DEVELOPMENT OF *IN VITRO* BIOASSAYS TO ASSESS EFFECTS ON ENZYMES INVOLVED IN STEROID SYNTHESIS AND METABOLISM AS MECHANISMS OF ENDOCRINE DISRUPTION

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Introduction

There is growing concern that certain environmental contaminants have the potential to disrupt endocrine processes, which may lead to reproductive problems and other toxicities related to sexual differentiation, growth and development. Current research has focused mainly on potential interactions with the sex hormone receptors, particularly the estrogen receptor (ER). Various chemicals have been shown to be agonists (or antagonists) for the ER, but usually with very low affinities relative to 17β-estradiol. The resultant biological potencies of these chemicals are invariably low and it appears unlikely that environmental concentrations are high enough to compete effectively with 17β-estradiol for the receptor. However, other mechanisms of potential interference with endocrine functions exist, including effects on enzymes involved in steroid synthesis and metabolism. Below we discuss some of the possibilities for interference with the biosynthetic pathway of steroid hormones, with particular emphasis on the synthesis and metabolism of estrogens. In addition, several cell systems are discussed with respect to their usefulness in the development of screening assays for the effects of xenobiotics on steroidogenesis.

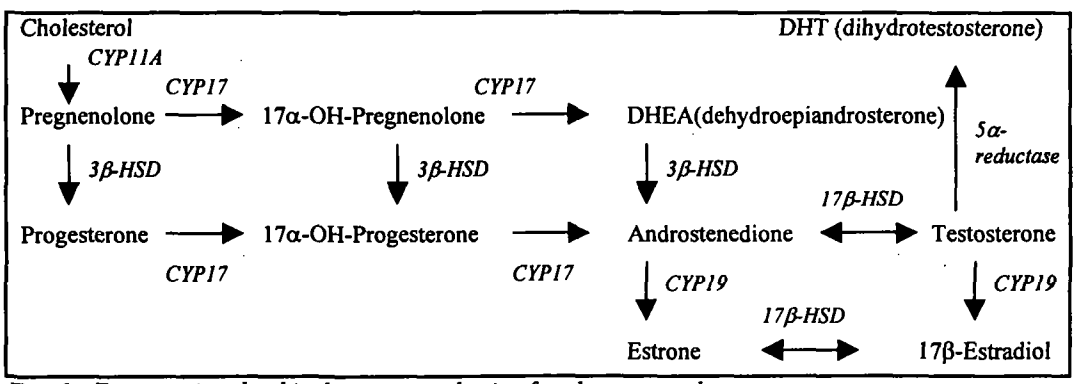

Fig. 1. Enzymes involved in de novo *synthesis of androgens and estrogens.*

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 cytochrome P-450 enzymes (CYPs), and several hydroxysteroid dehydrogenases (HSDs) and reductases (1). *De novo* synthesis of 17β-estradiol 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. 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, but also in tissues such as the brain and adipose, which utilize circulating levels of androstenedione or testosterone as precursors. Aromatase is expressed in various tissues and plays an important role in sexual differentiation, development and behavior, particularly in the brain (2), but is also involved in diseases such as estrogen-dependent tumors (3).

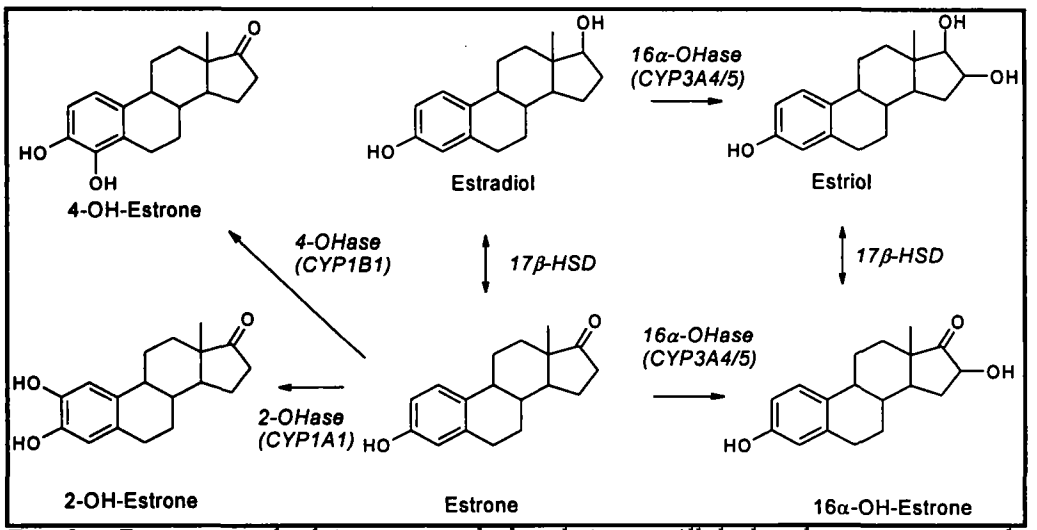

Fig. 2. Enzymes involved in estrogen hydroxylations. All hydroxyl groups can undergo methylation by COMT or conjugation to glucuronide or sulfate.

Enzymes involved in steroid metabolism

In addition to steroidogenic enzymes, steroid hormone homeostasis is regulated by enzymes that metabolize steroid hormones (4). The majority of estrogens are conjugated to sulfate or glucuronide which renders them hormonally inactive. However, a relatively small amount of estrogens are converted by CYPs to hydroxylated metabolites (catechol estrogens; exemplified in Fig. 2). Some of these metabolites, such as 4-OH-estradiol and 16α-OH-estrone are considered genotoxic and have been implicated in estrogen-mediated carcinogenesis, while other such as 2-OH-estradiol and -estrone are considered inactive (5). The catechol estrogens undergo further metabolism by catechol O-methyltransferase to methoxylated metabolites or conjugation by sulfotransferase and UDP-glucuronidyltransferase, all resulting in inactivated (non-estrogenic and non-genotoxic) 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 CYP3A, with a smaller contribution from CYP1A2. In extrahepatic tissues, where CYP3A expression is much lower, but expression of CYP1A1 and 1B1 are relatively higher, CYP1A1-mediated 2- and CYP1B1-mediated 4-hydroxylation of estrogens occurs.

Mechanisms of interference with steroid synthesis and metabolism

In contrast to a weak interaction with a sex hormone receptor, an interaction with a rate-limiting enzyme involved in sex hormone synthesis is likely to have a profound effect on hormone function and homeostasis. 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 require *in vivo* models, which fall outside the scope of this overview.

***In vitro* bioassays to screen for interferences with enzymes involved in steroid synthesis and metabolism**

Our laboratory has used several human cell lines to investigate the effects of xenobiotics on steroidogenic enzymes, including MCF-7 breast tumor, JEG-3 and JAR placental choriocarcinoma, and H295R adrenocortical carcinoma 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. They are useful for the study of estrogen hydroxylations as they express relatively high levels of CYP1A and 1B1, which are inducible by TCDD. The JEG-3 and JAR cells express high levels of aromatase, but appear relatively sensitive to cytotoxic effects of chemicals and appear more prone to apoptosis, rendering these cell systems difficult to use for screening purposes (6,7). H295R cells are somewhat less sensitive to cytotoxicity and have the major advantage that they express a wide range of steroidogenic enzymes, including all the enzymes required to produce mineralocorticoids, glucocorticoids, androgens and estrogens (8,9). 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.

Effects of xenobiotics on steroidogenic enzymes in various *in vitro* bioassays

2-chloro-s-triazine herbicides

Recently we have demonstrated the ability of the herbicides atrazine, propazine and simazine to induce aromatase activity about 2-fold in H295R cells (10). Induction is detected at concentrations as low as 300 nM. The same was true for two metabolites of atrazine, atrazine-desethyl and atrazine-desisopropyl, whereas the fully dealkylated and 2-hydroxylated metabolites of atrazine were in active (11). The mechanism of induction is not known, but the induction response may provide an explanation for some of the endocrine disrupting effects caused by exposure *in vivo* to triazine herbicides.

Organochlorines and metabolites

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 (6,7). 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 (6). Another methylsulfonated compound, the DDT metabolite 3-MeSO2-2,2-bis(4-chlorophenyl)-1,1-dichloroethene (MeSO2-DDE) is an inhibitor of CYP11B1 (11β-steroid hydroxylase) in Y-1 mouse adrenocortical tumor cells at concentrations above 3 μM (12). 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.

Diindolylmethane and structural analogs

Diindolylmethane (DIM) is an acid-catalyzed condensation product of indole-3-carbinol, found in cruciferous vegetables, such as broccoli and cabbage. DIM is known as an anticarcinogen and anti-estrogen and has been reported to alter estrogen metabolism to favour the formation of 2-OH-estrogens above 16α-OH-estrogens. Our laboratory has found that DIM is an inducer of both CYP1A1 and CYP1B1 in H295R cells, with an EC50 for EROD induction of 3 μM (13); this indicates that not only the route of estrogen 2-hydroxylation is increased, but also that of 4-hydroxylation resulting in a potentially genotoxic estrogen metabolite. DIM was also capable of inducing CYP19 (aromatase) at concentrations above 1 μM. In H295R cells, TCDD was a potent inducer of CYP1A1 and 1B1 and had an EC50 for EROD induction of about 0.3 nM. TCDD had no effect on aromatase activity at concentrations as high as 300 nM.

Future directions

There are many possibilities for interferences of xenobiotics with steroidogenic enzymes, potentially resulting in disruptions of various endocrine functions. Xenobiotics include substances from environmental contaminants to compounds found in foods, (veterinary) drugs or industrial and commercial chemicals. Numerous steroidogenic enzymes exist, responsible for very specific biotransformations, and their expression is variable and highly tissue-dependent. Here, we have given a brief overview of several enzymes involved in estrogen synthesis and metabolism with corresponding information about *in vitro* systems that may prove useful for their study. Clearly, this area of research can be expanded to include the study of enzymes involved in the synthesis and metabolism of androgens, glucocorticoids and mineralocorticoids. In *in vitro* studies of the latter two steroid families the H295R cell line could prove particularly useful.

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