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Review

Biomarker Genes for Detecting Estrogenic Activity of Endocrine Disruptors via Estrogen Receptors

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Abstract: Endocrine disruptors (EDs) are compounds used in various industrial products, drugs, and cosmetics. They can be found in the environment and disturb the endocrine and reproductive systems, resulting in adverse effects to humans and wildlife such as birth defects and developmental disorders. Since several EDs have a structure similar to that of endogenous steroid hormones such as estrogens, they intend to have an affinity for steroid hormone receptors and alter hormone-mediated metabolism by binding to these receptors. EDs are therefore a global concern and assays should be developed to efficiently determine whether these compounds are detrimental to biological systems. Diverse experimental methods may help determine the endocrine disrupting potential of EDs and evaluate the adverse effects of a single and/or combination of these reagents. Currently, biomarkers have been employed to objectively measure EDs potency and understand the underlying mechanisms. Further studies are required to develop ideal screening methods and biomarkers to determine EDs potency at environmentally relevant concentrations. In this review, we describe the biomarkers for estrogenicity of EDs identified both *in vitro* and *in vivo*, and introduce a biomarker, cabindin-D_{9k} (CaBP-9k), that may be used to assess estrogenic activity of EDs.

Keywords: endocrine disruptors; estrogen activity; biomarker; calbindin-D_{9k}

1. Introduction

Endocrine disruptors (EDs) are environmental chemicals that disrupt the endocrine system of wildlife and humans. Studies performed in the 1930s showed that a number of synthetic chemicals have estrogenic properties, and the feminizing effect of the pesticide dichlorodiphenyltrichloroethane (DDT) was demonstrated in roosters in the 1950s [1,2]. Hormonally active chemicals, (*i.e.*, diethylstilbestrol or DES) were widely used to prevent abortions until 1971; however, vaginal clear cell carcinogenesis was observed in young women due to use of synthetic estrogen during pregnancy [3]. Furthermore, female offspring from women who were exposed to DES had an increased risk of reproductive and immunologic abnormalities, and male offspring were at risk for genital anomalies and abnormal spermatogenesis [4,5]. It was also reported that EDs potentially cause fetal pathophysiology, abnormal maturation of the brain, learning disabilities, disorders associated with attention, motivation, emotion and cognitive development. In addition, reduction of sperm count and prostate enlargement in males; ovarian and uterine dysfunctions in females have been reported [6,7]. These findings indicate that hormonal disruption caused by EDs in animals and humans results in serious adverse effects.

EDs are widely found in drugs, pesticides, and compounds used for manufacturing plastics (see Table 1). Most EDs have a structure similar to that of endogenous steroid hormones and primarily act through nuclear hormone receptors including estrogen receptors (ERs), androgen receptors (ARs), and thyroid receptors (TRs) [8]. Having a structure similar to that of hormones permits EDs to exert abnormal effects that disrupt endocrine systems through diverse pathways including binding to hormone receptors, mimicking hormone action, and blocking the action of hormones [9-11]. Estrogen (E2) is hormone essential for the development of reproductive organs, bone, liver, and the cardiovascular system, and plays an important role in many physiological processes [12]. Diverse types of EDs have been shown to have estrogenic activity because of high binding affinity for ERs. ERs are steroid hormone nuclear receptors and act as ligand-activated transcription factors. ERs have two isoforms, ER-α and ER-β, which can bind a wide variety of EDs and activate the transcription of estrogen-responsive genes [13,14]. However, there are no standard methods to determine whether certain EDs have an estrogenic activity or not. In this review, we discuss diverse methods to evaluate EDs and discuss the use of CaBP-9k as a biomarker for identifying estrogenic EDs.

Table 1. List of estrogenic EDs.

Chemical Class	EDs	References
Industrial byproducts	Polychlorinated biphenyls (PCBs)	
	Polybrominated biphenyls (PBBs)	
	Dioxins	[15,16]
Plastics	Bisphenol A (BPA)	[17,18]
Plasticizers	Phthalates	[19,20]
Pesticides	Methoxychlor	
	Chlorpyrifos	
	Dichlorodiphenyltrichloroethane	[21,22]
Fungicides	Vinclozolin	[23,24]
Pharmaceutical agents	DES	[3,5]
Biodegradation products	Octyl-phenol (OP)	
	Nonylphenol (NP)	[25,26]
Phytoestrogens	Genistein	
	Coumestrol	[24,27,28]

2. Biomarkers for Measuring the Estrogenic Effect of EDs

2.1. Vitellogenin

Vitellogenin (VTG) is an egg yolk precursor protein and is normally produced by liver cells of female fish in response to E2 secreted by the pituitary gland [29]. It is released into the blood where it circulates until it reached the ovaries and promotes oocyte development. Male fish also carry the *VTG* gene, although VTG protein is normally not expressed because the circulation levels of E2 are extremely low in male blood plasma [30]. However, males still have the capability to express VTG, and male fish are known to produce the protein under the influence of estrogenic EDs [30-32]. E2, NP, and OP all induce the expression of VTG in male fish in a dose-dependent manner [33,34], suggesting that the *VTG* gene in male fish can be used as a biomarker for evaluating the effects of EDs [35-37].

2.2. Complement C3 and Ornithine Decarboxylase

The uterus is a general target organ for estrogen-mediated metabolism. A uterotrophic bioassay is widely used to measure increased uterine wet weight after EDs treatment [38]. However, this assay does not evaluate effects other than those associated with the estrogenic activity of EDs which could lead increased uterine weight via unknown pathways. In the past few years, several genes regulated in the uterus have been identified and used as marker genes to assess the estrogenicity of EDs. For example, genes for the gap junction connexin, such as connexin 26 and connexin 43, the plasma glycoprotein clusterin, and complement C3 were shown to be highly regulated by E2 in rat endometrium [39-41].

Complement C3, simply called C3, is a protein involved in the immune system which plays a central role in activating complement pathways and promotes innate immunity [42]. In adult female mice, C3 is exclusively expressed in the uterus. E2 administration to immature or ovariectomized mice significantly increases *C3* mRNA levels as well as immunoreactivity in the endometrium, indicating that the synthesis of this protein is regulated by E2 in mouse endometrium [43]. Phyto- and xenogestogens have been found to induce C3 expression in endometrium, and the sensitive parameter of C3 is highly suited to investigate the biological potential of natural and synthetic estrogens [39].

The enzyme ornithine decarboxylase catalyzes the decarboxylation of ornithine (a product of the urea cycle) to form putrescine, which is the committed step in polyamine synthesis [44]. The rapid growth and differentiation of uterus are concomitant with increased expression of the ornithine decarboxylase gene. Recent studies have shown that expression of ornithine decarboxylase gene in the uterus is augmented by EDs [45-48]. These estrogen-sensitive genes have therefore been used as markers for evaluating the estrogenic potential of EDs in the uterus

2.3. pS2 and Mucin 1

pS2 is a low molecular weight protein containing 60 amino acid. E2 and estrogenic compounds stimulate the expression of pS2 which was first observed in the MCF-7 breast cancer cell line into which the *pS2* gene has been cloned [49]. *pS2* mRNA production can be rapidly induced by E2 in certain breast cancers, but not in normal breast tissue nor in any other human cell lines. Therefore,

pS2 mRNA expression in MCF-7 cells is an ideal model for studying the effects of estrogenic compounds [49-51].

Cell surface mucins are a family of highly glycosylated glycoproteins found in the apical cell membranes of epithelial cells from the mammary gland, salivary gland, respiratory tract, digestive tract, uterus, and testis [52,53]. Mucin1 (MUC1), a mucin and well-known marker of breast cancer, is an extended rod-like molecule which protrudes above the cell surface of epithelial cells [54]. The MUC1 promoter region has half of an estrogen response element (ERE) and is regulated by E2. Therefore, MUC1 is known to be a direct E2 target gene due to specific ER binding in MCF-7 cells [55]. EDs, including NP, have been reported to induce the expression of pS2 and MUC1 in MCF-7 breast cancer cells [54].

2.4. Progesterone Receptor

The progesterone receptor (PR) is an intracellular steroid receptor that specifically binds to progesterone and is involved in a wide variety of physiological functions including the control of embryonic development, cell differentiation, and homeostasis [56-58]. The *PR* gene is a known target of E2 in certain cell lines including MCF-7 and GH3 cells that express ERs [59,60]. Recent studies have observed EDs-induced expression of PRs in GH3 rat pituitary cells. Several parabens and E2 significantly increased PR expression in these cells [60], demonstrating that PR levels may be augmented by EDs through an ER-mediated pathway in GH3 cells [61,62]. By measuring the expression levels of VTG, Complement C3, ornithine decarboxylase, pS2, MUC1 and PR genes, EDs estrogenicity can be efficiently evaluated in a cost- and time-effective manner.

3. *CaBP-9k* Gene Expression as a Biomarker for Assessing the Effects of EDs

3.1. Introduction of the CaBP-9k Gene

Calbindin-D_{9k} (CaBP-9k) is a 9-kDa cytosolic protein and a member of the S100 family of calcium-binding proteins that includes calmodulin, paravalbumin, troponin C, and calbindin-D_{28k}. These calcium-binding proteins were recently classified into different sub-families as they differ in the number of calcium-binding EF-hand sites [63,64]. CaBP-9k has two calcium-binding domains that have high affinities for calcium. The *CaBP-9k* gene is localized on the X chromosome and consists of three exons interrupted by two introns [65,66]. The CaBP-9k protein is composed of 79 amino acids. The *C*-terminal site (amino acids 54–65) is a normal EF-hand similar to that found in other proteins of the S100 family while the *N*-terminal site (amino acids 14–27) has a unique structure containing different amino acids. However, *N*-terminal site have not disrupted the calcium binding ability of CaBP-9k [67].

3.2. Regulation of CaBP-9k Gene Expression

CaBP-9k is an intracellular factor primarily known as a vitamin D-dependent calcium-binding protein found in the cytoplasm of intestinal cells [68,69]. CaBP-9k expression has been reported in intestine, kidneys, bone, lung, placenta, pituitary gland, and uterus [70]. Duodenal and renal CaBP-9k are involved in calcium absorption and re-absorption; its expression is regulated by 1,25-dihydroxyvitamin D3 [71,72]. Duodenal CaBP-9k is mainly expressed in enterocytes of the

duodenum in rodents and is involved in intestinal calcium absorption [73]. Additionally, the level of *CaBP-9k* mRNA is thought to be a marker of small intestine differentiation [74,75]. Renal CaBP-9k plays a role in calcium re-absorption in the kidney, which is important for maintaining calcium homeostasis in the body. Ionized and calcium enters the glomerular filtrate by convection and is re-absorbed by the renal tubules [72]. Renal CaBP-9k is expressed at the site of calcium re-absorption and localized in the distal convoluted tubules in rodents [76,77].

The regulation of CaBP-9k expression in other tissues other than duodenum and kidney is known to be accomplished through different mechanisms. For instance, uterine CaBP-9k is not under the control of 1,25-dihydroxyvitamin D3 despite the presence of 1,25-dihydroxyvitamin D3 receptors [78]. Uterine CaBP-9k is expressed mainly in the endometrial stroma and myometrium in non-pregnant rodents and regulated by sex steroid hormones [79,80]. An estrogen response element (ERE) in the CaBP-9k promoter mediates transcriptional regulation of CaBP-9k in the presence of E2 in rat uterus [81]. On the other hand, a progesterone response element (PRE) in the CaBP-9k promoter is responsible for responsiveness to progesterone (P4) in mouse uterus [82]. Interestingly, uterine CaBP-9k is up-regulated by E2 and down-regulated by P4 during the estrous cycle in rat uterus [83,84], while it has been shown to be decreased by E2 in mouse uterus [85-87].

3.3. In Vivo *Evaluation of Estrogenic Activity of EDs Using the* CaBP-9k *Gene*

Recently, *CaBP-9k* was suggested to be a novel biomarker for evaluating EDs activity [88,89]. *CaBP-9k* expression levels increased by exposure to EDs are considered to be effective tools for screening estrogenic compounds in *in vivo* models. Recent studies demonstrated that estrogenic compounds significantly increase the expression of uterine CaBP-9k in rats [78]. An *et al*. showed that E2, OP, NP, and BPA increased uterine *CaBP-9k* gene expression in rats. Furthermore, these estrogenic compounds also significantly increase uterine weight in rats [84,89]. Estrogenic compounds, which form a class of EDs that includes diethylstilbestrol [90], tetrabromodiphenyl ether [91], phthalates [92] genistein [93], and parabens [94], strongly induce uterine *CaBP-9k* mRNA and protein expression in the uterus of rats. Moreover, the ER antagonist ICI 182,780 prevents increased expression of uterine *CaBP-9k* when co-administered with estrogenic compounds. It has been confirmed that ER-mediated signaling is involved in the induction of *CaBP-9k* gene expression by estrogenic compounds [95]. These findings suggest that the *CaBP-9k* gene is a useful biomarker gene for assessing the estrogenic potential of EDs.

The ability of *CaBP-9k* for evaluating EDs in an *in vivo* system is summarized in Table 2. Triclosan, tetrabromodiphenyl ether 47 (BDE47), and genistein at concentrations less than 50 mg/kg have been found to have regulatory effects on uterine CaBP-9k expression [91,93]. Triclosan at a dose of 37.5 mg/kg up-regulates uterine *CaBP-9k* expression in an immature rat model [96]. Genistein has similar potential with triclosan at dose of 40 mg/kg. Treatment with BDE47 (50 mg/kg) led a significant increase in uterine CaBP-9k as determined by RT-PCR. OP, NP, and BPA (600 mg/kg) induce uterine *CaBP-9k* expression at in the rat; however, lower concentrations (100 to 250 mg/kg) of these compounds had a more prominent effect in mice [84]. Methoxychlor (250 mg/kg) was reported to have a moderate effect on *CaBP-9k* expression in rat uterus [97]. Butyl benzyl phthalate (BBP), dicyclohexyl phthalate (DCHP), 2-ethylhexyl phthalate (DEHP), di-*n*-butyl phthalate (DBP), and diethyl phthalate

(DEP) do not have any significant estrogenic effects on *CaBP-9k* gene expression in rat uterus [92]. Changes in CaBP-9k expression for assessing EDs activity are sufficient when compared to other biomarkers. For example, C3, a well-known biomarker for measuring EDs effects, shows a level of sensitivity similar to that of CaBP-9k at a concentration in the presence of OP and BPA (200 mg/kg) [40].

Table 2. Expression of uterine *CaBP-9k* in *in vivo* system by different EDs concentrations.

Endocrine disruptors	Doses (mg/kg)	Animal	Route	Method	Reference
Triclosan	37.5	rat	Oral	q-PCR/western	[96]
Genistein	40	rat	S.C injection	Western	[93]
Tetrabromodiphenyl ether 47	50	rat	S.C injection	RT-PCR	[91]
Octyl phenol	100/600	mouse/rat	S.C injection	Western	[84]
Methoxychlor	200	rat	Oral	Northern	[97]
Nonyl phenol	250/600	mouse/rat	S.C injection	Western	[84]
Bisphenol A	250/600	mouse/rat	S.C injection	Western	[84]

3.4. In Vitro *Evaluation of Estrogenic Activity of EDs Using the* CaBP-9k *Gene*

In 2004, Fujimoto *et al.* identified estrogen-responsive genes in the rat pituitary GH3 cell line [98]. GH3 cells were initially characterized as ones that can synthesize growth hormone and prolactin (PRL) in response to E2 stimulation [99]. It was suggested that the GH3 cell line is a good candidate for assessing the *in vitro* estrogenicity of EDs since it is E2-sensitive and expresses functional ERs [98]. Recent studies have reported that EDs induce CaBP-9k expression in this cell line. OP, NP, and BPA induce a significant increase in *CaBP-9k* expression in GH3 cells in addition to augmenting growth hormone and PRL production and gene expression [100]. Moreover, activation of extracellular signal-regulated kinases (ERKs), protein kinases B (Akt), or G proteins by OP, NP and BPA has been observed in these cells [99,100]. Significant induction in *CaBP-9k* and *PR* gene expression was also observed after treatment with various concentrations of parabens [61]. In this study, the effects of parabens on the regulation of *CaBP-9k* expression were blocked in the presence of ICI 182,780, indicating that CaBP-9k is induced by EDs via the ER pathway in GH3 cell line.

Evaluation of the *CaBP-9k* gene as a biomarker for EDs activity in *in vitro* systems is summarized in Table 3. OP, isobutyl phenol, and ethyl- and isobutyl parabens produce the strongest induction of CaBP-9k expression at a concentration of 0.1 μM in GH3 cells, while NP and isopropyl- and butyl-parabens increase *CaBP-9k* gene expression at a concentration of 1 μM [100]. The concentration of BPA and methyl-and propyl-parabens required for inducing *CaBP-9k* gene expression is 10 μM [61]. The efficiency of *CaBP-9k* gene for EDs effect was sensitive than other genes, since pS2, ER and MUC1 were induced at 10 μM of NP in human breast MCF-7 cells, although the cell lines and other experimental conditions were different [54].

Table 3. Expression of *CaBP-9k* on *in vitro* system under different EDs concentration.

Endocrine disruptors	Minimum concentration (μM)	Method	Reference
Octyl phenol	0.1	q-PCR	[62]
Ethyl paraben	0.1	q-PCR	[101]
Isobutyl paraben	0.1	q-PCR	[101]
Nonyl phenol	1	RT-PCR	[100]
Isopropyl paraben	1	q-PCR	[101]
Butyl paraben	1	q-PCR	[101]
Bisphenol A	10	RT-PCR	[100]
Methyl paraben	10	q-PCR	[101]
Propyl paraben	10	q-PCR	[101]

The sensitivity of the *CaBP-9k* gene for assessing estrogenic activity of EDs compared to other biomarkers is difficult to define because effect of intermediate concentrations (*i.e.*, OP: 0.01–1 μM) of EDs have not been tested, particularly in *in vivo* models. However, the *CaBP-9k* gene has been evaluated as a biomarker for EDs activity by a number of *in vivo* and *in vitro* studies as described above. Taken together, data from these studies have demonstrated that the *CaBP-9k* gene is a sensitive and valuable biomarker for assessing the potential estrogenic activity of EDs.

4. Conclusions

EDs are natural or synthetic chemicals with structures similar to those of endogenous hormones and have been shown to disrupt endocrine, nervous, and reproduction systems in animals and humans. There are growing concerns about health problems associated with exposure to EDs found in the environment. Chemicals that disrupt the endocrine system have been shown to have a high binding affinity to steroid hormone receptors, suggesting that exposure to EDs can seriously impact metabolism, development, reproduction, and behavior in mammals, including humans. Therefore, identification and characterization of EDs is urgent for predicting their detrimental effects. Efficient and accurate assays are required to measure EDs potency and to determine what chemicals can possibility be defined as EDs. However, methods for detecting the adverse effects of EDs are still limited. There are currently no sufficient standard biomarker systems or precise methods for evaluating the effects of EDs. Further research to determine the physiological concentration of various EDs (administered alone or in combination) necessary for disturbing biological systems and exerting adverse effects on human health is required. In this review, we discussed the properties of the *CaBP-9k* gene and its potential use as a biomarker for EDs activity. However, further studies to examine the accumulated, synergistic, and addictive effect of EDs using this biomarker are needed. In addition, identification of novel biomarkers that can determine the biologically and physiologically relevant concentrations of EDs is necessary.

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Conflict of Interest

The authors declare no conflict of interest.

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