Biochemical and Biophysical Research Communications **261,** 1–5 (1999)
Article ID bbrc.1999.0864, available online at http://www.idealibrary.com on IDEAL®

Distinct Effects on the Conformation of Estrogen Receptor α and β by Both the Antiestrogens ICI 164,384 and ICI 182,780 Leading to Opposite Effects on Receptor Stability

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Received May 18, 1999

Tissue-specific effects of 17β-estradiol (E_2) and synthetic estrogen receptor (ER) ligands on target gene regulation might, at least partly, be explained by a selective ligand-induced conformational change of their receptors (ERα and ERβ). In this study, the effects of E_2 and the synthetic ER ligands tamoxifen (TAM), ICI 164,384, and ICI 182,780 on the conformation of ERα and ERβ were examined using limited proteolytic digestion analysis. We found that E_2 induced a conformational change of ERα resulting in the protection of a 30-kDa product, whereas TAM protected a 28-kDa fragment. Strikingly, the ERα conformational change induced by both ICI 164,384 and ICI 182,780 did not result in protection but rather seems to induce a ligand concentration-dependent increase in proteolytic degradation of the 30- and 28-kDa products. Incubation of ERβ with E_2 resulted in an increased protection of a 30-kDa fragment, whereas with TAM protection of a 29-kDa fragment was observed. In contrast to the situation with ERα, ICI 164,384 and ICI 182,780 incubation induced the protection in a manner similar to 30-kDa fragment E_2. In addition, the ICI compounds also induced in a dose-dependent manner the preservation of a 32-kDa fragment. Our observations demonstrate that ICI 164,384 and ICI 182,780 have distinct effects on the conformation of ERα and ERβ, resulting in receptor subtype-selective opposite effects on receptor stability *in vitro*.

The estrogen receptor (ER) is expressed in two distinct forms, ERα (1) and ERβ (2, 3). Both ER subtypes bind estrogens and selective estrogen receptor modulators (SERMs) (2–4), form dimers (4, 5), interact with basal transcription factors and coactivators (4, 6), and bind to estrogen response elements (ERE) in the DNA (4, 5) eventually leading to modulation of target gene transcription (3, 4, 7). The distribution of both ER types varies among cells and tissues. Some tissues express predominantly one type of ER, while others express ERα and ERβ at more equal levels (8). Furthermore, the ER subtypes can be differentially expressed during cell differentiation (9). It is tempting to speculate that the tissue-specific effect of estrogens and SERMs is at least partly based on the differential distribution of ER subtypes and selective interactions of the various ligands with these receptor subtypes.

In view of the fact that the cascade of events resulting in target gene modulation is initialized by a conformational change of the receptor after ligand binding (10), we investigated whether the conformation of ERα and ERβ is changed differently by the estrogen 17β-estradiol (E_2) and the synthetic ER ligands tamoxifen (TAM), ICI 164,384 and ICI 182,780. TAM is a partial estrogen antagonist and considered a SERM, whereas the ICI compounds are generally denoted as pure antiestrogens, although some agonistic effects have been reported (7, 11–15).

MATERIALS AND METHODS

Chemicals. E_2 and TAM were purchased from Sigma Chemical Co. St.Louis, MO, USA. ICI 164,384 and ICI 182,780 were kindly supplied by Zeneca Pharmaceuticals, Macclesfield, UK.

In vitro transcription and translation. Human ERα cDNA (16) and ERβ cDNA (7) were *in vitro* transcribed and translated for 2 h at 30°C using a rabbit reticulocyte lysate system (Promega). Translation was performed in the presence of [^{35}S]methionine (Amersham) to produce radioactive receptor protein.

Conformational studies. Conformational studies were performed as described by Beekman *et al.* (17). Shortly, *in vitro* synthesized ERs were incubated with ligand (E_2, TAM, ICI 164,384 or ICI 182,780) for

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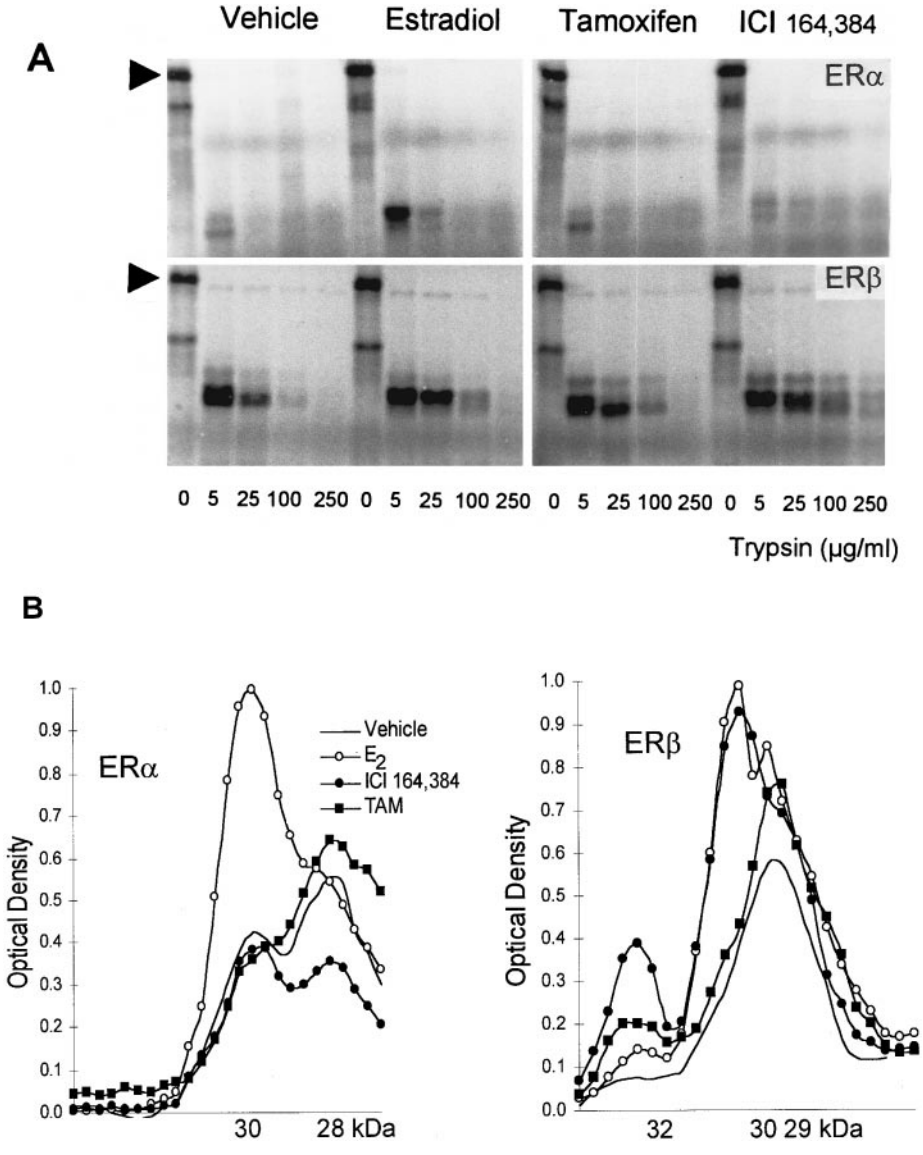

FIG. 1. Trypsin resistance of ERα and ERβ after incubation with vehicle, E_2, TAM, or ICI 164,384. *In vitro* synthesized ER was incubated with vehicle (0.01% ethanol) or ligand (10^{-6} M) and subsequently treated with increasing trypsin concentrations (0-250 μg/ml). The arrowhead indicates the intact ERs (A). Computerized optical density scans of the degradation products of ERα (at 5 μg/ml trypsin) and ERβ (at 25 μg/ml trypsin) are presented in B. The maximal optical density value measured in the E_2 scans was set at 1 and was used as a reference for the other optical density values. Each scan represents the mean of five independent experiments.

up to 30 min at 37°C in the presence of 10 mM Mg^{2+}. The co-incubation studies were performed with 10^{-8} M E_2 and increasing concentrations of either ICI 164,384 or ICI 182,780 added simultaneously to the ERs. After treatment with trypsin for 10 min at room temperature loading buffer was added. The samples were stored at −20°C or were directly analysed by SDS-polyacrylamide gel electrophoresis (12.5% wt/vol). The gel was dried and after radiography the bands were quantified by densitometry.

RESULTS

In general, ERβ was less sensitive to trypsin than ERα (Fig. 1A). Detailed analysis of the digestion products showed that trypsin treatment (5 μg/ml) of ERα resulted in the formation of a distinct 28 kDa fragment and a faint 30 kDa fragment. Increasing the trypsin concentration resulted in complete degradation of these fragments. Both E_2 and TAM enhanced the trypsin resistance of ERα. However, the size and intensity of the preserved fragments varied among the ligands studied. Incubation of ERα with E_2 had a preserving effect on the 30 kDa fragment, whereas incubation with TAM resulted in an increased protection of the 28 kDa fragment (Fig. 1). In contrast, incubation of ERα with both ICI compounds did not result in increased protection of distinct frag-

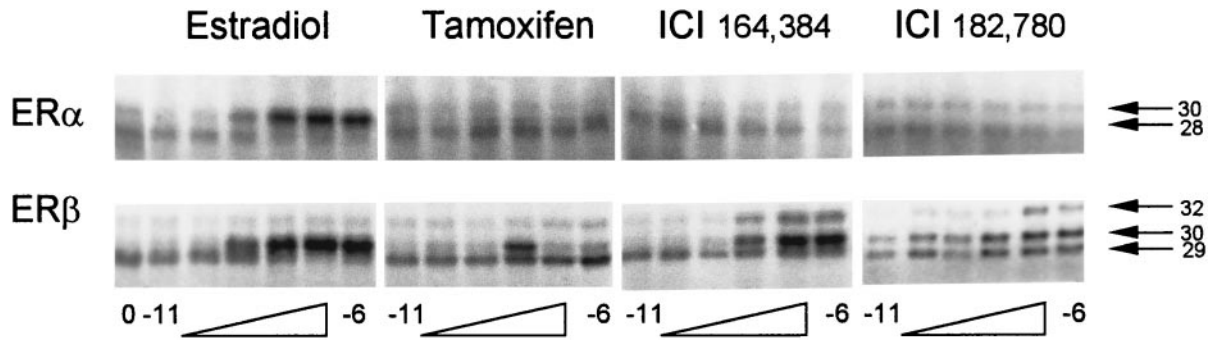

FIG. 2. E_2- and SERM-induced conformational changes of ERα and ERβ. *In vitro* synthesized ERα and ERβ were incubated with increasing concentrations of E_2, TAM, ICI 164,384, or ICI 182,780 (10^{-11}–10^{-6} M) as described under Materials and Methods and subsequently treated with 5 (ERα) or 25 μg/ml (ERβ) of trypsin.

ments, but rather led to a slightly enhanced degradation of the receptor (Fig. 2). The E_2- and synthetic ligands-induced effects on ERα conformation were ligand concentration-dependent (Fig. 2). Trypsin treatment of vehicle-incubated ERβ resulted in the formation of a 29 kDa fragment (Fig. 1). E_2 protected a 30 kDa fragment, whereas TAM mainly had an effect on a 29 kDa fragment. In contrast to the situation with ERα, both ICI 164,384 and ICI 182,780 induced a conformational change of ERβ resulting in a stabilization demonstrated by the increased protection of a similar 30 kDa fragment as seen with E_2. Again these effects were ligand dose-dependent (Fig. 2). Furthermore, the ICI compounds had a marked protective effect on an additional 32 kDa fragment. At higher TAM concentrations the 32 kDa fragment is also observed, whereas E_2 had hardly an effect on this fragment (Fig. 2). Co-incubation studies in which a fixed concentration of E_2 and increasing concentrations of the ICI compounds were added simultaneously to the receptors demonstrated that the E_2 effect on ER conformation can be overruled by the ICI compounds (Fig. 3).

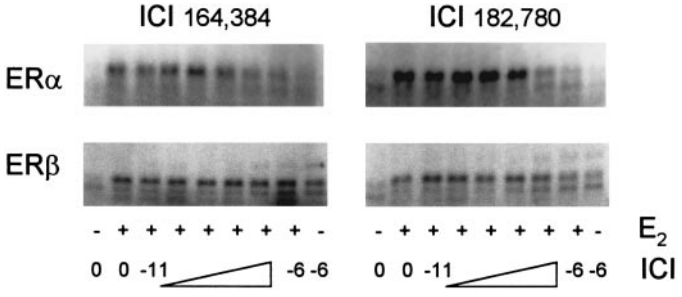

FIG. 3. The effect of co-incubation of E_2 (10^{-8} M) and increasing concentrations of ICI 164,384 or ICI 182,780 (10^{-11}–10^{-6} M) added simultaneously to *in vitro* synthesized ERα or ERβ. After treatment with 5 (ERα) or 25 μg/ml (ERβ) of trypsin, the protease resistant fragments were separated on a SDS-polyacrylamide gel and visualized by radiography.

DISCUSSION

Ligand-induced changes in receptor conformation are a common feature in steroid hormone action (17–21) and it is believed that this event initiates a cascade of processes, eventually resulting in modulation of target gene transcription. Therefore, ligand-specific-induced changes in receptor conformation might underly the cell- and tissue-specific effects of estrogens and SERMs (22–24). In addition, another aspect that could contribute to this was the identification of a second ER and the finding of a cell differentiation dependent- (9) and tissue-specific distribution of ER subtypes (8). The current study shows distinct effects of the antiestrogens ICI 164,384 and ICI 182,780 on the conformation of the ER subtypes, resulting in opposite effects on the stability of ERα and ERβ.

In general, we found that ERβ is more stable than ERα as exemplified by the increased protease resistance. This is not due to a diminished number of potential trypsin cleavage sites within the entire receptor molecule (about 60 in both receptor types) or ligand binding domain (about 20 in both receptor types), but is probably a direct consequence of its different conformation and resulting accessibility of trypsin cleavage sites. Whether the increased protease resistance of ERβ can also be observed in cells and tissues in terms of an increased receptor stability needs to be examined, but our previous conformational studies with the vitamin D receptor showed a close parallel between protease resistance of the receptor and its half-life in cells (20). This is further supported by our data obtained with ERα and the ICI compounds. The ligand-dose experiments showed that ICI 164,384- and ICI 182,780-incubated ERα was slightly more sensitive to trypsin compared to vehicle-incubated ERα while as it has been reported earlier ICI 164,384 impairs ERα dimerization (25), resulting in reduced half-life of the receptor (26). Also, the observed loss of ER in uterine tissue *in vivo* after ICI 164,384 treatment in mice was

ascribed to an ICI 164,384-induced conformational change of ER, resulting in a reduced stability and proteolysis of the receptor (27). The effect of both ICI compounds on ERβ is in marked contrast with the effect on ERα. With ERβ we observed a clear increased protease resistance. Interesting in this respect is the finding by Pace *et al.* (5) showing that ICI 182,780 was not effective in preventing loss of ERα binding to an ERE when the incubation temperature was increased from 4°C to 37°C whereas elevating the temperature did not affect ERβ-ERE binding. Loss of a specific ERα conformation suitable for DNA binding after incubation with this ICI compound was put forward as an explanation for the decreased ERα-ERE binding. Their observation that ICI 182,780 did protect ERβ from heat inactivation might result from a distinct effect on the conformation of ERβ in line with our findings presented in this study.

Both ICI 164,384 and ICI 182,780 are denoted as pure antagonists (28, 29), although some studies report an agonistic potency that could be based on an ER subtype-selective interaction (7, 11–15). However, it is yet not possible to directly translate the present data into a receptor subtype-selective agonistic- or antagonistic potency of the ICI compounds. In this respect one should take into account that the agonistic effects of SERMs may be cell type specific, dependent on the presence or absence of certain transcription factors (22). Also, studies performed by Watanabe *et al.* (30) showed that the agonistic effect of TAM was dependent on ER subtype, in combination with cell type and ERE promoter context. Therefore, conformational studies performed in a cellular context of different target cells might provide more insight.

Finally, our protease digestion assays clearly demonstrated different ER conformations induced by E_2 and the synthetic ER ligands with both ERα and ERβ. Furthermore, there was a marked difference between the ER conformations induced by ICI 164,384 and ICI 182,780 and the ER conformations induced by TAM (Figs. 1 and 2) and other synthetic ER ligands with partial antagonistic properties (4-hydroxytamoxifen, idoxifen and LY 117,018-HCl; data not shown). Also in line with observations by McDonnell *et al.* (21) we could not discriminate between TAM and other ER ligands with partial antagonistic activities (data not shown).

In conclusion, the present study shows that ERα and ERβ respond to both ICI 164,384 and ICI 182,780 with a distinct conformational change: ERα conformation changes into a less stable, more protease sensitive form, whereas ERβ conformation is changed into a more stable, less protease sensitive form. On basis of these clear opposite effects of the ICI compounds which were not observed with E_2 and TAM it is tempting to speculate that the ICI compounds are ER subtype selective ligands. The observed ligand- and receptor specific effect on receptor stability, together with the tissue specific distribution of ERα and ERβ may be part of the mechanism that determines tissue specific agonistic/antagonistic properties of ER ligands.

ACKNOWLEDGMENTS

We thank Dr. R. Docter for assistance with the densitometric computer analysis, Dr. A. E. Wakeling (Zeneca Pharmaceuticals) for the gift of ICI 164,384 and ICI 182,780, and Dr. P. Chambon for the supply of the human ERα expression vector.

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