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Estrogen- and xenoestrogen-induced ERK signaling in pituitary tumor cells involves estrogen receptor-α interactions with G protein-αi and caveolin I

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ABSTRACT

Multiple physiologic estrogens (estradiol, estriol, and estrone), as well as xenoestrogenic compounds (including alkylphenols and bisphenol A), can act via nongenomic signaling initiated by liganding of the plasma membrane estrogen receptor-α (mERα). We examined heterotrimeric G protein involvement leading to extracellular-regulated kinase (ERK) activation in GH3/B6/F10 rat anterior pituitary tumor cells that express abundant mERα, and smaller amounts of mERβ and GPR30. A combination of microarrays, immunoblots, and quantitative immunoassays demonstrated the expression of members of all α, β, and γ G protein classes in these cells. Use of selective inhibitors showed that the $G_{\alpha i}$ subtype was the primary initiator of downstream ERK signaling. Using antibodies against the GTP-bound form of G_{α} protein subtypes i and s, we showed that xenoestrogens (bisphenol A, nonylphenol) activated $G_{\alpha i}$ at 15–30 s; all alkylphenols examined subsequently suppressed activation by 5 min. GTP-activation of $G_{\alpha i}$ for all estrogens was enhanced by irreversible cumulative binding to GTPγS. In contrast, $G_{\alpha s}$ was neither activated nor deactivated by these treatments with estrogens. ERα and $G_{\alpha i}$ co-localized outside nuclei and could be immuno-captured together. Interactions of ERα with $G_{\alpha i}$ and caveolin I were demonstrated by epitope proximity ligation assays. An ERα/β antagonist (ICI182780) and a selective disruptor of caveolar structures (nystatin) blocked estrogen-induced ERK activation. *Conclusions:* Xenoestrogens, like physiologic estrogens, can evoke downstream kinase signaling involving selective interactions of ERα with $G_{\alpha i}$ and caveolin I, but with some different characteristics, which could explain their disruptive actions.

1. Introduction

The actions of the physiologic estrogen estradiol (E_2) in high pM to nM concentration ranges are most often associated with reproductive function, and with the development of cancers in reproductive tissues. Other endogenous estrogenic compounds can be more prevalent during other life phases where they can have significant effects on tissue development, function, and disease states. Estrone (E_1) is a significant estrogenic hormone contributor in both reproductive (~0.5–1 nM) and postmenopausal (150–200 pM) women and in men; estriol (E_3) levels are much higher in pregnant women (~10–100 nM) than in nonpregnant women (<7 nM) [1], and changes in free E_3 levels in pregnancy have been associated with complications of eclampsia [2] and the incidence of Down's syndrome in offspring [3]. All three of these physiologic estrogens are also produced by aromatases in a number of nonreproductive tissues where their effects may extend beyond reproductive functions [4,5]. Therefore, loss or enhancement of these physiological estrogenic effects due to interference by xenoestrogenic compounds could affect human and animal health in a large number of tissues and life stages. Nongenomic signaling actions of E_1, E_2, and E_3 at physiologic concentrations have been demonstrated [6–8], and we have shown that xenoestrogens can interfere with their activity via this mode of action [9,10].

Alkylphenol (AP) xenoestrogens are structurally related variants (in aliphatic side-chain lengths) and are commercially useful in a variety of processes requiring surfactants. The related bisphenol A (BPA) has a substituted phenol group instead of a long side chain, and is a widely used and environmentally distributed plastics monomer [11]. We have previously shown that these compounds also signal via nongenomic estrogen receptor pathways, but with distinct timing and nonmonotonic dose–response characteristics compared to that observed for physiological estrogens [12–14], suggesting a mechanism for the deleterious effects of xenoestrogens.

Rapid nongenomic actions can be triggered by estrogens and other steroids to generate a variety of second messengers and

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diverse pathway activities in many cell types [15]. One of the signaling paradigms known to be utilized by estrogens are those involving G proteins, as demonstrated in vascular tissues [16–18] and neurons [19]. The particular G_{α} protein involved in most of those responses is of the "i" subclass. Membrane versions of ERs (mERs) are located in caveolar membrane specializations in some tissues [20–22], making them available to partner with many other signaling proteins including G proteins involved in propagating nongenomic signals, leading to further downstream activation of kinases and phosphatases, including the mitogen-activated protein kinases (MAPKs) [23,24].

Traditionally, receptors that associate with heterotrimeric G proteins are seven-transmembrane receptors, such as the G protein-coupled receptor 30 (GPR30 or GPER) which binds estrogens and mineralocorticoids [25]. In oocytes and in ovarian and breast cancer cells GPR30 has been shown to activate $G_{\alpha s}$ or $G_{\beta\gamma}$ [26,27]. However, the classical "nuclear" ERα, has also been shown to interact with $G_{\alpha i}$ [16–19] in endothelial and brain tissues. In one case ERβ was specifically studied for this role, but was not found to be involved [18]. In our GH3/B6/F10 rat pituitary cells nongenomic estrogenic signaling operates via a membrane version of estrogen receptor-α (mERα) [28,29], with some mostly inhibitory participation by the other membrane ERs (mERβ and GPR30) shown by use of selective ligands in selective (nM) concentration ranges [10].

The functional endpoints for nongenomic signaling are varied, dependent upon cell type, and often involve rapidly generated second messengers (reviewed in [30]). All three mER types (mERα, mERβ, GPR30) are known to have some nuclear effects downstream of their initial peri-membrane signaling activations (examples in [31–33]). Nongenomic and genomic actions of estrogens are thus a continuum beginning at the plasma membrane and sometimes culminating in nuclear actions when sustained signaling warrants a commitment of the cell to new synthesis of macromolecules [34], though some nongenomic actions such as peptide secretion can terminate in actions devoid of nuclear participation [35]. We selected the GH3/B6/F10 rat pituitary tumor cell line for study based on its robust expression of mERα and previous demonstrations of a variety of rapid signaling responses to estrogens that culminate in functional changes (reviewed in [24]) such as prolactin release, cell proliferation, apoptosis, and changes in cell shape and attachment to substrate. We have previously shown that all MAPKs can be rapidly phosphoactivated by a variety of estrogens in these cells and that resulting functional responses can be blocked when extracellular-regulated kinases (ERKs) are inhibited ([9,24], and references therein). Changes in the activation of MAPKs are generally associated with functions such as changes in cell number and the mechanisms leading to that outcome (cell division, cell differentiation, cellular apoptosis) (reviewed in [36,37]). Though prolactin release in these cells is initiated by Ca^{++} signaling [14], other aspects of secretion (e.g. vesicle docking and refilling) probably involve other signaling cascades including those of kinases [38].

Our current studies examine if the actions of these three physiologic estrogens (E_1, E_2, E_3), and the APs/BPA operate proximally via mERα and G protein-coupled signaling mechanisms. We relate downstream activation of one class of MAPKs, the ERKs, to interactions with a specific G protein subclass – αi.

2. Experimental

2.1. Reagents and cell culture

We purchased phenol red-free Dulbecco modified Eagle medium (DMEM, high glucose) from Mediatech (Herndon, VA); horse serum from Gibco BRL (Grand Island, NY); defined supplemented calf sera and fetal bovine sera from Hyclone (Logan, UT); penicillin–streptomycin and trypsin EDTA from Mediatech (Manassas, VA); charcoal and Triton X-100 from Sigma (St. Louis, MO). NF023 and NF449 inhibitors were purchased from CalBiochem (San Diego, CA). All other materials were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma–Aldrich (St. Louis, MO).

GH3/B6/F10 cells were routinely cultured in phenol red-free DMEM containing 12.5% horse serum, 2.5% defined-supplemented calf serum, and 1.5% fetal bovine serum with penicillin–streptomycin (50 U/ml). Cells were used between passages 13 and 20 to stably maintain the robust mERα expression levels [35,39] needed for our assessment of these nongenomic responses. Because serum levels of steroids can mask the responses we monitor, we removed small hydrophobic molecules, including steroids, from serum by stripping four times with dextran-coated charcoal; cells were grown in welled plates pre-coated with poly-D-lysine in these media for 48 h before treatments. For some treatments (BPA) we used multiple concentrations to avoid discrepancies that exist in the literature about activating vs. inhibiting effects of xenoestrogens (for example [40,41]) due to complex nonmonotonic concentration–responses that we have seen previously.

2.2. Antibodies (Abs) and Ab assay reagents

Abs to GTP-$G_{\alpha i}$ and GTP-G_{as} were from NewEast Biosciences (Malvern, MA); Abs to ERα (MC-20) and caveolin-1 (N-20) were from Santa Cruz (Santa Cruz, CA); ERα C542 Ab was from Assay Designs, Enzo Life Sciences International (Plymouth Meeting, PA). Our other G protein Abs were from Santa Cruz or CalBiochem: $G_{\alpha s}$ (k20); $G_{\alpha q}$ (E-17); $G_{\alpha/12,13}$ (A-20); several anti-$G_{\alpha i}$ subtypes [3(C-10), o/t/z(D-15)]; $G_{\beta 3}$ (C-16). Vectastain kits with biotin-conjugated secondary Abs and ABC-AP color development reagents were from Vector Laboratories (Burlingame, CA); fish gelatin from Bio-Rad (Hercules, CA); FITC and Cy3 conjugated secondary Abs from Jackson ImmunoResearch Laboratories (West Grove, PA); Duolink reagents were from Olink Bioscience (Uppsala, Sweden).

2.3. pERK plate immunoassay

GH3/B6/F10 cells were treated with nM concentrations of all estrogens (plus 10 fM BPA) for 5 min, emphasizing this earlier time point as a known nongenomic response [12,42]. Briefly, 10,000 cells were plated in poly-D-lysine-coated wells of 96-well plates, deprived of serum steroids, and then treated with physiologic estrogens (E_1,E_2 or E_3), xenoestrogens (APs or BPA), 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 20 nM) as a positive control, or ethanol vehicle as a negative control. The cells were then fixed with 2% paraformaldehyde (PFA)/0.2% picric acid at 4 °C for 48 h, permeabilized with 0.1% Triton X-100 for 1 h at RT, blocked with 0.2% fish gelatin, and exposed to Ab for phospho-ERKs 1 and 2 overnight at 4 °C. Biotin-conjugated secondary Ab was then applied, followed by washing, development with Vectastain kit avidin-conjugated alkaline phosphatase, 0.1% Triton X-100 washes, and the addition of alkaline phosphatase substrate paranitrophenol phosphate (pNpp). The yellow product pNp was monitored at A_{405} nm in a model 1420 Wallac microplate reader (Victor, Perkin Elmer, Waltham, MA). The plates were then washed, dried, and stained with crystal violet solution as previously described [39,43] to estimate cell numbers for normalization. For inhibitor studies, selective G protein inhibitors used were: pertussis toxin (a $G_{\alpha i} + G_{\alpha o} + G_{\alpha z}$ inhibitor, 100 ng/ml or 9.5 nM), NF449 ($G_{\alpha s}$ inhibitor, 50 μM), and NF023 ($G_{\alpha i/o}$ inhibitor, 50 μM). The ICI182780 selective inhibitor for ERs α/β was used at 1nM, and the caveolae-disrupting chemical nystatin [44] at 50 μg/ml. Replicates of 7–8 wells were performed for pERK assays in each of 2–3 separate experiments.