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Predicting Health Effects of Exposures to Compounds with Estrogenic Activity: Methodological Issues

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Many substances are active in in vitro tests for estrogenic activity, but data from multigenerational and other toxicity studies are not available for many of those substances. Controversy has arisen, therefore, concerning the likelihood of adverse health effects. Based on a toxic equivalence factor risk assessment approach, some researchers have concluded that exposure to environmental estrogens is not associated with estrogen receptor (ER)-mediated health effects. Their rationale cited the low potency of these compounds in in vitro assays relative to estradiol, and the widespread exposure to pharmaceutical, endogenous, and dietary estrogens. This reasoning relies on two assumptions: that the relative estrogenic potency in in vitro assays is predictive of the relative potency for the most sensitive in vivo estrogen effect; and that all estrogens act via the same mechanism to produce the most sensitive in vivo estrogen effect. Experiment data reviewed here suggest that these assumptions may be inappropriate because diversity in both mechanism and effect exists for estrogenic compounds. Examples include variations in ER-ligand binding to estrogen response elements, time course of nuclear ER accumulation, patterns of gene activation, and other mechanistic characteristics that are not reflected in many in vitro assays, but may have significance for ER-mediated in vivo effects. In light of these data, this report identifies emerging methodological issues in risk assessment for estrogenic compounds: the need to address differences in in vivo end points of concern and the associated mechanisms; pharmaco-kinetics; the crucial role of timing and duration of exposure; interactions; and non-ER-mediated activities of estrogenic compounds. — Environ Health Perspect 105(Suppl 3):655–663 (1997)

Key words: estrogen, environmental estrogen, phytoestrogen, dose response, risk assessment, mechanism, pharmaco-kinetics

Introduction

A number of plant-derived and anthropogenic compounds are now known to have estrogenic activity (1–3). These compounds include plant-derived dietary consituents as well as plasticizers, surfactants, constituents of detergents, pesticides, and a variety of other chemicals (1,4–6). Identification of estrogenic activity is primarily based on results of in vitro assays that rely on indicators such as estrogen receptor (ER) binding, gene transcription, or cell proliferation, or on short-term in vivo assays such as uterine growth bioassays (7).

It is well known that estrogenic activity may be observed due to direct effects of a compound binding the ER and inducing gene transcription, and to indirect effects such as induction of enzymes involved in metabolism or synthesis of endogenous estrogens (8,9), effects on binding of endogenous estrogens to hormone binding proteins (10), effects on ER regulation (11), and others. Estrogenic compounds have also been reported to have other effects, such as involvement in other cell signaling pathways that may stimulate receptor-mediated estrogen action, e.g., protein kinase C (2,12,13), inhibition of microtubule polymerization (14), or inhibition of gap-junctional intercellular communication (GJIC) (15).

Exposure to estrogenic chemicals could be substantial because of their widespread use, yet data from sensitive in vivo studies are not available for many of them. Reproductive toxicity studies in animals are typically required for pesticides and can detect certain endocrine system effects; however, many chemicals in common use have not been screened for endocrine activity or examined in reproductive toxicity studies. Data from multigenerational toxicity studies in animals that allow identification of developmental effects are available for relatively few chemicals. Furthermore, new protocols for conducting multigenerational studies have identified more sensitive end points for endocrine effects on development than have been utilized in traditional protocols (16–18).

Public concern about the potential health effects of exposure to environmental pollutants with estrogenic activity, often referred to as environmental estrogens, has created pressure for scientists to make predictions about the significance of current exposures to environmental estrogens in the absence of good information on exposure, effects, or dose response (19–21). Predicting human health effects of exposure to estrogenic compounds involves synthesizing assay information on the activities of a broad spectrum of estrogenic compounds to which humans are simultaneously exposed, including endogenous and pharmaceutical estrogens, as well as phytoestrogens and environmental estrogens. Many types of compounds have been characterized as having estrogenic activity; predicting health effects associated with one class (environmental estrogens) requires consideration of the activities of, and interactions with, the other classes of estrogenic compounds.

To date, predictions of human health effects of exposure to these compounds have covered a wide range. Some researchers have hypothesized that there may be an association with serious health effects such as breast cancer and fertility (3,22–25). Others, however, have concluded that exposure to environmental estrogens will not be associated with any estrogen-mediated adverse health effects in humans because of...
the low potency of those compounds in the in vitro assays relative to endogenous estradiol, the high levels of circulating endogenous estrogen, and the fact that so many plant-derived estrogenic compounds are present at high concentrations in food (26). Although these factors are important to consider in predicting the health effects of exposure to compounds with estrogenic activity, the conclusion that environmental estrogens will not be associated with adverse health effects relies on several assumptions that must be carefully examined (27).

Review of these assumptions brings into focus a number of considerations that an empirically grounded risk assessment methodology for estrogenic compounds must address. These include the need to address differences between estrogenic compounds in in vivo end points of concern and the associated mechanisms; the impact of pharmacokinetics on dose to target tissue and type of response observed; the crucial role of timing and duration of exposure in determining type and severity of response; the adequate consideration of non-ER-mediated activities of estrogenic compounds; and the effects of mixtures, including interactions between endogenous hormones and substances that affect endocrine function.

**Toxic Equivalence Factors for Risk Assessment of Estrogenic Compounds**

Because of the paucity of comprehensive multigenerational toxicity studies for estrogenic compounds, the conclusion that exposure to environmental estrogens will not be associated with adverse health effects (26) relies on a toxic equivalence factor (TEF) risk assessment based on extrapolation from relative potency for ER-mediated activity in *in vitro* screening assays. The TEF risk assessment strategy was developed as part of the risk assessment for dioxin so that the *in vivo* toxic potency for the dioxin congeners could be expressed as 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) activity, based on relative potency in producing enzyme induction in *in vitro* assays (28).

In the case of dioxin, use of the TEF approach was predicated on the significant finding that the chemical has to bind to a receptor before causing any toxic effects (29). In addition, many chronic studies had been done to characterize the most sensitive end points for 2,3,7,8-TCDD, and to demonstrate similarity in mechanism and effects of 2,3,7,8-TCDD and other dioxin congeners (28,29). Use of the TEF for estrogenic activity relies similarly on two assumptions: that the relative estrogenic potency in *in vitro* assays for all compounds is predictive of the relative potency for the most sensitive *in vivo* estrogenic effect, and that all estrogens act via the same mechanism (e.g., binding ER) to produce the most sensitive *in vivo* effect.

One implication of making those assumptions for estrogenic compounds is the prediction that all estrogenic compounds, like the dioxin congeners, have a similar spectrum of ER-mediated effects and so are essentially interchangeable. In other words, diethylstilbestrol (DES), genistein, 17β-estradiol, chlordecone, nonylphenol, and other estrogenic compounds (1) are assumed to induce similar effects via the ER; and those effects are assumed to represent the most sensitive effects *in vivo*. These assumptions also allow the prediction that a high dose of a weak estrogen is equivalent to a low dose of a potent estrogen because, according to the TEF rationale, the compounds would differ from each other only in the dose required to produce the effects.

This article highlights emerging methodological issues in risk assessment for estrogenic compounds. It presents experimental data from *in vitro* and *in vivo* studies that suggest that the TEF assumptions may not be appropriate for all estrogenic compounds, and that additional *in vivo* and *in vitro* data are needed before such an approach can be applied with confidence. In particular, it considers mechanisms by which steroid receptors cause a diverse spectrum of effects depending on ligand, target tissue, timing of dose, presence of other stimuli, and other factors (30,31). It is possible that the factors responsible for this diversity of mechanism and effect may be important in differentiating the *in vivo* activities of estrogenic compounds. These considerations also suggest that the *in vitro* assays used to develop relative potency estimates have not been demonstrated to be representative of the mechanism that will be important for predicting the most sensitive *in vivo* effects.

Experimental data presented here also highlight examples in which timing or duration of dose, rather than simply size of dose, determines the magnitude and type of effect. The data also demonstrate the impact of pharmacokinetics on timing of dose, and consequently the impact of pharmacokinetics on type of response, rather than just size or duration of response. These factors are also important to consider when using a TEF approach to extrapolate from *in vitro* to *in vivo* effects.

In addition, many estrogenic compounds have multiple activities, both ER- and non-ER mediated (above). Although most chemicals have multiple mechanisms of toxicity, these may be particularly important to consider for estrogenic compounds because activity at more than one point in the steroid signaling system may not be identified in *in vitro* studies but may have profound implications *in vivo*.

Finally, although the question of how to fashion risk assessment techniques to address exposures to mixtures of chemicals has been discussed for some time, it may be a particularly important component of risk assessment for hormonally active compounds. This is important so that interactions between exogenous and endogenous hormonally active compounds can be considered, and so that synergistic or antagonistic interactions between estrogenic/antiestrogenic compounds can be addressed (22), as well as those between compounds with activity in more than one component of the endocrine system, such as estrogens and antiandrogens (32).

**Diversity in Mechanisms and Effects of Estrogenic Compounds**

The use of the TEF approach for risk assessment for estrogenic compounds relies on the assumption that all estrogens produce a similar spectrum of ER-mediated responses for the most sensitive *in vivo* effects and differ only in their potency relative to the reference compound. Exposure to environmental or pharmaceutical estrogens or phytoestrogens is assumed to be an extension of the exposure to endogenous estrogens; all estrogens are assumed to interact with the ER to increase or decrease biological responses in the same way as endogenous estrogens.

There are examples in the literature of the induction of somewhat different effects by different estrogenic compounds (30,31,33-43). Although it is well known that estrogenic compounds act on biological materials in different ways and have multiple effects, this information is reviewed here because of its relevance to risk assessment for these compounds. Specifically, these reports illustrate some of the difficulties in predicting *in vivo* health effects based on *in vitro* screening assays for estrogenic activity.
For example, the phytoestrogen coumestrol, a constituent of soy, has been established as an estrogen in MCF-7 cells (1) and immature rat uterus (33). In many assays, coumestrol acts very much like a typical estrogen. On the other hand, researchers have reported that coumestrol in ovariectomized rats behaved as an atypical estrogen. It has been known for many years that coumestrol has mixed estrogen agonist and antagonist activities (34,35). In more recent experiments, it increased uterine wet and dry weights but did not cause depletion of cytosolic ER, accumulation of nuclear ER, or uterine hyperplasia and DNA synthesis, all characteristic estrogen responses (36). Lack of uterine DNA synthesis was observed in these experiments even after multiple injections of coumestrol, indicating that coumestrol was not like estradiol or other short-acting or weak endogenous estrogens, which have been shown to mimic more potent estrogens following chronic dosing. Whitten et al. (37) investigated the influence of dietary coumestrol on estradiol action in the rat uterus and found that coumestrol acted additively with estradiol for some end points (increased uterine weight and decreased cytosolic ER binding) in these experiments but also dampened estradiol’s induction of progesterin receptors, uterine protein, and nuclear estrogen receptor binding (37).

Thus, in this system coumestrol’s activities do not appear to be identical to those of estradiol, even after multiple doses; and coumestrol modulates the activity of endogenous estradiol.

In the male mouse treated neonatally with DES, adult treatment with 17β-estradiol induces prostatic metaplasia while adult treatment with coumestrol or soy does not induce metaplasia or prevent 17β-estradiol-induced metaplasia (38). Makela et al. refer to coumestrol as a partial estrogen agonist in this system because it weakly induced c-fos expression, though estrogenic effects of coumestrol were generally missing in adult male neo-DES mice (38). These researchers also found that male mice fed a soy diet from fertilization onward and neonatally treated with DES had reduced dysplasia in the prostate compared with mice not fed soy diets; this effect was attributed to antiestrogenic activity of soy (39). In summary, coumestrol appears to be quite similar to 17β-estradiol in some systems under some conditions (e.g., MCF-7 cells, immature rat uterus), but acts as an incomplete estrogen or even modulates and dampens the effects of endogenous estrogens in other systems (male mouse, female rat). These variations in activity cannot necessarily be predicted from in vitro assays, but may have implications for in vivo response.

In another study, the effect of prenatal exposure to genistein, also a component of soy, was compared with that of DES and estradiol in rats (40). All animals except DES-treated females had smaller anogenital distance than controls. Genistein-treated females had decreased volume of sexually dimorphic nucleus in the preoptic area of the hypothalamus (SDN–POA) while DES and estradiol-treated females had increased SDN-POA volume compared to controls. In addition, genistein had a unique effect in that it delayed puberty onset. DES (but not estradiol or genistein) increased the incidence of atypical vaginal cycles (40). Thus, genistein, DES, and estradiol in this model induced different effects. The researchers also noted that the effects of prenatal and neonatal exposure to genistein were inconsistent. In previous experiments involving neonatal exposure to genistein, castrated female rats had decreased pituitary responsiveness to gonadotropin-releasing hormone (GnRH) and enlarged SDN–POA volume. However, prenatal genistein exposure in this experiment did not affect pituitary responsiveness to GnRH and caused a nonsignificant decrease in SDN–POA volume (40).

Although very little research has been done to compare activities of endogenous with environmental estrogens, one recent study compared the effects of two environmental estrogens, 4-tert-octylphenol (OP) and methoxychlor, in immature female rats (41). Both compounds accelerated vaginal opening. Methoxychlor exposure increased uterine weight but OP did not. In ovariectomized rats, 17β-estradiol and methoxychlor administered for 7 days induced uterine growth, vaginal opening, and elevated pituitary prolactin, while OP induced vaginal opening but did not stimulate uterine growth or pituitary prolactin (41). Thus, OP appears to exhibit a different spectrum of estrogenic responses than either estradiol or methoxychlor.

Although all the compounds discussed in these examples of variation in effects of estrogenic compounds were shown in the respective studies to bind to the ER, and most have been shown to induce cell proliferation in MCF-7 cells (1), there are many potential explanations for the different effects observed. For example, different pharmacokinetics, induction of enzymes involved in endogenous estrogen synthesis or metabolism, or other, unrelated effects of test chemicals may have accounted for the different responses observed. Timing of dose during development also seems to be an important modulator of estrogen action. These experiments involved complex physiologic responses involving multiple receptor-mediated processes, and sufficient data are not available to characterize the mechanisms responsible for the differences. However, the major point to note is that differences in responses to estrogenic compounds occur that could not be predicted by the TEF risk assessment approach that relies on relative in vitro potencies for a single effect.

Much more information is available on differences between activities of endogenous and pharmaceutical estrogens, such as DES. These experiments provide insight and support for the idea that different ER ligands may produce different patterns of ER-associated gene activation. A recent paper by Stancel et al. (31) discusses their hypothesis and that of others indicating that different estrogens may exhibit selective patterns in the activation of estrogen-responsive genes. These researchers report that the estrogen response element (ERE) for different genes varies in sequence and in location relative to the gene (31). They suggest that the ERs of different genes may be selectively or differentially activated by different ER–ligand complexes, and this may be responsible for the differential response patterns produced by different estrogens.

In support of this hypothesis, Stancel et al. (31) review a number of reports that suggest differential activation of ERs by different estrogens. Korach et al. (42) found that DES and a series of DES analogs, all of which bound to the mouse uterine ER with high affinity and caused nuclear ER retention and occupancy similar to that of estradiol, differed in their ability to induce responses such as induction of uterine DNA synthesis and stimulation of uterine glucose 6-phosphate dehydrogenase. These researchers speculate that the chemical nature of the ligand–receptor complex may influence its activity at different genetic sites. In addition, VanderKuur et al. (43) found that the relationship between ER binding affinity, nuclear binding of the ER complex, and induction of progesterone receptor was not consistent for a series of estradiol analogs examined.

Stancel et al. (31) discuss the toxicological implications of this proposed paradigm with respect to environmental and other
estrogens, noting that different conformations of ligand–ER could activate or repress genes differently than endogenous hormone and thus produce an imbalanced estrogenic response. For example, consider the possibility that estradiol produces hypothetical gene products A, B, and C in a 1:1:1 concentration ratio and another estrogen produces only products A and B and produces them in a ratio of 1:2. Because the signal from the two estrogens differs, it is possible that disruption of cell function could result that is not predicted by the effect of a slightly increased dose of estradiol, which in this hypothetical example would be expected to produce slightly more of A, B, and C in a 1:1:1 ratio. Thus, some estrogens could produce a state of estrogenization that is qualitatively different from that produced by endogenous hormone (rather than simply a quantitative extension of estradiol) and that perhaps cannot be compensated for by homeostatic mechanisms (37).

The examples cited here illustrate the difficulty of predicting the specific in vivo estrogen-related activities of compounds identified as estrogenic in screening assays. The many mechanisms by which steroid receptors bind a diverse set of ligands and produce an even more diverse spectrum of responses has been discussed by Fuller (30), who reviewed mechanisms by which specificity and diversity are generated at each step in the fundamental process of gene regulation by steroid receptors. Because these various mechanisms of creating diverse responses to signals involving a set of similar steroid hormone receptors are likely to be active in modulating the effects of various estrogenic ligands (above), they need to be considered in predicting the effects of exposure on human health.

**Can Screening Assays Help Predict in Vivo No-Effect Levels?**

Although these examples demonstrate that it is difficult to predict in vivo estrogenic effects at doses that are high enough to produce effects, it is worth considering whether relative potency in in vitro or short-term in vivo screening assays for ER-mediated activity could be useful in identifying a threshold dose, or no-effect level, for the activity of estrogenic compounds. A number of issues that would be important to address are briefly mentioned here. First, it would be necessary to identify the most sensitive in vivo effect and the conditions in which an in vitro test should be done to ensure that the relevant mechanism is captured. In other words, the normal parameters of the steroid signaling system in vivo, at critically sensitive times, would have to be understood, so that predictions could be made as to what level of perturbation to that signaling system would have no adverse effect. In evaluating whether a particular exposure to an ER ligand would result in binding and any subsequent effect, one would have to consider the many in vivo factors that would impact the ability of the ligand in question to bind the ER. Such factors might include sensitivity of target tissue, presence of another ligand, amount of receptor available, serum binding proteins, and others. Second, the recent reports of synergistic effects of multiple ER ligands applied as mixtures (22) suggest other important factors involved in regulating ER activity that need to be explored before the activity of a single ligand in an in vitro screening assay provides a basis for predicting in vivo estrogenic effects or lack of them.

In addition, consider the hypothesis of Stancel and others (31) that ER ligands vary in the specific pattern of genes activated. The in vivo end point associated with a test compound that activates one set of genes may be different from that expected from 17β-estradiol. Because the TEF approach is useful only when the most sensitive in vivo effect is similar for the test and reference compounds, it would no longer be valid to compare the potency of test compounds with the potency of estradiol. Furthermore, the in vivo assay in which relative potency is established would have to represent a mechanism relevant to the in vivo effect of concern, which in this hypothetical example would be a pattern of gene activation related to the end point of concern caused by the test chemical, not by estradiol.

Finally, biological systems closely regulate steroid hormone and receptor levels through multiple and complex mechanisms (30). Endogenous estrogens have multiple activities in this signaling system, so it is not surprising that the structural characteristics of some compounds that bind ER may also result in those compounds being active in other estrogen-sensitive processes, such as inhibition of enzymes involved in synthesis of endogenous estrogen (8). Most sensitive effects in vivo may occur due to compounds acting by multiple mechanisms in approximately the same dose range. The in vivo significance of these multiple activities is difficult to predict based on the study of one end point, such as ER binding. For example, polychlorinated biphenyls (PCBs) not only mimic thyroid hormones but may also bind thyroid hormone receptors in the pituitary, thus blocking thyroid-stimulating hormone release and inhibiting a mechanism that would compensate for reduced hormone levels (44). It is well known that different estrogenic compounds act through different mechanisms and produce multiple and varied effects. We are reviewing this information here because it needs to be considered when predicting in vivo health effects based on extrapolation from screening tests for estrogenic activity.

**ER Dynamics: Nuclear Accumulation and Impact of Pharmacokinetics**

The time course of nuclear accumulation of the ER–ligand complex has been studied in the context of efforts to understand mechanisms of estrogen-induced responses such as uterine hyperplasia (45–49). Generally, researchers using a mouse or rat uterine assay have shown a biphasic increase in nuclear ER levels to follow treatment with 17β-estradiol or DES. Increase in uterine wet weight follows the time course of the biphasic nuclear ER increase, with only the second phase described as "true uterine growth" due to increased cellular DNA synthesis. The first increase in nuclear ER accumulation and uterine weight is shown 1 to 3 hr after dose, and the second increase 7 to 9 hr after dose. Weak estrogens show the early response phase only, although multiple doses of some weak endogenous estrogens mimic the effect of more potent estrogens (7). Nuclear ER levels return to approximately control levels about 10 hr after treatment with estradiol (46). Cytosolic ER levels decrease as nuclear ER levels increase and ultimately increase above control levels (36,46).

Limited work has been done in these model systems to examine the time course of ER nuclear accumulation following treatment with a variety of estrogens. Katzenellenbogen et al. (50) evaluated temporal relationships between estrogen receptor binding and uterine growth for DES and stilbestrol derivatives. These researchers and others noted that increased retention of nuclear receptors correlated with prolonged elevation of uterine weight and stimulation of deoxycyclonate metabolism, or true uterine growth. The important role of pharmacokinetic factors in modulating the estrogenic effect of a weak
estrogen is apparent from their study. The weak estrogen dimethylsilbester (DMS) and its dimethylether DMS-(OMe)₂, which does not bind ER, were tested in the assay. Acting like a typical weak estrogen, DMS induced short-term but not long-term nuclear accumulation of ER and uterine growth. The DMS-(OMe)₂, which had to be metabolically activated to the weakly estrogenic DMS₁, induced long-term increases in nuclear ER and uterine growth because metabolic activation proceeded at a rate that simulated chronic dosing (50). Repeated dosing with some weak estrogens can cause long-term increases in uterine growth such as those caused by more potent estrogens (above). Thus, the pharmacokinetics of estrogens can modify not only the duration of a response, but also the type of estrogenic response observed.

Patterns of ER localization after treatment with coumestrol in similar assays shows some conflicting but interesting differences from ER localization after estradiol, estriol, or DES treatment. While Whitten et al. (33) found that coumestrol in the diet of immature rats increased nuclear ER concentrations, Markaverich et al. (36) reported that coumestrol failed to cause substantial nuclear accumulation of ER in ovariectomized rats, although it did cause increase in uterine wet and dry weights. Markaverich et al. (36) show a slight increase in nuclear ER after coumestrol treatment, with levels returning to control levels in less than 5 hr and then decreasing slightly below control levels through 24 hr. Treatment with a higher dose of coumestrol did not modify this pattern or increase nuclear ER, indicating that coumestrol was not able to function at a more potent estrogen in this system. Estradiol stimulation, on the other hand, caused a significant increase in nuclear ER that returned to control levels by 5 hr after treatment. Coumestrol appeared to induce long-term increases in cytosolic ER, which increased slowly but continuously over the 24 hr during which measurements were made.

In an experiment looking at coumestrol modulation of estradiol action in the rat uterus, Whitten et al. (37) found that animals that received coumestrol along with physiologic doses of estradiol for 90 hr had lower levels of nuclear ER than controls. When these animals were challenged with a single estradiol dose, the coumestrol-treated animals produced a smaller increase in nuclear ER than controls (37). Although there is conflicting evidence about whether coumestrol alone substantially increases nuclear ER (33,36), it does appear that a coumestrol diet diminished the nuclear ER accumulation after estradiol treatment (37). In another example of the differential abilities of various estrogens to induce nuclear accumulation, Martin et al. (51) reported that in MCF-7 cells, genistein and coumestrol (both soy derivatives) were less effective at translocating ER to the nucleus than zearalenol (a mycotoxin) and estradiol.

Hammond et al. (52) reported that the organochlorine pesticide chlordecone is estrogenic and interacts with rat uterine estrogen receptors. In experiments comparing estradiol and chlordecone, nuclear ER levels increased quickly following estradiol treatment and then decreased to nearly control levels by 12 hr posttreatment. Chlordecone, on the other hand, increased nuclear ER slowly, reaching maximum at 36 hr and maintaining that level through the end of the experiment at 48 hr. The long half-life of chlordecone, therefore, appears to moderate the relative potency of the compound in vivo, but may also moderate the qualitative estrogenic effect due to the potential importance of the time course of ER–ligand activity in the nucleus in determining the nature of the estrogenic response. For example, pharmacokinetics of the ER–ligand complex could affect the length of time that expression of some genes remain elevated (31).

In another example of how different estrogens exhibit different pharmacokinetics with respect to nuclear accumulation of ER, administration of o,p’-DDT to immature female rats caused translocation of ER to the nucleus that was maximal 3 hr after treatment; estradiol in this system caused maximal nuclear ER 1 hr after treatment (53). In experiments comparing the time course of uterine weight increases in rats following treatment with amosonic acid and DES, both test compounds induced an extended increase relative to estriol (6). Amosonic acid is an optical brightening agent that was tested for estrogenic activity after reports of sexual impotence among exposed factory workers (6).

It has been observed that the duration of nuclear ER accumulation affects the response observed, although this relationship has been explored only for a few endogenous and pharmaceutical estrogens (45–50). The fact that certain phytoestrogens and environmental estrogens show variation in timing of nuclear ER accumulation (33,36,37,52,53) strongly suggest that it may be important to consider timing and duration of dosing in determining effect. This is important because it is not clear that the effects of increasing the duration of nuclear accumulation of activated ER are equivalent to the effects of simply increasing the dose of estradiol. Pharmacokinetic considerations are also important, therefore, in determining not only dose to target tissue, but potentially also in characterizing the end point expected.

Consideration of Effects on Estrogen Synthesis, Metabolism, and Bioavailability

Because estrogen synthesis and metabolism in vivo are regulated by many factors including endogenous estrogen, it is not surprising that exogenous estrogens often also affect these regulatory mechanisms (8,23). In attempting to predict health effects of exposure to xenobiotic compounds, it is important to consider the ability of a compound to alter the endogenous hormone environment by influencing synthesis or metabolism of endogenous estrogens (and other endogenous steroids). Of course, these effects involve mechanisms that do not necessarily involve binding ER. Some compounds cause changes in levels of cytochrome P450 enzymes that are involved in estrogen metabolism (indirect estrogenic effects) (9,23,54), while other compounds that induce ER-mediated gene transcription also affect synthesis or metabolism of estradiol (8). Effects on the endogenous hormone environment cannot be accounted for by assuming that the consequences of exposure to exogenous estrogens are simply an extension of exposure to endogenous hormone, because the effects on hormone synthesis and metabolism vary among estrogenic compounds. Although it is not surprising that many compounds have multiple effects, consideration of multiple effects for estrogenic compounds may be particularly important because of the resulting difficulty of predicting interference with normal signaling processes.

For example, Bradlow et al. (23) have shown that exposure to a number of compounds, many of which are estrogenic, can affect the metabolism of 17β-estradiol by shifting the ratio of two metabolites, 2-hydroxyestrone and 16α-hydroxyestrone. This effect may be important for predicting health effects because the 16α-hydroxy metabolite is genotoxic and a potent estrogen, while the 2-hydroxyestrone metabolite is not reported to be genotoxic and is only very weakly estrogenic (55). Others have
reported that the plant-derived flavonoid quercetin, which increases the severity of estradiol-induced tumorigenesis in hamster kidney, operates by increasing the formation of the catechol estradiol metabolite 4-hydroxyestradiol, which may undergo redox cycling and generate free radicals (54).

Makela et al. (8) reported that several plant estrogens, including coumestrol and genistein, reduce the conversion of estrone to 17β-estradiol by inhibiting the estrogen-specific enzyme 17β-hydroxysteroid oxidoreductase Type 1 in vitro, but zearalenone and DES did not inhibit this enzyme. Thus, the phytoestrogens coumestrol and genistein, which have been reported by some as incomplete estrogens incapable of inducing all the effects of 17β-estradiol (36,40), may also decrease availability of active endogenous estrogen by inhibiting its synthesis. These types of differences in the combination of ER-mediated and other estrogen-related effects of exogenous compounds may have significant impacts on their potential health effects.

Differences in the bioavailability of compounds in vivo and effects of estrogenic compounds on the bioavailability of endogenous estrogens are additional factors that will modulate toxicity in vivo. These factors should also be considered in predictions of health effects of exposure to these compounds. One of the most important modulators of the availability of endogenous estrogens may be the serum-binding proteins like sex hormone-binding globulin (SHBG). While this protein can modulate the availability of endogenous estrogens, in most cases its ability to modulate the availability of exogenous estrogens remains to be explored. SHBG appears not to bind many environmental estrogens (16,56), but estrogenic compounds may affect SHBG binding to endogenous estrogens (57). This observation offers another mechanism by which exogenous estrogens may modulate endogenous estrogen activity.

The importance of the steroid hormone microenvironment within cells has been recognized and mechanisms of regulation of enzymes involved in estrogen metabolism and synthesis are being explored at the level of the target tissue (58–61). The effects of xenobiotics on systemic estrogen regulation may be different from their effects on estrogen regulation in the target tissue. For example, the cytochrome P450 enzymes that are inducible by different xenobiotics vary among tissues, which means that a compound may have different effects on estrogen metabolism in the liver and the breast, for example (61–64). Thus, it is important to consider the questions of synthesis, metabolism, and bioavailability at the level of the cell and target tissue, as well as at the systemic level. Of course, pharmacokinetics are also important in determining dose to target tissue. Concentrations of lipophilic chemicals in mammary adipose tissue, for example, may be much higher than serum concentrations, and exposure presented as body burden is often substantially different from exposure presented as daily intake due to pharmacokinetic considerations.

**Other Effects of Estrogens and Effects of Mixtures**

Environmental estrogens present a special challenge for risk assessment because they have the potential to be active in many different ways. Effects at multiple points in a signaling system in vivo may be difficult to predict from in vitro or short-term in vivo tests, as demonstrated for PCBs and thyroid hormones, discussed earlier (44).

In addition, researchers have reported the ability of DES, some stilbene estrogens, and the common environmental estrogen bisphenol-A, to inhibit microtubule formation (14). Endogenous estrogens and phytoestrogens tested in this cell-free assay did not have that effect. Inhibition of microtubules in intact cells may lead to the induction of micronuclei and aneuploidy, which may play a role in estrogen-mediated carcinogenesis (14).

The idea that estrogens may regulate cellular function at sites other than specific gene-regulating receptors has been explored recently (13,65). Plasma membrane-resident forms of ER have been proposed to explain observations of cellular responses to estrogen that occur within minutes and so cannot be explained through gene transcription (65). In addition, other researchers have shown that chemicals that activate peptide growth factor signaling systems, such as protein kinase-C activators, can also induce ERE-dependent transcription (12). These researchers showed that a protein kinase-C activator acted synergistically with 17β-estradiol to induce ERE-dependent transcription. They also showed that epidermal growth factor, which produces estrogen-like effects in the mouse reproductive tract, increases levels of nuclear ER (12). These researchers note that the potential health effects associated with exposure to exogenous estrogens may also be observed following exposure to chemicals that could activate peptide growth factor signaling systems.

Recently researchers showed that dieldrin, DDT, and toxaphene, all of which have been reported to be estrogenic, inhibited GJIC in normal human breast epithelial cells in a dose responsive manner. Effects of these compounds were additive, with subthreshold doses of individual compounds being effective when combined (15). Many tumor promoters have the ability to inhibit GJIC. It is hypothesized that inhibition of GJIC may release initiated cells from suppressing effects of signals passing from surrounding normal cells (15).

The effects of mixtures of compounds may be particularly striking for estrogenic and other hormonally active compounds, and thus particularly important for risk assessment. As illustrated above, certain phytoestrogens modulate the activity of endogenous estrogens (37). In addition, recent reports of synergistic activity of some environmental estrogen in vivo [(22); A Soto, personal communication], as well as information on the presence of environmental and dietary antiestrogens (26,66), suggest that interactions could be important.

Screening and exposure characterization needs to be comprehensive enough to identify all kinds of biological activity (67). Although o,p'-DDT was reported to be estrogenic before 1970 (68), the potent antiandrogenic activity of p,p'-DDE was not reported until 1995 (32). In addition, because activity of estrogen is also modulated by other hormones, such as progesterone (69), it is important that these biological activities are considered in a comprehensive manner.

**Conclusions**

The challenge of predicting health effects of exposures to estrogenic compounds is daunting because of the current limitations in our understanding. Chemicals have not been routinely screened for these endocrine activities before being introduced to commerce, and so the significance of current levels of exposure to environmental estrogens, or other hormonally active compounds, is unclear. In addition, data from multigenerational or other sensitive toxicity studies are not available for most compounds to provide information on hazard identification and dose response. The goal of this article is to suggest that it is simplistic to generalize that the effects of all estrogenic compounds can be predicted by assuming that their in vivo effects will necessarily be extensions of the effects of 17β-estradiol, based on a screening test for
estrogenic activity. Screening tests are useful to identify compounds for further study, but must be used with caution to predict health effects or no-effect levels. Although it is not clear whether current levels of exposure to estrogenic or other hormonally active compounds in the environment are associated with health effects, it is premature to dismiss exposure to environmental estrogens as a concern for human health effects based on relative in vitro potency.

A substantial body of experimental data provides insight into differences among estrogenic compounds in terms of mechanisms of action and end points. For example, data suggest variations between compounds in ER–ligand binding to ERs (31), time course of nuclear ER accumulation (36,52), patterns of gene activation (38), and other mechanistic characteristics. These and other data presented here suggest that the assumption that relative potency in in vitro screening assays is representative of relative potency for the most sensitive ER-mediated in vivo effect has not been demonstrated to be accurate.

Current toxicity testing protocols may not be adequate to identify endocrine effects, and may need to be expanded to accommodate the special challenges of risk assessment for estrogenic compounds. For example, for some estrogen-mediated end points the dose response curve is such that high-dose experiments are not likely to be predictive of low dose effects (16,56). Therefore, it may be necessary to broaden testing protocols to look at an extended dose–response curve. It may also be appropriate to modify protocols to evaluate an enhanced spectrum of end points, including more sensitive end points like delayed developmental or behavioral effects. For example, Vom Saal et al. (16) found teritorial behavior in male mice affected by prenatal exposure to 0.001 mg/day of DES or 1 mg/day of o,p‘-DDT; and Chapin et al. (17) have developed new protocols for testing a variety of endocrine, immune, and neurological effects of certain pesticides.

It is well known that timing of exposure has a substantial impact on the dose required to induce an effect. Testing protocols need to identify the most sensitive periods for exposure and to follow up for latency and multigenerational effects. In addition, timing of exposure can affect the type of response observed. For example, neonatal exposure of rats to genistein produced an enlarged SDN–POA, while prenatal exposure decreased SDN–POA volume (40). Duration of dosing also has an important impact on patterns of nuclear accumulation of ER and resulting effects (50), so pharmacokinetics and dosing regimes have an impact on the qualitative as well as quantitative nature of the response. Thus, consideration of time as a third axis on the dose–response curve may be particularly important for endocrine effects. The time axis could incorporate information on when in the lifecycle of the organism exposure occurs, as well as duration of exposure of the target tissue. The experimental data reviewed in this paper provide examples of the importance of both these factors in determining the toxicological end points observed.

Risk assessment for estrogenic compounds must consider, among other factors, the diversity in effects observed between classes of estrogens in various animal models, the importance of pharmacokinetics, timing, and duration of exposure in modulating the spectrum of toxicological end points, the diverse (ER- and non-ER-mediated) activities of many estrogenic compounds, and the interactions between multiple compounds to which individuals are simultaneously exposed, including interactions between exogenous and endogenous factors. Emerging questions about risk assessment techniques for hormonally active compounds, therefore, may require new methods.

We propose that a focused research strategy be developed to investigate the mechanisms of action, diversity of effects, and pharmacokinetics of endocrine disrupters. This research should integrate the study of endogenous, synthetic, anthropogenic, and phytoestrogens in a focused program that will not only increase our understanding of potential health effects associated with exposure to these compounds in diet and the environment, but will provide insight into the role of endogenous hormones in breast cancer and other major health concerns.

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