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Review

Endocrine disruption via estrogen receptors that participate in nongenomic signaling pathways☆

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ABSTRACT

When inappropriate (non-physiologic) estrogens affect organisms at critical times of estrogen sensitivity, disruption of normal endocrine functions can result. Non-physiologic estrogen mimetics (environmental, dietary, and pharmaceutical) can signal rapidly and potently via the membrane versions of estrogen receptors, as can physiologic estrogens. Both physiologic and non-physiologic estrogens activate multiple signaling pathways, leading to altered cellular functions (e.g. peptide release, cell proliferation or death, transport). Xenoestrogens' mimicry of physiologic estrogens is imperfect. When superimposed, xenoestrogens can alter endogenous estrogens' signaling and thereby disrupt normal signaling pathways, leading to malfunctions in many tissue types. Though these xenoestrogen actions occur rapidly via nongenomic signaling pathways, they can be sustained with continuing ligand stimulation, combinations of ligands, and signaling that perpetuates downstream, eventually also impinging on genomic regulation by controlling the activation state of transcription factors. Because via these pathways estrogens and xenoestrogens cause nonmonotonic stimulation patterns, they must be carefully tested for activity and toxicity over wide dose ranges. Nongenomic actions of xenoestrogens in combination with each other, and with physiologic estrogens, are still largely unexplored from these mechanistic perspectives.

Contents

Abbreviations: Ab, antibody; DAT, dopamine transporter; DES, diethylstilbestrol; E2, estradiol; E3, estriol; E1, estrone; ERK, extracellular-regulated kinase; ERR, estrogen receptor-related receptor; ER, estrogen receptor; GPER, G protein-coupled estrogen receptor; MAPK, mitogen-activated protein kinase; MEK, kinase activators of ERK; mER, membrane estrogen receptor; SERMs, selective estrogen receptor modulators; SmERMs, selective membrane estrogen receptor modulators.

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1. Introduction

Estrogens are triple-edged swords. If women have too little of them they can experience problems such as reproductive failure, bone loss, hot flashes, skin changes, and some cardiovascular system vulnerabilities and cognitive declines [1]. Too much of them can result in cancers such as for the breast, uterus, colon, and pituitary [2], or other malfunctions such as blood clots [3] and nausea/eating disorders [4,5]. Exposure to the wrong estrogens (xenoestrogenic mimetics) could result in endocrine disruption of functions normally mediated by physiologic estrogens [6]. There are many different types of estrogens to consider as candidates for estrogenic or estro-disruptive cellular actions. Since many tissues of males also have estrogen receptors, they will also respond to both physiologic estrogens and xenoestrogens. Some of these actions in both males and females could be of the organizational (nonreversible) types that occur during development [7].

Compounds that have estrogenic effects can act in several ways. Acting through an estrogen receptor (ER) in the cell nucleus, they can directly change the expression of genes via binding to DNA response elements, or binding to other transcription factors that bind to response elements [8]. Acting via an ER at the surface of the cell, they can rapidly initiate cascades of chemical signals (specific ions, lipids, cyclic nucleotides, etc.) which then percolate through a series of kinases and phosphatases to control their eventual targets by adjusting their phosphorylation levels [9,10]. While these membrane-initiated actions generally happen rapidly, they may take some time to travel to the functional end of the pathways or to build up levels of products that change function. They may also be sustained by repeated reactivation and perpetuation down signaling cascades. Post-translational modifications brought on by nongenomic signaling can have a variety and multiplicity of downstream effects on functional molecules. Of these (and other) possible estrogen-induced mechanisms, only the genomic pathway has yet been extensively examined, and xenoestrogens are very weak via that mechanism. Data are beginning to emerge indicating that xenoestrogens may be much more potent via the non-nuclear (nongenomic, membrane-initiated) mechanisms.

2. Different kinds of ERs, their different subcellular distribution, and association with different cellular signaling mechanisms

Historically, genomic (directly transcriptional) responses to steroids acting via their nuclear receptor mediators have been the most studied and thoroughly described with respect to signaling partners, modulators, and biochemical products (RNAs and proteins) [11]. Though very rapid responses to estrogens have been observed for decades [12–14], only recently have separate nongenomic receptor-mediated signaling mechanisms been assigned to them. A variety of ERs (α, β, and GPER) have been linked to nongenomic estrogenic responses, including some ERα splice variants [15,16]. Though ERs α and β are highly homologous in sequence and structure [17], the GPER (formerly known as GPR30) is of an entirely different receptor class homologous to other seven transmembrane G protein-coupled receptors [18]. Another class of so-called orphan (without clear ligand assignments) receptors, the estrogen receptor-related (ERR) receptors, has so far not been implicated in rapid responses and nongenomic signaling. It is still unclear why such a variety of ERs would be necessary to mediate the effects of estrogens. However, there are quite a few different estrogens (see Section 3) and this may offer one reason, as we learn more about selectivity of some ligands for certain receptor forms [19]. However, it is interesting that when more than one ER is present in the same tissue or cell type for either genomic or nongenomic responses, ERα tends to be the driver of responses, while ERβ and GPER, when in the presence of ERα, tend to antagonize its responses [20–22].

Among the unique correlations of rapid nongenomic actions with a receptor is one linking recognition of a specific receptor epitope (see Fig. 1) to a rapid nongenomic response time-frame. The hinge region epitope for the H151 antibody (Ab) has very interesting properties [23]. When this receptor region is blocked by Ab binding in live unpermeabilized cells (meaning that the Ab can only see the membrane form of receptor), rapid responses to estrogens in those cells are blocked [24]; however, when that same Ab is applied to cells after estrogens are administered, recognition of the epitope by the Ab is blocked for several minutes [25]. In addition, an Ab applied to a very nearby epitope (epitope R3/4; also recognized by Ab ER75 [26]), in the absence of estrogens, triggers the same estrogenic response (prolactin release) as does the E_2 ligand [24,25]. This is an interesting connection between membrane receptor specific subtype (ERα) recognition and a rapid functional response. Perhaps careful testing of the many Abs now available for different ER subtype epitopes can make some parallel connections and uncover some new therapeutic uses for such Abs.

3. So many different kinds of estrogens

3.1. Other physiological estrogens

Besides the most often studied estrogen – cycle-dominant estradiol (E_2) – there are other prominent physiologic estrogens with significant impact at different life stages, such as E_1 (estrone, elevated postmenopausally) and E_3 (estriol, elevated during pregnancy). There are also many modified physiologic estrogens or metabolites, such as catechol estrogens, methoxy estrogens, sulfated estrogens, etc. [27,28]. These other physiologic estrogens have long been labeled weak estrogens because they were tested exclusively via the genomic signaling pathway. Now we find that some of them (that have so far been tested) are actually quite potent via the nongenomic signaling pathway [29–32]. Their ability to act potently may relate to actions at particular life stages of women in which these hormones are quite prominent. In pregnancy E_3 levels climb steadily until at parturition they are the predominant estrogen available in the circulation; abnormally low amounts of E_3 are associated with fetal risk for diseases like Down's syndrome [33] and eclampsia [34]. In peri- and post-menopause, the levels of E_1 rise until they become a dominant hormonal influence [35]. It is at such times that lifelong exposures to some estrogens begin to cause tumors in a variety of estrogen target organs with high receptor numbers (the most sensitive). It is interesting that at this same time, signaling may switch from hormones that are predominantly known for their potent genomic actions (E_2) to those that act potently via only the nongenomic pathway (E_1 and E_3). Is this a protective mechanism at a tumor-prone time? Are the high levels of E_3 present at the end of pregnancy also protective—against eventual estrogen-induced tumor induction in exposed fetuses or pregnant women?

3.2. Environmental estrogens

There are also many different classes of environmental (toxic contaminant) estrogens. Products containing these compounds litter our landfills and leach into our land and water sources (plastics, industrial surfactants, and pesticides). Some xenoestrogens such as pesticides (e.g. dieldrin, endosulfan) and plastics monomers such as bisphenol A (BPA) have known disease associations [36]. Though the mechanisms are not well understood, BPA has become a frequent topic of news reports and regulatory agency debates because

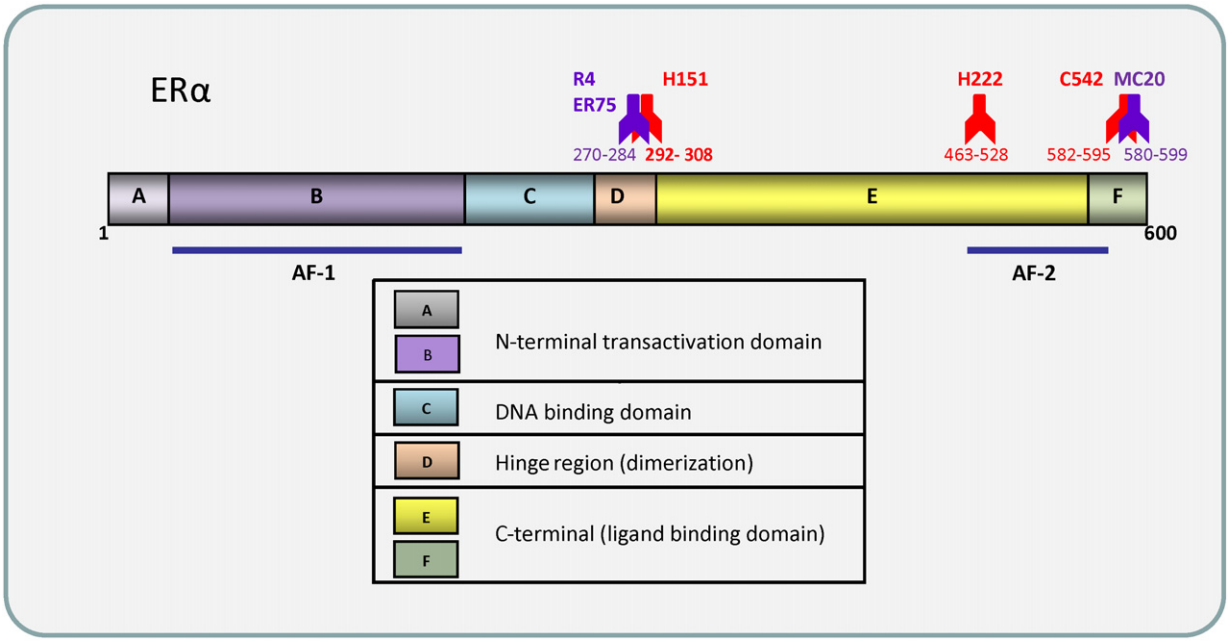

Fig. 1. An ERα domain map showing a number of different epitopes with functional properties. Hinge region epitopes for Abs R4 vs. H151 have special properties with respect to triggering or blocking (respectively) rapid actions, as described in the text. Although the 600 amino acid rat receptor is shown, some of the Abs were raised to the 595 amino acid human receptor (H151, H222, C542, symbols in red) or the 599 amino acid mouse receptor (MC20, rodent Ab symbols in purple), so these amino acid ranges (shown below each Ab symbol) will be approximate for the rat receptor. For reference to functional domain landmarks, AF-1 and AF-2 are the ligand independent and ligand-dependent transcription transactivation domains for nuclear ERα, though the function of these domains in the membrane receptor are likely to be interaction sites for proteins other than partnering transcription co-modulators. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of its prevalence in the environment [37], detectable levels in more than 90% of Americans and other populations [38], its actions on a variety of tissues and cell types [39,40], and at vulnerable developmental stages (e.g. developmental factors in asthma, breast cancer, and diabetes in rodent systems [41–43]). These compounds clearly affect functions in sensitive animal models [44], yet their activities could not be explained by genomic cellular mechanisms and the better known nuclear forms of ERs. We and others have recently started approaching this problem by studying the much-neglected atypical or nonclassical estrogenic signaling mechanisms—that is, the rapid membrane-initiated estrogenic signaling or nongenomic pathway [45]. We also have begun to ask these questions about ordered sets of environmental estrogen compounds that vary incrementally in their structural features, to try to decipher what makes a compound a good nongenomic pathway estrogen [46], as compared to the many studies already determining what makes a good nuclear signaling pathway estrogen.

3.3. Pharmaceutical estrogens

There are also a large number of pharmaceutical estrogens either purified from naturally occurring sources (e.g. equine estrogens) or produced synthetically, such as diethylstilbestrol (DES), contraceptive estrogens, antiestrogens (which are often tissue-selective estrogen receptor modulators [SERMs]). Again, how these estrogens mediate their actions is mostly known only for the nuclear transcriptional actions of ERs. Information about their membrane receptor-initiated signaling is nonexistent, or just beginning to be examined.

3.4. Phytoestrogens and their possible role as therapeutics

Many plant estrogens are available both via foods (many of them major components of typical Asian diets) [47] and as dietary supplements marketed by the health food industry. Prominent among these are the isoflavones (present in soy-based foods) and coumesterol (from sprouts and red clover). The prevalence of grape products (especially red grapes, red wine) and nuts in some diets are touted as the reason certain cultures gain health benefits from the actions of the phytoestrogen provided by these foods—resveratrol [48]. Molds that grow on grains can become dietary estrogens when they contaminate food items made from these grains (e.g. zearalenone [49]). Much speculation has arisen about the benefits or risks of these dietary components on disease incidences [47] and about the actual ingredient in foods that cause the effects (for instance isoflavones vs. other unidentified compounds that accompany isoflavones in soy-based foods [50]). Another complication in interpreting the beneficial vs. harmful actions of the soy isoflavone genistein is its well-known alternative role as an inhibitor of tyrosine kinases and other enzymes important to cellular signaling mechanisms [51].

Are there any estrogens that may alleviate the effects of estrogen withdrawal at surgical or natural menopause, while not promoting tumors? Phytoestrogens have been suggested as a possible subtype to fulfill this therapeutic role [47]. They have been called very weak estrogens because of their poor performance in transcription assays [52], and were originally thought to act by just replacing more potent estrogens on nuclear receptors. Yet they do have functional effects by themselves [53,54], both acute (e.g. genistein causing uterine bleeding) and more subtle long-term effects (across populations of women who eat lots of them and have better bone health, and lower cardiovascular and cancer risks). However, the exposure of nursing infants to high concentrations of phytoestrogens in soy-based formulas has been noted as a possibly inappropriate developmental exposure [55]. We recently found that though phytoestrogens do not have the same effects as a powerful tumor-inducing estrogen DES in a rodent model for pituitary cancer, even when present at high (though still dietarily obtainable) concentrations, they do, however, exacerbate increased cell nuclear size and size variability induced by the carcinogen DES,

perhaps related to induction of aneuploidy [56,57]. We will have to examine phytoestrogens and their combinations more carefully in many cancer-causing or -inhibiting tissue scenarios now that we know that they can operate potently via the nongenomic signaling pathway [57,58], with possible complications of nonmonotonic concentration-dependence (see Section 4).

3.5. Selective membrane estrogen receptor modulators (SmERMS)

We and others have studied a variety of estrogens and compared their estrogenicity across many different signaling assays. Though weak in the genomic pathway, many have recently been found to be very potent in the nongenomic signaling pathway (reviewed in [9,59,60]), dependent upon the tissue, developmental or reproductive stage, and other regulatory circumstances; so the story of their actions is very complex. By comparing their actions in different tissues we find that they are selective membrane estrogen receptor modulators, which we have dubbed SmERMs. This undoubtedly stems from the availability of different signaling partner molecules in different cell types, as they have been shown to directly interact with these partners in different tissues [61,62], just as nuclear ERs are well known to have different signaling partners (co-modulators) in different tissues [63].

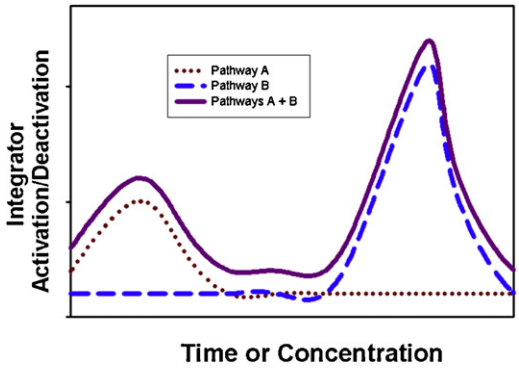

Fig. 2. The contribution of multiple pathways to a composite endpoint can result in oscillations. This theoretical diagram of the contributions of Pathway A in conjunction with Pathway B can be applied to both temporal and dose considerations. An example of a common endpoint could be the phosphorylation level of a MAPK. Here Pathway A ends in endpoint activation early, or in response to low concentrations of a triggering estrogenic ligand. Pathway B has the same activation endpoint, but that signaling cascade arrives at the endpoint later, or in response to higher concentrations of the estrogenic ligand. However, since both pathways are activated by the same ligand–receptor interaction, the endpoint integrates these actions, the composite of which oscillates.

4. Nonmonotonic and oscillating responses and their causes

A curious feature typical of the actions of both physiologic estrogens and xenoestrogens via nongenomic signaling mechanisms is their oscillating time courses and nonmonotonic concentration-responses. What is the basis of these patterns characteristic of these complex responses? Possible mechanisms have been previously reviewed including different receptor levels, subpopulations, oligomerization, compartmentalization, and non-receptor-mediated effects, [9,64–66]. However, we now know that estrogens activate a myriad of signaling cascades, probably dictated by the complement of ERs engaged, and by the availability of signaling partners in given tissues and cell types [10,59,67]. To borrow an analogy from the principles of electrical circuitry, the lesson of global signaling analysis is that cells seem to be hooked up "in parallel" instead of "in series," as we used to think of signaling pathways. We have examined several pathways that are simultaneously activated by estrogens in pituitary cells. By introducing pathway-specific inhibitors at different time points along the way toward eventual extracellular-regulated kinase (ERK) activation in these cells, we determined that signaling initiated by different estrogens passes down these pathways at different rates [68]. The same is probably true for activation of specific signaling cascades by different concentrations or combinations of estrogens. If one considers the contribution of multiple pathways in a time- and dose-dependent manner, then a composite picture of these results impinging upon the same target [for example, a downstream MAPK (mitogen-activated protein kinase) such as ERK], could sum to an oscillating pattern. See a theoretical example of this in Fig. 2. Here Pathway A ends in ERK activation (its signaling endpoint) early or in response to low concentrations of a triggering estrogenic ligand. Pathway B has the same ERK activation endpoint, but that pathway signal arrives at ERK-phosphorylating MEKs later, or in response to higher concentrations of the estrogenic ligand. But since both pathways are activated by the same ligand–receptor interaction, the endpoint integrates these actions. Therefore, apparent nonmonotonic dose responses and oscillating temporal patterns of activation are generated. Of course, more than two pathways could participate, making a more complex summation with even more potential oscillations, or even sustained activations.

In addition, other conditions could also contribute to nonmonotonic responses. Different receptor targets might oppose one another. For instance, phospho-ERK activation by estrogens could be opposed by phosphatase activation by estrogens, and the timing or concentration dependence of these effects could be different. We and others have determined that specific phosphatases are responsible for some of these fluctuating ERK activities induced by estrogens or other activators in cells [69–72]. It has long been observed that hormonal responses decrease again after they reach an asymptote, and this phenomenon is called the hormesis effect [65,73]. Therefore, the more potent are hormonal effects, the greater their potential for inhibition at higher exposure levels. This could also be brought on by combinations of hormones or their mimetics acting via the same receptor. Hormetic inhibitions are very common in hormonal responses of many kinds, and are thought to be safety mechanisms to prevent overstimulation or conserve cellular resources.

In the end, one of the most important practical questions to answer about the toxicity of these compounds is: at which concentration(s) are they active? Because everyone previously believed xenoestrogens to be weak estrogens (via the nuclear pathway), the large majority of earlier scientific studies about them have been at very high concentrations (μM-mM). Due to the nonmonotonic nature of their responses, many of the tested effects may have missed the concentration ranges in which these compounds are the most effective. Our work and that of others suggests that xenoestrogens can be active down to the pM and fM level in some cellular assays of signaling or function [46,74,75]. The ability to see actions at such low concentrations depends upon sensitive, quantitative cellular response assay systems devoid of contaminating estrogens in the control samples. In addition, use of non-transfected cell systems avoids artifacts due to overexpression and heteroexpression of receptors, perhaps with appropriate signaling partners in short supply. Low concentrations of many xenoestrogens are common in the environment, and without this information about low concentration-induced responses, we are incorrectly assuming that xenoestrogens are ineffective and harmless. With such complicated response patterns, these kinds of toxicity/effectiveness testing must be done with wider and more detailed examinations of doses and times. Extrapolation of results from very high doses to predict lowest effective doses is no longer acceptable.

5. Types of nongenomic or membrane-initiated functional endpoints and their relationship to the speed of the response

Nongenomic steroid responses are often characterized as rapid responses. However, just because such responses initiate rapidly does not mean they cannot be sustained over long periods of time. In addition, some responses are very proximal to the initial signal, and others further downstream, requiring more time to reach the eventual target. Rapid initiation and response progression speed is used experimentally to rule out a genomic mechanism, but may not describe the entire course or persistence of the response. The following sections supply examples of different types of nongenomic responses.

5.1. Simple activations and deactivations—e.g. transporters and enzymes

Some changes elicited by steroids and their mimetics are very rapid and have simple and immediate functional outcomes. One example is changes to the activity of a transporter. We found that estrogens can quickly reverse the direction of the dopamine transporter (DAT), which probably involves a conformational change caused either by direct interaction of the ER with the transporter, or by initiation of signaling cascades that in the end modify the transporter post-translationally [29,76]. In PC12 cells at concentrations where both E_2 and BPA can cause a high efflux via the DAT, they accomplish this in different ways. E_2 causes mERα (mediating stimulatory effects) to traffic to the membrane while mERβ (mediating inhibitory effects) leaves the membrane. But when BPA causes high efflux, it does so by causing more DAT to traffic to the membrane. In contrast, BPA at a concentration that inhibits efflux causes all three types of ERs (α, β, and GPER) and the DAT to leave the membrane [74]. DAT modulation usually results in changes to dopamine uptake, but strikingly, both estrogens [29,77] and amphetamines [78–80] instead cause efflux by reversing the transport direction. Another example of such a direct change to a functional protein is the nongenomic estrogenic regulation of enzymes, including enzymes that create the physiologic estrogenic hormones themselves and their metabolites [81,82].

5.2. Affecting multiple components of the nongenomic signaling system—e.g. secretion

Some functional endpoints may require several steps, and so could involve nongenomic actions on several components. For example, estrogens and xenoestrogens cause pituitary cells to release prolactin stored in secretory vesicles very rapidly. However, after this initial release from vesicles poised at the membrane, other vesicles must travel to and then dock at the membrane [83]. Our studies in this system indicate that while rising Ca^{2+} levels can trigger fusion of the secretory vesicle with the cell surface (release), probably other signaling mechanisms also contribute to the secretory response to estrogens. Other compounds that trigger Ca^{2+} changes very effectively, cannot release prolactin as effectively as estrogens [84]. So in this case several signaling pathways probably need to be engaged, driving multiple actions required to maximally release peptides from cells.

5.3. Complex combinations of genomic and nongenomic actions—e.g. cell proliferation and apoptosis

Estrogens and xenoestrogens can also cause cells to proliferate or die. Examples of estrogen-induced proliferative effects occur in the pituitary and the breast [21,31,57,85]; estrogens can cause prostate cancer cells to die [86,87]. Steroid-induced apoptosis via nongenomic mechanisms is also seen in other tissue types such as glucocorticoid-induced killing of lymphoma cells [88]. These kinds of responses to estrogens are a composite of both rapid (nongenomic) and slow (genomic) mechanisms. Membrane-initiated signaling travels downstream via multiple signaling pathways and is integrated at kinase "nodes" (MAPKs) that sum many incoming signals (including those coming from estrogens, xenoestrogens, and other ligands). MAPKs accomplish this summation by displaying a degree of phosphorylation (activation). Then targets downstream of the MAPK node eventually lead to cell division, cell death, or even retooling (differentiation) depending upon the tissue and types of MAPKs activated or inactivated [59]. Thus they coordinate complicated global cell changes. MAPKs that are thought to selectively activate cell death pathways (JNKs and p38) may take some time to manifest their ultimate functions because of the long series of enzymatic activations, target protein cleavages, resultant protein migrations, and degradations that eventually deconstruct cells.

In addition, major signaling modes can intermingle. Estrogens can either induce or inhibit these pathways, via a complex mixture of enzymatic mechanisms and transcriptional control. Some transcription factors are downstream targets of kinases, and can be rapidly activated by phosphorylation [57]. For example, ERKs activate (phosphorylate) downstream transcription factors such as Elk, ATF2, AP1, and CREB [89–92]. Such activated transcription factors thereby transform the initial nongenomic action into an eventual genomic consequence. Therefore, the terms genomic and nongenomic become somewhat inaccurate in following these actions to their final signaling and functional endpoints.

6. Combinations of physiologic estrogens with xenoestrogens

In real life xenoestrogens are rarely present by themselves; in humans and animals environmental or dietary estrogens usually signal on top of a pre-existing level of life stage-dependent physiologic estrogen signaling. Thus it is important to understand how added xenoestrogens affect endogenous physiologic estrogen signaling mechanisms. So far, we have learned that depending upon their concentration, alkylphenol and BPA xenoestrogens can either enhance or inhibit the signaling activities (ERK activation, Ca^{2+}) and functions (prolactin release, cell proliferation) elicited by endogenous estrogens in pituitary cells [93,94] spanning a wide exposure range (fM to μM). We also showed the ability of phytoestrogens to modify actions of physiological estrogens in pituitary [57]; and some xenoestrogens to block efflux via the DAT in PC12 cells [74]. It is unfortunately very difficult to predict disruption toxicities from single point assays, because of the combined nonmonotonic effects of each of the combined estrogens. In general, the lowest xenoestrogen concentrations enhance the activity induced by a physiologic estrogen, and the highest (nM concentrations in our studies) inhibit the actions of physiologic estrogens. But while this is generally true, it is not universally true. That is, sometimes xenoestrogens enhance the hormonal endpoint at every concentration, sometimes they inhibit the endpoint at every concentration, and sometimes they fluctuate back and forth between inhibition and enhancement for compounds with extreme nonmonotonic response curves. This means that each compound will have to be studied very carefully, over a range of concentrations, and in a range of cell types.

7. Summary

We cannot extrapolate from well-behaved dose-responses to predict the actions of xenoestrogens, either by themselves, or in disrupting the actions of physiologic estrogens. Xenoestrogens do

not follow the expected and simple "dose makes the poison" rules. In fact, the complexity of the signaling mechanisms makes it imperative for researchers to test the whole dose range of exposure to these compounds to decide which produce dangerous (or therapeutic) effects. In addition, these effects can vary in different cell types, tissues, and organs and for different xenoestrogens. Thus we are just beginning to learn how complicated the questions are, and mapping out a strategy for testing each potential xenoestrogen thoroughly, so we can make responsible predictions and set guidelines, especially for exposures during times of particular developmental or tissue vulnerabilities.

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