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Rapid actions of xenoestrogens disrupt normal estrogenic signaling

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ABSTRACT

Some chemicals used in consumer products or manufacturing (e.g. plastics, surfactants, pesticides, resins) have estrogenic activities; these xenoestrogens (XEs) chemically resemble physiological estrogens and are one of the major categories of synthesized compounds that disrupt endocrine actions. Potent rapid actions of XEs via nongenomic mechanisms contribute significantly to their disruptive effects on functional endpoints (e.g. cell proliferation/death, transport, peptide release). Membrane-initiated hormonal signaling in our pituitary cell model is predominantly driven by mERα with mERβ and GPR30 participation. We visualized ERα on plasma membranes using many techniques in the past (impeded ligands, antibodies to ERα) and now add observations of epitope proximity with other membrane signaling proteins. We have demonstrated a range of rapid signals/protein activations by XEs including: calcium channels, cAMP/PKA, MAPKs, G proteins, caspases, and transcription factors. XEs can cause disruptions of the oscillating temporal patterns of nongenomic signaling elicited by endogenous estrogens. Concentration effects of XEs are nonmonotonic (a trait shared with natural hormones), making it difficult to design efficient (single concentration) toxicology tests to monitor their harmful effects. A plastics monomer, bisphenol A, modified by waste treatment (chlorination) and other processes causes dephosphorylation of extracellular-regulated kinases, in contrast to having no effects as it does in genomic signaling. Mixtures of XEs, commonly found in contaminated environments, disrupt the signaling actions of physiological estrogens even more severely than do single XEs. Understanding the features of XEs that drive these disruptive mechanisms will allow us to redesign useful chemicals that exclude estrogenic or anti-estrogenic activities.

1. Introduction

With too little estrogenic activity, a species cannot reproduce, and non-reproductive tissues also supported by estrogens (Es) can malfunction. However, too much estrogenic activity, or imperfect mimicry of estrogenic activity, as with xenoestrogens (XEs), can also cause some responsive organs to malfunction or develop cancers [1]. Therefore, Es must be very tightly regulated, and there are multiple hormonal regulatory mechanisms to ensure this control. Our studies examine the cellular control mechanisms by which XEs interfere with this regulation via the relatively novel rapid nongenomic signaling pathways. As the relatively insensitive genomic pathways often require very high doses (μM–mM) of XEs to be affected, and this is incongruous with animal studies showing actions at environmentally relevant concentrations, nongenomic signaling initiated at membrane receptors for Es can better explain the potent effects of XEs on functions.

Contamination of our environment with chemicals that can disrupt endocrine functions by mimicking Es is a growing problem, with many new compounds being adopted for various industrial and consumer uses [2–4]. It will become very difficult to keep up with the potential health threats posed by these chemicals if we do not decipher the mechanisms and decode the chemical structures that contribute to endocrine disruption. Unfortunately, mixtures of different XEs are common in the environment, so we must also begin to understand how XEs acting at the same receptors, signaling initiators, signaling integrators, and downstream functions can have potentially additive or even synergistic impacts [5] on stimulations or inhibitions of function. Though the hormesis effect [6] offers various explanations for why physiological hormone effects do not simply plateau, but are depressed at higher concentrations, these safety mechanisms may also prevent overstimulation and harmful consequences from mixtures of XEs. These disruptions occur in multiple functional systems influenced by endogenous Es including development, reproduction, metabolism, behavior, and immunity.

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Hormonal influences are summed or "blended" together with actions caused by other important cellular regulators by funneling upstream signaling streams into downstream summative "nodes" such as the mitogen-activated protein kinases (MAPKs). The resultant activity determined by phosphorylation levels of a given signaling integrator in that class (like the extracellular regulated kinases [ERKs]) then goes onto deliver the message to downstream cellular machineries that coordinately control major cellular fates such as proliferation (together with malignant transformation), migration, differentiation, or death. This alteration of central kinase activation states by posttranslational modifications is a fundamental mechanism of cellular regulation. Such changes are differentially initiated by ligands (including Es and their analogs) binding at receptors at or near the cell surface [7]. MAPK responses oscillate with time and fluctuate up and down with concentration (are non-monotonic) [8–10]. Several types of mechanisms can be involved including different receptor populations and subtypes [11,12], phosphatase activations, and the engagement of different signaling cascades [13–17].

Here we summarize our demonstrations of the rapid nongenomic signaling mechanisms by which XEs act very potently with nonmonotonic patterns in a pituitary lactotrope cell line. We also present examples of how XEs alone and in mixtures oppose the actions of endogenous Es, how chemical modifications of XEs alter but do not diminish their effects on signaling, and finally how XEs also affect a functional response.

2. Experimental

2.1. Reagents, cell culture, and treatments

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

Our use of non-transfected cell systems avoids artifacts due to receptor or other component overexpression and hetero-expression, where partners can be in short supply, and results can therefore be harder to interpret. 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 4 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 XE treatments we used multiple concentrations to avoid discrepancies that exist in the literature about activating vs. inhibiting effects (for example [40,41]) due to complex nonmonotonic concentration–responses that we have seen previously. We challenged adult female levels of E_2 (1 nM) with various XEs singly and in combinations.

Antibodies (Abs) to GTP-$G_{\alpha i}$ and GTP-G_{as} were from NewEast Biosciences (Malvern, MA); Abs to unmodified G proteins were from Santa Cruz or CalBiochem. Abs to ERα (MC-20) and caveolin-1 (N-20) were from Santa Cruz (Santa Cruz, CA); ERK Abs were from Cell Signaling Technology, Beverly, MA. Vectastain kits with biotin-conjugated secondary Abs and ABC-AP color development reagents were from Vector Laboratories (Burlingame, CA). Duolink reagents were from Olink Bioscience (Uppsala, Sweden).

2.2. Co-localization by epitope proximity ligation assay (PLA)

We used a relatively new technique to determine the in situ association of two proteins of interest in our studies. The revised PLA protocol [44] determines the nearness of partnered protein epitopes [45]. Potentially near epitopes are tagged with primary Abs made in two different species, recognized in turn by two different anti-species Abs having attached complementary oligonucleotides. When the two epitopes are sufficiently close (⩽35 nm) the attached oligonucleotides hybridize, producing a template for a rolling circle DNA amplification, which is subsequently probed with oligonucleotides containing fluorescent nucleotides. Signals appear as discreet dots by fluorescence imaging. To visualize a single protein, epitopes from two parts of the same protein are chosen, and to show protein partnering, epitopes from each of the putative partners are probed.

GH3/B6/F10 cells were cultured on cover slips overnight and washed twice with PBS before fixation with 4% paraformaldehyde (PFA) for 20 min, which does not permeabilized cells [46]. The cells were then blocked with Duolink blocking buffer for 30 min at 37 °C, followed by incubation with primary Ab overnight at 4 °C and washed. In unpermeabilized cells proteins such as membrane ERα are expected to be exposed on the outside of the plasma membrane. Then the cells were re-fixed with 2% PFA for 5 min and permeabilized with 0.1% Triton X-100 for 10 min. Subsequent incubation with primary Ab for proteins inside the plasma membrane ($G_{\alpha i}$, caveolin-1) was for 2 h at RT, followed by washing. Appropriate anti-species secondary Abs to which oligonucleotides had been conjugated (anti-rabbit PLA probe PLUS and anti-mouse PLA probe MINUS) were then incubated with the preparation for 2 h at 37 °C, followed by treatment with Duolink ligation solution for 30 min at 37 °C. Finally, cells were incubated with the Duolink amplification-polymerase solution for 100 min at 37 °C, and then labeling oligonucleotides, followed by washing and mounting on slides with 4 μl of Duolink 2 Mounting Medium containing DAPI fluorescent dye for staining nuclei. The slides were kept at −20 °C before being viewed with confocal microscope.

Confocal images were acquired with a Zeiss LSM-510 Meta confocal microscope with a 63× water immersion objective (1.2 NA). Multi-track sequential acquisition was done with excitation lines at 364 nm for DAPI and 543 nm for the PLA red probe. Respective emissions were collected with 385–470 nm and 560–615 nm filters. Frame size was 512 × 512, and the final image was a collection of an 8-frame Kallman-averaging. The pinhole was properly adjusted to give the best confocal resolution according to the objective numerical aperture and wavelengths. The pixel size was 140 nm. Optical slices were kept constant in both channels (364 and 543 nm). *Z*-stack acquisition was done with 0.5 μm steps, and an additional optical zoom of 2.0 was applied over the region of interest. 3D renderings were done using Imaris 7.0 software.

2.3. pERK plate immunoassay

Briefly, 10,000 cells were plated in each well of a poly-D-lysine-coated 96-well plate, deprived of serum steroids, and then treated with physiological Es (E_1, E_2 or E_3), XEs (alkyl phenols [APs], bisphenol A [BPA], or bisphenol S [BPS]), 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% PFA/0.2% picric acid at 4 °C for 48 h, permeabilized with 0.1% Triton X-100 for 1hr 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