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REVIEW

# The Steroid Hormone Biosynthesis Pathway as a Target for Endocrine-Disrupting Chemicals

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**Various chemicals found in the human and wildlife environments have the potential to disrupt endocrine functions in exposed organisms. Increasingly, the enzymes involved in the steroid biosynthesis pathway are being recognized as important targets for the actions of various endocrine-disrupting chemicals. Interferences with steroid biosynthesis may result in impaired reproduction, alterations in (sexual) differentiation, growth, and development and the development of certain cancers. Steroid hormone synthesis is controlled by the activity of several highly substrate-selective cytochrome P450 enzymes and a number of steroid dehydrogenases and reductases. Particularly aromatase (CYP19), the enzyme that converts androgens to estrogens, has been the subject of studies into the mechanisms by which chemicals interfere with sex steroid hormone homeostasis and function, often related to (de)feminization and (de)masculinazation processes. Studies *in vivo* and *in vitro* have focussed on ovarian and testicular function, with less attention given to other steroidogenic organs, such as the adrenal cortex. This review aims to provide a comprehensive overview of the state of knowledge regarding the mechanisms by which chemicals interfere with the function of steroidogenic enzymes in various tissues and organisms. The endocrine toxicities and mechanisms of action related to steroidogenesis of a number of classes of drugs and environmental contaminants are discussed. In addition, several potential *in vitro* bioassays are reviewed for their usefulness as screening tools for the detection of chemicals that can interfere with steroidogenesis. Analysis of the currently scattered state of knowledge indicates that still relatively little is known about the underlying mechanisms of interference of chemicals with steroidogenesis and their potential toxicity in steroidogenic tissues, neither in humans nor in wildlife. Considerably more detailed and systematic research in this area of (endocrine) toxicology is required for a better understanding of risks to humans and wildlife.**

*Key Words:* **aromatase; steroidogenesis; H295R cells; cytochrome P450; endocrine disrupters; pesticides.**

There is increasing evidence that various chemicals introduced into the environment have the potential to disrupt the endocrine system in humans and wildlife. Endocrine-disrupting effects of environmental contaminants and commercial products have the potential to cause reproductive problems, increase the risk of the development of hormone-dependent cancers, and may result in other adverse effects on (sexual) differentiation, growth, and development. In the last decade, research has focused on potential interactions of chemicals with various hormone receptors, with particular emphasis on the thyroid hormone and estrogen receptor, and more recently the androgen receptor. Numerous chemicals have been shown to be agonists or antagonists for the estrogen receptor in various *in vitro* systems, although usually with very low affinities relative to endogenous hormones such as 17β-estradiol and estrone. However, many other mechanisms of interference with the endocrine system exist. The current definition of an endocrine disruptor is ''an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub)populations'' (IPCS, 2002). Inherent in this definition is the extremely wide scope of potential adverse effects and underlying mechanisms involved in the actions of endocrine-disrupting chemicals that need to be considered. Increasingly, key enzymes involved in steroid hormone synthesis and metabolism are being considered as important targets for endocrine-disrupting chemicals. Particularly, the cytochrome P450 (CYP) enzymes responsible for the highly specific reactions in the steroid biosynthetic pathway (Miller, 1988) are gaining interest as molecular 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 review will provide an overview of the enzymes involved in the synthesis of steroid hormones, their cellular regulation, and a few examples of their role in endocrine processes where relevant for the potential interaction with

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endocrine-disrupting chemicals. The mechanisms of action of various groups of compounds suggested or known to cause endocrine disruption by interfering with steroidogenesis will be discussed. This review will also give an update of the progress made in the development of sensitive *in vitro* bioassays for the screening of chemicals for potential interferences with steroidogenesis.

## STEROID BIOSYNTHESIS

Steroidogenic enzymes are responsible for the biosynthesis from cholesterol of various steroid hormones including glucocorticoids, mineralocorticoids, progestins, androgens, and estrogens. They consist of several specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases (Miller, 1988). Numerous organs are known to have the capacity to synthesize biologically active steroids, including the adrenal gland, testis, ovary, brain, placenta, and adipose tissue. Three endocrine organs that specialize in *de novo* steroid production, the adrenal gland, testis, and ovary, are described below in more detail.

*De novo* synthesis of all steroid hormones starts with the conversion of cholesterol to pregnenolone by CYP11A (cholesterol side-chain cleavage) (Parker and Schimmer, 1995). CYP11A is bound to the inner membrane of the mitochondrion and is found in all steroidogenic tissues (Miller, 1988; Reincke *et al.*, 1998) but is not or poorly expressed in nonsteroidogenic tissues. Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), one of several non-CYP450 enzymes involved in steroidogenesis and which is found in both mitochondria and smooth endoplasmic reticulum. 3β-HSD is widely distributed in steroidogenic and nonsteroidogenic tissues and consists of two isoenzymes, which are regulated in a tissue-specific manner (Gingras *et al.*, 2001; Leers-Sucheta *et al.*, 1997; Mason *et al.*, 1997; Simard *et al.*, 2005). The type 2 3β-HSD is predominantly expressed in steroidogenic tissues such as adrenal, testis, and ovary, whereas type 1 is found in placenta and in nonsteroidogenic tissues such as liver, kidney, and skin. Pregnenolone and progesterone form the precursors for all other steroid hormones.

### *Steroidogenesis in the Adrenal Cortex*

The adrenal gland is the most important steroidogenic tissue in the human body and, unlike the gonads, essential for survival (Addison, 1855). All steroidogenic processes take place in the adrenal cortex, which is histologically and functionally divided into three concentric zones: the outer zona glomerulosa, the intermediate zona fasciculata, and the central zona reticularis (Neville and O'Hare, 1985). Each zone is responsible for the synthesis of a specific set of steroid hormones, the mineralocorticoids, glucocorticoids, and weak androgens, respectively (Miller and Tyrell, 1995). Steroid production is regulated by specific external stimuli, such as adrenocorticotropic hormone (ACTH), which increases mainly glucocorticoid and weak androgen production via the cAMP–mediated protein kinase A (PKA) pathway (Rainey, 1999) that activates factors such as steroidogenic acute regulatory protein (Stocco, 2001) and steroidogenic factor-1 (SF-1) (Morohashi and Omura, 1996; Morohashi *et al.*, 1992), and angiotensin II and potassium, which selectively increase mineralocorticoid synthesis (Schimmer and Parke, 1996) via the inositol triphosphate/diacylglycerol–mediated protein kinase C (PKC) pathway (Rainey, 1999). Mineralocorticoids are essential for survival as they tightly regulate the Na+/K+ balance in extracellular fluids. Glucocorticoids are important in glucose homeostasis and the response of the organism to stressors and are also important in fetal lung development, immune modulation, and maintenance of normal function of a variety of tissues.

Steroid 21-hydroxylase (CYP21), an enzyme unique to the adrenal cortex, is essential for the biosynthesis of mineralo- and glucocorticoids. It is expressed in the smooth endoplasmic reticulum of all three adrenocortical zones and is responsible for the conversion of progesterone and 17α-hydroxyprogesterone to 11-deoxycorticosterone (mineralocorticoid pathway) and 11-deoxycortisol (glucocorticoid pathway), respectively (Sasano *et al.*, 1988). These precursors, in turn, are converted to the biologically active hormones aldosterone and cortisol by aldosterone synthetase (CYP11B2) and steroid 11β-hydroxylase (CYP11B1), respectively. These two mitochondrial enzymes are also unique to the adrenal cortex. CYP11B1, which is expressed in the zonae fasciculata and reticularis (Erdmann *et al.*, 1995; Ogishima *et al.*, 1992) has strictly 11β-hydroxylase activity, whereas CYP11B2, which is expressed only in the zona glomerulosa (Ogishima *et al.*, 1992; Pascoe *et al.*, 1995), has additional 18-hydroxylase/aldosterone synthetase activity, explaining the zone selectivity of adrenocortical steroid biosynthesis. The adrenal weak androgens are formed by CYP17, a single enzyme with both 17α-hydroxylase and 17,20-lyase activities. CYP17 is also found in ovary and testis but not in human placenta (Voutilainen and Miller, 1986). The two activities are due to distinct catalytic sites on the same enzyme which is expressed in the smooth endoplasmic reticulum (Miller, 1988). CYP17 hydroxylates pregnenolone and progesterone to form the respective 17α-hydroxysteroids, a process which occurs in the zonae reticularis and fasciculata but not in the zona glomerulosa (Reincke *et al.*, 1998). The 17,20-lyase activity of CYP17 is low in the adult adrenal cortex and is exclusive to the zona reticularis, where it converts the two 17α-hydroxylated steroids to the weak androgens dehydroepiandrosterone (DHEA) and androstenedione, respectively.

### *Steroidogenesis in the Testis*

The role of the testis is to produce fertile sperm for procreation and steroid hormones for sexual and reproductive function. Follicle-stimulating hormone (FSH) stimulates male germ cells (spermatogonial cells) to develop into mature

sperm, a process called spermatogenesis. These stem cells are continuously renewed by mitosis for most of the lifetime of human males. FSH binds to FSH receptors on Sertoli cells and stimulates the cAMP-mediated second messenger pathway resulting in the activation of various factors required for successful spermatid production. Luteinizing hormone (LH) binds to its receptor on the Leydig cell membrane, which is also coupled to the cAMP signaling pathway, to stimulate the production of testosterone *de novo* from cholesterol. In concert with the actions of FSH, this testosterone is required for optimal sperm production, as well as for sexual function. LH induces the various cytochrome P450 enzymes and dehydrogenases involved in testosterone synthesis in Leydig cells, including CYP17 17,20-lyase, the key activity directing the biosynthesis of steroids toward the sex hormones (Dharia *et al.*, 2004; Sasano *et al.*, 1989). The weak androgen androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD), and the balance between these androgens depends on the activity and type of 17β-HSD present. 17β-HSD types 3 and 5 catalyze the conversion of androstenedione to testosterone and are expressed in the testis Leydig cells (Mindnich *et al.*, 2004), whereas type 2 (found in prostate and placenta among others) performs the opposite reaction (Luu-The, 2001). Leydig, Sertoli, and germ cells further express low levels of aromatase, which converts testosterone originating from the Leydig cells into estradiol, a step that appears to be necessary for the successful initiation of spermatogenesis and mitosis of spermatogonia (Carreau *et al.*, 2003). It was found that treatment of bonnet monkeys (*Macaca radiata*) with a triazole-containing aromatase inhibitor related to letrozole blocked spermatid development (Shetty *et al.*, 1997). The most potent endogenous androgen dihydrotestosterone (DHT) is formed from testosterone by steroid 5α-reductase. This reaction is weak in the adult testis but is predominant in the epididymis and prostate where DHT has important physiological roles in maintaining sexual function. It is also expressed in other peripheral tissues such as skin and liver. Increased expression of steroid 5α-reductase has been associated with benign prostate hyperplasia and prostate cancer.

### *Steroidogenesis in the Ovaries*

The main role of the ovary is to produce eggs for fertilization and steroid hormones for sexual and reproductive function. The ovum inside the developing follicle is directly surrounded by layers of granulosa cells followed by thecal cells, which is where steroidogenesis predominantly takes place. The theca interna is highly vascularized and produces large amounts of progesterone and androgens, which act as precursor for estrogen synthesis in the granulosa cells. Androstenedione and testosterone diffuse into the neighboring poorly vascularized granulosa cells where they are converted to predominantly estradiol via the concerted action of aromatase and 17β-HSD types 1 and 7, which favor the conversion of estrone to estradiol (Luu-The, 2001; Mindnich *et al.*, 2004). In the preovulatory follicular stage, during which the follicle maturates, estrogen synthesis increases gradually due to upregulation of aromatase by LH and FSH. During this critical phase, estrogen appears to be responsible for the upregulation of LH receptors and the initiation of the positive feedback loop responsible for the LH and FSH surge which triggers ovulation (Greenwald and Roy, 1994). Interference with the synthesis of estrogens during this critical window of time would prevent ovulation. After the LH surge, the follicle enters the luteal phase and becomes a corpus luteum which predominantly synthesizes progesterone. Decreased LH concentration and subsequently decreased aromatase expression result in declining estrogen production (Fitzpatrick *et al.*, 1997), while a concurrent increase in CYP11A and 3β-HSD activity promotes the synthesis of progesterone which via its receptor initiates the process of follicle rupture.

## EFFECTS OF CHEMICALS THAT INTERFERE WITH STEROIDOGENESIS

### *Mitotane (o,p′-DDD): A Classic Example*

An early example of an environmental contaminant that specifically interferes with steroid biosynthesis is *o,p′*-DDD (mitotane; 1-chloro-2-(2,2-dichloro-1-(4-chlorophenyl)-ethyl)-benzene), which is found in *p,p′*-DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) formulations used extensively since the second world war, but was also used as an insecticide on its own accord. As early as 1949, *o,p′*-DDD (50 mg/kg) was found to be adrenotoxic in dogs (Nelson and Woodward, 1949). Mitotane appears to selectively affect the zona fasciculata where it inhibits mitochondrial steroid 11β-hydroxylase (CYP11B1) and cholesterol side-chain cleavage (CYP11A1) activity, thus reducing the production of glucocorticoids. This results in severe adrenal insufficiency, characterized by a loss of appetite, hypothermia, nausea, general weakness, and ultimately death. *o,p′*-DDD is believed to be bioactivated by a mitochondrial cytochrome P450, most probably CYP11B1, to reactive intermediates which cause extensive mitochondrial damage resulting in adrenocortical necrosis (Cai *et al.*, 1995; Martz and Straw, 1980). Its toxicity in bovine adrenocortical cells in primary culture was strongly correlated with the metabolism of *o,p′*-DDD to its major metabolite 1,1-(2,4′-dichlorodiphenyl)acetic acid via aliphatic oxidation of the 2,2-dichloroethyl moiety. The less toxic isomers, *p,p′*- and *m,p′*-DDD (Cai *et al.*, 1995), were less susceptible to aliphatic β-oxidation and preferred a conversion to ethylene derivatives via α-hydroxylation of the benzylic carbon of the 2,2-chloroethyl moiety and subsequent dehydrochlorination. The adrenotoxic actions of mitotane have made it of some clinical use in the treatment of nonoperable adrenocortical cancers (Chabner *et al.*, 1996).

*Other DDT Analogues and Metabolites*

Other analogues of *o,p′*-DDD, such as *p,p′*-DDT and several of its metabolites, *p,p′*-DDE 1,1-dichloro-2,2-bis(4-chlorophenyl)ethene and 3-methylsulfonyl-2,2-bis(4-chlorophenyl)-1,1-dichlorethene (3-Me$SO_2$-DDE) have also been studied for effects on steroidogenic enzymes. The 3-Me$SO_2$-DDE metabolite of *p,p′*-DDT has been found in various species including the Baltic grey seal (*Halichoerus grypus*) (Larsson *et al.*, 2004) and humans (Chu *et al.*, 2003) in a concentration range of anywhere from 0.5 to 22 ng/g lipid weight. Lund and coworkers observed a high incidence of bilateral adrenocortical hyperplasia in the declining Baltic gray seal population associated with elevated 3-Me$SO_2$-DDE contamination (Lund, 1994). 3-Me$SO_2$-DDE was shown to be a relatively potent inhibitor of CYP11B1 in Y-1 mouse adrenocortical tumor cells (Johansson *et al.*, 1998a; Lund and Lund, 1995). In an apparently similar way to mitotane, 3-Me$SO_2$-DDE acts as a substrate for CYP11B1, inhibiting synthesis of cortisol, and is bioactivated to a reactive intermediate that causes damage to mitochondrial proteins resulting in adrenocortical toxicity (Lund and Lund, 1995), also in human adrenocortical mitochondrial fractions and tissue slices (Jonsson and Lund, 1994; Lindhe *et al.*, 2002). Its inhibitory effect on CYP11B1 was further confirmed in human H295R adrenocortical carcinoma cells (Johansson *et al.*, 2002). It is plausible that the resistance of *p,p′*-DDT to aliphatic oxidation and preferred conversion to *p,p′*-DDE 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane explains the lack of adrenotoxicity of this insecticide and its inability to inhibit CYP11B1 activity. However, *o,p′*-DDT and in particular its highly persistent metabolite *p,p′*-DDE are potent reproductive toxins in nontarget species that are situated high in the food web, such as piscivorous birds, predator fish, and possibly humans. In the laboratory, *p,p′*-DDE has been established to be a relatively potent antagonist of the androgen receptor and to cause overt antiandrogenic effects on the developing rat fetus at 100 mg/kg/day (Kelce *et al.*, 1995). The interactions of xenobiotics such as *p,p′*-DDT with sex hormone receptors have been reviewed in detail in several publications (Gray *et al.*, 2001; Kelce and Wilson, 1997; Kelce *et al.*, 1998; Rosselli *et al.*, 2000; Sonnenschein and Soto, 1998) and are beyond the scope of this review.

Aromatase was also the subject of various *in vitro* and *in vivo* studies of the effects of *p,p′*-DDT and its metabolites. In H295R cells, neither *p,p′*-DDT nor *o,p′*-DDT, *p,p′*-DDE, and *o,p′*-DDE were capable of affecting the catalytic activity of aromatase unless cytotoxic concentrations ($\geq$ 10μM) were used (Sanderson *et al.*, 2002). Similarly, a study using an immortal testis cell line derived from the green sea turtle (*Chelonia mydas*) showed that *p,p′*-DDE did not reduce aromatase activity unless exposures were in the cytotoxic range (Keller and McClellan-Green, 2004). A recent *in vivo* study in Japanese medaka (*Oryzias latipes*), on the other hand, showed that *o,p′*-DDT, the only DDT analogue to have significant affinity for the estrogen receptor, was capable of increasing aromatase activity in the brain (Kuhl *et al.*, 2005). Medaka fry were exposed to increasing concentration of *o,p′*-DDT for up to 14 days. Increased brain aromatase activity, which was observed at an actual water concentration of 5.5 μg/l *o,p′*-DDT corresponded with an almost complete sex reversal (96% female). The authors hypothesize that *o,p′*-DDT, by activating the estrogen receptor, causes increased aromatase gene expression and catalytic activity due to increased transcription via estrogen-responsive elements (EREs) found in the promoter region of the brain-specific *cyp19b* gene (Kuhl *et al.*, 2005), a gene unique to teleost fishes. This mechanism has been shown to occur in Japanese medaka testis and ovary with 17α-ethynylestradiol (Scholz and Gutzeit, 2000) and in liver of both sexes with 17β-estradiol and the xenoestrogens, bisphenol A and nonylphenol (Min *et al.*, 2003). Estrogens together with androgens are also purported to activate (presumably via their respective steroid hormone receptors) brain-specific promoters of aromatase in the Japanese quail (*Coturnix japonica*) (Balthazart *et al.*, 2003). Overall, the observed reproductive toxicities of *p,p′*-DDE appear to be largely due to its ability to block androgen receptor signaling and do not appear to involve direct interferences with steroidogenesis.

*PCBs and Metabolites*

Several methylsulfonated metabolites of polychlorinated biphenyls (PCBs) were found to inhibit CYP11B1 in Y-1 mouse adrenocortical tumor cells (Johansson *et al.*, 1998). It was demonstrated that the Me$SO_2$ group on the third or fourth position of the PCB molecule was essential for this interaction, as the parent PCBs had no effect. The two most environmentally relevant metabolites, Me$SO_2$-CB-49 and -101, however, had no effect. This was also true in adrenal mitochondria of the grey seal (Lund, 1994) Also, this *in vitro* study failed to examine the potential cytotoxicity of the other Me$SO_2$ metabolites, all of which were tested at 20μM. The two most potent inhibitors, 4-Me$SO_2$-CB-64 and 4-Me$SO_2$-CB-110, caused strong decreases (34 and 23%, respectively) in cellular protein content at this concentration, suggesting that the decreased CYP11B1 activity may not be a selective effect but associated with general toxicity to the cell. Recently, it was found that 3-Me$SO_2$-CB-132, 4-Me$SO_2$-CB-132, 4-Me$SO_2$-CB-149, and 4-Me$SO_2$-CB-91 inhibited the catalytic activity of aromatase in H295R cells and in human mammary fibroblasts in primary culture (Heneweer *et al.*, 2005b), with $IC_{50}$ values between 1 and 2μM. The investigators concluded, however, that biologically significant aromatase inhibition was unlikely to occur *in vivo* at the then current environmental concentrations of PCB metabolites.

*TCDD*

*Bovine adrenocortical studies.* TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is known to interfere with steroid homeostasis

*in vitro* and *in vivo*. Early studies in cultured bovine adrenocortical cells indicated that preincubation (24 h) with 10nM TCDD caused a decrease in ACTH- or cAMP-mediated stimulation of cortisol synthesis, which appeared to coincide with enhanced depletion of cholesterol from the mitochondrion (DiBartolomeis *et al.*, 1986; Jefcoate *et al.*, 1987). No evidence for direct inhibition of CYP11A was found in these studies.

*Rat testicular studies.* In rats exposed to 100 μg/kg TCDD *in vivo* for 7 days, testicular testosterone secretion *ex vivo* was decreased by 30–75% after stimulation with human choriogonadotropin (hCG), relative to testes from control rats (Kleeman *et al.*, 1990). Addition of exogenous pregnenolone restored testosterone secretion to control levels, thus indicating that either cholesterol synthesis or mobilization to the mitochondria or inhibition of CYP11A is responsible for the decreased steroid synthesis. A follow-up study found that reductions in CYP11A activity could not explain the strong decrease testosterone production and that TCDD was in fact decreasing the ability of the cell to mobilize cholesterol from cholesterol esters (by cholesterol hydrolases), reducing its availability to the mitochondrion (Moore *et al.*, 1991). It has further been noted that the size and number of testicular Leydig cells were reduced in rats exposed to 50 μg of TCDD for 4 weeks (Johnson *et al.*, 1994). This reduction concurred with fewer organelles such as smooth endoplasmic reticulum and mitochondria, reducing the number of organelles where steroidogenesis occurs. In isolated mature rat Leydig cells in culture, TCDD (0.2 or 2 ng/ml; 0.6 and 6nM) was found to reduce CYP11A expression in rat Leydig cells by impairing the ability of hCG to increase intracellular cAMP levels (Gammon *et al.*, 2005), an effect which is fairly consistent with the observations by Moore *et al*. (1991). Reduced activity of the cAMP-mediated PKA pathway would also be expected to reduce the mobilization of cholesterol by cholesterol hydrolases.

*Rat ovarian studies.* TCDD, in relatively low dosages (1–10 μg/kg), is capable of disrupting estrous cycle and blocking ovulation in Sprague-Dawley rats (Gao *et al.*, 1999; Li *et al.*, 1995a,b; Son *et al.*, 1999), although mice appear less sensitive (Cummings *et al.*, 1996). Studies using gonadotropin-primed immature female rats as an ovulation model (Gao *et al.*, 1999; Li *et al.*, 1995b) demonstrated that TCDD and other polychlorinated dibenzo-*p*-dioxins (PCDDs) decrease ovarian weight before the whole body weight is affected. TCDD also caused an early (within 24 h and unrelated to gonadotropin injection) unscheduled surge in endogenous LH and FSH levels in serum, yet a decrease in the surge at the expected time of ovulation (72 h after gonadotropin injection). Serum levels of estradiol were consistently higher and those of progesterone lower in TCDD-treated rats than in controls. Also, estradiol levels in TCDD-treated rats did not decline in the hours just before ovulation as occurred in the controls. To address the question whether steroidogenic enzymes play a role as direct targets for the anovulatory action of TCDD, immature hypophysectomized rats were exposed to 20 μg/kg TCDD prior to priming with gonadotropins (Son *et al.*, 1999). In this ovulation model, TCDD had no effect on the serum levels of progesterone or estradiol during the 72 h until the time of expected ovulation. In granulosa cells in culture, 48 and 96 h TCDD exposures had no effect on the ability of LH and FSH to increase progesterone and estradiol. In thecal cells, TCDD had no effect on LH-stimulated progesterone and androstenedione secretion.

*Human luteinized granulosa cells.* In cultured human luteinized granulosa cells (hLGCs), TCDD has been shown to cause a concentration-dependent decrease in estradiol production in the 0.1–10nM range (Enan *et al.*, 1996; Heimler *et al.*, 1998). Progesterone secretion was not affected in these experiments, and addition of androgen substrate abolished the effect on estradiol production (Heimler *et al.*, 1998). These findings indicated that TCDD did not affect cholesterol mobilization or CYP11A inhibition but suggested a possible interference of TCDD with steps in the steroidogenesis pathway that occur after the biosynthesis of progesterone. Moran *et al.* (2000) exposed hLGCs in culture medium containing hCG to 10nM TCDD for 8 days to test the hypothesis that estradiol secretion was reduced through inhibition or downregulation of the enzyme aromatase. A decrease in neither immunoreactive CYP19 protein nor catalytic activity was observed despite a 75% lower estradiol secretion. Furthermore, addition of exogenous DHEA abolished the effect on estradiol production, supporting a lack of role for aromatase in reduced estradiol production. As there was no effect on progesterone or 17α-hydroxyprogesterone production, a follow-up study was conducted to examine the effects of TCDD on steroid 17,20-lyase activity and CYP17 expression levels (Moran *et al.*, 2003). This study found a strong correlation between CYP17 immunoreactive protein levels and E2 secretion levels among individual hLGC preparations. It also found that steroid 17,20-lyase activity was about 10 times lower than that of aromatase, indicating that the prior activity and not aromatase activity is rate limiting in the synthesis of estradiol in hLGCs. TCDD decreased CYP17 protein levels by over 50%, without affecting cytochrome P450 reductase levels (Moran *et al.*, 2003). It also decreased 17,20-lyase activity by about 65% without affecting aromatase as observed earlier (Moran *et al.*, 2000). The mechanism of selective inhibition of CYP17 17,20-lyase activity is not understood as it appears that CYP17 steroid 17α-hydroxylase activity is relatively unaffected.

TCDD and other persistent halogenated Ah receptor agonists are clearly capable of causing endocrine disruption and are known reproductive and developmental toxicants. They elicit potent antiestrogenic effects, disrupt ovulation and sperm production, and affect sex steroid levels in exposed organisms. However, a clear role for steroidogenic enzymes as direct targets for TCDD has not been established. TCDD is a highly potent agonist for the Ah receptor, which appears to mediate

most toxicities associated with TCDD. Recent studies indicate that this is also true for the mechanisms by which TCDD interferes with steroid hormone synthesis. Fukuzawa *et al.* (2004) found that the downregulation of CYP11A and LH receptor expression in TCDD-exposed mice was dependent on the presence of Ah receptor, as Ah receptor-null mice were not affected. The molecular mechanisms involved in the Ah receptor–mediated interference of TCDD with the cAMP-stimulated PKA pathway and other signaling pathways that control the expression of steroidogenic enzymes and other proteins in steroidogenic tissues require considerable further study.

### *Brominated Flame Retardants*

Brominated flame retardant such as the polybrominated diphenylethers (PBDEs) are used in large quantities in various commercial and industrial products. Concentrations of several PBDE congeners have been increasing steadily over the last few decades in the biotic and abiotic environments. Their widespread use and ubiquitous presence in the environment has raised concern about possible adverse effects in humans and wildlife. Several PBDEs have been found to disrupt the endocrine system. Pregnant rats exposed to a single dose of 60 or 300 μg PBDE-99/kg body weight on gestation day 6 resulted in decreased spermatogenesis in male offspring (Kuriyama *et al.*, 2005). Exposure of male rats to the commercial PBDE mixture DE-71 during the peripubertal period caused a delay in the onset of puberty, together with decreased seminal vesicles and ventral prostate weights, indicating an antiandrogenic mechanisms of action of one or more components of the mixture (Stoker *et al.*, 2004). An elaborate study set out to determine the mechanisms of these antiandrogenic effects revealed that adult rats exposed for 3 days to 3, 30, or 60 mg DE-71/kg/day caused a significant increase in serum LH levels at the highest dose (Stoker *et al.*, 2005). It had little impact on testosterone or estrone levels, suggesting that not steroidogenesis but possibly the androgen receptor is a target for DE-71–mediated antiandrogenicity. In the same study, male rats exposed peripubertally to 60 or 120 mg DE-71/kg/day for 30 days (PND23-53) had impaired growth of the seminal vesicles and of the ventral and lateral prostate. Here too there were no effects on serum levels of testosterone or LH, although there was a statistically significant reduction in thyroxin levels at both dose levels. In the Hershberger assay (using castrated testosterone supplemented rats), DE-71 caused a typical antiandrogenic profile at all doses tested (30–240 mg/kg/day for 9 days) (Stoker *et al.*, 2005). Furthermore, this study demonstrated the ability of DE-71 and several individual PBDE congeners to bind to the prostate cytosolic androgen receptor and to activate the human androgen receptor in the stably transfected MDA-kb2 breast cancer cell line. The DE-71 components BDE-47 and -100, but not -99, -153, and -154, showed antagonism for the androgen receptor *in vitro*. These findings indicate that DE-71 and several of its components are antiandrogenic endocrine disruptors by acting as competitive antagonists for the androgen receptor. The implications and underlying mechanisms of the observed hypothyroxemia is not yet understood. Direct or indirect effects on steroidogenesis do not appear to play a role in the endocrine disruptive effects caused by DE-71. A recent study determined the potential effects of several individual PBDE congeners and hydroxylated metabolites on aromatase activity in H295R human adrenocortical carcinoma cells. None of the compounds had an effect, except for the PBDE metabolite 6-OH-BDE-99 which caused inhibition of aromatase activity at concentrations above 5μM (Canton *et al.*, 2005). This latter observation is of interest as a study in 2006 demonstrated that male rats exposed perinatally to 1 and 10 mg/kg of BDE-99 (administered to pregnant dams on GD10-18) exhibited feminization based on reduced anogenital distance and increased sweet preference (Lilienthal *et al.*, 2006). Plasma sex steroid concentrations were also concentration dependently suppressed in males at PND21, and this effect was still observed on PND160. These observations could be explained by inhibition of CYP17 and/or 19, as such inhibition would be expected to diminish the androgen imprinting process in the male rat during the perinatal period. Whether sufficient concentrations of 6OH-metabolite of BDE-99 would be formed to cause such an effect is not known, but the ability of hydroxylated metabolites of PBDEs to interfere with perinatal imprinting through inhibition of steroidogenesis warrants further investigations.

### *Azole Fungicides and Antifungal Drugs*

Azole fungicides (compounds containing an imidazole, triazole, or pyrimidine moiety) and azole antifungal drugs block the biosynthesis of ergosterol in yeasts and fungi by inhibiting the cytochrome P450 enzyme 14α-lanosterol demethylase (CYP51). These fungicides are not 100% selective for CYP51 and demonstrate various extents of cross-selectivity for mammalian (human) hepatic drug metabolizing CYP enzymes (Zhang *et al.*, 2002) and steroidogenic CYP enzymes, such as CYP17 (steroid 17α-hydroxylase and/or 17,20-lyase activity) (Ayub and Levell, 1987, 1989; Mason *et al.*, 1987), CYP11B (steroid 11β-hydroxylase) (Ayub and Levell, 1989; Mason *et al.*, 1987), and aromatase (Ayub and Levell, 1988, 1990; Mason *et al.*, 1987; Sanderson *et al.*, 2002; Vinggaard *et al.*, 2000).

*Inhibition of steroidogenic enzymes* in vitro. Several azole-containing chemicals used in either agriculture (fungicides) or medicine (antifungals) have been shown to inhibit the catalytic activity of aromatase in human placental microsomes (Ayub and Levell, 1988; Mason *et al.*, 1987; Vinggaard *et al.*, 2000). Inhibitory potencies for the antifungal drugs econazole, tioconazole, bifoconazole, miconazole, isoconazole, and clotrimazole were found in the upper nanomolar range ($IC_{50}$

values between 0.25 and 0.67μM), whereas ketoconazole and nimorazole were less potent ($IC_{50}$ values of 7.3 and 66μM, respectively) (Ayub and Levell, 1988). Considerably less effective inhibitors in this study were astemizole, metronidazole, carbimazole, mebendazole, tinidazole, and thiabendazole that inhibited aromatase activity less than 50% at 100μM. Vinggaard *et al.* (2000) determined $IC_{50}$ values for the fungicides prochloraz, imazalil, propioconazole, fenarimol, triadimenol, and triadimefon of 0.04, 0.34, 6.5, 10, 21, and 32μM, respectively. Ayub and coworkers also found that several azole antifungals were capable of inhibiting CYP17 17α-hydroxylase and 17,20-lyase activities in human adrenal microsomes. Inhibition potencies ($IC_{50}$ values) for bifoconazole, clotrimazole, ketoconazole, miconazole, econazole, isoconazole, and tioconazole ranged from 0.6 to 2μM for 17,20-lyase activity and from 1 to 4μM for 17α-hydroxylase activity. This study further observed weaker but still lower micromolar inhibition potencies of these azole compounds toward CYP11B1 and CYP21. These findings indicate that the class of azole fungicides and antifungal drugs has the general capability to interfere with the CYPs involved in steroidogenesis.

In human cell culture experiments, results for aromatase inhibition by azole fungicides were found to be similar to those in human placental microsomes preparations. Examining more closely the inhibition kinetics of the various azole structures in H295R cells (Sanderson *et al.*, 2002), the commonly used imidazole fungicides imazalil and prochloraz were determined to be potent mixed-type inhibitors ($K_i/K'_i = 0.04/0.3$ and 0.02/0.3μM, respectively), whereas the triazole fungicides propiconazole, difenoconazole, and penconazole were less potent competitive inhibitors ($K_i = 1.9$, 4.5, and 4.7μM, respectively). Prochloraz and imazalil are structurally similar to various imidazole-containing drugs used clinically, such as the potent aromatase inhibitor fadrozole ($K_i$ in lower nanomolar range) and numerous antifungal drugs shown to reversibly (although not necessarily competitively) inhibit aromatase activity in human placental microsomes (Ayub and Levell, 1988). It is not clear whether imidazole structures are generally noncompetitive or mixed-type inhibitors; e.g., the imidazole-containing aromatase inhibitor fadrozole has been reported to have competitive (Moslemi and Seralini, 1997) and noncompetitive properties. Consistent with the competitive nature of the triazole fungicides, the clinically used aromatase inhibitor letrozole, notwithstanding its far greater potency, was also found be a competitive inhibitor of microsomal aromatase ($K_i = 1.2$nM) in guinea pig brain (Choate and Resko, 1996).

Several of these antifungals compounds also inhibited aromatase activity in rainbow trout ovarian microsomes with $IC_{50}$ values for clotrimazole, imazalil, prochloraz, and ketoconazole of 0.5, 5, 5, and 50μM, respectively (Monod *et al.*, 1993). Prochloraz was shown to inhibit estradiol secretion in rainbow trout ovarian follicles in culture, indicating the potential of these azole fungicides to block natural estrogen–mediated responses such as vitellogenin synthesis in female oviparous species during reproduction.

*Effects of azole fungicides and other azole-containing aromatase inhibitors* in vivo. A study examining the effects of aromatase inhibition on embryonic development found that exposure *in ovo* of chickens to the nonsteroidal aromatase inhibitor fadrozole led to ''masculinization'' of females (Elbrecht and Smith, 1992). In other words, aromatase inhibition during the critical time of embryonic development caused genotypical females to develop as phenotypical 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 (Elbrecht and Smith, 1992). A later study in chicken and turkey chicks exposed *in ovo* to fadrozole found that female hatchlings had testes-like gonads and the presence of atypical seminiferous tubules, also indicating a strong masculinizing effect (Burke and Henry, 1999).

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 (Jost, 1983). Thus in mammals, including humans, it can be suggested that during critical (irreversible) developmental periods, such as embryonic, perinatal, and pubertal development, aromatase inhibition may result in inappropriate demasculinization responses in males and defeminization in females.

*Prochloraz.* Although azole fungicides such as prochloraz and imazalil are particularly potent inhibitors of aromatase activity *in vitro*, the toxicological consequences of exposures *in vivo* have not been fully investigated. Recently, prochloraz was found to be a weak antagonist for the androgen receptor *in vitro* in an androgen receptor reporter assay in Chinese hamster ovarian cells transfected with human androgen receptor (Andersen *et al.*, 2002; Vinggaard *et al.*, 2002). $IC_{50}$ values for antiandrogenicity in this *in vitro* system ranged from 4 to more than 10μM. These values are between one and two orders of magnitude greater than the $IC_{50}$ values observed *in vitro* for inhibition of aromatase activity, which ranged from 0.04 to 0.7μM. A rat study investigating the antiandrogenic effects of prochloraz found a relatively weak effect on androgen-dependent seminal vesicle size in intact young rats exposed to 250 mg/kg prochloraz. In castrated rats supplemented with testosterone propionate prochloraz had a weak effect on androgen-dependent ventral prostate weight, seminal vesicles, and bulbourethralis, effects similar in profile to the potent antiandrogen flutamide. However, the researchers point out that several variables such as seminal vesicle weight are also dependent on estrogens, so a possible involvement of prochloraz-mediated CYP19 inhibition (possibly locally in the prostate or seminal vesicle) could not be ruled out. A perinatal exposure study recently demonstrated the ability of prochloraz to feminize male rat offspring (Vinggaard *et al.*, 2005). Dams

exposed to 30 mg/kg prochloraz by oral gavage from gestational day 7 to postnatal day 17 produced male offspring that had strongly reduced plasma and testicular testosterone levels as determined on gestational day 21. Bulbourethral glands were also lower in weight when examined on postnatal day 16, and nipple retention was significantly elevated upon examination on postnatal day 13. It is suggested that these effects are due to the capability of prochloraz, just like several other imidazole-containing chemicals (Ayub and Levell, 1987, 1989; Mason *et al.*, 1987), to inhibit CYP17 activity and are not related to the potential antiandrogenicity of prochloraz (at this dose at least). This *in vivo* observation appears to be consistent with the relatively potent inhibitory effect of prochloraz on steroidogenic enzymes and its lesser potent antagonistic effect on the androgen receptor.

A recent study in fathead minnows (*Pimephales promelas*) exposed to the imidazole-containing medicinal aromatase inhibitor fadrozole for 21 days demonstrated numerous perturbations of the male and female endocrine system (Ankley *et al.*, 2002). Brain aromatase was inhibited to about 20% of control levels in both sexes at 50 μg/l fadrozole. In males, plasma levels of testosterone and 11-ketostestosterone and the gonadal-somatic index were increased concentration dependently by fadrozole (2–50 μg/l). Marked changes in gonadal histopathology included enlargement of the seminiferous vesicles and accumulation of sperm. In females, a concentration-dependent decrease in plasma levels of estradiol and vitellogenin was observed, with no effect on testosterone. Furthermore, ovary maturation was delayed, and spawning appeared to be completely inhibited at 10 and 50 μg/l fadrozole. Fecundity was decreased dramatically by fadrozole as determined by the daily number of eggs produced by each female during the exposure regime.

A follow-up study (Ankley *et al.*, 2005) demonstrated that the imidazole fungicide prochloraz inhibited aromatase activity in the brain of the male but not female fathead minnow by about 35% at the highest test concentration of 0.3 mg/l, making it at least an order of magnitude less potent than fadrozole. It further showed that prochloraz inhibited female brain and ovarian microsomal aromatase activity *ex vivo*. This study found that prochloraz caused the same suite of endocrine-disrupting effects as fadrozole (Ankley *et al.*, 2002), providing support that the mechanism of endocrine disruption by prochloraz is largely mediated by aromatase inhibition.

### *Carboximide Fungicides*

Vinclozolin together with iprodione, chlozolinate, and procymidone are dicarboximide fungicides with a different structure and mechanism of fungicidal action from the azole-containing ergosterol biosynthesis inhibitors. They have been reported to have more or less potent antiandrogenic effects (Gray *et al.*, 1994, 1999; Kelce *et al.*, 1994, 1997; Ostby *et al.*, 1999). Perinatal exposure to vinclozolin with doses ranging from 100 to as high as 400 mg/kg/day (from gestational day 14 to postnatal day 3) induced nipple retention, cleft phallus, hypospadias, occurrence of vaginal pouch, and atrophy of seminal vesicles and ventral prostate gland (Gray *et al.*, 1994; Wolf *et al.*, 2000). These effects are typical of that caused by flutamide, a known antiandrogen (Imperato-McGinley *et al.*, 1992). Although several of the above effects could also be explained by inhibition of testosterone or DHT synthesis, the suite of effects caused by vinclozolin differs from that of the known 5α-reductase inhibitor finasteride (Imperato-McGinley *et al.*, 1992). Furthermore, inhibition of 5α-reductase was experimentally ruled out in a study by Kelce *et al.* (1994). In a recent study (Sanderson *et al.*, 2002), vinclozolin was identified as an inducer of aromatase activity and mRNA expression in H295R human adrenocortical cells. It is possible that vinclozolin may exert additional antiandrogenicity via aromatase induction if this mechanism were to occur *in vivo*. However, no studies with vinclozolin have so far been performed to examine this. Vinclozolin is not known to interact with the estrogen receptor or cause CYP17 inhibition, indicating that antagonism of the androgen receptor is its main mechanism of endocrine disruption.

### *Triazine Herbicides*

Atrazine, simazine, and propazine belong to the 2-chloro-*s*-triazine family of herbicides which are used in large amounts worldwide to control weeds, mainly on maize crops. The estimated use of atrazine in the United States was almost 35,000 tons in 1993 (USEPA, 1994). More recent estimates are not readily available but are expected to be higher in the United States and lower in Western Europe where atrazine usage has been gradually banned. As a result, it is found in relatively high concentrations in surface waters in large parts of the North American continent (Solomon *et al.*, 1996). It is relatively persistent to abiotic and biotic breakdowns (Khan and Foster, 1976; Solomon *et al.*, 1996). Epidemiological studies have associated long-term exposures to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (Donna *et al.*, 1989) and increased risk of breast cancer in the general population of Kentucky in the United States (Kettles *et al.*, 1997).

In experiments with female F344 rats, dietary atrazine (0, 375, and 750 mg/kg for 126 weeks) was shown to induce tumors of the mammary gland and reproductive organs (Pinter *et al.*, 1990); increased mammary tumors were also found in males. In female Sprague-Dawley rats, 22.5 mg/kg atrazine caused lengthening of estrous cycle and a dose-dependent increase in plasma levels of estradiol (Wetzel *et al.*, 1994). Atrazine also resulted in an earlier onset of the incidence of mammary and pituitary tumor (Wetzel *et al.*, 1994) responses typical of exposure to exogenously administered estrogens (Brawer and Sonnenschein, 1975; Geschickter and Byrnes, 1942). A more recent study observed disruption of estrous

cycle (extended diestrus) and delayed onset of puberty in female Wistar rats exposed by daily oral gavage from PND22-41 to 100 or 200 mg/kg atrazine (Laws *et al.*, 2000). The same study found a delay in vaginal opening at 50 mg/kg atrazine. Atrazine exposure (12.5 mg/kg/day for 4 days) during lactation has been shown to suppress suckling-induced prolactin release in female Wistar rats (Stoker *et al.*, 1999). Also, the lactationally exposed male offspring of the atrazine-exposed dams had an increased incidence of prostatitis (Stoker *et al.*, 1999), an effect known to be induced by exposure to exogenous 17β-estradiol (Tangbanluekal and Robinette, 1993). 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 (Cooper *et al.*, 2000).

Atrazine has been associated with various endocrine-disrupting effects observed in Florida alligators in areas contaminated with numerous pesticides including atrazine, DDT and metabolites, dicofol, and vinclozolin (Guillette *et al.*, 1994). 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 Lake 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 (Connor *et al.*, 1996; Tennant *et al.*, 1994a,b). 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 (Babic-Gojmerac *et al.*, 1989). Weak inhibitory effects were observed on testosterone 5α-reductase (20–37%) in pituitaries in tissue culture at an atrazine concentration of 0.5mM; a similar observation was made for the deethylated metabolite atrazine desethyl. Triazine herbicides are known to be metabolized in various mammals (Hanioka *et al.*, 1999; Lang *et al.*, 1996, 1997) and chickens (Khan and Foster, 1976). In human liver microsomes, the major metabolites formed are the monodealkylated forms of parent triazines, atrazine desethyl, and atrazine desisopropyl, and hydroxylation of the isopropyl groups present in atrazine and propazine also occurs but to a lesser extent. Some endocrine-disrupting properties of atrazine have been linked to one or more of its metabolites (Eldridge *et al.*, 1994; Laws *et al.*, 2003; Stoker *et al.*, 2002). Studies 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) induced human aromatase activity and gene expression *in vitro* in H295R adrenocortical carcinoma cells (Sanderson *et al.*, 2000, 2001a). A possible explanation for the structure-activity relationship for aromatase induction may be related to the reduced ability of the ever more polar metabolites to enter the cells in the *in vitro* test system. It was further shown that none of the triazine herbicides or their metabolites induced estrogen-dependent vitellogenin production in male carp hepatocytes (Sanderson *et al.*, 2001a). Nor did they antagonize the induction of vitellogenin by 17β-estradiol. Increased synthesis of vitellogenin, a yolk-precursor protein in oviparous animals such as fish and birds, is a response highly sensitive to estrogens and occurs after exposure to xenobiotics that are agonists for the estrogen receptor. These experimental findings together 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*.

*Atrazine and frogs.* The large-scale agricultural use of pesticides such as atrazine has been implicated as a factor involved in the decline of amphibian species worldwide (Clark *et al.*, 1999; Houlahan *et al.*, 2000). Given the important role of hormonal balance in amphibian sexual development and their aquatic habitat, it has been suggested that they would be particularly sensitive to the effects of endocrine-disrupting chemicals introduced into agricultural areas. Numerous gonadal abnormalities have been observed in amphibians (frogs) the wild (Hayes *et al.*, 2002a, 2003; Reeder *et al.*, 1998). Exposing tadpoles of the African clawed frog (*Xenopus laevis*) to 21 μg/l atrazine for as little as 48 h demonstrated a 57% reduction of testicular volume and a 70% decrease in germ cells (spermatogonial cell nests) in males (Tavera-Mendoza *et al.*, 2002a). In females, an increased frequency of secondary oogonia and increased oogonial resorption (atresia) were observed (Tavera-Mendoza *et al.*, 2002b). In both males and females, atrazine clearly decreased germ cell populations, thus impairing future reproductive capacity. Recent laboratory studies by Hayes *et al.* (2002b) have observed that atrazine at (nominal) concentrations as low as 0.1 ppb (about 0.5nM) caused gonadal abnormalities and hermaphroditism in African clawed frogs. Laryngeal size in males was reduced at atrazine concentrations above 1 ppb (5nM), as were testosterone concentrations at 25 ppb. In leopard frogs (*Rana pipiens*) exposed to atrazine from hatching until tail resorption, males developed oocytes (29 and 8% of males at 0.1 and 25 ppb, respectively) (Hayes *et al.*, 2003). An explanation for the lack of dose-response relationship is not given but is made difficult by the choice of only two concentrations that are 250-fold apart. The observation of adverse effects at such remarkably low concentrations as 0.5nM has spurned several follow-up studies. Hayes and coworkers suggested that induction of aromatase activity in the male frog is responsible for the

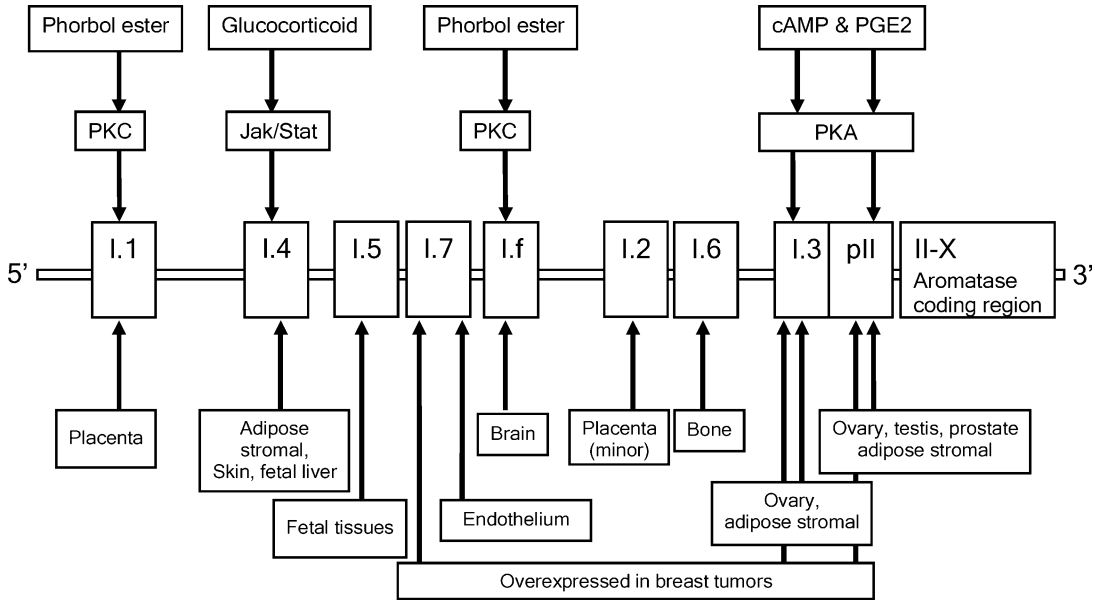


**FIG. 1.** Promoters and signaling pathways involved in the tissue-specific regulation of human aromatase expression (Bulun *et al.*, 2003).

decreased plasma concentrations of testosterone due to increased conversion to estradiol. In an attempt to verify this suggestion, Hecker *et al.* (2005) exposed male adult African clawed frogs to 1, 25, or 250 µg/l atrazine for 36 days. They found statistically significantly decreased plasma levels of testosterone only at 250 µg/l (about 1µM), with no effect on either estradiol levels, testicular aromatase activity, or CYP19 mRNA levels, at any of the tested concentrations. Gonadal-somatic index was also not affected. The same investigators studied the effects of atrazine (10 and 100 µg/l) and estradiol (0.1 µg/l) on the ultrastructure of testicular cells in combination with effects on testicular aromatase activity and plasma sex hormone levels in male *X. laevis* (Hecker *et al.*, 2005). Ultrastructural changes observed by estradiol (lack of chromosome condensation, fewer dividing germ cells, and increased number of vacuoles and inclusion bodies) were not observed with atrazine or controls. Testosterone and estradiol levels were not affected by atrazine at either concentration, whereas estradiol strongly decreased testosterone and more than doubled estradiol levels. However, neither estradiol nor atrazine had an effect on the catalytic activity of aromatase in testicular homogenates. These studies indicate that atrazine does not cause the same profile of endocrine-disrupting effects as exogenous estradiol.

### *Potential Mechanisms of Aromatase Induction by Atrazine and Other Compounds in Humans and Wildlife*

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* or in which target tissues it would occur. Given the recent evidence that plasma estradiol and estrone levels are increased about twofold in atrazine-treated male Wistar rats (Stoker *et al.*, 2000), 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* (Stoker *et al.*, 2000) 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 that contain relatively greater levels of androstenedione than testosterone as precursor; tissues such as adrenal cortex and adipose. However, the highly tissue-specific nature of the regulation of aromatase expression is a complicating factor. In humans, CYP19 is expressed in ovary, testis, brain, adrenal, placenta, adipose, bone, and skin, where its products estradiol and estrone have various functions. The regulation of CYP19 is highly tissue specific and complex (Agarwal *et al.*, 1996; Bulun *et al.*, 1994; Kamat *et al.*, 2002; Mahendroo *et al.*, 1993; Zhao *et al.*, 1997). Unlike other steroidogenic enzymes, CYP19 expression is controlled by numerous alternate promoters that are regulated tissue specifically via various second messenger pathways (Bulun *et al.*, 2003; Simpson *et al.*, 2002) (Fig. 1). Studies in H295R cells demonstrated that atrazine increased intracellular cAMP levels after only several hours of exposure (Sanderson *et al.*, 2002). This intracellular messenger is known to stimulate CYP19 gene transcription in H295R cells, as do the adenylate cyclase stimulant forskolin (Sanderson *et al.*, 2002; Watanabe and Nakajin, 2004) and the phosphodiesterase inhibitor isobutyl methylxanthine (Sanderson *et al.*, 2002). Sanderson *et al.* (2002) postulated that atrazine and other chloro-*s*-triazine herbicides induce aromatase activity by inhibiting phosphodiesterase activity in H295R

human adrenocortical carcinoma cells. This hypothesis was tested by Roberge *et al.* (2004) who, using a fluorescence polarization method, found that atrazine could inhibit isolated bovine heart phosphodiesterase activity with an $IC_{50}$ value of 1.8μM, whereas isobutyl methylxanthine was less potent with an $IC_{50}$ of 4.6μM. Classical binding studies on the other hand showed a weaker interaction of atrazine with phosphodiesterase with a Kd value of 85μM (Roberge *et al.*, 2004). Additional studies using human phosphodiesterase isoenzymes are required to assess the inhibitory potency of atrazine and other pesticides that are capable of cAMP-mediated aromatase induction, such as in H295R cells. The effects of various triazine metabolites on phosphodiesterase activity also warrant further investigation to explain their structure-activity relationship for aromatase induction in responsive cell systems.

The mechanisms of regulation of CYP19 and other steroidogenic enzymes in wildlife are still poorly understood. It is known that in teleost fish, two differentially regulated aromatase genes exist, with *cyp19a* predominantly expressed in the ovary and *cyp19b* in brain (Callard *et al.*, 2001; Kuhl *et al.*, 2005). *Cyp19b* has EREs in the promoter region and can be upregulated by estrogens; whether antiandrogens have the opposite effect is not clear. Expression of the *cyp19a* gene is under control of SF-1 which in turn is activated by the cAMP-mediated PKC pathway, opening up the possibility of cyp19a as a target for induction by atrazine. CYP19a expression can be downregulated by dioxin-like compounds, presumable via an interaction between the activated Ah receptor pathway and dioxin-responsive elements found in the promoter region of *cyp19a*. In amphibians and reptiles, ambient temperature strongly influences aromatase expression during a critical thermosensitive sex-determining period (Crews *et al.*, 2001; Kuntz *et al.*, 2004). It appears that amphibians produce two differentially regulated isoforms of aromatase (in brain and in gonad) coded by a single gene through a splicing mechanism similar to that in humans (Kuntz, 2004). Birds appear to express only one aromatase gene, yet they appear also to produce two identically coding mRNA species with different promoter-specific 5′-untranslated regions (for gonad and brain, respectively) (McPhaul *et al.*, 1993). Underlying mechanisms of regulation of aromatase expression in the various tissues of amphibians, reptiles and birds are thus far not well understood, but appear to differ considerably from mammals.

### *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 molluscs by inhibiting aromatase activity (Fent, 1996). Organotins have further been reported to inhibit cytochrome P450 (CYP) activities such as CYP1A1 and aromatase in fish. However, little evidence supports aromatase inhibition as a mechanism of organotin-mediated imposex. Inhibitory effects on CYPs *in vitro* (Fent and Stegeman, 1991; Fent *et al.*, 1998) and *in vivo* (Fent and Stegeman, 1993) were generally reported at environmentally unrealistic concentrations (upper micromolar range). Historically, concentrations of tributyltin in contaminated surface waters such as boat harbors have been found to range anywhere from 0.001 to as high as 7.2 μg/l (Bruschweiler *et al.*, 1996), corresponding to concentrations of 3pM to 22nM. Concentrations in fish have been found to be as high as 2 mg/kg (wet weight), which would correspond to about 6μM if wet weight is equalled to that of water. A recent study reported 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 (Sanderson *et al.*, 2002). Similarly, in human ovarian granulosa-like tumor cells, decreased aromatase activity by tributyltin was entirely explained by decreased cell viability (Ohno *et al.*, 2004). In agreement with these findings, various measures of impaired cellular energy status and general health, such as decreased ATP production, loss of mitochondrial membrane integrity, and apoptosis were also caused by tributyltin (concentrations ranging from 50 to 1000nM) in various other cell systems (Fent, 1996). Thus, it could not be concluded that the organotin compounds selectively inhibited aromatase activity. Several recent publications also do not support the aromatase inhibition hypothesis of imposex. A field study in gastropods (*Bolinus brandaris*) in Spain (Morcillo and Porte, 1999) showed that a population highly polluted with organotin compounds (100% incidence of imposex in females) and had 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 pointed out that the reductions in steroid levels occurred in the later stages of imposex development and appeared to be a consequence rather than a cause of imposex (Oberdorster, 2001). Instead, it was suggested that certain peptide hormones are more likely to play an important role in masculinization of molluscs (Oberdorster, 2001). The above studies indicate that the development of imposex and the action of organotin compounds occur via mechanisms other than inhibition of aromatase activity.

## *IN VITRO* BIOASSAYS FOR THE MECHANISM-BASED DETECTION OF DISRUPTION OF STEROIDOGENESIS

### *Potential* In Vitro *Bioassays for the Detection of Interferences with Steroidogenesis*

In contrast to relatively weak (ant)agonism of steroid hormone receptors, interactions with key enzymes involved

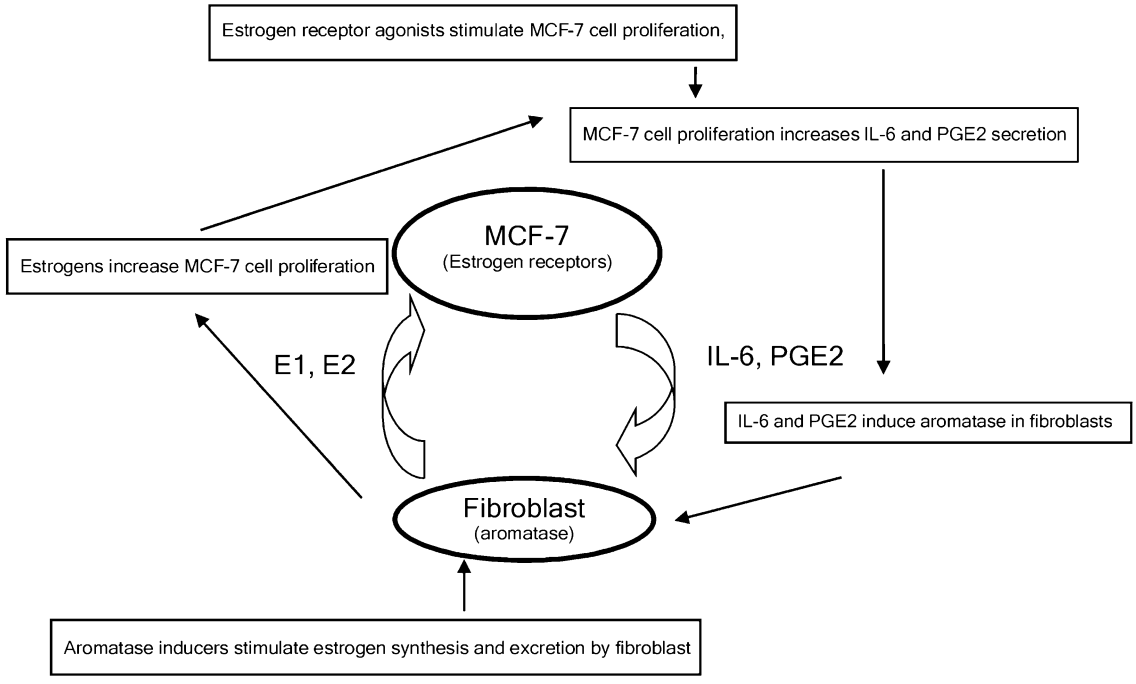


**FIG. 2.** A simplified representation of the interaction between aromatase inducers and estrogen receptor agonists in a coculture of MCF-7 cancer cells and primary human mammary fibroblasts. A positive feedback loop is maintained through estrogen-mediated stimulation of MCF-7 cell proliferation, which in turn results in greater secretion of interleukin 6 and its receptor (IL-6 and IL-6sR) and prostaglandin E2 (PGE2). These factors increase aromatase transcription in fibroblasts which results in continued or increased synthesis of estrogens, completing the positive feedback loop that allows for maintenance and growth of the epithelial tumor cells. Exposure to estrogen receptor agonists or aromatase inducers would be able to accelerate this process. Conversely, aromatase inhibitors and estrogen receptor antagonists would disrupt the loop and block or even reverse the ability of the tumor cells to grow (Heneweer *et al.*, 2005a). The latter interventions are commonly practiced in the treatment of estrogen-responsive breast tumors.

in steroid hormone synthesis have the potential to dramatically affect endogenous steroid hormone concentrations and their functions. Catalytic activity is one of the most functional endpoints of steroidogenesis, which can be measured accurately using selective substrates for the enzyme in combination with specific inhibitors of the enzyme under study, as well as inhibitors of subsequent reactions in the steroidogenesis pathway. Up- and/or downregulation of enzyme expression can be determined using techniques such as northern blotting or RT-PCR to determine levels of mRNA expression. Real-time RT-PCR is a particularly powerful method as it is highly selective, sensitive, and quantitative once optimized appropriately. Two recent studies have demonstrated the versatility of this approach by developing quantitative RT-PCR methods to screen the effects of xenobiotics on the relative levels of mRNA expression of 10 steroidogenic enzymes in H295R human adrenocortical carcinoma cells (Hilscherova *et al.*, 2004; Zhang *et al.*, 2005). Another approach is to use immunoblotting techniques such as western blotting for the quantitative detection of protein levels of the enzyme. In the case of CYP enzymes, the challenge is to raise highly selective (preferably monoclonal) antibodies that detect the protein under study without significant cross-reactivity with other structurally related CYPs. Another, less direct 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. 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.

Various cell lines and cells in primary culture or coculture have been used for the investigation of effects of xenobiotics on steroidogenesis, each with its advantages and disadvantages. Primary cell cultures, although offering more mechanistic and environmental/species relevance than cancer cell lines, have the disadvantage that they are difficult to obtain, maintain, and standardize, and loss of gene expression may occur over time. Nevertheless, the power of primary cultures when applied in combination with cancer cell lines was demonstrated recently using a coculture of human mammary fibroblasts (adipose stromal) and MCF-7 cells (Heneweer *et al.*, 2005a). These experiments were able to demonstrate a positive feedback loop between the two cell types in which stimulation of aromatase activity in fibroblasts resulted in increased estrogen synthesis, which in turn stimulated MCF-7 cell-specific pS2 expression, a marker of estrogenic activity, resulting in cell proliferation in this estrogen receptor–positive cancer cell line (Fig. 2). Furthermore, in this coculture, which mimics more closely the environment of an epithelial breast tumor (MCF-7 cells

surrounded by fibroblasts), estrogenic compounds such as bisphenol A were shown to be considerably more estrogenic (inducing pS2 expression at lower concentrations) than in MCF-7 cells alone (Heneweer *et al.*, 2005b). This coculture study indicates that effects of aromatase inducers and estrogenic chemicals may occur at lower exposure levels in the intact organism than in single cell-type screening assays.

Human placental JEG-3 and JAR choriocarcinoma cells express high levels of aromatase, but are relatively sensitive to the cytotoxic effects of chemicals and appear more prone to apoptosis, rendering them difficult to use for screening purposes (Drenth *et al.*, 1998; Letcher *et al.*, 1999). H295R cells, which appear less sensitive to cytotoxicity, have been used successfully as a bioassay to screen for interferences of xenobiotics with steroidogenesis (Canton *et al.*, 2005; Heneweer *et al.* 2004, 2005b; Hilscherova *et al.*, 2004; Johansson *et al.*, 2002; Letcher *et al.*, 2005; Li *et al.*, 2004; Ohno *et al.*, 2002; Sanderson *et al.*, 2000, 2001a,b, 2002, 2004; Zhang *et al.*, 2005). The H295 and H295R (a subpopulation of H295 that forms a monolayer in culture) cell lines have been characterized in detail and shown to express all the key enzymes necessary for steroidogenesis (Gazdar *et al.*, 1990; Rainey *et al.*, 1993, 1994; Sanderson *et al.*, 2000; Staels *et al.*, 1993). These include CYP11A, CYP11B1, CYP11B2, CYP17 (17α-hydroxylase and 17,20-lyase), CYP19, CYP21, and 3β-HSD. 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 (Gazdar *et al.*, 1990; Staels *et al.*, 1993). H295R cells are suitable for the study of mechanisms of inhibition (competitiveness/reversibility) (Heneweer *et al.*, 2004; Sanderson *et al.*, 2002, 2004) and induction (involvement of signaling pathways and tissue-specific promoters) of aromatase (Sanderson *et al.*, 2000, 2001b, 2002, 2004; Watanabe and Nakajin, 2004; Watanabe *et al.*, 2006). Several tissue-specific promoters appear to be active in the regulation of aromatase expression in H295R cells (Heneweer *et al.*, 2004; Watanabe and Nakajin, 2004). Forskolin, 8-bromo-cAMP, and prostaglandin E2 induced aromatase activity and increased levels of the CYP19 mRNA transcripts specific for promoter regions pII and 1.3, which are normally active in gonadal tissues (Fig. 1). It was also found that aromatase expression could be induced by dexamethasone, phorbol-12-myristate-13-acetate. Glucocorticoids and phorbol esters are stimulants of aromatase expression in (breast) adipose tissue (via the 1.4 promoter) and placenta (via the 1.1 promoter), respectively (Fig. 1). However, 1.4 promoter-specific transcript was not detected, suggesting that this promoter region may have been altered in this cancer cell line (Heneweer *et al.*, 2004). The H295R cell line has also been used to develop a quantitative RT-PCR method for the detection of chemicals that can up- or downregulate the expression of 11 steroidogenic enzymes (Hilscherova *et al.*, 2004; Zhang *et al.*, 2005). These included CYP11A, CYP11B1, CYP11B2, CYP17, CYP19, CYP21, 3β-HSD1, 3β-HSD2, 17β-HSD1, 17β-HSD4, and hydroxymethylglutaryl-CoA reductase. These studies demonstrate the versatility of the H295R cell line as a bioassay tool for the assessment of effects on steroidogenic enzymes. It should be kept in mind that alterations in gene expression do not necessarily reflect or result in alterations of catalytic activity. Inhibition of catalytic activity (which this review makes clear is an important mechanism by which chemicals interfere with steroidogenesis and endocrine function) will generally not be detected in such an assay. For example, known inhibitors of aromatase activity such as ketoconazole and aminogluthetimide have no effect on CYP19 expression (Hilscherova *et al.*, 2004; Zhang *et al.*, 2005), and it is further known that changes in mRNA levels may in fact reflect an opposite compensatory effect on the catalytic activity of the enzyme. It is also difficult to interpret the importance of slight changes in gene expression caused by a chemical perturbation without the measurement of additional functional effects. A human ovarian granulosa-like tumor cell (KGN) bioassay has recently been used to examine the effects of chemicals on aromatase activity (Ohno *et al.*, 2004). This bioassay is also capable of detecting inhibitors and inducers of aromatase activity, although it is not clear if the mechanisms of induction of aromatase are the same as in H295R cells or comparable to normal granulosa cells. Nevertheless, KGN cells may provide a useful ovary-relevant tool for screening endocrine-disrupting chemicals. Clearly, a combination of the measurement of steroid hormone production and the analysis of steroidogenic gene expression, in combination with determination of subsequent hormone signaling events, using several endocrinologically relevant cell systems, will provide a powerful battery of tools for the assessment of interferences with the function of steroidogenic enzymes and function.

## CONCLUSIONS

The ability of xenobiotics to disrupt steroidogenesis and the mechanisms by which these compounds interfere with the function of steroidogenic enzymes is a relatively unexplored area of (endocrine) toxicology. This review has shown that structurally highly divergent groups of chemicals can interfere with steroidogenesis and cause endocrine-disrupting effects. In many cases, perturbations of certain endocrine endpoints have been observed, but their consequences are unknown. Clearly, certain chemicals such as the azole fungicides and systemically used antifungal drugs directly interfere with steroidogenesis by acting as potent inhibitors of steroidogenic enzymes and are known to cause endocrine disruption mainly via this mechanism. Other classes of compounds such as the TCDD-like chemicals have less consistent effects on steroidogenic enzymes and hormone synthesis, although they are well known endocrine-disrupting compounds and interfere with steroidogenesis to some extent in various *in vitro* and *in vivo*

systems. Further elucidation of the interaction between the Ah receptor–mediated pathway and the steroid biosynthesis pathway is needed to understand in more detail the effects of Ah receptor agonists such as TCDD on steroidogenic enzymes. Although catalytic inhibitors of steroidogenesis are likely to have similar effects across species due to the conserved nature of the catalytic activity of these enzymes, the effects of inducers and/or inhibitors of gene expression are likely to differ greatly due to the highly tissue- and species-specific mechanisms of regulation involved. Various chemicals will have a combination of effects on the endocrine system, as they may act as steroid receptor (ant)agonists, steroidogenic enzyme inducers/inhibitors, and via other less well understood mechanisms to cause net effects on the endocrine system which will be highly concentration and endpoint dependent. Thus, it is to be expected that for many endocrine-disrupting compounds, more than one mechanism will play a role, inevitably resulting in complex dose-response relationships for many different endocrine parameters. Increased efforts need to be made to interpret the relevance of slight endocrine perturbations in isolated *in vitro* systems for the situation in intact organisms. Biologically relevant bioassays need to be developed and environmentally realistic dose ranges need to be chosen for the assessment of the toxicological hazard of various endocrine-disrupting chemicals for humans and wildlife. Potentially large tissue and species differences in the regulation of steroidogenic enzyme expression also require more consideration and fundamental investigations in endocrine toxicology research. Several *in vitro* bioassays discussed in this review provide a promising basis for a set of tools for the initial screening of compounds for their potential to interfere with the function of steroidogenic enzymes in various tissues and organisms and for the study of mechanistic bases of disruption of steroidogenesis.

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