Xenoestrogens can mimic or antagonize the activity of physiological estrogens, and the suggested mechanism of xenoestrogen action involves binding to estrogen receptors (ERs). However, the failure of various in vitro or in vivo assays to show strong genomic activity of xenoestrogens compared with estradiol (E2) makes it difficult to explain their ability to cause abnormalities in animal (and perhaps human) reproductive functions via this pathway of steroid action. E2 has also been shown to initiate rapid intracellular signaling, such as changes in levels of intracellular calcium, cAMP, and nitric oxide, and activations of a variety of kinases, via action at the membrane. In this study, we demonstrate that several xenoestrogens can rapidly activate extracellular-regulated kinases (ERKs) in the pituitary tumor cell line GTH/F10, which expresses high levels of the membrane receptor for ER-α (mER). We tested a phytoestrogen (coumestrol), organochlorine pesticides or their metabolites (endosulfan, dieldrin, and DDE), and detergent by-products of plastics manufacturing (p-nonylphenol and bisphenol A). These xenoestrogens (except bisphenol A) produced rapid (5–30 min after application), concentration (10^{-11}–10^{-8} M)-dependent ERK-1/2 phosphorylation but with distinctly different activation patterns. To identify signaling pathways involved in ERK activation, we used specific inhibitors of ERs, epidermal growth factor receptors, Ca^{2+} signaling, Src and phosphoinositide-3 kinases, and a membrane structure disruption agent. Multiple inhibitors blocked ERK activation, suggesting simultaneous use of multiple pathways and complex signaling web interactions. However, inhibitors differentially affected each xenoestrogen response examined. These actions may help to explain the distinct abilities of xenoestrogens to disrupt reproductive functions at low concentrations. Key words: environmental estrogens, ERks, estradiol, phytoestrogens, prolactinoma cell line, rapid estrogen effects. Environ Health Perspect 112:1481–1487 (2004). doi:10.1289/ehp.7175 available via http://dx.doi.org/[Online 28 July 2004]
ERK (pERK) monoclonal antibody (Ab), anti-mouse horseradish peroxidase–linked Ab, and lysis buffer were obtained from Cell Signaling Technology (Beverly, MA). Paraformaldehyde was from Fisher Scientific (Fair Lawn, NJ). BAPTA-AM (B-BA) was from Molecular Probes (Eugene, OR), and PP2, Ag 1468 (AG 14), and Ly294002 (Ly) were from Calbiochem (San Diego, CA). ICI 182,780 (ICI) was from Tocris (Ellisville, MO). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

**Materials and Methods**

Phenol red–free Dulbecco’s modified Eagle medium (DMEM) was purchased from Mediatech (Herndon, VA). Horse serum was obtained from Gibco BRL (Grand Island, NY); defined supplemented calf sera and fetal bovine sera were from Hyclone (Logan, UT). Endosulfan and DDE were purchased from Ultra Scientific (North Kingstown, RI). From Vector Laboratories (Burlingame, CA), we purchased biotinylated universal anti-mouse/rabbit IgG, Vectastain ABC-AP (avidin/biotinylated enzyme complex with alkaline phosphatase) detection systems, levamisol (endogenous alkaline phosphatase subtype inhibitor), and para-nitrophenol phosphate (pNpp; the substrate for our alkaline phosphatase reaction). Phospho-p44/42 ERK (pERK) monoclonal antibody (Ab), anti-mouse horseradish peroxidase–linked Ab, and lysis buffer were obtained from Cell Signaling Technology (Beverly, MA). Paraformaldehyde was from Fisher Scientific (Fair Lawn, NJ). BAPTA-AM (B-BA) was from Molecular Probes (Eugene, OR), and PP2, Ag 1468 (AG 14), and Ly294002 (Ly) were from Calbiochem (San Diego, CA). ICI 182,780 (ICI) was from Tocris (Ellisville, MO). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

**Cell culture.** Our clonal rat prolactinoma cell line GH3/B6/F10 was selected for high expression of mER-α (Pappas et al. 1994). Cells were routinely subcultured in DMEM containing 12.5% horse serum, 2.5% defined supplemented calf serum, and 1.5% fetal calf serum. For individual experiments, cells were deprived of steroids for 48 hr after plating by substituting DMEM containing 1% charcoal-stripped (4×) serum. All test estrogens were dissolved in ethanol (EtOH) at a 10−2 M concentration to create a stock solution and then
diluted into experimental media to yield final concentrations from $10^{-8}$ to $10^{-12}$ M. The EtOH concentration used as the vehicle control was 0.0001%.

**Fixed cell–based ELISA.** To estimate ERK phosphorylation quantitatively, we used a cell-based ELISA, which we previously developed and described (Bulyeva et al. 2004). Briefly, cells ($10^4$ cells/well) were plated in 96-well plates (Corning Incorporated, Corning, NY) and withdrawn from serum hormones by incubation in medium containing 1% charcoal-stripped serum for 48 hr before experiments began. The cells were next treated with hormones and estrogen mimetics for 3–30 min, and then fixed with 2% paraformaldehyde/0.2% picric acid at 4°C for 48 hr. After fixation, the cells were incubated with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 hr at room temperature (RT), and then with primary Ab against pERK (1:400 in PBS/1% BSA/0.1% Triton X-100) overnight at 4°C. After a wash with PBS, biotin-conjugated secondary Ab (1:300) in PBS/1% BSA was added for 1 hr at RT. The cells were again washed in PBS and incubated with Vectastain ABC-AP solution (100 µL/well) for 1 hr at RT, and then Vectastain alkaline phosphatase substrate (pNpp solution) with levamisole was added to each well (100 µL). Plates were incubated in the dark for 30 min at 37°C, and the signal from *para*-nitrophenol (pNp) was read at A$_{405}$ in a model 1420 Wallac microplate reader (Perkin Elmer, Boston, MA). The pNp signal was normalized to cell number, determined by using the crystal violet (CV) assay (Campbell and Watson 2001). Briefly, after washing away alkaline phosphatase reaction reagents with double-distilled H$_2$O, the plate was completely dried at RT. CV solution (0.1% in water, filtered) was added at 50 µL/well, incubated for 30 min at RT, and washed out with double-distilled H$_2$O. Dye was released from the cells with 50 µL/well acetic acid (10% in water) at RT for 30 min. The A$_{590}$ signal was then read in the microplate reader.

**Statistics.** Data were compared for significance of differences using Sigma Stat 3 (Jandel Scientific, San Rafael, CA) and one-way analysis of variance (significance accepted at $p \leq 0.05$).

**Results**

Xenoestrogens can cause unique time-dependent patterns of ERK phosphorylation. E$_2$ ($10^{-9}$ M) produced rapid (3, 15, and 30 min after application) and bimodal (with apparent periods of dephosphorylation between activation periods) ERK phosphorylation. Xenoestrogens at $10^{-9}$ M also caused ERK activations but with distinct temporal patterns (Figure 1). According to these patterns, compounds could be divided into several groups. Endosulfan and nonylphenol did not cause an initial (3 min) stimulation, but instead caused only a delayed single ERK phosphorylation peak at 30 min (which we designated slow-phase-only responders). DDE and dieldrin caused a single peak of activation at 6–10 min and were unable to cause a second sustained activation at 30 min (fast-phase-only responders). Coumestrol produced a rapid response (significant by 6 min), but the phosphorylation levels never declined after the activation, as was seen with the other active compounds. Bisphenol A did not produce any significant changes from the basal level of ERK phosphorylation during the 30 min assessment time and was not examined further in this study. All active xenoestrogens produced only a monophasic activation, failing to mimic the bimodal E$_2$ activation.

**Xenoestrogens can be potent activators of ERK phosphorylation but with unique concentration-dependent patterns.** At optimal stimulation time points (Figure 1), different concentrations of E$_2$ and xenoestrogens were compared in their ability to activate ERKs (Figure 2). E$_2$ (tested at 3 min) was active in two concentration ranges: very low levels ($10^{-14}$ M) and higher, but still physiological, levels ($10^{-9}$–$10^{-8}$ M). Nonylphenol and coumestrol showed similar patterns of potency, with dual ranges of activation similar to that seen with E$_2$. Endosulfan was able to produce phosphorylation at almost all tested concentrations but still showed an apparent loss of activity centered on the $10^{-10}$ M concentration. DDE and dieldrin were not active at low concentrations (picomolar and lower) but were active in the concentration range centering on $10^{-9}$ M. Thus, although some subtle differences were observable between activation patterns for each compound, basically two patterns of stimulation were seen: compounds active in both the subpicomolar and nanomolar ranges (E$_2$, endosulfan, nonylphenol, coumestrol, dieldrin, and dioxin) at 3 min, and compounds active in both the picomolar and lower nanomolar ranges (E$_2$, endosulfan, nonylphenol, coumestrol, dieldrin, and dioxin) at 30 min.

![Figure 2. Concentration-dependent changes in the phosphorylation status of ERK. Each compound was tested at its previously determined time optimum (Figure 1): E$_2$ (A) at 3 min, coumestrol (B) at 6 min, p-nonylphenol (C) and endosulfan (D) at 30 min, and DDE (E) and dieldrin (F) at 6 min. Data are presented as percentage (mean ± SE) of control values (which were set to 100); n = 78–85 wells from three different 96-well plates.

*Statistically significant ($p < 0.05$) compared with vehicle (0.0001% ethanol)-treated controls.
and coumestrol) versus compounds active only in the nanomolar range (DDE and dieldrin).

Possible pathways for ERK activation for different compounds. To detect possible signaling pathways through which E2 and xenoestrogens could affect pituitary tumor cells, we used inhibitors that have been described in the literature to pinpoint various mechanisms leading to ERK phosphorylation (Belcheva and Coscia 2002; Lowes et al. 2002). ICI and AG 14 are specific antagonists of estrogen and EGFRs, respectively. Nystatin (Nys) is a cholesterol-binding antibiotic that disrupts membrane architecture (Ushio-Fukai et al. 2001). B-TA is a Ca<sup>2+</sup> chelator. PP2 is a Src kinase inhibitor, and Ly is a PI3K inhibitor. An example of each type of xenoestrogen based on temporal activation patterns shown in Figure 1 (fast-phase activator DDE, slow-phase activator endosulfan, and sustained-activator coumestrol) was examined for each of these inhibitor actions. All time points in their activation profiles were examined to determine when each mechanism might come into play (Figures 3–5). Inhibitor data were divided into two groups for clarity of presentation. Figures 3A, 4A, and 5A group together the responses to inhibitor compounds that can interfere with receptors (ERs, EGFRs) or disrupt membrane structures housing receptors: ICI, AG 14, and Nys (group A). Figures 3B, 4B, and 5B group together data for compounds whose substrates are mostly localized in the cell’s cytoplasm or are adjacent to the cell membrane and part of the downstream signaling cascades initiated at the membrane: B-TA, PP2, and Ly (group B).

Inhibition of endosulfan-stimulated ERK activation is shown in Figure 3. In these assays, endosulfan stimulated ERK significantly only at 30 min (as in Figure 1). Only ICI and Ly inhibited the endosulfan-provoked ERK activation at 30 min. The activity of ICI implicates ER-α in this process [because this subline does not express ER-β (Campbell and Watson 2001; Norfleet et al. 1999)]. However, even at times when endosulfan could not significantly elevate basal phosphorylation of ERK (3–15 min), all tested inhibitors were able to further deactivate basal ERK activity levels at some of these time points (e.g., AG 14 at 15 min; all group B compounds were effective at 3 and 15 min: PP2 at 6 and 10 min; Ly, ICI, and Nys at 6 min). Such inhibitions are xenoestrogen dependent because the inhibitors alone do not cause these dephosphorylations (Bulayeva et al. 2004).

DDE produced ERK activation only at 6 min (Figure 4), as expected from earlier studies (Figure 1). At this time point, ERK phosphorylation was inhibited by AG 14, PP2, and Ly. Although at other time points DDE did not raise ERK activation levels above basal, the addition of inhibitors nevertheless did lower activity to subbasal levels (all at 3 min; AG 14, PP2, and Ly at 15 min). Altogether, all tested compounds had an effect on basal ERK activity levels at some time point, but some tended to affect this outcome earlier in this time frame compared with others.

Coumestrol activated ERKs from 6 min onward in our assay (as shown in Figure 1F and in Figure 5). During the preactivation phase (3 min), basal levels of phosphorylation were further lowered by ICI, Nys, and B-TA. During the 6 min onward coumestrol activation phase, ICI was never effective at lowering ERK phosphorylation levels. AG 14 was effective at 6–15 min time points, and PP2 during the entire stimulation phase, which suggests early involvement of EGFR and Src kinase. Nys disruption of membrane structure (15–30 min) and Ly inhibition of PI3K (15 min) were effective only during these short temporal windows. B-TA’s chelation of Ca<sup>2+</sup> was effective only very late in this sequence, at 30 min. Therefore, most inhibitors were effective at some point, although some later than others.

**Discussion**

An important and surprising conclusion from our studies was that all tested estrogenic compounds, except bisphenol A, elicited rapid membrane-initiated actions at very low concentrations compared with their reported potencies in classical genomic pathways (Gutendorf and Westendorf 2001; Hodges et al. 2000; Inoue et al. 2002). All active compounds were able to produce rapid (3–30 min) ERK phosphorylations in the nanomolar concentration range, and some (E2, coumestrol, nonylphenol, and endosulfan) were also active in the subpicomolar range. Compounds from different classes of endocrine disruptors with dissimilar chemical structures (e.g., endosulfan...
as an organochlorine compound vs. nonylphenol as a simple phenolic detergent) can produce the same time-dependent activation pattern for ERKs. Coumestrol, a phytoestrogen, initiated a sustained ERK activation that had no temporal pattern similarity with any of the other tested compounds, including E2. None of the tested compounds was able to precisely repeat the E2 pattern of activation, which may contribute to their disruptive effects on estrogen-mediated endocrine functions.

The bimodal E2 time-dependent response seems to superimpose the patterns from both groups of other response-producing compounds: fast phase (during the first 10 min) and slow phase (not until 30 min). Interestingly, the most potent endocrine-disrupting chemical in genomic action assays, bisphenol A (Cappelletti et al. 2003; Recchia et al. 2004; Sato et al. 2003), was unable to produce time-dependent ERK activation. However, studies in progress show that bisphenol A, although unable to trigger ERK activation, nevertheless is somewhat effective at triggering Ca2+ influx, resulting in prolactin secretion (Wozniak et al., unpublished data). Thus, there are likely to be specific pathways within the nongenomic signaling network that individual compounds will trigger, leading to different functional end points. Therefore, each xenoestrogenic compound must be tested for an array of possible mechanistic routes of action.

Several tested xenoestrogenic compounds (coumestrol, nonylphenol, and endosulfan) demonstrated a bimodal dose–response curve for ERK activation similar to that seen with E2. This is reminiscent of the same bimodal dose–response pattern reported previously for rapid prolactin release after E2 (Watson et al. 1999b) and E2-BSA (Watson et al. 1995) treatment. The reason for this gap in dose responsiveness at intermediate concentrations is still not understood, but it is interesting that other estrogens in the present study demonstrate the same phenomenon. These very low effective doses for xenoestrogens demonstrate that many environmental contamination levels previously thought to be subtoxic may very well exert significant signal-and endocrine-disruptive effects, discernable only when the appropriate mechanism is assayed. Possible reasons for these potent effects not being noted previously are that little testing of the nongenomic pathway has been done, many tests did not examine such low concentrations, and some test conditions probably did not adequately remove endogenous estrogen levels (as we have done by use of low quantities of extensively charcoal-stripped serum) to reveal effects of these low concentrations. The potent effects we see on nongenomic signaling mechanisms could explain why concentrations previously determined to be inactive via genomic mechanisms still have toxic and teratogenic effects on wildlife (Brucker-Davis et al. 2001). Therefore, the threat levels of these compounds to wildlife, and probably humans, need to be reconsidered.

The complexity of multiple signaling pathways triggered simultaneously is probably related to the organization of ERs within membrane substructures (caveolae or membrane rafts), where they encounter many signaling machineries (Chambliss et al. 2000; Nadal et al. 2000; Razandi et al. 2002). Our data indicate that the disruption of a nongenomic signaling cascade midway in its time course caused by Nys (e.g., for coumestrol) probably corresponds to disruption of this cholesterol-rich meeting place for ligands and receptors with their down-stream signaling partners. Interestingly, only endosulfan effects failed to be inhibited by disruption of cholesterol-rich membrane structure, perhaps implicating different membrane sub-domains as locations for the actions of different compounds. Alternatively, endosulfan signaling may move into the intracellular compartment rapidly after initiation and earlier than 3 min (and earlier time point assessment using these methods would be technically difficult).

Although here we have only directly assessed ERK activation as a signaling cascade end point, the participation of upstream signaling repertoires was implicated by our specific inhibitor assays. We found that all examined pathways can participate in ERK activation but that different xenoestrogens use different subsets of these pathways. Table 1 summarizes the vulnerability of E2- (Bulayeva et al. 2004), endosulfan-, DDE- and coumestrol-initiated actions to inhibitors of different signaling components. E2- or xenoestrogen-treated cells showed inhibitions of both stimulated ERK phosphorylation levels and background levels of phosphorylation. Our time course measurements allowed an analysis of when pathway inhibitions affected the outcome of ERK phosphorylation, and we noted whether this was very early after treatment (3 min) or later (≥ 6 min). Although these times are arbitrary cutoffs, they allowed us to highlight some possible temporal differences in the effects of compounds’ pathways. All xenoestrogens shared activation via all pathways, although compounds differed in their timing of pathway engagement. For example, inhibitors of action via the EGFR and ER were sometimes effective only after 6 min. This could mean that the activation

Figure 5. Effects of different inhibitors on coumestrol (Coum)-induced ERK activation. (A) Inhibition effects for membrane levels components ICI, Nys, and AG 14. (B) Effects for postmembrane signaling system components B-TA, PP2, and Ly. Cells were pretreated with inhibitors at optimal effective concentrations and for optimal times of action: 1 μM ICI for 40 min, 50 μg/ml Nys for 40 min, 10 μM B-TA for 40 min, 10 μM PP2 for 20 min, 10 μM Ly for 40 min, 250 nM AG 14 for 20 min, or 0.01% DMSO vehicle (control) for 40 min, and then stimulated with coumestrol at 1 nM. Values shown are mean ± SE; n = 39–79 wells from three to six 96-well plates.

**Table 1. Xenoestrogens each have unique signaling pathway inhibition patterns during rapidly initiated estrogenic actions.**

| Inhibitors | E2 | Endosulfan | DDE | Coumestrol |
|------------|----|------------|-----|------------|
| Ca2+       | +  | +          | +   | +          |
| Membrane   | +  | +          | +   | +          |
| Src        | +  | +          | +   | (+)        |
| PKC        | +  | +          | (+) | (+)        |
| EGFR       | (+) | (+)      | +   | (+)        |
| ER         | (+) | (+)      | +   | +          |

*, inhibition effect at 3 min; †+, inhibition effect at ≥ 6 min. For comparison, the E2 response inhibition data summarized here are taken from Bulayeva et al. (2004). All time points where the combination of xenoestrogen and inhibitor showed a significant reduction in ERK phosphorylation levels below the hormone-untreated background level are shown.
sequence took some time to reach the level of a receptor (EGFR is downstream) or that a unique conformation of receptors in the plasma membrane could initially prevent binding by antagonists (ER). Although all xenoestrogens shared activation via the PI3K pathway, PI3K inhibitors could not lower DDE- or coumestrol-mediated ERK phosphorylation levels until ≥ 60 min, so perhaps progression to this level of signalings required variable amounts of time depending upon the compound initiating the response. A possible complication to our interpretation of these data is the recent demonstration that Ly can have antiestrogenic activity by binding to ER (Pasapera Limon et al. 2003).

Inhibitors also interfered with ERK phosphorylation levels that were not stimulated by xenoestrogens above untreated background levels. For example, endosulfan, which elevates ERK phosphorylation levels that were not stimulated by 17beta-estradiol (Pasapera Limon et al. 2003). For this reason, we conclude that 17beta-estradiol and environmental estrogens significantly affect mammalian sperm function. Hum Reprod 18:100–107.

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