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Transcriptional activities of estrogen receptor alpha and beta in yeast properties of raloxifene

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Abstract

Raloxifene represents a potent compound for the prevention and treatment of osteoporosis and cardiovascular disease in postmenopausal women. Raloxifene exhibits targeted antiestrogenicity in breast and uterus, but acts as an agonist in bone and liver. This synthetic selective estrogen receptor modulator binds both estrogen receptors α and β. The molecular mechanisms by which raloxifene exerts agonistic or antagonistic activity are still not resolved. Therefore, the binding behavior of raloxifene to estrogen receptors and its effects on DNA binding and transactivation were studied. The equilibrium binding affinity of raloxifene by displacing radiolabeled 17β-estradiol exhibited a similar affinity behavior to that of its natural ligand. Using BIACORE technology with an immobilized estrogen response element, we showed that 17β-estradiol and raloxifene increased the binding of estrogen receptor α to the DNA, suggesting a ligand-dependent dimerization. The influence of the ligands to the binding of estrogen receptor β was lower. We may conclude that unliganded estrogen receptor α binds as a monomer whereas in the presence of 10^{-8} M 17β-estradiol or higher, homodimers are formed that interact with the estrogen response element. Transactivation studies in a yeast reporter system in a ligand-dependent manner resulted in a similar potency of raloxifene to estrogen receptor β compared to the control testosterone. Subeffective doses of raloxifene combined with 17β-estradiol did not shift the efficiency, whereas saturating concentrations of 17β-estradiol combined with increasing concentrations of raloxifene altered the response induced by 17β-estradiol. In this pure system, the antagonistic activity of raloxifene could not be detected as was expected by the results from ligand competition analysis.

Keywords: Raloxifene; Transcription; Agonist; Antagonist; Human estrogen receptor α and β; BIACORE

1. Introduction

Estrogens exert a great impact on the development and maintenance of reproductive tissues as well as on the regulation of cellular processes unrelated to reproduction, such as bone density, the cardiovascular system, and the central nervous system [1]. The onset of menopause causes a deficiency in estrogens, resulting in a loss of bone density [2]. Estrogen replacement therapy counteracts this decrease in estrogens, relieving symptoms of menopause such as hot flushes, sweating, and a rapid decrease in bone loss. Further beneficial effects on the cardiovascular and central nervous systems are ascribed to these hormone mimics. Nevertheless, the relative risk of breast cancer is increased in women taking estrogen replacement therapy [3].

SERMs are being developed to function in a tissue-selective manner. Until recently, various SERMs have been synthesized, e.g. idoxifene, droloxifene, CP336-156, GW5638, and LY353381; the most prominent representatives are tamoxifen and RAL, although they are not specifically designed as SERMs [1]. Tamoxifen is a non-steroidal antiestrogen, frequently used as an adjuvant agent in breast cancer therapy as well as an agent decreasing the incidence of collateral breast cancer [4–7]. Tamoxifen can function as a tissue-selective estrogen agonist/antagonist, but its uterotrophic activity results in increased risk for developing uterine cancer [8]. In 1998, the new SERM RAL was approved by the FDA for prevention of osteoporosis in postmenopausal women. RAL acts as an estrogen agonist in bone and liver, but lacks uterotrophic activity [9]. Crystal structure

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Abbreviations: ERE, estrogen response element; E2, 17β-estradiol; RAL, raloxifene; ERα, estrogen receptor α; ERβ, estrogen receptor β; and SERM, selective estrogen receptor modulator.

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studies of ERα and ERβ either liganded to E2 or RAL have been performed, suggesting a distinct conformation in the transactivation domain of the ligand-binding domain, providing structural evidence of the mechanism of antagonism [10,11]. Further differences in the relative ligand-binding affinities and tissue distribution of SERMs to both estrogen receptor subtypes are likely to contribute to either agonistic or antagonistic action in different tissues [12]. Using yeast as a bipartite transactivation system [13], the agonistic behavior of RAL at high concentrations in respect to ERα and ERβ was assessed in this study. Differences between various SERMs in terms of their antagonistic/agonistic behavior on the two receptor subtypes were detected [14]. An ERE-reporter gene assay also resulted in slight agonistic activity of RAL for ERα. High concentrations of RAL were not able to antagonize nanomolar concentrations of E2 [15]. Real-time biosensor analysis was performed measuring kinetic constants of ER–ERE interaction. Complexes formed with no ligand or E2 appeared to be less stable than those with RAL, although E2 induced the formation of a larger number of ER–ERE complexes [16].

In order to gain more insight into the molecular mechanisms of RAL binding to ERα and ERβ, we compared the binding properties of RAL to the receptors and its transactivational activity. We investigated the properties of RAL exclusively on ERα and ERβ using *Saccharomyces cerevisiae* to overexpress hERα [17–19] and full-length hERβ [20] fused to ubiquitin for ligand competition binding and transactivation analysis. From ligand competition data, one would expect transactivational properties of RAL comparable to E2, which could not be verified. Our results demonstrate that in the presence of E2 ERα is able to form homodimers which subsequently bind to ERE. This effect could not be observed on ERβ. RAL also influenced ERβ interaction, but this effect seems to be dependent upon ligand concentration.

2. Materials and methods

2.1. Materials

Buffer reagents and dextran-coated charcoal were purchased from Merck or Sigma and [^{3}H]17β-estradiol from NEN Life Science Products. For yeast media preparation, yeast nitrogen base was purchased from Difco, aminoacids and uracil from Serva Feinbiochemica and adenine from Sigma. 17β-estradiol and testosterone were obtained from Sigma, RAL was a gift from Eli Lilly. Glass beads (0.25–0.5 mm diameter) were purchased from Merck. For studies with BIACORE instrument, SA chips were obtained from BIACORE AB and ERα and ERβ from Panvera.

2.2. Plasmids and yeast strains

BJ3505, a protease-deficient *Saccharomyces cerevisiae* strain, was transformed either with YEpE12 encoding the human ERα gene or YEpFERβH3 encoding the full-length human ERβ gene fused to the FLAG tag [21]. Both genes are expressed as a fusion to ubiquitin [17–19]. Yeast extracts were used for competition binding analysis. For all transactivation assays, the yeast strain 188R1, a derivative of RS 188N [22], was used. The strain was transformed with YEpE12 or YEpFERβ, then a β-galactosidase reporter plasmid YRpE2 was introduced [19,22]. Transformation of yeast cells was performed as described previously [19,23].

2.3. Competition assay

Extracts of yeast-expressed ERα and ERβ were incubated with 3.96 nM [^{3}H]E2 and increasing amounts of RAL for 16 hr at 4°. Ratios of RAL to [^{3}H]E_2 were in the range of 0.001- to 100-fold. 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 uL) was added to remove unbound [^{3}H]E_2, incubated for 15 min at 4°, and finally removed by centrifugation. One hundred microliters of the supernatant was counted by scintillation in a Beckman scintillation counter.

2.4. Kinetic analysis using BIACORE technology

Wild-type ERE (5′ GTCCAAAGTCAGGTCACAGT-GACCTGATCAAAGTT 3′) was conjugated with biotin at the 5′ prime end (MWG-Biotech AG). Single-stranded oligonucleotides were annealed by heating at 95° for 5 min, followed by a cooling process at room temperature overnight. For immobilizing, 1 μM ERE was injected onto the surface of a streptavidin-coated sensor chip for 4 min at a flow rate of 5 μL/min.

The analysis was performed at a flow rate of 10 μL/min using 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.01% Tween 20 (TNMT buffer). Two hundred microliters of ERα and β was employed at concentrations of 24 nmol/L in TNMT buffer. Incubation with ligand was carried out at 4° for 45 min. Bound sample was washed with buffer for an additional 800 sec. After each protein injection, the surface was regenerated by two one-minute pulses of 0.1% SDS. All experiments were performed at 25°.

Data were analyzed using BIAEvaluation Software 3.1 relying on the so-called Marquardt–Levenberg algorithm. 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 is the fitted value at a given point, and r_x is the experimental value at the same point. 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

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

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

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

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

with

$$R_{eq} = \frac{k_aC}{k_aC + k_d} * R_{\max} \quad (4)$$

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

2.5. Transactivation assays

Transactivation assays were performed as described previously [19]. The transactivation test was performed in 5-mL cultures (in 50-mL jars), and the overnight culture was then diluted to $OD_{600} = 0.5$. hER expression was induced by addition of 10 μmol/L of $CuSO_4$. For all preparations, the same volume of dimethylsulfoxid was added to the yeast cultures. After inducing for 4 hr at 30° and 150 rpm, the cells were extracted. Fifty microliters of dimethylsulfoxid alone was used as a blank. A calibration curve was made with E2. For testing the synergism between RAL and E2 on ERα, RAL and E2 were mixed in two ways. First (a), RAL and E2 were applied simultaneously to the yeast test system and incubated for 4 hr (after inducing). Second, (b), RAL was added 20 hr before induction and incubated with E2 for 4 hr.

2.6. Curve fitting

Data derived from transactivation assays were fitted using a logistic dose–response equation to approximate the concentration-dependent effect of a ligand on transactivation. Curve 2D software (Jandel Scientific) was used for calculation. The function is described as

$$Y = a + \frac{b}{1 + \left(\frac{x}{c}\right)^d} \quad (5)$$

where parameter a equals the baseline and b the plateau of the curve designated as the ligand efficiency. Parameter c gives the transition center and equals the ligand potency, which is the concentration that causes 50% efficiency. Absolute values or values normalized in respect to maximal response from at least two independent experiments were fitted. Each ligand concentration step was done in duplicate for transactivation assays.

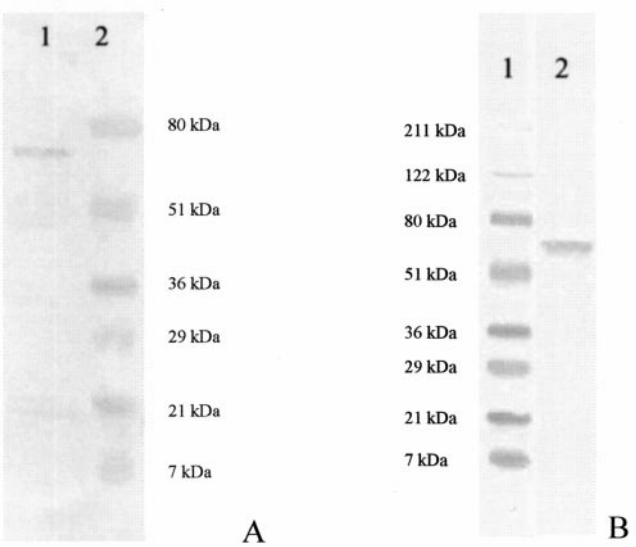

Fig. 1. Western blot analysis of ERα (A) and FLAG-tagged ERβ (B). Full-length estrogen receptors can be expressed in *Saccharomyces cerevisiae*. Samples (1–2 μg receptor protein per lane) were loaded onto a 4–20% Tris-Glycine gel and subsequently blotted onto nitrocellulose membrane. Anti-ERα AER 320 was used in blot A: ERα (lane 1), Broad Range Marker (lane 2). Flag-tagged proteins were identified with anti-Flag M1 monoclonal antibody: Broad Range Marker (lane 1), ERβ-extract (lane 2) (B).

3. Results

Agonistic behavior of RAL, recently approved as a drug for the prevention of osteoporosis, was evaluated. It was shown that yeast is a suitable host for expression of nuclear hormone receptors. A functional expression product could be obtained, as demonstrated earlier for ERα by reconstitution of a bipartite transactivation system [24]. Agonistic behavior of various ligands could be shown [19,25,26]. Barkhem *et al*. [14] and Graumann *et al*. [26] have reported that yeast may act differently than mammalian-based test systems. Nevertheless, pure action on the receptor can be studied with yeast systems. The expression of full-length ERα and ERβ in *Saccharomyces cerevisiae* was demonstrated by Western blots (Fig. 1). The binding of the receptors to the vitellogenin ERE was verified by electrophoretic mobility shift assays (data not shown).

It was first tested whether RAL displaces radiolabeled E2 in a ligand competition assay. It could be shown that RAL bound with a similar affinity to ERα as E2 (Fig. 2A). As previously reported, an equilibrium binding constant of E2 to ERα of 1×10^{-10} M^{-1} was obtained [25]. Dissociation

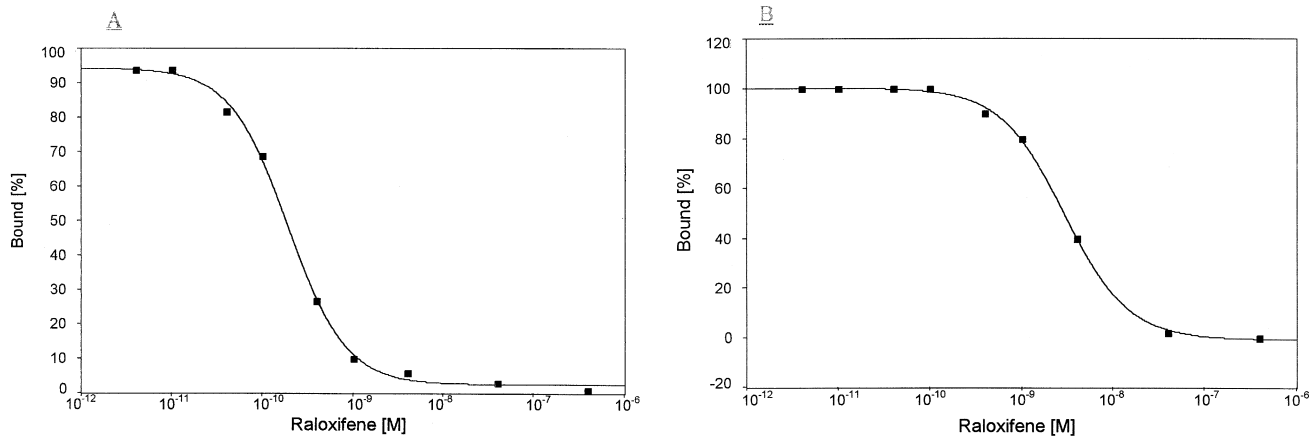

Fig. 2. Ligand competition assay of RAL on ERα (A) and ERβ (B). The ligand competition assay was performed with 3.96×10^{-9} M [^{3}H]E2 and increasing concentrations of RAL. Receptor and ligand were incubated for 16 hr at 4°, unbound radiolabeled ligand removed by dextran-coated charcoal, and bound ligand measured by scintillation counting. A logistic dose–response fit was applied in order to calculate affinity constants. The response is displayed as a percent of the maximum response to E2 with no RAL added. Normalized data of two or more independent experiments were combined.

constants of 1.9×10^{-10} M^{-1} could be obtained. In contrast, binding of RAL to ERβ was lower, exhibiting a dissociation constant of 2.7×10^{-9} M^{-1} (Fig. 2B).

Second, kinetic analysis to determine binding and dissociation rate constants were performed by applying BIACORE technology. Vitellogenin ERE containing a single repeat of the palindromic sequence was immobilized onto a sensor chip via strong biotin–streptavidin binding. ERα and ERβ were injected onto the surface with and without ligand. Increasing concentrations of RAL were compared to increasing concentrations of E2. Data were fitted using BIAEvaluation Software 3.1. This applies minimization of the sum of the squared residuals for characterization of rate constants. Segments selected for fitting were chosen in order to achieve low values (preferably <10) for χ^2, i.e. the mean square of the signal noise. For each receptor subtype, four injections with 0, 10^{-9}, 10^{-8}, and 10^{-7} M ligand were injected over the sensor chip with a constant amount of immobilized ERE. Overlaying sensograms of increasing concentrations of E2 unequivocally demonstrate a ligand-specific rise in resonance units (Fig. 3, A and C). Subtracting the injection jump at the beginning and after the end of the injection resulted in the amount bound onto the ERE immobilized chip. When ERα was incubated with 10^{-8} M E2, a doubling in resonance units compared to no ligand could be observed. Addition of 100 nM E2 still increased the signal. Incubation of ERα with increasing concentrations of RAL also resulted in a specific rise in resonance signal, although saturation at 10^{-8} M RAL could be observed (Fig. 3B). The complexes formed with E2 appeared to be more unstable, as indicated by the dissociation rate constant of 1.5×10^{-4} sec^{-1} than those incubated with RAL (2.9×10^{-5} sec^{-1}) or no ligand (6.8×10^{-5} sec^{-1}) (Table 5).

Association rates obtained from complexes either liganded with E2, RAL, or no ligand did not differ, suggesting homodimer formation induced by E2 and RAL, respectively. On the other hand, incubation of ERβ with increasing concentrations of E2 did not alter the signal of resonance units. When ERβ was incubated with RAL, an association curve exhibiting serial transitions could be obtained. The applied model did not allow calculation of consistent association rate constants (Fig. 3D).

For transactivation tests, RAL was tested alone and in combination with E2. The effect of increasing concentrations of E2 in a range from 1×10^{-11} M to 1×10^{-3} M resulted in a 14-fold higher response for ERα than ERβ (Fig. 4). A half-maximal response could be obtained at 2.9×10^{-10} M for ERα and 1.1×10^{-10} M for ERβ when E2 was used as a ligand (Tables 1 and 2).

RAL showed lower efficiency and potency in this test system (Tables 1 and 2, Fig. 5). A 39% response compared to E2 could be achieved when ERα was expressed. When incubating yeast with RAL for 20 hr before induction, an increase in efficiency up to 61% could be observed. Potency values (Table 1, Fig. 5) were also altered. This indicates a difference in the yeast cell wall for incorporating RAL into the cytoplasm compared to 17β-estradiol, presumably depending on the chemical structure of RAL. Incubating yeast with 17β-estradiol alone for a period of more than 4 hr did not alter potency and efficiency values (data not shown). Still, an 85% relative response was obtained with ERβ. As we expected, RAL was about 10,000 times less potent than E2, exhibiting 2.2×10^{-6} M for ERα compared to 6.4×10^{-7} M for ERβ.

In both estrogen receptor assays, testosterone was used as a negative control for estrogenic activity. Although efficiencies achieved were higher than when RAL was used as ligand, potencies exerted by testosterone were 2.0×10^{-5} M for ERα and 2.5×10^{-6} M for ERβ, concentrations approximately 10 times higher. In this system, E2, RAL, and testosterone exhibited an approximate 10-fold higher affinity for ERβ than for ERα (Fig. 6, A and B). All dose–response relationships were approximated by the LDR

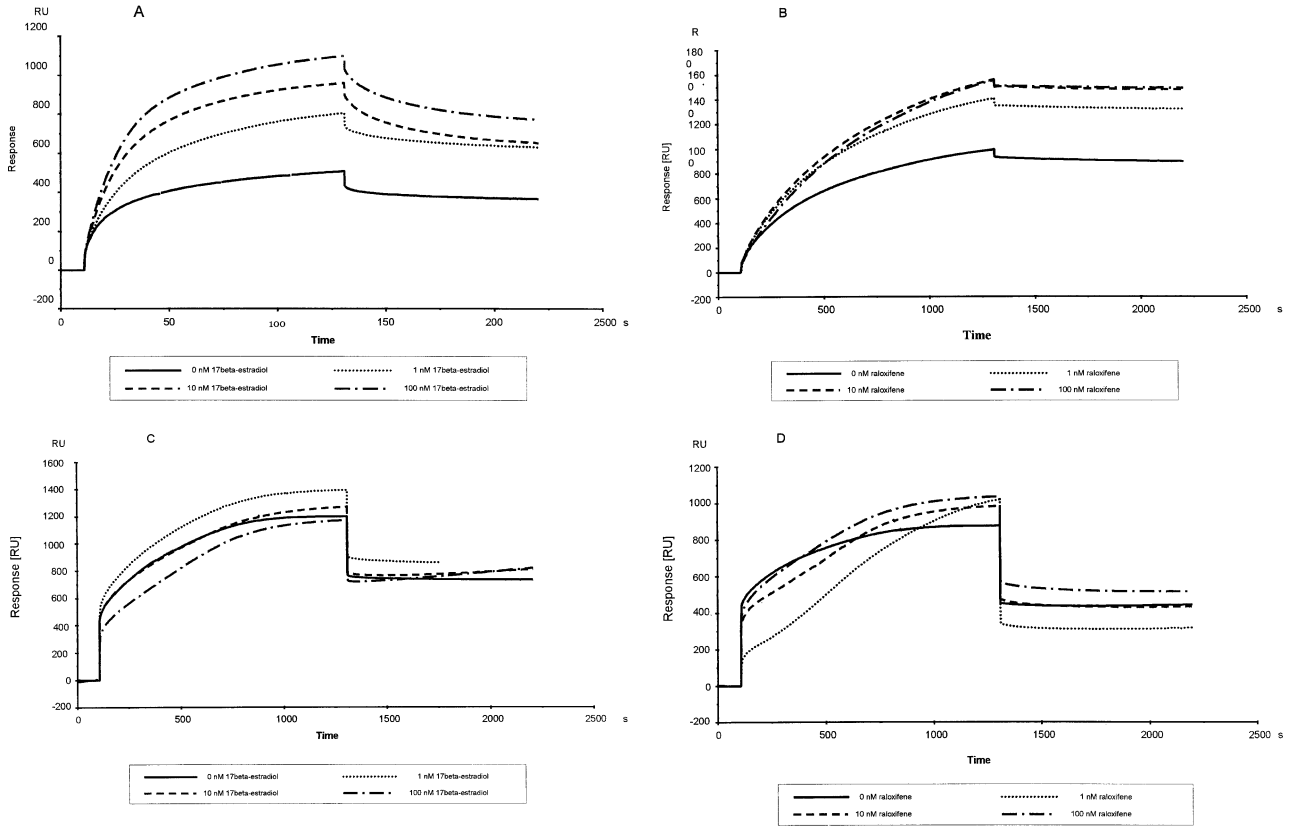

Fig. 3. Association and dissociation rate constants of estrogen receptors to ERE were determined by surface plasmon resonance technology. Recombinant ERα and ERβ were injected onto a biosensor surface immobilized with ERE at various concentrations of either E2 or RAL: A: ERα with E2; B: ERα with RAL; C: ERβ with E2; and D: ERβ with RAL. As a control, estrogen receptors were injected without preincubation with ligand. Data were collected at 1 Hz and analyzed using BIAEvaluation 3.1.

function. Parameters *b* (efficiency) and *c* (potency) are given in Tables 1 and 2.

Graumann *et al*. reported synergistic behavior for E2 and subeffective doses of tamoxifen in an ERα-containing two-plasmid system [19]. To assess possible antagonisms or synergism between E2 and RAL, increasing concentrations of E2 were combined with subeffective doses of RAL (Table 3 and 4). RAL (1×10^{-7} M) was considered as subeffective, since no significant transactivation could be obtained below this value. In contrast to the studies with

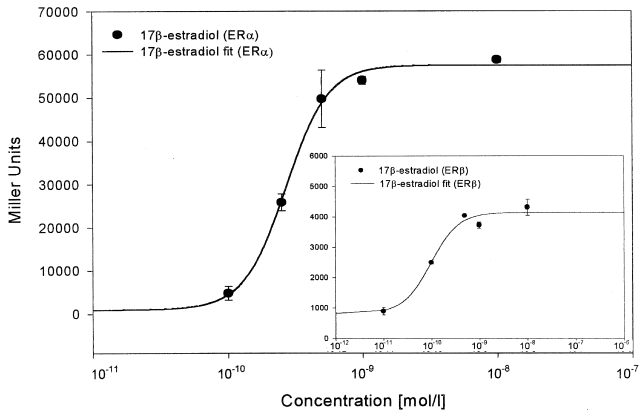

Fig. 4. Dose–response to E2 in the ERα (A) or ERα (B) yeast assays system. Yeast cells were exposed to the indicated concentrations of E2 for 4 hr before measuring β-galactosidase activity. Normalized data of two or more independent experiments were combined. Values are the means of duplicate determinations for each concentration of E2, with error bars (standard deviation) indicated for each value.

Table 1
Potencies and efficiencies of various ligands with human ERα (A) and ERβ (B) measured by a yeast transactivation system

A Ligand	Efficiency [Miller Units]	Potency [M]	Rel. response (%)
E2	56213	2.90×10^{-10}	100
RAL	21987	2.20×10^{-6}	39
RAL (20 hr)	34290	9.95×10^{-7}	61
Testosterone	38008	2.00×10^{-5}	67
B **Ligand**	**Efficiency [Miller Units]**	**Potency [M]**	**Rel. response (%)**
E2	2377	1.09×10^{-10}	100
RAL	2040	6.40×10^{-7}	85
Testosterone	2993	2.50×10^{-6}	126

Table 2
Spiking human ERα (A) ERβ (B) measured by a yeast transactivation system

A Ligand	Efficiency [Miller Units]	Potency [M]	Rel. response (%)
E2	56213	1.55×10^{-10}	100
E2 + RAL	59134	3.40×10^{-10}	105
B Ligand	**Efficiency [Miller Units]**	**Potency [M]**	**Rel. response (%)**
E2	2377	1.09×10^{-10}	100
E2 + RAL	2680	9.50×10^{-11}	113

tamoxifen, no shift or decrease in efficiency could be observed, either for ERα or ERβ.

Further, assays combining 4×10^{-9} M E2 with RAL in a concentration range from 4×10^{-11} M to 3.7×10^{-6} M were carried out. The use of RAL alone exhibited transactivation activity at concentrations of 4×10^{-8} M or higher. In contrast in the competition mode, RAL was not able to abolish the response induced by E2 as was expected by the results of the ligand competition assay (Fig. 7 A and B).

In this pure yeast system, RAL is not able to compete for E2. Additional adapters (coactivators and corepressors) may be necessary to obtain a full antagonistic behavior of RAL.

4. Discussion

Estrogen replacement therapy is important for treating symptoms accompanying menopause in women. Recently, a novel SERM, RAL, was approved by the FDA to serve as an alternative to conventional estrogen replacement therapy, circumventing negative side effects such as uterotrophic activity. RAL interacts with estrogen receptors exhibiting agonistic activity in bone and liver, but antagonistic activity in breast and uterus [9].

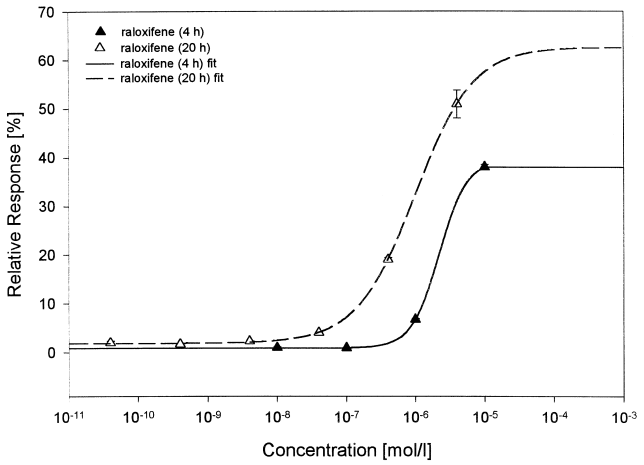

Fig. 5. Response of yeast reporter cells on ERα-mediated β-galactosidase activity to increasing concentrations of RAL spiked with a constant amount of 17β-estradiol (4×10^{-9} M). The level of expressed β-galactosidase was assayed for 4 and 20 hr, respectively, after the addition of the ligands. The response is displayed as a percent of the maximum response to E2 in the transformed cell lines. Normalized data of two or more independent experiments were combined.

In this paper, we compared the binding and transactivational properties of RAL on ERα and ERβ. In radioligand competition assays using yeast-expressed estrogen receptors, RAL was able to displace E2, exhibiting dissociation constants comparable for E2 on ERα (1.9×10^{-10} M^{-1}). The binding affinity of RAL to ERβ turned out to be lower, resulting in a K_d value of 2.7×10^{-9} M^{-1}. Although RAL binds ERα and ERβ obtained from various tissues with the same affinity as does E2 [27,28], transactivational properties seem to vary within mammalian and yeast cells [14,15]. In order to evaluate the impact of these ligands on dimer formation and ERE-binding properties, binding of ERα and ERβ onto the surface of a biosensor chip immobilized with ERE using surface plasmon resonance technology was applied. For ERα, a ligand-dependent increase in complexes bound to ERE could be observed (Fig. 3A). Saturating concentrations for homodimerization are reached at 10 nM, resulting in twice the amount of bound complexes in resonance units (RU). This indicates binding of unliganded ERα to ERE as a monomer. Addition of increasing concentrations of ligand induces homodimer formation and subsequently more binding in absolute mass to ERE, resulting in a higher resonance signal. This could also be shown for ERα ligated to RAL, even though at the concentrations used only a 1.5-fold increase could be achieved (Fig. 3B). ERβ seems to act differently from ERα when liganded to E2. No increase in RUs was observed, indicating no dimerization (Fig. 3C). This is in contrast to several publications [29–31] reporting homodimerization of ERβ even in the absence of ligand. RAL may influence ERβ conformation dependent upon concentration, since a biphasic curve was obtained for low concentrations of RAL (Fig. 3D).

We have shown that E2 was able to activate β-galactosidase activity in both receptor systems (Fig. 5), although activation was about 14 times less for ERβ than for ERα. This phenomenon can be attributed to the dependency of gene transcription on activation function domains 1 and 2 (AF1 and AF2). It was reported that the activity of AF1 contributing to transactivation in ERβ is negligible compared to ERα when an ERE-based reporter system was applied [32]. In the past, various coactivators and corepressors have been found to play a crucial role in estrogen receptor target gene transcription [33–36]. Cell-type-specific modulation will occur upon availability of certain cofactors in defined cell systems. In addition, it is likely that ERα and ERβ require different cofactors for efficient transcription. Using a yeast two-plasmid system, it was shown that ERE-dependent transactivation could be enhanced 100-fold for E2 and other xenoestrogens in the presence of RIP140 [37]. Lack of specific coactivators in yeast and lack

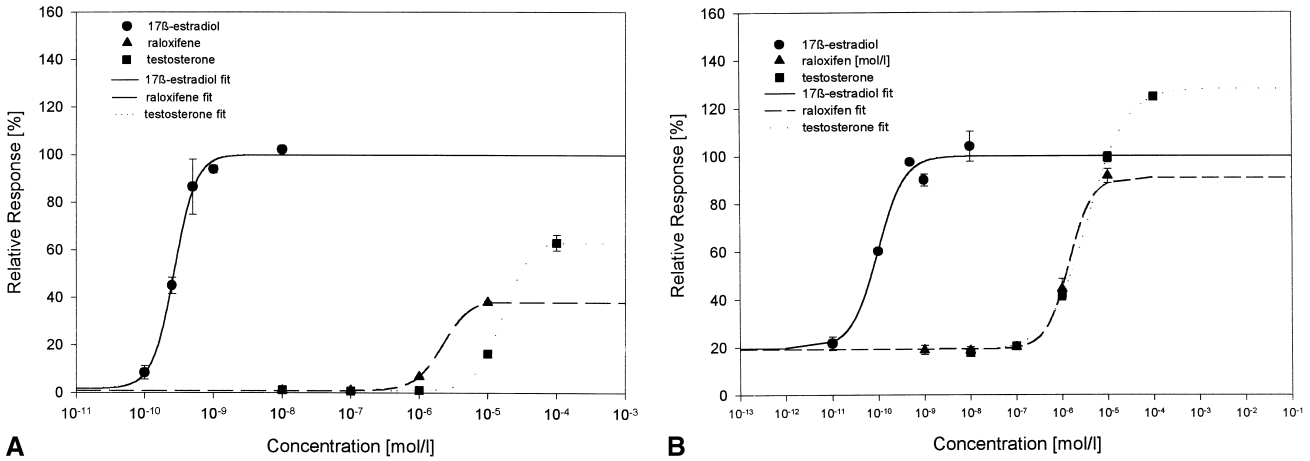

Fig. 6. Response of yeast reporter cells to increasing concentrations of E2, RAL, and testosterone. The transactivational behavior of these compounds on ERα (A) and ERβ (B) was tested at the concentrations indicated. The level of expressed β-galactosidase was assayed for 4 hr after the addition of the ligands. The response is displayed as percent of the maximum response to E2 in the transformed cell lines. Normalized data of two or more independent experiments were combined. The response values for each concentration of ligand are the means of duplicate determinations with the mean standard deviation for each value indicated.

of particular AF1 function in ERβ may be responsible for the reduced activity exerted in ERβ.

We could observe a higher background β-galactosidase expression for ERβ than for ERα in the absence of ligand (Fig. 4), an observation in agreement with a study performed in human kidney epithelial cells [14].

The amino acid sequences between ERα and ERβ were reported to be very homologous [20,38]. Crystallographic analysis showed very similar structures for the ERα- and ERβ-ligand binding domains. Upon interaction of the ligand with a unique set of residues within the ligand-binding cavity, a distinct orientation in the AF2 helix (H12) is induced. When E2 is bound within the hydrophobic cavity, H12 is allowed to align over the ligand. Upon this conformational change, the specific binding site for nuclear cofactors is accessible. In contrast, the bulky side chain of RAL prevents the correct assembly of H12 and therefore the availability of the nuclear coactivator binding site [10,11]. This structural difference raises a possible mechanism of distinct recruitment of coactivators and corepressors for agonistic and antagonistic ligands exhibiting varying transactivational properties.

Therefore, we tested the agonistic behavior of RAL in our yeast system at increasing concentrations of RAL. We compared 4 and 20 hr of incubating the yeast cells with RAL (Fig. 5). It was reported that yeast cells were impermeable to antiestrogens like ICI, but in the yeast strain SPH246 RAL could compete with E2 [15]. In our test system, RAL was not able to compete with E2 either after 4 or after 20 hr of incubation (Fig. 7, A and B). This is an indication that a mass transport limitation is not responsible for the lower potency. From a ligand binding assay, one would expect a similar potency as for E2.

We report no agonistic activities of RAL in ERα at concentrations below 1 and 0.1 μM after 4 and 20 hr of incubation, respectively (Fig. 5). By applying concentrations higher than 1 μM, a slight increase in transactivation could be observed. Testosterone was used as a negative control, exhibiting a transactivational response starting at concentrations of 10 μM. Comparing RAL and testosterone leads to the conclusion that in this yeast model system RAL exerts no agonistic activity. Although efficiencies and potencies achieved with ERβ were slightly higher compared to testosterone, effects cannot be regarded as agonistic. These results are in contradiction to those observed by Barkhem *et al.*, when a human kidney epithelial cell line transformed with estrogen receptor expression plasmids as well as a reporter plasmid was applied. They obtained slight agonistic properties in ERα, but none in ERβ. Potency to antagonize

Table 3
Determination of rate and equilibrium constants for ERE–estrogen receptor interaction using real-time biosensor technology

Compound	k_a (1/Ms)		k_d (1/s)		K_A (1/M)		K_D (M)	
	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ
no ligand	9.9E + 4	9.6E + 4	6.8E − 5	2.2E − 5	1.7E + 9	1.9E + 9	7.4E − 10	2.3E − 10
17β-estradiol	7.8E + 4	7.8E + 4	1.5E − 4	6.7E − 5	7.0E + 8	1.3E + 9	1.8E − 9	7.5E − 10
Raloxifene	7.4E + 4	4.2E + 4*	2.9E − 5	8.0E − 5*	3.0E + 9	5.0E + 8*	3.8E − 10	2.1E − 9*

* Values for kinetic constants for ERβ incubated with RAL have to be handled with care, since the association curve suggests a biphasic binding phase.

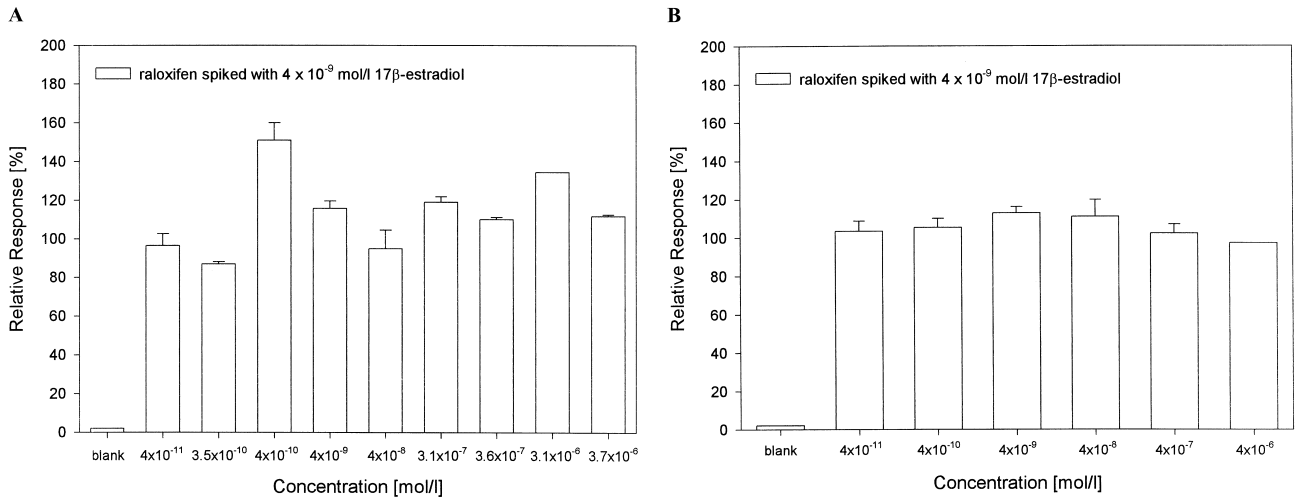

Fig. 7. Properties of ligand-induced transactivation of combined RAL with E2 on ERα. Increasing concentrations of RAL were combined with 4×10^{-9} M of 17β-estradiol. First, induction and addition of 17β-estradiol and RAL were simultaneous (A). Second, addition of RAL took place 20 hr before induction and addition of 17β-estradiol (B). Normalized data of two or more independent experiments were combined.

E2-dependent transactivation was about 15-fold higher for ERα than for ERβ [14].

Graumann *et al*. reported synergistic behavior for E2 and subeffective doses of tamoxifen in an ERα-containing two-plasmid system [19]. To further study the transcriptional effects of RAL, we performed titrations of E2 in the presence of a subeffective dose of RAL. Neither in ERα nor in ERβ synergistic effects could be observed as reported with tamoxifen. Likewise, no antagonistic effects could be observed, in contradiction with the finding of Barkhem *et al*. [14]. They reported competitive antagonism of RAL in the presence of increasing concentrations of E2. These differences in agonism and antagonism of RAL and other SERMs might be due to the different cellular environment in yeast cells.

We may conclude that in the first level RAL is able to bind within the ligand-binding pocket of the estrogen receptors since it efficiently displaces E2. Affinity constants comparable to those obtained for E2 could be measured.

In the two-plasmid system, RAL exhibited about 10,000 times lower potency than E2. Surprisingly, when constant concentrations of E2 were spiked with increasing concentrations of RAL, RAL was not able to exhibit its antagonistic activity. This is in contrast to our expectations from the competition ligand-binding experiments. Unlike previous results assessing the agonistic properties of tamoxifen [19], the potencies of RAL and testosterone to activate an ERβ-dependent reporter gene construct were the same. Spiking E2 with nanomolar levels of RAL did not alter the efficiency. This suggests no estrogenic activity of RAL in this pure bipartite system. From our BIACORE binding studies with immobilized ERE, we can conclude that unliganded ERα binds as a monomer whereas at concentrations of 10^{-8} M or higher, homodimers are formed and interact with the ERE.

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