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# Toxicological Mechanism of Endocrine Disrupting Chemicals: Is Estrogen Receptor Involved?

**Eui-Bae Jeung[1] and Kyung-Chul Choi[2]**

*[1]Laboratory of Veterinary Biochemistry and Molecular Biology and*
*[2]Laboratory of Veterinary Biochemistry and Immunology, College of Veterinary Medicine,*
*Chungbuk National University, Cheongju, Chungbuk 361-763 Korea*

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Endocrine disrupting chemicals (EDCs) have been shown to interfere with physiological systems, i.e., adversely affecting hormone balance (endocrine system), or disrupting normal function, in the female and male reproductive organs. Although endocrine disruption is a global concern for human health, its impact and significance and the screening strategy for detecting these synthetic or man-made chemicals are not clearly understood in female and male reproductive functions. Thus, in this review, we summarize the interference of environmental EDCs on reproductive development and function, and toxicological mechanism(s) of EDCs in *in vitro* and *in vivo* models of male and female reproductive system. In addition, this review highlights the effect of exposure to multiple EDCs on reproductive functions, and brings attention to their toxicological mechanism(s) through estrogen receptors.

***Key words***: Endocrine disrupting chemicals, estrogen receptor, toxicological mechanism

## INTRODUCTION

Endocrine disrupting chemicals (EDCs) are environmental chemicals which may interfere with physiological systems, adversely affecting hormone balance (endocrine system), or disrupting normal function in the organs that hormones regulate or modulate, in particular, the female and male reproductive systems (Daston *et al.*, 2003). Thus, EDCs can lead to serious reproductive, developmental, and metabolic dysfunction in humans, animals, and plants. This class of EDCs is comprised of both naturally occurring and man-made compounds which are structurally similar or distinct to estrogen, and can interfere with the actions of this endogenous steroid hormone via estrogen receptor (ER) (Daston *et al.*, 2003). To date, the World Health Organization (WHO), United States National Institute of Environmental Health Sciences (NIEHS) and Environmental Protection Agency (EPA), Health Canada, the UN Environment Programme, UNICEF, and the World WildLife Fund have all identified endocrine disrupting chemicals as a critical environmental and health issue in the 21st Century (Daston *et al.*, 2003; Hoyer, 2001). In addition, of particular relevance to women and children are EDCs which are associated with an increased risk and incidence of reproductive dysfunction, breast and ovarian cancers.

The suspected environmental estrogenic EDCs include those biological reagents or drugs which have been specifically designed to treat hormone imbalance in humans, i.e., diethylstilbestrol (DES) initially used a post-coital contraceptive agent. In addition, synthetic compounds, including octyl-phenol (OP), nonyl-phenol (NP), bisphenol A (BPA), and methoxychlor (MXC), can also be transferred through the placenta to the fetus and through breast milk to infants in our previous and other studies (An *et al.*, 2003; Hong *et al.*, 2003; Laws *et al.*, 2000). BPA is recognized as a potential environmental estrogenic chemical which can interfere with endogenous estrogen (Laws *et al.*, 2000). BPA is widespread in the environment, and commonly ingested by humans, because it is used in the manufacture of polycarbonate plastics and food-storage containers, and other plastics (Choi and Jeung, 2003). Other man-made chemicals with hormone-like activity include pesticides such as dichlorodiphenyltrichloroethane (DDT), vinclozolin, endosulfan, toxaphene, dieldrin, and industrial chemicals and byproducts such as polychlorinated biphenyls (PCBs), and dioxins (Crisp *et al.*, 1998). The effluent from sewage treatment may also contain a variety of natural and man-made endocrine disrupting chemicals, i.e., natural hormones from animal and human waste. Although chemical structures of

Correspondence to: Kyung-Chul Choi, Laboratory of Veterinary Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763 Korea
Email: kchoi@cbu.ac.kr

these estrogenic phenol compounds, OP, NP, and BPA, are different to those of 17beta-estradiol (E2) and progesterone (P4), they can bind to the receptors for steroid hormones, i.e., estrogen receptors (ER), progesterone receptors (PR) or androgen receptors (AR), and induce or modulate a steroid hormone receptor-mediated response (Park *et al.*, 2009).

A few *in vitro* and *in vivo* methods have been developed for determining whether a chemical is endocrine disrupting or not. Most results from *in vitro* and *in vivo* data are derived from assays which measure estrogenic and androgenic activity, and far less is known for progestogenic effects. Although endocrine disruption is a global issue for human health, little is known of its impact and significance, and strategies for detecting offending chemicals are not well described. Thus in this review, we described the results from *in vitro* and *in vivo* experiments to show the possible impact of man-made chemicals on human reproductive health. Although some of results are derived from *in vitro* and animal models, they could impact human studies or human reproductive health. We also review investigations of use for biomarkers and screening methods, although these are few in mammals. Finally, we introduce a novel *in vivo* model for detecting EDCs, a method which uses immature rats and mice to follow the ability of estrogenic chemicals or progestogenic compounds, which are not well characterized in other studies, to induce Calbindin-D9k (CaBP-9k; a cytosolic calcium binding protein) in the uterus.

***Estrogen and its biological function.*** E2 plays an important role in regulating many physiological functions and in the development of diverse organs *in vivo* (Charpentier *et al.*, 2000). E2 is thought to be of essence in development of reproductive organs, bone, liver and cardiovascular systems (Watanabe *et al.*, 2003). In humans, E2 exerts reproductive function and regulates the development of secondary sexual characteristics (Leung *et al.*, 2004). In addition, E2 plays many roles in a number of physiological effects such as modulation of inflammation (Marino *et al.*, 2006). This sex hormone exerts its roles through binding to E2-specific ERs, and the mechanisms underlying E2-activity are recently understood. Previous studies showed that ERs are localized into diverse intracellular structures within cells.

As abovementioned, E2 exerts genomic effects by binding to nuclear ERs and consequently regulates transcription of estrogen-responsive genes through a complex series of molecular events, i.e. binding to estrogen responsive elements (EREs) in promoter regions or via interactions with other transcription factors (Dos Santos *et al.*, 2002). A cross talk between membrane and nuclear ERs has been demonstrated, and receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and insulin-like growth factor type 1 (IGF-I) receptor (IGFR), are required for signal integration (Filardo *et al.*, 2002; Levin, 2005). Furthermore, activation of membrane tyrosine kinase receptors by complexes of E2-bound membrane ERs results in activation of secondary messengers and downstream kinase pathways, including extracellular regulated kinase (ERK) and protein kinase B (Akt) (Tokunaga *et al.*, 2006). To date, both genomic and non-genomic mechanisms of E2-activity have been described. Non-genomic responses occur rapidly, often within minutes of exposure, whereas genomic responses are delayed and require at least several hours to become established (Filardo, 2002; Kelly and Levin, 2001). Membrane-bound ERs play important roles in modulation of cell function (Revelli *et al.*, 1998), and in the regulation of target gene transcription through non-genomic interactions (Qin *et al.*, 2004; Watters *et al.*, 2000). Most recently, the rapid physiological actions of E2 in nervous system have also been explored by which an interaction between membrane ERs and metabotropic glutamate receptors is required to elicit changes in cell signaling pathways (Micevych and Mermelstein, 2008). Although there is increasing evidence that different signaling pathways are activated by E2, the potential impacts of E2 remain distinct with respect to the types of target cells (Levin, 2005; Marino *et al.*, 2005).

Diverse evidence indicates a pivotal role of estrogen in the development and function of the male reproductive axis. In rodents, the biological actions of estrogen include the masculinization of brain structures involved in the neuroendocrine control of gonadal function and reproductive behavior (Arnold and Gorski, 1984; MacLusky and Naftolin, 1981), regulation of the hypothalamic expression and release of gonadotropin-releasing hormone (GnRH) (Roy *et al.*, 1999), direct control of pituitary hormone secretion (Sharpe, 1998b), modulation of the differentiation and function of Leydig cells within the testis (Abney and Myers, 1991) and regulation of luminal fluid resorption in the epididymis (Hess *et al.*, 1997). The pituitary gland is a heterogeneous tissue consisting of the anterior, intermediate and neural lobes. The anterior lobe is comprised of five hormone-producing cells and the supporting folliculo-stellate cells. The intermediate lobe contains primarily melanotrophs, whereas pituicytes and nerve endings make up the neural lobe. Several pituitary cell types are known targets for estrogens, including lactotrophs (Lieberman *et al.*, 1982; Sarkar *et al.*, 1992), gonadotrophs (Turgeon and Waring, 1981), folliculostellate (Allen *et al.*, 1997) and intermediate lobe cells (Ellerkmann *et al.*, 1991; Steinmetz *et al.*, 1997).

Classical ERα, which is localized in most pituitary cells (Keefer *et al.*, 1976), is thought to mediate the direct effects of estrogen in the pituitary. The impact of endogenous and exogenous ligands with estrogenic activity on the formation of the male reproductive system and on reproductive function in adulthood, remains the subject of intensive research activity and debate (Sharpe, 1998a, b; Toppari *et al.*, 1996). In this context, characterization of the molecular mechanisms underlying the effects of estrogen exposure

during critical periods of rodent development has drawn considerable attention. Recently, the critical effects of E2 on the pituitary gland have been shown including cellular proliferation and the regulation of hormone synthesis mediated by ER (Nishihara *et al.*, 2000). E2 regulates the expression of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), two forms of the gonadotropins in the anterior pituitary gland (Levine, 1997; Wilson *et al.*, 1998). In the somatolactotrophs, E2 activates prolactin transcription through the ERE located in the 5'-upstream regulatory region and also promotes cell proliferation in somatolactotrophs (Mitchner *et al.*, 1999). In addition, both the anterior lobe and the intermediate lobe are a target for E2-actions (Pelletier *et al.*, 1988).

***Estrogen receptors.*** Actions of estrogen are mediated through an interaction with its intracellular receptor, a member of the steroid/thyroid/retinoid receptor gene superfamily. The classical estrogen receptor (ER, now referred as ERα) was thought to be the only form of nuclear receptor able to bind to estrogen, and mediate its hormonal effects in their target tissues. However, the cloning of a second form of ER, now referred to as ERβ, has caused a reexamination of the estrogen signaling system (Mosselman *et al.*, 1996). Recent studies have revealed different tissue distributions and expression levels of ERα and ERβ in the ovary, suggesting different biological roles of ERα and ERβ in this tissue (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996).

In estrogen target tissues, a wide variety of physiological cellular processes are affected by E2. It is reported that multiple signaling pathways are involved in E2 activity in the human body, including genomic and non-genomic pathways. E2 exerts its biological effects via a complex series of molecular events, in which the interaction between E2 and ERs may be an important factor in E2-response induction. In particular, E2-mediated regulation of gene expression is believed to occur via a nuclear complex of E2-bound ERs that induce a conformational change in the receptors, causing dissociation from chaperones, dimerization and activation of the receptor transcriptional domain (Hall and McDonnell, 2005; Nilsson *et al.*, 2001). Two types of ERs, ERα and ERβ, share a common structural architecture (Katzenellenbogen and Katzenellenbogen, 1996; Turner *et al.*, 1994) and some functional characteristics (Frasor *et al.*, 2003). It has been reported that ERα activates gene transcription in the presence of E2, while a binding complex of E2-bound ERβ inhibits activator protein 1 (AP-1)-dependent transcription (Aranda and Pascual, 2001). In addition, the molecular mechanisms underlying expression of ERα and ERβ are distinct, and their distribution differs among estrogenic target tissues as previously described (Williams *et al.*, 2001).

The expression of ERα and ERβ is different in most tissue types (Couse *et al.*, 1997) and they differentially activate target genes in different environments (Paech *et al.*, 1997). It has been known that ERα is predominantly expressed in ovarian cancer cell lines, while ERβ was mainly expressed in normal ovaries (Pujol *et al.*, 1998) and involved in the induction of apoptosis or anti-tumoral effects on ovarian cancer cells (Treeck *et al.*, 2007). The previous studies indicated that EDC-induced estrogenic effect is mediated through ERá in an animal model (Dang *et al.*, 2007c), indicating that EDCs may increase cell growth at a high concentration by binding to ERα. However, further research is required to clarify the precise roles of ERá and ERâ involved in EDC-induced cell proliferation.

***Estrogenic actions of EDCs.*** A variety of environmental EDCs have endocrine disrupting properties which may endanger human health and the ecosystem. Until recently, there has been no standardized assay to determine whether an environmental chemical is an EDC or to determine its EDC-potency (Choi and Jeung, 2003). Thus, efficient and sensitive *in vitro* and *in vivo* assays are essential for detecting ED-activity, rating EDC-potency and investigating the mechanisms underlying the detrimental effects of EDCs in humans and wildlife. An ideal assay for screening and rating EDC-potency would be costly- and timely effective. In our previous studies, we employed single-treatment *in vivo* and *in vitro* methods to determine the biological effects of OP, NP or BPA in the induction of CaBP-9k (Dang *et al.*, 2007b). CaBP-9k is a novel biomarker of EDC-action which shows an induced increase in mRNA and protein levels following a single *in vivo* or *in vitro* exposure; the biological pathway of this response to EDC-exposure may involve ER and ER physiological-mediated responses (Dang *et al.*, 2007a, b, c, d, 2009a, b; Tinnanooru *et al.*, 2008; Vo *et al.*, 2009a, b). However, recent studies have indicated that non-genomic pathways may also contribute to the potency of EDCs to disrupt endocrine system(s) function in a genomic-independent induced response (Dang *et al.*, 2010; Waring and Harris, 2005).

Such environmental compounds may exert their effects by altering hormone synthetic pathways or endogenous hormone availability. Other evidence indicates that E2 and estrogenic EDCs may induce rapid ERK phosphorylations via non-genomic responses (Bulayeva *et al.*, 2004). In addition, the activation of PI3K and ERK1/2 evoked by these EDs, including OP, NP and BPA, was previously shown in MCF-7 cells (Cobb and Goldsmith, 1995). Some EDCs exhibiting weak estrogenic activity appear to be act as potent EDCs through some non-genomic responses (Watson *et al.*, 2007). Non-genomic responses may occur rapidly, within minutes after exposure, whereas genomic responses are delayed, requiring at least several hours to be established (Dang *et al.*, 2010; Filardo, 2002). Thus, genomic effects may play an important role in later stage responses to EDC and/or xenoestrogen exposure. In addition, treatment with BPA, NP, OP and MXC resulted in an increase of cell pro-

liferation and increased the ERE activity in ovarian cancer cells, BG-1 (Park *et al.*, 2009). This increase of cell proliferation and activation of ERE was reversed in the presence of an estrogen receptor antagonist, ICI 182780, suggesting that ER is involved in EDC-mediated pathway in ovarian cancer cell growth. In addition, BPA rapidly induced activation of ERK1/2 and p38 MAPK, but the effect of BPA on stimulation of cell growth was not blocked by pretreatment with inhibitors of MEK (PD98059) or p38 (SB203580) in these ER-positive ovarian cancer cells (Park *et al.*, 2009).

***EDCs actions through ERs.*** In our previous study, cDNA microarray was employed to determine the expression levels of approximately 13,000 genes and expressed sequence tags (ESTs) after treatment with OP and/or DES in the uteri of both maternal and neonate groups. In the study, the results indicated that placental exposure to OP or DES may cause temporal changes in gene expression in the uteri of dams and neonates and may provide useful indicators of the adverse effects of EDCs and prove particularly important in elucidating the effects of xenoestrogens on estrogen-responsive tissues, such as the developing reproductive tract (Dang *et al.*, 2007a). Cotreatment with the ER antagonist ICI 182,780 completely prevented the EDC-induced uterine weight gain. Taken together, these results demonstrate that a single injection of OP, NP, or BPA results in an increase in CaBP-9k mRNA and protein levels via an ER-dependent pathway in the uterus of immature rats (Dang *et al.*, 2007b).

Polybrominated diphenyl ethers (PBDEs), a class of organic brominated flame retardants, have been increasing in the environment and in the tissues and milk of animals, including humans. Among these, 2,2',4,4'-tetrabromodiphenyl ether (BDE 47) is the dominant congener found in humans and animals. We employed immature rats as a developmental model to examine the potential involvement of BDE 47 in the induction of CaBP-9k, which is a novel biomarker for screening estrogenic compounds. In this study, treatment with a high dose of BDE 47 resulted in a significant increase in CaBP-9k mRNA and protein at 24 h after injection, while cotreatment with ICI 182,780, an ER antagonist, completely reversed the BDE 47-induced increases in uterine wet weight and induction of CaBP-9k mRNA and protein (Dang *et al.*, 2007c). To determine which ER subtype is involved in CaBP-9k regulation in the pituitary, the immature rats were treated with propyl pyrazole triol (PPT, an ERalpha-selective ligand) or diarylpropionitrile (DPN, an ERbeta-selective ligand) for 3 days (Tinnanooru *et al.*, 2008). Pituitary CaBP-9k expression was mainly mediated by PPT in immature male rats, whereas no significant alteration of pituitary CaBP-9k gene expression was observed after DPN treatment. suggesting that pituitary CaBP-9k is induced by E2 in male rats and its expression is predominantly regulated by ERalpha, but not ERbeta (Tinnanooru *et al.*, 2008).

*In vivo* exposure to EDCs can induce growth hormone (GH) mRNA and protein expressions in the rat pituitary gland and that their activities may involve an ER-mediated signaling pathway. These results may provide critical evidence for EDC-induced dysregulation of pituitary GH expression and thus may be important for elucidating the potential impacts of EDs in altered body growth and development and for predicting the health risks of EDC exposure in humans and wildlife (Dang *et al.*, 2009a). Cotreatment with ICI 182,780 antagonized E2-induced prolactin (PRL) secretion (Bulayeva *et al.*, 2005), suggesting that ERs play a physiologically-mediated role in the PRL release. Although some functional characteristics are shared, ERα and ERβ genes have distinct molecular control mechanisms (Frasor *et al.*, 2003).

In addition, pregnant Sprague Dawley (SD) rats were treated with testosterone propionate (TP), flutamide (Flu) and di-(2-ethylhexyl) phthalate (DEHP) from gestation days (GD) 11 to 21 (Vo *et al.*, 2009a). In this study, we examined differential gene expression patterns by microarray analysis following EDC exposure, particularly in sex determination-related genes. Although Flu and DEHP are considered to be identical with regard to their anti-androgenic effects, their effects on developing male reproductive organs were distinct, suggesting that Flu competes with endogenous testosterone, while DEHP influences a different step in androgenesis (Vo *et al.*, 2009a). In our previous study, GH mRNA levels and GH release in GH3 cells was significantly increased at 24 h in response to OP, NP or BPA, whereas EDC-induced GH mRNA and GH secretion was significantly attenuated by cotreatment with ICI 182,780. However, complete inhibition of GH transcription was observed following co-administration with a pure ER antagonist (Dang *et al.*, 2009b). We further examined the effects of OP, NP and BPA on the induction of non-genomic response in these cells and observed an increase in the expression levels of ERK1/2, Akt1/2/3 and/or $G_{\alpha\ i\text{-}2}$ proteins following EDs-exposure. Additionally, rapid and significant activation of p-ERK was observed at 5 or 15 min after these EDCs-exposure, indicating that various signaling pathways may be involved in EDCs-induced molecular events in GH3 cells (Dang *et al.*, 2009b). A further research warranties to illustrate the potential role of these non-genomic signaling pathways evoked by estrogen- like EDCs in these cells.

## CONCLUSION

An impact and significance of endocrine disruption and the screening strategy for detecting these synthetic or man-made chemicals are not well described in female and male reproductive functions. Although EDCs may interfere with the endocrine system(s) of our body and have estrogenicity or androgenicity, the exact mechanism(s) underlying their

detrimental effects is not clearly understood. Thus, we and others have investigated the effect(s) of EDCs on the induction of a biomarker in cellular models and diverse tissues of *in vivo* animal models. These results indicate that biochemical pathway(s) of EDCs-induced activity may involve the ER-mediated signaling pathway and provide new insights into the toxicological effects of EDCs at a critical stage of reproduction and development in both female and male. A more clear study warranties to describe exact toxicological mechanism(s) of EDCs-induced activities using rapid and economic biomarkers in various *in vitro* and *in vivo* models.

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