RAPID COMMUNICATION

Distinct effects of the antiestrogen Faslodex on the stability of estrogen receptors- α and - β in the breast cancer cell line MCF-7

N T Peekhaus, T Chang, E C Hayes, H A Wilkinson, S W Mitra, J M Schaeffer and S P Rohrer

Department of Atherosclerosis and Endocrinology, Merck Research Laboratories, Rahway, New Jersey 07065, USA

(Requests for offprints should be addressed to N T Peekhaus; Email: norbert_peekhaus@merck.com)

Abstract

The effects of estrogen receptor (ER) ligands on the stability and transcriptional activity of ER β in the breast cancer cell lines MCF-7 and HeLa were examined. We found that ER β was degraded in the presence of 17 β -estradiol. Tamoxifen and Faslodex (ICI 182,780) prevented ER β receptor destabilization. In contrast to ER α , ER β degradation was not abolished by inhibitors of the proteasome-mediated protein degradation pathway. Furthermore, single point mutations in helix 12 of the receptor dramatically affected the stability and subsequent transcriptional activation of ER β .

Journal of Molecular Endocrinology (2004) **32**, 987–995

Introduction

Estrogens regulate growth and cellular differentiation in a variety of female tissues, including breast, uterus, bone, cardiovascular systems, and the brain. There are two known estrogen receptor (ER) subtypes, ER α and ER β (Kuiper *et al.* 1996, Cowley *et al.* 1997, Katzenellenbogen & Katzenellenbogen 2000, Katzenellenbogen *et al.* 2000). After hormone binding, ERs form dimers, interact with basal transcription factors and numerous co-activators, and bind to estrogen responsive elements (ERE) to effect transcription. ERs can also modulate expression of target genes by interacting indirectly with AP-1, Sp1, or CRE to modulate expression in a tissue-specific manner. Despite their similar action at EREs where both receptors activate when complexed to 17 β -estradiol (E2), the activation at AP-1 or Sp-1 response elements is somewhat different (Paech *et al.* 1997, Webb *et al.* 1999). ER α trans-activates gene transcription via AP-1 and ER β trans-represses. Besides the action of the hormone, the ER

concentration is a key component to its responsiveness in different cell tissues. The ER α level changes with the varying physiological state of the tissue and may be regulated by the ligand itself (Read *et al.* 1989, Preisler-Mashek *et al.* 2002).

Studies with ER knockout mice have shown that ER α but not ER β is essential for proliferation in mammary gland, uterus, and prostate (Hewitt & Korach 2003). E2-stimulated proliferation was shown to be normal in the ER β but absent in the ER α knockout mice (Couse *et al.* 2001, Hewitt & Korach 2003). However, even if ER α is the proliferative inducer in most tissues, a possible impact of ER β on proliferation has been suggested (Couse *et al.* 2001, Frasor *et al.* 2003). While the majority of E2 action in the uterus is mediated by ER α , ER β does play a role in modulating ER α effectiveness (Weihua *et al.* 2000) in a dose-dependent manner. Additional studies with knockout animals have shown that, in bone, in the absence of ER α , ER β can stimulate ER α -regulated genes. In the presence of ER α , ER β almost always reduces the stimulation by ER α (Lindberg *et al.* 2003).

It has been proposed that ER β modulates the proliferative action of ER α and negatively affects cell proliferation in breast and uterus (Lazennec *et al.* 2001). Previous studies have shown that ER α is a short-lived protein with a rapid turnover rate due to proteasome-mediated degradation. In contrast to ER α , there are very limited data available regarding the regulation and stability of ER β .

In our study, we have evaluated the influence of ER ligands on the stability and activity of transiently transfected ER β overexpressed in MCF-7 and HeLa cells. We also identified critical amino acids involved in modulating ER β protein stability using mutant forms of the receptor.

Materials and methods

Reagents

E2, 4-hydroxytamoxifen (Tam), lactacystin, and insulin were purchased from Sigma (St Louis, MO, USA). ICI 182,780 (Faslodex) was purchased from Tocris Cookson Inc. (Ballwin, MO, USA). Proteasome inhibitor I (PI) was purchased from Calbiochem (San Diego, CA, USA). ER β antibody D7N was obtained from Zymed (San Francisco, CA, USA), ER α antibody Ab-15 from NeoMarkers (Freemont, CA, USA), and β -actin antibody AC-15 from AbCam (Cambridge, Cambs, UK). Tissue culture reagents such as Hepes, L-glutamine, non-essential amino acids (NEAA), fetal bovine serum (FBS), pheno red-free minimum essential medium (MEM), and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA).

Expression and reporter vectors

AP-1-luc reporter plasmids were a gift from Azi Schmidt (Merck Research Laboratories). ER β expression vector phER β wt was generated by subcloning a 1.8 kb *Bam*H1-*Apa*I DNA fragment, carrying the full-length human ER β cDNA, into vector pCDNA3.1To/myc-HisA (Invitrogen). The resulting construct was sequenced. Two silent mutations were detected within the receptor gene. The ER α expression vector phER α was constructed by subcloning a 1.9 kb *Eco*RI-*Spe*I DNA fragment, containing the full-length human ER α , into pCDNA3.1.

Mutagenesis

ER β mutants were generated by site-directed mutagenesis using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the plasmid phER β wt was used as template. The following primers were used: D489A (5'-GTG GTCCCAGTGTATGCCCTGCTGCTGGAGA TG-3' and 5'-CATCTCCAGCAGCAGGGCATA CACTGGGA-3'), L491Q (5'-CCAGTGTATGAC CTGCAGCTGGAGATGCTGAATGCC-3' and 5'-GGCATTTCAGCATCTCCAGCTGCAGGTC ATACACTGG-3'), E493A (5'-GACCTGCTGCT GCGGATGCTGA-ATGCCCCAC-3' and 5'-GTG GGCATTTCAGCATGCGCAGCAG-CAGGTC-3'). All constructs were confirmed by sequencing.

Transient transfection and reporter assay

MCF-7 and HeLa cells were cultured in phenol red-free MEM supplemented with 10 mM Hepes, 2 mM L-glutamine, NEAA, 10% FBS, and penicillin/streptomycin. Cells were grown to 50–60% confluency and transfected in 6-well or 12-well plates using lipofectamine-2000 as per the manufacturer's instructions (Invitrogen). For the luciferase reporter assay, 0.5 μ g expression vector (phER β wt and mutant), 1.0 μ g reporter plasmid, and 1.0 μ g normalizing β -galactosidase plasmid were used. One volume of media with 20% FBS and compound was added 4 h after transfection. The media were replaced with fresh media and compound after 12 h and cells were grown for another 6 h. The cells were harvested, washed twice with PBS, and stored at -80°C .

Cell extract and Western blotting

The frozen cell pellet was resuspended in 200 μ l cell extraction buffer (50 mM Tris-HCl, pH 8.0; 0.4 M NaCl; 5 mM EDTA; 1% Nonidet; 0.2% sarcosyl; 100 μ M sodium vanadate; 10 mM sodium molybdate; 20 mM NaF) containing a 1:100 dilution of protease inhibitor cocktail (Sigma) on ice, passed several times through a small micro-pipette tip, and incubated on ice for 30 min. The extract was centrifuged at 10 500 *g* for 30 min at 4°C and the supernatant was transferred to a new tube. Protein concentration was measured using the BCA protein assay reagent A from Pierce (Rockford, IL, USA). Cell extract was subject to

SDS-PAGE (10%) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer (PBS; 10% glycerol; 0.2% Tween 20; 10% non-fat milk) for 1 h. Blots were probed with antibodies overnight at 4 °C. Blots were washed in blocking buffer and incubated with HRP-conjugated anti-rabbit or anti-mouse (Pierce) antibodies. The membranes were washed and developed with Supersignal West Dura ECL substrate (Pierce).

Luciferase assay

Luciferase and β -galactosidase activity were measured using luciferase (Promega, Madison, WI, USA) and β -galactosidase (Applied Biosystems, Bedford, MA, USA) assay kits. Transfection and assays were performed in triplicate and luciferase activity was normalized for β -galactosidase activity. Data are reported as relative light units.

Real-time RT-PCR

Total RNA was extracted from cells using Trizol according to the manufacturer's instructions (Invitrogen) and cDNA was synthesized using the multiscribe reverse transcriptase kit (Applied Biosystems, Foster City, CA, USA). The following Taqman probes and primers were used: ER β -forward (GACCACAAGCCCAAATGTGTT), reverse (AACTGGCGATGGACCACTAAA), probe (6 FAMTGGCCAACACCTGGGCACCTTTC). ER β mRNA levels were normalized to β 2-microglobulin and 18S mRNA levels using probes obtained from Applied Biosystems.

Results

ER β protein level in MCF-7 and HeLa cells is sensitive to ligand

All of the known breast cancer cell lines exhibit only very low endogenous ER β protein levels. To examine ER β protein stability in MCF-7 cells, we transiently transfected ER α -positive MCF-7 cells with the ER β expression vector phER β wt, expressing the full-length receptor (530 amino acids). Transfected MCF-7 cells were grown for 24 h in the absence and presence of the ligand, and cell extracts were analyzed by immunoblotting (Fig. 1A). In the absence of ligand, moderate

expression of ER β was detected. In the presence of E2, the apparent protein level of ER β was slightly lower. In contrast, treatment with either Tam or ICI 182,780 caused an increase of ER β protein.

Similar results were obtained in ER-negative HeLa cells (Fig. 1B), which suggested that the observed effect is not cell-type specific. To exclude the possibility that the ligand-mediated effects are due to a regulation of mRNA level, we performed RT-PCR analysis on total RNA extracted from the same treated cells as were used for the Western blot analysis (Fig. 1C). No significant changes in ER β mRNA level were observed in the different treatment groups, which indicated that the ligand-dependent effects on ER β are mediated at the post-transcriptional level. Our results suggested that ICI 182,780 and Tam increase the stability of ER β protein.

Lactacystin and PI do not block ER β degradation in MCF-7 or HeLa cells

In order to assess the potential involvement of the proteasome-mediated pathway in ER β degradation, we evaluated the effects of specific and non-specific inhibitors of this pathway on ER β stability (Fig. 2). In agreement with previous data (Dick *et al.* 1997, El Khissini & Leclercq 1999), lactacystin and PI, highly specific proteasome inhibitors, abrogated ER α degradation in MCF-7 cells transiently transfected with the ER β expression vector (Fig. 2A) and in HeLa cells co-transfected with the ER β and ER α expression vectors (Fig. 2B) significantly in the presence of E2. Interestingly, lactacystin and PI also increased the ER α pool in the absence of ligand.

In contrast to their action on ER α , lactacystin and PI did not block ER β turnover. In fact, the presence of the proteasome inhibitors was associated with an even stronger degradation of ER β in the presence of E2 in MCF-7 and HeLa cells. In the absence of ligands, PI caused a slight decrease in ER β protein level in MCF-7 cells. Insulin, a non-specific inhibitor of cellular proteolysis, failed to block estrogen-dependent ER α or ER β degradation.

Mutation of helix 12 alters the ligand-induced degradation profile of ER β

Recent studies have shown that specific amino acid residues in helix 12 of ER α affect the stable

Figure 1 Protein and mRNA levels of ER β . Western blot of ER β protein levels in (A) MCF-7 and (B) HeLa (B) cells. Cells were transiently transfected with control vector (c) or ER β expression vector phER β wt. Cells were treated with ethanol (EtOH) (n), 10 nM E2 (E), Tam (T), or ICI 182,780 (I) for 24 h. Western blot: equal amounts of cell extract (10–20 μ g) were loaded onto SDS-PAGE gels and analyzed by immunoblotting and densitometry. The level of ER β protein in the graph is expressed as a ratio of ER β to β -actin protein. (C) RT-PCR analysis of ER β mRNA levels in MCF-7 and HeLa cells. Total RNA was extracted and RT-PCR was performed as described in Materials and methods. Three independent experiments were performed and the ratio of ER β to β 2-microglobulin mRNA is shown.

Figure 2 Effect of proteasome inhibitors on ER protein levels. MCF-7 cells transiently transfected with (A) phER β wt and (B) HeLa cells transiently transfected with phER β and phER α were grown with EtOH (control) or 10 nM E2 for 20 h and treated with EtOH (n), 2 μ M insulin (I), 25 μ M lactacystin (L), or 50 μ M PI (P) for 4 h. Cell extracts were analyzed by immunoblotting. ER levels were quantified by densitometry. Cumulative data from at least three independent experiments are plotted as a ratio of ER and β -actin protein level. A representative Western blot from each experiment is shown.

Figure 3 Western blot analysis of ER levels in ER β mutants D489A, L491Q, and E493A in MCF-7 cells and HeLa cells. Cells were treated with EtOH (n), 10 nM E2 (E), Tam (T), or ICI 182,780 (I) for 24 h; c, control vector. Western blot: equal amounts of cell extract were loaded onto an SDS-PAGE gel and analyzed by immunoblotting and densitometry. ER protein level was normalized to β -actin level. Data are presented as the means $\pm$ S.D. of three independent experiments.

expression of the receptor (Mahfoudi *et al.* 1995, Pearce *et al.* 2003). To examine the role of those well-conserved sites in the regulation of ER β stability, we generated analogous mutants in ER β and tested these mutants with regard to their stability and activity in the presence of different ER ligands in MCF-7 and HeLa cells (Fig. 3). The E2- and Tam-mediated stability of ER β mutant D489A was similar to that of ER β wild type in both cell lines. However, this mutant showed enhanced ICI 182,780-mediated degradation. ER β mutant L491Q, which is analogous to ER α L540Q, showed a different degradation profile compared with wild type. In this mutant, treatment with E2 increased

ER β protein to a level significantly higher than that with Tam or ICI 182,780. The expression of ER β mutant E493A was low in untreated cells and remained unchanged with different ligands.

ER β mutants exhibit different transcriptional activity of ERE and AP-1 reporter genes

Previous studies have shown that, in mammary cells, ER α and ER β bind if complexed with estrogen directly to EREs within promoters and activate gene expression. In contrast, ER α activates at AP-1 sites exclusively with estrogen and ER β only with antiestrogens (Paech *et al.* 1997, Webb

Figure 4 ER β mutants display differential activation of ERE and AP-1 reporter. Expression of (A and C) synthetic ERE and (B and D) AP-1 luciferase reporter in (A and C) MCF-7 and (B and D) HeLa cells expressing wild type (wt) and mutant forms of ER β . Cells were transiently transfected with an ERE or AP-1-luciferase reporter plasmid, ER β expression vector (wild type or mutant form) or control vector, and normalizing β -galactosidase vector. Cells were grown for 24 h in the presence of 10 nM E2 (solid bars) or 10 nM Tam (shaded bars) and luciferase activity was measured in crude extracts. Data are presented as the means $\pm$ S.E. of three independent experiments. * P < 0.05 compared with control.

et al. 1999). We evaluated the effects of the ER β mutations on the transcriptional activity of ER β using an ERE as well as an AP-1-luc reporter gene construct containing three synthetic ERE or AP-1 sites respectively (Fig. 4).

In agreement with previous results, estrogen showed a more than fourfold ER α -mediated induction of ERE in MCF-7 cells (Fig. 4A). In cells transiently transfected with ER β expression vector the induction by estrogen was less than twofold. These results are similar to previous results where ER α was more transcriptionally active than ER β , which acts as a trans-dominant modulator of ER α (Liu *et al.* 2002). Only a basal ERE induction was detected in HeLa cells transfected with the control vector (Fig. 4C). All three ER β mutants showed a distinct phenotype regarding the activation at ERE, which was similar in both cell lines. Mutation D489A caused a 2.5-fold and fourfold increases in estrogen-dependent induction compared with the ER β wild type in MCF-7 and HeLa cells

respectively. In contrast, mutation E493A exhibited a strong decrease in transcriptional activation of the ERE reporter gene. Mutation L491Q had no significant effects on ER β activity in both cell lines.

In contrast to E2, Tam showed a strong induction of the AP-1-luc reporter in the presence of ER β (Fig. 4B and D), which is in agreement with previous results (Paech *et al.* 1997). ER β mutation D489A caused a significantly higher induction of AP-1 with Tam compared with ER β wild type and only a slight increase in the presence of E2. This is in sharp contrast to the effect of the analogous mutation (D538A) in ER α , which lacked any estrogenic responsiveness to Tam (Table 1) (El Khissiin & Leclercq 1999). Interestingly, ER β mutant L491Q showed a six- and eightfold induction of transcription at AP-1 in the presence of E2, compared with wild type, in MCF-7 and HeLa cells respectively. The AP-1 activation of mutant E493A was similar to that of the wild type with E2 and Tam in both cell lines.

Table 1 Stability of homologous ER β and ER α mutants. The protein levels of ER β (this study) and ER α (stable transfectants of MDA-MB-231 cells; adapted from Pearce *et al.* (2003)) wild type (wt) and mutants in the presence of none, E2, Tam or ICI 182,780 were compared (+++: highest, ++: modest, +: lowest level)

	ER α * wt	ER β wt	ER α * D538A	ER β D489A	ER α * L540Q	ER β L491Q	ER α * E542A	ER β E493A
None	++	+	+	+	+++	+	++	+
E2	+	+	+	+	+++	+++	++	+
Tam	+++	+++	+	+++	+++	++	++	+
ICI	–	+++	–	+	+++	++	+	+

*1 nM E2; 1 μ M Tam or ICI 182,780 (ICI).

Discussion

One of the novel findings of this study was that the pure antagonist ICI 182,780 caused a stabilization of ER β in MCF-7 and HeLa cells. The ability of ICI 182,780 to promote stable expression of ER β stands in contrast to its ability to promote degradation of ER α (Gibson *et al.* 1991, Dauvois *et al.* 1992). ICI 182,780 was found to decrease the half-life of ER α from 4–5 h to 0.5 h (Gibson *et al.* 1991, Dauvois *et al.* 1992), whereas Tam increased the half-life of ER α . It is well documented that ER α degradation is mediated via a proteasome-mediated pathway in which ER α is ubiquitinated and degraded by the 26S proteasome in an ATP-dependent reaction (Hershko & Ciechanover 1998, Alarid *et al.* 1999, El Khissiin & Leclercq 1999).

It has been shown that the pattern and the extent to which ER α is ubiquitinated determine the rate at which the receptor is degraded (Wijayaratne & McDonnell 2001). ICI 182,780-bound ER α was found to be the most heavily ubiquitinated, whereas Tam-bound ER α was the least ubiquitinated.

So far there are no data available regarding the stability of ER β *in vivo*. However, *in vitro* proteolytic digestion analysis revealed that ER ligands have distinct effects on the conformation of both receptors, resulting in receptor subtype-selective opposite effects on receptor stability *in vitro* (Van Den Bemd *et al.* 1999). This suggests that there may be fundamental differences in the mechanism by which both receptors are recognized by the protein degradation machinery when complexed with the ligands.

It was shown that specific inhibitors of the proteasome-mediated pathway, like lactacystin and PI, are able to block ER α turnover (Dick *et al.*

1997). A recent study showed that lactacystin is effective at inhibiting ER α as well as ER β turnover in HUAEC cells (Tschugguel *et al.* 2003). We tested these inhibitors and found that they do not abrogate ER β degradation of MCF-7 or HeLa cells. In fact, lactacystin and PI caused an even higher turnover of ER β in the presence of E2 in MCF-7 cells. In contrast, the inhibitors had no effects on ER β stability in HeLa cells. However, the protein levels of ER β under these conditions were overall very low. The data suggested that the mechanism of ER β degradation is likely to be different from that of ER α and might not be mediated via the same proteasome-dependent pathway. Our results further suggest that inhibition of the ER α -specific proteasome pathway may negatively affect ER β stability in MCF-7 cells. Blockade of the proteasome pathway could lead to an increase of an unknown factor which decreases ER β stability.

Furthermore, we evaluated the effects of point mutations in helix 12 of ER β on the stability of the receptor (Dauvois *et al.* 1992). We found that some of the mutations changed the stability profile of the receptor. A key residue involved in ICI 182,780-mediated stability of ER β is D489 because mutation of this residue results in loss of protein stability in the presence of ICI 182,780. ER α D538A and E542A were both shown to have similar ligand-mediated modulating effects on receptor stability (Table 1) (El Khissiin & Leclercq 1999). ER β E493A showed reduced expression of ER β protein in the absence and presence of all tested ligands. This is in contrast to the analogous ER α mutation E542A, which showed only slightly reduced degradation with ICI 182,780 but wild-type-like stability in the presence of Tam (Table 1) (El Khissiin & Leclercq 1999). Interestingly, the

L491Q mutant displayed E2-mediated stabilization of ER β expression. Together these observations support and extend the idea that helix 12 plays a major role in the regulation of the stability of ER α and ER β . D489 and L491 appear to be directly or indirectly involved in the recognition and degradation of the ER β receptor–ligand complex.

We also tested the effect of those mutants on the transcriptional activity of the receptor to activate an ERE and AP-1-luc reporter (Fig. 4). In agreement with previous results (Paech *et al.* 1997, Webb *et al.* 1999), we observed that ER β expression in MCF-7 cells caused a three- to fourfold induction of the AP-1-luc reporter by Tam (Fig. 4B). Expression of ER β in MCF-7 cells resulted also in a strong decrease in the ER α -mediated ERE activation (Fig. 4A). This can be explained by the lower transcriptional activity of ER β compared with ER α at the ERE site (Liu *et al.* 2002). Mutant D489A showed a sixfold increase in activation at AP-1 response elements in the presence of Tam. In contrast, in the presence of estrogen this mutant exhibited a significantly higher activation of an ERE reporter compared with ER β wild type. Further, mutant L491Q, which was stabilized by E2, was able to activate the AP-1 reporter with E2 fivefold (Fig. 4B). However, this mutation had no effect on ERE activation. Mutation E493A showed, in contrast to the homologous mutation in ER α , a strong effect on the transcriptional activation of an ERE reporter. The ER β mutation caused a threefold decrease in estrogen-mediated activation and a two-fold decrease with Tam (Fig. 4A). Therefore, mutations in helix 12, which increased the stability of ER β , also increased its transcriptional activity. This pattern of activation correlates with the increased stability of ER β under these conditions and shows that ligand-mediated stability and transcriptional activity are linked.

Acknowledgements

We thank Azriel Schmidt (Merck Research Laboratories) for kindly supplying the AP-1 reporter plasmid and for his helpful suggestions.

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Received 31 December 2003

Accepted 30 March 2004