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XENOESTROGEN EXPOSURE AND MECHANISMS OF ENDOCRINE DISRUPTION

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1. ABSTRACT

Environmental xenoestrogens can be divided into natural compounds (e.g. from plants or fungi), and synthetically derived agents including certain drugs, pesticides and industrial by-products. Dietary exposure comes mainly from plant-derived phytoestrogens, which are thought to have a number of beneficial actions. However, high levels of exogenous estrogens including several well-known synthetic agents are associated with harmful effects. Chemicals like xenoestrogens, which can mimic endogenous hormones or interfere with endocrine processes, are collectively called endocrine disruptors. Adverse effects by endocrine disrupting chemicals (particularly xenoestrogens) include a number of developmental anomalies in wildlife and humans. Critical periods of urogenital tract and nervous system development in-utero and during early post-natal life are especially sensitive to hormonal disruption. Furthermore, damage during this vulnerable time is generally permanent, whereas in adulthood, ill effects may sometimes be alleviated if the causative agent is removed. The most commonly studied mechanism in which xenoestrogens exert their effects is through binding and activation of estrogen receptors a and ß similar to endogenous hormone. However, endocrine disruptors can often affect more than one hormone (sometimes in opposite directions), or different components of the same endocrine pathway, therefore making it difficult to predict effects on human health. In addition, xenoestrogens have the potential to exert tissue specific and nongenomic actions, which are sensitive to relatively low estrogen concentrations. The true risk to humans is a controversial issue; to date, little evidence exists for clear-cut relationships between xenoestrogen exposure and major human health concerns. However, because of the complexity of their mechanism and potential for adverse effects, much interest remains in learning how xenoestrogens affect normal estrogen signaling.

2. INTRODUCTION

Many naturally occurring and synthetic agents have the potential to disrupt normal endocrine processes regulating cell growth, homeostasis and development. These structurally diverse endocrine disrupting chemicals (EDCs) have been defined as exogenous compounds that interfere with the synthesis, storage/release, metabolism, transport, elimination, or receptor binding of endogenous hormones (1). Although controversial, there is concern over potential human health risks imposed by exposure to EDCs. The uncertainty stems from complex mechanisms in which these agents exert their effects, making it difficult to define acceptable levels and determine true risk. Nonetheless, there are several well-documented examples illustrating adverse effects on wildlife and humans. The environmentally persistent pesticide dichlorodiphenyltrichloroethane (DDT) and its metabolites were related to reproductive defects in birds and amphibians (2). Prenatal exposure to the potent estrogen Diethylstilbestrol (DES) was associated with reproductive tract malformations in infants and development of cancer in young women (3). Exposure of fresh-water aquatic species to a variety of EDCs has been linked to deleterious effects (4). The most commonly studied EDCs are compounds that mimic the actions of endogenous estrogen 17-β estradiol (E2). These include plant derived phytoestrogens (isoflavones, lignans), drugs (DES), pesticides (DDT, methoxychlor, other organochlorines), and industrial by-products (polychlorinated biphenyls, alkylphenols, bisphenol-A). Some synthetic xenoestrogens such as

alkylphenols, DDT derivatives, and polychlorinated biphenyls (PCBs) are extremely persistent in the environment, accumulating in the food chain and in human biological matrices. Exposure is most commonly by ingestion of contaminated food or water, or through isolated occupational contact. Of particular concern, however, is fetal and early postnatal exposure to xenoestrogens during critical periods of neurological and urogenital tract development when tissues are especially sensitive to steroid hormone regulation. In this brief review, we will describe the means of exposure to various xenoestrogens, their modes of action, and potential or confirmed adverse *in-vivo* effects.

3. ENDOGENOUS ESTROGEN ACTION

Estradiol plays an important role in development of many tissues such as the nervous system, heart, and reproductive tract. In addition to developmental functions, E2 also promotes bone maintenance and cardiovascular health, as well as having a critical role in female reproductive physiology. At the cellular level, E2 stimulates proliferation or differentiation through induction of target genes. A few examples of estrogen-regulated genes include protoocogenes (c-fos, c-jun, c-myc), progesterone receptor, cyclin D1, vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (ENOS). The classic mechanism involves ligand binding and activation of estrogen receptors (ER alpha and beta isoforms), which are members of the steroid hormone nuclear receptor superfamily (reviewed in 5, 6). In its inactive form, ER remains associated with heat shock protein hsp 90 and exhibits a diffuse nuclear localization. However, when bound by ligand, ER dimerizes, becoming an active transcription factor and interacting with estrogen responsive elements (EREs) in target genes. In addition, a number of ancillary factors known as coactivators (7) are recruited to the complex, which interact with ER and participate in remodeling of chromatin. This process is thought to provide access for general transcription machinery to associate with the promoter. Besides direct binding to EREs, ER also affects transcription at other enhancer elements including AP1, NF Kappa B and SP1 sites, typically through protein:protein interaction (8).

3.1. Estrogen Receptors

Estrogen receptors ERa and ß, which are coded for on separate genes, show tissue specific expression and are thought to possess distinct physiological roles (9). ERα is highly expressed in breast, uterus, pituitary, testis, and kidney whereas β shows a somewhat wider distribution, in the cardiovascular system, prostate, hypothalamus, GI tract, ovary (granulosa cells), kidney and lungs (10-14). The two ER subtypes can form homo- or heterodimers allowing for three distinct dimer species. Although most tissues show a predominance of either α or β, there may be coexpression of both receptors in many cells, suggesting potential *in-vivo* heterodimer functions. The relative roles of the three possible dimer combinations are of great interest in terms of differences in target genes, ligand specificities, and coactivator recruitment. These distinct physiologic functions are not well characterized, but ERß was proposed to have a regulatory role in attenuating mitogenic actions of ERa (9, 15). For the most part, the receptor types bind a wide variety of ligands with similar affinities (16, 17). However, a few compounds show a preference for one subtype, or differences in transcriptional activity depending on dimer composition. For example, the demethylated product of the pesticide methoxychlor, (HPTE) shows ERα agonist activity and ERβ antagonism in transfected human hepatoma cells (18). Phytoestrogens and bisphenol-A have significantly greater binding affinity for ERβ relative to α (16, 17). Certain synthetic drugs known as selective estrogen receptor modulators (SERMs) (19) are ER agonists in some tissues and antagonists in others. These include the clinically used antiestrogens tamoxifen and raloxifene. In addition to the predominant ER species, several polymorphisms (20), mutations (21) and splice variants (22, 23) have been described, providing further complexity for estrogen signaling. Furthermore, at least one ERa point mutant is known that shows hypersensitivity to E2 and is associated with hyperplastic lesions in the breast (24). This mutation, which produces a lys-to-Arg substitution at amino acid residue 303, is currently under investigation as a risk factor for breast cancer.

3.2 Nongenomic Actions

Besides the well known transcriptional actions of E2, many nongenomic effects have been characterized (25), which typically involve rapid modulation of signal transduction pathways. A number of investigators have proposed the existence of a sub-population of ER localized at the cell surface, which mediates nongenomic actions (26, 27). The mechanism and role of nongenomic activity with respect to overall estrogen action is unclear. However, certain of these rapid effects including activation of MAP kinase (ERK1/2) and phosphatidyl inositol (PI3) kinase have been linked to proliferation and/or antiapoptosis in various cell systems (28-31). Xenoestrogens have not been characterized with regard to nongenomic activity, but because these rapid effects are sensitive to relatively low estrogen levels, it is likely that exogenous agents may influence them.

4. EXPOSURE TO ENVIRONMENTAL ESTROGENS

4.1. Phytoestrogens

The most common mode of exposure to estrogenic compounds is through dietary sources containing phytoestrogens. Human ingestion of phytoestrogens is quite significant, especially in soy supplemented diets such as infants taking soy-based formulas. Typically, the most important of these compounds in terms of human consumption are the isoflavones (genistein and daidzein), found mainly in soy products, but also present in fruits and nuts (32). In addition there are several other dietary phytoestrogens the most common of which are lignans and coumesterol. The Lignans, enterolactone and enterodiol, are formed in the gut from bacterial fermentation of plant precursors (33). Coumesterol is present in bean, clover and alfalfa spouts. Phytoestrogens such as these are increasingly marketed as over-the-counter, natural products, for use as an alternative to hormone replacement therapy in post-menopausal women. As with other

xenoestrogens, their potential for *in-vivo* endocrine disruption is not completely understood, but phytoestrogens have actually shown a protective effect against some cancers, cardiovascular disease, and osteoporosis (33). Adults on soy-supplemented diets have shown plasma concentrations of 50-500 nM daidzein and up to 900 nM genistein (34). These levels of phytoestrogens have not been associated with any ill- effects, and could be related to lower incidences of certain cancers in Japan. However, infants taking soy-based formulas have shown plasma levels of 2.5 μM genistein, and 1.2 μM daidzein (35). Since this age represents a critical period in neurological development especially vulnerable to changes in steroid hormones, there is legitimate concern about high levels of these exogenous estrogens.

4.2. Synthetic Xenoestrogens

Some of the more prevalent synthetic estrogens in the environment include DDT metabolites and polychlorinated biphenyls (PCBs). Organochlorines such as PCB are extremely resistant to biological transformation and are known to accumulate in the food chain. Therefore they could be transferred from fish and wildlife to humans. Human blood, adipose tissues and milk contain specific PCB types (cogeners), whose physical and chemical characteristics allow them to persist in biological matrices, (termed bioaccumulation). Plasma levels of PCBs and the DDT metabolite DDE were approximately 20 nM in U.S. women from several geographical areas (36). Although PCBs and DDT are very persistent, a recent survey in Germany has suggested that their environmental and endogenous burden is declining (37). There is some thought that these contaminants may be related to an increasing incidence of breast cancer, however, epidemiological evidence has not supported this (38).

Bisphenol-A (BPA) is an industrial monomer used in production of polycarbonates and epoxy resins. There is considerable potential for human exposure to this compound because traces of it are known to leach from the lining of food cans, plastic ware, and from dental sealants (39, 40). Estimates of oral exposure to BPA are 90-930 μg during the first hour following administration of dental sealant (40) and up to 6.3 μg per day from food cans (41-43). BPA is less likely to bioaccumulate than some xenoestrogens because it is readily metabolized through glucuronidation followed by excretion (44). Nonetheless, there have been numerous in-vitro and *in-vivo* studies of the estrogen-like effects of BPA, which illustrate its potential for endocrine disruption, and adverse effects on development.

Other widely distributed environmental contaminants include the alkylphenolic surfactants used in textile manufacturing. In particuluar, nonylphenol and octylphenol are thought to be related to the deleterious effects in aquatic species found close to sewage effluents and other contaminated sources. In addition to being found in high concentrations in certain sediments, traces of these pollutants have been detected in many water sources including U.S. tap water (45). Daily intake of nonylphenol is estimated to be 10-160 μg (46, 47), and alkylphenols have the potential for bioaccumulation. However, rats receiving low and high doses of octylphenol did not show accumulation unless doses exceeded the animals' detoxification capacities (48, 49).

In addition to xenoestrogen exposure through dietary means, there are numerous isolated occupational incidents, or clinically related exposures in which synthetic estrogens have demonstrated adverse effects. Examples include effects on workers involved with production of oral contraceptives, in which active ingredients were likely absorbed through the skin. Crop dusters with high exposure to DDT showed high incidence of oligospermia (50). Indeed, there has been speculation that the decreased reproductive capacity seen in males since the 1950's may be related to persistent xenoestrogen exposure, although this has not been substantiated (51, 52). There is also the unfortunate diethylstilbestrol (DES) incident, in which clinical use of this potent estrogen lead to significant morbidity in males and females exposed *in utero*.

5. MECHANISMS OF XENOESTROGENS

5.1. Direct Effects On The Estrogen Receptor

Considering the widespread tissue distribution of ER subtypes and complexity of estrogen action, a number of mechanisms and *in-vivo* locations exist for xenoestrogens to perturb normal signaling. The most commonly studied mechanisms are those directly affecting steroid receptor activity. Most xenoestrogens are ligands for ER, and may or may not compete with E2 for binding. Like E2, these agents are thought to exert their actions by promoting an active ER conformation, which regulates target genes accordingly. Alternatively, xenoestrogens may function as mixed ER antagonists/agonists in a manner similar to SERMs. In general, xenoestrogens require 100-1000 fold greater concentration to show a similar biological effect relative to endogenous estradiol (16). However, their potential for biological actions is complex and depends on a number of factors including pharmacokinetics (absorption, bioavailability, metabolism and clearance) and tissue specific cofactor and receptor accessibility. For example, there is evidence that some organochlorine compounds can be concentrated in fat tissue and subsequently released to promote estrogenic effects (53). Furthermore, it is not known if xenoestrogens, even at low concentrations that alone do not activate ER, might potentiate the activity of estradiol, or exhibit additive effects if present as mixtures. Indeed, Arnold *et al.* proposed that combinations of weak environmental estrogens have a synergistic effect in activating ER (54, 55). On the other hand, more recent work has suggested only additive effects for estrogenic compounds (56-59).

As discussed earlier, ligand dependent activation of ER involves dimerization and conformational changes within the receptor allowing recruitment of coactivators. Paige and co-workers showed that structurally diverse peptides that bound to ER could promote different conformations leading to recruitment of various sets of coactivators (60). Since human tissues show different expression patterns of coactivator subsets, this could

explain the tissue specific activities (agonist or antagonist) of xenoestrogens. Using the yeast two-hybrid system, we assessed the ability of several environmentally persistent and prevalent xenoestrogens to promote ERα dimerization and stimulate ERE regulated transcription (61). Estrogen receptor α was expressed in yeast as GAL4-DB-hER and GAL4-TA-hER fusion proteins and grown overnight with varying concentrations of E2, octylphenol, bisphenol A, o-p'-DDT, or o-p'-DDE. These structurally diverse compounds (except for o-p'-DDE) induced ER dimer formation as well as stimulating ERE mediated transcription (61). Expression of the coactivator RIP-140 amplified the effects of the xenoestrogens illustrating the potential for tissue specific actions. Using a GST pull down protocol, Routledge and co-workers investigated recruitment of coactivators TIF2 and SRC-1 to ERα or β in the presence of xenoestrogens (62). Genistein, DES, OP, BPA and PCB were each able to displace tritiated E2 from ERα/β in a dose dependent fashion. Binding affinities for each receptor were similar except for genistein, which showed 20 fold greater affinity for ERβ relative to α. However, there were ligand and receptor dependent differences in coactivator binding. In short, binding affinities did not correlate with the ability of xenoestrogens to stimulate recruitment of TIF2 or SRC-1. ERβ generally showed greater coactivator binding in response to xenoestrogens (relative to α), which correlated with enhanced reporter gene activity. The authors concluded that differential recruitment of coactivators by various estrogens contributes to complex tissue-dependent agonistic/antagonistic responses *in vivo* (62). In partial agreement with these data, Matthews *et al.* observed displacement of tritiated E2 from ERα and β by BPA, however, BPA competed much more effectively for binding to ERβ (63). Interestingly, BPA induced ERα and β mediated reporter gene activities to a similar extent, thus again illustrating the non-correlation of ligand:receptor affinity with coactivator recruitment and transactivation. It is noteworthy to mention that xenoestrogens could also affect nongenomic ER pathways, resulting in endocrine disruption at potentially much lower concentrations.

5.2. Other Mechanisms

Several examples are known in which EDCs can alter circulating levels of endogenous hormones. For instance, some hydroxylated PCB metabolites inhibit the sulfotransferase required for estradiol metabolism, thus increasing bioavailability of E2 (64). In apparent contrast with their ability to activate ER, many isoflavanoid phytoestrogens inhibit human aromatase activity in cancer cells, leading to lower E2 and preventing growth of estrogen dependent tissues (65). Some endocrine disrupting agents can interfere with binding of endogenous hormones to plasma transport proteins. An example of this is the displacement of thyroid hormone by dioxin and organochlorines, resulting in more rapid clearance and lower hormone levels (66). Also, it is important to note that EDCs may affect more than one component of the endocrine system, with different potencies. Genistein is a phytoestrogen that also inhibits thyroperoxidase activity, necessary for thyroid hormone synthesis (67). Metabolites of DDT and methoxychlor as well several other xenoestrogens are ER agonists as well as androgen receptor (AR) antagonists (68, 69). In the case of DDT, the *in vivo* metabolite (p,p'-DDE) has greater affinity for AR, such that it binds only this receptor at lower concentrations, and both ER and AR at higher concentrations. Based on these observations, it is clear that the effects of an endocrine disruptor at the high end of its dose-response spectrum result from perturbation of more than one pathway, whereas at lower concentrations, only a single endocrine component may be involved (70).

6. ADVERSE *IN-VIVO* EFFECTS

6.1. Reproductive Tract

Diethylstilbestrol (DES) although not an environmentally persistent EDC per se, provides a good example of how endocrine disruption can adversely affect development. DES is a potent estrogen analogue used clinically several decades ago in pregnant women for prevention of miscarriages. The deleterious effects on male and female offspring of mothers taking this drug are well documented, (for reviews see 3,71,72). Briefly, "DES-sons" showed significantly increased incidence of urogenital tract abnormalities including epididymal cysts (21% vs 5% in controls), hypospadias (4.4% vs 1.1% in controls), and cryptorchidism (11.4% vs 2.1% in controls), (73). Animal studies of pregnant mice receiving DES showed similar anomalies in male offspring (74). Daughters of women taking DES exhibited increased incidence of urogenital tract abnormalities as well as the occurrence of a rare tumor (clear cell carcinoma of the vagina) in young adulthood (75). Fortunately, the risk of cancer was still relatively small (1 in 1000). Obviously, inadvertent exposure to weaker xenoestrogens is not directly comparable to use of this potent drug. Also, one must consider that DES is readily absorbed through oral administration, crosses the placenta, and was taken in high doses (typically 5 to 125 mg per day) (3).

Metabolites of pesticides such as DDT are thought to be a causative factor in many instances of reproductive anomalies in amphibians and birds. An example is the report of alligators in Florida's Lake Apopka, which showed altered sexual differentiation of male reproductive tract (2), presumably resulting from the spill of a DDT-like pesticide into the lake in the early 1980's (76, 77). The estrogenic/antiandrogenic effects of DDT metabolites were also seen in humans who showed a high incidence of oligospermia as a result of working around high levels of this agent (50). PCBs, other estrogenic contaminants, and dioxins have each been linked to a number of deleterious events in freshwater aquatic species. These include high mortality in Lake Ontario trout, vitellogenin induction in fish near sewage outfalls, congenital defects in Lake Michigan cormorants, and changes in sex steroid profiles in fish found near kraft-mill outfalls (4).

Recently there has been concern about the endocrine disruptive potential of a group of industrially produced non-ionic surfactants, alkylphenols (nonylphenol

and octylphenol), which are present in surface waters and sediments. These products have been shown to bioaccumulate in several species (78), and may be related to reproductive defects in fish (79, 80). Octylphenol (OP) and nonylphenol are ER ligands but no clear causality has yet been established between them and adverse effects in humans. However, because of widespread industrial use of OP, and potential for human exposure from water sources, many laboratory studies are currently underway to investigate the estrogenic mechanism of this environmental contaminant. For similar reasons, the synthetic product BPA, used in plastics and resins, is also under close scrutiny. Katsuda *et al*, noted several reproductive tract defects in newborn female rats exposed to high doses of OP, including early vaginal opening, uterine hyperplasia and persistent estrous (81). Other estrogen-like actions have been found for OP in developing hypothalamic neurons (82). BPA was shown to cause reproductive effects in rodents (83), and stimulation of MCF-7 human breast cancer cell proliferation (84). However, as with other environmental estrogens, more evidence is needed to determine if BPA and OP impose any true risk to human health.

6.2. Nervous System

Central nervous system development is acutely sensitive to sex hormone levels, particularly in the perinatal period just prior to and after birth (85). During this time, hormonal regulation is responsible for sexual dimorphic organization of the brain, i.e. feminization or masculinization. This process allows for sex-typical activational effects (e.g. behavioral) by gonadal hormones later on following puberty. Many of the developmental endpoints related to sexual dimorphism in the CNS are regulated by estrogen receptors. This is particularly true for rodents, whereas in primates, androgens appear to play a more critical role (86, 87). Furthermore, steroid hormones are known to affect brain function after development through typical activation of cognate receptors and interactions with neurotransmitter pathways. ERα and β show distinct expression patterns in the mammalian CNS (88) with β showing a wider distribution and expression of several splice variants whose relative functions are currently unknown (89, 90). Although not substantiated, there is suspicion that EDCs may pose a risk for developmental neurotoxicity. Few studies have addressed this issue, but epidemiologic associations have linked perinatal exposure to PCBs, pesticides, and polychlorinated dibenzofurans with cognitive and behavioral deficits (85). Because of the prevalence and bioaccumulation of PCB mixtures (e.g. in adipose tissue, blood and milk) these compounds in particular are under suspicion (91, 92). PCBs are known to activate estrogen receptors, alter thyroid hormone status, and affect dopamine signaling and related behaviors in rodents. Commercial mixtures of PCBs as well as individual PCB cogeners are typically used to study the neurologic effects of these compounds. The PCB mixtures Aroclor 1221 and 1254 were shown to alter female sex behaviors (93), however, other studies have shown that bioaccumulated PCBs are more toxic than the commercial mixtures. Therefore, mixtures that approximate bioaccumulated PCBs may prove more informative. Several highly bioaccumulated forms of PCB were shown to compete with E2 binding, and alter ER mediated gene transcription in cell-based assays (94). In another study, male monkeys were exposed for 20 weeks following birth to a mixture of PCBs similar to that found in human breast milk (50 parts per billion) (95). These animals showed marked behavioral abnormalities 2.4 to 5 years after exposure. Male rats exhibited permanent changes in motor function and emotional behavior following exposure in-utero to specific PCB cogener #77 (96). It should be emphasized that PCBs, like many endocrine disruptors, can affect several signaling mechanisms, therefore the phenotypic effects described above may not be attributed just to the estrogen receptor perturbations. There is some suspicion that xenoestrogens may play a role in carcinogenesis in sex steroid sensitive tissues such as breast, uterus and prostate. However, to date, no definitive causal relationships have been established; therefore, this discussion will be limited to the effects described above.

7. PERSPECTIVES

One of the most difficult problems related to endocrine disrupting chemicals, is establishing risk assessment strategies for potential adverse effects on human health. Toxicological assessments take into consideration both the biologic potency of a chemical as well as possible or known exposure scenarios (47). For xenoestrogens, there are many complicating factors: gender and age as related to periods of development with high susceptibility to permanent damage, involvement of multiple receptors and endocrine components, additional exogenous burden from natural estrogens in foods, and complicated pharmacokinetics. In addition, recently described nongenomic effects of estrogen could provide mechanisms for xenoestrogen action at concentrations several orders of magnitude lower than those required for other modes of action. However, cause-effect relationships between xenoestrogens and ill effects on human health have not been established except in cases of occupational exposure or the former use of DES. Furthermore, it must be realized that the estrogenicity of a chemical should not necessarily be equated to adverse health effects, which is reflected by the significant dietary consumption of phytoestrogens due to their presumed beneficial effects. Nonetheless, because of the persistence of many xenoestrogens in the environment, high potential for human exposure, and accumulation in biological matrices, interest should be maintained both in determining modes of action, and establishing the true risks from these chemicals.

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