Elucidation of estrogenic endocrine-disrupting chemicals responsive genes related with male genital disorders

男児生殖疾患に関与するエストロゲン様内分泌かく乱物質応答遺伝子の解明

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1. Introduction

Increases in the prevalence of hypospadias and cryptorchidism have been reported in various countries in the past few years[1]. It is hypothesized that exposure to environmental factors (i.e. endocrine-disrupting chemicals (EDCs)), especially to those with estrogenic activity, affecting androgen homeostasis during the early life, may cause these diseases[2]. Several epidemiological studies have suggested the association between perinatal exposure to environmental chemicals, such as organochlorine pesticides, PCBs, phthalates and xenoestrogens with male genital disorders (MGDs)[3, 4]. It is known that estrogenic EDCs can bind to nuclear estrogen receptors (ESRs), regulate the expression of certain genes and cause adverse problems on human reproductive health[5]. Today, the early events of ESRs binding have been widely studied, but the following story is still not very clear. To make clear this mechanism, it is important to find out what genes could response to estrogenic EDCs exposure and how these genes take part in the development of MGDs. Recently, molecular epidemiological studies indicated that variants of several particular genes may be related to the prevalence of MGDs[6], which provides an approach to screen genes that might be included in the EDCs-mediated pathway to cause MGDs. Sone *et al.* (2008) had systematically selected single-nucleotide polymorphisms (SNPs) in the nuclear receptor genes interacting with ESR1 and examined the association of certain SNPs with MGDs[7]. Case-control analysis revealed differences in 3 genes (*ARNT2*, *CYP17A1*, and *CYP1A2*), which may be candidates as modifiers of those diseases.

The concrete goal of this present study is to elucidate the estrogenic EDCs responsive genes among the susceptible genes. It is a part of the ultimate purpose of study to elucidate the potential mechanism for the effect of estrogenic EDCs exposure on male genital health at the molecular levels, which is a candidate topic in the doctor's course.

2. Materials and Methods

2-1. Test chemicals and Cell lines

All the test chemicals examined in this study are shown in Table1. The LNCaP human prostate cancer cell line (LNCaP) and HEV0091 human lymphocyte cell line (HEV) were purchased from the cell bank of RIKEN, Japan. The recombinant BG1Luc4E2 human ovarian cancer cell line (BG1) was a gift from Dr. M. Denison (University of California, Davis, CA).

2-2. Estrogenic chemically activated luciferase gene expression (E-CALUX) bioassay

An ERE-luciferase reporter gene system (E-CALUX bioassay) was used to measure the estrogenic activity of EDCs. The ESR-positive BG1 cells were maintained in estrogen-stripped media for a week before they were plated to 96-well plates at 40,000cells/well and allowed to attach

for 24 hr. Cells were then incubated with EDCs for 24 hr at 37°C. Luciferase induction was measured by ATTO AB-2100.

2-3. Taqman Real-time quantitative PCR

The responsiveness of MGDs related genes to estrogenic EDCs exposure was examined by Taqman real-time PCR. The human cell lines were cultured in steroid free medium (DMEM+5%FBS) before incubated with the test chemicals for 24 hr at 37°C. Total RNA was extracted by the RNeasy kit (Qiagen, Valencia, CA) and quantified by RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA). cDNA was synthesized by High-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Gene expressions were detected using a Taqman® gene expression master mix (Applied Biosystems, Foster City, CA) with an ABI Prism 7000 sequence detection system.

2-4. Statistics

All the statistics analysis was performed using Microsoft Excel 2003. Relative analysis between the estrogenic activities of EDCs and MGDs related genes expression was performed by Pearson Test.

Table 1. Summary of test chemicals

	Common name	Abbreviation
Phenols	Bisphenol A	BPA
Phthalates	Butyl benzyl phthalate	BBP
	Di-n-butyl phthalate	DBP
	Di(2-ethylhexyl) phthalate	DEHP
Persistent Organic Pollutants	2,3,3',4',5-pentachloro-4-biphenylol	4-OH-PCB107
	2,2',3,4',5,5'-hexachloro-4-biphenylol	4-OH-PCB146
	2,2',3,4',5,5',6-heptachloro-4-biphenylol	4-OH-PCB187
	2,3,7,8-tetrachlorodibenzo-p-dioxin	TCDD
Pesticides	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane	*p, p'*-DDT
	1,1,1-Trichloro-2,2-bis(2-chlorophenyl-4-chlorophenyl)ethane	*o, p'*-DDT

3. Results and Discussion

3-1. Estrogenic activity of test chemicals in BG1 cells

The agonistic effects of the test compounds on ER were analyzed in BG1 cells using the E-CALUX bioassay (Table 2). BPA, BBP and *o, p'*-DDT elicited the most strong potency and OH-PCBs and *p, p'*-DDT weakly induced the ERE-luciferase activity. TCDD showed significant antiestrogenic activity at high concentrations.

3-2. MGDs related genes expression in human cell lines

In the previous study, polymorphisms of gene *ARNT2, CYP17A1* and *CYP1A2* were related with

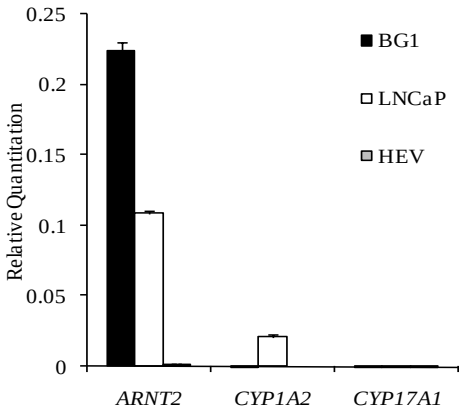

Fig. 1. MGDs related genes expression in three human cells

increasing risk of MGDs. These genes are related to dioxin binding (*ARNT2*), dioxin induction (*CYP1A2*), and estrogen synthesis (*CYP17A1*), which may indicate a possible role of chemical exposure in the development of MGDs. In this study, I measured the expression of gene *ARNT2*, *CYP17A1* and *CYP1A2* in 3 human cell lines. The result (Fig. 1) indicates that *CYP17A1* gene has very weak expression in these human cell lines. *ANRT2* gene has a very high expression in LNCaP and BG1 cells and a very low expression in HEV cells. Based on this result, I decided to use LNCaP cells for the dose-response examination between test chemical exposure and expression of *ARNT2* and *CYP1A2* genes.

Table 2. Estrogenic activity of EDCs in the E-CALUX bioassay

Estrogenic effect	Chemicals	LOEC (M)	EC50 (M)	MOEC (M)	% of control (max)
Significant +	E2	4.1×10^{-13}	4.8×10^{-13}	1×10^{-10}	263
	DHT	8.0×10^{-10}	4.8×10^{-8}	1×10^{-7}	200
	BPA	1.23×10^{-7}	2×10^{-7}	3.33×10^{-6}	220
	BBP	1.23×10^{-7}	2.45×10^{-7}	1×10^{-5}	295
	o, p'-DDT	3.13×10^{-7}	8.92×10^{-7}	5×10^{-6}	285
Significant -	TCDD	4.96×10^{-11}	6.0×10^{-10}	1.55×10^{-7}	43
Weak	*p, p'*-DDT	3.13×10^{-7}	-	5×10^{-6}	175
	OH-PCB107	1.4×10^{-7}	-	1.4×10^{-7}	117
	OH-PCB146	2.3×10^{-10}	-	1.3×10^{-7}	122
	OH-PCB187	4.8×10^{-8}	-	2.4×10^{-8}	125
No effect	DBP, DEHP	-	-	-	-

LOEC: lowest observed effect concentration; EC50: half of maximum effect concentration; MOEC: maximum observed effect concentration; +: induce ERE-luciferase activity; - : inhibit ERE-luciferase activity; M: mol/ml

3-3. Effect of estrogenic EDCs exposure on *ANRT2* and *CYP1A2* genes expression

Dose-sensitive regulations of EDCs exposure on these genes expression were found. This might indicate that environmental chemicals could affect human health by a complicated cross-talk molecular mechanism, which includes multiple nuclear receptor signaling pathways.

Relative analysis

Relative analysis was performed to study the relationship between estrogenic activities of test chemicals and *ARNT2* and *CYP1A2* expression. As the scatter diagram (Fig. 2) shows, a strong positive correlation was found between *ARNT2* expression and estrogenic activities of E2, DHT, BPA, BBP, *o, p'*-DDT, and antiestrogenic activity of TCDD (r=0.77, $p < 0.05$) in LNCaP cells. No correlation was found between *CYP1A2* expression and estrogenic activities of test chemicals.

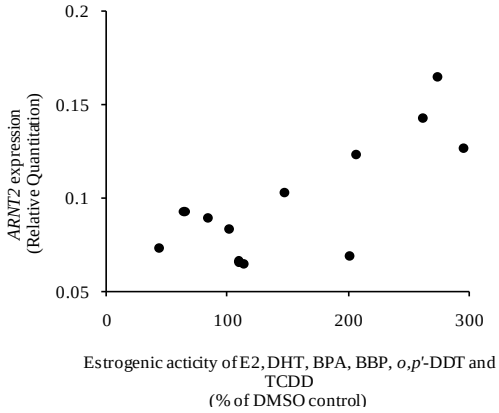

Fig. 2. Association between *ARNT2* expression and estrogenic activity of EDCs

ARNT2 gene encodes a member of the basic

helix-loop-helix Per/ARNT/Sim (bHLHPAS) family of transcription factors. It is known that the encoded protein acts as a partner for several sensor proteins of the bHLH-PAS family, such as the aryl hydrocarbon receptor (AHR) and hypoxia-inducible factors 1α (HIF-1α), forming heterodimers with the sensor proteins that bind regulatory DNA sequences in genes responsive to developmental and environmental stimuli[8]. The present finding arises a question that whether *ARNT2* also participates in the pathways of the other bHLH-PAS proteins involved in developmental processes, such as ESR pathways. Further elucidation of the role of this gene is needed.

3-4. Dose-response relationship between estrogenic activity of EDCs and *ARNT2* expression

To further investigate the relationship between estrogenic test chemicals and *ARNT2* expression on individual chemical basis, I exposed LNCaP cells to E2, DHT and TCDD at high, middle, and low does (1×10^{-10}M, 3×10^{-11}M, 1.11×10^{-11}M for E2; 1×10^{-7}M, 2×10^{-8}M, 4×10^{-9}M for DHT; 1.55×10^{-7}M, 7.78×10^{-8}M, 3.89×10^{-8}M for TCDD, respectively). As the results (Fig. 3), dose-response relationships were found between *ANRT2* expression and E2, DHT and TCDD exposure. Strong correlations were found between relative gene expression of *ARNT2* and the estrogenic activities of E2 ($r=0.80$, $p < 0.05$), DHT ($r=0.89$, $p < 0.05$) and TCDD ($r=0.69$, $p < 0.05$).

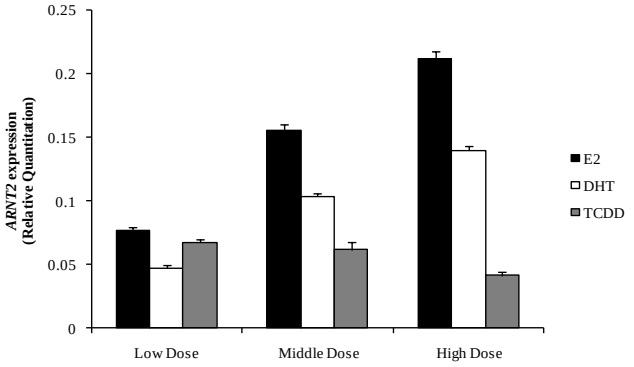

Fig. 3. Dose-response relationship

4. Conclusion and Perspectives

In the present study, I found that *ARNT2* gene expression was affected by the exposure to some EDCs with estrogenic activity with dose-response manner, which may suggest that estrogenic EDCs may affect human health by regulating *ARNT2* gene related signaling pathway.

There is a potential mechanism that estrogenic EDCs might bind to ESRs, regulate certain genes and cause MGDs. According to this present study, *ARNT2* gene might play an important role in this mechanism. The next step to this finding is to elucidate what role does *ARNT2* gene play. For example, it is reported that genetic polymorphisms could affect metabolism of EDCs and increase individual cancer susceptibility[9]. In this study, to further determine whether the genetic variants of *ARNT2* gene could affect individual susceptibility to estrogenic EDCs and then increase the risk to develop MGDs, more biological and epidemiological studies are necessary to completely understand this mechanism.

References

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