Environmental Estrogens Stimulate Gene Transcription in the Prolactin Promoter

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Abstract

The ability of environmental estrogens to regulate gene transcription on model and physiologically complex promoter was examined in GH_3 cells, a pituitary cell line. In transient transfection studies, the pGL3 model promoter and the physiologically complex prolactin promoter were both responsive to the xenoestrogen bisphenol A and the phytoestrogen daidzein in a dose related manner. These transcriptional responses were mediated by estrogen receptors, as responses were ameliorated in the presence of ICI 180, 782, a pure antiestrogen. Cotransfection of Pit-1 significantly enhanced the transcriptional response of the prolactin promoter to stimulation by environmental estrogens. The nature and magnitude of transcriptional responses to estradiol sensitive genes following challenge by environmental estrogens is likely dependent on regulatory elements found in the promoter and their ability to recruit transcription factors.

Keywords: Pituitary, Bisphenol A, Daidzein, Transcription, Activation, Promoter, Genistein

1. Introduction

The steroid hormone 17 -estradiol (E_2) is the primary estrogen generated in the ovaries (Gruber et al., 2002) and regulates gene expression in a number of target tissues. This gives way to physiological effects which include cell differentiation, cell proliferation, and protection from pathological insult (Gruber et al., 2002, Heldring et al., 2007). Arguably, the most significant reproductive neuroendocrine event mediated by E_2 is the initiation of the luteinizing hormone (LH) surge in mammals during the estrous or menstrual cycle. This LH surge is driven by positive feedback

effects of estrogen on the pituitary and hypothalamus and these events subsequently trigger ovulation (de Ziegler et al., 2007).

Estrogen receptors alpha and estrogen receptor beta (ER , ER) mediate the action of E_2 in a target cell (Kuiper et al. 1996; Shughrue et al., 2007). Like other nuclear receptors, ERs can be divided into several functional domains that govern ligand dependent and independent gene activation and DNA binding (Dahlman-Wright et al., 2006). Cell and/or promoter specific ligand independent gene activation (as is the case of stimulation of the estrogen receptor by growth factors) is mediated at the amino terminus of the ER via activation function-1 (AF-1). The central receptor domain is characterized by the presence of two zinc fingers mediating DNA binding. The carboxyl terminus of the ER, containing the AF-2 domain, is required for ligand binding and ligand dependent transcription. ER and ER differ primarily in the amino acid sequence identity between them, particularly in the AF-1 domain (Tora et al., 1989; Zhao et al., 2008) and the respective distribution of the proteins in target tissues. In the rat pituitary, ER and ER can be detected but ER is the predominantly expressed in lactotrophs and gonadotrophs (Gonzalez et al., 2008).

The reproductive effects of E_2 are largely initiated via a genomic mechanism of action (Greeley et al., 1975). Ligand binding induces the estrogen receptor (ER) dimerization and dissociation from stabilizing heat shock proteins (Dahlman-Wright et al., 2006; Heldring et al., 2007). The receptor complex translocates to the nucleus to bind an estrogen response element (ERE), in the promoter region of a responsive gene (Gruber et al., 2004; Dahlman-Wright et al., 2006; Heldring et al., 2007). The displacement of corepressor proteins and the recruitment of coactivators and integrator proteins to the receptor results in chromatin remodeling and the establishment of a preinitiation complex (Moggs & Orphanides, 2001). With the subsequent recruitment of RNA polymerase I, transcription the target gene commences.

The actions of endogenous E_2 may be mimicked by environmental estrogens (EEs) which can bind ERs and alter gene transcription. Phytoestrogens are non-steroidal plant derived compounds with estrogenic activity and include lignans, coumestans, and isoflavones. Of particular interest to this study, the abundant isoflavones tend to be introduced into the body as a dietary component or generated as bioactive products during metabolic processing (Atkinson et al., 2005). Xenoestrogens are non-steroidal, synthetic estrogen mimics that may enter the environment via a number of routes as components of plastics, industrial pollutants or pesticides (Safe, 2004). Xenoestrogens are endocrine disruptors—their negative effects on physiology, particularly reproduction, are well documented in a number of non-mammalian species (Pickford & Morris 1999; Olmstead & LeBlanc, 2000; Cheshenko et al., 2008).

Collectively, phytoestrogens and xenoestrogens act as selective estrogen receptor modulators—their specific actions (as agonists or antagonists) on a given gene may depend on number of factors: dose, length of exposure, cell type, and the ability to recruit various regulatory promoter elements in the targeted gene (Nowakowski et al., 1994; Gould et al., 1998; Lascombe et al., 2000). Thus, the prevalence of EE and our frequent exposure to them may have significant transcriptional effects on E_2 regulated genes. In that E_2 regulates the coordinated release of pituitary hormones, it is critical to characterize the consequences of environmental estrogen exposure on transcriptional regulation of gene expression in anterior pituitary gland cell types. We examined the ability of EEs to regulate the transcriptional activity in a relatively simple model promoter and a pituitary specific, complex, estrogen regulated promoter.

2. Materials and Methods

2.1 Cells

The mammosomatotroph GH_3 cell line (ATCC® CCL-82.1) secretes prolactin (PRL) and growth hormone and was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 5% charcoal-stripped newborn calf serum (NCS) (HyClone, Logan, UT) at 37C in 95% O_2/5% CO_2.

2.2 Plasmids

The model ERE reporter plasmid (250 ng/well in all experiments) used in transfection experiments was pGL3 containing 2 consensus EREs fused to the SV40 viral promoter (gift of Dr. Margaret Shupnik). Plasmid constructs 2.5 kb of the 5' flanking region of the rat PRL gene (PRLluc, gift of Dr. Margaret Shupnik) was also used. Rat Pit-1 was inserted into the pCDNA 3.1 expression vector (Invitrogen, San Diego, CA). Total plasmid DNA concentrations used in transfections were normalized with pcDNA 3.1.

2.3 Transient transfection

GH_3 cells were seeded to 1×10^6 6 well plates (BD Falcon, San Jose, CA) with phenol red-free DMEM (Mediatech, Manassas, VA) supplemented with 5% charcoal-stripped newborn calf serum and 100U/ml penicillin, and 100 ug/ml streptomycin to a final concentration of 1×10^6 cells/well. 250 ng PRL-luc or pGL3 per well was transiently transfected into cells via GeneJuice® transfection reagent (Novagen, Gibbstown, NJ) according to manufacturer's instructions. To assess the effect of Pit-1 on EE induced transcriptional activity, 100 ng/well of Pit-1 expression vector was cotransfected with PRL-luc. Following a 16 hour transfection, media was changed and cells were treated with vehicle or

10nM E_2. In addition, some cells will be treated with a range of doses of bisphenol A (BPA, a xenoestrogen), kepone (K, a xenoestrogen), genistein (G, a phytoestrogen), or daidzein (D, a phytoestrogen) for 24 hours. All treatments were purchased from Sigma (St. Louis, MO) with the exception of BPA (Oakwood Products, Columbia SC) and K (Chem Service, West Chester, PA). Cells were washed twice with PBS (pH 7.4) and collected in 200ul 1X lysis buffer (Promega, Madison, WI). 50 l lysate was assayed for luciferase activity. Luciferase assays of lysate samples were performed with a Turner TD-20E luminometer and protein content determined by total lysate protein using protein dye (Promega, Madison, WI). Promoter activity is expressed as ALU/ g protein.

2.4 Data analysis

Values are expressed as mean ± SEM. Transfections were performed in triplicate; experiments were performed at least 4 times. To determine if there are differences in promoter activity as a function of treatment, a one way analysis of variance (ANOVA) was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). The Tukey's post hoc test was used to determine significant differences between the means. Statistical significance was achieved at $p<0.05$.

3. Results

3.1 EEs stimulate E2 sensitive promoters in a dose related fashion

We first examined the ability of two EEs, BPA, a xenoestrogen and D, phytoestrogen to stimulate pGL3, a model ERE-containing promoter (Figure 1). Untransfected cells exhibited negligible basal transcriptional activity, which increased only slightly upon transfection of reporter (Figure 1, Panel 1). The pGL3 promoter exhibited a 3.43 fold increase in transcriptional activity following challenge with 10nM E_2. As cells were treated with increasing concentrations of BPA (10^{-15}M-10^{-6}M), promoter activity was observed to increase in a dose related fashion, though response to highest dose of BPA was not significantly different that of E_2 alone (3.23 fold increase in activity as compared to vehicle treated control). In subsequent experiments, 1 M BPA was used as a stimulatory dose. Daidzein was more effective at stimulating promoter activity than BPA (4.6 fold at maximum dose as compared to control) and stimulated a slightly more robust response increase in pGL3 activity than E_2.

The E_2 regulated PRL promoter was also stimulated by EE (Figure 1, Panel B) with the exclusion of the lowest dose tested (10^{-15}M). BPA and D significantly increased PRL promoter activity as compared to vehicle treated controls and E_2 treated cells (3.6 and 4.2 fold respectively at the highest doses), however, BPA and D were equally as effective at stimulating promoter activity.

3.2 EEs regulate promoter activity via interactions with ERs

To demonstrate that EE indeed interact with ERs to bring about a transcriptional response, transfected cells were co treated with stimulatory doses of estrogens (including genistein (G), a phytoestrogen) and kepone (K), a xenoestrogen) in the absence and presence of ICI 182, 780 a pure ER antagonist. ICI 182,780 reduced basal pGL3 activity and prevented BPA- or D-induced increases in transcriptional activity (Figure 2, Panel A). Similar results were observed in the PRL promoter. While, ICI had no effect on basal activity of the PRL (Figure 2, Panel B) promoter, estrogen-induced activity was soundly prevented in cells co treated with ICI and E, BPA, or D. Thus, the activation of promoter activity by EE is mediated by ER and/or ER.

3.3 Stimulation of PRL promoter by EEs is enhanced in the presence of the transcription factor Pit-1

Having observed some basic aspects of EE activity on model promoters, we also examined their effects in physiologically complex promoters. The PRL promoter contains an imperfect ERE in the distal promoter region. GH_3 cells were transfected with 250 ng PRL promoter fused to and ERE and treated for 24 hours with E_2, BPA, and D. Promoter activity was significantly increased by all estrogenic compounds, with D being especially potent (4.2 fold). Cotransfection of the pituitary transcription factor Pit-1 significantly enhanced the stimulatory effects that were observed. BPA-induced transcription in the PRL promoter was increased by 40%, while D-induced transcription was increased by 20%.

4. Discussion

The goal of this study was to assess transcriptional activity in a model gene promoter and physiologically complex gene promoter following exposure to EEs. A wealth of *in vitro* and *in vivo* studies clearly demonstrates that xenoestrogens and phytoestrogens can exert significant effects on E_2 regulated target tissues. For example, isoflavones (phytoestrogens) bind and activate ER and ER in uterine and breast cancer cell lines with varying degrees of potency depending on cell and promoter context (Willard & Frawley, 1998, Massaad & Barouki, 1999; Bowers et al., 2000), but preferentially bind ER (Kuiper et al., 1994; Mueller et al., 2004; Harris et al., 2005). Regarding pituitary effects in whole animal studies, ingestion of BPA (via drinking water) in rats reduces serum LH levels, suggesting that BPA may act on the hypothalamus or pituitary gland (Rubin et al., 2001). Subcutaneous injection of BPA increases PRL levels in rats (Goloubkova et al., 2000).

In primary, transfected pituitary cultures, BPA treatment stimulates an ERE and PRL promoter and increases PRL secretion in cell culture and whole animal studies (Steinmetz et al., 1997). Oral administration of G to rats for 21 days or the D metabolite equol for 2-3 months significantly increased serum levels of PRL (Santell et al., 1997; Rachon et al., 2007). Whole animal studies are less abundant, but ingestion of the daidzein itself increases serum PRL levels while decreasing serum concentrations of LH (Rachon et al., 2007). We focus here more closely on the physiology of the PRL promoter as a complex, E_2 sensitive pituitary promoter and challenged it with both the xenoestrogen BPA and the phytoestrogen D in the presence and absence of Pit-1, a pituitary specific transcription factor, and assessed transcriptional activity via reporter assay.

Our results are consistent with the results of Steinmetz et al. (1997). In our system, transcriptional activity in both promoter constructs were stimulated by EEs in a dose related fashion. These stimulatory effects are mediated via AF-2 interactions between the EEs and the ER, since ICI (which competitively inhibits the binding of estrogen to the ER) is observed to abolish these effects. Relative to the potency E_2, BPA, G and D proved to be more effective at stimulating transcription in the PRL promoter. Hormonal regulation of the PRL gene is mediated by transcription factors that bind in the proximal promoter and distal enhancer; these upstream regions then act synergistically to facilitate gene expression (Crenshaw et al., 1989). ERs, acting as ligand inducible transcription factors, are tethered to an imperfect ERE in the distal portion of the PRL promoter to stimulate gene transcription. The ability of the PRL promoter to be stimulated by estrogen requires the binding the transcription factor Pit-1 in the proximal and distal regions of promoter (Nelson et al., 1988) with the Pit-1 binding site adjacent to the imperfect ERE being of particular importance (Schaufele, 1999).

In our experiments, the cotransfection of Pit-1 resulted in a moderate increase in EE-induced transcription. Similar results have been seen in the stimulation of the PRL promoter using E_2 (Day et al., 1990) and confirmed here. Thus, occupancy of the ER by EEs may elicit responses in the PRL promoter that require Pit-1 for full transcriptional activity. Collectively, these data indicate that EEs indeed behave similarly as E_2 in the stimulation of the prolactin promoter with regard to mechanism but not magnitude. These data in these studies are significant because they demonstrate the effects of EEs on a pituitary specific gene promoter that is not regulated by a palindromic ERE, but multiple promoter elements.

How can differences in promoter response and magnitude of stimulation be explained? It is likely that sensitivity of the two promoters to EEs appears to be due to cell/promoter context. Routledge et al. (2000) demonstrated in transfection studies and GST pull down assays an increased recruitment of ER coactivators SRC-1 and TIF-2 by ER   (as compared to ER) in the presence of BPA and G. While E_2 and EE utilize the same receptor and their respective abilities to stimulate transcription in sensitive genes is bolstered by a common transcription factor, Pit-1, it is possible the ligands may initiate discrepant interactions between other proteins within the nucleus that drive changes in gene transcription; EEs may initiate the recruitment of a different amounts or different/additional transcription factors to the nucleus upon binding ERs.

PRL is of physiological significance for its ability to initiate and maintain lactation in mammals, but also regulates the reproductive cycle, general body growth, and maternal behavior (Ben-Jonathan et al. 2008). The prolactin gene, however, is not the only E_2 sensitive gene expressed in the pituitary. Indeed, E_2 regulation gene expression and hormone release in the anterior pituitary is evidenced by the ubiquitous expression of ERs in the APG (Shupnik, 2002). Thus, the ability of xenoestrogens and phytoestrogens to stimulate complex promoter activity in pituitary cells may present some particular implications for overall health, but reproductive health in particular (Adeyoa-Osiguwa et al., 2003). With detailed information on specific nuclear mechanisms of EE action, we may be better able to assess risk of incidental or deliberate exposure to such compounds.

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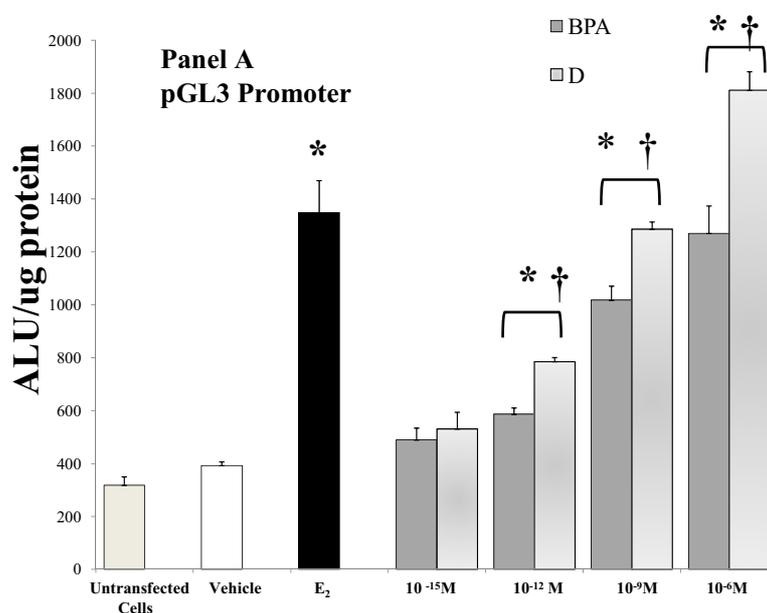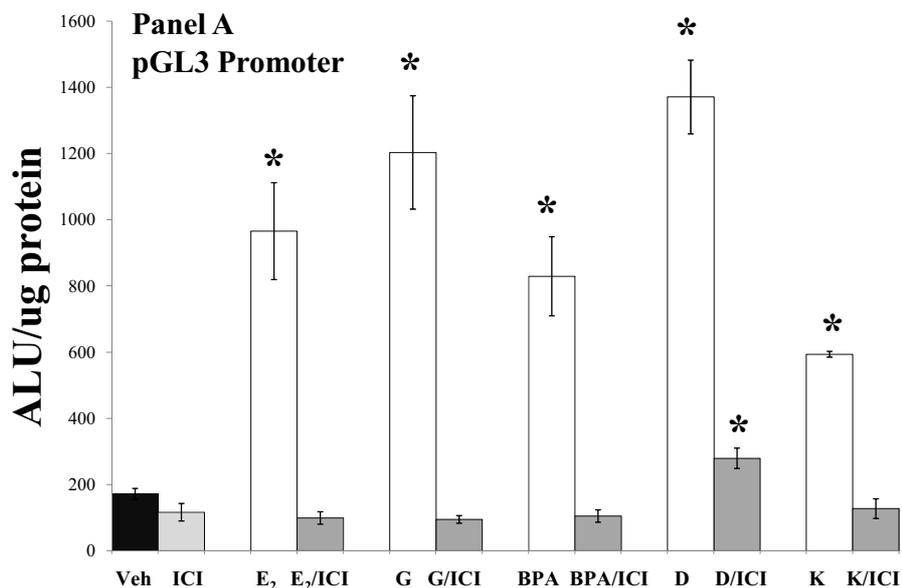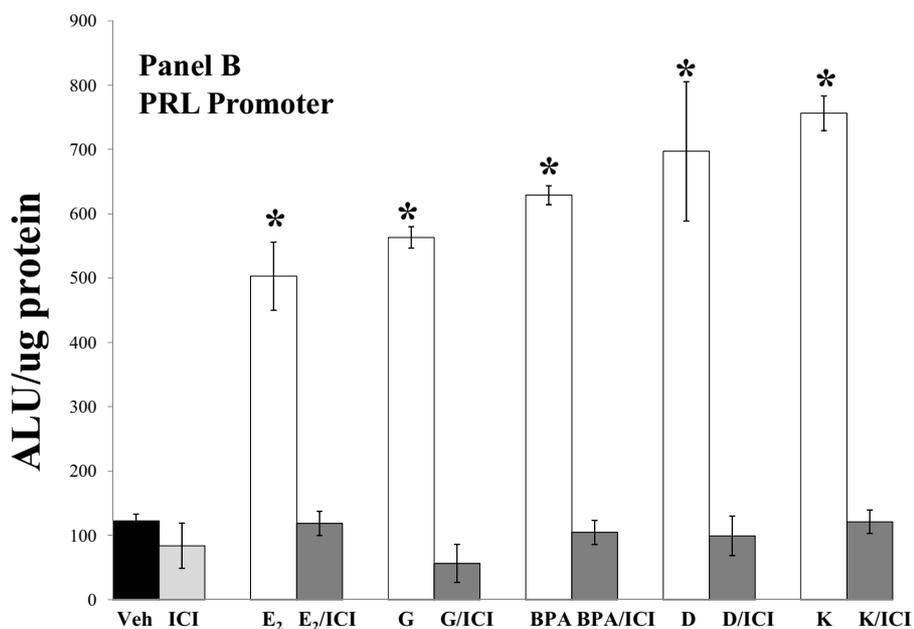
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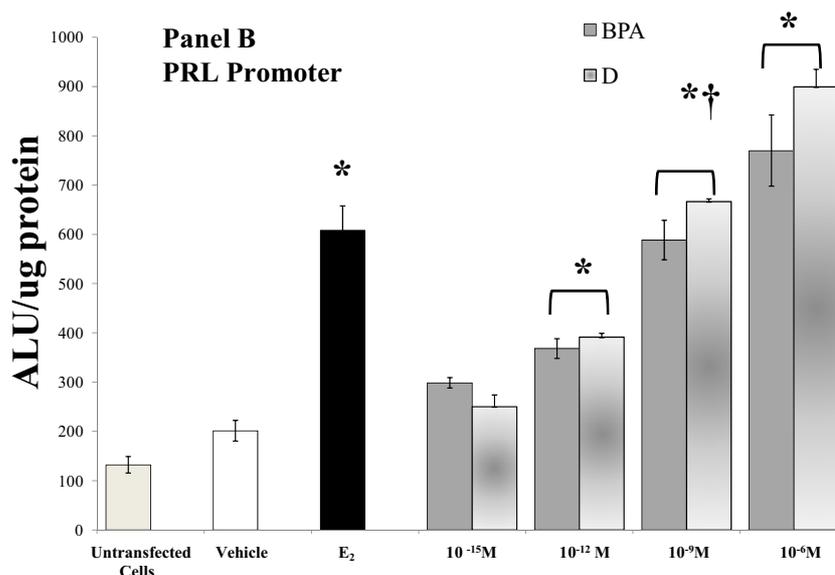
Panel B

Figure 1. Environmental estrogens stimulate transcription in a dose related manner. GH_3 cells were transfected with the 250 ng pGL3 model promoter (Panel A) or 250 ng of the physiologically complex PRL promoter (Panel B). Cells were treated for 24 hours with BPA (solid grey bars) or D (shadowed bars) at the doses indicated in the figure. Cells were collected, lysed and subjected to luciferase assay. Data are expressed as arbitrary light units per microgram of protein. Bars represent mean ± SEM for 4 experiments. For purposes of comparison, response of transfected cells to E_2 is also shown (black bar).*, significantly different from vehicle treated control, $P<0.05$. †, significant difference in respective responses of EEs, $P<0.05$.

Panel A

Panel B

Figure 2. ERs mediate EE-induced transcription of a model and complex promoter. GH_3 cells were transfected with the 250 ng pGL3 model promoter (Panel A) or 250 ng of the physiologically complex PRL promoter (Panel B). Cells were treated for 24 hours with stimulatory doses of E_2, G, BPA, D, and K in the absence (white bars) or presence (gray bars) of ICI 182, 780, a pure estrogen antagonist. Cells were collected, lysed, and subjected to luciferase assay. Data are expressed as arbitrary light units per microgram of protein. Bars represent mean ± SEM for 4experiments. *, significantly different from vehicle treated control (black bar), $P<0.05$.
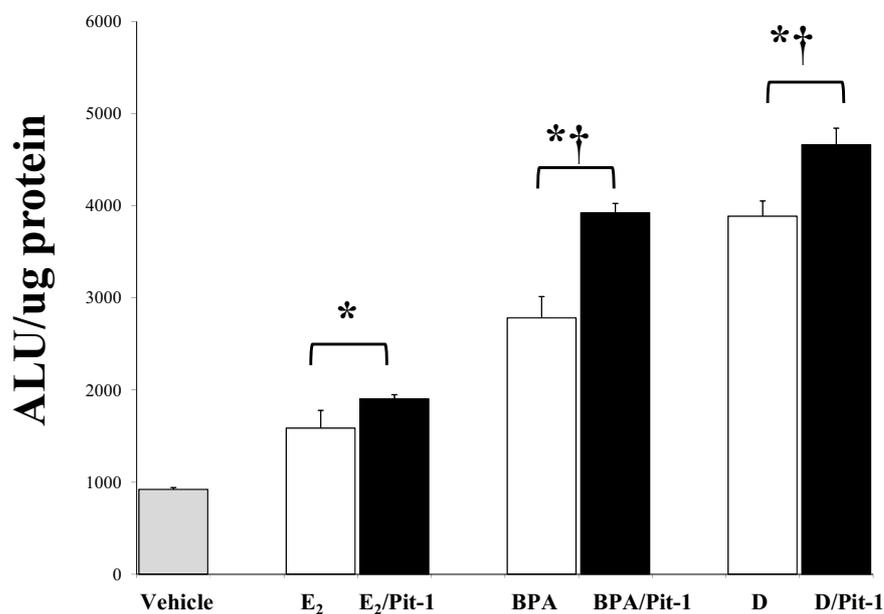
Figure 3. The nuclear transcription factor Pit-1 enhances EE-induced transcriptional responses in the PRL promoter. GH_3 cells were transfected with the 250 ng of the PRL promoter (white bars). Additionally, some cells were cotransfected with Pit-1 (black bars). Cells were treated for 24 hours with stimulatory doses of E_2, BPA or D. Cells were collected, lysed and subjected to luciferase assay. Data are expressed as arbitrary light units per microgram of protein. Bars represent mean ± SEM for 4 experiments. For purposes of comparison, response of transfected cells to E_2 is also shown (black bar). Bars represent mean ± SEM for 4 experiments.*, significantly different from vehicle treated control, P<0.05. †, significant difference in transcriptional responses in presence of Pit-1, P<0.05