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Topic 3.1

Interactions of xenobiotics with the steroid hormone biosynthesis pathway*

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Abstract: Environmental contaminants can potentially disrupt endocrine processes by interfering with the function of enzymes involved in steroid synthesis and metabolism. Such interferences may result in reproductive problems, cancers, and toxicities related to (sexual) differentiation, growth, and development. Various known or suspected endocrine disruptors interfere with steroidogenic enzymes. Particular attention has been given to aromatase, the enzyme responsible for the conversion of androgens to estrogens. Studies of the potential for xenobiotics to interfere with steroidogenic enzymes have often involved microsomal fractions of steroidogenic tissues from animals exposed in vivo, or in vitro exposures of steroidogenic cells in primary culture. Increasingly, immortalized cell lines, such as the H295R human adrenocortical carcinoma cell line are used in the screening of effects of chemicals on steroid synthesis and metabolism. Such bioassay systems are expected to play an increasingly important role in the screening of complex environmental mixtures and individual contaminants for potential interference with steroidogenic enzymes. However, given the complexities in the steroid synthesis pathways and the biological activities of the hormones, together with the unknown biokinetic properties of these complex mixtures, extrapolation of in vitro effects to in vivo toxicities will not be straightforward and will require further, often in vivo, investigations.

INTRODUCTION

There is increasing evidence that certain environmental contaminants have the potential to disrupt endocrine processes, which may result in reproductive problems, certain cancers, and other toxicities related to (sexual) differentiation, growth, and development. Research has focused mainly on interactions with sex hormone receptors, particularly the estrogen receptor. Numerous chemicals have been shown to be agonists (or antagonists) for the estrogen receptor, although usually with very low affinities relative to 17β-estradiol. Many of these chemicals, except for certain halogenated compounds, do not bioaccumulate in the environment. The resultant biological potencies of these chemicals are invariably low, and, in most situations, it appears unlikely that environmental concentrations are sufficiently high to compete effectively with 17β-estradiol and other endogenous estrogens for the receptor. However, other mechanisms of interference with endocrine functions are increasingly being considered, including effects on enzymes involved in steroid hormone synthesis and metabolism. Particularly, the cytochrome P450 (CYP) enzymes responsible for the highly specific reactions in the steroid biosynthetic pathway

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[1] are of interest as potential targets, given their key role in the formation of various highly potent endogenous steroid hormones. It is possible for certain chemicals to cause or contribute to hormonal disruption and subsequent reproductive and developmental toxicities by interfering with the function of key enzymes involved in steroid synthesis and breakdown. This proceeding discusses the potential targets in the steroid biosynthetic pathway; the development of sensitive techniques and bioassays for the rapid screening of chemicals; the types of chemicals that may be expected to interfere with steroidogenesis, with the emphasis on the enzyme aromatase (CYP19); and the implications of such interferences. A final section will briefly discuss potential effects of chemicals on steroid metabolism. The emphasis throughout will lie on the synthesis and metabolism of estrogens from androgens.

ENZYMES INVOLVED IN STEROID SYNTHESIS

Steroidogenic enzymes are responsible for the biosynthesis of various steroid hormones, including glucocorticoids, mineralocorticoids, progestins, and sex hormones (Fig. 1), and consist of various specific CYPs, and several hydroxysteroid dehydrogenases (HSDs) and reductases [1]. De novo synthesis of estrogens starts with the conversion of cholesterol to pregnenolone by CYP11A (cholesterol side-chain cleavage). In the subsequent steps, 3β-HSD, CYP17 (17α-hydroxylase and 17,20 lyase activity), 17β-HSD and CYP19 (aromatase) are involved. Several key enzymes in steroidogenesis are described in more detail below.

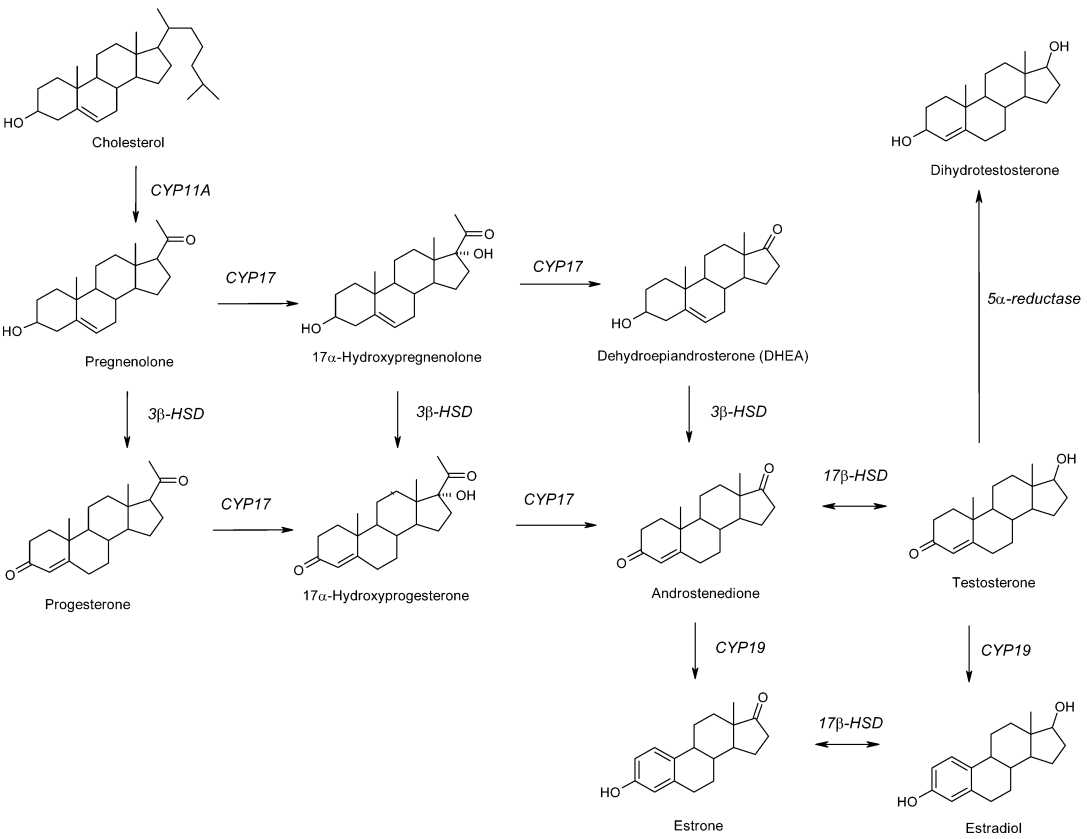


Fig. 1 Enzymes involved in de novo synthesis of androgens and estrogens.

Cholesterol side-chain cleavage (CYP11A)

CYP11A converts cholesterol to pregnenolone via a three-step reaction: 20α-hydroxylation, 22R-hydroxylation, and C20-C22 lyase, which occurs at a single catalytically active site on the CYP450 molecule [2]. CYP11A is bound to the inner membrane of the mitochondrion and is found in all steroidogenic tissues, but is not expressed in nonsteroidogenic tissues. The CYP11A product pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), one of the few non-CYP450 enzymes involved in steroidogenesis and which is found in both mitochondria and microsomes (smooth endoplasmic reticulum). 3β-HSD is widely distributed in steroidogenic and nonsteroidogenic tissues.

Steroid 17α-hydroxylase/17,20-lyase

CYP17 is responsible for the 17α-hydroxylation and the C17-20 lysis of steroid structures, which takes place in the endoplasmic reticulum [1]. Its C17-20 lyase activity is the key activity involved in directing the biosynthesis of steroids toward sex steroids and is highly expressed in testicular Leydig and ovarian follicle cells. In the adrenal cortex, CYP17 is responsible for the 17α-hydroxylation of pregnenolone and progesterone, which are found specifically in the zonae reticularis and fasciculata, but not in the zona glomerulosa. The C17-20 lyase activity is low in the adrenal cortex. Both activities appear to be due to distinct catalytic sites on the enzyme. CYP17 hydroxylates the 17α position of pregnenolone or progesterone to form the respective 17α-hydroxysteroids. The two 17α-hydroxylated steroids can be converted by CYP17 to the weak androgens dehydroepiandrosterone and androstenedione, respectively.

Aromatase

The 17α-hydroxysteroids are further converted to androstenedione and testosterone in the testis and ovary. The balance between these androgens depends on the activity of 17β-HSD; high 17β-HSD (type 1) activity favors the formation of testosterone. In both these organs, CYP19 or aromatase is expressed in the endoplasmic reticulum and is capable of converting androstenedione and testosterone into estrone and 17β-estradiol, respectively.

Implications of interferences with aromatase

CYP19 is of particular interest as it is the rate-limiting catalyst in the formation of estrogens, not only in cells engaged in de novo synthesis of estrogens, such as ovarian granulosa cells and the human adrenal cortex, but also in tissues such as the brain, adipose, and placenta, which utilize circulating levels of androstenedione or testosterone as precursors. Aromatase plays an important role in sexual differentiation, development, reproduction, and behavior, particularly in the gonads and the brain [3], but is also involved in diseases such as estrogen-dependent tumors [4]. Thus, interferences with the catalytic activity or expression of aromatase activity may be expected to result in disruptions of endocrine-regulated processes, such as estrous cycle, sperm production and maturation, development of puberty, masculinization/feminization of (sexual) behavior, and the inhibition or stimulation of the development and growth of hormone-dependent tumors of the breast, ovary, and prostate.

However, the prediction of biological effects of interferences with steroidogenic enzymes in intact organisms is highly complex. Induction of steroidogenic enzymes is highly tissue- and cell-type-specific and is controlled by different promoters and second messenger pathways, which, in turn, provide various targets for interaction with xenobiotics. Inhibition of steroidogenic enzymes may occur by mechanisms such as substrate competition, or mechanism-based inactivation and other forms of non-competitive inhibition. Although inhibition by xenobiotics is likely to be less cell-type- and organism-dependent, it may be differentially influenced by cell-type- and organism-specific biokinetics.

Further complicating matters, the resultant hormone products have various physiological functions dependent on the tissue of formation and the stage of development of the organism. For example, estrogens are involved in determining sex-dependent behavior in the brain, whereas, peripherally, they control growth of bone, lipid metabolism and distribution, and the reproductive cycle in tissues such as the ovaries and uterus. In some tissues, aromatase plays a crucial role by forming the required estrogens locally using circulating levels of androgens; in others, circulating estrogens are required that originate mainly from the ovaries. Thus, effects of chemicals on aromatase activity may result in altered estrogen synthesis, but the ultimate tissue and organism responses will be harder to predict.

MECHANISMS OF INTERFERENCE WITH STEROID SYNTHESIS AND TECHNIQUES OF DETECTION

In contrast to a weak interaction with a sex hormone receptor, an interaction with a rate-limiting enzyme involved in sex hormone synthesis has the potential to profoundly affect the function and homeostasis of the highly potent endogenous steroid hormones. Among interactions with steroidogenic enzymes, various mechanisms can play a role. These include direct reversible or irreversible catalytic inhibition, and up- or down-regulation of enzyme expression (e.g., induction or inhibition of gene expression). Other, less direct effects on steroidogenic enzyme activities, such as modulation by the hypothalamic-pituitary-gonadal axis, may also occur.

To investigate effects on steroidogenic enzymes, various methods are available, starting with the choice of a biological system. This can be the simple isolated enzyme, if available, or a microsomal fraction of tissues that express the enzyme of interest. On an increasingly more complex level, cell lines, primary cells in culture, tissues slices, or whole animals may be used. To answer the straightforward question whether a compound can inhibit a specific enzymatic reaction, simple systems, such as purified enzyme, microsomes, or cell lines, may suffice. For questions regarding the effects of chemicals on the expression of steroidogenic enzymes, more complex systems are required. Cell lines and primary cultures may provide information on intracellular regulation, co-cultures may shed light on intra and intercellular regulation, and in vivo studies will be necessary to investigate possible effects of chemicals on steroidogenesis by affecting the hypothalamic-pituitary-gonadal/adrenocortical axes.

Once the choice of a suitable biological system has been made, various aspects of enzyme function can be investigated. Catalytic activity is one of the most functional endpoints that can be measured using selective substrates for the enzyme. To obtain reliable estimates of catalytic activity, disappearance of substrate or formation of product may be determined. However, disappearance of substrate does not necessarily implicate the involvement of a specific enzyme, and estimates of product formation can be hampered by further metabolism of the product, particularly in the steroidogenesis pathway. Ideally, catalytic measurements should involve the measurement of product formation in the presence of selective inhibitors that block further metabolism. In the case of aromatase, its catalytic activity is determined by measuring the release of tritiated water during the aromatization of 1β-^{3}H-androstenedione [5,6], in combination with the measurement of estrone formation, in the presence and absence of the selective aromatase inhibitor 4-hydroxyandrostenedione [6,7].

Up- and/or down-regulation of enzyme expression can be determined using techniques such as northern blotting or RT-PCR to determine levels of mRNA expression. RT-PCR and real-time RT-PCR are particularly powerful methods as they are highly selective and sensitive once optimized appropriately. Another approach is to use immunoblotting techniques, such as western blotting, that require selective antibodies developed to detect levels of enzyme protein quantitatively.

An indirect way to measure effects on steroidogenic enzyme function is to measure alterations in the ability of cell lines to excrete certain steroid products as an indicator of potential effects of xenobiotics on steroidogenesis. Such experiments would involve measuring a large number of steroid products in the presence of the xenobiotic of interest, possibly in combination with a stimulating concentration of an early precursor in the steroidogenic pathway, such as pregnenolone. An advantage of this

approach is that alterations in the profile of the steroid hormones secreted provide an indication of the identity of the enzymes affected by the xenobiotic treatment, without the need to examine each enzyme activity individually.

IN VITRO BIOASSAYS TO SCREEN FOR INTERFERENCES WITH ENZYMES INVOLVED IN STEROID SYNTHESIS AND METABOLISM

Our laboratory has evaluated several human cell lines for the investigation of effects of xenobiotics on steroidogenic enzymes, such as MCF-7 breast tumor, JEG-3 and JAR placental choriocarcinoma, and H295R adrenocortical carcinoma cells. We also have experience with rat cell lines, such as R2C and LC540 Leydig tumor cells. Each cell line has its advantages and disadvantages. MCF-7 cells are not capable of de novo synthesis of estrogens and generally do not express aromatase, although there have been conflicting reports [8–11]. They are useful for the study of estrogen hydroxylations (discussed later in this chapter) as they express relatively high levels of CYP1A1 and 1B1, which are inducible by 2378-tetrachlorodibenzo-*p*-dioxin (TCDD). The JEG-3 and JAR cells express high levels of aromatase, but appear relatively sensitive to cytotoxic effects of chemicals and seem more prone to apoptosis, rendering these cell systems difficult to use for screening purposes [12,13]. H295R cells are somewhat less sensitive to cytotoxicity and have the major advantage that they express a wide range of steroidogenic enzymes, including all of the enzymes required to produce mineralocorticoids, glucocorticoids, androgens, and estrogens [14,15]. Experiments in our laboratory have recently shown that they also express (TCDD-inducible) CYP1A1 and 1B1, albeit at lower levels than in MCF-7 cells [7].

H295R human adrenocortical carcinoma cell line

The H295R human adrenocortical carcinoma cell line has demonstrated to be a useful bioassay to screen for interferences with steroidogenesis. The H295 and H295R (a subpopulation of H295 that forms a monolayer in culture) human adrenocortical carcinoma cell lines have been characterized in detail and shown to express all the key enzymes necessary for steroidogenesis [14–17]. These include CYP11A (cholesterol side-chain cleavage), CYP11B1 (steroid 11β-hydroxylase), CYP11B2 (aldosterone synthetase), CYP17 (steroid 17α-hydroxylase and/or 17,20 lyase), CYP19 (aromatase), CYP21B2 (steroid 21-hydroxylase), and 3β-hydroxysteroid dehydrogenase. The cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells, with the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex [14,16]. It expresses numerous steroidogenic enzymes, including aromatase [6,14,15].

EFFECTS OF VARIOUS CLASSES OF COMPOUNDS ON AROMATASE

Pesticides

Several classes of (relatively) persistent pesticides, such as organotin compounds, DDT, and several metabolites, a number of azole fungicides, and several 2-chloro-*s*-triazine herbicides, are suspected or have been shown to interfere with steroidogenesis. Particular attention has been given to the enzyme aromatase (CYP19), which catalyzes the final, rate-limiting step in the conversion of androgens to estrogens.

Organotin compounds

Organotin compounds are highly toxic chemicals and ubiquitous environmental contaminants due to their persistence and wide use in industry, agriculture and antifouling paints. It has been postulated that organotin compounds may cause endocrine disruptive effects such as "imposex" (penis development in

females) in mollusks by inhibiting aromatase (CYP19) activity [18]. Organotins have further been reported to inhibit cytochrome P450 (CYP) activities, such as CYP1A1 and aromatase (CYP19), in fish. However, little evidence supports aromatase inhibition as a mechanism of organotin-mediated imposex. Inhibitory effects on CYPs in vitro [19,20] and in vivo [21] were generally reported at environmentally unrealistic concentrations (upper micromolar range). Recently, we have published detailed concentration-response experiments in H295R cells demonstrating that, although the organotin compounds dibutyl-, tributyl-, and triphenyltin chloride decreased the activities of both CYP1A and CYP19 in the upper nanomolar range, the decrease occurred concomitantly with quantitatively similar decreases in various measures of cell viability [22] (Fig. 2). Thus, it could not be concluded that the organotin compounds could selectively inhibit aromatase activity. Several recent publications also do not support the aromatase inhibition hypothesis of imposex. A field study in gastropods (*Bolinus brandaris*) in Spain [23] showed that a population highly polluted with organotin compounds (100 % incidence of imposex in females) and having strongly decreased estradiol levels compared to a relatively uncontaminated population (37 % imposex) did not have altered aromatase activities compared with the less polluted population. Furthermore, a recent report points out that the reductions in steroid levels occur in the later stages of imposex development and appear to be a consequence rather than a cause of imposex [24]. Instead, it is suggested that certain peptide hormones are more likely to play an important role in masculinization of mollusks [24]. The above studies indicate that the development of imposex and the action of organotin compounds occur via mechanism(s) other than inhibition of aromatase activity.

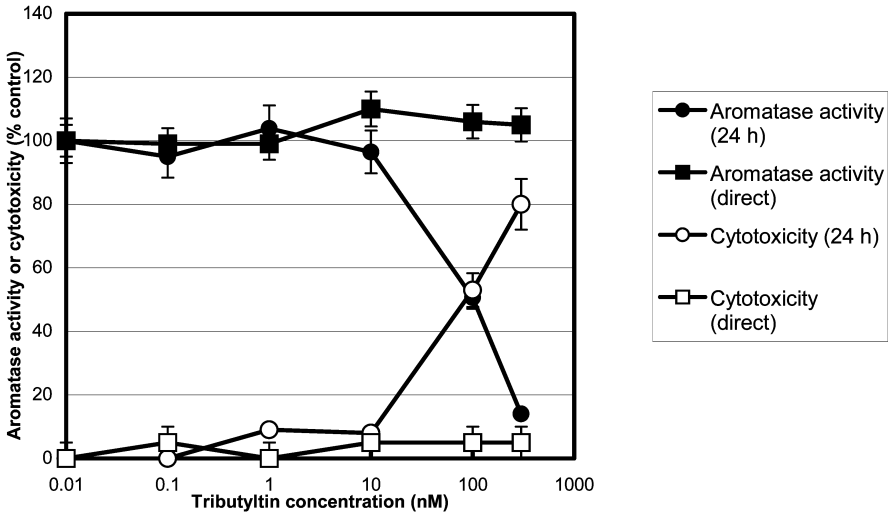


Fig. 2 Effects of tributyltin (TBT) on aromatase activity and cytotoxicity in H295R human adrenocortical carcinoma cells. Cells were exposed to TBT either for 24 h or only for the duration of the aromatase assay (1.5 h; direct catalytic inhibition).

Azole fungicides and vinclozolin

The class of azole fungicides targets the ergosterol biosynthesis pathway in yeasts and fungi by inhibiting the CYP enzyme 14α-lanesterol demethylase. The selectivity of these fungicides is variable, and some are known to inhibit several human CYP enzymes, including the steroidogenic CYP enzymes CYP17 (steroid 17β-hydroxylase activity) [25] and aromatase [25–27]. They have also been shown to inhibit aromatase activity in rainbow trout ovarian microsomes [28], indicating their potential to block natural estrogen-mediated responses, such as vitellogenin synthesis in female oviparous species during reproduction.

Several studies have shown that the commonly used fungicides imazalil and prochloraz (for various structures, see Fig. 3) are potent inhibitors of human, rodent, and fish aromatase activity [25–27]. A recent study using H295R cells [22] demonstrated that these two imidazole fungicides are mixed-type inhibitors ($K_i/K_I' = 0.04/0.3$ and 0.02/0.3 μM, respectively), whereas the triazole fungicides propiconazole, difenoconazole, and penconazole were less potent competitive inhibitors (K_i = 1.9, 4.5, and 4.7 μM, respectively). In the same study, the fungicide vinclozolin, which is structurally unrelated to the azoles, was identified as an inducer of aromatase activity and mRNA expression. Although vinclozolin has been shown to weakly antagonize the androgen receptor [29], it is possible that vinclozolin may exert additional anti-androgenicity via aromatase induction if this mechanism were to occur in vivo.

A study examining the effects of aromatase inhibition on embryonic development found that exposure in ovo of chickens to a nonsteroidal azole aromatase inhibitor led to "masculinization" of females [30]. In other words, aromatase inhibition during the critical time of embryonic development causes genotypical females to develop as fenotypical males. Also, coadministration of exogenous estrogen prevented the observed masculinization of females but led to "feminization" of males, which are the "default" sex in avian species [30]. Estrogens are key hormones involved in feminization of the central nervous system in birds, while, in contrast, they lead to defeminization and masculinization of the mammalian central nervous system [31]. Thus, it can be suggested that, during critical (irreversible) developmental periods, such as embryonic, perinatal, and pubertal development, aromatase induction may result in inappropriate (de)feminizing responses, depending on the type of species, gender, and the tissues in which local estrogen concentrations have been increased.

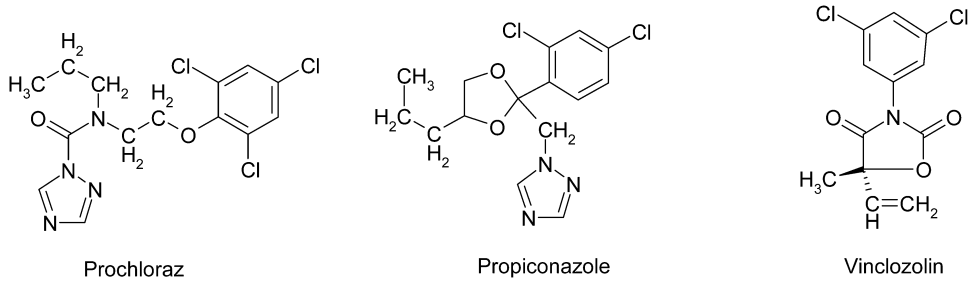


Fig. 3 The structures of the fungicides prochloraz (imidazole), propiconazole (triazole), which are aromatase inhibitors, and vinclozolin, which is an aromatase inducer.

Triazines

The 2-chloro-*s*-triazine family of herbicides is used in large amounts to control weeds, particularly on maize crops, in North America and Europe. The estimated use of atrazine in the United States was almost 35 000 tons in 1993 [32]. As a result, it is found in relatively high concentrations in surface waters in large parts of the North American continent [33]. It is relatively persistent to abiotic and biotic breakdown [33,34]. Epidemiological studies have associated long-term exposures to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy [35] and increased risk of breast cancer in the general population of Kentucky in the United States [36]. In experiments with female F344 rats, atrazine has been shown to induce tumors of the mammary gland and reproductive organs [37]. In female Sprague–Dawley rats, atrazine caused lengthening of estrous cycle and a dose-dependent increase in plasma levels of estradiol [38]. Atrazine also resulted in an earlier onset of the incidence of mammary and pituitary tumors [38], responses typical of exposure to exogenously administered estrogens [39,40]. Recently, atrazine exposure during lactation has been shown to suppress suckling-induced prolactin release in female Wistar rats [41]. Also, the lactationally exposed male offspring of the atrazine-exposed dams had an increased incidence of prostatitis [41], an effect known to be induced by exposure to exogenous 17β-estradiol [42]. A subsequent study in Long–Evans and Sprague–Dawley

rats has attributed the effects of atrazine on serum prolactin levels to alterations in the hypothalamic control of the release of this hormone by the pituitary [43].

It has further been observed that atrazine causes various endocrine-disrupting effects in Florida alligators in areas contaminated with numerous pesticides including atrazine, DDT, dicofol, and vinclozolin. Male and female alligators from the contaminated Lake Apopka had elevated estradiol to testosterone plasma concentration ratios relative to a control site (Lake Woodruff), indicating a disturbance of the balance of androgens and estrogens, which is partly regulated by the activity of aromatase. In addition, females from Apopka had an abnormal ovarian morphology, with increased numbers of polyovular follicles and polynuclear oocytes. Apopka males had poorly organized testes and abnormally small penises.

Initially, investigations into the mechanism of these apparent estrogenic effects were directed toward the estrogen receptor. However, consistent interactions of triazine herbicides with the estrogen receptor or effects on receptor-mediated responses were never demonstrated [44–46]. Effects on enzymes involved in steroid metabolism have been limited to a study of the inhibition of testosterone metabolism in the anterior pituitary of rats exposed in vivo or of whole anterior pituitaries exposed in vitro to atrazine [47]. Weak inhibitory effects were observed on testosterone 5α-reductase (20–37 %) at an atrazine concentration of 0.5 (mM); a similar observation was made for the deethylated metabolite atrazine-desethyl. Studies in our laboratory of effects on enzymes involved in steroid synthesis demonstrated that several 2-chloro-*s*-triazine herbicides (atrazine, simazine, and propazine) and a number of their common metabolites (atrazine-desethyl and atrazine-desisopropyl) (Fig. 4) induced human aromatase activity and gene expression in vitro in H295R adrenocortical carcinoma cells [6,48]. It was fur-

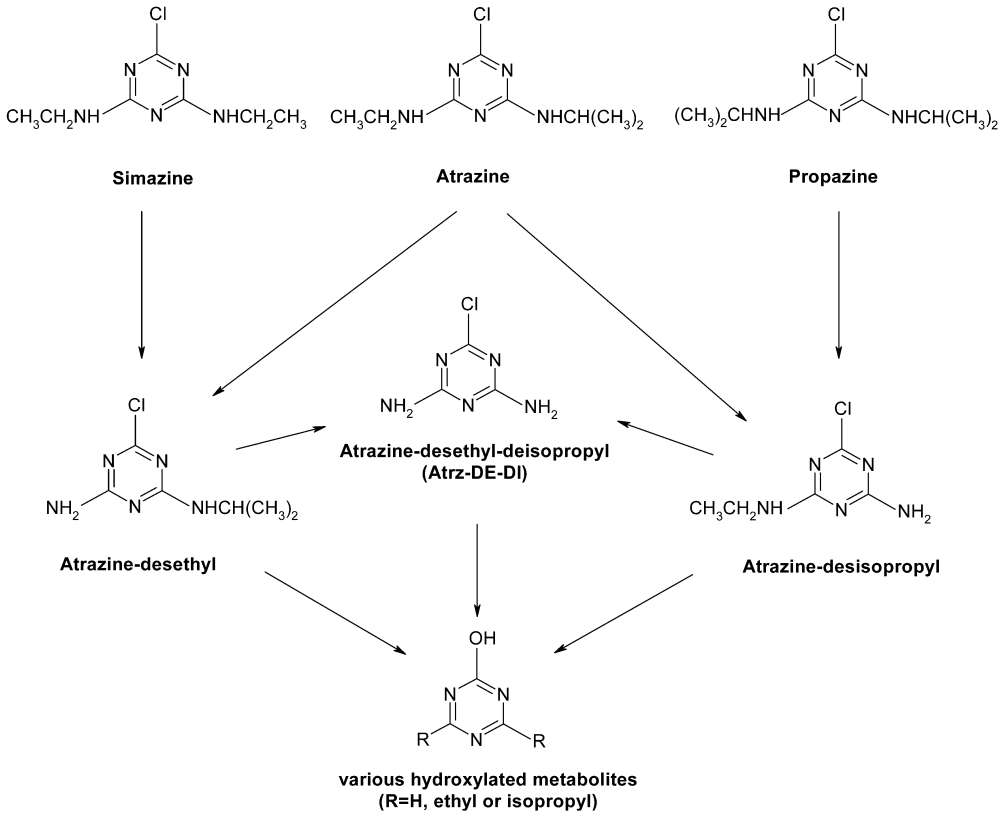


Fig. 4 Various routes of metabolism of the 2-chloro-*s*-triazines herbicides atrazine, simazine, and propazine to several common dealkylated and hydroxylated metabolites.

ther shown that none of the triazine herbicides nor their metabolites induced estrogen-dependent vitellogenin production in male carp hepatocytes. Nor did they antagonize the induction of vitellogenin by 17β-estradiol. Increased synthesis of vitellogenin, a yolk-precursor protein in fish and birds, is a response highly sensitive to estrogens and occurs after exposure to xenobiotics that are agonists for the estrogen receptor. Together, these experimental findings indicate that the estrogenic effects associated with triazine herbicides or their major metabolites in vivo are unlikely to be estrogen receptor-mediated, but may be partly explained by their observed ability to induce aromatase in vitro.

More experimental evidence is necessary to support the hypothesis that aromatase induction may play a role in vivo to explain the estrogenic effects of various chemicals. It is not clear whether aromatase induction occurs in vivo, nor in which target tissues it would occur. Given the recent evidence that plasma estradiol and estrone levels are increased about two-fold in atrazine-treated male Wistar rats [49], it is apparent that the presence of ovarian aromatase is not essential for the effects of atrazine. The further observation that estrone levels appear to be preferentially increased in vivo [49] may be an indication of a tissue-specific effect on aromatase. If aromatase induction is shown to play a role in vivo, it may be hypothesized that the induction would occur in tissues, such as adrenal cortex and adipose, that contain relatively greater levels of androstenedione than testosterone as precursor. Preliminary results from a collaboration between the laboratory of Dr. Susan Laws (U.S. Environmental Protection Agency) and our own laboratory suggest that, although slight increases in aromatase activity are measured, they are not statistically significant due to high interindividual variability. Nevertheless, good correlations ($p < 0.05$; $n = 24$) existed between aromatase activity and either estradiol or estrone plasma levels when using the entire data-set of 12 control and 12 atrazine-exposed animals, thus indicating that aromatase activity is a good predictor of the variation in plasma estrogen concentration even after two-fold induction by atrazine.

ORGANOCHLORINES AND METABOLITES

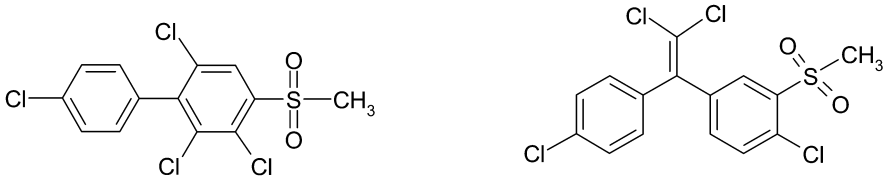
Polyhalogenated aromatic hydrocarbons

Highly persistent contaminants, such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) and the pesticides DDT and its metabolites, have undergone extensive toxicological studies over the last few decades. Certain members of theses classes of contaminants are potent endocrine disruptors. PCDDs, such as TCDD and several TCDD-like PCDFs and PCBs, are known to cause reproductive toxicities and various forms of endocrine disruption, including disturbances in sex steroid homeostasis and action, most notable anti-estrogenicity. Most of these effects are mediated by binding of TCDD-like compounds to the aryl hydrocarbon (Ah) receptor [50,51]. Ah receptor activation results in the induction of various enzymes, including CYP1A1, 1A2, and 1B1, which are involved in estrogen metabolism, and in "negative cross-talk" with the estrogen receptor-mediated pathway [52]. Effects of TCDD-like chemicals on steroidogenic enzymes, however, have either shown very weak or inconsistent effects.

TCDD has been shown to inhibit adrenocorticotropin hormone- (ACTH-) stimulated adrenocortical steroid synthesis in bovine primary cultures, although not by altering the activity of steroidogenic enzymes. TCDD appeared to interfere with the availability of cholesterol to CYP11A [53]. In another report, TCDD was shown to reduce the conversion of precursor steroids to progesterone in porcine luteinizing granulosa cells [54]. TCDD decreased estradiol synthesis in human luteinizing granulosa cells without affecting the activity of aromatase [55]. A decrease in aromatase activity was observed in vitro in TCDD- and PCB-126-exposed JEG-3 cells, although possible aselective cytotoxic effects could not be ruled out [13]. In H295R cells, TCDD had no effect on basal aromatase or cholesterol side-chain cleavage activity, but did reduce the inducibility of both activities by 8-bromo-cyclic AMP [7,56].

Exposures of JEG-3 or JAR cells to various organochlorines and some of their hydroxylated or methylsulfonated metabolites, such as MeSO2-PCBs, have demonstrated no specific effects on the

aromatase enzyme [12]. However, 4-hydroxy-2,4,6-trichlorobiphenyl, tris-(4-chlorophenyl)-methanol, and several MeSO2-PCBs, such as the 3- and 4-MeSO2-PCB-52, -70, -87 and -101, were highly cytotoxic to the placental cells, possibly via an apoptotic mechanism [12]. Several methylsulfonated metabolites of PCBs were found to inhibit CYP11B1, the enzyme responsible for glucocorticoid synthesis, in Y-1 mouse adrenocortical tumor cells [57]. It was demonstrated that the methylsulfone group on the 3- or 4-position of the PCB molecule (Fig. 3) was essential for this interaction.



2,3,4',6-tetrachloro-4-methylsulfonyl-biphenyl 3-Methylsulfonyl-DDE

Fig. 5 The structures of two methylsulfonated metabolites of persistent organochlorine chemicals that have inhibitory effects on the glucocorticoid synthesizing enzyme CYP11B1.

DDT and metabolites

Another methylsulfonated compound, the DDT metabolite 3-$MeSO_2$-2,2-bis(4-chlorophenyl)-1,1-dichloroethene (MeSO2-DDE), is a relatively potent inhibitor of CYP11B1 (11β-steroid hydroxylase) in Y-1 mouse adrenocortical tumor cells at concentrations above 3 μM [57,58]. Here, it acts as a substrate for the enzyme, inhibiting glucocorticoid synthesis, and is bioactivated to a reactive intermediate that binds to proteins, ultimately resulting in adrenocortical cytotoxicity.

In addition, *p,p′*-DDE, which has anti-androgenic properties by acting as an antagonist for the androgen receptor [59], has been reported to increase aromatase protein in rat liver [60]. However, this induction was not confirmed in rat hepatocytes in primary culture [60]. This study did not rule out the possibility that the increase in immunoreactive protein observed in vivo may have been due to the known ability of *p,p*-DDE to induce hepatic CYP2B and 3A in rat liver but not in rat hepatocytes. Also, the tritiated water release assay used to measure aromatase activity can be confounded by hydroxylation of 1β-^{3}H-androstenedione at the 1β-position. This reaction is catalyzed partly by CYP3A1 and possibly CYP2B1 [61], enzymes that are highly induced by *p,p′*-DDE in rat liver [62]. It was recently also shown that *p,p*-DDE, unlike compounds such as atrazine and vinclozolin, was not capable of inducing aromatase activity in H295R cells [22].

Industrial chemicals

Various mass-produced industrial chemicals are known to cause endocrine disruption and reproductive toxicities in laboratory studies. Phthalates are used on a large scale as plasticizers and are found in various polyvinylchloride packages used in the medical and food industry. Phthalates leach out of the packages, contaminating the surrounding area. Particular attention has been given to the endocrine-disrupting properties of di-(2-ethylhexyl) phthalate (DEHP) and its metabolite, mono-(2-ethylhexyl) phthalate (MEHP). DEHP and MEHP are not [63] or are very weakly [64] estrogenic in vitro, but demonstrate ovarian [65] and testicular [66–68] toxicities in rats at very high concentration. Several studies have indicated that DEHP and, more effectively, MEHP interfere with estradiol synthesis, disrupting the estrous cycle in rats. MEHP, but not DEHP, was shown to decrease the activity of aromatase in rat granulosa cells in vitro. The mechanism of this decrease did not appear to be catalytic inhibition, but down-regulation of CYP19 mRNA expression [69].

Several high-volume production chemicals are suspected or known endocrine disruptors, including the alkylphenols and bisphenol A. 4-*tert*-octylphenol, nonylphenol, and bisphenol A are weakly estrogenic and appear to have inhibitory properties toward the 17α-steroid hydroxylase activity of CYP17 [70]; the same appeared to be true for octylphenol and bisphenol A [71,72]. However, a drawback of these studies is that the potential for aselective cytotoxic effects by the chemicals was not accounted for. Octyl- and nonylphenol, and bisphenol A exhibit signs of decreased cell function at concentrations between 1 and 30 mM in vitro, dependent on the cell system and exposure time used [73].

Effects of chemicals on enzymes involved in steroid metabolism

In addition to steroidogenic enzymes, steroid hormone homeostasis is regulated by enzymes that metabolize steroid hormones [74]. Although not the focus of this review chapter, chemically induced alterations in steroid metabolism represent an important mechanism of endocrine disruption and warrant a brief introduction, using estrogen metabolism as example.

In human females, the main sites of synthesis of 17β-estradiol are the ovaries, whereas the liver is one of the major sites of metabolism of estrogens [75]. However, estrogens are synthesized in various other organs and are metabolized extensively throughout the body. Circulating estrogens, such as 17β-estradiol and estrone, are metabolized in the liver either by direct conjugation to sulfate to form estrone- or 17β-estradiol-2-sulfate, or by sulfate conjugation subsequent to hydroxylation by cytochrome P450 enzymes (CYP) [74]. Although the majority of estrogens are conjugated to sulfate or glucuronide, rendering them hormonally inactive, a relatively small amount of estrogens are converted by CYPs to catechol estrogens. CYP enzymes can hydroxylate estrogens at various positions, most commonly the 2-, 4-, and 16α-position of the 17β-estradiol or estrone molecule, to form 2,3- and 3,4-catechol estrogens, and estriol (16α-OH-estradiol) or 16α-OH-estrone, respectively. Some of these metabolites, such as 4-OH-estradiol and 16α-OH-estrone, are considered genotoxic and mutagenic and have been implicated in estrogen-mediated carcinogenesis [76]. 16α-OH-estrone can undergo covalent binding to proteins such as the estrogen receptor, albumin, and various proteins containing free amino-groups, and to DNA [74]. 4-OH-estradiol appears to be just as carcinogenic as 17β-estradiol in Syrian hamster kidney,

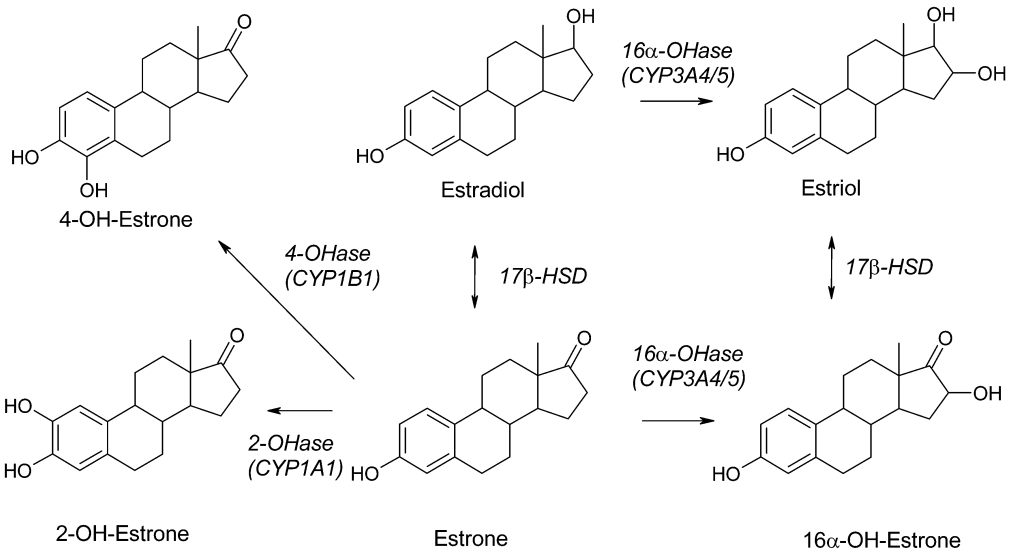


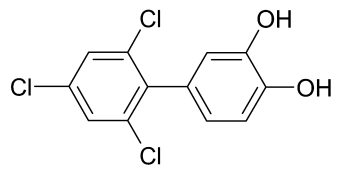
Fig. 6 Enzymes involved in estrogen hydroxylations. Catechol estrogens can undergo methylation by catechol *O*-methyltransferase (COMT), and all hydroxyl groups can be conjugated to glucuronide or sulfate.

whereas 2-OH-estradiol is not carcinogenic [77,78]. The same may be true for mammary carcinogenesis in humans [79]. 4-OH-estrogens can be converted to quinone structures that may undergo redox cycling to produce reactive free radicals and oxygen species, resulting in genotoxic damage [80]. The catechol estrogens undergo further metabolism by catechol *O*-methyltransferase (COMT) to methoxylated metabolites or conjugation by sulfotransferase and UDP-glucuronidyltransferase, all resulting in inactivated (nonestrogenic and nongenotoxic) products. The isoforms of CYP enzymes involved in estrogen hydroxylation are species- and tissue-dependent. In the human liver, which is the main site of estrogen metabolism, 2- and 16α-hydroxylations are catalyzed by CYP3A4 and/or 3A5, with a smaller contribution from CYP1A2 [74,81]. In extrahepatic tissues, where CYP3A expression is much lower [82], but expression of CYP1A1 and 1B1 is relatively higher, CYP1A1-mediated 2- and CYP1B1-mediated 4-hydroxylation of estrogens occurs. Thus, certain routes of estrogen metabolism, such as 4-hydroxylation, that are minor in the liver, may play a considerably greater role in tissues such as the breast or uterus.

Various environmental chemicals can affect the catalytic activity of the cytochrome P450 enzymes involved in estrogen metabolism. The most prominent groups of compounds are polyhalogenated and polycyclic aromatic hydrocarbons. Many of these compounds are more or less potent inducers of the enzymes CYP1A1, 1A2, and 1B1 in humans, via activation of the Ah receptor. Most potent Ah receptor agonists, such as TCDD, 23478-PCDF, and PCB-126, generally induce CYP1A1 to a greater extent than CYP1A2 or 1B1, thus favoring the induction of estrogen 2-hydroxylation above 4- and/or 16α-hydroxylation in extrahepatic cell systems. It was further found that, in MCF-7 breast cancer cells, PCB 169, although an Ah receptor agonists capable of inducing CYP1A1 and 1B1 gene expression [83], is a selective inhibitor of the 4-hydroxylation of estradiol [84].

In human liver, where the majority of estrogen hydroxylations are performed by CYP3A4 and 3A5 [74,85], it would be expected that inducers of these enzymes would enhance oxidative estrogen metabolism. Typically, human CYP3A4 and 3A5 inducers belong to the class of phenobarbital-type inducers, which includes environmental contaminants such as di-*ortho*-PCBs (non-TCDD-like), and organochlorine pesticides, such as aldrin, dieldrin, DDT and certain metabolites, and toxaphene.

COMT, a crucial enzyme in the detoxification of catechol estrogens and catecholamines [86], has been reported to be inhibited by hydroxylated metabolites of PCBs [87]. The PCB metabolites 2,4,6-trichloro-3′,4′-dihydroxybiphenyl, 2,5-dichloro-3′,4′-dihydroxybiphenyl, and 2,4-dichloro-3′,4′-dihydroxybiphenyl were substrates for methylation by COMT with K_m and V_{max} values similar to those for 2- and 4-hydroxyestradiol, and adrenaline (epinephrine). The study further showed that these three catechol PCBs inhibited COMT-catalyzed methylation of 2- and 4-hydroxyestradiol with mixed-type kinetics. K_i values were estimated to be around 0.3 to 0.5 micromolars (μM).



2,4,5,-trichloro-3',4'-dihydroxybiphenyl

Fig. 7 The structure of the catechol PCB metabolite 2,4,6-trichloro-3′,4′-dihydroxybiphenyl, which is an inhibitor of catechol *O*-methyl transferase and competes with catechol estrogens for methylation.

CONCLUSIONS AND RECOMMENDATIONS

Steroidogenic enzymes are important targets for chemicals that can interfere with the endocrine system. Until recently, this mechanism of endocrine disruption has received relatively little attention in com-

parison with steroid hormone receptors. Nevertheless, several classes of environmental contaminants have been shown to inhibit the function of certain enzymes involved in either steroid hormone synthesis or metabolism, based on the results of in vivo and in vitro studies. Induction of certain steroidogenic enzymes has also been demonstrated, but only in a limited number of in vitro studies.

So far, relatively few chemicals have been screened for their potential to interfere with steroid hormone synthesis and/or metabolism, and relatively few steroidogenic enzymes have been investigated. There are relatively few bioassays available to perform such initial screenings, but the H295R cell line and possibly other in vitro systems may prove to be a useful tools for future investigations in this area.

Very little is also is known about the consequences in vivo of interferences with steroid biosynthesis. Future animal studies are required to interpret and extrapolate in vitro studies of xenobiotic interferences with steroidogenic enzymes to the in vivo situation. Given the complexities in the steroid synthesis pathways and the numerous biological activities of the resultant steroid hormones, together with the often unknown biokinetic properties of the xenobiotics, this will be a formidable but important task.

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