—Review—

Reproductive Endocrine Disruption by Environmental Xenobiotics that Modulate the Estrogen-Signaling Pathway, Particularly Tetrachlorodibenzo-*p*-dioxin (TCDD)

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Abstract. Environmental pollutants are known to exert endocrine-disrupting effects on several hormonal axes of animals, including reproduction and development. Many of these xenobiotics modulate the estrogen-receptor signaling pathway(s) in agonistic or antagonistic ways. Some of the compounds are themselves estrogenic (so-called xenoestrogens, environmental estrogens, or ecoestrogens), and are classified as synthetic estrogens, phyto- or fungal estrogens, alkylphenol ethoxylates; certain non-coplanar polychlorinated biphenyls (PCBs), *etc.* Other molecules are anti-androgenic, *e.g.*, *p,p'*-dichloro-diphenyl-dichloroethylene (DDE); while still others disrupt estrogen function in a manner that is dose, species and tissue specific, *e.g.*, certain co-planar PCBs and dioxin-like molecules (*e.g.*, tetrachlorodibenzo-*p*-dioxin [TCDD]). Exposure to some compounds has been correlated with skewing of sex ratios in aquatic species, and feminization and demasculinization of male animals; declines in human sperm counts; and overall diminution in fertility of birds, fish, and mammals. This review will cover these various xenobiotics and evaluate them for their estrogen-modulating effects; and then further concentrate on TCDD specifically. Dioxins are produced as by-products of herbicide overuse, from paper bleaching, plastics manufacture, and waste incineration. TCDD has been correlated with altered fecundity and endometriosis in monkeys, and with certain cancers in experimental animals and humans. TCDD is also correlated with reproductive deficits in many laboratory species. In summary, we believe that some of the reproductive deficits from endocrine-disrupting xenobiotics may be attributable to the modulation of the estrogen-signaling pathway, in positive and negative manners, depending on dose, species, and tissue specificity.

Key words: Xenobiotic estrogens, Dioxin, Endocrine disruption.

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Environmental Estrogens: General Considerations

The contention that hazards in the environment are responsible for reproductive and developmental defects in animals was brought to our

consciousness most dramatically over 30 years ago by popular books primarily aimed at the lay public by Rachel Carson such as “The Silent Spring” and “The Sea Around Us”; and has been strengthened more recently by the writings of Dr. Theo Colborn [1, 2].

Many accounts substantiate that estrogens exert toxic effects on certain, non-inconsequential segments of the general population, be it human or non-human animal. This estrogen toxicity has been

documented by several laboratories [3]. The ubiquity and deleterious effects of estrogenic compounds, both “native” and environmental xenobiotics, were stressed in a recent editorial and in a subsequent article [3, 4] published in the journal *Endocrinology*. From this and many other publications collectively, it has become apparent that our world in general has received a large estrogenic exposure due to *e.g.*, diethylstilbestrol (DES, shown to increase the incidence of cervical dysplasia in primates after exposure to reduce spontaneous abortion) [5], plant or phytoestrogens, and other man-made estrogen-like chemicals [6].

Interestingly, recent reports revealed high concentrations of ethinyl estradiol (the active estrogen in most contraceptive pills) and native estradiol- 17β (E_2) (from cycling and post-menopausal women) [7] in waste water effluent from some metropolitan areas of England; and that these may be responsible for the skewed sex ratios of fish in such areas [8]. In addition, human populations carry in breast milk many non-coplanar polychlorinated biphenyls (PCBs) (*e.g.*, PCB153, which is estrogen like) and co-planar PCBs (*e.g.*, PCBs 77,126,169, which are dioxin like, bind the dioxin- or aromatic hydrocarbon receptor (AHR), and in some instances are anti-estrogenic), especially those such as Native American populations who subsist almost entirely on fish heavily laden with these compounds [9].

Although some of the aforementioned environmental estrogens and anti-estrogenic compounds are no longer as prevalent in the “developed First World” as they were some 20–30 years ago, this and other societies still bear the scars of excessive exposure to estrogens and estrogen-like compounds. Certain PCBs, *e.g.*, although only loosely linked to diminished fertility rates in humans, are quite strongly documented with regard to reproductive impairment in birds, fish and fur-bearing mammals [10]. In this literature review, we will delineate the reproductive effects of several xenobiotics with estrogen-modulating actions, including TCDD.

O,p'-dichloro-diphenyl-trichloroethane (o,p'-DDT) has been the prototypical xenobiotic estrogen (for review, see 11). O,p'-DDT mimics the action of estrogen in diminishing reproduction, inducing persistent estrus in rats, inhibits luteinizing hormone (LH) secretion by the anterior pituitary, and competitively inhibits binding of radiolabeled es-

trogen [11]. O,p'-DDT, paralleling estrogen's effects, augments the induction of progesterone-receptor (PR), the activity of creatine kinase, ornithine decarboxylase, pentose shunt enzymes and uterine DNA synthesis, all of which are estrogenic biomarkers. With the increasing use of DDT in countries struggling to industrialize, output of environmental xenobiotics exerting both estrogenic and antiestrogenic effects has increased dramatically. (It should be noted here that although in the United States we have substituted methoxychlor for DDT as an insecticide, some reports suggest that this compound may be equally as estrogenic as the former [12].) *E.g.*, in attempts to eradicate malarial hosts, increased use of the pesticide DDT in developing countries has allowed this agent to show up in animal and human breast milk which is then transferred to offspring; and has been shown to exert deleterious effects on wild-life populations, such as eagle egg shell thinning. Previously it was reported that DDT might be linked to breast and other cancers in women exposed to this pesticide as long as 20 years ago [13, 14], although the association is controversial [15, 16]. Metabolites of DDT are also effective endocrine-disrupting agents; *e.g.*, while o,p'-dichlorodiphenyl-dichlorophenylethylene (DDE), which is a minor metabolite of DDT (p,p'-DDT makes up roughly 70% of commercial DDT) is in some systems estrogenic; p, p'-DDE appears to be primarily anti-androgenic [17].

Other Environmental Estrogens

A brief overview of the aforementioned and other environmental estrogens follows. The potent non-steroidal estrogen agonist diethylstilbestrol (DES) was in the 1960's and 1970's used to a significant extent by an estimated 2 million women to maintain embryonic implantation and subsequent pregnancy, thereby preventing spontaneous abortion [18]. However, it later became evident that the daughters of these women manifested an increased incidence of cervical dysplasia and neoplasia [19]. Additionally, DES is still used in certain situations as a bolus injection postcoitally for victims of rape. DES probably exerts effects through multiple mechanisms [20].

More pervasive today, however, is the use of synthetic and conjugated, slowly metabolizable es-

trogens such as ethinyl estradiol in contraceptive pills. Although the total amount of estrogen per pill has been drastically reduced from mg to μg quantities from the 1950's to the 1980's, the total body burden per woman is still relatively high [21]. Certainly, male workers in the contraceptive pill industry showed evidence for environmental contamination, including gynecomastia and reduced fertility [22]. Also, the use of unopposed conjugated equine estrogens ("premarins") taken by post-menopausal women with or without ovaries but with intact uteri is correlated with increased risk for breast and other reproductive tract cancers [21]. Whether the addition of progestins ameliorates these deleterious effects is to some investigators still equivocal and controversial, but gaining credence [21].

Phytoestrogens provide another environmental, though less-potent source of estrogenic substances. Sheep have shown inhibited fertility due to feeding on *Trifolium subterraneum*, or Australian clover; this source is rich in the isoflavone formononetin, which is metabolized in the digestive tract to the weak estrogen, equol [23]. Conversely, some plant estrogens appear to exert beneficial effects. *E.g.*, the isoflavones diadzein and genistein (found in soy), may reduce the risk for osteoporosis in postmenopausal women [24].

Estrogens are still used in the cattle industries of various countries as growth promotants (*e.g.*, estradiol-17 β and zearanol [a fungal or mycoestrogen, presently forbidden by the European Union] in the U.S., and DES elsewhere). These compounds are still found in beefsteak [25]; and effects on human consumption of these are poorly understood [26]. Accidental exposures in scientific and clinical laboratories can lead to disrupted menstrual cycles in women (Hutz, personal observations). Such compounds are also recognized to bioaccumulate in animals exposed most directly in water, *i.e.*, aquatic species such as fish [26].

Effects of Endocrine-disrupting Xenobiotics on Animal Hormonal, Behavioral and Gender Systems, Specifically Aquatic Populations

Many of the aforementioned agents and others have been shown to specifically affect fish populations in many areas of the world. *E.g.*, American Great Lakes' fish living downstream of pulp mills

showed reduced gonad weights and sex steroid hormone concentrations [27] due evidently to the wood-derived phytoestrogen β -sitosterol (which induces vitellogenin synthesis in male fish, which normally do not possess this estrogen-sensitive marker). Peterson and others [28] have shown that the reduced survivability of newly hatched lake trout in Lake Ontario, Canada, is inversely proportional to the toxic equivalency factors (TEFs) of the AHR agonists involved (*e.g.*, dioxins, some PCBs, *etc.*) relative to the potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Whether this effect is a result of endocrine disruption is not yet clearly established.

Elsewhere, other endocrine-disrupting molecules may be at work. Alkylphenol polyethoxylates (APEs) are widely used as components of detergents, paint, herbicides, and pesticides. Effluent of sewage-treatment works in England identified these alkylphenols as having estrogenic properties. It was estimated that 60% of APEs found their way into the aquatic environment as the major degradative products nonylphenol (NP) and octylphenol, with concentrations reaching up to 3000 PPB in some US rivers and lakes [8]. NP was first reported to exert estrogenic bioactivity based on induction of proliferation in ER-positive breast tumor cell lines and endometrial epithelium in rat, and increased progesterone receptor (PR) in breast tumor. NP's estrogenic activity using molecular biomarkers was also found leaching from polystyrene flasks in *in-vitro* culture laboratories [29].

Importantly, molecules such as DDT and DDE are carried thousands of miles on wind and exported in fish and other animals [10]. These and other studies have shown that bioaccumulation can magnify from parts per quadrillion (PPQ, pg/liter of water) in water to parts per billion (PBB, mg/kg animal tissue) (*i.e.*, millions of times) in fish fat and other tissues; and Great Lakes fish are indeed high in PCBs [30]. Regarding linear transmission of these xenobiotics--in fish, the mother deposits some of the toxic load into her eggs, resulting in specific effects. *E.g.*, although embryonic Coho salmon exposed to benzo(a)pyrene, a dioxin-like molecule that binds not the estrogen receptor (ER) but the AHR were normal morphologically, their ability to orient and navigate upstream was compromised [31]. Reproductive deficits have also been seen as an increase in spontaneous embryonic loss peri-implantation in harbor seals (in the

Wadden Sea off the Netherlands coast) fed a Wadden fish diet containing 1.5 mg PCBs compared with those fed cleaner North Atlantic fish (equivalent to 0.22 mg/day) [32].

Xenobiotic Endocrine Disruption of Reproduction/Development

Powerful evidence for endocrine disruption in fish was shown by Sumpter [33] in that effluent running into certain English streams skewed the sex ratio of fish dramatically toward the female gender; and that much of this effect was due to the contaminant 4-NP and other molecules that bind the ER in an agonistic manner [8, 36]. Work by McLachlan, Guillette, Vonier and others showed a similar relative increase in female turtles and alligators in Lake Apopka, FL, USA [34–36]. This correlated with an industrial spill of the insecticides DDT and dicofol, and coincided with a decline in the population of alligators, concomitant with a reduction in phallus size and an increase in multinuclear oocytes and polyovular follicles [34]. Some of these effects were similar to those observed for diethylstilbestrol (DES) exposure [5]. Certain of the effects are not estrogenic at all, but rather anti-androgenic, achieving some similar effects of de-masculinization as opposed to feminization [17].

Recently, it was shown that the insecticide methoprene is metabolized to methoprene acid in insects and prevents metamorphosis by acting as a juvenile hormone analogue [37], and binds the retinoid X receptor (RXR), suggesting that it may be responsible in part for the disrupted limb development/severe limb deformities observed in frogs of some northern American states.

This myriad of molecules (*e.g.*, some non-coplanar PCBs and DDT acting as ER agonists; and AHR agonists acting at times antagonistically toward estrogen) are all found in one great environmental “soup” surrounding us; and because much of this material settles in water, the inundation of the aquatic environment on this planet--factory waste effluents, defoliant overuse, waste incineration and forest fire emissions into the atmosphere--all affect the general fish and, hence, the predator (including human) populations. But how the effects are generated is for the most part unknown, except for a few molecules in *in-vitro* systems.

Mechanistically, it is known that *o,p'*-DDT and *o,p'*-DDE can competitively displace to a significant extent estradiol-17 β (E_2) from the ER in radioligand exchange assays [35]. And the binary combination of several of the environmental chemicals including endosulphan, dieldrin, toxaphene, and chlordane initially appeared to exert greater than additive effects in competitive binding assays, therefore showing synergy [38]; but this paper has since been retracted. However, because of inter-laboratory variability and inconsistent results, definitive answers have not yet arisen and so there is still a great need to screen for such estrogenic-like effects. Since some compounds such as *p,p'*-DDE and the fungicide vinclozolin [39] may actually be potent androgen receptor antagonists, and some of the apparently estrogen-mimicking effects of such molecules are probably not feminizing effects at all, but rather de-masculinizing effects, it is incumbent upon us to search for appropriate biomarkers of steroid modulatory activity.

Biomarkers Used to Evaluate Xenobiotic Endocrine Disruption

Besides ER which is up or down regulated depending upon dose or time, and PR which is usually up-regulated by estrogenic compounds, there are numerous other endpoints.

Since fish liver and other organ systems synthesize estrogen-inducible egg yolk precursor protein (vitellogenin) necessary for oocyte maturation and ovarian development, this is an important estrogenic endpoint. And nonylphenol has indeed been shown to exert estrogenic effects in trout *in vivo* [8], as it induces vitellogenin A2 mRNA transcription. Dioxins and β -naphthoflavone (BNF) (both AHR agonists) reduced vitellogenin synthesis in fish primary hepatocyte cultures [42] and demonstrated a negative correlation between cytochrome P450 1A1 (*cyp1A1*)-inducing capability and antiestrogenicity. This fits with our results where presumed greater *CYP1A1* activity with increasing BNF dosage administered to rainbow trout resulted in reduced circulating E_2 concentrations in a dose-dependent manner at 48 h of exposure (Hutz *et al.*, in press).

Chronic dietary exposure to the chlorinated pesticide and *cyp1A1*-inducing compound Arachlor

1254, reduced fish plasma vitellogenin response to estradiol-17 β (E₂) [43]. Similarly exposed Atlantic croaker salmon showed reduced blood E₂ concentrations and gonadal function in general [44], which might have been due to direct steroidogenic impairment at the ovary or disruption of the hypothalamic-pituitary-gonadal axis.

Other examples of biomarker assessments are BNF's 30-fold increase in *cyp1A1*-mediated ethoxresorufin-o-deethylase (EROD) activity in striped bass [45]. O,p'-DDT and o,p'-DDE displaced competitively ³H-moxestrol in juvenile rainbow trout at concentrations of 5–15 mg/kg and induced immunoreactive vitellogenin synthesis as assessed by enzyme-linked immunosorbent assay (ELISA) as biomarkers for xeno/eco-estrogenic activity [46].

Very recent evidence suggests that p,p'-DDE can in addition to displacing 5-alpha-dihydrotestosterone (DHT) from the androgen receptor (100% at 100 mM), also displace competitively estrogen from ER; similarly, 4-NP is known to act estrogenically at the ER (100 mM reduces competitive specific binding 75%), but is also shown to displace 5-alpha-DHT from androgen-binding protein (ABP) by 40% at a concentration of 100 mM. As can now be observed, some of these xenoestrogens and estrogen-signal modulating compounds may exert a multiplicity of endocrine-receptor disrupting effects, opposite to that deduced from the literature [47]. These compounds are complex and it is presently difficult to evaluate their mechanism(s) of action. Hence, the rational use of a battery of molecular biomarker endpoints is of utmost necessity in elucidating the exact nature of the action of these compounds. One estrogen signal-modulating xenobiotic that exerts disparate effects on many different biomarkers is tetrachlorodibenzo-*p*-dioxin (TCDD).

TCDD as Modulator of the Estrogen Signal

Although modulation of the estrogen- or ER-signaling pathway is recognized for many xenobiotic compounds, little is known in the scientific literature as to the mechanism whereby TCDD exerts its reproductive defects, particularly on women and other female animals. There are scarce data available regarding the nature of the reproductive effects of TCDD on females, except that it is deleterious and it is not a competitive receptor antagonist for

ER. As it is known that some of the action of native estrogens is attributed to a local effect at the ovary, and that TCDD may alter estrogen's action, we hypothesize that the action of TCDD is at a molecular level on the ability of estrogen to interact with its receptor and/or the genome. Before we explore a mechanism for such an estrogen-modulatory action of TCDD, we must first recount facts concerning TCDD.

TCDD is one of the most potently toxic chemical tumor promoters and carcinogens known to man; and still accumulates and persists in the biosphere as a by-product of herbicide overuse (e.g., from production of 2,4,5-trichlorophenoxy acetic acid [2, 4, 5-T]), from paper processing and of plastics manufacture particularly polyvinyl chloride (PVC), waste incineration, forest fires, and other wood burning; and has recently been re-classified by the United States Environmental Protection Agency (US EPA) as a "known human carcinogen". TCDD has been shown to bioaccumulate in individuals exposed to the defoliant Agent Orange during the Vietnam War. The risk posed by TCDD to humans is still unappreciated and for the most part unknown, as the only information available is not of an experimental nature but one of anecdotal accounts of accidental exposure in spills in Japan, China, and most recently (1976), in Seveso, Italy [48, 49]. But, certainly, reproductive effects are of major concern and only in the past year have we for the first time observed an increase in ovarian cancer in the women exposed at Seveso [49]; and a skewing of the sex ratio toward females born of these exposed women [50]. Other reproductive deficits include a recent documentation of endometriosis for female rhesus monkeys given TCDD orally [51, 52]. Chronic dietary intake of 25 ppt of TCDD reduced infant survival in mother rhesus monkeys whose peripheral and central fat contained greater than 200 ppt. Such long-term studies in monkeys are extremely useful in extrapolation to the human, but excessively difficult to perform, due to costs and logistics. Mabley *et al.* [53, personal communication] have shown that 0.15 ppb administered to pregnant rats on d 15 of gestation resulted in a diminution of reproductive organ weights in male offspring, and demasculinizing and feminizing behaviors are manifested; this is true of other mammals and birds also [10]. (It has recently been reported that human sperm counts have decreased by 42% over the last 50 yr,

possibly due to environmental insults such as organochlorines (DDT, PCBs) that can pass the placental barrier [54], or might possibly be attributed to mixtures of dioxins and other compounds. It should be noted here that the report of this reduction in human sperm count is still extremely controversial [55,56].)

Mechanistically, TCDD increases cell differentiation [57]; enhances epithelial cell keratinization through mediation of the epidermal growth factor-receptor (EGF-R) [58] at sebaceous glands, (hence the accompanying association with chloracne); and augments activity of hepatic monooxygenase (cytochrome P450IA1 [*cyp1A1*] and P450IA1-dependent aryl hydrocarbon hydroxylase (AHH) and other liver enzymes in mouse and analogues in rat, rabbit and human. TCDD's "trade-mark" effect on inducing epithelial keratinization appears to be by diminishing plasma membrane receptor density for EGF through alteration of tyrosine kinase autophosphorylation of the EGF-R [59]; and subsequent down-regulation of EGF-R by increased TGF- α (an EGF-R ligand). Interestingly, however, TCDD increases binding of dexamethasone to glucocorticoid-receptor (GR) in some cells and augments GR number [60]. Although TCDD drastically affects testis structure and function directly in rat and guinea pig [61]; there is at present a paucity of information regarding the effect(s) of halogenated aromatic hydrocarbons such as TCDD on ovarian structure and function [62], although it appears that Chinese hamster ovary (CHO) cells possess the AHR [63].

The Ah- or dioxin-receptor is a soluble intracellular protein and a member of the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) family of transcription factors, which includes several proto-oncogene products such as c-myc, myo-D and neuro-D developmental proteins; and Sim and Per *Drosophila* periodicity proteins, among others. This receptor now appears to be similar to members of the so-called "orphan-receptor" group, as the natural endogenous ligand has not yet been discovered. AHR, is, as are the steroid-receptors, a ligand-inducible transcription factor that in one situation binds a xenobiotic- or dioxin-responsive transcriptional enhancer element (XRE/DRE) upstream from the *cyp1A1* gene [59].

Transduction of TCDD Effects

Certainly, TCDD reduces circulating concentrations of estrogens in mammals, including women who have been victims of accidental exposure to TCDD [48]; although, in contrast, this is not the case for CD1 mice [64]. TCDD appears to bind AHR and activate a heterotetramer comprised of AHR, a hsp90 dimer, and another protein of less than 50 kDa [63]; the aromatic hydrocarbon (AH)-AHR complex is then translocated from cytoplasm to nucleus via a nuclear translocator factor (ARNT) resident in the nucleus [65]; this phenomenon is similar to that espoused for glucocorticoid interaction with its receptor [66]. There is some evidence for receptor phosphorylation being important in the transduction of TCDD's effects (which is also the case for steroid receptors, including estrogen). TCDD may reduce activity of estrogen-synthetic enzymes and P450s in mouse ovary, as well as diminishing progesterone and testosterone [67]. This appears to be a direct effect without affecting circulating gonadotropins [68]. TCDD is also known to inhibit the estrogen-induced increase in uterine weight, and to reduce uterine estrogen-receptor number by 30% in mouse strains that are AHR+ [69]; (the AHR locus is transmitted as a simple autosomal dominant on chromosome 7 in human) [70]; increases 2-hydroxylation of estrogen in female rat liver microsomal fraction [69, 71-73]; and favors 4- (*cyp1B1*-mediated) rather than 2-hydroxylation (*cyp1A1*-mediated) in human breast tissue [74]. The catecholestrogens may then be responsible for some of the observed untoward effects. For example, a certain AHR agonist in vegetables (indole-3-carbinol in *Brassica* [broccoli, Brussel sprouts, cabbage]) is metabolized to indolocarbazole (ICZ) in the digestive tract and augments 2-hydroxylation, which has been suggested to reduce breast cancer risk vs. augmented risk with 16- α -hydroxylation (as with dioxin exposure). However, these associations are still considered to be controversial [16, 75]. TCDD and estrogen may at times act synergistically, *i.e.*, "estrogenically"; or cancel each other's effect to varying degrees (acting "anti-estrogenically")—all of which depends on dose and duration given and tissue type. Both compounds are metabolized to glucuronide conjugates [73]; with TCDD manifesting a biologic half-life of two weeks in rat, and upwards of 11

years in man. To illustrate the various actions of TCDD and apparent cross-talk between the two signaling systems, Umbreit *et al.* [76] showed that estradiol diminished TCDD-induced toxicity in fish; and Fernandez and Safe [59] showed that TCDD diminished estrogen's mitogenic effects (*i.e.*, is antiestrogenic) in inducing tumor activity of human breast cancer cell lines, *e.g.*, MCF-7 cells at 10^{-11} to 10^{-13} M [68, 76].

Estrogen and TCDD may serve as co-mutagens with tumor inducers, promoters and progressors, and are therefore genotoxic, although this activity may not necessarily be receptor mediated [78]. It is abundantly evident that TCDD does not exert a universal, generalized and clear-cut effect in all tissues and systems, as ovarian hormones appear to be required for TCDD-promotion of tumor growth in liver, but not in lung; although human lung has AHR mRNA [79] and lung of stump-tail macaque has been shown to possess AHR by gel-shift assay [80]; and TCDD is a potent hepatocarcinogen in female, but not in male rats [61]. Therefore, it is of paramount importance that the interactive effects of TCDD and estrogenic substances be further elucidated for female mammals.

To this end, other environmental estrogenic xenobiotics may bind the ER (such as DDT); or as in the case of TCDD and other halogenated aromatics and polycyclic aromatics (PAH, such as benz(a)pyrene), bind the AHR agonistically, probably resulting in receptor "cross-talk". Some of these congeners down-regulate ER number in certain tissues by binding the AHR [81, 82]; or bind the dioxin-response element(s) (DRE) of the human ER gene [82] and/or diminish binding affinity of estrogen for its receptor [66, 83, 84], or potentially of ER for its cognate response element (ERE) [84]; but this effect, however, may be uncoupled from induction of specific cytochromes P450 [78]. (Interestingly, liganded ER interferes with AHR binding to DRE, showing reciprocal receptor cross-talk [85]). Yet, TCDD *does* increase P450IA1-mediated enzymatic activity (EROD) [78]; and increases activity of P450IA2 and CYP1B1, which are involved in the hydroxylation of endogenous estrogens to catecholestrogens [86]. In MCF-7 cells, very low concentrations of TCDD still reduce occupied nuclear ER without altering P450-dependent monooxygenase enzyme [77]. This may occur at transcriptional or posttranscriptional levels. In an attempt to resolve some of the apparent

inconsistencies with regard to TCDD action, our investigations led us to use and/or develop several model systems.

In a series of recent publications we have demonstrated the presence of the ER mRNA and protein by western immunoblot and gel mobility-shift assay (GMSA) in monkey ovary, and in human ovarian granulosa cells (GC) by reverse-transcription polymerase chain reaction (RT-PCR); and ER mRNA by ribonuclease protection assay (RPA) in rat ovary, hypothalamus, pituitary and uterus; and the AHR to macaque ovary by GMSA and western immunoblots [80, 87]. In conjunction with Dr. Richard Peterson of the University of Wisconsin, we have shown that oral administration of 1 μ g TCDD/kg body mass (*i.e.*, one part per billion [PPB]) to a pregnant rat on day 15 of gestation resulted in a 60% diminution of circulating estradiol-17 β (E_2) concentrations (but not in progesterone or androstenedione) in female offspring at post-natal day (PND) 21–25; and an increase in the expressed mRNA encoding the ER by RPA and the gene product by GMSA in ovary and uterus [87]. This paradigm also resulted in an increase in circulating growth hormone (GH) concentrations possibly via a rise in GH-releasing hormone (GHRH) mRNA and decrease in somatostatin mRNA as measured by RPA [88]; this might have been responsible for the increase in the ratio of wet ovarian mass to overall body mass. The reduction in circulating E_2 did not appear to be due to an alteration in circulating LH or FSH or *in-vitro* output of gonadotropins by cultured hemipituitaries, although FSH- β subunit mRNA was reduced [89]; nor was there a perceptible change in LH- or FSH-receptor mRNAs. However, because there was no expected augmentation in LH with lowered E_2 , we believe that the pituitary-gonadal axis was compromised. We do not yet have information on modulation of gonadotropin-releasing hormone (GnRH) pulsatility with TCDD, as these experiments are currently underway. The lowered E_2 was expected to be reflected in reduced folliculogenesis of certain size groups of both pre-antral and antral follicles, and this was in fact the case [90]; although classical apoptosis did not appear to be the culprit. AHR mRNA and AHR DNA binding was in this paradigm regulated in a tissue-specific manner by TCDD [91]. Most recently, we and others have investigated the reduction in E_2 using an *in-vitro* human luteinizing granulosa

cell (hGC) model [92]; and show that hGC indeed show reduced synthesis and secretion of E_2 at picomolar TCDD concentrations, without compromising androgen and progestin output [93, 94]. This drastic reduction is completely abolished by the replenishment of aromatizable androgen substrate to the culture medium. We believe, therefore, that either TCDD depletes bioavailability of androstenedione (A_4) substrate for E_2 synthesis (e.g., by some type of intracellular sequestration in the *in-situ* situation); or that E_2 is preferentially converted to another metabolite such as 2- or 4-hydroxyestrone, unrecognizable by the antibody used in our radioimmunoassay. Enzyme kinetic studies (using conversion of tritiated-androstenedione) show no apparent effect of TCDD on the aromatase enzyme *per se* (encoded by *cyp19*) [92]; although we are currently corroborating this with competitive, quantitative RT-PCR of enzyme message. TCDD therefore appears to disrupt in a tissue-specific fashion reproductive function at all levels of the hypothalamic-pituitary-ovarian-uterine axis, both directly at these end organs and indirectly by altered hormonal feedback.

The importance of and expressed need for studies in the area of endocrine disruption is made evident in the following passage from the Wingspread "conference on the environment" held recently in Racine, Wisconsin, USA [95]: "Measurable endpoints (biologic markers) as a result of exposure to exogenous endocrine disruptors are needed that include a range of effects at the molecular, cellular, organismal and population levels. Molecular and cellular matters are important for the early monitoring of dysfunction".

In conclusion, it is apparent that in the environ-

ment there exists a plethora of xenobiotics, mostly man made, that can exert endocrine-disrupting effects on all animals, including man; and that many of these compounds interfere with or somehow alter the estrogen-receptor signaling pathway(s), thereby causing defects in reproductive anatomy and function, in development, and induces "gender switching". The effects of the compounds, however, are difficult to ascertain, and change depending upon type of molecule, dose, and tissue analyzed; and effects are species and strain specific. Some molecules behave estrogenically in certain tissues under certain circumstances, while the same molecules may act as antiestrogens in other situations. For that reason, these xenobiotics should be referred to as estrogen-signal modulatory or altering. It is, therefore, of utmost importance to analyze these substances in several experimental paradigms before drawing any conclusions as to a "universal" effect.

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