness that may be observed among different ER+ breast cancer cell lines.

The objective of this study was to examine whether growth factors can augment HPTE-induced ERE-mediated responses in two estrogen sensitive breast cancer cell lines (MCF-7 and T47-D). Methoxychlor

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is a broad spectrum insecticide against a wide variety of insects, however following exposure in mammalian systems the liver transforms methoxychlor into 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is the biologically active metabolite that can act as a xenoestrogen via interactions with ER [3]. In this study, we address the interactions of HPTE with three growth factors important to cancer progression (TGF, EGF and IGF-1) that may interact, directly or indirectly, with ER-ERE stimulated pathways. Two classical estrogen-sensitive (ER+) breast cancer cell lines (MCF-7 and T47-D) have been used in these investigations to address whether observed interactions between HPTE and growth factors on ERE-mediated gene transcription are cell line-specific and/or if differential responsiveness between cell lines might be observed.

MATERIALS AND METHODS

Estrogen Receptor and β Binding Assays: Fluorescence Polarization methodologies were used to conduct estrogen receptor (ER) competitor binding assays (Panvera Corp., Madison WI) to ascertain relationships between HPTE and ER and ER-β in relation to estradiol (E_2). Briefly, recombinant ER and β were diluted in combination with varying concentrations of fluorescein-labeled E_2 and incubated at room temperature for 60 min. Fluorescence polarization values were measured using a Beacon 2000 (Panvera Corporation, Madison WI) with 490 nm excitation filter and a 530 nm emission filter. This competitive assay operates on the basis that a molecule bound to the ER is large and thus rotates slowly in the plane of light (revealing a high polarization value), whereas small molecules or unbound ER undergoes rapid rotation thus resulting in a low polarization value. In the ER competitor assays, an ER/ES2 complex with a high polarization value was added with 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE; 10 pM to 100 μM; Cedra Corp., Austin, TX) or estradiol (E_2; 10 pM to 100 μM; Sigma-Aldrich, St. Louis, MO). The shift in polarization in the presence of HPTE and E_2 (positive control) was used to determine the relative affinity for ER or ER-β. These were used to determine theoretical "low" (initial binding) and "high" (saturation) concentrations for HPTE to be used in combinatorial *in vitro* investigations (i.e., HPTE treatment with growth factors on estrogen-mediated gene transcriptions in breast cancer cells).

T47-D and MCF-7 Cell Culture: Cultures of T47-D and MCF-7 human breast adenocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD). T47-D cells were maintained in phenol-free RPMI-1640 medium and MCF-7 cells in phenol-free MEM, both mediums supplemented with 10 μg/ml bovine insulin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM 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 mM 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 1.5×10^5 cells/well in phenol free medium (RPMI-1640 and MEM for T47-D and MCF-7 cells respectively) supplemented with 10% charcoal-stripped FBS (cs-FBS) for 24-h prior to chemical transfection. Following transfection, cells were maintained in serum-free medium for an additional 24 h until the administration of treatments.

Chemical Transfection and Treatments: T47-D and MCF-7 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 [4], and contains five tandem copies of a 33-bp vitellogenin ERE, which was inserted into the plasmid ΔMTV-LUC for use as a generic estrogen-responsive reporter. This reporter plasmid has been used previously by the author [5,6,7,8] and others [4,9] as an estrogen responsive reporter. Briefly, lipofectamine (10 μl/well) and the MMTV-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 (incubated) for 5 h. Next, transfection medium was removed and serum-free medium (RPMI-1640 and MEM for T47-D and MCF-7 cells respectively) was added to each well. Twenty-four hours later, cells were treated with one, or a combination, of the following compounds: 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE; 1 nM and 1 μM; Cedra Corp., Austin, TX), estradiol (E_2; 1 nM; Sigma-Aldrich, St. Louis, MO), TGF (10 ng/ml; R & D Systems, Minneapolis, MN), EGF (10 ng/ml; R & D Systems, Minneapolis, MN) or IGF-I (10 ng/ml; R & D Systems, Minneapolis, MN). Treatment doses for HPTE were selected based on binding affinity assays (see Results) where initial binding and subsequent saturation of ER was observed. Cells were incubated

with treatments for 6 h (short-term) or 24 h (long-term) to ascertain any biphasic effects of growth factors; which has been observed previously for other estrogenically active compounds (e.g., phytoestrogens and TGF; [7]). Following the incubation of cells with respective treatments, cells were lysed and the extracts assayed for luciferase activity. Control experiments using a promoterless ("null") MMTV-LUC reporter have been conducted previously, which demonstrated no transcriptional activation of the null-MMTV-LUC reporter in the presence of growth factor (e.g., TGF), E_2 or combinations thereof [7].

Luciferase Assays and Data Analysis: Cell lysate extracts were assayed for luciferase activity (Luciferase Assay System; Promega, Madison, WI) in a luminometer for 10 s (TD 20/20; Turner Designs, Sunnyvale, CA). Results were expressed as actual luciferase activity or as fold increases above control luciferase activity values (RU; Relative Units), since preliminary experiments indicated no differences ($P > 0.10$) in well protein content (bicinchoninic acid protein assay; Pierce, Rockford, IL) or cell number (trypsinization of cells followed by trypan blue exclusion) between treated (e.g., 1 nM E_2) and control wells within the time-frames under study (data not shown). Data from at least three independent experiments with six replicates per treatment within each experiment were normalized as fold increases relative to serum-free controls. Statistical analysis was performed using factorial ANOVA with treatment and incubation time as model parameters within cell lines. Significant differences between treated and control wells and time-points were established at the $P < 0.05$ level, and tendencies or trends established at $P < 0.10$, using the Student's *t*-test (SAS, Cary, NC).

RESULTS AND DISCUSSION

Using the ER binding assay described (see Methods), E_2 bound ER-a and ER-b at 1 nM and 100 pM concentrations, respectively. Relative to E_2, HPTE began binding ER and ER-β at a 10-fold and 100-fold greater concentration (10 nM), respectively, than that of E_2. HPTE began to reach saturation at 1 uM for both ER and ER-β. Thus, the binding affinity of HPTE for ER and ER-β was less ($P < 0.05$) than for E_2 at equimolar concentrations; as expected and as reported previously. For *in vitro* studies, a 1 nM concentration (i.e., 10-fold less than low-dose binding of HPTE at 10 nM) was selected to evaluate the combinatorial effects of growth factors for synergizing with HPTE to initiate responses that may not have been observed with HPTE alone, and the 1 mM HPTE (saturation) concentration was used to evaluate the effects of growth factors for inducing augmented HPTE responses.

Addition of EGF in combination with E_2 resulted in a synergistic effect at 6 h for both MCF-7 (Figure 1 – Panel A) and T47-D (Figure 2 – Panel A) cells, whereas at 24 h no effect of EGF above that of E_2 alone was observed for either cell line. Within the MCF-7 cell line (Figure 1 – Panel A), addition of EGF for 6 h resulted in a 139.3%, 151.8% and 98.2% increase ($P < 0.05$) in ERE-mediated gene expression above that of estradiol at 1 nM and HPTE at 1 nM and 1 μM concentrations alone, respectively. EGF addition at 24 h had no effect ($P > 0.10$) on ERE-mediated gene transcription. For T47-D cells (Figure 2 – Panel A), addition of EGF for 6 h resulted in a 390.6%, 514.0% and 263.4% increase ($P < 0.05$) in ERE-mediated gene expression above that of E_2 at 1 nM and HPTE at 1 nM and 1 μM concentrations alone, respectively. EGF addition at 24 h resulted in a 31.1% decrease ($P < 0.05$) on ERE-mediated gene transcription in the HPTE 1 nM treatment group, but did not alter ($P > 0.10$) the E_2 or 1 μM HPTE groups. With respect to 6 versus 24 h time-points, for both the MCF-7 and T47-D cell lines, the 6 h treatment with E_2 or HPTE (1 nM or 1 μM) with EGF addition exhibited a greater ($P < 0.05$) induction of ERE-mediated gene transcription than the 24 h treatment period; which was not different ($P > 0.10$) from the respective non-EGF-treated controls.

Addition of IGF-1 in combination with E_2 resulted in no effect ($P > 0.10$) on ERE-mediated gene transcription above that of E_2 alone at 6 and 24 h for MCF-7 (Figure 1 – Panel B), and at 6 h for T47-D cells (Figure 2 – Panel B). However at 24 h, addition of IGF-1 resulted in a 281.3% increase ($P < 0.05$) over E_2 alone (Figure 2 – Panel B). While differences in levels of gene expression were observed at 6 h versus 24 h of treatment ($P < 0.05$), the addition of IGF-1 did not increase ($P > 0.10$) ERE-mediated gene expression in MCF-7 cells (Figure 1 – Panel B). For T47-D cells, addition of IGF-1 to 1 nM HPTE did not alter ($P > 0.10$) ERE-mediated gene expression at 6 or 24 h of treatment, while IGF-1 caused a 97.0% increase ($P < 0.05$) over 1 μM HPTE treatment alone (Figure 2 – Panel B). The 1 nM HPTE + IGF-1 treatment of T47-D cells for 24 h did not differ ($P > 0.10$) from the 6 h time-point, whereas in all other 24 h treatment groups receiving IGF-1 the 24 h time-point was significantly greater ($P < 0.05$) than the 6 h treatment period.

Combined treatment of E_2 with TGF resulted in a 249.4% and 131.4% increase ($P < 0.05$) at 6 h for MCF-7 and T47-D cells, respectively, but not at 24 h for either cell line ($P > 0.10$). For MCF-7 cells,

addition of TGF with HPTE at 1 nM and 1 μM resulted in a 291.4% and 188.0% increase ($P < 0.05$), respectively, in ERE-mediated gene transcription at 6 h, and 195.1% and 62.9% increase ($P < 0.05$), respectively, at 24 h (Figure 1 – Panel C). For T47-D cells, addition of TGF at 6 h resulted in a 369.1% and 191.8% increase ($P < 0.05$) in ERE-mediated gene transcription above that of HPTE alone at 1 nM and 1 μM concentrations, respectively. The addition of TGF-a at 24 h did not increase ($P > 0.10$) ERE-mediated gene transcription in T47-D cells. Differential responses between MCF-7 and T47-D cell lines was observed with respect to the effect of treatment time, with MCF-7 cells treated with HPTE + TGF being greater ($P < 0.05$) at 24 h than at 6 h whereas T47-D cells exhibited greater ($P < 0.05$) ERE-mediated gene expression at 6 h than 24 h with HPTE + TGF treatment.

To address the effects of TGF further, given the 6 h results for both MCF-7 and T47-D and 24 h results for MCF-7, we chose to investigate "low dose" HPTE treatment (1 pM to 1 nM) in the presence of TGF on ERE-mediated gene transcription to further elucidate the influence of this growth factor in augmenting HPTE treatment (see Figure 3 – Panels A and B). Treatment with E_2 (1 nM) and TGF resulted in a 204.3% and 162.4% increase ($P < 0.05$) at 6 h for MCF-7 and T47-D cells, respectively, but not at 24 h for either cell line; as observed previously (see Figures 1 and 2 – Panels A and B). However for MCF-7 cells, only at the 1 nM HPTE treatment did TGF-a increase ($P < 0.05$) ERE-mediated gene transcription at 6 and 24 h (224.3 and 129.7%, respectively), as observed previously (Figure 1 – Panel A), but not at pM concentrations ($P > 0.10$; Figure 3 – Panel A). For T47-D cells, treatment with 1 nM HPTE with TGF-a for 6 h resulted in a 178.3% increase ($P < 0.05$), however no effect ($P > 0.10$) was observed at 6 or 24 h for TGF with pM HPTE treatment.

Discussion: Previous studies have demonstrated a lower binding affinity of HPTE to ER than endogenous estrogen, demonstrating ER binding but at a much higher concentration. Gaido *et al.* [10] reported that the relative binding affinities of ER and ER-β for HPTE were 0.004 and 0.02, respectively ($E_2 = 1.0$) when competitive binding assays were performed. Bolger and others [11] reported the relative binding affinity of ER for HPTE was 1.7 ($E_2 = 100$) when using a fluorescence polarization binding assay similar to that utilized in the present investigation. Not surprisingly, the binding affinity of HPTE for ER and ER-β in the present study was similarly less than E_2 at equimolar concentrations; which was expected, and as reported previously. However a central question regarding introduced (e.g., pesticides or herbicides) environmental endocrine disruptors (EEDs) or E_2 mimics is whether alternate mechanisms exist that may enhance estrogenic effects above those normally reported for the native compound (e.g. HPTE treatment alone), and these interactions were the focus of the current study.

Evidence for intracellular cross-talk between the nuclear estrogen receptor and membrane bound peptide growth factor receptor has been demonstrated in the normal uterus of mice [12]. It has been stated previously by Ring and Dowsett [13] that growth factor receptor pathways may up-regulate and stimulate growth independently of the ER, or could communicate by cross-talk with the ER and affect cell growth and patterns of resistance. It has been previously shown that the growth factors, such as those used in this study (TGF-α, EGF and IGF-1), can act synergistically with estrogen to accelerate cancer cell growth and enhance ERE mediated gene expression [2,14]. The communication between a cell surface peptide hormone receptor and an intracellular steroid hormone receptor can take different routes as dictated by the physiology (i.e., state) of the cell and other influencing factors. Thus responses could even be different for individual cells within the same tumor as a result of tumor progression and during the development of transitional hormone resistance [15]. In the current study, we demonstrate synergistic combinatorial effects of growth factors on HPTE-induced ERE-mediated gene expression. Moreover, differential effects between MCF-7 and T47-D cells, two estrogen dependent breast cancer cell lines, were noted in both the time-course and extent of ERE activation; suggesting heterogeneity in responsiveness among breast cancer cell lines to cross-talk between growth factors and E_2 or HPTE responsiveness. While the interaction with HPTE is the focus of the current investigation, previous studies have similarly demonstrated that cross-talk occurs among breast cancer cells in combination with estrogenic agents on ERE mediated gene expression, and several have also confirmed differential responses among MCF-7 and T47-D cell lines. In a study by Lichtner *et al.* [15], growth factors (EGF, IGF-1) were tested for their mitogenic activity on MCF-7 and T47-D cells. Only T47-D cells were stimulated by EGF, whereas proliferation of both cell lines was stimulated by IGF-1. In the current study, EGF stimulated ERE gene expression in both cells lines (MCF-7 and T47-D) at 6 hr but the T47-D cells showed a greater increase than the MCF-7 cell line; suggesting both time and magnitude differences among the cell lines. With the addition of IGF-1, only T47-D cells were stimulated at the 24 hour time point. No other increase in ERE gene expression was observed at the 6 hour time point

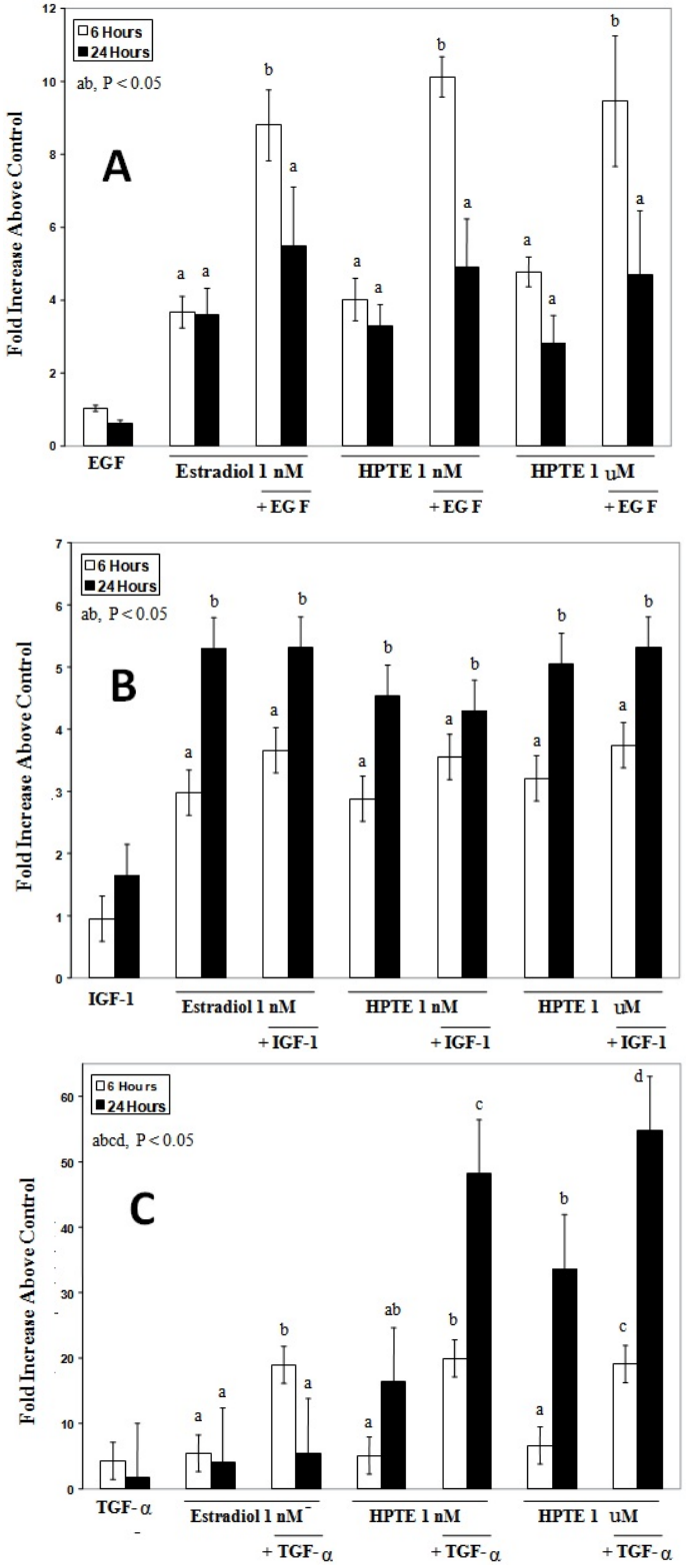

Fig. 1: Treatment of MCF-7 cells with estradiol and HPTE alone and in combination with epidermal growth factor (EGF, 10 ng/ml; Panel A), insulin-like growth factor-1 (IGF-1, 10 ng/ml; Panel B) and transforming growth factor (TGF, 10 ng/ml; Panel C) on ERE-mediated gene expression at 6 and 24 hours post-treatment.

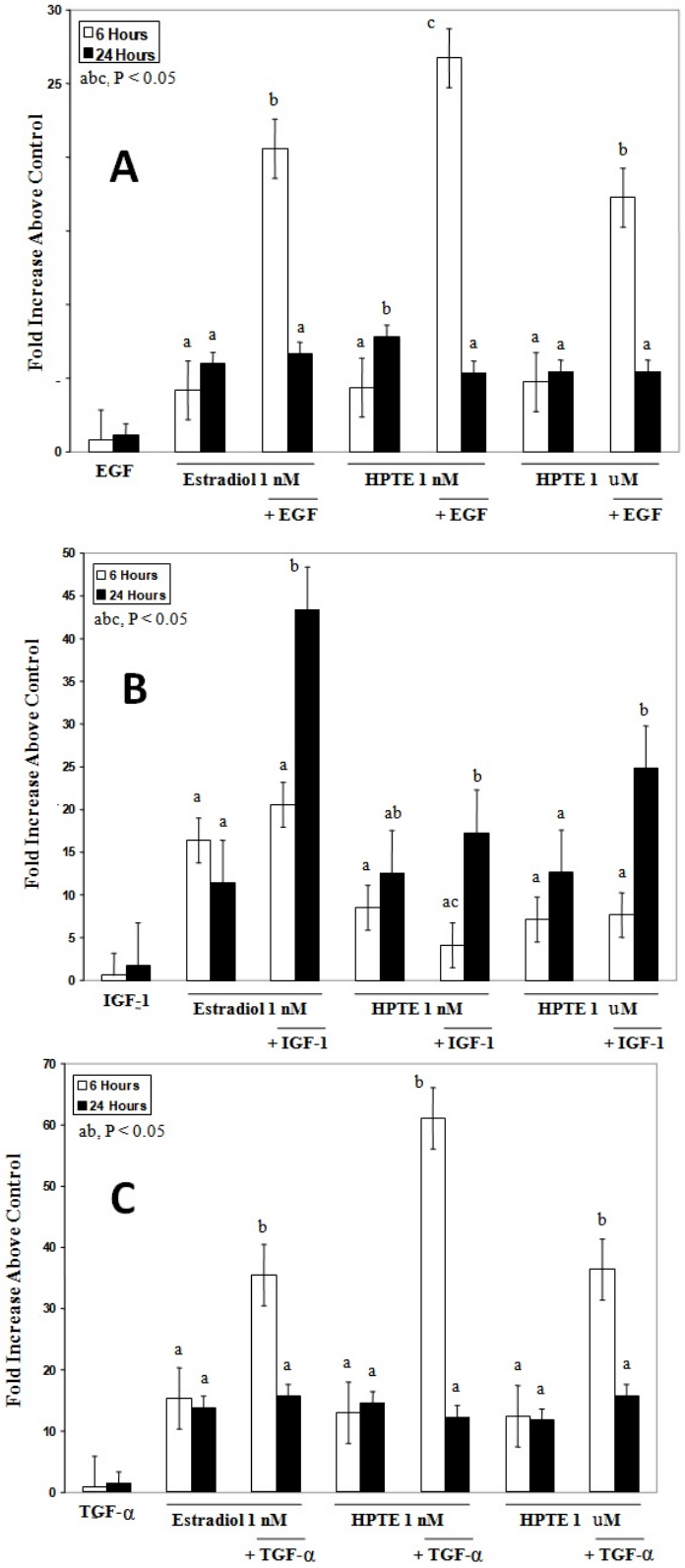

Fig. 2: Treatment of T47-D cells with estradiol and HPTE alone and in combination with epidermal growth factor (EGF, 10 ng/ml; Panel A), insulin-like growth factor-1 (IGF-1, 10 ng/ml; Panel B) and transforming growth factor (TGF, 10 ng/ml; Panel C) on ERE-mediated gene expression at 6 and 24 hours post-treatment.

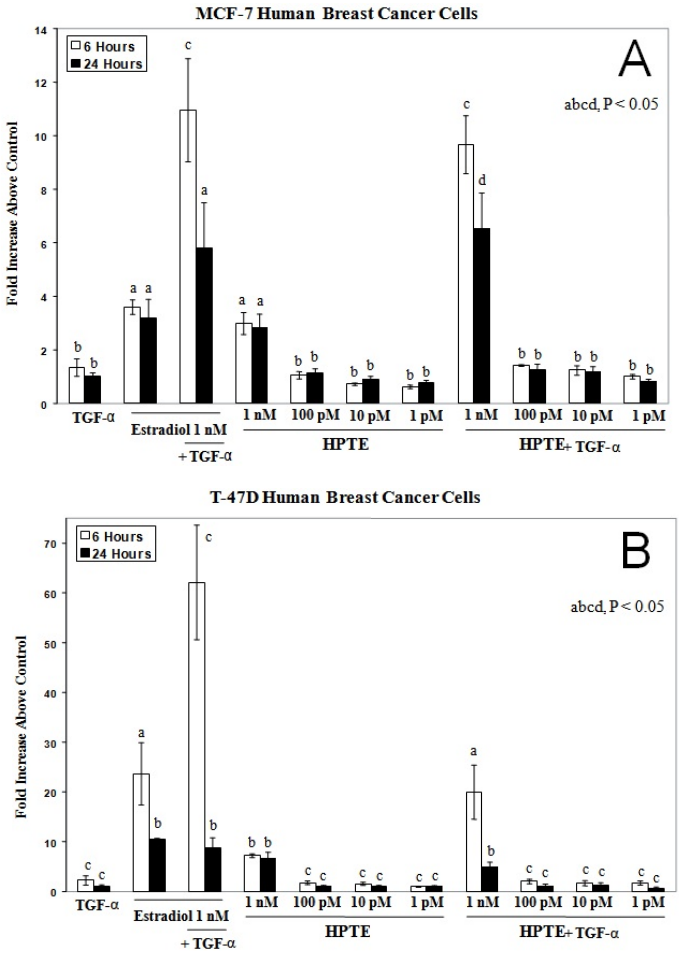

Fig. 3: Treatment of MCF-7 (Panel A) and T47-D (Panel B) cells with estradiol and low-dose (pM) concentrations of HPTE alone and in combination with transforming growth factor (TGF, 10 ng/ml) on ERE-mediated gene expression at 6 and 24 hours post-treatment.

or at the 24 hour time point for the MCF-7 cells. Other studies have reported that concomitant treatment of MCF-7 cells with E_2 and IGF-1 enhanced proliferation and DNA synthesis in a synergistic manner [16]. Lichtner et. al. [15] also investigated the interference of the pure antiestrogen ZM 182780 with growth factor signaling pathways. The pure antiestrogen ZM 182780 was effective in interfering with growth factor action in the MCF-7 cell line but it did not interfere with growth factor action in T47-D cells. The authors suggest differential cross-talk between ER and growth factor receptors in MCF-7 and T47-D breast cancer cell lines [15] which could explain why differences in ERE gene expression are noted among different cell lines and even within the same cell line using different time points.

Similar to our results with EGF and IGF-1, previous studies by Willard and Frawley [7] using the T47-D cell line demonstrated that TGF-α has synergistic effects on E_2 regulated gene expression that are time and dose dependent. The investigators observed short-term (within a 4-hour treatment period) synergistic effects of TGF-α with E_2 on ERE mediated gene expression. In addition, it was shown that TGF-α in combination with phytoestrogens (genistein, daidzein, and equol) produced a much greater increase of ERE mediated gene expression than any of the phytoestrogens alone [7]. In the current study, the addition of TGF elicited a response in the MCF-7 cells at the 6 and 24 hr time points. However the T47-D cells only showed an increase in expression at the 6 hr time point. This led to incorporation of an additional experiment to examine low-dose (pM) HPTE in combination with TGF to determine whether TGF addition would result in an estrogenic response that is not observed with pM HPTE treatment alone; similar to examination of EGF and IGF-1 addition reported previously. Our results indicate that low-dose HPTE

treatment (i.e., below the threshold of a major estrogenic response) in combination with TGF did not augment ERE-mediated gene expression at these concentrations. El-Tanai and Green [17] found that the mRNA levels of LIV-1 and pS2 (estrogen-induced genes) were increased in the absence of estradiol by EGF, TGF- α and IGF-1. In addition when the MCF-7 cells were exposed to estradiol and one of the growth factors, the increases in the mRNAs were additive. This was not the case in the current investigation using a reporter-based (luciferase) indicator of gene transcription via ERE-mediated interactions.

Conclusion: Collectively, these data demonstrate synergistic combinatorial effects of growth factors on HPTE-induced ERE-mediated gene expression. We suggest that the actions of EEDs may be enhanced by cross-talk with alternate signaling pathways (such as those initiated by growth factors), therefore assessments of EED stimulation alone may not reveal the true nature or magnitude of the actions of these agents on physiological mechanisms. Moreover, differential effects between MCF-7 and T-47D cells were noted among HPTE and HPTE plus growth factor addition in time-course and in the extent of ERE activation; suggesting heterogeneity in responsiveness among cancer cell lines.

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