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Cellular and biochemical mechanisms by which environmental oestrogens influence reproductive function

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The biology and physiology of the male as well as female reproductive system is hormonally regulated. Abnormalities in the dynamics of hormone production, metabolism and elimination, as well as their binding to certain target tissues, has been associated with pathophysiological conditions of the reproductive system. Although oestrogens are known to be one of the major hormone groups in regulating the reproductive function and the fertilization process, the cellular and biochemical mechanism or mechanism(s) via which oestrogens induce their effects are still not fully defined. Moreover, in a modern environment we are also exposed to a wide battery of environmental agents which are structurally similar to oestrogens, and termed 'environmental oestrogens'. Because environmental oestrogens have been shown to mimic some of the effects of oestradiol, it has been postulated that these exogenous chemicals may influence or interfere with the oestrogen-dependent reproductive processes, and may be associated with beneficial as well as deleterious effects on the reproductive system. In this regard, two classes of environmental oestrogens have been widely studied, i.e. phyto-oestrogens (plant-derived dietary oestrogens) and xeno-oestrogens (industrial chemicals, including polychlorinated biphenyls, DDT, TCDD, dioxins, etc.). The main focus of this review is to provide an overview on the cellular and biochemical mechanism(s) by which xeno-oestrogens and phyto-oestrogens influence the oestrogen-dependent reproductive functions and induce their deleterious or protective effects on the reproductive system.

Key words: biochemical and cellular mechanisms/environmental oestrogens/exposure/reproductive function

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Introduction

Oestrogens influence cell growth and cell differentiation of the male and female reproductive tissues, and in this regard are known to regulate the development of mammary glands, uterus, vagina, ovary, testes, epididymis and prostate (Korach, 1994). Oestrogens also play an important role in the biology as well as physiology of the vascular system which is strictly related to the reproductive processes. (Korach, 1994; Iafrati *et al*., 1997; Dubey *et al*., 1999a). Findings during the past decade have provided evidence that oestrogens induce their biological effects via genomic, as well as non-genomic, mechanisms that are triggered by oestrogen receptor (ER) -dependent as well as ER-independent mechanisms (Figure 1). Hence, we first delineate the mechanisms by which endogenous oestrogens themselves induce their cellular and biochemical effects.

Role of oestrogen receptors

To mediate their receptor-dependent effects, oestrogens diffuse through the plasma membrane of cells and bind to the specific, high-affinity ER within the target cell (Figure 1). The hormone–receptor complex is activated by undergoing surface-charge and conformational changes, triggered by changes in intracellular temperature and salt. The activated oestrogen–receptor complex

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translocates to the nucleus and binds to chromatin at specific regions of the DNA, called hormone response element, and stimulates or inhibits specific genes resulting in the production of the respective messenger RNAs and subsequently synthesis of specific proteins. Changes in the generation of intracellular proteins result in activation of a cascade of events which finally influences the metabolic processes and translates into cell growth and differentiation. In this regard, it is well documented that 17β-oestradiol induces uterine growth (hypertrophy and hyperplasia; Martin *et al*., 1973). Moreover, it has been shown that 17β-oestradiol rapidly induces the expression of proto-oncogenes which may play a role in oestrogen-induced cell growth and proliferation. In uterine tissue cells, treatment with 17β-oestradiol rapidly induces the steady-state expression of uterine N-*myc* and c-*myc* mRNAs (Murphy *et al*., 1987a; Travers and Knowler, 1987), c-*ras*- mRNA and c-*fos* mRNA (Travers and Knowler, 1987; Loose-Mitchell *et al*., 1988). The uterine epidermal growth factor (EGF) receptor, which is a homology of the *erb*-B oncogene, is also induced by 17β-oestradiol (Mukku and Stancel, 1985). Apart from up-regulating the expression of proto-oncogenes, 17β-oestradiol has been shown to up-regulate receptors of growth factors such as EGF, insulin-like growth factor (IGF) (Berthois *et al*., 1989; Stewart *et al*., 1990), and to stimulate the levels of growth-promoting peptides which can act in an autocrine fashion to induce cell growth (Rochefort *et al*., 1980; Dickson and Lippman, 1987; Murphy *et al*., 1987b; Murphy and Ghahary, 1990). Alternatively, oestrogens can synergize with growth factors by inducing their synthesis, activating their receptors as well as their effects on the proto-oncogenes. In MCF-7 cell lines, oestradiol has also been shown to increase EGF-induced activator protein-1 (AP-1) independent of its effect on c-*fos* and c-*jun* (Philips *et al*., 1993), which suggests that there may be cross-talk between the ER and AP-1 activity. In this regard, it has been shown that oestradiol can interfere with the growth factor pathway not only by inducing its own synthesis (Dickson and Lippman, 1987) or that of its receptors (Berthois *et al*., 1989; Stewart *et al*., 1990), but also by facilitating its action at the nuclear level. It has been shown (Van der Burg *et al*., 1990) that oestradiol, by inducing c-*fos*, could synergize with low concentrations of insulin, which is able to induce c-*jun* but not c-*fos*. Subsequently, it was shown (Philips *et al*., 1993) that even when c-*fos* and c-*jun* synthesis and AP-1 activity are maximally induced by growth factors, oestradiol can still enhance AP-1-dependent transcriptional activity. It has also been demonstrated (Gaub *et al*., 1990) that AP-1 DNA sequence in the chicken ovalbumin gene promoter can be a target for both oestrogen activation and co-operation for *fos/jun*. Moreover, in-vivo studies with ovariectomized rats, have shown that EGF enhances the nuclear localization of the ER, suggesting that it activates the ER to bind to oestrogen response element (ERE) sequences as does oestrogen (Ignar-Trowbridge *et al*., 1992). Hence, cross-talk between EGF-triggered pathways would activate ER-mediated transcription (Ignar-Trowbridge *et al*., 1992), and this would stimulate oestrogen-induced activation of ER which stimulates AP-1-dependent transcription (Philips *et al*., 1993).

The above findings provide evidence that ER plays a key role in mediating the cellular effects of oestradiol on cell growth and differentiation. Recent studies (Kuiper *et al*., 1996) have shown that apart from the classical ER receptor cloned more than a decade ago (Green *et al*., 1986; Greene *et al*., 1986) and now classified as ERα, cells from rat, mouse and humans also express another ER called ERβ (Kuiper *et al*., 1996; Mosselman *et al*., 1996; Enmark *et al*., 1997; Tremblay *et al*., 1997).

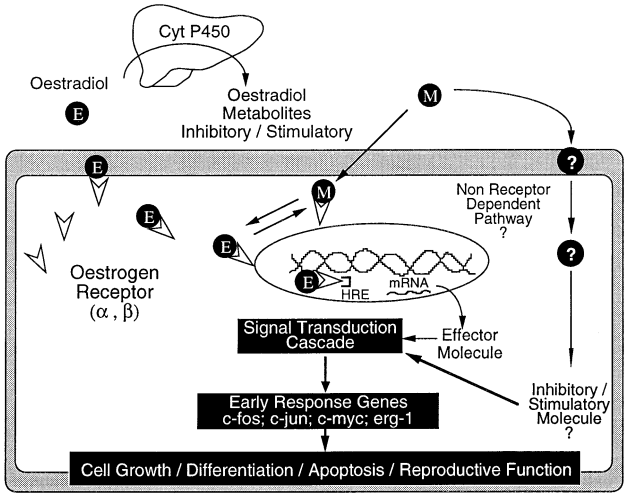

Figure 1. Schematic representation of the various mechanisms via which oestrogen (oestradiol) can induce its biological effects. E = oestradiol; M = metabolite of oestradiol; Cyt P450 = cytochrome P450 (drug-metabolizing enzymes); HRE = hormone response element; mRNA = messenger RNA.

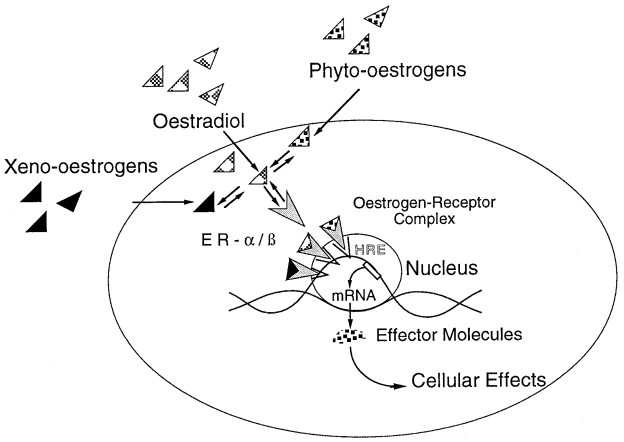

Figure 2. Environmental oestrogens (phyto-oestrogens and xeno-oestrogens) can compete with oestradiol for the oestrogen receptor (ER) and consequently influence the biological response of oestradiol. Depending on the chemical nature of the environmental oestrogen, it can induce oestrogenic or anti-oestrogenic effects by directly inducing effector molecules or inhibiting the generation of oestradiol-induced molecules, respectively. ER-α/β = oestrogen receptor alpha and beta; HRE = hormone response element; mRNA = messenger RNA.

Whether ERα and ERβ play a similar or differential role in mediating the physiological effects of oestradiol is still unclear. However, differential expression of ERα and ERβ has been observed in some tissues (Glatstein and Yeh, 1995; Korach *et al*., 1996; Kuiper *et al*., 1996, 1997; Byers *et al*., 1997; Saunders *et al*., 1998), which may help define the physiological role of these receptors. In this regard, as compared with ERα, high amounts of ERβ mRNA were identified in fetal ovaries, testes, adrenals and spleen of the midgestational human fetus (Brandenberger *et al*., 1997). Moreover, differential ligand activation of ERα and ERβ

Table I. Binding affinities of oestradiol and some environmental oestrogens to the oestrogen receptor (ER) or aryl hydrocarbon receptor (AhR)

Compound	ER-affinity	ERα-affinity	ERβ-affinity	AhR-affinity
Oestradiol	+++++ (Zava *et al.*, 1997; Kuiper *et al.*, 1998)	+++++ (Kuiper *et al.*, 1998)	+++++ (Kuiper *et al.*, 1998)	nda
Xeno-oestrogens				
TCDD	–	–	–	++++ (Lu *et al.*, 1996)
PCBs (209 possible congeners)	+/++ (Zava *et al.*, 1997; Reinhart *et al.*, 1999)	nda	nda	+++ (Kafafi *et al.*, 1993; Safe, 1994; Rowlands and Mekenyan, 1996; Gustafsson, 1997)
4-OH-PCB	++ (Korach *et al.*, 1988; Kuiper *et al.*, 1998; Kramer and Giesy, 1999; Reinhart *et al.*, 1999)	+ (Kuiper *et al.*, 1998)	+ (Kuiper *et al.*, 1998)	nda
Phyto-oestrogens				
Genistein	+++ (Miksicek, 1993; Zava *et al.*, 1997; Reinhart *et al.*, 1999)	+++ (Kuiper *et al.*, 1998)	+++ (Kuiper *et al.*, 1998)	nda
Equol	++ (Petit *et al.*, 1997; Zava *et al.*, 1997)	nda	nda	nda
Daidzein	++ (Markiewicz *et al.*, 1993; Miksicek, 1995; Hopert *et al.*, 1998)	++ (Kuiper *et al.*, 1998)	++ (Kuiper *et al.*, 1998)	nda
Quercetin	+ (Kuiper *et al.*, 1998)	+ (Kuiper *et al.*, 1998)	+ (Kuiper *et al.*, 1998)	+++ (Kuiper *et al.*, 1998)

Data from various cell types.
nda = no data available; PCB = polychlorinated biphenyls; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

has been shown to occur at site AP-1 (Paech *et al.*, 1997) and xeno-oestrogens differentially activate different ERE when linked to ERα and ERβ (Pennie *et al.*, 1998). Because environmental oestrogens (phyto-oestrogens and xeno-oestrogens) have been shown to bind differentially to both ERα and ERβ (Kuiper *et al.*, 1997, 1998) (Table I), it is feasible that they may compete with oestradiol for the ER and interfere with its receptor-mediated biological effects (Figure 2).

Apart from the cytosolic/nuclear ER, oestradiol has also been shown to bind with high affinity to membrane fractions prepared from isolated uterine cells (Pietras and Szego, 1975; Szego, 1984) and pituitary (Bression *et al.*, 1986). The functional role of the membrane receptors for oestrogen is evident from the findings that oestradiol stimulates adenylate synthase activity in membranes prepared from secretory human endometrium (Bergamini *et al.*, 1985). Moreover, oestradiol has been shown to induce rapid changes in intracellular Ca^{2+} concentrations/flux, K^+ conductance and cAMP levels (Pietras and Szego, 1975; Nabekura *et al.*, 1986; Aronica *et al.*, 1994). These findings suggest that oestradiol can induce several cellular effects via non-genomic ER-independent effects. Another important non-receptor-mediated effect of oestrogen is linked to its chemical property of being a phenol with anti-oxidant effects. Because free radicals are known to play a key role in regulating cell growth it is feasible that oestradiol may induce some cellular effects by acting as a free radical scavenger or anti-oxidant. Additionally, oestrogens have been shown to cause changes in membrane fluidity, induce rapid membrane responses, and bind to enzymes such as tyrosine hydroxylase (Weiss and Gurpide, 1988).

Role of oestrogen metabolism

Several lines of evidence suggest that some of the effects of oestradiol may be mediated via its metabolites (Figure 1). Oestradiol is eliminated from the body by metabolic conversion by the drug-metabolizing enzymes, which constitute a battery of cytochrome P450 enzymes (CYP450; Martucci and Fishman, 1993). The CYP are a superfamily of mono-oxygenases which are involved in the metabolism of both exogenous and endogenous compounds. Many isoforms of CYP450 have been identified; however, the isoenzymes that metabolize steroids, such as 17β-oestradiol, are CYP1, CYP2 and CYP3 (Martucci and Fishman, 1993; Zhu and Conney, 1998). Although most of the metabolites formed are hormonally less active, water-soluble and excreted in the urine, some have been shown to have strong growth-regulatory effects (Zhu and Conney 1998). Oestradiol is largely

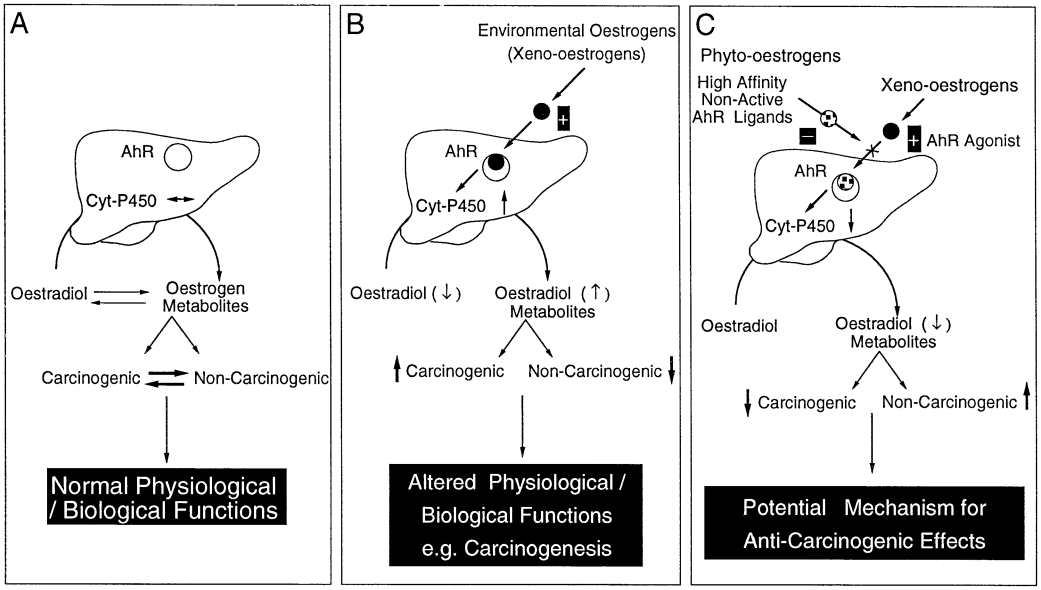

Figure 3. Environmental oestrogens can influence the biological effects of oestradiol by modulating its metabolism and disrupting the balanced generation of metabolites. Under normal conditions, oestradiol is metabolized by cytochrome P450 (Cyt-P450) into metabolites which are known to induce both carcinogenic and non-carcinogenic effects. Balanced generation of these metabolites maintains normal reproductive function and protects against cancer (**A**). Environmental oestrogens (xeno-oestrogens in particular) bind to the aryl hydrocarbon receptor (AhR) and activate Cyt-P450. Activation of Cyt-P450 results in increased metabolism of oestradiol to carcinogenic metabolites. Increased metabolism of oestradiol, as well as increased generation of carcinogenic effects, may consequently induce reproductive disorders as well as carcinogenesis (**B**). Several phyto-oestrogens bind to the AhR with high affinity, but are inactive in inducing the Cyt-P450. These environmental oestrogens may protect against carcinogenesis by competing for the AhR with xeno-oestrogens which are known to induce oestradiol metabolism via AhR and Cyt-P450 (**C**). Additionally, these agents may preserve normal reproductive function by blocking the abnormal metabolism of oestradiol.

metabolized within the liver via oxidative metabolism to form hydroxylated metabolites such as 2- and 4-hydroxyoestradiol (Martucci and Fishman, 1993); glucuronidation to form glucuronide conjugates (Ritter *et al*., 1990; Zhu *et al*., 1996; Musey *et al*., 1997); sulphation to form sulphates (Brooks and Horn, 1971; Watanabe *et al*., 1988; Hernandez *et al*., 1992); esterification to form fatty acid esters (Zhu and Conney, 1998); and O-methylation of catechol oestrogens to form O-methylated catechols (Guldberg and Marsden, 1975; Ball and Knuppen, 1980). Even though oestradiol is largely metabolized within the liver, cells in several other tissues—including the reproductive organs—have been shown to contain CYP450 and to metabolize oestradiol as well as its metabolites (Zhu and Conney, 1998). The metabolism of oestradiol locally within a tissue may be of immense importance in mediating several physiological as well as pathophysiological effects (Figure 3). In this regard, it should be noted that inhibition of CYP450 enzymes by cimetidine increases oestrogen concentrations, and high doses of cimetidine have been shown to cause gynaecomastia (Michnovicz and Galbraith, 1991; Garcia-Rodriguez and Jick, 1994). Moreover, several studies have provided evidence that the catechol oestradiols can induce several biological effects. 2-Hydroxyoestradiol has been shown to regulate multiple mechanisms associated with the reproductive process: 2-hydroxyoestradiol can also bind to ER (Ball and Knuppen, 1980; MacLusky *et al*., 1983b; Van Aswegen *et al*., 1989), and has been shown to antagonize the growth-stimulatory effects of 17β-oestradiol on MCF-7 cancer cells lines (Schneider *et al*., 1984; Vandewalle and Lefebvre, 1989); increased generation of 2-hydroxyoestradiol has been observed in human term placenta (Fishman and Dixon, 1967; Osawa *et al*., 1993); 2-hydroxyoestradiol modulates the physiological effects of arachidonic acid and prostaglandins during pregnancy by stimulating the metabolic co-oxidation of arachidonic acid to prostaglandins in the uterus during certain periods of pregnancy (Castracane and Jordan, 1976; Kelly and Abel, 1981; Pakrasi and Dey, 1983). 2-Hydroxyoestradiol also inhibits the catabolism of catecholamines by inhibiting catechol O-methyltransferase (COMT) activity, and this may modulate neurophysiological/pharmacological effects of catecholamines within the central nervous system (Ball *et al*., 1972; Breuer and Koster, 1974; Fishman and Norton, 1975; Ghraf and Hiemke 1983; Parvizi and Ellendorff, 1983); 2-hydroxyoestradiol has been shown to alter prolactin, LH/FSH release by the anterior pituitary (Fishman and Tulchinsky, 1980; Linton *et al*., 1981). Moreover, oestradiol-2-hydroxylase activity is markedly increased in the anterior pituitary and hypothalamus of female rats during lactation (Hersey and Weisz, 1983), and modulates the interaction of dopamine with its receptors (Schaeffer and Hsueh, 1979; Paden *et al*., 1982), suggesting that it may be regulating prolactin formation (Fishman and Tulchinsky, 1980; Linton *et al*., 1981). 2-Hydroxyoestradiol is present in the ovarian follicular fluid (Dehennin *et al*., 1984) of humans as well as animals, and has been suggested to play an autocrine/paracrine role in regulating follicular development (Spicer and Hammond, 1989); 2-hydroxyoestradiol is a potent anti-oxidant and protects membrane phospholipids against peroxidation (Dubey *et al*., 1999b), and may protect follicles against oxidative damage.

Similar to 2-hydroxyoestradiol, the generation of 4-hydroxyoestradiol plays an important role within the reproductive system.

Even though it is not the dominant metabolite formed by the liver, it is a major metabolite formed in some extrahepatic tissues such as rat pituitary, human uterine myometrial as well as myoma tissue, human breast (Bui and Weisz, 1988; Liehr *et al*., 1995; Hayes *et al*., 1996). Similar to oestradiol, 4-hydroxyoestradiol binds to and activates ER, though its dissociation rate from the receptor is much lower than that observed for oestradiol (Barnea *et al*., 1983; MacLusky *et al*., 1983b); hence any increase in the levels of 4-hydroxyoestradiol formation may interfere with receptor-mediated effects of oestradiol. Within the reproductive system, 4-hydroxyoestradiol has been shown to stimulate uterine growth in animals; however, its uterotrophic potency is marginally weaker than that of oestradiol (Martucci and Fishman, 1976; Franks *et al*., 1982). 4-Hydroxyoestradiol is more effective than oestradiol in inducing progesterone receptor in the rat pituitary (MacLusky *et al*., 1983a). Administration of 4-hydroxyoestradiol alters the secretion of LH/FSH by the anterior pituitary (Martucci and Fishman., 1979). Similar to oestradiol, 4-hydroxyoestradiol has been shown to facilitate embryo implantation in mice (Ball *et al*., 1979; Hoversland *et al*., 1982; Kantor *et al*., 1985); moreover, an increased selective expression of oestradiol 4-hydroxylase activity occurs in the pig blastocyst during the preimplantation period (Mondschein *et al*., 1985; Chakraborty *et al*., 1988; Dey *et al*., 1989) and in the uteri of rabbits and mice during embryo implantation (Chakraborty *et al*., 1990; Paria *et al*., 1990). Taken together, these findings suggest that 4-hydroxyoestradiol actively participates in the implantation process. Similar to 2-hydroxyoestradiol, 4-hydroxyoestradiol also acts as a co-oxidant and increases the formation of prostaglandins from arachidonic acid within the uterus during pregnancy (Castracane and Jordan, 1975, 1976; Kelly and Abel, 1981; Pakrasi and Dey, 1983); it also prevents inactivation of catecholamines by inhibiting COMT activity, thereby regulating the neurophysiological/pharmacological effects on the central nervous system (Ball *et al*., 1972; Breuer and Köster, 1974; Ghraf and Hiemke, 1983; Parvizi and Ellendorff, 1983). However, in contrast to 2-hydroxyoestradiol, 4-hydroxyoestradiol has been shown to induce carcinogenic effects (Liehr, 1994; Yager and Liehr, 1996). In this regard, recent studies provide evidence for reduced 2-hydroxylation and increased 4-hydroxylation of 17β-oestradiol in subjects with cancer, suggesting that 2-hydroxyoestradiol or its methylated metabolite (2-methoxyoestradiol) may be anti-carcinogenic, where as 4-hydroxyoestradiol and its metabolite 4-methoxyoestradiol may be carcinogenic (Liehr *et al*., 1995; Liehr and Ricci, 1996). Moreover, differential metabolism of oestrogens may in part be responsible for the negative effects of oestradiol on the reproductive system.

Role of binding proteins

The above findings provide convincing evidence that the biological as well as the pathophysiological effects of oestradiol can be mediated via ER-dependent as well as independent pathways for example, via the generation of metabolites. In addition to these mechanisms, the effects of oestradiol can also be influenced by factors regulating its transport to the target tissues. In this regard, oestradiol is known to bind to sex hormone binding globulin (SHBG), the synthesis of which is influenced by other hormones (Horvath, 1992). The unbound (free) fraction of oestradiol is important for inducing its biological activity; moreover, the extent of binding also defines the rate of oestradiol metabolism as well as its elimination from the body, thereby influencing its half-life and pharmacological effects. Hence, any factors that influence the levels of SHBG will subsequently influence the biological effects/activity of oestradiol. In this regard it has been shown that, within the circulation, 40% of oestradiol is bound to SHBG; moreover, the concentration of SHBG is increased by oestrogens such as oestradiol, oestriol, oestrone sulphate, ethinyl oestradiol, etc. (von Schoultz, 1988), and decreased by androgens (Horvath, 1992), progestins (Horvath, 1992) as well as under pathological conditions such as polycystic ovarian syndrome (PCOS) (Horvath, 1992). Additionally, it is interesting to note that metabolites of oestradiol, i.e. 2- and 4-methoxyoestradiols and methoxyoestrones, do not bind to the classical ER, but have a higher binding affinity to SHBG than does oestradiol (Dunn, 1983). This finding suggests that the methoxy metabolites of oestrogen may have a longer half-life, and may be present within the circulation at much higher concentrations. Indeed, in pregnant women the concentration of unconjugated 2-methoxyestrone is approximately 4000 pg/ml (Ball and Knuppen, 1990). Apart from SHBG, oestrogens can also bind to albumin and membrane proteins (Horvath, 1992), and this may also influence the biological effects of oestrogens. Taken together, the above findings provide evidence that factors influencing the concentrations of SHBG or albumin may eventually also influence the effects of oestradiol on the reproductive system by influencing its metabolism and elimination, as well as the availability of the free (active) oestrogen or its metabolites to the target tissue.

Role of aryl hydrocarbon receptors (AhR) in regulating the biological effects of oestrogen

Environmental oestrogens have been shown to play a critical role via the AhR; however, whether the AhR influences oestradiol effects under physiological conditions is unclear. As shown in Figure 4, the unoccupied AhR is a basic helix–loop–helix (HLH) protein which is localized within the cytosolic compartment of the cells. Based on molecular properties, the AhR is comparable with steroid hormone receptors, and was thought to belong to the steroid receptor family (Poellinger *et al*., 1985). However, based on the amino acid sequence analysis it was revealed that AhR is a member of the HLH superfamily of proteins whose members include AhR nuclear translocator protein (ARNT), the *Drosophila* proteins single minded and period, as well as the hypoxia-inducible factor 1 alpha (Ema *et al*., 1992). In addition to the lung, pancreas, brain, heart, liver, kidney, vasculature, tonsils and B lymphocytes, the AhR receptors have also been found in the reproductive tissues, including placenta, ovary, endometrium, blastocytes, cervix and breast cancer cells (Stols and Iannaccone, 1985; Landers and Bunce, 1991; Safe *et al*., 1991b; Wang *et al*., 1992; Rowlands *et al*., 1993; Enan *et al*., 1998).

The inactive cytosolic AhR is found complexed with two heat shock protein 90 (hsp90) molecules and a 43 kDa protein (p43) molecule. Binding of halogenated aromatic hydrocarbons/environmental oestrogens to the AhR initiates the sequential transformation process which involves shedding of the hsp90 and p43 molecules and formation of a dimer with ARNT

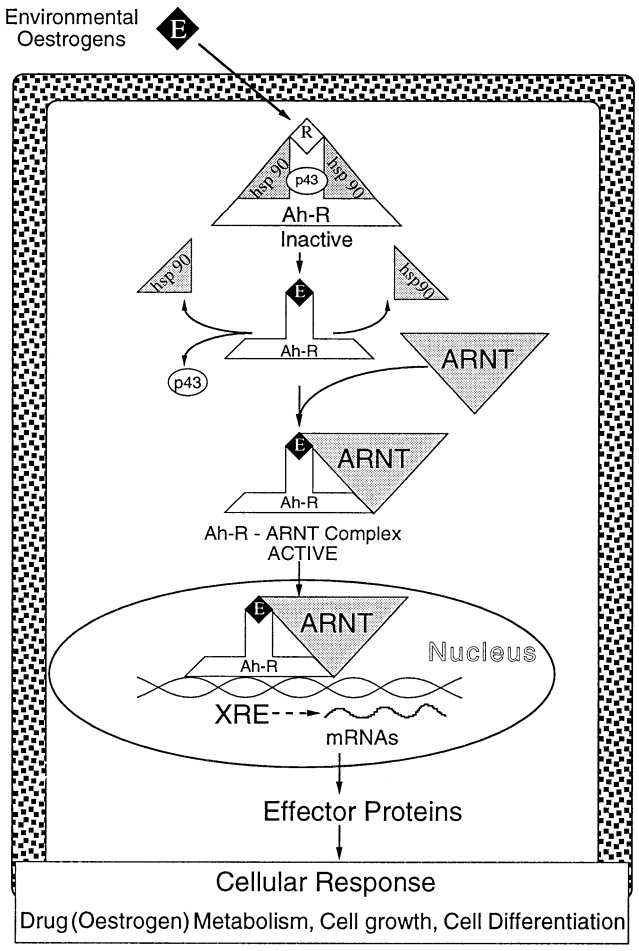

Figure 4. Model depicting the mechanism by which AhR-ARNT mechanisms are activated by environmental oestrogens. E = environmental oestrogens; Ah-R = aryl hydrocarbon receptor; ARNT = aryl hydrocarbon receptor nuclear translocator; R = receptor; XRE = xenobiotic response element; hsp 90 = 90 kDa heat shock protein; p43 = 43 kDa protein.

(Rowlands and Gustafsson, 1997). The AhR–ligand–ARNT complex is active, translocates to the nucleus, and binds to the DNA at the xenobiotic regulatory elements (XRE) in the target enhancer and induces the expression of several genes including CYP450 (Schmidt and Bradfield, 1996; Rowlands and Gustafsson, 1997). Because, the CYP450 is involved in oestradiol metabolism, the activation of AhR can result in rapid metabolism of oestradiol and influence its biological activity. Moreover, depending on the type of CYP450 isoenzyme (CYP1A1, CYP1A2, CYP1B1, etc.) induced, the generation of oestradiol metabolites which are carcinogenic or non-carcinogenic may be influenced and result in deleterious or altered effects of the reproductive system (see Figure 3).

Ligand-activated AhR has been shown to induce CYP1A1 activity in MCF-7 cell lines and induce oestradiol metabolism via increase in oxidative metabolism by activating the 2- and 4-hydroxylation as well as the 15α and 16α hydroxylases (Gierthy *et al*., 1987; Spink *et al*., 1990, 1992, 1994), suggesting that AhR may influence the biological effects of oestradiol by increasing its metabolism. Alternatively, it may also alter the effects of oestradiol by generating biologically active metabolites of oestradiol such as 2-hydroxy- and 4-hydroxy-oestradiol and their methylated products 2-methoxy- and 4-methoxy-oestradiol. In contrast to the above observation, ligand-induced activation of AhR has been shown to induce anti-oestrogenic effects, without lowering the circulating concentrations of oestradiol (Shiverick and Muther, 1982; DeVito *et al*., 1992), thus suggesting that pathways other than oestrogen metabolism may also participate in AhR-mediated anti-oestrogenic effects.

Several lines of evidence suggest that cross-talk between ER and AhR may play a critical role (Vickers *et al*., 1989; Safe *et al*., 1991a,b; Thomsen *et al*., 1994). It has been well established that cell lines that express AhR are also positive for ER, whereas ER-negative cell lines (MDA-MB-231) are non-responsive to AhR ligands (Vickers *et al*., 1989)—an activity that is restored when the cell lines were transfected with AhR. Similarly, loss of AhR does not necessarily translate into loss of ER responsiveness (Thomsen *et al*., 1994).

Environmental oestrogens and reproductive disorders: potential mechanism(s) of action

Environmental oestrogens constitute a class of chemical agents that structurally resemble 17β-oestradiol and which possess oestrogenic activity. The environmental oestrogens can be divided into two main categories: (i) phyto-oestrogens, which are plant-derived oestrogenic molecules and have co-evolved with humans; and (ii) xeno-oestrogens, which are chemically synthesized man-made molecules (phenols, pesticides, plastics, polychlorinated biphenyls, etc.) released into the environment. Because oestrogens play a key role in regulating several reproductive processes, it is feasible that exposure of humans to environmental oestrogens in an modern environment may influence the reproductive system. In this regard, ample evidence has accumulated during the past decade to support the notion that environmental oestrogens may cause reproductive disorders. However, less clear are: (i) the mechanisms by which xeno-oestrogens and phyto-oestrogens induce their pathophysiological effects on the reproductive process; (ii) whether the influence of phyto-oestrogens and xeno-oestrogens on the reproductive system are similar or different; and (iii) how some environmental oestrogens induce oestrogenic effects, whereas some are anti-oestrogenic.

Xeno-oestrogens

The list of chemicals within this group of oestrogen-like chemicals is large, and includes pesticides and herbicides, phthalates, organic solvents and drugs. With regard to the effects of xeno-oestrogens on the reproductive system, data from human and animal studies provide evidence that exposure to oestrogenic and anti-oestrogenic chemicals can influence sexual differentiation, offspring sex ratio, gonad development (size, morphology, weight), accessory sex organ development (size, morphology, weight), accessory sex organ function (secretory products production), secondary sexual characteristics (muscle and body mass, hair/fur, etc.), sexual development and maturation (vaginal opening, testes descent, preputial separation, anogenital distance, nipple development), fertility, fecundity (litter size and the number of litters), time of mating, mating and sexual behaviour, ovulation, oestrous cyclicity, gestation length, abortion, premature delivery, spermatogenesis, sperm count and production, LH

Table II. Plasma concentrations of xeno-oestrogens in humans

Xeno-oestrogen	Subjects (*n*) (reference)	Plasma concentration
PCBs median (range)	Women (36)	<1.0 ng/g (10/15)[c]
	USA	(118: <1.0–369.2 ng/g)
Two individual congeners[a]	(Archibeque-Engle *et al.*, 1997)	(153: <1.0–1000.0 ng/g)
p,p'-DDE median (range)		967.0 ng/g (35)[c]
		(<1.0–2265.5 ng/g)
		(plasma residues, lipid adjusted)
PCBs median (range)	42-month-old children (126)	0.81 μg/l (0.23–2.2 μg/l) breast-fed
Four congeners[b]	The Netherlands	0.18 μg/l (0.07–1.49 μg/l) formula-fed
	(Lanting *et al.*, 1998)	
PCBs median (range)	Women with breast cancer (240)	4.49 ppb case patients
	USA (Hunter *et al.*, 1997)	(5.08 ± 2.51 ppb: mean ± SD)
		4.68 ppb controls
		(5.16 ± 2.26 ppb: mean ± SD)
DDE median (range)		4.71 ppb case patients
		(6.01 ± 4.56 ppb: mean ± SD)
		5.35 ppb controls
		(6.97 ± 5.99 ppb: mean ± SD)
		(values adjusted to cholesterol)
PCBs median; range	Neonates (80)	1.37 μg/l (born 1984/5); <0.3–3.18 μg/l
	Germany (Lackmann *et al.*, 1996)	0.96 μg/l (born 1994/5); <0.3–3.14 μg/l
HCB median; range		2.03 μg/l (born 1984/5); <0.1–12.36 μg/l
		0.61 μg/l (born 1994/5); <0.1–4.38 μg/l
PCBs (range)	Lactating women (7)	2.6–5.8 ng/g serum
	USA (Greizerstein *et al.*, 1999)	
DDE (range)		0.5–3.6 ng/g serum
HCB (range)		0.03–0.29 ng/g serum
PCBs (range of means)	Women (406)	PCB 118: 0.15–0.20 ng/g plasma
	The Netherlands, various areas	PCB 138: 0.58–0.66 ng/g plasma
	(Koopmann-Essboom *et al.*, 1994)	PCB 153: 0.88–0.99 ng/g plasma
		PCB 180: 0.50–0.58 ng/g plasma

[a]PCB congener 118, 153; [b]Σ of PCB congeners 118, 138, 153, 180 (calculated); [c]Number of samples with congener 118 (10), 153 (15) at or above quantitation limit ($n = 36$).
PCB = polychlorinated biphenyls; HCB = hexachlorobenzene; DDE = metabolite of DDT.

and FSH concentration, androgen and oestrogen concentrations, gross pathology of reproductive tissue, histopathology of reproductive tissue, anomalies of the genital tract, malformation of the genital tract, viability of the conceptus and offspring growth (body weight) of the conceptus and offspring (see review by Daston *et al.*, 1997). High concentrations of xeno-oestrogens were required to induce their deleterious effects on the reproductive system; however, whether the reproductive disorders could be caused by concentrations of xeno-oestrogens found in humans (Table II) and animals is less clear, and currently under investigation. It has been argued that the cumulative concentrations of various xeno-oestrogens may reflect the effects of high concentrations of xeno-oestrogens (Danzo, 1998); however, this notion could only be supported if all the xeno-oestrogens were either oestrogenic or anti-oestrogenic in nature. Hence, the main aim of this review is to highlight the various cellular and biochemical mechanisms by which xeno-oestrogens (oestrogenic or anti-oestrogenic) induce their deleterious effects on the male and female reproductive systems.

Within the male reproductive system, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to induce deleterious effects. Regarding the mechanism(s) via which TCDD induces such effects, a number of possibilities exist. Testosterone plays a key role in maintaining the physiological functions in males. Since TCDD has been shown to interact with AhR receptors and induce CYP450 enzymes which are involved in steroid metabolism, it may induce these deleterious effects by interfering with the biosynthetic pathway for steroid synthesis in the testis.

In rats, exposure to TCDD has been shown to reduce plasma testosterone and dihydrotestosterone concentrations (Moore *et al.*, 1985), without altering plasma LH concentrations (Moore *et al.*, 1989). In order to investigate the mechanism via which TCDD reduces testosterone synthesis, one group (Kleeman *et al.*, 1990) studied the effect of the induction of human chorionic gonadotrophin (HCG), an LH analogue, on the synthesis of testosterone in testes obtained from sexually mature Sprague–Dawley rats treated with TCDD (100 μg/kg bodyweight). As compared with untreated controls, testosterone synthesis was inhibited by between 30% and 75% in testes obtained from TCDD-treated rats. Moreover, TCDD treatment neither increased the HCG-stimulated secretion of any testosterone precursor, nor significantly decreased the efficiency with which testes converted

the pregnenolone into testosterone. Additionally, TCDD treatment did not influence the formation of testosterone from exogenous pregnenolone. Based on these findings, the authors concluded that TCDD does not inhibit the conversion of pregnenolone to testosterone. Instead, the inhibition of testosterone biosynthesis results from inhibition of pregnenolone formation, which was supported by their observation that the formation of pregnenolone was significantly inhibited in TCDD-treated testis. These findings suggested that TCDD interferes with some biochemical step upstream from pregnenolone synthesis. Subsequent studies showed that TCDD inhibits steroidogenesis by inhibiting the mobilization of cholesterol to CYP450scc (a mitochondrial enzyme which converts cholesterol into pregnenolone), and is not due to the inhibition of mitochondrial enzyme activity (Moore *et al*., 1991).

The effects of TCDD on plasma LH concentrations seem to be time-dependent, i.e. a significant decrease (50–60%) in LH concentration was observed after 1–3 days of TCDD treatment (Ruangwises *et al*., 1991), but no changes in LH concentration and LH receptors were observed in rats after 7 days of treatment (Kleeman *et al*., 1990). The early decreases in LH concentration in response to TCDD were also accompanied by 17α-hydroxylase and C17-20 lyase activity, as well as decreases in testicular microsomal haem and CYP450. However, no changes in mitochondrial CYP450 content or activity of cholesterol side-chain cleavage was observed. Moreover, treatment with HCG prevented TCDD-induced depression of the activities of testicular microsomal 17α-hydroxylase and C17-20 lyase activity and serum testosterone concentrations (Mebus *et al*., 1987). Taken together, the above findings suggest that TCDD decreases serum testosterone by interfering with the microsomal CYP450 enzymes in the testis.

TCDD influences the CYP450 enzymes by activation of AhR receptors via expression of ARNT and the formation of ligand–AhR–ARNT complex. However, in the testis of TCDD-treated rats, no induction of CYP1A1 activity and expression of ARNT protein was observed; moreover, a reduction of between 5% and 51% in AhR expression occurred (Roman *et al*., 1998). This finding suggests that TCDD—a known ligand of AhR—may be mediating its effects on testicular function via some alternative pathway, such as regulation of cell growth. Indeed, treatment of rats during pubertal development with TCDD alters both signalling kinase activities (c-Src kinase, protein tyrosine kinase, mitogen-activated protein2 kinase, protein kinase A and protein kinase C) and EGF receptor binding in the testis (El-Sabeawy *et al*., 1998). Because cellular function is importantly regulated by the signalling kinases, and TCDD inhibited all the kinases (except c-Src), it is feasible that the effects of TCDD on the testis as well as androgen synthesis are secondary to its effects on the testicular cells participating in steroidogenesis. In this regard, it is important to note that TCDD has been shown to cause dose-dependent reduction in Leydig cell function and Leydig cell volume per testis; moreover, within individual Leydig cells there was a reduction in smooth endoplasmic reticulum and mitochondria (Johnson *et al*., 1994; Wilker *et al*., 1995). Interestingly, the effects of TCDD on Leydig cells and androgen synthesis were abrogated when HCG was co-administered, suggesting that the effects of TCDD may be directly on the testicular cells.

Apart from the above mechanisms, TCDD has also been shown to induce lipid peroxidation, decrease superoxide dismutase and glutathione peroxidase activities, and decrease divalent cations in rat testis (Al-Bayati *et al*., 1988). Similar to TCDD, administration of polychlorianted biphenyls (PCBs), polychlorinated naphthalenes, as well as inhalation of cigarette smoke, induces lipid peroxidation and decreases anti-oxidant enzyme activities in rat testis (Peltola *et al*., 1994), thereby suggesting that the xeno-oestrogens may induce their deleterious effects via free radical generation. Although TCDD is known to induce anti-oestrogenic effects, its effects on the testis are neither AhR receptor- nor ER-mediated (Roman *et al*., 1998). Moreover, similar to TCDD, treatment with diethylstilboestrol (DES) and 4-octylphenol (both of which bind to the ER) resulted in reduced CYP450 17α-hydroxylase/C17-20 lyase (a key enzyme for testosterone) activity in the Leydig cells of rat fetal testis (Majdic *et al*., 1996). Taken together, the above results provide evidence that the deleterious effects of environmental oestrogens on the testis, testosterone biosynthesis and consequently on masculinization may be mediated via different mechanisms, and may not necessarily fit the classical requirement of interaction with ER, oestrogen metabolism, etc. This is supported by recent studies (Gray *et al*., 1999) which showed the effects of various anti-androgenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p-DDE and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169 and ethane dimethane sulphonate) on malformations in the male rat during sexual differentiation, and found that these agents produce diverse profiles.

Maternal exposure to DES or 4-octylphenol has also been shown to alter the expression of steroidogenic factor-1 (SF-1/Ad4BP) in the testes (Sertoli and interstitial cells)—but not ovaries—of rat fetuses (Majdic *et al*., 1997). Because SF-1/Ad4BP is essential for gonadal differentiation, it is feasible that the xeno-oestrogens may influence reproductive function in males via this mechanism. It is important to note that in fetal gonads the expression of SF-1 is accompanied with the appearance of steroidogenic P-450 (P450scc), and both SF-1 and CYP450 play a key role in the steroidogenesis (androgen synthesis) process (Hatano *et al*., 1994). Moreover, maternal treatment with DES and 4-octylphenol has been shown to reduce the expression of the mRNA and protein for CYP450 17α-hydroxylase/C17-20-lyase, a key enzyme which regulates testosterone synthesis in the Leydig cells (Majdic *et al*., 1996). A striking reduction in the amount of CYP450 17α-hydroxylase/C17-20-lyase and SF-1 in the fetal testes was also reported by others (Saunders *et al*., 1997). The above findings, together with the fact that TCDD does not bind to ER (Zacharewski *et al*., 1991), whereas DES binds to ER with high affinity, provides evidence that the xeno-oestrogens can induce their deleterious effects within the male reproductive system via ER-independent effects.

It is important to point out that even though TCDD and DES differ in their binding characteristics to ER, both of the agents have been shown to down-regulate the expression as well as production of ER (Korach and McLachlan, 1985; Safe and Krishnan, 1995). Whether the effects on ER mRNA expression and synthesis has any common link to the effects of these agents on androgen synthesis remains undefined. In addition to the above mechanisms, environmental oestrogens may also induce their

deleterious effects by disrupting the physiological effects of androgens. In this regard, several environmental oestrogens have recently been shown to possess anti-androgenic activity (Sohoni and Sumpter, 1998; Sonnenschein and Soto, 1998).

The above findings provide evidence that xeno-oestrogens can influence the male reproductive system via multiple mechanisms, and without the involvement of classical oestrogen-regulated mechanisms. However, within the female reproductive system the same xeno-oestrogens interfere with the oestrogen-regulated processes/mechanisms more intimately. In this regard, it is well established that xeno-oestrogens influence growth and function of the uterus, vagina/cervix, breast, ovary, placenta and oviduct (Daston *et al*., 1997).

In the ovary, TCDD treatment results in diminution in the number of antral and preantral follicles of certain size classes in rats exposed during critical periods of development (Heimler *et al*., 1998). In human luteinized granulosa cells, treatment with TCDD has been shown to perturb oestradiol secretion by depleting the androstenedione precursor, and increases apoptotic cell death of granulosa cells in dose- and time-dependent manner (Heimler *et al*., 1998). Using normal and DBA/2J (D2) mice (which possess the non-responsive allele of the AhR), it has been demonstrated (Tian *et al*., 1998a) that treatment with TCDD suppresses the gene expression of the ER mRNA and unspliced ER transcript within the ovary and uterus, as well as the liver, of normal but not DBA/2J (D2) mice. These findings suggest that such suppression of gene expression occurs by a decrease in transcription, and that AhR plays an important role in mediating this response (Tian *et al*., 1998a). In a separate study, the same group also demonstrated that down-regulation of ER mRNA by TCDD treatment occurs in the ovary (Tian *et al*., 1998b); therefore, TCDD can induce its anti-oestrogenic effects by down-regulating ER and interacting with AhR within the target tissues. Indeed, the relative potencies of a series of polychlorinated dibenzo-*p*-dioxins to decrease constitutive cytosolic ER levels in the uterus (Romkes *et al*., 1987) has been shown to parallel their rank order potency to induce AhR-mediated responses (Mason *et al*., 1986; Safe, 1990).

Other lines of evidence supporting the anti-oestrogenic effects of TCDD come from the observations that in the uterus, TCDD abrogated oestrogen-induced effects on cytosolic and nuclear progesterone receptor binding (Romkes *et al*., 1987; Astroff and Safe, 1988; Romkes and Safe, 1988); peroxidase activity (Astroff and Safe, 1990); EGF receptor binding and mRNA levels (Astroff *et al*., 1990) and uterine wet weight.

Although the above findings provide evidence that TCDD influences the female reproductive system by acting as an anti-oestrogenic agent, other xeno-oestrogens may not share the same characteristic. In this regard, DES and bisphenol A mimic the effects of oestradiol, and induce deleterious effects on the female reproductive system (Gray *et al*., 1996; Colerangle and Roy, 1997; Steinmetz *et al*., 1998). Treatment of hamsters neonatally with DES has been shown to induce uterine tumours, and this was not associated with changes in the physiochemical/functional properties of the ER system, but rather with increased metabolism of oestradiol (Hendry and Leavitt, 1982). In contrast to the above findings, it has been demonstrated (Medlock *et al*., 1988) that postnatal DES treatment of the rat uterus resulted in a significant decrease in total ER concentrations, and this was accompanied with abnormal patterns of uterine growth and morphogenesis. In the same vein, the effects of DES and certain chemically structural derivatives and analogues were compared (Korach and McLachlan, 1985). These authors found that, as compared with DES, DES analogues (indenestrol A, indenestrol B, indanestrol and pseudo-DES) had poor uterotrophic activity *in vivo*, even though their capability to bind to ER, translocate ER to the nucleus and effect nuclear ER retention was similar to that observed for DES and oestradiol. Moreover, their effects on glucose-6-phosphate dehydrogenase activity, uterine progesterone receptors and DNA synthesis were differential. These observations provide clear evidence that not all xeno-oestrogens may induce similar effects on the reproductive system, and that in uterine tissue ER stimulates certain biochemical responses independently, and not in concert. Moreover, the ability of a particular response to be increased may depend on the chemical nature of the ligand–receptor complex and its interaction at the genomic sites.

Other mechanisms by which xeno-oestrogens may induce their deleterious effects on the reproductive system (or may act as endocrine disrupters) include metabolism of the xeno-oestrogen to a more toxic metabolite. In this regard, it is well established that hydroxymetabolites of DES can be oxidized by nuclear enzymes (peroxide-supported nuclear CYP450) to quinones (2,3-oestradiol quinone and DES quinone, respectively), and that the quinones may be reduced back to hydroquinones and these metabolites can bind covalently to nuclear proteins and DNA (Roy and Thomas, 1994) to induce deleterious effects (toxicity/cancer/tetratogenicity). In this regard, DES has been shown to induce chromatid gaps and breaks, chromosome breaks, and chromosomal aberrations in Syrian hamster renal cortical cells (Banerjee *et al*., 1994). Moreover, DES induced p53 expression in human endometrial cells, and this was accompanied by a down-regulation of ER expression (Rinehart *et al*., 1996). Up-regulation of IGF-1 and its receptor are associated with cell growth as well as the aetiology of oestrogen-induced cancers. Because the uterotrophic effects of oestradiol as well as tamoxifen (an ER ligand with partial agonistic characteristics) are accompanied by an increase in IGF-1 gene expression, and ICI182780 (a pure ER antagonist; Wakeling and Bowler, 1991) blocks the uterotrophic effects of oestradiol as well as tamoxifen by down-regulating IGF-1 expression (Wakeling and Bowler, 1991; Huynh and Pollak, 1994), it is feasible that carcinogenic xeno-oestrogens may also mediate their effects via up-regulation of IGF-1. Indeed, DES—a known carcinogenic environmental oestrogen—has been shown to induce nuclear IGF-1 receptors (Chen and Roy, 1996).

Recent studies have provided evidence that weak environmental oestrogens such as bisphenol A can induce profound growth of the mammary gland of Noble rats (Colerangle and Roy, 1997), as well as of the uterus and vagina of Fischer 344 rats and Sprague–Dawley rats (Steinmetz *et al.*, 1998). Therefore, the oestrogenic effects of xeno-oestrogens may not correlate with their affinity to bind to the ER. In this regard, the effects of DES and its analogues (indenestrol A, indenestrol B, indanestrol and pseudo-DES) on uterine growth were compared (Korach and McLachlan, 1985). These authors observed that even though all compounds except indanestrol interacted with the mouse uterine ER with high affinity, they were able to translocate similar levels of ER to the nucleus *in vivo*, and had comparable nuclear

retention and ER occupancy times. Only indenestrol A stimulated uterine glucose-6-phosphate dehydrogenase to a similar degree to DES and oestradiol. The uterine progesterone receptor was induced to varying degrees by all compounds, with indenestrol A and indenestrol B being the most active. Uterine DNA synthesis was marginally stimulated by the DES derivatives and analogues except for indenestrol B, which showed a response increase comparable with that of DES or oestradiol. Taken together, these data provide strong evidence that the ER stimulate certain biochemical responses independently and not in concert, and the ability of a particular response to be increased may depend on the chemical nature of the ligand–receptor complex and its interaction at genomic sites. Similar to the uterine tissue, the effects of xeno-oestrogens in other reproductive tissues may not correlate to their binding affinity to the ER. Indeed, methoxychlor—another weak xeno-oestrogen—has also been shown to induce morphological and biochemical alterations in the reproductive tracts of neonatal female mice (Eroschenko and Cooke, 1990).

Explanations for the lack of association between ER binding affinity and the oestrogenic effects of xeno-oestrogens are not well defined. It is well known that molecular (c-*fos* induction) and morphological (growth in vagina and uterus) alterations induced by bisphenol A are identical to those induced by oestradiol (Steinmetz *et al*., 1998). Moreover, in pituitary cells, similar to oestradiol, bisphenol A mimics the effects of oestradiol in inducing prolactin release and inducing ERE activation (Steinmetz *et al*., 1997). The finding that environmental oestrogens bind differentially to ERα and ERβ (Kuiper *et al*., 1998) suggests that the differences in the biological effects may depend on interaction of the molecule with a specific receptor type. Indeed, bisphenol A has been shown to interact with ERα in a distinct manner from oestradiol, and antagonized the stimulatory effects of oestradiol on peroxidase activity and progesterone levels, but not the effects on uterine growth (Gould *et al*., 1998). Moreover, differential activation of ER by xeno-oestrogens, including bisphenol A, of ERα and ERβ when linked to different response elements (vitellogenin gene or LH beta gene) has been demonstrated (Pennie *et al*., 1998).

Similarly, extensive evidence suggests that the anti-oestrogenic effects of xeno-oestrogens which are AhR ligands [i.e. TCDD-related compounds such as: 2,3,7,8-tetrachlorodibenzofuran; 2,3,4,7,8-pentachlorodibenzofuran (PCDF); 1,2,3,7,9-PCDF; 1,2,3,7,8-pentachlorobibenzo-*p*-dioxin; 3,3',4',4'-tetrachlorobiphenyl; 3,3',4,4',5-pentachlorobiphenyl; and 3,3',4,4',5,5'-hexachlorobiphenyl] correlate with their binding affinity to AhR (Gierthy *et al*., 1987; Harris *et al*., 1990; Krishnan and Safe, 1993; Harper *et al*., 1994; Spink *et al*., 1994; Zacharewski *et al*., 1994). However, recent data provide evidence that the binding affinity of some AhR receptor ligands (xeno-oestrogens) may not correlate with their anti-oestrogenic effects, and the biological effects of several xeno-oestrogens may vary within different tissues and cell types. In this regard, 6-methyl-1,3,8-trichlorodibenzofuran (MCDF), a TCDD-related xeno-oestrogen which binds to the AhR with moderate affinity, had weak agonistic effects on AhR-mediated biochemical and toxic responses. In the uterus, MCDF does not block the anti-oestrogenic effects of TCDD, but rather acts as an agonist (Safe, 1990), and as compared with TCDD, MCDF is 300–570 times less active in inducing its anti-oestrogenic effects. Moreover, MCDF inhibits TCDD-induced hepatic CYP450 activity. Similar to the MCDF, halogen-substituted flavones which exhibit competitive Ah receptor binding affinities (IC_{50} = 0.79–2.28 nmol/l) comparable with that of TCDD (1.78 nmol/l) were >1000 times less active than TCDD in inducing their biological effects on CYP1A1 gene expression in MCF-7 human breast cancer cells. Similarly, for other substituted flavones, there was no correlation between AhR binding affinities and their activities as an AhR agonist (Lu *et al*., 1996). Because reproductive disorders have been reported in AhR knockout mice (Abbott *et al*., 1999), it possible that interaction of different xenobiotics which are AhR ligands may interfere with the normal reproductive process, and enable the interaction of environmental oestrogens. Nonetheless, it must be emphasized that the exact role of AhR in regulating the reproductive process remains largely unknown.

Taken together, the above findings suggest that the oestrogen-like effects of xeno-oestrogens may not be mediated via the same receptors or pathways. Moreover, in order to define the mechanism of action of xeno-oestrogens on the reproductive system one may have to study and compare the effects of each individual xeno-oestrogen, as they may not follow the same mechanisms or induce the same biological effects. Additionally, the effects of an individual xeno-oestrogen may be differential in various reproductive tissues.

Because polypeptide growth factors are known to play a key role in regulating the cellular process, and the effects of oestradiol are known to be associated with EGF, transforming growth factor (TGF)-1, TGF-2, TGF-3, IGF and platelet-derived growth factor (PDGF), the direct effects of xeno-oestrogens on the synthesis of the growth factors, as well as expression of their receptors, may influence the reproductive process. In this regard, a distinctive mRNA expression profile for the PDGF ligand and receptor genes has been shown within the uterus and vagina of mice treated with DES (Gray *et al*., 1995). The same group also provided evidence for increased incidence of DES-induced vaginal adenosis, uterine endometrial hyperplasia, uterine polyps, hypospadia, benign ovarian cysts and pituitary adenomas in TGF-α transgenic mice (Gray *et al*., 1996), suggesting that a balance between constitutively expressed growth factors such as TGF-α and xeno-oestrogen-induced biochemical changes defines the pathological effects of an agent on the reproductive organ. Because pathways independent of AhR and ER can regulate the synthesis of growth factors, this provides an alternative pathway via which xeno-oestrogens can influence the growth of cells within the reproductive organs and induce their pathophysiological effects.

Regarding the growth effects, one parameter of utmost importance and sensitivity is the embryo. Right from the onset of the fertilization process, the development of the embryo can be influenced directly or indirectly by the influence of the agents on the oviduct or on the uterus. It is interesting to note that the cavitation rates of in-vitro-dosed murine embryos was significantly higher for TCDD than controls, suggesting that TCDD accelerated the differentiation of murine preimplantation embryos (Blankenship *et al*., 1993), and that xeno-oestrogens can act directly on preimplantation embryos. Evidence has also been provided (Tsutsumi *et al*., 1998) that the dioxins are present in human follicular fluid, and that they influence the development of the preimplantation embryo in a stage-specific manner. It has also been shown (Dey *et al*., 1989) that during the early postimplanta-

tion stage, dioxin-inducible CYP1A1 mRNA is expressed in the extra-embryonic tissues of the mouse, and that this expression did not occur between 5.3 and 14.5 days of gestation. These findings suggest that the metabolic activity of the inducible CYP1A1 enzyme may play an important role in protecting the embryo and fetus from exogenous chemicals, i.e. the xeno-oestrogens. In the same vein, it is feasible that agents such as TCDD (which are known to induce CYP1A1 activity) and anti-oestrogenic xeno-oestrogens (which act as AhR antagonists) may disrupt the time-dependent physiological up-regulation of CYP1A1 and render the embryo susceptible to chemical/xeno-oestrogen insult and toxicity. AhR receptors have also been identified in the preimplantation embryos of mice and rabbits. Moreover, PCBs which are AhR ligands have been shown to cause direct embryotoxicity (Peters and Wiley, 1995; Lindenau and Fischer, 1996). The presence of operational metabolite-detoxifying pathways within the late preimplantation embryos have been reported (Filler and Lew, 1981). The ability of preimplantation embryos to activate directly polynuclear aromatic hydrocarbons to reactive intermediates provides another mechanism whereby carcinogenic or teratogenic chemicals may induce deleterious/ontological effects on the developing embryo.

In addition to the above findings, recent data from our laboratory provide evidence that xeno-oestrogens can also influence oviduct mechanisms that are essential for the development of the preimplantation embryos and the implantation process. In this regard, we have shown that, similar to 17β-oestradiol, treatment of bovine as well as human oviduct cells with phyto-oestrogens (genistein, equol, biochanin A and daidzein) and PCBs (4-OH-trichlorobiphenyl and 4-OH-dichlorobiphenyl) significantly induced the synthesis of leukaemia inhibitory factor (LIF). Moreover, the stimulatory effects of both phyto-oestrogens and xeno-oestrogens were completely blocked by the presence of ICI182780, suggesting that xeno-oestrogens can, under these in-vitro conditions, induce their effects on LIF synthesis via the ER. In contrast to oestradiol and xeno-oestrogens, tamoxifen (an ER ligand) also induces LIF synthesis in the oviduct cells; moreover, these effects are enhanced by oestradiol, suggesting that these effects are not ER-mediated but rather occur via an alternative mechanism (Reinhart *et al*., 1999). LIF is a glycoprotein essential for embryo implantation, development and differentiation. The oviduct provides the microenvironment for fertilization and early embryo development, and its overall physiology is regulated by 17β-oestradiol. Our findings suggest that environmental oestrogens may act as endocrine modulators/disrupters, and may induce deleterious effects on the reproductive processes by influencing LIF synthesis in a non-cyclic fashion, and thus be responsible for abnormal development, differentiation and implantation of the embryo, eventually leading to infertility. This notion explains, in part, why high concentrations of PCBs were observed in women who suffered repeated miscarriage (Leoni *et al*., 1989).

Early embryo development and its transport from the oviduct to the uterus is critically dependent on the ratio of the concentrations of oestradiol and progesterone (Harper, 1988). Moreover, increased concentrations of oestradiol during the period of ovum transport have been shown to be associated with ectopic pregnancy (Coutinho, 1971; Morris and van Wagenen, 1973), and high expression of LIF mRNA has been reported within the oviduct of patients with ectopic pregnancy (Keltz *et al*., 1996). Because environmental oestrogens mimic the effects of oestradiol and induce LIF synthesis, it is feasible that—similar to oestradiol—the increased or continuous presence of environmental oestrogens may inhibit transport of the ovum to the uterus. Moreover, they may induce LIF synthesis abnormally within the oviduct, which may in turn induce biochemical changes that promote the growth and implantation of the embryo within the oviduct, leading to ectopic pregnancy and infertility.

Certain environmental oestrogens have been shown to disrupt the immune system and are immunotoxic (Voccia *et al*., 1999). Because abnormal immune responses are considered to cause implantation failure (Edwards, 1995), environmental oestrogens may also induce their deleterious effects via this mechanism. An impact of the environmental oestrogens on the immune system has also been suggested to play a key role in endometriosis (Osteen and Sierra-Rivera, 1997). Endometriosis is a serious disorder associated with chronic pain and infertility which affects 6 million women in the USA, and is characterized by the growth of endometrial cells at sites outside the uterus. Several studies have provided evidence that TCDD—which is known adversely to influence immunocompetence—also induces endometriosis in monkeys exposed to this compound (Rier *et al*., 1995). In mice treated with various xeno-oestrogens, endometriosis was found to be promoted only by halogenated hydrocarbons which were AhR ligands, suggesting that the AhR-mediated mechanisms may play a key role in endometriosis (Johnson *et al*., 1997). Since AhR are present within the endometrium, and agents that bind to AhR can interact with several cellular processes (including the regulation of growth factor synthesis), the interaction between AhR and growth factors/cytokines and the role of these interactions in context to endometriosis needs to be further investigated.

Phyto-oestrogens

Plant-derived phenolic compounds which exhibit oestrogen-like activity are classified as phyto-oestrogens, and could be divided into three main classes: isoflavones, coumestans and lignans. Oestrogen-like compounds are also produced by moulds and termed myco-oestrogens (resorcylic acid lactones). A single plant can be the source of multiple phyto-oestrogens. For example, the soy bean is rich in isoflavones, whereas the soy sprout is a potent source of coumestan. Within the plant, the isoflavones genistein and daidzein exist as inactive glucosides, and after consumption these are converted into heterocyclic phenols which are structurally similar to oestrogens and capable of inducing oestrogenic effects (for a review, see Murkies *et al*., 1998). Various studies have documented the presence of significant levels of various phyto-oestrogens in humans (Table III).

Even though phyto-oestrogens are consumed on a daily basis and form part of a daily diet, very little is known about their effect on the reproductive system. Several lines of evidence suggest that phyto-oestrogens induce anti-carcinogenic effects without inducing any adverse effects on the reproductive system. However, many studies also provide evidence that phyto-oestrogens can induce carcinogenic effects as well as deleterious oestrogenic effects on the reproductive system (Welshons *et al*., 1987; Lamartiniere *et al*., 1995; Kuiper *et al*., 1997; Zava and Duwe, 1997; Strauss *et al*., 1998; Willard and Frawley, 1998). Therefore,

Table III. Plasma concentrations of phyto-oestrogens in humans

Phyto-oestrogen	Subjects/ diet (reference)	Plasma concentration	
		nmol/l	ng/ml
Daidzein	Finnish women, vegetarians	18.5[a]	4.7[a]
	(Adlercreutz *et al.*, 1994)	6.6–51.9[b]	1.7–13.2[b]
	Japanese men, typical diet	58.3–924	14.8–234.9
	(Adlercreutz *et al.*, 1994)		
	Postmenopausal women, soy-supplemented diet	10.6–544.4	2.7–138.4
	(Morton *et al.*, 1994)		
	4-month old infants, soy-based formula	1160[a]	294.9[a]
	(Setchell *et al.*, 1997)	236.2[c]	60[c]
Equol	Finnish women, vegetarians	0.7[a]	0.17[a]
	(Adlercreutz *et al.*, 1994)	0.3–1.8[b]	0.07–0.44[b]
	Japanese men, typical diet	0.15–20.7	0.036–5.0
	(Adlercreutz *et al.*, 1994)		
	Postmenopausal women, soy-supplemented diet	5.4–220.4	1.3–53.4
	(Morton *et al.*, 1994)		
Genistein	Finnish women, vegetarians	17.1[a]	4.6[a]
	(Adlercreutz *et al.*, 1994)	7.0–42.5[b]	1.9–11.5[b]
	Japanese men, typical diet	90.4–1204	24.4–325.4
	(Adlercreutz *et al.*, 1994)		
	4-month old infants, soy-based formula	2530[a]	684[a]
	(Setchell *et al.*, 1997)	1641[c]	443[c]

[a]Mean; [b]Confidence interval; [c]SD.

it is important to delineate the mechanism(s) via which phyto-oestrogens induce their effects on the reproductive system, and this will be discussed further.

Similar to oestradiol, phyto-oestrogens bind to the ER (see Table I), and have been shown to displace oestradiol competitively from the ER (see Figure 2). The binding affinity of a single class of phyto-oestrogens can vary dramatically. For example, genistein binds with high affinity to the ER, whereas biochanin A does not (Kuiper *et al.*, 1998). In the same report, these authors provided evidence that phyto-oestrogens bind to both ERα and ERβ and that, as compared with xeno-oestrogens, phyto-oestrogens bind with high affinity to the ER (Table I). Several studies suggest that even though phyto-oestrogens such as genistein bind with high affinity to ER, its effects on MCF-7 cancer cells are not reversed by ICI182780, a selective ER antagonist (Wang *et al.*, 1996). This notion is further supported by the findings that genistein inhibits growth in both ER-positive and ER-negative cell lines (Wang *et al.*, 1996). The effects of genistein on the growth of MCF-7 cells is biphasic in nature (Wang and Kurzer, 1998). At low concentrations genistein induces cell growth, whereas at higher concentrations it inhibits cell growth—an effect that is not reversed by ICI182780 (Wang *et al.*, 1996). Genistein has been shown to inhibit tyrosine kinase activity (Akiyama *et al.*, 1987) and mitogen-activated protein kinase activity (Dubey *et al.*, 1999a), and to down-regulate EGF receptor autophosphorylation (Akiyama *et al.*, 1987), all of which are key processes that regulate cell growth. Therefore, it is feasible that the growth effects of genistein are largely mediated via its inhibitory effects on these signal transduction pathways.

Because of its high binding affinity to the ER, genistein may also induce anti-oestrogenic effects by competing with oestradiol for ER and blocking the effects of oestradiol. Indeed, it has been shown (Wang *et al.*, 1996) that in MCF-7 cells treated simultaneously with both genistein and oestradiol, the expression of oestrogen-stimulating protein (ps2) was lower than when each compound was added alone. Similarly, in MCF-7 cells treated for 6 days with genistein, the effects of oestradiol on pS2 activity was significantly reduced (Wang *et al.*, 1996). The decrease in the pS2 activity in the MCF-7 cells was found to be associated with down-regulation of ER by 60%, in cells treated with genistein, suggesting that genistein induces its anti-oestrogenic effects by down-regulating ER levels. Alternative mechanisms via which genistein may induce its anti-oestrogenic effects include inhibition of oestrogen-specific hydroxysteroid oxidoreductase type 1 which metabolizes oestrone to oestradiol, thereby reducing the formation of oestradiol (Makela *et al.*, 1995).

As with the xeno-oestrogens, the effects of various phyto-oestrogens may vary considerably. In this regard, equol—which has a relatively weak affinity for the ER—does not stimulate uterine weight gain and increase progesterone receptors in macaques (Thompson *et al.*, 1984). Infertility associated with clover disease is presumably caused by increased ingestion of equol (Bennetts *et al.*, 1946); hence, it is likely that effects of phyto-oestrogens may also differ between various species.

Coumestrol, which binds with high affinity to both ERα and ERβ (Kuiper *et al.*, 1998) has been shown to act as an atypical oestrogen that does not stimulate uterine cellular hyperplasia. Although acute administration of coumestrol significantly increased uterine wet and dry weights, it failed to increase DNA content (Markaverich *et al.*, 1995). The lack of true oestrogenic effects of coumestrol were also supported by its inability to cause cytosolic ER depletion, nuclear ER accumulation, or the

stimulation of nuclear type II sites which characteristically precede oestrogenic stimulation of cellular DNA synthesis and proliferation. Interestingly, in rats treated with coumestrol there was a 3-fold induction of cytosolic ER without corresponding cytosolic depletion and nuclear accumulation of this receptor, and this increased the sensitivity of the uterus to subsequent stimulation by oestradiol. Taken together, these findings suggest that the oestrogenic effects of phyto-oestrogens such as coumestrol may be mediated via increased sensitivity of the tissue to endogenous oestradiol.

Most of the plant flavonoids have been shown to possess oestrogenic activity, although the potency of the individual flavone may vary considerably (Miksicek, 1993). Hence, based on the reproductive effects of phyto-oestrogens, it seems that most of the phyto-oestrogens bind to the ER and mimic the effects of oestradiol, albeit in atypical fashion (Markaverich *et al*., 1995). The explanation for the anti-oestrogenic effects of phyto-oestrogens may lie in the capability of certain phyto-oestrogens such as genistein, which can counteract the growth effects by inhibiting tyrosine kinase activity an effect independent of ER-mediated pathways. In the same vein, the phyto-oestrogens may induce their anti-oestrogenic effects by altering the metabolism of oestradiol. In this regard, several studies provide evidence that phyto-oestrogens increase plasma concentrations of steroid binding protein and decrease the plasma concentrations of free oestrogens (Adlercreutz *et al*., 1987, 1993; Pelissero *et al*., 1993). Equilibrium binding studies also show that lignans and isoflavonoid phyto-oestrogens inhibit the binding of oestradiol and testosterone to steroid binding protein (Martin *et al*., 1996). Because clearance and metabolism of the steroids depends on the unbound fraction, the decreased binding of oestradiol would result in increased clearance and metabolism of these steroids and reduced biological effects. It is feasible that the anti-carcinogenic effects in Asian women taking dietary phyto-oestrogens may, in part, be due to increased clearance and metabolism of oestradiol, and generation of anti-carcinogenic and anti-tumorigenic oestradiol metabolites such as 2-methoxyoestradiol (Fotsis *et al*., 1994). Alternatively, phyto-oestrogens also bind to the ER and can abrogate the ER-mediated effects of oestradiol, or inhibit the synthesis of oestradiol by inhibiting aromatase activity (Kellis and Vickery, 1984)

As discussed earlier, the metabolism of oestradiol may be the major mechanism via which many environmental oestrogens may induce their anti-oestrogenic effects. This concept is well established with respect to the effects of TCDD, though in the case of phyto-oestrogens the effects on oestrogen metabolism are less clear. It is possible that, similar to the xeno-oestrogens, the phyto-oestrogens may also act as AhR ligands and influence the CYP450 enzyme activities that are responsible for oestradiol metabolism (Figure 3). In this regard, it is well established that bioflavonoids can activate the CYP450-dependent activities and inhibit or enhance carcinogen-induced tumours or DNA binding (DiGiovanni *et al*., 1978; Huang *et al*., 1981; Testa and Jenner, 1981; Lasker *et al*., 1982; Nakadate *et al*., 1984; Le Bon *et al*., 1992). Flavones and their analogues are known to bind to AhR receptors; however, their structure–induction (CYP1A1) relationship does not parallel their structure–binding relationship (Lu *et al*., 1996). Some of the flavones which bind to the AhR with high affinity (3′-methoxy-4′-nitroflavone) were unable to induce CYP450 activity and blocked TCDD-induced activation of CYP1A1, whereas some flavones (3′-amino-4′-methoxyflavone) induced CYP1A1 activity via the AhR receptor (Lu *et al*., 1996). Because activation of CYP1A1 plays a key role in oestradiol metabolism, it is feasible that phyto-oestrogens (flavones) may induce anti-oestrogenic effects by inducing oestradiol metabolism, thereby reducing the active concentrations of oestradiol. Also, increased metabolism of oestradiol may generate metabolites of oestradiol, such as 2-methoxyoestradiol, that may induce anti-carcinogenic/oestrogenic effects. In the same vein, it is important to know that induction of CYP450 may also generate carcinogenic metabolites, and hence the overall biological effects of oestradiol metabolism may depend on the balance of the metabolites generated, i.e. carcinogenic (4-methoxyoestradiol) versus anti-carcinogenic (2-methoxyoestradiol) (Figure 3). Also, the oestrogenic and anti-oestrogenic effects of individual phyto-oestrogens may vary considerably, and may depend largely on their chemical characteristics as well as their tissue specificity. Therefore, to fully understand the effects of phyto-oestrogens on the reproductive system it may be necessary to evaluate the effects of individual phyto-oestrogens on individual organs and cell types.

Finally, phyto-oestrogens form a relatively large part of our daily diet, and their concentrations within the body may be quite high. Since some of the soy-derived phyto-oestrogens (genistein, etc.) induce oestrogenic effects at low concentrations, but induce anti-oestrogenic effects at higher concentrations (Wang *et al*., 1996), the levels of phyto-oestrogens within the body may be the decisive factor in governing the oestrogenic or anti-oestrogenic effects. This may also explain why Asian and Oriental women who consume diets with a high phyto-oestrogen content are less prone to reproductive cancers (Murkies *et al*., 1998), when compared with their Western counterparts. In contrast to the above contention, a high incidence of infertility has been observed in sheep consuming clover which contains high concentrations of the phyto-oestrogen, equol (Thompson *et al*., 1984). Differences in the binding of the phyto-oestrogens to ER or AhR, their biochemical characteristics, as well as tissue and species specificity may be largely responsible for these differences.

Dietary phyto-oestrogens have been shown to modify the hormonal status in premenopausal women. Consumption of conjugated isoflavones has been shown to prolong significantly the length of the menstrual cycle, specifically during the follicular phase (Cassidy *et al*., 1994, 1995; Lu *et al*., 1996). This delay was accompanied by a delay in peak luteal-phase progesterone concentration, as well as suppression in the surges of gonadotrophins, LH and FSH during the mid-cycle. Phyto-oestrogens also reduce the incidence of hot flushes and protect postmenopausal women against disorders associated with oestrogen deficiency (Murkies *et al*., 1995; Brzezinski *et al*., 1997; Albertazzi *et al*., 1998). These findings suggest that soy-derived phyto-oestrogens do induce oestrogenic effects and influence female reproductive physiology. However, whether individual phyto-oestrogens mimic the effects of oestrogen is unclear, and this point requires further investigation.

The above findings suggest that, similar to xeno-oestrogens, phyto-oestrogens can bind to ER and AhR, induce CYP450

activities and regulate enzymes and pathways independent of their binding affinity for ER/AhR, and induce both oestrogenic and anti-oestrogenic effects. In this regard, in a recent study we compared the effects of various phyto-oestrogens and xeno-oestrogens on LIF synthesis by oviduct cells. We found that both xeno-oestrogens and phyto-oestrogens mimicked the effects of oestradiol and induced LIF synthesis, and that the effects of both the xeno- and phyto-oestrogens were blocked by the ER antagonist, ICI182789. Because LIF plays a major role in embryo implantation as well as in the development of preimplantation embryos, it is feasible that the xeno- and phyto-oestrogens induce similar effects on these processes. Whether these effects may result in adverse effects on the implantation process or embryo development is difficult to explain. It is feasible that unlike the cyclic generation of oestradiol, the continuous presence of xeno- and phyto-oestrogens may induce abnormal synthesis of LIF, leading to early development of the embryo and tubal implantation. It is feasible that the direct effects of these agents on the growth of the embryo may be responsible for their deleterious or beneficial effects, and future studies are needed to evaluate and compare the effects of individual phyto- and xeno-oestrogens on embryo development and to evaluate whether LIF plays a role in mediating these effects. Finally, phyto-oestrogens have been shown to inhibit the activities of a number of enzymes, including protein kinases (Graziani *et al*., 1983; Cunningham *et al*., 1992), porcine-5-lipoxygenase (Voss *et al*., 1992), ornithine decarboxylase (Nakadate *et al*., 1984) and MAP-kinase (Dubey *et al*., 1999a), all of which are involved in the regulation of cell growth. Therefore, phyto-oestrogens may also mediate their effects on the reproductive system by directly influencing these enzymes.

Conclusion

Xeno-oestrogens, as well as phyto-oestrogens, are capable of inducing both oestrogenic and anti-oestrogenic effects which are not only ER- or AhR-mediated, but could also be indirect and independent of these receptors. A review of the data in the literature does not provide any convincing evidence that phyto-oestrogens may induce beneficial effects, whereas xeno-oestrogens induce deleterious effects on the reproductive system. One fact that is evident is that the effects of individual phyto-oestrogens and xeno-oestrogens can vary considerably, and that the effects are species- as well as tissue-dependent. Hence, the effects of any particular xeno- or phyto-oestrogen may not necessarily reflect the effects of a group of environmental oestrogens. In order to determine the mechanisms and the effects of phyto-oestrogen/xeno-oestrogen on the reproductive system, one would have to study each environmental oestrogen individually in different organs and in different species. Thus, the verdict on the effects of environmental oestrogens on the reproductive system remains inconclusive. However, one clear point is that multiple mechanisms are involved in mediating the oestrogenic and anti-oestrogenic effects of environmental oestrogens.

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