PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 141–148

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Kinetic analysis of estrogen receptor homo- and heterodimerization in vitro☆

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Abstract

The coexistence of ERα and ERβ suggests that active receptor complexes are present as homo- or heterodimers. In addition each of three forms of active receptors may trigger different cellular responses. A real-time biosensor based on surface plasmon resonance was used as instrument to determine binding kinetics of homo- and heterodimerization of estrogen receptor α and β. Partially purified full-length estrogen receptor α was expressed intracellularly as a C-terminal fusion to a hexa-histidine tag using the baculovirus-expression system. Purified estrogen receptor α and β without tags were used as partners in the dimerization process. An association rate constant of 3.6×10^3 to 1.5×10^4 M^{-1} s^{-1} for the homodimer formation of ERα and 5.7×10^3 to 1.5×10^4 M^{-1} s^{-1} for the heterodimer formation was found assuming a pseudo first-order reaction kinetic. The equilibrium dissociation constant for homodimerization of ERα was 2.2×10^{-8} to 5.4×10^{-8} and 1.8×10^{-8} to 2.6×10^{-8} M for the heterodimer formation. The homo- and heterodimer formation was characterized by a slow association kinetics and kinetic rate constants were within the same range.

Keywords: BIACORE; Human estrogen receptor α and β; Homodimer; Heterodimer; 17β-Estradiol

1. Introduction

Estrogen receptors are ligand-activated transcription factors located in the nucleus of target cells. They mediate the effects of estrogens by binding specific DNA sequences called estrogen response elements and subsequently activating target gene transcription [1,2]. These effects result as an interaction of two estrogen receptor subtypes called ERα and ERβ. Since a second estrogen receptor subtype called ERβ has been discovered [3–5], investigations in designing potent and potential ligands was further extended. Estrogens and estrogen-like compounds can bind to ERs inducing homo- and/or heterodimerization. Upon ligand binding ERs forms dimers in vivo [6,7]. In contrast, in the absence of ligand accessory proteins such as heat-shock proteins enclose the ER in order to stabilize the unbound and therefore inactive receptor [8]. Upon addition of ligand the heat-shock protein complex is shed off and activated ER binds to DNA and activates target gene transcription [8–13]. Estrogens induce dimerization of the receptors which subsequently bind to the ERE as dimers. Various gel shift experiments have been performed to confirm this hypothesis [14,15]. In addition further techniques such as a yeast two-hybrid system [7], fluorescence anisotropy [16] and BIACORE technology [17] have been employed to confirm homodimer binding of ERα to ERE. Applying an antibody-based DNA binding assay it was demonstrated that ERα bound its ERE as a monomer [18]. Antiestrogens and selective estrogen receptor modulators were shown to act differently on ERα dimerization either not influencing dimerization [18–21] or altering this process [17,22,23]. The existence of ERβ complicated this unclear situation even more by the formation of homo- and heterodimers [5,24,25]. The transcriptional response of ER is further influenced by various of co-activators and -repressors [26–31].

Receptor, dimerization is a crucial step in the effect of a therapeutic ligand. Since antiestrogens and selective estrogen receptor modulators were shown to influence receptor dimerization, assessment of their association and dissociation constants may represent an easy tool to distinguish between ligands exerting their function via homo- or heterodimers. The BIACORE system has been selected as the best suited instrument for determination of parameters of binding kinetics and equilibrium binding constants and become a standard technique [32–35]. The BIACORE system

Abbreviations: ERE, estrogen response element; E_2, 17β-estradiol; ERα, estrogen receptor α; ERβ, estrogen receptor β; SPR, surface plasmon resonance

☆ Poster paper presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, "Recent Advances in Steroid Biochemistry and Molecular Biology", Munich, Germany, 17–20 May 2002.

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0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00023-2

utilizes the optical principle of the so-called "surface plasmon resonance" for determination of adsorption/desorption processes in real-time. Plane polarized light is totally internally reflected from the gold-coated sensor chip where the molecular interaction takes place. SPR in the gold layer results in extinction of the reflected light at a specific angle varying with the refractive index of the solution. Upon binding of molecules onto the chip, the refractive index changes and this effect can be monitored as a change in the SPR angle.

Serially ligand and the receptor partner, ERα or ERβ, are injected onto the sensor chip. Binding of this partner to immobilized baculo-expressed and partially purified his-tagged ERα occurs simultaneously with its homodimerization. Assuming that these homodimers are not capable of binding to immobilized ERα this reaction will not interfere with the kinetic assessment of heterodimers. Dissociation rate (k_d) and association rate constants (k_a) for formation of the heterodimer complex can be simply extracted by approximating the dissociation phase using a pseudo first-order kinetic reaction. When different concentrations are injected, then R_{max} is depending upon different ERβ monomer and ERβ/ERβ homodimer concentrations. Following equilibrium conditions will be obtained:

$$\frac{^{*}\mathrm{ER}\alpha}{\mathrm{ER}\beta} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{ER}\beta \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \frac{\mathrm{ER}\beta}{\mathrm{ER}\beta}$$

The immobilized reaction partner is indicated by an asterisk.

In our experiments we demonstrate that ERα is presented as a homodimer in crude insect cell extracts. We determined the association rate and dissociation rate constants of ERα/ERα and ERα/ERβ formation in the presence of 17β-estradiol.

2. Materials and methods

2.1. Plasmids and host cells

Spodoptera frugiperda cells (Sf9, CRL 1711; ATCC, Rockville, MD, USA) were transfected with pVL1392-UERaHis6 encoding the human ERα gene fused to a hexa-histidine tag. The ERα-gene is expressed as a fusion to ubiquitin [36–38]. Viruses were isolated and plaque-purified by standard procedures (18). Sf9 cells were infected with virus at an m.o.i. of 1 for 72 h and harvested by centrifugation (173 × *g*, 5 min Hereaus Megafuge 1.0). Cells were resuspended in lysis buffer Tween 20 containing 20 mM Tris–HCl, pH 7,5, 150 mM NaCl, 10 mM Na_2MoO_4, 10% glycerol, 10 mM monothioglycerol, 10 μM PMSF, 10 μM TPCK and 10 μM TLCK. Urea (1 M) was added for enhanced yield. Lysis was either carried out by homogenization using a Polytron PT1200C (Novodirect, Germany). Supernatants were harvested and processed for further analysis. All procedures were carried out at 4 °C.

2.2. Ligand binding assay

ER-activity was quantified by radioligand binding assay. Extracts of ERα and ERβ expressed in Sf9 cells were incubated with 3.96 nM [^{3}H]E_2 for 16 h at 4 °C. In parallel non-specific binding was determined by adding a 300-fold excess of diethylstilbestrol. Casein (1 mg/ml) was used as a carrier protein in the reaction buffer. Dextran-coated charcoal (300 μl) was added to remove unbound [^{3}H]E_2, incubated for 15 min at 4 °C and finally removed by centrifugation. A volume of 100 μl of the supernatant were counted by scintillation in a Beckman Scintillation counter.

2.3. Kinetic analysis using BIACORE technology

NTA chips were used to bind histidine-tagged ERα. To saturate the NTA with nickel a 1 min impulse of Ni-solution containing 500 μM $NiCl_2$ in eluent buffer was injected at 20 μl/min. Partially purified ERα was diluted in eluent buffer to a final concentration ranging between 58 nM and 580 nM.

The analysis was performed at a flow-rate of 2 μl/min using 10 mM HEPES pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.005% Tween 20 (HBS-E buffer) as eluent buffer. As an extra buffer to increase the efficiency of the autosampler HBS-E buffer was used containing 3 mM EDTA (HBS-D). Purified ERα and ERβ (Panvera, Madison, WI) were employed at various concentrations in eluent buffer indicated in the respective experiment. For experiments testing the influence of salt on dimer formation samples were incubated in eluent buffer supplemented with 1 M NaCl and incubated at 4 °C for 60 min. After each protein injection proteins and Ni^{2+} were stripped by a three 1 min pulse of regeneration buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 350 mM EDTA, 0.005% Tween 20). During all experiments the sample racks were cooled to 10 °C, the IFC was incubated at 25 °C.

Data were analyzed using BIAEvaluation Software 3.1 based on the so-called Marquardt–Levenberg algorithm for fitting of the experimental data. It optimizes parameter values by minimizing the sum of the squared residuals for characterization of rate constants:

$$S = \sum_{1}^{n} (r_f + r_x)^2 \quad (1)$$

where S is the sum of squared residuals, r_f the fitted value at a given point and r_x the experimental value at the same point. The k_a and k_d were fitted separately applying a first-order association and dissociation kinetic. The model assumes a 1:1 interaction of ligand and ligate. The integrated form of the equation describing the dissociation is:

$$R = R_0\, e^{-k_d(t-t_0)} + \mathrm{Offset} \quad (2)$$

where k_d represents the dissociation rate constant (s^{-1}), R_0 the response at the start (RU), t_0 the time at start (s) and Offset is the response at infinite time.

The association phase of a 1:1 interaction is described by the following equation:

$$R = R_{eq}(1 - e^{-(k_a C + k_d)(t - t_0)}) + RI \quad (3)$$

with

$$R_{eq} = \frac{k_a C}{k_a C + k_d} \times R_{max} \quad (4)$$

The k_a describes the association rate constant ($M^{-1}\,s^{-1}$), R_{max} the maximum analyte binding capacity (RU), C the analyte concentration (M), t_0 the injection start time (s) and RI the bulk refractive index contribution (RU). R_{eq} represents the steady-state binding level.

3. Results

3.1. Partially purified his-tagged ERα binds specifically onto Ni-NTA chips

ERα was expressed as a C-terminal fusion to a hexa-histidine tag in order to facilitate immobilization onto an NTA sensor chip. Baculo-infected Sf9 cells were harvested by centrifugation and cell extracts prepared by homogenization with a Polytron PT1200C. Extracts were clarified by centrifugation and ligand binding activity assessed by radioligand binding assay. ERα was partially purified by heparin–sepharose affinity chromatography and eluates used for BIACORE experiments. Activities of eluates ranged between 5.7 and 9.0 pmol/ml.

A volume of 30 μl of undiluted heparin-purified ERα (7.4 pmol/ml) was bound to the Ni^{2+}-saturated surface of an NTA sensor chip and a specific association curve could be observed. Bound ERα was confirmed by injection of a monoclonal antibody AER320 2 μg/ml (Fig. 1). In addition an ERα-positive eluate was compared to an ERα-negative one. As expected the response in RU of the inactive eluate was negligible in comparison to the active fraction (data not shown).

3.2. Recombinant ERα is presented as a monomer

From association and dissociation behavior of ERα bound to an NTA surface it cannot be distinguished if the receptors are present as monomers or homodimers. Therefore, an approach was chosen where possible homodimers should be annihilated. This process would lead to a decrease of bound protein on the sensor chip-surface since free monomer is liberated. Cell extracts were incubated at 85 °C for 5 min and an aliquot injected onto the sensor chip. No association could be measured after this treatment suggesting that this harsh treatment completely denatured ERα. Therefore, various dilutions of cell extracts (740, 370 and 74 fmol/ml) were incubated in eluent buffer containing 1 M NaCl at 4 °C. This concentration of salt is supposed to be high enough to prevent homodimer formation. The binding behavior of samples incubated with high concentrations of NaCl showed a remarkable different binding curve (Fig. 2). The response curve reached a peak that already decreased during injection. This may be due to NaCl altering the binding behavior on the chip surface. In addition when sample without NaCl

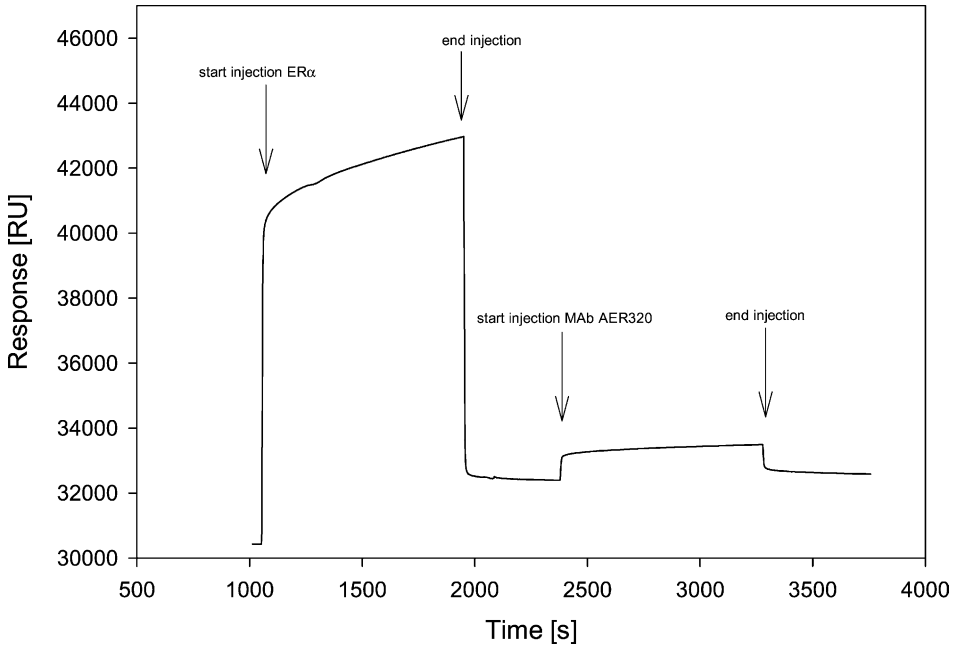


Fig. 1. Serial injection of his-tagged ERα and AER320. ERα was bound onto a Ni^{2+}-saturated NTA surface and subsequently recognized with specific anti-ERα monoclonal antibody. Association curves confirmed binding of antibody to ERα. Data were collected at 1 Hz and analyzed using BIAEvaluation Software 3.1.

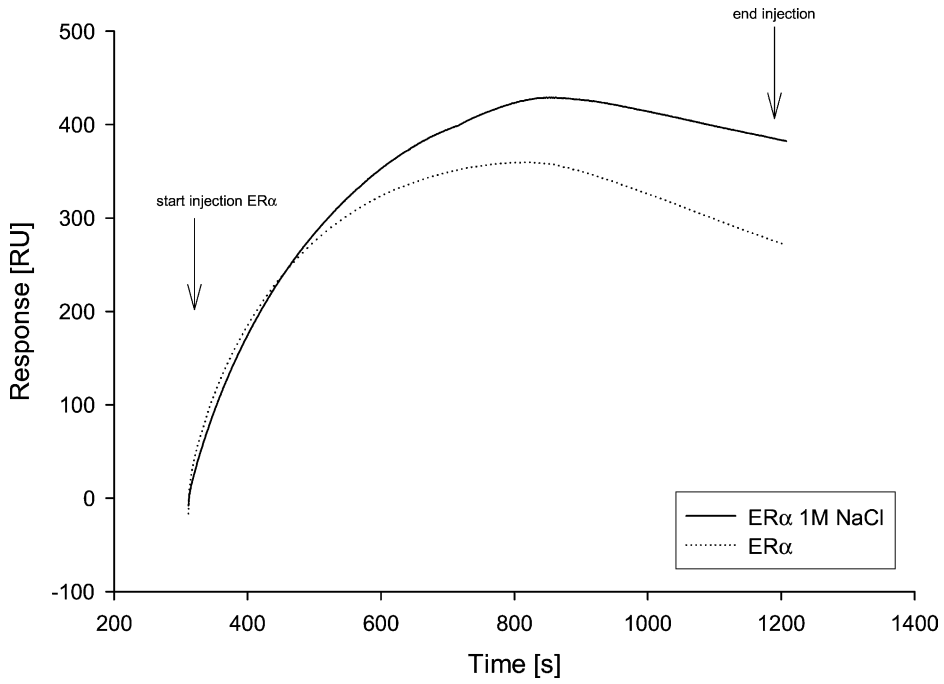


Fig. 2. Assessing ERα as monomers or homodimers. ERα was bound onto a Ni^{2+}-saturated NTA surface in the presence and absence of 1 M NaCl. ERα was incubated with 1 M NaCl at 4 °C. Association curves and absolute response units bound were compared and suggest the existence of ERα as monomers in insect cell extracts. Data were collected at 1 Hz and analyzed using BIAEvaluation Software 3.1.

was injected the same effect could be observed. Remainders of NaCl might be still present inducing the same effect and therefore supporting this assumption. However, the amount of bound protein was reduced up to 30% when 370 fmol/ml his-tagged ERα was first incubated with 1 M NaCl. Although a decrease could be observed this is suggested to be due to interference of salt with the chip surface. The reduction is not consistent enough over a range of various ERα concentrations (74 and 740 fmol/ml) leading to the conclusion that ERα exists as monomers in partially purified insect cell extracts.

3.3. Estrogen is necessary for homo- and heterodimerization

The fact that ERα is present as a monomer in insect cell extracts enables to determine association and dissociation binding kinetics of homo- and heterodimerization in the absence and presence of ligand. The only competing process taking place is the homodimerization of the ER partner. Without estrogen neither homo- nor heterodimerization could be observed confirming that ligand was necessary for dimerization of steroid hormone receptors. E_2 (10 nM) in

Table 1
Determination of rate and equilibrium constants for ERα/ERα and ERα/ERβ interactions using real-time biosensor technology

	ERα/ERα (nM)			ERα/ERβ (nM)	
	580	290	116	300	150
(A) k_a ($M^{-1}\,s^{-1}$)					
Coinject	5.1E+3 ± 1.2E+3	3.6E+3 ± 2.0E+3	3.6E+3 ± 5.1E+2	5.8E+3 ± 4.9E+3	5.7E+3 ± 7.2E+2
Serial	3.9E+3 ± 8.8E+2	7.8E+3 ± 2.3E+3	1.5E+4 ± 1.7E+3	1.1E+4 ± 1.4E+2	1.5E+4 ± 1.9E+3
(B) k_d (s^{-1})					
Coinject	1.1E−4 ± 2.5E−5	1.4E−4 ± 3.5E−5	3.1E−4 ± 1.9E−4	1.7E−4 ± 7.5E−5	1.7E−4 ± 5.9E−5
Serial	1.1E−4 ± 7.0E−6	1.4E−4 ± 2.6E−5	4.0E−4 ± 2.0E−4	1.7E−4 ± 1.0E−4	1.5E−4 ± 4.5E−5
(C) K_A (M^{-1})					
Coinject	4.4E+7	4.1E+7	3.3E+7	5.9E+7	1.2E+8
Serial	4.1E+7	5.3E+7	4.1E+7	8.0E+7	8.8E+7
(D) K_D (M)					
Coinject	2.7E−8	5.0E−8	5.4E−8	2.5E−8	2.6E−8
Serial	2.6E−8	2.2E−8	5.4E−8	1.8E−8	1.8E−8

(A), association rate constant k_a; (B), dissociation rate constant k_d; (C), equilibrium association constant K_A; (D), equilibrium dissociation rate constant K_D.

eluent buffer was used in experiments when dimerization was performed in the presence of ligand. Shortly before the sample was injected 30 μl of ligand solution at a flow rate of 2 μl/min was injected onto the surface. Retardation and blending of this pulse should assure the presence of E_2 when the sample to bound was injected. For this purpose either an automatical coinjection step was performed compared to a manually operated serial injects of first E_2 buffer and then sample. For a control experiment, eluent buffer replaced the first injection of the respective receptor.

From these data some interesting information can be extracted. From BIAEvaluation Software 3.1 association rate

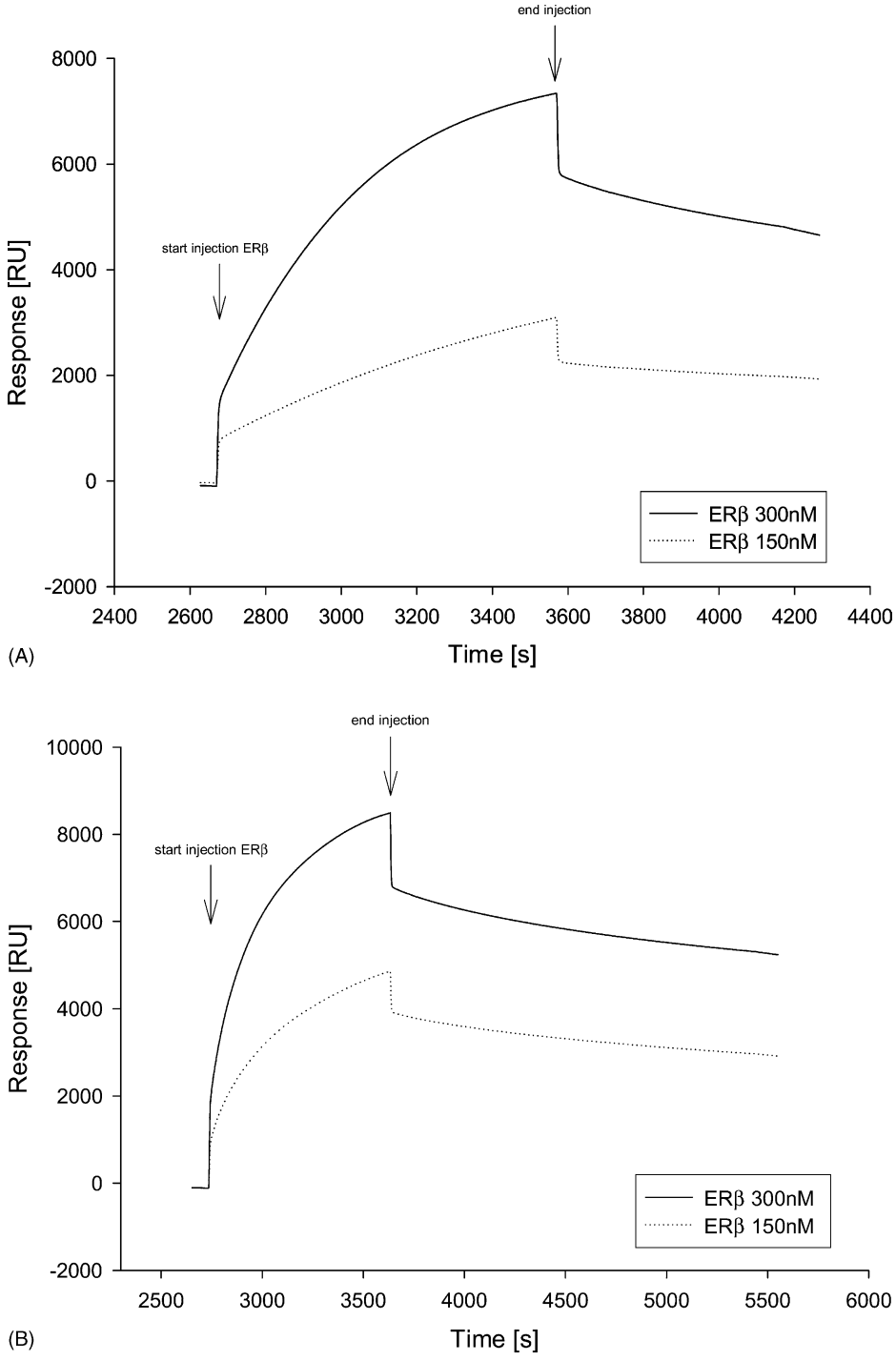
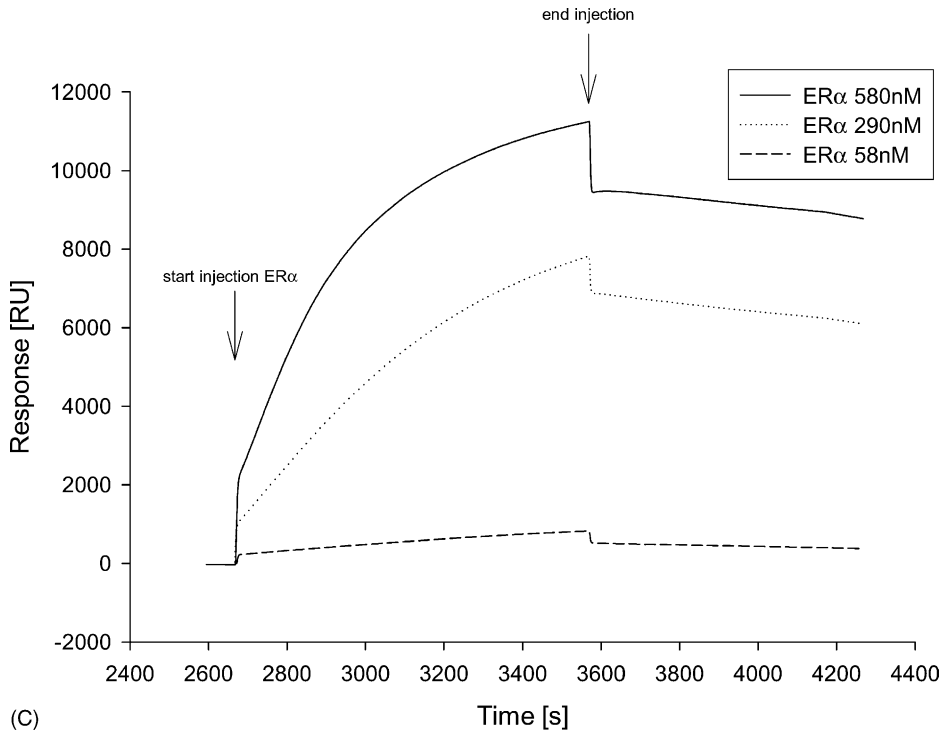
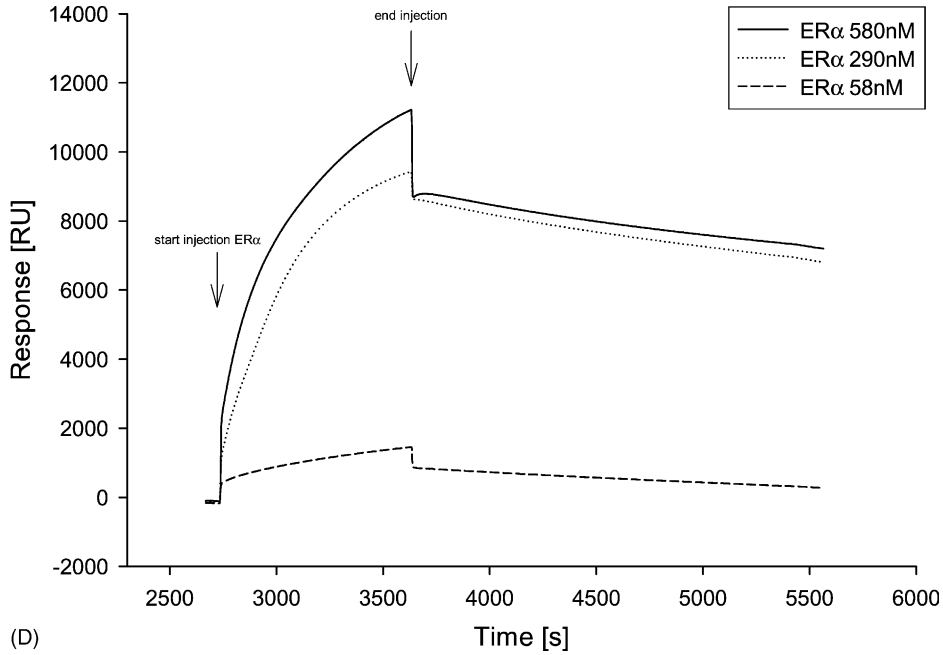


Fig. 3. Association and dissociation constants were determined for ERα and ERβ binding to immobilized ERα. Recombinant purified ERα and ERβ were injected onto a biosensor surface immobilized with his-tagged ERα at various concentrations: (A) ERβ was coinjected with 10 nM E_2; (B) 10 nM E_2 and ERβ were injected serially; (C) ERα was coinjected with 10 nM E_2; (D) 10 nM E_2 and ERα were injected serially. Data were collected at 1 Hz and analyzed using BIAEvaluation Software 3.1.



(C)



(D)

Fig. 3. (*Continued*).

constants k_a in the range of 3.6×10^3 to $1.5 \times 10^4\ M^{-1}\ s^{-1}$ for ERα and 5.7×10^3 to $1.5 \times 10^4\ M^{-1}\ s^{-1}$ for ERβ could be measured when dimerization with immobilized his-tagged ERα was employed. In general higher association rate constants were obtained when E_2 and sample were injected in a mode where first ligand and afterwards the receptor sample was injected. This indicates faster binding of ligand when E_2 had more time to bind immobilized ERα than when it was coinjected with the partner receptor. Absolute k_a-values for ERα and ERβ were found in the same range over the concentrations tested (Table 1A). The dissociation rate constants k_d varied between 1.1×10^{-4} to $4.0 \times 10^{-4}\ s^{-1}$ for ERα and 1.5×10^{-4} to $1.7 \times 10^{-4}\ s^{-1}$ for ERβ (Table 1B).

Consequentially equilibrium dissociation constants could be calculated exhibiting dissociation constant values of 2.2×

10^{-8} to 5.4×10^{-8} M for ERα and 1.8×10^{-8} to 2.6×10^{-8} M for ERβ (Table 1C and D, Fig. 3).

4. Discussion

In the present study, both homo- and heterodimerization between immobilized ERα and the respective partner exhibit rather low binding affinities presenting equilibrium dissociation constants K_D in a range of 1.8×10^{-8} to 5.4×10^{-8} M. Real-time analysis using a BIACORE 2000 was used for these studies. This technique has been implemented for determining the kinetic interaction of heterodimerization between bone morphogenetic proteins exhibiting relatively fast association rate constants in the range of $3.8\times10^{4}\,M^{-1}\,s^{-1}$ [39]. For the Vitamin D receptor it was shown that SPR-technology was suitable to measure kinetic constants [40]. 1,25-Dihydroxyvitamin D3 binding favors both VDR–RXR heterodimerization, but conversely, the ligand reduces VDR homodimerization in solution. These ligand-dependent influences also alter DNA binding. The K_D-values for VDR–RXR heterodimerization resulted in 2.8×10^{-7} M, for VDR–VDR homodimerization values of 4.2×10^{-7} M were measured. For ER receptors it was shown that ligands modulate interaction with its ERE [17,23]. These ligands altered homo- and heterodimerization of ERs and subsequently affected DNA binding. Many studies have been performed determining binding behavior between steroid receptors an their co-activators in the presence and absence of various ligands using SPR-technology [8,41–43].

Our results demonstrate that using the baculovirus expression system native ERα monomers could be obtained (Fig. 2). Incubation with high concentration of NaCl confirmed this assumption. The presence of high concentrations of salt is a mechanism by which dimers can be converted into monomers. For glucocorticoid receptors it was shown that in fish living in salt water compared to freshwater adapted fish the majority of receptors are presented in the monomeric form [44]. In order to gain information of the molecular mechanisms by which ligands modulate responses of ERs, the dimerization process is a crucial step. Ligands even influence dimerization as can be assumed from altered DNA-binding behavior. We immobilized one partner of this dimerization procedure, and measured the change in refractive index for the respective partner in the presence of E_2. The K_D-values in the range of 1.8×10^{-8} to 5.4×10^{-8} M were obtained consisting of association rate constants from 3.6×10^{3} to $5.8\times10^{3}\,M^{-1}\,s^{-1}$ for both ERα and ERβ (Fig. 3 and Table 1A and D). This indicates a relatively slow association rate resulting in a low affinity of the receptors to each other. Somehow it was remarkable that different injection modes of ligand and receptor resulted in different association rate constants. This change occurs in the same dimension of k_a-values, but an up to two-fold increase was observed when E_2 was incubated with the immobilized ERα for a longer time (Table 1B). An explanation for this effect can be that due to longer incubation of E_2 with the immobilized ERα, a conformational change of ERα takes place. This structure represents a better binding partner for the respective receptor resulting in faster binding. Consequently equilibrium dissociation rates decrease showing tighter binding when the receptor is able to obtain a more appropriate conformation.

These studies enable fast analysis of homo- and heterodimerization between ER molecules. This technique is a fast and universal tool to screen for new ligands designed to bind to one receptor-dimer-subtype. Such ligands called selective estrogen receptor modulators play an important role in the treatment of endocrine-related diseases.

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