PREFACE

The sharp decline in ovarian steroidogenesis occurring at the time of the menopause results in immediate adverse events that impair quality of life. These can include vasomotor instability and urogenital atrophy, as well as more long-term sequelae such as increased morbidity and mortality from cardiovascular disease and osteoporotic fractures. Hormone replacement therapy (HRT) with estrogen and progesterone (in the presence of an intact uterus) is clearly effective in alleviating symptoms of hot flashes and urogenital atrophy. Case control and cohort studies have also indicated that HRT reduces the risks of cardiovascular disease and osteoporotic fractures. However, results from the Heart and Estrogen/Progestin Replacement Study (HERS), which failed to demonstrate a benefit in cardiovascular mortality in women with established heart disease, emphasize the difficulty in drawing conclusions from epidemiological data. Despite its proven or implied benefits, HRT is associated with a variety of significant drawbacks that include increased risks of breast cancer, uterine cancer, deep vein thrombosis, gallbladder disease, and breast enlargement/tenderness. Because of these disadvantages, HRT is restricted to a relatively small fraction of postmenopausal women, and long-term compliance with treatment is estimated to be only 15–40%.

Agents that retain the benefits of estrogens but at the same time avoid the risks are urgently needed to provide postmenopausal women with an optimal form of HRT. Selective Estrogen Receptor Modulators (SERMs) are a class of drugs with mixed estrogen agonistic/antagonistic activity that holds promise in fulfilling this need. Tamoxifen, the first and most studied of these compounds, has been in clinical practice for over 20 years in the treatment of women with hormone-responsive breast cancer. As a result of its antiestrogenic action in the breast, tamoxifen may, indeed, be effective as a chemopreventive agent for hormone-responsive breast cancer, while its partial estrogen agonistic effects on the skeletal system and on serum lipoproteins may offer protection from osteoporosis and cardiovascular disease. Although demonstration of these clinical benefits is still preliminary or lacking (e.g., reduction in heart disease risk), such mixed agonistic/antagonistic properties of tamoxifen provide proof of principle for the feasibility of developing new SERMs with an improved pharmacologic and therapeutic activity profile. A possible improvement in this regard may have been the introduction of raloxifene, which, in contrast to tamoxifen, has minimal estrogen-like activity in the uterus. As a result, its use has not been associated with an increased risk of endometrial cancer.

Over the last several years, our knowledge of the basic cellular mechanisms governing estrogen action has grown exponentially. The simple model of estradiol binding to its cognate receptors (ER) followed by binding of the complexed receptor to estrogen-responsive elements of target genes has significantly expanded to include multiple additional interactive components. Several chapters in the Basic Studies section address in detail the cellular mechanisms of action of estrogens and SERMS, focusing on important aspects such as distinct ligand-dependent conformational changes in the ER that play a

critical role in the recruitment of coactivators and corepressors and the bidirectional crosstalk between estrogen receptor and growth-factor signaling. Differences in tissue distribution and function of ER-α and -β are also reviewed and discussed. Understanding of these basic mechanisms is critical for the design of new SERMs with improved tissue-specific estrogen agonistic/antagonistic activity resulting in maximal health benefits and minimal risks. The chapters in the Basic Studies section will provide a comprehensive updated review of the preclinical studies with currently available SERMs focusing on their effects on critical target organs such as the cardiovascular system and the brain.

The Clinical Studies section will compare and contrast the influence of estrogens and currently available SERMs (primarily tamoxifen and raloxifene) on the major clinical endpoints, such as incidences of breast cancer, cardiovascular disease, osteoporosis, and cognitive impairment. Based on our current state of knowledge, a tentative approach to menopause-related health issues will be provided both for normal women as well as for women with a previous diagnosis of localized breast cancer. We believe that *Selective Estrogen Receptor Modulators: Research and Clinical Applications* will be of interest to basic scientists in endocrinology, tumor biology, and pharmacology, as well as a wide range of clinicians, including endocrinologists, medical oncologists, gynecologists, and family practitioners. We wish to thank the many contributors, who are distinguished leading experts in their fields and without whose major efforts this book would not have been possible.

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2 Ligand-Induced Conformational Changes in Estrogen Receptors-α and -β

Elizabeth A. Allegretto, PhD

Contents

ABSTRACT

Since the cloning of the second known estrogen receptor (ER), termed ERβ, there have been efforts to reevaluate estrogen signaling. ERα and ERβ are generated from separate genes and have marked nucleotide- and protein-sequence differences. Human ERα and ERβ share approx 96% amino-acid sequence identity in the DNA-binding domain (DBD), approx 53% sequence identity in the ligand-binding domain (LBD), and only about 30% identity in the amino terminal region. While both receptors bind to 17β-estradiol with equal affinity (K_d ~ 0.5 nM) there are compounds that bind with varying affinities to the two receptors. The biology of ERα and ERβ are likely to be quite different based on their tissue distribution. Additionally, transgenic mice that do not express either ERα or ERβ display distinct phenotypes.

Because ERα and ERβ bind to endogenous estrogens with apparent equal affinity, their ability to activate genes differently based on promoter context and/or cell-type context might be mediated by their ability to assume different conformations upon binding to the same and/or different ligands, thereby attracting different cofactor proteins and resulting in distinct biological activities. Partial proteolytic enzyme digestion has been used to detect differences in agonist-bound versus antagonist-bound receptor conformations. Additionally, the X-ray crystal structures of ligand-occupied ERα and ERβ LBDs show that clear changes occur in the receptors on binding to different classes of compounds. To date, however, the most sensitive technique for garnering

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information about subtle conformational changes induced by ligands in ERα and ERβ has been peptide phage display.

INTRODUCTION

ERα and ERβ are similar to each other in that they both bind to endogenous estrogens with approximately equal affinity and they both stimulate transcription from an estrogen-responsive element (ERE) in the presence of estradiol in cotransfection experiments *(1–3).* Differences between the two have emerged as efforts to study the receptors have increased. ERα and ERβ have been found to regulate transcription from activator protein 1 (AP-1) elements in a different manner *(4).* While estradiol acted as an agonist through ERα to stimulate transcription from a synthetic AP-1 element, it was inactive through ERβ, while ER antagonists such as 4-hydroxy-tamoxifen (4-OH-Tam), raloxifene, and ICI-164,384 acted as agonists through ERβ from an AP-1 site *(4).* Since then, several other groups have shown that ERα and ERβ exhibit different characteristics on various promoters in cotransfection assays *(5–8).*

Cell and tissue distribution of ERα and ERβ are also quite different, with ERα being highly expressed in classical estrogen target tissues (mammary gland, uterus) and ERβ showing high levels of expression in ovary, prostate, thymus, and testis *(2,9).* Both receptors are also expressed in other cells such as those from brain, bone, and breast cancer *(10–15).* The differential promoter activity and cell-type expression of ERα and ERβ imply that various ligands may induce distinct conformational changes in ERα and ERβ that then allow binding of different coactivators or corepressors (*see* ref. 16 for review), ultimately accounting for their unique pharmacology.

Different classes of ligands are known to bind to ERα with similar affinity, but exert different activities depending on the promoter or cell context. For example, the known ER antagonists exhibit various profiles of activities. Some are classified as pure ERα antagonists, such as ICI-164,384 and ICI-182,180, which seem to block the actions of estradiol in all tissues tested *(17).* Other ER antagonists such as raloxifene and tamoxifen are classified as selective estrogen receptor modulators (SERMs) since they act as antagonists in the breast, but agonists in bone *(18–22).* Additionally, tamoxifen is a partial agonist in uterus *(23)* while raloxifene is not *(19,21).* The hypothesis that different ligand-induced receptor conformations correlate with diverse biology has been tested by various methods. Protease digestion was the first method utilized to study this prior to cloning of the ERs *(24).* With the cloning of ERα in 1986 *(25)* and ERβ in 1996 *(1,2)* protein overexpression and crystal structure determination was made possible and yielded information on the overall structures of the ER LBDs with agonists or antagonists bound *(26–28a).* More recently, the use of peptide phage display technology *(29)* has enabled the mapping of minute changes in receptor conformation induced by different ligands *(30–36).*

PROTEASE DIGESTION EXPERIMENTS

Workers first started to probe intracellular receptor structure/function relationships by use of limited proteolytic enzyme digestion in the late 1970s. Glucocorticoid receptor *(37),* progesterone receptor *(38),* ER *(39),* and vitamin-D receptor *(40)* were subjected to partial digestion and the resulting fragments were analyzed in the effort to gather information on functional domain alignment and modularity prior to the cloning of the

receptors. The first study in which this method was used to probe structural aspects of ERα in the presence of different ligands was performed in 1986 by Attardi and Happe *(24)*. Rat uterine ER was radiolabeled in vivo with either the ER agonists [^{3}H]-estradiol or [^{3}H]-DES, or the ER antagonist [^{3}H]-4-OH-Tam. Protein extracts were prepared and submitted to partial digestion with chymotrypsin and the resultant fragments from each labeled receptor were then separated by sucrose gradient sedimentation. Attardi and Happe found that the DES- and estradiol-labeled ER sedimentation patterns were similar to each other, but different from that of the 4-OH-Tam-ER complex. They concluded that because the agonist- and antagonist-bound ERs were differentially sensitive to limited proteolysis, the two classes of ligands were inducing different conformations in the receptor protein. They proposed that these different conformations might influence the interaction of receptor with DNA or chromatin, and hence their biological activity.

Beekman et al., and McDonnell et al. extended these early findings using *in vitro* translated, [^{35}S]-labeled recombinant ERα *(41,42)*. ERα was incubated with estradiol or with ER antagonists of varying biological properties, including 4-OH-Tam, ICI-164,384, and raloxifene, and then subjected to limited protease digestion and SDS-PAGE *(41,42)*. These three ER antagonists showed different activities in cotransfection assays in a promoter- and cell-dependent manner and therefore were hypothesized to induce different conformations in ERα *(42,43)*. Discrete differences were observed in the digestion patterns of ER bound to estradiol versus ER complexed with any of the ER antagonists. However, the technique was unable to discriminate between any of the three antagonists based on the protease digestion patterns of their complexes with receptor *(41,42)*.

With the cloning of ERβ, workers compared ERα and ERβ using protease digestion techniques. Two groups showed that ligand-bound ERβ was more resistant to proteolytic cleavage than holo ERα *(44,45)*. There was not a clear difference between the proteolytic digestion patterns of ERα and ERβ bound to any of the three ER antagonists tested (tamoxifen, ICI-164,384, and ICI-182,780) *(44)*. With the discovery of ligands that interact differentially with each of the ER subtypes, work was done to compare these ligands using tryptic mapping. One study tested compounds with selectivity for ERα versus ERβ and vice versa using [^{35}S]-labeled ERα and ERβ *(45)*. These compounds included a pair of tetrahydrochrysenes, S,S-THC and R,R-THC, the S,S being an agonist on both ERα and ERβ and the R,R version an agonist through ERα, but an antagonist on ERβ. Also tested was propyl pyrazole triol (PPT) which is a potent and efficacious ERα agonist and a weak ERβ antagonist. The three compounds were indistinguishable from estradiol in a tryptic digest of ERα. The R,R-THC and PPT compounds yielded similar patterns to ICI-182,780 when bound to ERβ whereas the S,S-THC-ERβ pattern was similar to estradiol bound to ERβ. Therefore, these experiments were able to differentiate antagonists from agonists bound to each of the receptors, but there were no discernable differences observed between the three agonists bound to ERα or between the two antagonists bound to ERβ. Although there were observed differences in the ability of these compounds to recruit coactivators to the receptors, any potential correlative conformational changes in the receptors were not detectable using protease digestion experiments *(45)*.

In summary, protease digestion experiments with ERα and ERβ bound to various ligands enabled the observation of crude conformational changes induced in the receptors by agonists versus antagonists. However, this method has not been useful to date in

discerning potential subtle differences in receptor conformation that would be hypothesized to take place based on the different biological activities of the various ligands within each compound class.

ER CRYSTAL STRUCTURES

The crystal structures of the ERα LBD bound to estradiol or raloxifene were solved in 1997 *(26)*. In 1998 ERα LBD was cocrystallized with a peptide from the receptor coactivator, glucocorticoid receptor interacting protein (GRIP-1), in the presence of the ER agonist DES, and the ERα LBD structure with 4-OH-Tam was solved *(27)*. In 1999 the ERβ LBD structures with genistein and raloxifene were solved *(28)*, and in 2001 the structure of the ICI-164,384-ERβ LBD complex was determined *(28a)*. These structural studies demonstrated directly that there are dramatic differences between ER bound to agonist and ER bound to antagonist. The agonists and antagonists bind in the same pocket of the protein core, but result in distinct conformations. ERα LBD bound to estradiol or DES resulted in a structure with helix 12 of the ERα LBD fitting tightly over the binding domain cavity where the ligand is situated *(26,27)*. In the ERα LBD antagonist-bound structures (4-OH-Tam and raloxifene) the binding of ligand prevented the alignment of helix 12 over the core and it is repositioned *(26,27)*. The agonist-induced position of helix 12 is thought to be necessary for formation of a competent activation-function-2 domain which allows interaction of receptors with coactivators *(16,27)*. The DES-bound ERα LBD cocrystallized with an ER-interacting peptide sequence from GRIP-1 showed that the peptide bound to a hydrophobic groove formed in part by helix 12 on the surface of the DES-liganded ERα LBD *(27)*. In the ER-4-OH-Tam structure, however, helix 12 of the LBD blocked this coactivator recognition groove by mimicking the interaction of the peptide with the receptor *(27)*.

The ERβ-raloxifene structure *(28)* is very similar to the ERα-raloxifene structure in that helix 12 is in the typical antagonist position not allowing coactivator to interact with receptor *(26,27)*. In contrast to raloxifene, the binding of the "pure" antagonist, ICI-164,384, to ERβ prevented helix 12 from interaction with the ERβ LBD, hence completely destabilizing helix 12, which may help to explain its full antagonist profile *(28a)*. It will be of interest to compare this structure with that of ERα bound to pure antagonist, once it is determined. Genistein binds with higher affinity to ERβ than ERα *(9,46)* and has been shown to be an ERβ-selective agonist in transfection assays *(47)*. Genistein also exhibits estrogenic activities *in vivo*, causing increased uterine weight and decreased serum LDL *(48)*, protection of smooth muscle vasculature *(49)* and protection against bone loss *(50)*. The genistein-ERβ structure shows that helix 12 lies in a position more similar to antagonist-bound receptor than agonist-bound ERα *(28)*. It is not clear why this would be the case, although it has been proposed that since genistein has shown less than 100% efficacy in certain assays it may be a partial agonist *(28)*. Additional crystal structures of ERβ bound to other agonists such as estradiol and ERα bound to genistein will be informative in this regard.

The crystal structures of the ERs have afforded a molecular picture of how ligands interact with the receptor LBDs and have shown that clear conformational changes take place on binding of receptors to agonists versus antagonists. Although these structures have been instrumental in our understanding of receptor structure/function relationships, they don't explain why raloxifene and 4-OH-Tam have different activities

in certain tissues or why estradiol can exert different activities through ERα and ERβ (*see* Introduction). One explanation would be that different receptor conformations are indeed induced by these ligands, but are not detectable by the protease digestion or crystal structure methods performed to date. To test this hypothesis, peptide phage display methodology was utilized.

PEPTIDE PHAGE DISPLAY TECHNOLOGY

Peptide phage display methodology *(29)* has been used as a way to study distinct receptor-conformational changes induced in ERα and ERβ by a variety of ligands *(30–36).* It has been exquisitely sensitive to detecting subtle changes in receptor conformation induced with different ligands. The technique involves screening of libraries of M13 phage-displayed peptides using purified preparations of ERα or ERβ that have been immobilized on streptavidin-coated plates through a biotinylated ERE in the presence and absence of various ligands *(30).* Phage that were affinity selected in this manner were then tested for interaction with each ER in the presence or absence of ligands by the use of anti-M13 antibodies in a phage ELISA. Time-resolved fluorescence (TRF) assays were employed to demonstrate that the peptide portion of the phage was binding to the ERs by use of europium-labeled peptides *(30).* TRF was also used to perform dose-response studies of ligands in recruitment of peptides to ERα and ERβ and to quantitate the extent to which the various peptide-interacting surfaces are exposed in the ligand-ER complexes.

The affinity selection of phage by estradiol-bound ERα resulted in the identification of several peptides that contained LXXLL motifs, the motif that is present in various receptor coactivator proteins that have been shown to interact with agonist-bound ER *(16).* Various other peptides that did not contain LXXLL motifs were also identified that preferred binding to unoccupied (apo) receptors or to 4-OH-Tam-bound ERs *(30).* Additionally, peptides that bound preferentially to either ERα or ERβ were detected. These peptides were then tested for binding to ERα and ERβ in the presence of several ER ligands. Each ligand tested altered the binding pattern of the peptides, yielding a distinct fingerprint which was indicative of the different conformations induced by each of these ligands upon binding to the receptors *(30,31).* Additionally, peptides were identified that interacted differentially with ERα or ERβ in the presence of 4-OH-Tam or raloxifene *(30).* Several peptides showed preference for 4-OH-Tam-bound ERα over raloxifene-bound ERα, indicating for the first time that there are conformational differences in the receptor structures induced by these two SERMs (*see* Table 1). Several of the SERMs are structurally similar triphenylethylene derivatives (4-OH-Tam, clomiphene, idoxifene, GW5638, GW7604), but induce different conformations in ERα as assessed by their ability to interact with various unique peptides, suggesting that even modest changes in ligand structure can affect receptor conformation *(30–31a).* GW5638 elicits distinct biology in comparison with these other compounds, and in fact it is in development for tamoxifen-resistant breast cancer *(31a).*

This methodology has been extended to test the ability of ER-interacting peptides to affect receptor biology *(32–35).* In one study, peptides that interacted with ERα or ERβ in the presence of estradiol or 4-OH-Tam were tested for their ability to modulate ER-dependent transcriptional activity. Peptide-GAL4-DBD fusions were constructed and tested for their ability to inhibit ER transactivation from luciferase reporters driven

Table 1
Biological Activity and ER-Peptide Interaction Induction of Various ER Ligands

		17β-Estradiol	*Genistein*	*4-OH-Tam*	*Raloxifene*	*ICI-182,780*
Breast		Agonist	Agonist *(52)*	Antagonist *(18)*	Antagonist *(19)*	Antagonist *(17,53)*
Uterus		Agonist	Agonist *(48)*	Partial Agonist *(23)*	Antagonist *(19,21,54)*	Antagonist *(17,53)*
Bone		Agonist	Agonist *(50)*	Agonist *(20)*	Agonist *(22)*	? *(53a)*
Brain		Agonist	Agonist *(52)*	Antagonist *(55)*	Antagonist *(56)*	Not active[a] *(53)*
Peptide α/β I *(30,31)*	ERα	++	ND	0	0	0
	ERβ					
Peptide α/β III *(30,31)*	ERα	–	ND	++	+	–
	ERβ					
Peptide α/β V *(30,31)*	ERα	0	ND	++	0	0
	ERβ					
Peptide α II *(30,31)*	ERα	++	ND	+	+	+
EBIP-49 *(34)*	ERβ	++	++	0	0	0
EBIP-53 *(34)*	ERβ	++	+	0	0	0
EBIP-92 *(34)*	ERβ	+	++	0	0	0

[a]ICI-182,780 is thought to be inactive in the brain because of its inability to cross the blood-brain barrier *(57)*. Plus and minus signs indicate approximate relative efficacy in changing interaction of peptide with receptor by the designated ligand versus vehicle control (++: higher relative fluorescence units (RFU) induced, +: lower efficacy of induction, 0: no change in interaction, –: decrease in RFU, ND: not determined).

by the C3 promoter or the collagenase promoter in transfected Hep G2 cells. 4-OH-Tam and estradiol each act as agonists from these promoter constructs in this assay *(32)*. Peptides that had been shown to interact with 4-OH-Tam-bound ERα in the phage ELISA or TRF assay inhibited 4-OH-Tam-induced luciferase activity, whereas those that interacted with estradiol-bound ERα *in vitro* had no effect on 4-OH-Tam-induced luciferase activity from either of the promoters *(32)*. Likewise, the peptides that interacted with estradiol-bound ERα inhibited the estradiol-induced luciferase activity from these promoters, but not that stimulated by 4-OH-Tam. Additionally, a peptide that interacted with ERβ, but not ERα, was found to block estradiol-induced luciferase activity through ERβ from an ERE, but not through ERα *(33)*. These experiments showed that the peptides that were identified by affinity selection of phage libraries to interact *in vitro* with ERα or ERβ also interacted with the receptors in cells and were able to inhibit their transcriptional activity. Therefore, these peptides acted as ER antagonists by blocking the receptor/cofactor interaction in cells, opening up the possibility that deliverable peptides such as these might be useful as drugs for breast cancer or other conditions *(32–34)*.

The conformational changes observed in the ERs on binding to different ligands is thought to result in the recruitment of specific cofactors. Whereas a number of these cofactors have been shown to interact with multiple members of the intracellular receptor (IR) family *(16)*, studies have shown that specificity for individual receptors can be conferred by the flanking regions of the coactivator LXXLL motifs *(51)*. While these types of mutational studies are informative, they are limited by the number of permutations that can be generated. The use of combinatorial phage display has been useful in circumventing this problem. One study involved screening a phage display library with more than 10^8 variations of peptides containing LXXLL motifs *(33)*. Three classes of LXXLL-containing peptides were selected by ERα in the presence of estradiol. These peptides were tested against ERβ, and several other IRs. Each class of peptide showed preferences for different receptors, indicating that the flanking regions of the LXXLL core sequence are important for specificity of IR/cofactor interactions.

Although peptides had been identified that interacted with ERβ without binding to ERα *(33)* or to TRβ *(36)*, peptides that were specific to ERβ without interacting with several other IRs were identified by screening the LXXLL-containing peptide-combinatorial phage library with ERβ protein *(34)*. These peptides disrupted the action of ERβ in cells, but did not affect the activity of any of the other IRs, including ERα. These reagents may prove to be instrumental in deciphering the action of ERβ versus ERα in cells or animals and may be useful in searching for novel ERβ-specific coactivators. Differences were observed in the ability of genistein and estradiol to interact with some of the ERβ-specific peptides, suggesting that there may be differences in the receptor conformations induced by genistein and estradiol *(34, see* Table 1). This is intriguing in light of the unique crystal structure of genistein-ERβ *(28)* and the interesting biological properties of genistein *(28,48–50,52)*. Therefore, unique conformations are induced in ERα and ERβ with various compounds, resulting in the exposure of different receptor surfaces, some of which may be bona fide interaction regions for specific coactivator proteins. This work supports the hypothesis that the diverse biological activities of various ER ligands may be caused in part by different receptor conformations induced by those compounds.

The three techniques described have contributed to the concept that different ligands

can induce distinct conformational changes in the ERs, allowing differential interaction with transcriptional accessory proteins, which may ultimately help determine the pharmacology of those ligands. The use of peptide phage display methodology could be extended in the future for screening libraries of compounds against a battery of identified ER-interacting peptides to find unique fingerprints. The ideal SERM is still an elusive entity, and several clinically useful SERM molecules might be designed with varying biological profiles, depending on the disease being targeted. New compounds with unique receptor conformational fingerprints may help lead the way to discover novel SERMs with desirable profiles of activity.

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