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Environmental endocrine disruptors: does a sex-related susceptibility exist?

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TABLE OF CONTENTS

1. ABSTRACT

Several substances present in the environment, now classified as endocrine disruptors (EDs), strongly interfere with both androgen and oestrogen actions in reproductive tissues. However, nowadays it is well recognized that these sex steroid hormones are more than regulators of gonadal functions. In fact, they, in synergy with genes, are responsible of sex-related differences in anatomical, physiological, and behavioral traits which characterize males and females of many vertebrate species, including humans. Thus, even if EDs are present in minute amount (part for trillion) in environment, their effects in male and female physiology could be greater than before expected also prejudicing the sex-steroid hormone-induced integrated physiological responses in women and men. In addition, differences in male and female susceptibility to EDs could be present even if scarce information on this aspect is still available. Here we have reviewed the state of the art on the sex-related susceptibility to EDs underlying the mechanism at the root of these effects.

2. INTRODUCTION

Differences between males and females in anatomical, physiological, and behavioural traits characterize many vertebrate species, including humans. The literature on these differences now encompasses everything from variations in gene expression between male and female mice, to a higher susceptibility to adverse drug reactions in women compared with men. Moreover, hormones made by the gonads are known to influence symptoms in human diseases ranging from multiple sclerosis to epilepsy.

Some sex-dependent differences are already apparent at the sixth week of pregnancy, resulting from the influence of a cascade of genes, such as the sex-determining gene on the Y chromosome (the SRY) of the father. In addition, from the earliest stages of foetal development onwards, many cells throughout the entire body already have receptors for sex steroid hormones.

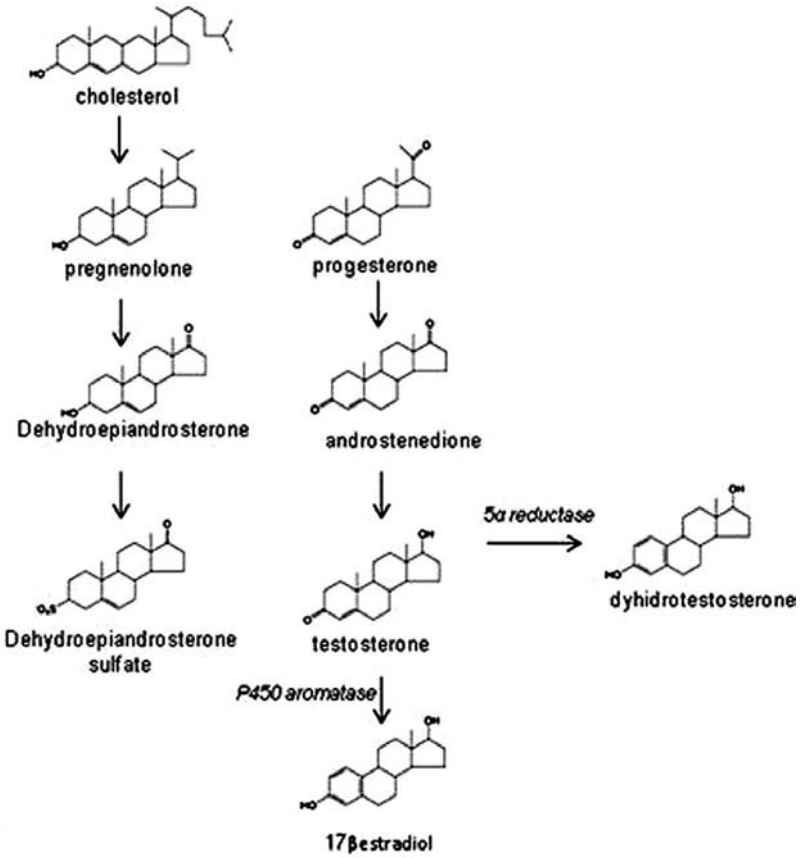

Figure 1. Biosynthetic pathway of sex steroid hormones.

The production of the androgens, i.e., testosterone (T), and its peripheral conversion into 5α dihydro-testosterone (DHT) by a boy's testes is necessary for the differentiation of the sexual organs between weeks 6 and 12 of pregnancy. However, once the differentiation of the sexual organs into male is settled, other organs start the differentiation program (e.g., the brain) under the influence of sex steroid hormones (1). This involves (permanent) organizational changes in several organs and functions.

The development of the female sexual organs in the womb is primarily based on the absence of androgens (1). Girls and boys are protected against the effect of circulating estrogens from the mother by the α-fetoprotein, which is produced by the foetus and strongly binds to estrogens but not to testosterone (2). At the end of the pregnancy, when α-fetoprotein declines, the foetus is more exposed to the estrogens released from the placenta which, in turn, inhibit the hypothalamus–pituitary–gonadal axis of the child. This inhibition is lost once the child is born causing a peak in testosterone in boys and a peak in estrogens in girls. Other striking sex differences between males and females emerge at or around the time of sexual maturation (3). Indeed, during puberty the organs and tissues which developed in the womb, are activated by sex hormones (activational effects) (3).

Natural and man-made exogenous substances present in the environment, now classified as endocrine disruptors (EDs), could strongly interfere with permanent and activational effects of sex steroid hormones, seriously impairing male and female physiology (4, 5). Several mechanisms have been implicated in ED effects (e.g., DNA adduct formation, epigenetic modification, protein-interactions) (6-9). Among others, the ability of EDs to impair hormone effects by binding to their receptors have been reported (10-12) even if the putative presence of sex-related differences in susceptibility to EDs has not yet been elucidated.

Here we have reviewed the state of the art on the action mechanisms of sex steroid hormone receptors focusing on the role exerted by EDs in modulating receptor activities to define the possible sex-related susceptibility to these compounds.

3. SEX STEROID HORMONES

The first steroid hormone, oestrone, was isolated in 1929 at a time before the characteristic ring structure of the steroid nucleus had been elucidated. In mammalian systems, there are six families of steroid hormones that can be classified on both a structural and biological basis. They are mineral-corticoids, gluco-corticoids, vitamin D metabolites, sex steroids (estrogens, progestins, and androgens). All these steroids are biologically derived from cholesterol (Figure 1) (13). In this paragraph, synthesis,

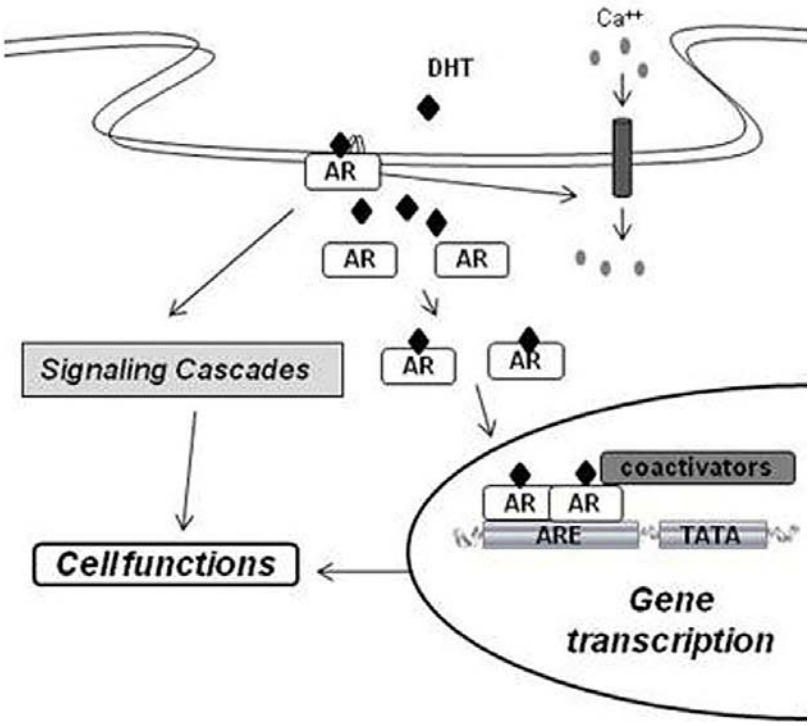

Figure 2. Androgen receptor action mechanisms. Upon DHT binding to AR, active androgen receptor dimerizes, recruits co-activators, and binds to ARE sequence on DNA, allowing androgen responsive gene transcription. DHT binds to plasma membrane localized AR, allowing AR-dependent activation of rapid signaling cascades. DHT: 5α-dihydro-testosterone; AR: androgen receptor; ARE: androgen response element.

effects, and action mechanisms of androgens and estrogens will be discussed.

3.1. Androgens

Androgens are all steroids with 19 carbons. The major naturally occurring steroids with androgenic activity (in decreasing order of relative potency) are DHT (150-200%), T (100%), androstanediol (65%), androst-4-ene3,17-dione (25%), androsterone (10%), and deydroepiandrosterone (DHEA, 10%) (13).

Over 95% of T is secreted by the testicular Leydig cells, the remaining 5% is produced in peripheral tissues by conversion of precursors (i.e., DHEA, DHEA sulphate, and androstenedione) secreted by testes and adrenals (14). In men, normal circulating T levels range from 10 to 30 nM, whereas much lower levels (0.6 to 2.5 nM) are found in women. T is enzymatically converted to DHT by the enzyme 5α-reductase, an NADPH-dependent enzyme (Figure 1). DHT and T bind to the same specific intracellular receptor, although with different dissociation affinity constants (DHT K_d= 2 nM, T K_d= 8 nM) (15, 16).

As told above, androgens are critical for the differentiation of male gonadal structures prior to birth, for sexual maturation during puberty, and for the maintenance of male secondary sexual characteristics and genital function in adulthood. Because men have larger skeletons and greater muscle mass than women, it is assumed that this sexual dimorphism is due, at least in part, to androgens. In addition, androgens exert anabolic effects on muscle. The falling of testosterone levels with advancing age is associated with the loss of muscle mass (sarcopenia) and strength. Accordingly, T supplementation of hypogonadal men results in muscle differentiation and hypertrophy (17, 18). While developmental effects of sex hormones on brain cognition in the pre- and early postnatal period have been demonstrated, their activational effects in later life are still a focus of contemporary research. In normally ageing men, the age-related cognitive decline is accompanied by gradual but marked decreases in androgen levels, however, the evidence for activational effects of androgens in healthy men brain throughout adult life remains inconsistent (19). In addition, the lower testosterone levels present during ageing are associated with several risk factors including diabetes, hypertension, heart disease, psychological stress, and obesity. Men with proven coronary atherosclerosis have lower levels of testosterone and sex hormone binding globulin, which results in negative correlation with very low-density lipoprotein, triglycerides, body mass index and body fat mass (20, 21).

3.1.1. Androgen receptor action mechanisms

The androgen receptor (AR), a 110 kDa phospho-protein, is member of nuclear receptor super-family, functioning as a ligand-activated transcriptional factor. AR is localized in the cytoplasm and in the nucleus of androgen-target cells where it is associated, in the resting state, to heat shock proteins (Figure 2).

Like other nuclear receptors, the AR has three main domains, a *C*-terminal ligand binding domain (LBD), an *N*-terminal transcriptional activation domain and a central DNA-binding domain consisting of two zinc finger motifs and a hinge domain. Binding of androgen induces the conformational change, the receptor dimerization, and the nuclear translocation of the hormone-receptor complex (22). This complex binds to specific androgen response elements (ARE) in the upstream promoter of target genes and along with several co-regulator proteins (co-activators or co-repressors), leads to transcriptional activation of androgen-regulated genes (Figure 2). ARE motifs are 15 bp palindromic units (AGAACAnnnTGTTCT) and are found in a wide range of differing genes (23). The best characterised ARE are those in the 5' prostate specific antigen (PSA) promoter/enhancer (24). However, only 5-10% of potential DNA-AR binding sites has the canonical 15 bp ARE, 16-22% has no ARE motif and a further 68-79% has a non-canonical 15 bp or 6 bp half site ARE. These non-canonical sites, some of which are frequently repeated, are important for enhancer promoter function (25, 26). Many of these binding sites are located down-stream from the start of transcription, in intronic regions or a long way upstream from the transcription start site. Hence, ARE provides little evidence of selective pressure on gene promoters, but may allow for combinatorial regulation of transcriptional factors (23).

In addition to these slow genomic modes of action, increasing evidence suggests that androgens, as well as other steroid hormones, exert rapid extra-nuclear effects (27-34) (Figure 2). Extra-nuclear effects of androgens have been implicated in a number of cellular effects, including intracellular Ca^{2+}-homeostasis, gap junction communication, aortic relaxation, neuronal plasticity, and neurite outgrowth (34).

These rapid effects of androgens typically involve cell membrane ion-channels and transporters (e.g., Ca^{2+}, Na^{+}-K^{+}-ATPase) (34) and/or the indirect modulation of conventional second messenger signal transduction cascades [e.g., intracellular Ca^{2+}, activation of protein kinases A/C (PKA/PKC), extra-cellular regulated kinases 1/2-mitogen-activated protein kinase (ERK1/2-MAPK)] (35, 36). Androgen-induced second messenger cascades lead to the phosphorylation of numerous cytoplasmic targets including kinases, phosphatases, transcriptional factors (e.g., Elk-1), and cytoskeletal proteins. Rapid mechanisms also mediate the transcription of non-ARE containing genes (37-39). An increase of ERK1/2 phosphorylation comparable to that of free T has been reported after target cell stimulation with T-conjugated with bovine serum albumin which does not cross plasma membrane (40). This activation requires a plasma membrane localized AR. Recently, it has been reported that AR is subjected to post-translational modification with the fatty acid palmitate. Palmitoylation occurs in a conserved cysteine residue located in the AR LBD. Mutation occurring in this sequence completely prevents, receptor membrane localization and, in turn, the activation of rapid signals (41).

As a whole, the pleiotropic effects elicited by androgens are obtained by different signal transduction pathways (i.e., genomic and extra-nuclear) which activation depends on the cellular context of the target cell, the receptor location within cells (i.e., membrane, cytosol, nucleus), as well as the ligand itself (i.e., T versus DHT) (42).

3.2. Estrogens

Estrogens [oestrone, oestriol, and 17β-estradiol (E2)] are all 18 carbons steroids which derive from the aromatization of A ring of either testosterone to yield E2 or androst-4-ene-3,17-dione to yield oestrone (Figure 1). This reaction is mediated by a member of P450 family, aromatase which is present in the endoplasmic reticulum of the ovary, placenta, brain, and adipose tissue (Figure 1). The main sources of sex steroids in females come from the adrenal gland and the ovary (13).

Besides the central role in the development of the secondary sexual features in females, estrogens are well known for bone turnover inhibition, by reducing osteoclast-mediated bone resorption, and for the ability to enhance osteoblast-mediated bone formation. Estrogens induce several effects in the vasculature, including vasodilatation and relaxation of vascular smooth muscle, due to oestrogen-dependent nitric oxide (NO) released by vascular endothelial cells (43). Both metabolism and location of white adipose tissue present strong sex-differences which are regulated by estrogens. In fact, these hormones play a role in adipogenesis, adipose deposition, lipogenesis, lipolysis, and adipocyte proliferation (44). In addition, protective effect of E2 against colon cancer growth (45), neurodegenerative diseases (46), atherosclerosis (47), as well as in skeletal muscle mass loss (48) have been reported.

Oestrogen effects have been also reported in male individuals. Testosterone conversion to 17β-oestradiol by aromatase is fundamental for the masculinization of developing male brain, for prostate growth, for male bone mineralization, and for male fertility (19, 20, 29, 32, 49, 50-52).

3.2.1 Estrogen receptor action mechanisms

As well as androgens, E2 effects in living cells are mediated by various pathways rather than by a single uniform mechanism. E2 binds to oestrogen receptors, ERα and ERβ (NR3A1 and NR3A2, respectively), which are the products of separate genes (*ESR1* and *ESR2*, respectively) present on distinct chromosomes (locus 6q25.1 and locus 14q23-24.1, respectively) (47). Human ERα and ERβ, like all the members of the nuclear receptor super-family, are modular proteins sharing common regions. These regions participate in the formation of independent but interacting functional domains: the *N*-terminal transactivation domain, the DBD, the dimerization domain(s), the nuclear localization sequence, and the LBD (47).

The pioneering work by O'Malley demonstrated that ERs function as ligand-activated transcriptional factors (53). In fact, the biological effects of ERs result from modifications in the pattern of expression of specific target genes. These transcriptional regulations are achieved

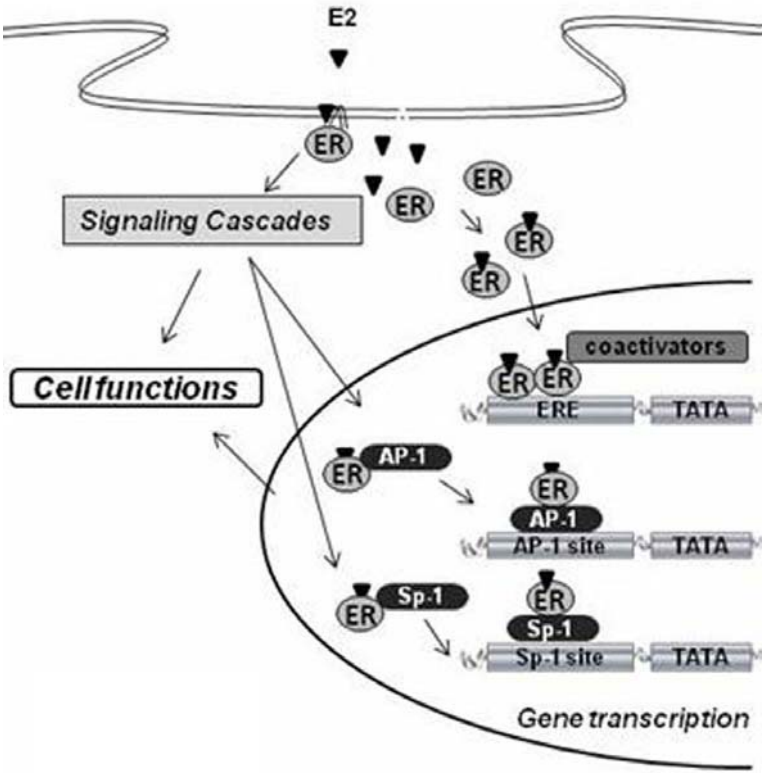

Figure 3. Estrogen receptor action mechanisms. Upon E2 binding, activated ER dimerizes, translocates in the nucleus, and binds to ERE sequence on DNA (direct genomic mechanism). E2:ER complex could interact with Sp1 and AP-1 transcriptional factors responsible of ER indirect transcriptional activity. E2 binding to plasma membrane localized ER, leads to the activation of rapid signaling cascade necessary for E2-induced cell functions. E2: 17β-estradiol; ER: estrogen receptor; AP-1, activating factor-1; Sp-1, stimulating factor-1; ERE: estrogen response element.

through recruitment of ERs to the promoter region of the target genes, either directly through interaction with cognate DNA sequences (*i.e.*, oestrogen response element, ERE) or through protein-protein indirect interaction with other transcriptional factors (Figure 3). The consensus palindromic element ERE was initially described based on the oestrogen-responsive sequence in the *Xenopus laevis* vitellogenin A2 promoter: 5'-GGTCAnnnTGACC-3' (54). Only a fraction of the known mammalian oestrogen-responsive palindromic EREs reflects this 'perfect' ERE sequence consensus (55). Both in the direct and indirect action modes, the agonist-activated ER is not the controller of transcription. In fact, ER needs to interact with a complex of co-regulatory proteins (co-activators or co-repressors) to form a macromolecular complex which provides a platform upon which additional proteins are assembled (56, 57) (Figure 3).

The current set of E2 response genes have been mainly identified in breast cancer cells, while only a few studies have been performed in bone [*e.g.*, progesterone receptor, c-*fos*, insulin-like growth factor-1 receptor (IGF-1-R)], vascular (*e.g.*, vascular endothelial growth factor, progesterone receptor, and c-*fos*), neuronal (*e.g.*, oxytocin, and *N*-methyl-D-aspartate receptor 2D subunit), or liver cells [*e.g.*, low density lipoprotein (LDL) receptor] (47, 55). Among E2-responsive genes the expression of ERα and ERβ should be mentioned. In the case of ERα, exposure to E2 results in a ligand-dependent down-regulation, however, it is not known if all distinct ERα promoters are responsive to E2 (58). On the other hand, a time dependent oestrogen-induced increase in ERβ mRNA and protein levels in the human breast cancer cell-line T47D and human colon cancer cells has been reported (59, 60).

The 'genomic action' of steroid hormones occurs after a time-lag of at least 2 hours after E2 stimulation and explains some hormone functions in physiological and pathological situations. Only seconds are requested for an E2-induced increase of intracellular calcium level in granulose cells (61) and to increase inositol trisphosphate production in the liver and in liver derived HepG2 cells (62, 63). This mechanism is insensitive to inhibitors of transcription (*e.g.*, actinomycin D) and translation (*e.g.*, cycloheximide) (64). It is interesting to note that the cell membrane impermeable E2-bovine serum albumin conjugate mimics the E2 effects in activating rapid signal transduction pathways (65, 66).

These E2-induced rapid effects have been attributed in most cells to a population of ERs present on

the plasma membrane. We recently demonstrated that ERα undergoes to *S*-palmitoylation on a cysteine residue (Cys447) present in the LBD which allows ER anchoring to plasma membrane, association to caveolin-1, and which accounts for the ability of E2 to activate different signalling pathways (67). The Cys399 residue present in the LBD of ERβ is also subjected to *S*-palmitoylation (45) indicating that a similar mechanism also works for ERβ localization to the plasma membrane and association to caveolin-1 (68) (Figure 3).

The physiological significance of ER-dependent rapid pathways is quite clarified, at least for some E2 target tissues. E2 actions on proliferation have been assumed to be exclusively mediated by ERα-mediated rapid membrane-starting actions (*e.g.,* phosphatidylinositol 3-kinase, PI3K/AKT and ERK/MAPK pathway) (47, 69), whereas E2 induces cancer cell death through ERβ non-genomic signalling (*i.e.* p38/MAPK pathway) (70). E2 affects neural functions, both in male and in female brain, in part by inducing such rapid responses (64, 71). In the skeleton, ERα-dependent Src/Shc/ERK pathway transmits survival signals and prolongs the life span of osteoblasts (72). At the same time, E2 delivers a pro-apoptotic signal to bone-resorbing osteoclasts, shortening their life span (72-74). In the liver, rapid E2-induced signals (i.e., phospholipase C, PLC/PKC) are strongly linked to the increased expression of the LDL receptor which leads to a decreased level of LDL-cholesterol in the plasma (75, 76). E2-activated PI3K/AKT pathway is responsible for E2-induced survival signals (70) and for activation of endothelial nitric oxide synthase, which is at the root of E2-induced vascular protection in ischemia/reperfusion injury *in vivo* (77, 78). ERα-dependent PI3K/AKT activation is also essential for E2-induced skeletal myoblast differentiation (48). E2:ERα complex interacts with growth factor receptors [e.g., epidermal growth factor receptor (EGFR), IGF-1-R) to activate down-stream extra-nuclear signals via guanosine triphosphate-binding proteins (66, 79).

Data from cell culture, gene expression, and knockout mice indicate that E2-activated ERβ can function as a dominant negative suppressor of the proliferative activity of ERα (80, 81). These studies support a functional antagonism between ERα and ERβ with respect to the E2-induced cell proliferation, but do not clarify the signal transduction pathway involved. Recently, the ability of the ERβ:E2 complex to activate rapid non-genomic mechanisms has been reported. In colon cancer cells, E2:ERβ complex rapidly induces a persistent membrane-initiated activation of p38/MAPK which in turn is involved in caspase-3 activation and cleavage of poly(ADP-ribose) polymerase, driving cells to the apoptosis (45). Consequently, besides its role as a negative modulator of ERα activities, our findings indicate that ERβ directs the anti-proliferative effects of E2 sustaining the tumor suppressor functions of ERβ (70). More recently, E2 protective effect from oxidative stress of skeletal muscle cells has been associated to the ERβ-dependent p38 kinases activation (Marino, unpublished results).

Remarkably, it has been recently demonstrated that AR inhibits ERα, but not ERβ, activities, potentially *via* a direct interaction which decreases both ERα mRNA and protein expression in breast cancer cells (82). The activity of AR in prostate and breast cancer cell lines seems to be also enhanced by a direct interaction between AR and breast cancer 1 protein (83, 84), whereas breast cancer 1 protein interaction with ERα has been demonstrated to inhibit ERα transcriptional activity (85, 86). Thus androgens may oppose to oestrogen signalling in breast tissue controlling cellular proliferation and maintaining tissue homeostasis in the breast (87).

4. ENDOCRINE DISRUPTORS

In the early 1900s, pig farmers in the USA complained of fertility problems in swine herds fed on mouldy grain (88), and concern was stimulated in the 1940s by reports of infertility in sheep grazing on certain clovers in Western Australia (6). Over the following two decades, masculinization of bivalves and gastropods as well as estrogenic actions in birds and mammals were evidenced (4, 5, 89). Endocrine disruption in wildlife is now acknowledged to be a widespread problem, much resulting from environmental pollution, and, in the case of aquatic forms of wildlife, from the continuous exposure to these chemicals in the water. Extrapolation of the results of these researches resulted in concern that the same compounds could interfere with human health mimicking, antagonizing or altering the physiological actions of endogenous hormones mainly sex steroid hormones. These compounds have been termed endocrine disruptors and defined as “exogenous substances that cause adverse health effects in an intact organism or in its progeny, consequent to change in endocrine function” (5, 6). EDs, even if present in minute amounts (part per trillion), could interfere with the synthesis, secretion, transport, metabolism, binding, action, or elimination of natural hormones responsible for homeostasis maintenance, reproduction, and developmental processes (4-6, 89).

Currently more than 100 chemicals have been identified as EDs. Within this heterogeneous group of molecules we find: (a) synthetic chemicals used in industry and agriculture; (b) synthetic chemicals used as pharmaceutical drugs; and (c) natural chemicals found in human and animal foods. About half of these compounds are substituted with halogen groups, mostly chlorine and bromine, and include dioxins, polychlorinated biphenyls, organochlorine pesticides, methoxychlor, dieldrin, and hexachlorocyclohexane (6).

EDs have long environmental half-life resulting in a continue increase of their global concentration in the environment and can be detected and may concentrate at great distances from where they are produced, used or released. EDs have very low water solubility and extremely high lipid solubility, leading to their bioaccumulation in adipose tissue. Exposure to EDs can occur from a number of different sources: humans and animals can be exposed involuntarily by drinking contaminated polluted water, breathing contaminated air, ingesting food, contacting contaminated soil or even in the workplace (6).

4.1. Endocrine disruptor effects

EDs can exert both short and long term effects in many physiological states. Animals, including humans, are especially sensitive to EDs at early stages of development, but some effects exerted at these stages may be expressed only in adult life or even in subsequent generations.

ED interferences have been reported in immune function (90, 91), bone structure (92-95), thyroid function (5, 96-98), obesogenic systems (5, 98, 99), mammary gland structure and function (5, 98, 100, 101), cardiovascular function (102, 103), as well as social and other behaviours (91, 104). Perturbation of any one of these systems has the potential to adversely affect an animal reproductive success and competitiveness, and therefore the potential viability of the population.

4.1.1. ED effects in developing nervous system

EDs affect a huge number of sexually dimorphic neural systems influencing, later in life, the development and functions of reproductive tract (105). As told before, since the pivotal role of E2 and DHT in developing brain, neuronal circuits are particularly vulnerable to disruption by EDs (106). As examples, the isoflavone genistein (GEN) prevents the masculinisation effect of sex steroid hormones on male hypothalamus nuclei (107-110). Bisphenol A (BPA) and GEN mimic E2 in masculinising female in anteroventral periventricular nucleus, while males are unaffected by any treatment (111, 112). Exposure to polychlorobiphenyls (PCB) results in more dramatic neuro-developmental and behavioural changes in male neonates (113). Consistently with the de-masculinising effects of these compounds, several adult male offspring exhibits changes in their social and sexual behaviour following ED exposure (114, 115). EDs also modulate the catecholamine and indolamine neurotransmitters, peptides such as arginine vasotocin, and the gonadotropin releasing hormone. The EDs impact on these neural systems will be translated, subsequently in the adult life, into delayed or impaired reproductive, metabolic, and endocrine functions, altered behavioural responses, and in reducing immuno-competence and stress resistance (105). As a whole, since both E2 and DHT are required for the masculinising of male developing brain, whereas femminilisation does not require sex hormones, the male brain seems to be more susceptible to the interference of EDs. However, these data do not allow to discriminate if ED effects are mediated by binding to AR or ERs. Recently, it has been evidenced that environmental levels of phthalates, well known anti-androgens, act to feminize infant boys exposed during pregnancy (116, 117) lowering testosterone concentrations (118). According to these data, plasma free testosterone levels in dibutyl phthalate-exposed workers is significantly reduced (119). These data indicate that ED effects in developing brain could be related to a decrease in circulating T levels and, consequently, in both E2 and DHT intracellular levels.

4.1.2. ED effects in reproductive system

Several studies demonstrated the ability of the major man-made EDs, found abundantly in the environment [*e.g.,* dichlorodiphenyltrichloroethane (DDT), dioxin, PCB, BPA, polybrominated biphenyls, phthalate esters, endosulfan, atrazine and zeranol)], to impair development and functions of reproductive organs (120).

In animal models, atrazine causes de-masculinisation in males and delay in puberty (121) by altering E2 secretion. PCB showed a significant delay in puberty in boys, like reduction in the pubic hair stage and genitals and lower testicular volume while BPA has shown to cause an increase in genital abnormality (122, 123). It has been suggested that exposure of phthalates, known for the anti-androgenic properties, can cause testicular dysgenesis syndrome, reduction in testosterone production, and decrease in the anogenital distance (124). However, very few reports are available on phthalate esters ability to affect pubertal boys.

In girls, DDT, dioxin, PCB, and BPA have been linked to earlier menarche, roughly 3-4 years earlier than normal (125), abnormal breast development, and an increased risk of breast cancer latter in the life (126) In addition, the effects of phthalate esters on pubertal girls include de-feminization (124).

4.1.3. ED effects in adipose tissue

White adipose tissue (WAT) is the main site of energy storage in mammals and birds. Nowadays WAT is considered as a highly dynamic organ, being involved in a wide range of physiological and metabolic processes far beyond the paradigm of fuel storage. Several sex-related differences in WAT have been reported including the greater adipose stores in women than men and the different distribution of body fat in men and women bodies. These sex related differences in WAT are already present at birth, and are further strengthened during puberty. They stem from metabolic and hormonal differences between the sexes contributing to differences between women and men in obesity risk (127).

Although gestational exposure to BPA does not alter either pregnancy or sex ratio or litter size at the birth, WAT stores and, consequently, body weight is increased of about 3-fold in these animals (128). This excess of WAT has been associated with adipocyte hypertrophy and over-expression of lipogenic genes (128). These *in vivo* observations are in accordance with previous *in vitro* observations, showing that BPA can accelerate the differentiation of 3T3-L1 fibroblasts into adipocytes (129). After weaning, peri-natal BPA exposure predisposes to overweight in a sex- and diet-dependent manner. Finally, a direct oral exposure to isoflavone GEN in 4-week-old mice increases fat pad weights of male, but not in female (130). Taken together, these findings provide a clear direct evidence of early gene expression alterations in adipose tissue after peri-natal exposure to EDs. This is really inter-esting since the prevalence of childhood obesity is increasing worldwide (131).

4.1.4. ED effects on pathology onset

There is increasing evidence both from epidemiology studies and animal models that specific EDs may influence the development or progression of several

type of cancer. Particularly, prostate and breast cancers have become very frequent in male and female, respectively, both of these cancers still express androgen and oestrogen receptors (132, 133). Prostate cancer rates, the most common solid cancer in males (134), today are markedly higher than rates observed three decades ago. Human and rodent prostate expresses both ERs during development and into adulthood with ERα primarily found in stromal cells (135) and ERβ in differentiated epithelium (136). In addition to epidemiologic studies, *in vitro* and *in vivo* studies indicate the association between EDs and prostate cancer, prostate carcinogenesis, and/or increased prostate cancer susceptibility. An extensive epidemiologic study of workers highly exposed to PCB has revealed a strong exposure–response relationship for prostate cancer mortality (137).

However, the most compelling data come from the established occupational hazard of farming and increased prostate cancer rates (138, 139). The most likely explanation is the chronic or acute farming exposure to pesticides (138, 140). The exposure to six pesticides (*i.e.,* chlorpyrifos, fonofos, coumaphos, phorate, permethrin, and butylate) out of 45 common agricultural pesticides has been correlated to increased prostate cancer in men with a familial history (141). Importantly, there is a heightened sensitivity of the prostate to EDs during puberty, thus infants and children may be considered a highly susceptible population for ED exposures and increased risk of prostate cancers with aging (142).

Early-life exposure to BPA has also been demonstrated to increase the susceptibility to mammary gland tumorigenesis later in the life, but there is less evidence for carcinogenic activity of BPA when administered to adult animals (100). However, epidemiological data have highlighted the correlation between the increase of BPA level in the environment and the incidence of cancer in humans (143). Several other EDs have been demonstrated to promote mammary carcinogenesis: DDT, pesticides, hexachlorobenzene (HCB,), lindane, heptachlor, aldrin and dieldrin, DDT cocktails, and PCB are clearly associated to the increased incidence of breast tumors (144-148). Since EDs cause transient and persistent effects on mammary gland development, they alter or inhibit mammary gland functional differentiation leading to malnutrition or increased mortality of their offspring. For example, dioxin exposed animals show an impairment of terminal end bud development and the delay of both their formation and differentiation (100).

In contrast, plant-derived flavonoids represent a particular class of EDs able to reduce the onset of several cancer types (*e.g.,* colon, breast and prostate cancer). In fact, epidemiological data show a lower incidence of cancer in Asian countries and in vegetarians (149). Furthermore, the migrants from Asia to Western countries who lose their traditional diet have rates of cancer similar to the Western population (150). These epidemiological data are supported by *in vitro* experiments which show that "anticancer" flavonoids effects require ER expression (see par. 4).

As well as for some type of breast cancer, endometriosis is a sex steroid hormone-dependent disease defined as the growth of endometrial glands and stroma at extra-uterine sites (151). Oestrogen dependence and immune modulation are established features of endometriosis but do not adequately explain the cause of this disorder. Until now epidemiological studies have examined the association between EDs and endometriosis but, when taken together, these studies are inconsistent. Thus, it is difficult to conclude that women with higher toxicant exposures are at increased risk of endometriosis (151). However, recent evidence indicates that exposure to environmental toxicants, with estrogenic activity, results in the insurgence of endometriosis (152). Furthermore, it has been proposed that mice exposed to BPA during foetal life develop endometriosis-like structure later in the life (153, 154). Besides BPA, also TCDD and dioxin-like chemicals modulate endometrial physiology, exerting adverse effects on endometrial function in monkeys (155).

5. ACTION MECHANISMS OF ENDOCRINE DISRUPTORS

It is now clear that EDs possess several action mechanisms all of which have been claimed to be involved in their endocrine disrupting effects. Indeed, the disrupting effects of pesticides on oestrogen synthesis have been attributed to their significant capacity as P450 aromatase inhibitors (141) or as inhibitor of testosterone synthesis (116, 117). Moreover, EDs can increase or block the catabolism of naturally occurring steroid hormones (156) Thus, the modification of E2 and DHT balance and availability into target cells could contribute to some of the reported effects.

In addition, EDs affect DNA structure and gene expression (8). Intriguingly, recent studies with *in uterus* or early life exposure to EDs (*i.e.,* vinclozolin or BPA) show that these compounds induce DNA epigenetic modification altering DNA methylation pattern later in the life (9, 142, 157, see also par 3.1.1). Although epigenetic modifications have originally been thought to be highly regulated and stable, there is emerging evidence to suggest that they are much more dynamic than previously thought. One possibility is that hormonal perturbations can affect epigenetic regulation of specific hormone receptors (*e.g.,* ER), which may then alter their expression or subsequent activity in a direct or indirect manner. Since hormone receptors are required for the functional activity of hormones, a change in their expression and activity may explain the occurrence of hormone-related diseases later in life. The dynamic nature of epigenetic alterations upon hormone treatments further complicates the nature of how hormonal perturbations and epigenetic changes in early life may predispose to disease in adulthood (9).

Collectively, these studies suggest that early alterations in the hormone-dependent pathways together with epigenetic modifications may contribute to disease susceptibility in adulthood (9). Therefore, deregulated expression of specific genes as a result of early hormonal

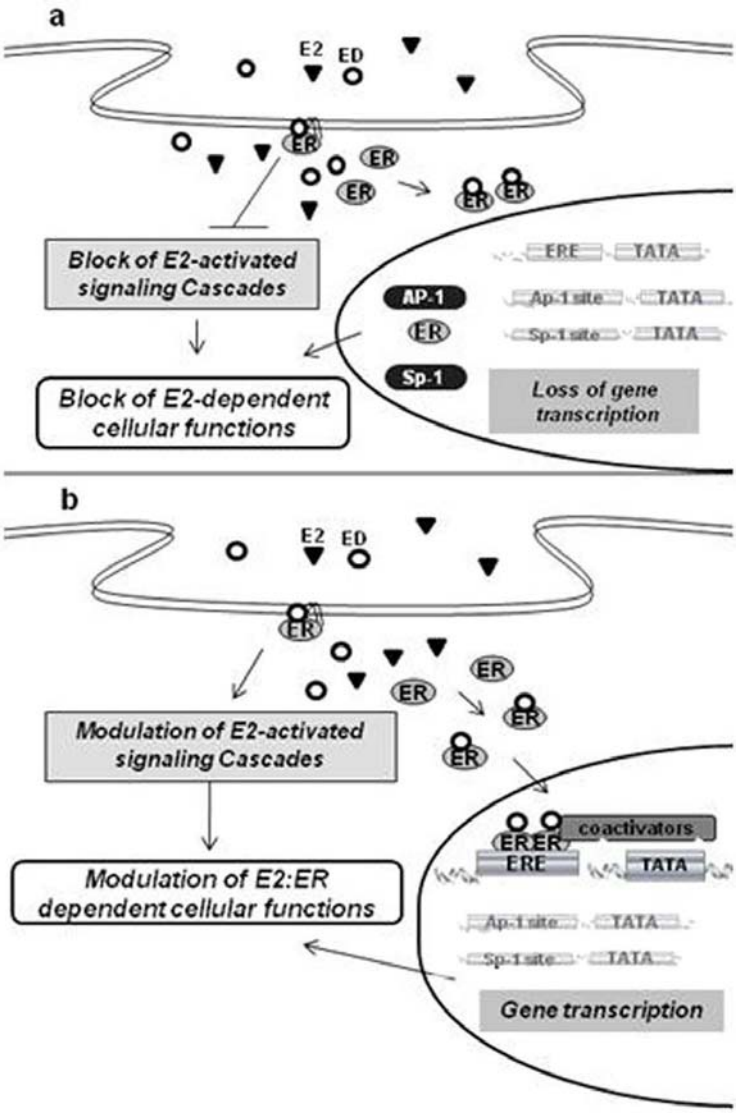

Figure 4. EDs action mechanisms through ERs. EDs could act as E2 antagonist by blocking genomic and extra-nuclear ER activities (panel a). EDs could also act as E2 agonists or as mechanism-specific ligand of ER by blocking specific ER activities (panel b). ED: endocrine disruptor; E2: 17β-estradiol; ER: estrogen receptor; ERE: estrogen response element.

perturbations may not manifest until adulthood, resulting in the onset of disease.

Despite of epigenetic changes, EDs, in particular plant derived flavonoids, modulate a wide spectrum of responses due to their chemical structure compatible with putative antioxidant properties (7). However, flavonoids also possess pro-oxidant properties which could contribute to the activation of pro-apoptotic cascades important for their anti-cancer activities (158).

EDs are also able to modulate the activity of several kinases and protein functions (*e.g.,* receptors) via competitive or allosteric interactions. Just to mention some, inhibition of the EGFR, PKC, PI3K, ERK, a block of enzyme activity modulating redox-sensitive transcriptional factors have been described (159, 160). The ED ability to block the nuclear receptor activities has also been reported. In particular, the thyroid hormone receptors (161), the retinoid X receptor (RXR), the peroxisome proliferator-activated receptors (128, 162), the pregnane X-receptor, and the constitutive androstane receptor (163, 164) are all considered target of ED interference.

A profound discussion on these ED action mechanisms is far from the goal of this review as any sex-related difference in these mechanisms has been reported to date.

5.1. Oestrogen receptors as target of EDs

A plethora of papers, supported by epidemiological and experimental data, indicates the ability of EDs to bind to ER leading to oestrogen mimetic or anti-oestrogenic effects (159, 165, 166) (tab1, Figure 4, Table 1).

Table 1. Values of the dissociation affinity constant for various ligand binding to ER

Compounds	ERα k_d (M)	ERβ k_d (M)	Ref.
17β-estradiol	4×10^{-11}	1.1×10^{-10}	196
Tamoxifen	3.4×10^{-9}	2.5×10^{-9}	197
Diethylstilbestrol	4×10^{-11}	5×10^{-11}	197
Genistein	1.26×10^{-7}	1.28×10^{-8}	196
Naringenin	2.4×10^{-7a}	5.9×10^{-7b}	a: 12 b: 165
Bisphenol A	1.2×10^{-6a}	3.5×10^{-8b}	a: 166 b: 197
ICI-164384	2×10^{-10}	8.0×10^{-11}	197

The ligand binding pocket of ERα and ERβ, in fact, may accommodate different ligands inducing conformational changes in receptor structure (47). In particular, ED binding to ER induces the repositioning of LBD of ERα, in a conformation that may favor or impair co-activators recruitment and, in turn, receptor transcriptional activity. This mechanism would contribute to the complex tissue-dependent agonistic or antagonistic responses observed with exposure to endocrine disruptors (167).

Similar to E2, natural compounds such as the isoflavones daidzein and GEN, the flavanone naringenin, and the flavonol quercetin increase the activity of ERE-luciferase reporter gene construct in cells expressing ERα or ERβ (168-171), but impair ERα interaction with the transcriptional factors Sp1 and AP-1 (170-172). Since Sp1 and AP-1-dependent gene expression, is strictly dependent on PI3K/AKT and ERK1/2 pathway activation, our groups postulated that different conformational changes of ERs could also preclude the activation of rapid signalling cascades (172) (Figure 4b). The flavonoids quercetin and naringenin hamper ERα-mediated rapid activation of signalling kinases (*i.e.,* ERK/MAPK and PI3K/AKT) and cyclin D1 transcription only when HeLa cells, devoid of any ER isoforms, were transiently transfected with a human ERα expression vector (171). In particular, naringenin induces conformational changes in ERα which include receptor de-palmitoylation, its rapid dissociation from plasma membrane, and inability to activate signalling cascades (*i.e.,* ERK/MAPK and PI3K/AKT) (172). Accordingly, in rat skeletal myoblasts (L6 cells), Nar stimulation decouples ERα mechanism of action impeding the E2-dependent myoblast differentiation important for the E2-protective effects in muscle (48; Marino unpublished results). On the other hands, flavonoids are E2 mimetic on both genomic and extra-nuclear ERβ activities (169; Marino unpublished results). Thus, the same chemical compound could selectively prevent rapid ERα mechanisms specifically impairing body functions induced by E2:ERα complex (see 1.2.1.) and stimulate the functions regulated by E2:ERβ complex.

It has been reported that BPA binds to ERα and ERβ with a lower affinity than E2 (*i.e.,* 10 μM BPA versus 10 nM E2) inducing E2-responsive gene expression. Interestingly, the set of genes induced by BPA and E2 seems to be quite different. Most of them being unique for BPA (167, 173). Moreover, BPA induces cell proliferation via ERα-mediate signal transduction pathway activation (*i.e.,* ERK and AKT phosphorylation) suggesting that BPA acts as an E2 agonist in the presence of ERα (166). On the contrary, discordant effects of BPA are reported in presence of ERβ. Our recent experiments in colon cancer cells expressing only ERβ isoform indicates that BPA acts as a full E2 antagonist by blocking both genomic and extra-nuclear ERβ activities (174). This area of research requires further investigation taking into the account the fact that ERβ mediates most of E2 protective effects in females (*e.g.,* neuroprotection, colon cancer protection), this result point to an increased susceptibility of females to BPA interference. However, due to the role played by E2:ER complexes in male physiology (see 1.2.1), it should be considered that specific ED interferences on male functions could also be dependent by the ER isoform present.

Very recently it has been suggested the ED action could be also mediated by the interaction between the aryl hydrocarbon receptor (AhR), its partner aryl hydrocarbon receptor nuclear translocator (ARNT), and ERs. AhR is a transcription factor belonging to the sensory factors of the basic helix-loop-helix Per-ARNT-SIM family. AhR activities can be induced by a wide spectrum of synthetic and naturally occurring chemicals (175, 176) and AhR activation leads to translocation of AhR into the nucleus where it dimerizes with its partner protein ARNT (177) which, in turn, increases the expression of genes involved in metabolism of EDs, as well as of many endogenous substances (178). In breast cancer cells as well as in mouse uterus, the ED-induced estrogenic effects are due to the recruitment of AhR:ERα complex, suggesting that AhR could act as ERα co-activator leading to the activation of receptor transcriptional activity even in the absence of E2 (179, 180). One intriguing finding is that ARNT has a stronger impact on ERβ than on ERα activities (181). However, these data consider only the direct transcriptional activity of ERs leaving out the ER indirect transcriptional activity and the extra-nuclear mechanism of action.

5.2. Androgen Receptor as target of EDs

Even if DHT and E2 share common mechanisms of action (*e.g.,* genomic and rapid mechanisms), very few data are available on ED mechanism of action underlying the disruption of AR-mediated pathways. Several endocrine disrupting compounds have been hypothesized to alter androgen action or growth and development of prostate. In fact, developmental (*in utero* or neonatal) exposure to BPA low doses and to other compounds, including PCB, HCB, and the synthetic estrogen diethylstilbestrol, has been reported to increase size and weight of prostate, suggesting that these agents early influence prostate growth (167). Although the mechanism of ED action in the prostate is yet to be determined, recent studies have shown its ability to influence AR function and activity. Anti-androgenic compounds has been divided into those that have a direct receptor antagonistic activity, those that disturb synthesis

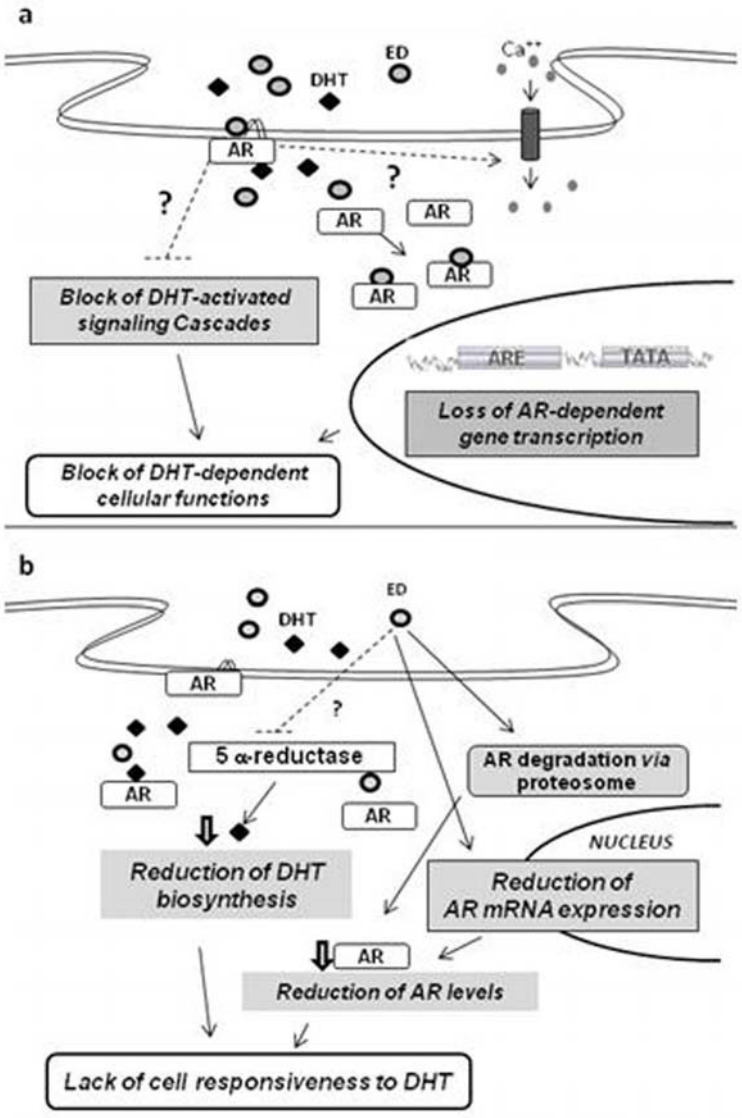

Figure 5. EDs action mechanisms through AR. EDs binding to AR can lead to a block of both genomic and extra-nuclear AR (panel a). EDs can impair both DHT biosynthesis and AR levels leading to a loss of cell responsiveness to DHT (panel b). DHT: 5α-dihydro-testosterone; ED: endocrine disruptor; AR: androgen receptor.

and/or metabolism of androgens, and those that can have a dual effect on synthesis and receptor interaction (*e.g.,* the fungicide prochloraz and the herbicide linuron) (182, 183) (Figure 5).

Phthalates and their metabolites impair both synthesis and metabolism of androgens and androgen receptors levels. However, phthalates do not bind to AR (83). For example the di(2-ethylhexyl) phthalate causes anti-androgenic effects by inhibiting Leydig cell function in foetal life (83). Contrarily, the above mentioned pesticides, vinclozolin, procymidone, DDT, and linuron as well as BPA exhibit strong anti-androgenic activity by inhibiting androgen binding to AR and, consequently, androgen-induced gene expression (10, 167). In particular, rat exposition to linuron *in utero* leads to a reduction in male offspring, ventral prostate weight, seminal vesicle, and epididymal weights resulting in malformations of the epididymides and testis in the adults (184). Interestingly, vinclozolin significantly alters the sub-cellular distribution of AR (185).

Several PCB congeners affect the transcriptional activity of AR in human prostate cell line (11). However, some congeners have also been shown to alter AR protein levels in the prostate when animals are exposed during development, while other PCB increase the metabolism of androgens *in vivo*. Thus, it is difficult to discern which specific congeners, or combination of congeners, are responsible for the observed results. It has been postulated that a compound with the non planar orientation and two

ortho substitutions, combined with one *meta* and *para* substitution, respectively, could bind to AR with high affinity (11). Surely, this structure: activity relationship could be possibly used to establish which PCB congeners exhibit the highest androgen-modulating effects, presumably because of their strong interaction with AR (11). However, such structure-dependent prediction does not take in account the possibility of heterotrophic allosteric phenomena characterizing nuclear steroid receptors. Therefore, the effect of each PCB congener on AR activity should be experimentally assessed in order to clarify the contribution of each compound in the observed PCB-induced anti-androgenic actions (11).

Some studies also have highlighted the ability of the catechin, (-)-epigallocatechin-3-gallate (EGCG), a natural compound occurring in green tea, to inhibit the development and progression of prostate cancer in mice and in men. Particularly, in LNCaP cells EGCG prevents androgen-induced cell proliferation, PSA and kallikreins 2 secretion, AR expression, and AR transcriptional activity (186, 187). Interestingly, EGCG had little or no effect on AR mRNA and protein levels in these cells in the absence of androgen, while in the presence of androgen, EGCG reduced AR protein levels by 30% (188). Besides EGCG effect on AR expression, EGCG suppresses the serum PSA level in castrated mice. Since PSA gene expression is regulated by AR (186), the EGCG-induced reduction of PSA expression suggests that EGCG inhibits AR signalling (188).

On the contrary to EGCG, the organotins trybutyltin and tryphenyltin enhance the proliferation of androgen-dependent and -independent human prostate cancer cells and the transactivation of AR (189). However, AR antagonist flutamide cannot inhibit organotin-mediated AR transactivation and these organotins do not function as AR agonists in yeast two hybrid assay. Recently RXR has been found to function as a novel co-regulator of AR, and 9-cis-Retinoic Acid (9cRA), a RXR ligand, was found to inhibit AR activity trough the activation of RXR (190). It remains unclear whether the co-regulators recruited by organotin-activated RXR are different from those recruited by 9cRA, but RXR activation by organotins might be involved in the AR transactivation (191).

As a whole, the combined effect of EDs on AR-dependent gene expression and, more general, on animal physiology may be more complex than suggested by these simplified experimental models because, as well as for ER, many EDs can act as modulator of AR, leading to the activation and the interaction of multiple signalling pathways, and in turn, EDs can affect reproduction and development by more than one mechanism. In spite of the huge number of studies evaluating the anti-androgenic properties of EDs, only androgen metabolism and AR transcriptional activity have been take into consideration, while a lack of knowledge is still present on the ability of these compounds to interfere with androgen dependent extra-nuclear signals (192). Since the alteration of androgen signalling can induce a variety of endocrine disruptive responses, further studies are required to identify the downstream targets of ED-modulated AR signalling, in order to elucidate their specific impact on male health (192).

6. PERSPECTIVES

EDs has recently drawn immense attention to the scientific community as they are now recognized as potentially hazardous factors for human health. Over the years these concerns grew with the advancement of biochemical, biomedical, and biotechnological industries and with the increasing possibility of bioterrorism and chemical-warfare. These man-made chemicals are found abundantly in the environment on residential buildings, cars, furniture, plastics, products such as baby feeding bottles, lining, tin-food containers, and even in children's toys.

These compounds can exert prominent effects during vulnerable developmental stages as *in uterus* or during puberty where EDs may pose a risk of developing disease later in life. It has been theorized that the insurgence of different pathologies may be due to the exposition to EDs during a critical window of pre-natal development (193). Studies have confirmed that the exposure during prenatal period could alter the gender-specific characteristics, the developmental programming, and could delay pubertal development without the need for a second exposure (100). If confirmed, these data indicate that *in utero* exposition to EDs could be more critical for males which development is mainly dependent from testosterone produced by testis in the prenatal period.

Data obtained from epidemiologic evidence both in human and wildlife*, in vivo* studies but also genomic, proteomic and metabolomic studies give us a picture of the effect of these compounds. However, most studies focused on one sex exclusively, or the experimental design does not include the evaluation of sex-dependent characteristics in particular window of exposure. Last but not least, data on ED action mechanisms are still unclear, confused and sometimes contrasting. Particularly, most studies on ED mechanisms are performed in the absence of the endogenous hormone. These experimental approaches can surely highlight their underlying disrupting action mechanism, however, if endogenous hormones are not included in the studies the physiological relevance of these findings is not clear. Since the final outcome of the exposure to a single or a mixture of these compounds, is strictly dependent on the interaction of the ED-activated and hormone-activated signals, it should be take in account the contribution of both hormones and EDs.

A huge number of papers describes ED ability to interfere with ER action mechanism, and, even if AR shares some action mechanisms with ER, few information is available on the ability of EDs to affect androgen signalling. The reported data suggest that ED effects in male are mainly due to ED ability to reduce cellular responsiveness to androgens and in modulating ER activities.

Finally, the precise nature of ED effects depends on the extent to which they are taken up by the animals and then on the extent to which they are degraded, excreted or metabolized; each of these factors depends on the individual animal species and on the chemical nature of compounds (*e.g.,* hydrophilic or lipophilic compounds). Accordingly, EDs can accumulate in animal tissues. Thus EDs can be biologically active at concentrations well below that at which they are toxic in the conventional sense (167).

Furthermore, a synergy between different pollutants has been proposed (12, 194, 195), so that biological responses may be induced even when the concentrations of individual chemicals appear to be too low to exert an effect. Since sub-lethal effects of EDs may not immediately manifest, compared with toxicological impacts, major accuracy should be taken in choosing experimental ED dose and way of administration.

In conclusion, EDs are differently distributed in environment, thus, some class of people could result more exposed to a specific group of EDs than others. Therefore, ED exposure must be effectively considered as a "sex and gender matter". As we will learn more about the actions of EDs the basis for making sound decisions for their use will emerge.

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Abbreviations: 9cRA, 9-cis-retinoic acid; AhR, aryl hydrocarbon receptor; AR, androgen receptor; ARE, androgen response element; ARNT, aryl hydrocarbon receptor nuclear translocator; AVPV, anteroventral periventricular nucleus of hypothalamus; BPA, Bisphenol A; DDT, dichlorodiphenyltrichloroethane; DHEA, deydroepiandrosterone; DHT, di-hydro-testosterone; E2,17ß-estradiol; ED, endocrine disruptor; EGCG, (-)-epigallocatechin-3-gallate; EGF-R, epidermal growth factor receptor; ERE, estrogen response element; ERK, extracellular regulated kinase; ERα, estrogen receptor α; ERβ, estrogen receptor β; GEN, genistein; HCB, hexachlorobenzene; IGF-1-R, insulin-like growth factor 1 receptor; LBD, ligand binding domain; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PCB, polychlorobiphenyl; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RXR, retinoid X receptor; T, testosterone; WAT, white adipose tissue

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