Unexpected Estrogenicity of Endocrine Disruptors may Evoke a Failure of Pregnancy Derived from Uterine Function: Overview of Their Possible Mechanism(s) through Steroid Receptors

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ABSTRACT

Although many studies have focused on the biological and toxicological effects of phenol products, in particular, in reproductive tracts, the data about their effects in this estrogenic responsive tissue are much less clear. In addition, the *in vitro* and *in vivo* data concerning ED-adverse impacts in other endocrine organs, i.e. pituitary gland, are not understood well either. Thus, a further study is needed for providing a new insight into possible impacts of estrogenic EDs including phenol products in humans and wildlife. A combination of *in vitro* and *in vivo* system for examining EDs may bring better understanding into the regulatory mechanisms underlying EDs-induced events. In addition, this information may support for developing optimal screening methods of estrogenic EDs, in particular, phenol products.

(Key words : endocrine disruptors, estrogenicity, cDNA microarray, uterotrophic)

INTRODUCTION

It has been reported that a number of environmental chemicals, both synthetic and naturally occurring, can alter functionally the endocrine system(s) of humans and wildlife. While some of these chemicals may bind to the receptors of hormones, mimic or block the action of these hormones and others may stimulate or inhibit various enzymes that play essential roles in the synthesis of a hormone (Dang *et al.*, 2007a, b, c, d). The potential consequences of these actions may cause abnormal hormone regulation and gene expression, thus affecting adversely function(s) of reproductive, immunological and neurological system (Waring and Harris, 2005). It has been suggested that EDs, even at very low concentrations, can cause their harmful effects via a complex series of molecular events in which the interaction of these chemicals with hormone receptor system may be important in the ED-responses. In addition, it has been reported that the classical mode of action of EDs, including estrogen-, androgen- or progesterone-like compounds, are mediated via their high affinity receptors. Also these receptors are necessary to induce and/or modulate an ED-response. Recent studies indicated that non-genomic pathway may also contribute to the potency of EDs to disrupt functionally endocrine system(s) (Watson *et al.*, 2007). These environmental compounds can exert their effects by altering the synthesis or availability of endogenous hormones (Waring and Harris 2005). E2 and xenoestrogens may induce rapid extracellular signal-regulated protein kinase (ERK) phosphorylations via non-genomic responses (Bulayeva *et al.*, 2004), suggesting that EDs may possess the potential to induce a non-genomic response. Some of EDs referred to be weak estrogenic activity, but they appear to be potent via non-genomic responses (Watson *et al.*, 2007). However, the mechanisms via which EDs may exert their biological and toxicological effects in body remain unclear.

It has been demonstrated that estrogen refers to be an important factor in the control of many physiological processes and development of various organs *in vivo* including reproductive tract (Charpentier *et al.*, 2000). In addition, estrogen also plays an essential role in bone, liver and cardiovascular systems (Watanabe *et al.*, 2003). However, a variety of environmental contaminants possess hormonal properties, including estrogen-like activities, so called xenoestrogens. The estrogenic EDs, diethylstilbestrol (DES), phthalate acid ester, alkylphe-

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nols (APs), polychlorinated biphenyls (PCBs), phytoestrogens, and methothoxychlor are rapidly metabolized *in vivo* (Elsby *et al.*, 2001a, b). It has been documented that phthalates, some PCBs, DDT and its derivatives, certain insecticides and herbicides such as Kepone and methoxychlor, plastic components such as bis-phenol A (BPA), and components of detergents and their biodegradation products, such as alkylphenols, can bind to estrogen receptors (ERs) to induce or modulate an ER-mediated response (Choi and Lee 2004; Laws *et al.*, 2000; Gray *et al.*, 1997; Roy *et al.*, 1997). Some of them can easily pass through maternal-placental barrier during pregnancy and cause adverse effects in normal function(s) of neonatal reproductive system, including octyl-phenol (OP), nonyl-phenol (NP) and BPA (Hong *et al.*, 2003, 2004a, b, 2005). It has been demonstrated that alky phenols are branched typically octyl-, nonyl-, or dodecyl- chains, forming a variety of isomers mostly in the para-position of a phenolic ring (Dang *et al.*, 2007a; Hong *et al.*, 2004a). In addition, alkyl-phenols have been demonstrated rapid conjugation and excretion in the rodent, particularly in oral route (Upmeier *et al.*, 1999; Certa *et al.*, 1996). *In vitro* and *in vivo* experiments indicate that these estrogenic EDs are considered to be a weak estrogenic agonist (Bolger *et al.*, 1998; Soto *et al.*, 1995). The potency of OP or NP is about 10^{-5} to 10^{-7} M relative to E2 (Watanabe *et al.*, 2004; Nagel *et al.*, 1997; Arnold *et al.*, 1996; White *et al.*, 1994). OP and NP were demonstrated to be equally potent EDs with greater activity than BPA (Laws *et al.*, 2000). In anterior pituitary GH3 cells, the potency of BPA is 1,000 to 5,000-fold lower than that of E2 in the induction of prolactin gene expression, release and cell proliferation (Steinmetz *et al.*, 1997). This ED is structurally similar to DES, a synthetic chemical with high potent estrogenicity. However, the mechanism underlying BPA-induced estrogenic activity remains unclear (Dang *et al.*, 2007b; Papaconstantinou *et al.*, 2001).

Many of experimental research, including *in vivo* and *in vitro* studies, have been developed to elucidate the biological and toxicological effects of estrogenic EDs in humans and animals. *In vitro* methods include ER-binding assay, Michigan Cancer Foundation (MCF) cell proliferation assay, and the yeast-estrogen-screen cell assay (Miller *et al.*, 2000; Seifert *et al.*, 1988). *In vivo* methods include an uterotrophic bioassay in ovariectomized (OVX) adult and immature female rodents, age at vaginal opening in prepubertal rats, vaginal cytology in adult OVX female rats, and estrous cyclicity in intact rats (Balaguer *et al.*, 1999; Gray *et al.*, 1997). The biomarkers to assess EDs, i.e., pS2, Muncin1 (MUC1), androgen receptor, progesterone receptor (PR), ER, clusterin, complement C3, lactoferrin, vitellogenin, and cathepsin B, have been developed for examining the interactions of EDs and hormone system in humans and animals (Ren *et al.*, 1997; Heppell *et al.*, 1995). Recently, a genome-wide analysis technique, DNA microarray technology, has been developed to screen EDs, which facilitates the rapid monitoring of a large number of gene alterations and its successful application for screening EDs in our previous study (Dang *et al.*, 2007a).

In this review, we described the *in vitro* and *in vivo* results from three screening systems for estrogenicity of phenol products reported by us and others. Furthermore, the information concerning with biological and toxicological effects of EDs or xenoestrogens is very important for elucidating the mode of actions of EDs *in vitro* and *in vivo* and strategy of ED- screening methods.

UTEROTROPHIC STUDIES

Many previous studies investigated the biological and toxicological effects of OP, NP and BPA on female reproductive system using *in vivo* model. The uteri of rodents undergo three important stages during the postnatal development. Among these stages, the quiescent period (postnatal day 17 to 26) refers to be a critical window stage of sensitivity for uterotrophic bioassay (Owens and Ashby, 2002). Exposure to EDs during certain developmental stages of female reproductive system is critical step to assess the potential effects of EDs. Based on an increase in uterine weight in immature rodents after treatment with environmental chemicals, an uterotrophic bioassay is considered to be a reliable method *in vivo* for screening estrogenic agonists and antagonists (Dang *et al.*, 2007c; Owens and Ashby, 2002). It has been indicated that that the metabolism of phenol products may be affected by exposure route in the induction of uterotrophic responses (Laws *et al.*, 2000). While NP was reported to be more potent in increase of uterus weight by oral route, subcutaneous exposure to OP and BPA caused the highest response in uterine weight compared to oral treatment (Laws *et al.*, 2000; Gray and Ostby, 1998). The previous evidence suggested that oral administration of OP and NP to immature rats resulted in a significant increase in uterus weight (Laws *et al.*, 2000; Odum *et al.*, 1997). A positive uterotrophic response following OP, NP and/or BPA exposure was previously reported in which OP, NP and/or BPA induced

an elevation in rat uterine weight at 24 h after 3 day treatment (An *et al.,* 2002). The similar results also were observed after a single injection with these compounds (Dang *et al.,* 2007b), suggesting that uterotrophic bioassay is an excellent indicator for estrogenicity of EDs in both single and/or multi-exposures within 24 h. Moreover, co-treatment with an ER antagonist, ICI 182,780, prevented completely the ED-induced uterine weight gain in this model. It has been shown that the action of E2 is, especially involved in estrogen-responsive tissues (Owens and Ashby, 2002). The effect of BPA in rodent uterus has been demonstrated in which an induction in hypertrophy of the luminal epithelium, stroma and myometrium following BPA exposure is noted (Papaconstantinou *et al.,* 2001). In immature mice, BPA may cause a modest uterotrophic response under specific testing conditions (Tinwell *et al.,* 2000). The growth of uterus evoked by this ED was completely inhibited by co-treatment with ICI 182,780, suggesting the physiological involvement of ERs in uterotrophic response to BPA (Dang *et al.,* 2007b). Although the potential of BPA refers to be much lower than that of E2, a higher dose of ICI 182,780 is required to prevent BPA-induced effects on uterine epithelium (Papaconstantinou *et al.,* 2001).

The uterus of rodents refers to be ideal organ for bioassay to detect estrogenicity. However, the term of false negative or false positive in uterotrophic bioassay is also mentioned in which very weak agonists may generate a false negative, whereas a false positive may occur in the case of negative chemicals as described previously (Owens and Ashby, 2002). In addition, certain substances which are well-known as non-estrogens may cause a positive uterotrophic response (Nelson *et al.,* 1991; Gardner *et al.,* 1989; Mukku and Stancel, 1985; Jones and Edgren, 1973; Velardo, 1959). It has been reported that the physiological effects of estrogen action on target tissues may induce changes in expression patterns of specific target genes (Dang *et al.,* 2007b; Kos *et al.,* 2000). The appearance of molecular and biochemical events linked with increased uterine weight was previously demonstrated in which the mode of estrogenic ED-action in the rodent uterus would be initiated by the transcription of the ER-mediated genes (Bolger *et al.,* 1998; Gould *et al.,* 1998; Danzo, 1997; Gaido *et al.,* 1997; Bulger *et al.,* 1978), and then resulted in uterine growth (Owens and Ashby 2002). In order to understand the relationship of molecular and cellular events with uterotrophic response, an induced biomarker system and/or a genome-wide analysis (Naciff *et al.,* 2002; Watanabe *et al.,* 2002) are required to provide new insight into molecular and biochemical mechanism(s) in the uterus in response to estrogenic EDs. In addition, an ideal assay for assessing the potential estrogenicity of EDs would be an accurate-, cost- and time-effective. Thus, a combination of uterotrophic bioassay and induced biomarker or gene array is very required to determine whether an environmental chemical is ED or not.

INDUCED BIOMARKER SYSTEM

Recently, induced biomarker system was widely used to assess and characterize an estrogenicity of EDs, in particular, at low dose exposures. Patterns of potency in ED panels determined using assays of biomarker induction in response to ED exposure may provide insights into understanding the mode of ED-induced action. The linkage of cell division biomarkers and uterine growth has been described in previous studies (Kirkland *et al.,* 1979; Gorski *et al.,* 1977; Clark, 1971; Galand *et al.,* 1971; Ham *et al.,* 1970; Martin and Finn, 1970). Based on the interaction between EDs and specific target genes, induced biomarker activity is a very sensitive and powerful tool to identify estrogenic compounds in the environment (Choi and Jeung, 2003). These marker genes include pS2, MUC1, androgen receptor, progesterone receptor, ER, clusterin, complement C3, lactoferrin, vitellogenin, cathepsin B (Ren *et al.,* 1997; Heppell *et al.,* 1995) and CaBP-9k (Dang *et al.,* 2007b, c, d; Choi and Jeung 2003; An *et al.,* 2003; An *et al.,* 2002). Among them, Calbindin-D9k (CaBP-9k) has been shown to be a useful biomarker for screening EDs or xenoestrogens including phenol products.

CaBP-9k, a cytosolic protein, is a member of the family of vitamin D-dependent calcium-binding proteins with high affinity for calcium (Christakos *et al.,* 1989; Kumar *et al.,* 1989). It has been reported that CaBP-9k gene is localized on the X chromosome and consists of three exons and carries four Alu repeats (Jeung *et al.,* 1994; Jeung *et al.,* 1992). It has been demonstrated that CaBP-9k is primarily expressed in the intestine, kidney, uterus, bone (Armbrecht *et al.,* 1989; Mathieu *et al.,* 1989; Seifert *et al.,* 1988; Delorme *et al.,* 1983), lung (Dupret *et al.,* 1992) and pituitary gland (Nguyen *et al.,* 2005). Functionally, CaBP-9k is involved in intestinal calcium absorption and regulated at the transcriptional and post-transcriptional levels by 1,25-dihydroxyvitamin D3, the hormonal form of vitamin D (Darwish and DeLuca, 1992; Wasserman and Fullmer, 1989; Roche *et al.,* 1986). Since the hormonal

mechanism of CaBP-9k by E2 is well understood in rats (Krisinger *et al.,* 1992), CaBP-9k mRNA and protein levels induced by EDs are considered to be a very useful tool for screening environmental estrogenic compounds in the immature rat model (Choi and Jeung, 2003). In the previous study, we also demonstrated that a single injection with OP, NP and BPA resulted in an increase in uterine weight and the induction of uterine CaBP-9k mRNA and protein in immature rats and the biological pathway of these actions may involve the ER and ER-mediated pathway *in vivo* (Dang *et al.,* 2007b). In addition, utero exposure to these compounds resulted in an significant increase in CaBP-9k protein expression as previously shown (An *et al.,* 2003). In addition, a positive correlation between uterotrophic assay and CaBP-9k mRNA or protein expression was observed following OP, NP or BPA treatment in the uterus of immature rats. Although placenta refers to be a physiological barrier, which may protect the fetus from some harmful substances, a variety of environmental chemicals including phenol products may cross this barrier and exert their adverse effects on fetal health (Hong *et al.,* 2003, 2004a, 2005). The previous studies reported that maternal exposure to OP, NP or BPA resulted in an increase of CaBP-9k mRNA and protein in maternal and fetal uterus, suggesting that this chemical group can easily pass through the placenta during the pregnancy (Dang *et al.,* 2007a, b, d). Moreover, a transfer of OP, NP and BPA from mother to pups via breast milk also was detected in which a high expression level of CaBP-9k mRNA was observed in neonatal uterus when the dams were exposed to these compounds (Hong *et al.,* 2004b).

It has been suggested that the estrogenic mode of ED-induced action is mediated ER and ER-mediated pathway. The ER is known well as a member of the steroid receptor family, which includes estrogen, androgen, progesterone, glucocorticoid and mineralocorticoid receptors (Thornton, 2001; Baker, 1997; Escriva *et al.,* 1997; Laudet, 1997; Mangelsdorf *et al.,* 1995). In the previous study, we demonstrated that the estrogenic effects of OP, NP and BPA in the induction of uterine CaBP-9k expression were completely attenuated by co-treatment with an antiestrogen, ICI 182,780, suggesting that phenol products exert their effects via an ER-mediated pathway *in vivo*. In the uterus, two forms of ER have been identified as ER α and ER β. Although both receptors share some functional characteristics, district molecular mechanisms control their genes (Frasor *et al.,* 2003). Additionally, the distribution of these ERs also differs in this estrogenic target tissue (Williams *et al.,* 2001; Kuiper *et al.,* 1996). In the anterior lobe of pituitary gland, the expression level of ER α is higher than ER β (Kuiper *et al.,* 1997). A previous study in immature rats described that uterine CaBP-9k is evoked by EDs via ER α pathway, but not ER β-mediated mechanism (Lee *et al.,* 2005), indicating that ER α is a predominant form in the uterus.

In order to contribute to understanding of the relationship between the molecular events caused by EDs and its biological effects, *in vitro* models to employ rat pituitary cells, a GH3 cell model have been developed, and the estrogen responsiveness of GH3 cells has been demonstrated by Fujimoto (Fujimoto *et al.,* 2004). A marked up-regulation of CaBP-9k gene following E2 treatment indicated that GH3 line is good candidate for investigating the estrogenicity of EDs. Recently these cells have been selected to study the biological effects of phenol group in the induction of CaBP-9k as a biomarker (Dang *et al.,* 2007d). Treatment with OP, NP and BPA induced a significant increase in CaBP-9k at both transcriptional and translational levels at 24 h. A significant increase in CaBP-9k mRNA expression was detected as early as 6 h after OP, NP and BPA exposure, whereas these chemicals caused a high level of CaBP-9k protein expression at 1 h in these cells. Taken together, these results indicated that a combination of uterotrophic bioassay and induced biomarker system is very critical to elucidate the biological ad toxicological effects of estrogenic EDs, in particular, phenol groups. In addition, a combined assessment of uterotrophic response and one or set of induced biomarkers may provide valuable information involved in estrogenic endocrine disrupting activities of EDs or xenoestrogens.

GENOME-WIDE ANALYSIS

A variety of screening methods have been developed to investigate EDs. An induced biomarker system has been used widely to screening EDs, but this assay can detect only one of many potential hormonal responses. Data from one or a set of biomarkers fail to accurately reflect whole organism responses to EDs (Dang *et al.,* 2007a). Recently, cDNA microarray has been successfully applied for the characterization of EDs. A genome-wide analysis may monitor a large number of gene alterations and provide better insight into the regulatory mechanisms underlying EDs-induced events (Francois *et al.,* 2003). In addition, the information from cDNA microarray may complement perfectly the result obtained from the utero-

trophic bioassay (Owens and Ashby, 2002). Estrogenic EDs can bind to ER, and induce or modulate an ER-mediated physiological response involving a complex series of molecular events that may lead to changes in gene expression patterns (Laws *et al.*, 2000; Gray *et al.*, 1997; Korach *et al.*, 1991). The identification of estrogen-induced genes is a necessary step to elucidate mechanisms underlying biological and toxicological effects of EDs (Watanabe *et al.*, 2003). The studies which assess altered gene expression profiles evoked by OP, NP or BPA have been previously carried out in estrogen target tissues. A genuine response to estrogen or estrogenic EDs can be observed while the female reproductive tract is still undergoing development (Dang *et al.*, 2007a; Naciff *et al.*, 2002). A recent study in the uterus of immature rats pointed out that district altered gene expression patterns following treatment with E2 and/phenol products were noted in which elevated expression level of the genes (over 2-fold) were detected as 8.81 % for OP, 9.51 % for NP and 8.26 % for BPA. These include CaBP-9k, oxytocin, adipocyte completement related protein, lactate dehydrogenase A and calcium biding protein A6 (Hong *et al.*, 2006). The similar gene expression patterns were observed following E2 and NP treatment in the uterus. In addition, an agreement of gene expression profiles was observed and obtained from uterotrophic bioassay caused by NP. However, the relative activity of this ED from gene expression levels is about 10^{-5} to 10^{-7} when compared to E2 (Watanabe *et al.*, 2004).

Estrogenic EDs induce an identifiable transcript profiles. These gene expression changes may reflect the molecular and biochemical mechanism underlying its action in target tissues at different stages of developing reproductive system. In pregnant rats, a relative potency of OP and NP is similar and higher than BPA (Hong *et al.*, 2006). Maternal exposure to phenol products may cause temporal changes in gene expression in the uterus of dams and neonates. Treatment with high dose of OP (600 mg/kg body weight per day) during late stage of pregnancy may cause alterations in gene expression in both maternal and neonatal compartments. However, the magnitudes of these alterations differed markedly between dams and neonates, reflecting the temporal susceptibility of reproductive tract to EDs (Dang *et al.*, 2007a) in which some estrogen responsive genes i.e. complement C3, c-fos or CaBP-9k (also called calbindin 3) expressed markedly in the uterus of neonates. It has been demonstrated that complement C3 refers to be well-known estrogen responsive gene in the uterus of rats (Jefferson *et al.*, 2000; Hasty and Lyttle, 1992; Sundstrom *et al.*, 1989) and consequently this gene is used as a common biomarker to screen estrogenic ED-activity (Strunck *et al.*, 2000; Hopert *et al.*, 1998; Bigsby and Young, 1994; Leiva *et al.*, 1991; Brown *et al.*, 1990; Howe *et al.*, 1990). C-fos oncogene plays an important role in cell proliferation and transformation (Angel and Karin, 1991) and is known to be one of sex steroid hormone-regulated genes (Nephew *et al.*, 1995; Nephew *et al.*, 1994). An estrogenic *in vitro* and *in vivo* induction of cell proliferation is a critical stage in carcinogenesis of gynecologic tissues (Bardin *et al.*, 2004). In addition, the identification of tumor-associated genes is very important for clarifying carcinogenic effects evoked by EDs. Tumor associated genes have been up-regulated i.e. TTF2, tumor protein D52 like 2, tumor protein p53, Bcl-2 or nucleostemin (Wang *et al.*, 2005; Yang *et al.*, 2005; Dhar *et al.*, 2003; Tsai and McKay, 2002; Charpentier *et al.*, 2000; Byrne *et al.*, 1996; Jensen *et al.*, 1995; Wenger *et al.*, 1993) following maternal exposure to EDs (Dang *et al.*, 2007a), suggesting that maternal exposure to estrogenic EDs may play a part in the pathogenesis of estrogen-dependent tumor progression. Another study using developing female reproductive system indicated that BPA possesses a weak estrogenic like actions, which also may cause altered gene expression profiles in both uterus and ovaries of rat fetuses (Naciff *et al.*, 2002). These findings may provide useful information on the risks associated with estrogenic ED exposure and the prediction of ED-induced adverse effects in later of life.

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