Toxicity of Endogenous and Environmental Estrogens: What Is the Role of Elemental Interactions?

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Many naturally occurring and man-made chemicals present in the environment possess estrogenic activity. Examples include plant and fungal products, pesticides, plasticizers, and other agricultural and industrial chemicals. These environmental estrogens as well as endogenous ovarian estrogens are thought to initiate their physiological actions in target tissues largely via interactions with a nuclear receptor system. The resultant estrogen–receptor complex in turn affects transcription via its interactions with nucleotide sequences known as estrogen response elements (EREs) present in the regulatory regions of hormone responsive genes. A "consensus" ERE sequence GGTCAnnnTGACC was originally identified in the vitellogenin genes of birds and amphibians, but it is now clear that most naturally occurring EREs differ from this sequence in one or more bases. We and others have obtained both *in vivo* and *in vitro* data suggesting a differential interaction of receptor complexes containing different ligands with the multiple EREs present in mammalian systems. This raises the possibility that the toxicity of environmental estrogens may arise in part from a differential pattern of ERE activation by environmental compounds relative to endogenous ovarian estrogens. The experimental basis for such a paradigm and its toxicological implications are discussed in this paper. — Environ Health Perspect 103(Suppl 7):29–33 (1995)

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Introduction

It has been recognized for some time that a number of chemicals in the environment possess estrogenic activity in a variety of biological systems. These compounds include plant and fungal products, pesticides, plasticizers, estrogenic agents administered to livestock, and a variety of other chemicals (*1,2*). As these agents are capable of perturbing the normal hormonal milieu in humans and other animals, they can be properly considered as environmental toxicants. Similarly, it is clear that the pharmacological use of ovarian estrogens and synthetic compounds such as diethylstilbestrol (DES) can cause toxicity, e.g., the endometrial cancer seen in women receiving unopposed estrogens (*3*) and reproductive tract abnormalities in women exposed to DES *in utero* (*4*). For ease of discussion, we will refer to all such environmental and pharmacological compounds as environmental estrogens. What then is the mechanism of toxicity of such environmental estrogens?

One straightforward possibility is that these compounds alter the physiological patterns of target tissue function, proliferation, and development normally regulated by ovarian estrogens such as estradiol. The basic idea here is that exposure to these compounds is simply like being exposed to too much of an endogenous estrogen produced by the ovary. For example, environmental estrogens could interact with estrogen receptors in the hypothalamic/pituitary axis to alter circulating gonadotropin levels and affect reproductive function in both males and females. Such effects on differentiated function have been suggested as a possible mechanistic basis for the decrease in human sperm counts recently reported (*5*). A second example would be the increase in endometrial cancer resulting from the pharmacological use of unopposed estrogens for hormone replacement therapy in postmenopausal women (*3*). Since estrogens increase endometrial proliferation, this toxicity could result simply from an increased level of DNA replication by increasing the probability of introducing mutations into the genome due to mismatch errors. Alternatively, one could envision that estrogens might be promoters and act by expanding a clone of altered endometrial cells to form a tumor. In either case, estrogens would be acting by an extension of their physiological action to stimulate endometrial proliferation in preparation for implantation. Similarly, a third possibility is that inappropriate exposure to estrogens such as DES during development alters target cell differentiation and predisposes the tissue to subsequent tumor development. This again could be viewed as a case in which toxicity results largely from an extension of normal hormonal function—in this case the control of tissue differentiation.

It is implicit in this paradigm that environmental estrogens interact with the

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Abbreviations used: DES, diethylstilbestrol; ERE, estrogen response element; kb, kilobases; ER, estrogen receptor; ERR, estrogen receptor–receptor.

estrogen receptor to increase or decrease a biological response in a manner similar to that of endogenous ovarian steroids. In other words, all estrogens (environmental or endogenous) function in the same way to convert the receptor from an inactive state to an active state. The basic paradigm here is that the receptor is like a simple switch, i.e., it is "off" in the absence of an estrogen but is turned "on" by ligand binding, and all ligands convert the receptor to the same basic "on" state.

In the nucleus of target cells, estrogen receptors interact with the so-called estrogen response elements (EREs) of target genes. The EREs are DNA sequences of approximately a dozen nucleotides that enable the receptor to bind to target genes. The activated receptor bound to the ERE then interacts with other nuclear factors (e.g., other transcription factors) and the resulting interactions enhance or repress transcription. In this model the basic function of the ERE is to serve as the binding site on the DNA for the receptor-ligand complex, and little thought has been given to other possible regulatory roles for this element (*6–8*).

Given this perspective most research to date on environmental estrogens has focused on *a*) identifying environmental compounds that bind to the estrogen receptor, *b*) analyzing their pharmacokinetic profiles and bioaccumulation, *c*) developing bioassays predictive for estrogenic actions such as cellular proliferation, and *d*) defining the relationships between their chemical structures, receptor-binding properties, and subsequent biological effects.

More recently however, we have begun to consider the pharmacology of estrogen action from a somewhat different perspective. Our work and that of others suggests that the EREs of different genes may be differentially activated by the complex of the estrogen receptor (ER) with any given estrogen, and the complexes formed between the ER and different estrogens may exhibit selective patterns of ERE activation *in vivo*. This led us to consider a paradigm in which one may view the ERE and associated factors (e.g., chromosomal proteins, other transcription factors, etc.) as a functional receptor or estrogen receptor–receptor (ERR) for the traditional estrogen receptor, and the complex between the traditional receptor and an estrogenic ligand as the agonist in the system. While clearly speculative, this paradigm suggests some additional issues related to estrogenic substances in the environment. In the remainder of this paper we will discuss some of the experimental findings that led us to consider this paradigm and its potential implications for the toxicity of environmental estrogens.

Estrogen Response Elements Are Diverse

The paradigm noted above originally occurred to us from our studies on the EREs of hormone target genes. By way of background, the majority of experimental studies on EREs have used the so-called consensus ERE (c-ERE) originally identified in the vitellogenin genes of xenopus and chickens (*9,10*). This element is a 13-mer palindromic sequence, GGTCAnnnTGACC, that binds the estrogen receptor as a homodimer, confers estrogen inducibility to target genes, and is located in the 5′-flanking region of the endogenous genes (*9–12*). More recently, we and numerous other groups initiated studies to identify the EREs in other genes regulated physiologically by estrogens.

Our own work has focused on c-*fos*, c-*jun*, and other members of the AP-1 family of transcription factors, whose expression is regulated by physiological levels of estradiol in intact animals. Transcript levels of these genes are rapidly activated in the rodent uterus following *in vivo* administration of estradiol, and several studies suggested that this was a direct transcriptional effect of the hormone (*13,14*). Based upon the location of EREs in the vitellogenin gene and other genes, we initially searched the 5′-upstream region of the murine c-*fos* gene for a functional element but were unsuccessful. We next examined other regions of the gene and identified a functional ERE in the 3′-untranslated region of the gene approximately 6 kilobases (kb) from the start site of transcription (*15*). This element binds the estrogen receptor in cell-free band-shift assays (*15,16*) and confers hormonal responsiveness to both homologous and heterologous promoters (*15*). Interestly, the *fos* ERE has the sequence GGTCAnnnCAGCC, which contains one-half site (GGTCA) identical to that in the c-ERE but a second half site that is quite different (TGACC in the c-ERE vs CAGCC in the *fos* ERE).

More recently, we have identified a functional ERE in the rat c-*jun* gene that binds the estrogen receptor and confers hormonal inducibility (*17*). This element is located in the single exon of the *jun* gene and has the sequence GCAGAnnnTGACC. This sequence again has one-half site (TGACC) that is identical to that in the c-ERE, but the other half site differs in 3 of 5 positions.

Our results with c-*fos* and c-*jun* indicated that the EREs for these genes vary considerably in sequence and location from that of the consensus element and prompted us to examine the literature for EREs located in other hormone responsive genes. Table 1 lists a number of EREs that have been reported to date. Upon viewing this information, it was somewhat surprising to us to realize that the consensus ERE is present only in the vitellogenin gene of xenopus and chicken and that all other elements identified to date contain one to three changes in the 10 bases comprising the palindromic half sites of the c-ERE. The most common pattern, seen in all but two cases, is an element with one-half site identical to the c-ERE, and a second nonidentical half site.

Table 1. Identified estrogen response elements.

Source	Species	Sequence[b]	Location[c]	References
Vitellogenin A2	Xenopus	GGTCANNNTGACC[a]	5′-P	(*10*)
Vitellogenin II	Chicken	GGTCANNNTGACC[a]	5′-P	(*9*)
Ovalbumin	Chicken	GGTCA	5′-P	(*18*)
Calbindin	Rat	GGTCANNNTGATC	5′-P	(*19*)
Prolactin	Rat	GGTCANNNTGTCC	5′-p	(*20*)
LHb	Rat	GGACA(N)$_5$TGTCC	5′-p	(*21*)
c-*jun*	Rat	GCAGANNNTGACC	Exon	(*17*)
Creatinine kinase B	Rat	GGTCANNNCACCC	5′-P	(*22*)
Uteroglobin	Rabbit	GGTCANNNTGCCC	5′-P	(*23*)
MCF-7 pS2	Human	GGTCANNNTGGCC	5′-P	(*24*)
Oxytocin	Human	GGTGANNNTGACC	5′-P	(*25*)
Progesterone receptor	Human	GGTCGNNNTGACT	5′-*	(*26*)
c-*fos*	Human	CGGCANNNTGACC	5′-P	(*27*)
c-*fos*	Mouse	GGTCANNNCAGCC	3′-UT	(*15*)
Lactoferrin	Mouse	GGTCANNNTAACC	5′-P	(*28*)

[a]This sequence represents the consensus. [b]Underlined bases represent deviation from the consensus.In the case of ovalbumin gene, only a fully conserved first half-site is required for ER mediated induction. [c]Locations are 5′-P = 5′, promoter region; 3′-UT = 3′, untranslated region; 5′-* = 5′, untranslated and overlapping initiation of transcription.

Dose–Response Relationships for Estrogenic Responses Are Variable

What then are the possible biological ramifications of this diversity of hormone response elements? One obvious possibility is that different EREs interact differentially with the estrogen receptor. We have recently examined the relative binding affinity of the estrogen receptor to oligodeoxynucleotides containing the sequences of the consensus ERE and the c-*fos* ERE in an *in vitro* band-shift assay system (*16*). The results indicate that there is about a 10-fold difference in affinity of these two naturally occurring elements. Similarly, another set of measurements we recently performed indicate that there is a 3- to 5-fold difference in the relative affinity of the estrogen receptor for the c-*fos* and c-*jun* ERE sequences (unpublished observations). These results are consistent with several studies illustrating that minimal alterations (i.e., changes in one to two nucleotides) in hormone response elements can dramatically alter receptor binding and the ability to confer hormone inducibility to reporter constructs (*29–31*).

This raises the interesting possibility that differential affinities of the estrogen receptor for different EREs could lead to different dose–response curves for activation of different genes and hence lead to different dose–response curves for various biological responses to estrogens. This possibility prompted us to examine the literature for such differential dose–response relationships.

As much as 20 years ago, Clark and his colleagues (*32–34*) emphasized that different responses of the rodent uterus exhibit different dose–response curves for estrogens. For example, early tissue responses occurring 3 hr after estradiol administration exhibit a different dose–response curve than growth responses occurring at later times (*32–34*). This was thought to be due to the differential interaction of the estrogen receptor with different "nuclear acceptor sites" present in uterine chromatin (*35*) since specific ERE sequences had not been defined at this time. In other studies, Clark and his colleagues (*33*) also showed that different uterine responses (e.g., glucose oxidation vs organ weight) occurring at the same time (3 hr) after estradiol treatment also exhibit different dose–response curves. A possible explanation of these data is that ER interactions with EREs having different receptor affinities are responsible for the different dose–response curves observed.

Interpretation of such studies is complicated, however, because they used complex processes (e.g., glucose oxidation, tissue weight, etc.) as indices of estrogen action, and these processes are likely to be affected by many parameters that are difficult to analyze *in vivo*. More recently, however, several studies have examined the dose–response curves for different genes using the rodent uterus as an experimental system. For example, Pentecost et al. (*36*) noted a substantial difference in the dose–response curves for estradiol induction of actin and creatinine kinase. Similarly, we (*37*) observed approximately a 4-fold difference in the dose–response curves for induction of c-*fos* and c-*jun* in the uterus. A consequence of this difference is that a dose of estradiol (0.4 μg/kg) which increases c-*jun* expression to only 20 to 25% of its maximum increases c-*fos* expression to 75 to 80% of its maximum.

Given the traditional view of estrogen receptors as molecules containing a single class of binding sites for hormone agonists, it is very difficult to explain observations such as these without some type of differential interaction of the receptor–hormone complex with different target genes. This suggested to us a slightly different way to view the overall control of complex processes such as endometrial proliferation that are likely to involve the regulation of multiple genes by estrogens. One can envision the basic 13-mer ERE and its associated factors (e.g., chromosomal proteins, general transcription factors, nonreceptor regulatory proteins, etc.) as a functional complex that we shall refer to as estrogen receptor–receptor (ERR) in the following discussion. In a pharmacological sense one could view the ERR as the functional receptor in the system since it is interactions at this site that initiate the biological response, i.e., altered transcription. Concomitantly, one could view the complex between the classical receptor and an estrogenic ligand as the functional agonist. The ERR is thus the receptor in the pharmacological sense because it performs the dual roles of a traditional receptor, i.e., it binds the agonist and initiates the biological response. While somewhat semantic in nature, this paradigm offers a slightly different conceptual view of how steroids regulate complex biological processes such as growth and differentiation that probably involve many different genes.

The ERRs of different estrogen regulated genes have different ERE sequences (Table 1), and it is reasonable to expect that they may also contain a diversity of protein factors *in vivo*. If the ERRs of different genes have different structures, might they also have different properties such as affinities for the receptor–hormone complex? Such a paradigm readily allows one to explain the types of observations noted above in a system that contains only a single estrogen receptor. In addition, such a view of estrogen receptor signaling has potentially important ramifications for the toxicity of environmental estrogens.

Differential Response Patterns Produced by Different Estrogens

In situations where multiple receptor subtypes exist for a single ligand (e.g., multiple adrenergic and cholinergic receptors), pharmacological agents may exhibit selective patterns of receptor activation. Is it possible that this can occur in the estrogen receptor system? For example, do complexes formed between estrogen receptors and different ligands exhibit different patterns of responses, i.e., do they selectively activate different ERR receptors?

A possible example of such differential selectivity was provided by the studies of Korach and his colleagues (*38–40*) who examined several different uterotropic responses (induction of DNA synthesis, glucose-6-phosphate dehydrogenase activity, and progesterone receptor levels) following *in vivo* administration of a series of diethylstilbestrol (DES) analogs. They observed that the measured parameters showed differential patterns of responsiveness to the series of DES analogs and suggested that "the ability of a particular response to be increased may depend on the chemical nature of the ligand receptor complex and its interaction at genomic sites" (*38*). These results are certainly consistent with the possibility that complexes formed between the estrogen receptor and different DES analogs exhibit selective activation of distinct ERRs present in various hormone-responsive genes.

We (*41*) and Galand et al. (*42*) studied the ability of the antiestrogen nafoxidine to block different estrogen-induced responses in the intact animal and similarly concluded that the ER–nafoxidine complex exhibited selective interactions with different receptor binding sites within target tissue nuclei. This implied that the antiestrogen–receptor complex was able to block the ER–estradiol complex from interacting at some nuclear sites but not others. These intact organ responses were again somewhat complex and probably involved activation of multiple genes, but

more recent examples have appeared in studies of the effects of the antiestrogen tamoxifen and analogs of 17β-estradiol on expression of individual genes.

While tamoxifen is an antiestrogen in some systems, in the rat uterus it acts primarily as an estrogen agonist following acute administration; several studies have recently reported that tamoxifen and estradiol elicit different patterns of expression of *fos* and *jun* family members in this system (*43,44*). Some of these differences, such as the length of time that expression of some genes remains elevated, can be explained on the basis of pharmacokinetic differences in the two compounds. However, differences in the initial patterns of gene expression are more difficult to explain without postulating selective patterns of gene activation by the estrogen–receptor versus tamoxifen–receptor complexes.

Perhaps the clearest example of this possiblity to date comes from a recent series of experiments from Brooks and his colleagues (*45–47*). They examined the ability of a series of estradiol analogs to bind to the estrogen receptor and elicit biological responses including induction of the progesterone receptor, pS2 mRNA, cathepsin D mRNA, and CAT activity from reporter constructs containing the c-ERE. They found clearly different patterns of activation of these responses by the different estrogen analogs (*45–47*) and emphasized the possible biological significance of these observations.

Discussion and Implications for Toxicity of Environmental Estrogens

Taken together, these findings suggest that the toxicity of environmental estrogens could involve a number of factors and could occur via several related but distinct mechanisms. The first and most obvious type of toxicity could be that in which an environmental estrogen binds to the estrogen receptor and produces an estrogenic response. Toxicity in this case is simply due to hyperestrogenism, i.e., an extension of the physiological effects of estrogenic hormones.

A second type of toxicity, which has been proposed, is that some estrogens could also produce toxicities by virtue of their chemical rather than their hormonal properties. For example, it has been shown that administration of estrogens to certain species leads to the formation of DNA adducts (*48*). While not discussed in this manuscript, we note this possibility for completeness.

In this manuscript we suggest that a third, more subtle, type of toxicity could result if an environmental estrogen produced an imbalanced estrogenic response in a target tissue. As discussed above, this could occur in one of two basic ways. First, an environmental estrogen such as a pesticide, a phytoestrogen produced by plants, a plasticizer, etc., might bind to the estrogen receptor and produce a different conformation than estradiol. In this case, the resulting receptor–ligand complex might selectively activate/repress estrogen responsive genes differently than the naturally occurring ovarian hormone. Rather than simply creating a situation of too much estrogenic stimulation (which might be ameliorated by compensatory homeostatic mechanisms involving the hypothalamic-pituitary-gonadal axis), the result might be a qualitatively distinct state of estrogenization.

An imbalanced estrogenic response might also occur due to the possibility that estrogenic responses may exhibit different dose–response curves as illustrated in Figure 1. Consider for example the relative stimulation of genes A and B by estradiol alone or by the presence of estradiol plus an environmental estrogen. As drawn in this example, the ratio of expression of A:B in the presence of estradiol alone (point E_2) would be approximately 2:1. If, however, an environmental estrogen is also present

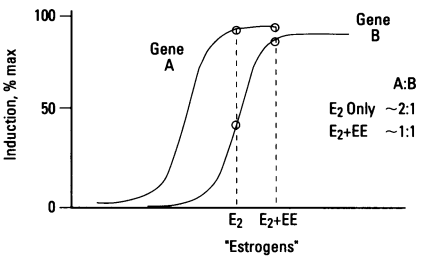

Figure 1. Hypothetical dose–response curves for two estrogen-regulated genes in the presence of estradiol alone (E_2) and in the presence of estradiol plus an environmental estrogen (E_2 + EE).

in the system (point E_2 + EE), the ratio of the two genes products now becomes 1:1.

The net result of such effects could be an imbalance in the levels of hormonally regulated growth factors, growth factor receptors, protooncogenes, and other key molecules in target cells. Such an altered profile of regulatory molecules could lead to abnormal proliferative and differentiative properties of hormone responsive cells. This might be particularly deleterious during critical developmental periods when cells may be especially sensitive to alterations in regulatory systems.

Many of the suggestions outlined in this discussion are admittedly hypothetical and speculative and will require much additional work to substantiate or refute. We also recognize that one can propose various other models which could equally well explain some of the observations such as multiple dose–response curves that we have discussed, e.g., the existence of cell membrane as well as intranuclear receptors as suggested by others. However, the ideas we have discussed stem from ongoing projects in our labs, which indicate that estrogen response elements are more diverse than we had previously appreciated, and we elected to offer them here to stimulate discussion and offer new perspectives on the possible mechanisms involving the toxicity of environmental estrogens.

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