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Additive Effects of Estrogenic Activity by the Combination of E₂ and Pesticides

Mihoko KOJIMA^{1*}, Mari MANABE¹, Seiji KANDA¹, Kenji FUKUNAGA^{1**},
Masafumi NAKAMURA², Motohiro TUJI³ and Toshimasa NISHIYAMA¹

¹Department of Public Health, Kansai Medical University, Osaka, Moriguchi, 570-8506 Japan

²Hiyoshi Corporation, Engineering Department analysis research section, Shiga, Oumihachiman, 523-8555 Japan

³Shiga Prefectural Institute of Public Health and Environmental Science, Shiga, Otsu, 520-0834 Japan

*To whom request for reprints should be addressed

**Present Address, Department of Engineering, Kansai University, Osaka, Suita, 564-8680 Japan

Abstract

The E-CALUX assay has been established to measure the estrogenic activity of agents such as exogenous endocrine disrupting chemicals. The method utilizes human ovarian carcinoma cells, and is able to evaluate estrogenic activity as a reporter gene assay by using estrogen receptors (ERs) expressed endogenously. However, we have no information about the subtypes of ERs that ovarian carcinoma cells express. Therefore we detected the expression of both ER- α and ER- β by RT-PCR, and determined the ratio of ER- α to ER- β to be 5.7:1 using semi-quantitative real-time PCR. Further, the protein expression of each ER subtype was detected using immunocytochemistry. These results suggest that estrogenic activity detected with the E-CALUX assay is mainly ER- α dominant and the additive estrogenic activity was observed when cells were stimulated with the combination of 17 β -estradiol (E₂) and pesticides (pyriproxyfen, thiabendazole (TBZ) and *o*-phenylphenol (OPP)), respectively.

Key words: estrogen receptor (ER), E-CALUX assay, estrogenic activity, subtype, mRNA, 17 β -estradiol (E₂)

Introduction

Exogenous endocrine disrupting chemicals are known to have estrogenic activity and can cause serious environmental problems. The E-CALUX assay has been established as a screening method to detect estrogenic activity¹⁾. This assay is able to detect weak estrogenic activity using a reporter gene assay. Hormones such as estrogen bind to hormone receptors, and form a dimer. This dimer combines with the estrogen responsive element (ERE), and the complex induces the synthesis of RNA for a specific gene downstream. The assay is based on this mechanism, and is a method for measuring the affinity of chemical substances for estrogen receptors using the transcriptional activation of the target gene in vitro. The assay utilizes human ovarian carcinoma cells (BG1) transfected with an estrogen-responsive luciferase reporter gene plasmid and cloned (BG1Luc4E2) to respond to estrogenic chem-

icals or exogenous endocrine disrupting chemicals.

It has been reported that the physiological effects of estrogen were mediated by specific estrogen receptors, part of the nuclear receptor superfamily²⁾. Estrogen receptor (ER)- β was identified in 1996³⁾, and differences in ligand-binding ability and transactivation properties were detected between ER- α and ER- β ^{4,5)}. For example, 17 β -estradiol (E₂) shows high affinity for ER- α , while phytoestrogens such as genistein and coumestrol show high affinity for ER- β .

ER- α and ER- β also differ in their distribution, ER- α is detected in endometrium, breast cancer cells, ovarian interstitial cell, and ER- β detected in kidney, small intestine, mucosa, bone marrow, bone, brain, and prostate gland, etc⁶⁾. The physiological effects also differ depending on whether the chemical agents bind to ER- α or ER- β . Estrogen accelerates the growth of breast cancer and the

endometrium when it binds to ER- α . On the other hand, estrogen prevents or improves of the osteoporosis, activates cells in various organs, and suppresses the generation of large bowel cancer when it binds to ER- β . To evaluate the possibility of endocrine disruption in the environment, it is necessary to clarify which ER subtype is involved. Recently, we attempted to detect the estrogenic activity of pesticides using the E-CALUX assay.

In this study, we identified the subtypes of ER by semi-quantitative RT-PCR and immunocytochemistry, and determined the proportion of each by real-time PCR in human ovarian carcinoma cells used in the E-CALUX assay. Therefore, the effects of estrogenic activity were evaluated by this assay stimulating with E₂ and pesticides (pyriproxyfen, TBZ and OPP).

Materials and Methods

Cell line

The assay utilizes human ovarian carcinoma (BG1) cells transfected with an estrogen-responsive luciferase reporter gene plasmid and cloned (BG1Luc4E2) to respond to estrogenic chemicals or exogenous endocrine disrupting chemicals. The cells were cultured in RPMI 1640 medium (Nacalai Tesque 30264-85) supplemented with 8% FBS and penicillin/streptomycin at 37°C in a CO₂ incubator until 60–80% confluent. For the assay, the cells were treated with a 0.25% trypsin–EDTA solution for 5 min at 37°C and collected by centrifugation.

RNA extraction and RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) following the instructions of the manufacturer. The extracted RNA was treated with DNase I (Takara Bio) to remove DNA and quantified with an Ultraspec 2100 (Amersham Biosciences). After the inactivation of DNase I, 1 mg of total RNA was used to synthesize first strand cDNA with random primers and reverse transcriptase (Revertra Ace: Toyobo) for 1 h at 42°C. For PCR analysis, 0.5 mg of cDNA was used as a template and amplified using the following specific primers: ER- α , 5'-AATTCT-

GACAATCGACGCCAG-3' and 5'-GTGCTTCAAC-ATTCTCCCTCC-3', ER- β , 5'-TTCCCGGCAGCAGTAAC-3' and 5'-TCCCTCTTTGCGTTTGGA-CTA-3'. General PCR conditions were 94°C for 4 min, then 94°C/30 sec, 61°C/30 sec, and 72°C/1 min for 30 cycle, and the reaction was performed using an i-Cycler (Bio-Rad). The amplified fragments were 345 bp for ER- α and 262 bp for ER- β . The PCR products were electrophoresed in 1.5% agarose gels with ethidium bromide and specific bands were detected with a Kodak digital camera DC290 system (Invitrogen).

Real-time PCR

Real-time PCR was performed using a DyNAmo HS SYBR Green qPCR kit (MJ Research) according to the directions of the manufacturer. Briefly, 10 ng of template DNA and 5 pmol of each primer for ER- α and ER- β were used. General PCR conditions were 95°C for 15 min, then 95°C/10 sec, 61°C/20 sec, and 72°C/20 sec for 40 cycles and the reaction was performed using a (MJ Research). To quantify transcripts of the genes precisely, G3PDH was monitored as a quantitative control and each sample was normalized based on the amount of G3PDH transcript content. The parameter C_t represents the number of cycles at which a significant increase in the fluorescence signal above a baseline can first be detected and is used to calculate the ratio of ER- α to ER- β . The experiment was conducted in triplicate for each sample, and a mean value was used for calculation of the mRNA levels.

Immunocytochemistry

Human ovarian carcinoma cells (BG1) were plated on 8-well chamber slides (parmanox, Nunc). After 2 days, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and indirect immunofluorescence microscopy was carried out using standard protocols. Non-specific antibody binding was blocked with 5% normal goat serum in PBS and the primary antibody was used under optimized conditions. ER- α : monoclonal antibody against estrogen receptor- α (Santa cruz, D-12 1:100), ER- β :

Fig. 1. Expression of estrogen receptors (ER)- α and β in rat pituitary cell lines by RT-PCR

Human ovarian carcinoma (BG1Luc4E2) cells were immediately processed for total RNA isolation, and random-primed cDNA was synthesized. RT-PCR products were run on ethidium bromide-stained 1.5% agarose gel. M: 100 bp ladder marker, lane1: ER- α , 345 bp, lane 2: ER- β , 262 bp

Typical digital photograph of the fractionation of RT-PCR products amplified from cDNA of transcripts for either ER- α or ER- β in a human ovarian carcinoma cell.

polyclonal antibody against estrogen receptor- β (Santa cruz, H-150, 1:100). The fluorescent-labeled antibody rhodamine-anti-mouse IgG (CHEMICON, 1:400) or FITC-anti-rabbit IgG (CHEMICON, 1:400) was used as a secondary antibody. Cells were photographed using a confocal microscope, Fluoview (Olympus).

Evaluation of Estrogenic activity by Reporter gene assay

E-CALLUX assay is in vitro reporter gene bioassay and the method was described in our previous report⁷⁾. Briefly, human ovarian carcinoma (BG1) cells stably transfected with Luciferase reporter gene and ERE (estrogen response element) was cultured in 96 well plates at 2.0×10^5 cells/ml. After 24 hours, medium was removed and the cells were cultured with E_2 (1.0×10^{-11} M) and/or pesticides (5.0×10^{-5} M) for additional 24 hours. Cells were

lysed with lysis buffer and measured the luciferase activity in microplate luminometer. The results showed as a relative light units (RLU).

Results

ER- α and ER- β mRNA expression in human ovarian carcinoma cells.

To assess the expression of estrogen receptors at the RNA level, RT-PCR was performed. After RNA was extracted from human ovarian carcinoma cells, cDNA was synthesized and PCR was performed. Specific bands were detected ER- α and ER- β (Figure 1). Therefore, human ovarian carcinoma cells expressed both ER- α and ER- β at the mRNA level. A quantitative analysis was performed to determine the ratio of ER- α to ER- β using real-time PCR (Figure 2 and Table 1). The mean threshold cycle (Ct value) was 23.1 for ER- α and 25.6 for ER- β .

The quantity of (Y) after n cycles is given by the equation,

$$Y = I \times 2^n$$

Therefore, the initial ratio of ER- α to ER- β is

$$\begin{aligned} ER\alpha:ER\beta &= Y/2^{23.1} : Y/2^{25.6} \\ &= 2^{(25.6-23.1)} : 1 \\ &= 5.7:1 \end{aligned}$$

Immunohistochemistry

The results of immunostaining using the anti-ER- α antibody and anti-ER β antibody are shown in Figures 3. Mouse anti-ER- α -Ab as a primary antibody and Rhodamine-anti-Mouse IgG as a secondary antibody were used in ER α (α). Rabbit anti-ER- β -Ab as a primary antibody and FITC-anti-

Table 1. The threshold cycle (Ct value) for ER- α and ER- β in real-time PCR

	cycle (T)	mean
α -1	23.2	23.1
α -2	23.3	
α -3	22.8	
β -1	25.5	25.6
β -2	25.1	
β -3	26.2	

The results showed threshold cycle resulted in three different experiments and the mean of Ct was calculated.

Fig. 2. The growth curve for ER- α and ER- β determined by semi-quantitative real-time PCR
The curve showed three different results normalized based on the amount of G3PDH transcript content. The parameter C_i represents the number of cycles at which a significant increase in the fluorescence signal above a baseline can first be detected. The curve showed ER- α : blue, green and red, ER- β : pink, orange and light blue, respectively.

Fig. 3. Immunofluorescence micrograph of ER- α and ER- β in the human ovarian carcinoma cell BG1Luc4E2
ER- α : Mouse anti-ER- α -Ab (primary antibody) and Rho-anti-Mouse IgG (secondary antibody), ER- β : Rabbit anti-ER- β -Ab (primary antibody) and FITC-anti-Rabbit IgG (secondary antibody). Merge: merged image of ER- α and ER- β .

Fig. 4. Estrogenic activities of pesticides and in combination with E₂

Human ovarian carcinoma cell line (BG1) was cultured with E₂ (1.0×10^{-11} M) and/or each pesticides (5×10^{-5} M) and the estrogenic activity was evaluated by E-CALLUX assay. The level of activity showed as the relative light units (RLU). Grey column showed the estrogenic activity of each pesticide and open column showed that of E₂ and pesticide (Pyriproxyfen, TBZ and OPP) mixture.

Rabbit IgG as a secondary antibody was used in ER- β (β). This result showed that this cell line expressed both ER subtypes, α and β . These results confirmed that ER α and β protein (Figure 3) as well as their mRNAs (Figure 1) were expressed in BG1 human carcinoma cells.

E-CALLUX assay

Human ovarian carcinoma cell line (BG1) was cultured with E₂ and/or pesticides and the estrogenic activity was calculated as the relative light units. The estrogenic activity of E₂ only was 4,653 RLU and that of each pesticide was from 2,544 to 2,762 RLU (Figure 4). On the other hand, the estrogen activity stimulated with the mixture of pesticides and E₂ was always higher than that of each pesticide or E₂ alone. These results suggested that the estrogenic activity showed the additive effects by the combination of two chemical compounds (E₂ and pesticides).

Discussion

Endocrine-disrupting chemicals play a role in the toxicity of human reproduction and development⁸⁾. Little is known about the potential risks of exposure to the known xenoestrogenic effect. Therefore, many researchers have studied the xenoestrogenic activity of chemical compounds and pesticides in

vivo and in vitro^{9,10)}. The recent discovery of a second estrogen receptor ER- β ³⁾ was distinguished from the classical ER- α and the two receptors may have different roles in gene regulation¹¹⁾. Kojima et al.¹⁰⁾ reported that organophosphorous prothiofos had a higher affinity to ER- α than ER- β , while tolclofos-methyl had a lower affinity to ER- α than ER- β in the transactivation assay. These results indicated the estrogen receptor is thought to be a key target of endocrine-disrupting chemicals. The expression of ER at the RNA level has clarified the subtypes of ER expressed in BG1Luc4E2 cells used in the E-CALUX Assay. In the results, these cells express both α and β subtype and the ratio of ER- α to ER- β was 5.7 : 1. Therefore, the estrogenic activities of the chemical compounds in this cell line reflect the activity of both ER- α and ER- β .

The additive effect in the estrogenic activity was observed with the combinations of E₂ and each of pesticides (pyriproxyfen, TBZ and OPP) (Figure 4). It has been reported that the estrogenic activities of bisphenol A (BPA) were detectable via ER- α and ER- β , but the combination of E₂ and BPA exhibited different actions depending on the subtype of ER¹²⁾. It was indicated that the action toward the subtype of ER might differ with the chemical substance. The mechanism of this phenomenon is still unknown, we

consider that the actions of pesticides to the estrogen receptor might be changed by the coexistence of the chemical compound with the strong estrogenic activity.

The tissue distribution differs between ER- α and β . The action is greatly different depending on which subtype is bound. In addition, the action might also differ when material with estrogenic activity present. We will try to evaluate the estrogenic activities of its respective subtypes, and must be investigated these activities coexistence of pesticides and other materials.

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