Screening of Endocrine Disrupting Chemicals Using a Surface Plasmon Resonance Sensor

Kazunobu ASANO,[†] Atsushi ONO, Setsuko HASHIMOTO, Tohru INOUE, and Jun KANNO

New Business Development, Biacore K.K., Koyo Bldg. 5F, 2-9-5 Shiba Koen,
Minato-ku, Tokyo 105-0011, Japan

Because concern over endocrine disrupting reactions caused by chemicals to humans and animals is growing, a rapid and reliable screening assay for endocrine disrupting chemicals is required. We have developed an *in vitro* screening assay based on a hormone receptor mechanism using a surface plasmon resonance (SPR) sensor. The interaction between an estrogen receptor α (ER) and an estrogen response element (ERE) is monitored in real time, when ER is injected over the SPR sensor chip on which a DNA fragment containing ERE is immobilized. In the presence of a chemical with estrogenic activity, the ER-ERE interaction is enhanced and the kinetic parameters are altered. We have validated the assay in terms of its specificity, dose dependency, optimal reaction conditions and reproducibility. It has been shown that the assay is very reliable as a rapid and quantitative screening method to judge the estrogenic activities of chemicals.

Introduction

Recently, concern has grown that some chemicals, such as organic chloride insecticides, plasticizers and detergents can cause endocrine disrupting effects to wild animals and humans.¹ Many of them are supposed to pose endocrine disrupting activities through direct interaction with the hormone receptors, such as estrogen receptor, thus modifying or inhibiting the physiological hormonal activities.² Chemical safety is evaluated by a set of the toxicological tests, such as carcinogenicity, teratogenicity, mutagenicity, reproduction tests. However, they have limitation to evaluate the chronic toxicity of chemicals. Moreover, the mechanisms of the endocrine disrupting activities are yet to be elucidated and the test methods to evaluate the effects are not well established.³

Several test methods have been reported to detect the endocrine disrupting activities caused by hormone receptors, *i.e.* competitive receptor binding assay, a cell growth assay using breast cancer cells expressing the estrogen receptors (MCF7),⁴ cell-based reporter assay,⁵ an *in vivo* rodent uterotrophic test,^{6,7} and a vitellogenin assay using medaka fish (*Oryzias latipes*).⁸ Many of these methods require a long time to obtain results. Furthermore, the endocrine disrupting activities can not always be detected when the chemicals are administered to the animals due to physiological regulations concerning the animal bodies. It is not easy to detect the hormonal effects of chemicals. The existing toxicological methods are not always the best way to detect the effects which have the feedback mechanism or the effects *via* the receptors. Therefore, a novel approach is sought for the rapid assessment of the endocrine disrupting activities of the chemicals.

The hormone receptors are the ligand dependent transcription factors. For example, estrogen receptor (ER) changes its

conformation upon binding of the endogenous ligand, estrogen, and binds to the specific sequence of the DNA located upstream of the target genes and activates transcription of the genes (Fig. 1). Many chemicals with diverse structures have been reported to have estrogenic activities. Due to the variety of the structure, it is unlikely that all the chemicals act with the same mechanism. Each chemical may pose a different regulatory effect on the gene expression.⁹

Recently, a surface plasmon resonance (SPR) sensor is emerging as a novel analytical instrument.¹⁰ The SPR sensor has features that it can monitor molecular interaction without labeling the molecules in real time. It is, therefore, suitable for high throughput screening assays. Compared to the existing technologies which monitor the binding amounts at the end of the interactions, the SPR sensor is unique to be able to detect the processes throughout the association and the dissociation of the interaction. This feature enables detailed analyses of chemical effects to receptors.

We have established a cell free screening assay focusing on the hormone receptor mechanism as a high throughput screening method for the endocrine disrupting chemicals. In order to measure the interaction of the biological molecules using the SPR sensor, one of the test molecules is immobilized

Fig. 1 Functional mechanism of the estrogen receptor in gene regulation.

[†] To whom correspondence should be addressed.

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Fig. 2 Scheme of the ER-ERE assay with an SPR sensor.

on the sensor chip surface and a solution containing the other molecule is passed over the sensor surface at a constant flow rate through the microfluidics. A small mass change, resulting from the binding and the dissociation of the two molecules on the sensor surface is monitored as SPR signals. The time course of the changes in the SPR signals is displayed as a curve called a sensorgram. Unlike from the conventional technologies, the SPR sensor can measure the interactions using a small amount of non-labeled samples within a short time. With regard to the interaction of the molecules, the SPR sensor can give not only the affinity of the two molecules at the equilibrium (as the dissociation constant, K_D or the affinity constant, K_A) but also the information on two molecules binding or dissociation velocity, namely the association rate constant (k_a) and the dissociation rate constant (k_d).

We have designed an assay to monitor the interaction of estrogen receptor α (ER) and the estrogen response element (ERE)¹¹ that is located in the promoter region of the estrogen target genes by immobilizing the DNA fragment containing the ERE sequence on the sensor chip and injecting purified ER over the sensor chip (Fig. 2). Thirty chemicals were tested for the estrogenic activities.

Experiments

Reagents and instruments

Reagent. Tricine, CaCl_2 , MgCl_2 , KOH, Tween 20, NaOH and HCl were purchased from Nacalai Tesque and DMSO from Sigma. Estrogen receptor (ER) was purchased from PanVera. ER was aliquoted into 5 μl and stored at -80°C . Biotinylated estrogen response element (ERE) DNA (5'-biotin-tcgagcaagtgcaggtcacagtgacgtgatcaat-3') of viterogenin gene and the anti-strand DNA have been synthesized by Nisshinbo. The synthesized oligomers were diluted with MilliQ water to 1 mg/ml and stored at -20°C . The running buffer for Biacore 3000 was prepared by filtering a solution of 25 mM Tricine, 160 mM KCl, 5 mM MgCl_2 (pH 7.8), 0.05% Tween 20.

Instrument. The assay was performed using Biacore 3000 (Biacore AB), the heat block (EYELA) and the circulator (Asone). Sensor chip SA (Biacore AB) was used. Through the assay, the sample rack of Biacore instrument was cooled to 4°C by connecting the circulator to the instrument and the reaction was run at 25°C .

Operation

Immobilization of biotinylated ERE. For the immobilization of biotinylated ERE to the sensor chip, a streptavidin preimmobilized sensor chip (Sensor chip SA) was set to the Biacore 3000 instrument and the instrument was equilibrated with running buffer. In order to stabilize the sensor surfaces, 100 mM NaOH and 50 mM HCl were injected for 30, 5 times.

Fig. 3 Dose-dependent responses of E2.

After checking the baseline stability, we performed immobilization of the biotinylated ERE. Biotinylated ERE (1 mg/ml) was diluted hundred thousand times with the running buffer. Then, 100 μl of the ERE solution was heated in boiling water for 5 min and chilled rapidly to denature the biotinylated ERE. The solution was injected over the sensor surface to immobilize approximately 60 RU onto the SA sensor chip surface. Then, the complementary ERE (1 mg/ml) was diluted to 100 times with the running buffer and denatured by the same method. This solution was injected for 2 min over the sensor surface where the biotinylated ERE was immobilized to form double stranded ERE on the surface. Biotin (1 $\mu\text{g}/\text{ml}$) was injected to block free SA on the sensor surface. A separate flow cell was used as a blank cell on which only biotin was immobilized.

Preparation of the test chemicals. Each chemical was dissolved with 100% DMSO to make 0.1 M stock solution, and stored at -80°C . Immediately before the assay, 1 μl of the stock solution was diluted 500 times using the chilled running buffer. Also ER stock solution was diluted to 40 nM using a chilled running buffer. A 50- μl volume of the ER solution and 50 μl of the chemical solution of each concentration were mixed to give final concentrations of 20 nM ER and 10 μM to 1 nM of the chemical. The samples were kept at 4°C in a sample rack to maintain the ER activity. 17β -Estradiol was used as a positive control. First, we prepared the various concentration of 17β -estradiol (1 μM to 1 nM) and measured the binding of ER to ERE (Fig. 3). As the binding activity of ER to ERE was plateaued over 100 nM 17β -estradiol, we decided to use 100 nM 17β -estradiol as a positive control in the following experiments. We also prepared a negative control solution which did not contain any chemicals. After the preparation of samples, the samples were treated at 37°C , 5 min and rapidly cooled. The samples were then set on the sample rack for measurements.

Assay of ER and ERE. The prepared samples were injected for 2 min at a flow rate of 20 $\mu\text{l}/\text{min}$ over the immobilized ERE and the blank flowcell. Injection command of "kinject" was used and the dissociation phase was monitored for 2 min. The "kinject" command is one of the injection commands specially designed for the kinetic analysis in the Biacore instrument. Upon injecting the samples using "kinject" command, the sample solution was clearly separated by two air plugs at the both ends of the sample solution from the running buffer in order to prevent the sample solution from being diluted by the running buffer. The command is also designed to monitor dissociation of the bound molecule without being disturbed by the movement of the injection needle for the set period of time. After monitoring the binding and dissociation, 100 mM NaOH and 50 mM HCl were injected 30 s each for regeneration of the sensor surfaces. All the measurements were run automatically.

Fig. 4 Data evaluation of chemical screening.

Data evaluation

A set of the assay consisted of 5 concentrations of the test chemical, a negative control (no chemical) and a positive control (100 nM 17 β -estradiol). The results were compared as the ratio to the positive control (% activation). ER was unstable and lost its binding activity to ERE during the assay period in spite of optimizing the assay conditions. We have developed an assay design to correct for any loss of the binding activity of ER over time. A positive control cycle and a negative control cycle were run at the beginning and the end of the assay. The binding responses were recorded. Based on the rate of loss in the positive and negative control samples, the binding responses of the positive and negative controls for each cycle were calculated. The enhancement of ER binding by the test chemical was expressed as the ratio to those by the positive control of 100 nM 17 β -estradiol, namely as a relative activation (% activation) using the formula and the corrected binding signals, as shown in Fig. 4.

Results

Validation of the ER-ERE assay using Biacore

In order to confirm the significance of the ER assay, the binding of ER to ERE was tested with a varying concentration of ER. The binding signals increased in relation to the increasing concentrations of ER. ER did not bind to the sensor surface where no ERE was immobilized (Fig. 5). A 1 μ M volume of BSA did not show any significant binding to ERE surfaces (Fig. 6). These observations indicate that the assay monitors the specific binding of ER to ERE. Comparing the results with 10, 20 and 40 nM ER, we often observed relatively low binding signals with 10 nM ER. Higher binding signals were obtained by adding a final concentration of 1 μ M BSA to 10 nM ER. Due to the low protein concentration, ER was lost by absorption to the surfaces of the plastic vials and tips and the actual concentration of ER became lower than 10 nM. Based on

Fig. 5 Dose-dependent responses of ER-1.

Fig. 6 Dose-dependent responses of ER-2.

Fig. 7 Reproducibility of the ER-ERE assay.

those results, we decided to run the following assays with the final ER concentration of 20 nM.

It is important to regenerate the sensor surfaces to achieve reproducible results in the Biacore assay. We have repeated 30 cycles of the assay (Fig. 7a) and monitor the end point levels (Fig. 7b). It was confirmed that the sensor surfaces were properly regenerated and the assay showed high reproducibility.

Fig. 8 Precision of the ER-ERE assay.

Fig. 9 Three types of chemical responses.

Fig. 10 Results of Bisphenol A.

The difference in the end point level was within 4 RU and the annealing level of anti-ERE was kept constant at around 25 RU throughout 30 cycles (Fig. 7c). We tested the reproducibility by repeating positive and negative controls for 16 cycles. It was shown that the results were with high precision with a CV% value of 4.68%, as shown in Fig. 8.

Screening results of 30 chemicals

We tested 30 chemicals to check the dose-dependent activation of the ER binding. One cycle of the assay took 15 min and the screening of one chemical was completed with 9 cycles in 2.5 h, including 5 different concentrations of the test chemicals, the positive and negative controls repeated twice for

each control. We calculated the relative activation (% activation) using the formula shown in Fig. 4 for 30 chemicals. Based on the values of % activation at 100 nM of each chemical, chemicals could be classified into three groups (Fig. 9): the chemicals that showed more than 50% of the activation as “high responders”, those with 20–50% as “low responders” and those less than 20% as “non-responders”. The results obtained with two independent sets of screening were summarized in Table 1. 28 out of 30 chemicals showed the same results in the first and the second screening. 17β -Estradiol and its derivatives were classified to “high responders”, while male hormones (progesterone) were “non-responders”. Bisphenol A which is regarded as one of the endocrine disruptors, was classified among “low responders” (Fig. 10).

Furthermore, the differences in the effect of the chemicals on the ER binding activities were observed in the different shapes of the sensorgrams among those of 17β -estradiol, bisphenol A, 17α -estradiol, diethylstilbestrol (DES), tamoxifen and progesterone (Fig. 11). We have plotted the binding level at the end of the injection of ER in the presence of 1 μ M of the test chemical (Y axis) versus the binding stability 2 min after the end of the ER injection (X axis), as shown in Fig. 12. We found that the agonists and the antagonists had significantly different patterns. The antagonists (such as tamoxifen) had a tendency to stabilize the binding of ER to ERE. The assay using Biacore indicated the possibility not only to detect the estrogenic activities of the chemicals, but to distinguish the antagonists from the agonists.

Fig. 11 Different kinetic patterns in the ER-ERE interaction.

Table 1 Results of ER-ERE screening with SPR sensor

No.	CAS No.	Name	1st Screening	2nd Screening
1	000050-28-2	Estradiol	H	H
2	000057-91-0	Estra-1,3,5(10)-triene-3,17-diol (17 α)	H	H
3	000053-16-7	Estrone	H	H
4	000057-63-6	19-Nor-17-alpha-pregna-1,3,5(10)-trien-20-yne-3,17-diol	H	H
5	000362-05-0	Estra-1,3,5(10)-triene-2,3,17-beta-triol	H	H
6	000362-07-2	Estra-1,3,5(10)-triene-3,17-diol, 2-methoxy-, (17 β)-	L	L
7	000068-22-4	19-Nor-17-alpha-pregn-4-en-20-yn-3-one, 17-hydroxy-	L	L
8	000063-05-8	Androst-4-ene-3,17-dione	N	N
9	000057-83-0	Progesterone	N	N
10	000501-24-6	3-Pentadecylphenol	N	N
11	005153-25-3	Benzoic acid, 4-hydroxy-, 2-ethylhexyl ester	N	L
12	001034-01-1	Gallic acid, octyl ester	N	N
13	006807-17-6	4,4'-(1,3-Dimethylbutylidene)bisphenol	L	L
14	027955-94-8	Phenol, 4,4',4"-ethylidynetri-	N	N
15	000081-92-5	Benzenemethanol, 2-[bis(4-hydroxyphenyl)methyl]-	L	L
16	000081-90-3	<i>o</i> -Toluic acid, .alpha.,.alpha.-bis(p-hydroxyphenyl)-	N	N
17	000978-86-9	4-(Triphenylmethyl)phenol	L	L
18	062625-31-4	Phenol, 4,4'-(3H-1,2-benzoxathiol-3-ylidene)bis 3-methyl-, S,S-dioxide, monosod	N	N
19	005384-21-4	Phenol, 4,4'-methylenebis[2,6-dimethyl-	L	L
20	005613-46-7	2,6-Xylenol, 4,4'-isopropylidenedi-	L	L
21	000084-16-2	Phenol, 4,4'-(1,2-diethylethylene)di-, meso-	H	H
22	000084-17-3	Phenol, 4,4'-(diethylideneethylene)di-	L	H
23	56-53-1	diethylstilbestrol	H	H
24	006893-02-3	Alanine, 3-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl)-, L-	N	N
25	000500-38-9	Nordihydroguaiaretic acid	N	N
26	023239-51-2	Benzyl alcohol, p-hydroxy-alpha-(1-(p-hydroxyphenethyl)amino)ethyl-, hydrochlo	N	N
27	001050-28-8	L-Tyrosine, N-L-tyrosyl-	N	N
28	000145-50-6	1(4H)-Naphthalenone, 4-.alpha.-(4-hydroxy-1-naphthyl)benzylidene -	L	L
29	000446-72-0	Genistein	L	L
30	000080-05-7	Bisphenol A	L	L

H, High responder; L, low responder; N, non-responder.

Fig. 12 Binding level vs. binding stability plot.

Discussion

We established a cell free screening method while focusing on the mechanism of the hormone receptor using a surface plasmon resonance sensor. We developed an assay method to detect estrogenic activities of the chemicals with changes in the binding level of ER to ERE by preincubating the chemicals with ER.

It was also suggested that the agonists and the antagonists had different effects on the interaction of ER and ERE from an analysis of the binding level of ER during the association and dissociation processes. With the conventional end point assay used to monitor only the binding signals, it was impossible to distinguish the agonists from the antagonists. The real time analysis, which is the main feature of the surface plasmon resonance sensor enabled the classification of the agonists and the antagonists. When running the cell based hormone assay, it must be taken into account any unexpected effects of the chemicals to the other components than the receptors of the cells. On the other hand, the cell free assays simply show the

direct effects of chemicals to the receptor-signal transduction systems. Our new assay, based on the hormone receptor mechanism, can rapidly screen a large number of the chemicals for their hormonal activities.

Since other hormone receptors employ similar mechanism as ER for the activation of the gene expression, it is possible to develop same assays for other hormone receptors. A newly developed ER assay is both reliable and efficient as a primary screening method of chemicals for estrogenic activities.

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