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Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology☆

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Abstract

Estrogens exert profound effects on the physiology of diverse target cells and these effects appear to be mediated by two estrogen receptor (ER) subtypes, ERα and ERβ. We have investigated how ER ligands, ranging from pure agonists to antagonists, interact with ERα and ERβ, and regulate their transcriptional activity on different genes. Mutational mapping-structure activity studies indicate that different residues of the ER ligand binding domain are involved in the recognition of structurally distinct estrogens and antiestrogens. We have identified from ligands of diverse structure, several particularly interesting ones that are high potency selective agonists via ERα and others that are full agonists through ERα while being full antagonists through ERβ. Antiestrogens such as hydroxytamoxifen, which are mixed agonist/antagonists through ERα, are pure antagonists through ERβ at estrogen response element-containing gene sites. Studies with ERα/β chimeric proteins reveal that tamoxifen agonism requires the activation function 1 region of ERα. Through two-hybrid assays, we have isolated an ER-specific coregulator that potentiates antiestrogen antagonist effectiveness and represses ER transcriptional activity. We have also focused on understanding the distinct pharmacologies of antiestrogen- and estrogen-regulated genes. Although antiestrogens are thought to largely act by antagonizing the actions of estrogens, we have found among several new ER-regulated genes, quinone reductase (QR), a detoxifying phase II antioxidant enzyme, that has its activity up-regulated by antiestrogens in an ER-dependent manner in breast cancer cells. This response is antagonized by estrogens, thus showing 'reversed pharmacology'. Increased QR activity by antiestrogens requires a functional ER (ERα or ERβ) and is, interestingly, mediated via the electrophile response element in the QR gene 5′ regulatory region. The up-regulation of QR may contribute to the beneficial effects of tamoxifen, raloxifene, and other antiestrogens in breast cancer prevention and treatment. Estrogens rapidly up-regulate expression of several genes associated with cell cytoarchitectural changes including NHE-RF, the sodium hydrogen exchanger regulatory factor, also known as EBP50. NHE-RF/EBP50 is enriched in microvilli, and may serve as a scaffold adaptor protein in regulating early changes in cell architecture and signal transduction events induced by estrogen. Analyses of the regulatory regions of these primary response genes, and the antioxidant and other signaling pathways involved, are providing considerable insight into the mechanisms by which ligands, that function as selective estrogen receptor modulators or SERMs, exert their marked effects on the activities and properties of target cells. The intriguing biology of estrogens in its diverse target cells is thus determined by the structure of the ligand, the ER subtype involved, the nature of the hormone-responsive gene promoter, and the character and balance of coactivators and corepressors that modulate the cellular response to the ER–ligand complex. The continuing development of ligands that function as selective estrogens or antiestrogens for ERα or ERβ should allow optimized tissue selectivity of these agents for menopausal hormone replacement therapy and the treatment and prevention of breast cancer.

Keywords: Estrogen; Estrogen receptor pharmacology; SERMS

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1. Introduction

Estrogens exert profound effects on the growth, differentiation, and functioning of many reproductive tissues. They also exert important actions on other tissues outside of the reproductive system including bone, liver, the cardiovascular system, and brain [1–3]. Most of the actions of estrogens appear to be exerted via two estrogen receptor (ER) subtypes, denoted ERα and ERβ, intracellular proteins that are members of a large superfamily of proteins that function as ligand-activated transcription factors [4,5]. Because of the diversity of estrogen target tissues, and the fact that the levels and proportion of ERα and ERβ differ in different target cells, much current interest focuses on trying to understand the basis for the cell and promoter context-dependent actions of estrogens and antiestrogens, and on the development of ligands showing optimal tissue-selective agonist/antagonist effectiveness for hormone replacement therapy and breast cancer prevention and treatment.

The action of estrogen receptors is tripartite, involving the receptor, its ligands, and its coregulator proteins (Fig. 1) [6–9]. The important interactions shown in Fig. 1 include the interaction of ligands, which can be either natural or synthetic, with the two ER subtypes and with various isoforms and splice variant forms of the ER in both normal and tumor cells, and the interactions of these ligand–receptor complexes with effectors that include different DNA response elements, and important coregulator proteins that regulate the magnitude of transcriptional response to the ER. In addition to well-documented effects on gene transcription, as discussed further below, there is increasing evidence

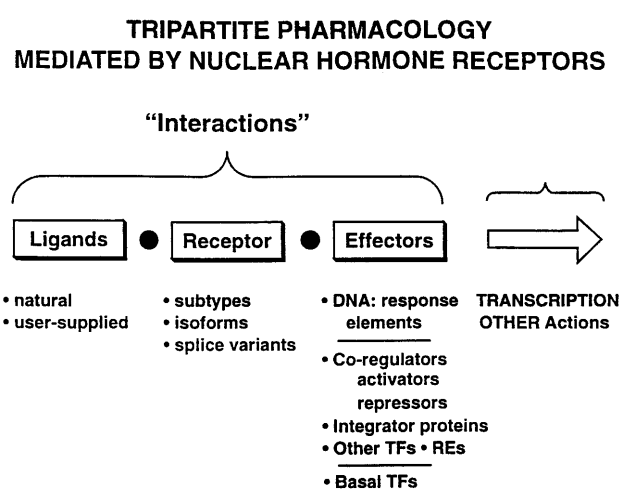

Fig. 1. The tripartite nature of nuclear hormone receptor action, involving ligand, receptor, and effector interactions. The flow of information from ligand–receptor–effector to actions initiated by the binding of ligands with the estrogen receptor is indicated. TF, transcription factor; RE, response element. See text for details.

that ERs and other estrogen-binding proteins are involved in some of the rapid, non-genomic effects of estrogens in target cells. These include, among others, effects of estrogens on calcium fluxes in bone and breast cancer cells, and rapid activation of MAP kinase pathways [10–14].

Recent efforts in this laboratory have focused on the fine discrimination in ER–ligand interrelationships that allow different ligands to strongly discriminate between ERα and ERβ, and the marked modulation of ER activity by coregulators. In the development of optimal selective estrogen receptor ligands, one is aiming for tissue selective ligands that will be estrogen antagonists in the breast and uterus, thereby not stimulating cell proliferation in these tissues, while being good estrogen agonists in bone, the cardiovascular system, in terms of lipid profiles, and in the brain where cognitive functions and other activities may be regulated by estrogens.

Our recent work, described in this report, continues to highlight the exquisite precision in receptor activity regulation by ligand. Small changes in ligand structure can result in major changes in the biological character of the receptor. Also, the activity of different ligands via ERα or ERβ shows distinct pharmacology at different target genes. These factors no doubt underlie the cell-specific and promoter-specific activities of estrogens in different target cells and at different gene sites. There has been tremendous progress in understanding the biochemical nature of ERα and more recently ERβ, and how they interact with estrogen and antiestrogen ligands and with coregulator proteins that modulate receptor transcriptional activity.

2. Results and discussion

2.1. Estrogen receptor structure–activity relationships

In order to address the question 'How does the estrogen receptor discriminate among different ligands?', we undertook studies in which we mapped the ERα ligand binding domain by affinity labeling and mutational analyses from which we have determined regions in ER critical for hormone binding and discrimination between antiestrogens and estrogens [15–21]. Further, we identified ligand contact sites between ER and antiestrogens versus estrogen, and showed that different estrogens contact a distinct set of different amino acids in the ER [22,23]. These findings defined the orientation of ligands within the ERα ligand binding pocket, with the A-ring phenol of the ligand hydrogen bonded to E353 in helix 3 of the ER and the D-ring like portion of the ligand in contact with H524 in helix 11 [22–24].

Notably, some mutations in activation function-2 (AF-2) or those impacting on AF-2 function resulted in

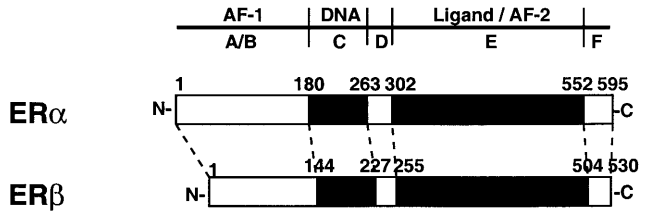

Fig. 2. Schematics of the human estrogen receptor alpha and human estrogen receptor beta. The structural domains of these receptors (A/B, C, D, E, and F), as well as the hormone binding, DNA binding, and transactivation (AF-1, AF-2) functional domains are shown, as is the percent amino acid identity of each domain between the two estrogen receptors (numbers in parentheses). AF, activation function.

transcriptionally inactive ERs that functioned as very effective dominant negative ERs. These dominant negative ERs could effectively suppress ER-positive breast cancer cell proliferation and estrogen-stimulated gene expression [15,25–27], while a single amino acid change near the start of helix 12 in the ER generated strong, ligand-independent constitutive activity of the receptor and ligand-independent association with coregulators [28–32].

2.2. Development of estrogen receptor alpha- and estrogen receptor beta-selective ligands

The cloning in 1996 of ERβ, a new ER subtype encoded by a gene different from that encoding ERα, greatly excited the ER field [33–35]. Estrogen receptors alpha and beta differ most markedly in the N-terminal A/B domain, having only 18% amino acid identity. They also differ substantially in the hormone binding domain, showing only 56% amino acid identity (Fig. 2). The great differences in the A/B domains suggested that the transcriptional activation of different estrogen-responsive genes by ERs alpha and beta might show distinctly different patterns, because it is now appreciated that gene activation is influenced by promoter- and cell-specific factors [6,36–38] and by synergistic interaction between N- and C-terminal receptor activation domains [39].

We therefore studied the ER subtypes alpha and beta, as well as chimeric constructs we prepared with ERα and ERβ, to examine the bioactivities of these receptors and their responses to estrogen and antiestrogen ligands [40]. These investigations showed that transcriptional activity of ERβ is highly dependent on cell/promoter context and on the nature of the ligand. Most interestingly, antiestrogens such as tamoxifen and 2-phenylbenzofuran, which show some agonistic activity with ERα, exhibited no agonistic activity with ERβ. We found that replacing the A/B domain of ERβ with the A/B domain of ERα resulted in a receptor chimera exhibiting an improved transcriptional response to estrogens and, interestingly, that this ERα/β chimera was also now able to activate transcription upon treatment with these antiestrogens. As antiestrogen agonism was lacking in ERβ and in the ERβ/α chimera containing the amino-terminal A/B domain of ERβ fused to domains C through F of ERα, but was restored in the ERα/β chimera containing the A/B domain of ERα, antiestrogen agonism was shown to depend on the A/B domain (activation function-1-containing region) of ERα. These results indicate that the differences in the amino-terminal regions of ERα and ERβ contribute to the cell- and promoter-specific differences in the transcriptional activity of these receptors, and their ability to respond to different ligands, thus providing a mechanism for differentially regulated transcription by these two ERs [40].

Because ERα and ERβ have significantly different primary sequences in their ligand binding domains, it seemed reasonable that these ER subtypes might bind some ligands with different affinity and that these ligands might also have different agonist or antagonist characters mediated by the two receptors. Because ERα and ERβ have some overlapping but also some different tissue distributions [33,34,41], differences in ligand interaction or activity with the two ERs could translate into important differences in their biological actions at the tissue level.

To identify compounds that might have ER subtype-selective activity, we examined some structurally diverse ER ligands, from which we have identified several novel, nonsteroidal ligands that show pronounced subtype-selective differences in ligand binding and transcriptional potency or efficacy for ERα and ERβ. An aryl-substituted pyrazole was found to be an ERα potency-selective agonist, showing 120-fold higher potency in stimulation of ERα versus ERβ in transactivation assays in cells. A different non-steroidal compound, a tetrahydrochrysene (THC), was found to be an ERα agonist but a complete antagonist on ERβ [42]. Further investigations have revealed that the antagonist character in THC ligands for ERβ depends in a progressive way on the size and geometric disposition of substituent groups [43]. These studies also highlight that there is a different set point for change between agonist and antagonist in ERα and ERβ, and indicate that it is easier to shift to the antagonist conformation in ERβ. Furthermore, antagonists that are selectively effective on ERβ can have structures that are very different from the typical antiestrogens (tamoxifen, raloxifene, ICI182780) that are antagonists on both ERα and ERβ. Other compounds we have identified are even more ERα-potency selective than the pyrazole, while others are ERβ-selective agonists. Investigations with these ERα and ERβ selective agonist and antagonist ligands, along with studies in ERα and/or ERβ knock out animals [44–48], should be very useful in

defining the distinct roles played by these two receptors in the biological effects of estrogens in the many tissues (e.g. mammary gland, vascular system, bone, brain, etc.), in both males and females, in which estrogens act.

2.3. An estrogen receptor selective coregulator that potentiates antiestrogen inhibitory effectiveness and represses estrogen activity

Because of the role of estrogens in promoting the growth and progression of breast cancers, there is currently great interest in exploring ways to functionally inactivate the ER, so as to suppress ER-mediated gene expression and cell proliferation. These approaches have involved the use of antiestrogens such as tamoxifen, which is used widely in breast cancer treatment [2,49,50], and may in the future utilize optimized antiestrogens as well as dominant negative ERs.

Our analysis of the mechanisms that determine dominant negative ER effectiveness [25,51] showed that dominant negative ERs exerted their inhibitory effectiveness by heterodimerizing with the wild-type ER, and their potency implied that an active mechanism was involved in their suppression of wild-type ER activity [26,51]. We therefore hypothesized that the dominant negative ER might recruit a repressive protein into the dominant negative ER complex. We imagined as well that a similar mechanism might be involved in mediating the inhibitory effects of antiestrogens operating through wild-type ER. Because the activation function-2 region of the ER has been shown to be important in its interaction with other protein factors, and our dominant negative ER was altered by a point mutation in this AF-2 region (L540Q ER), we thought it likely that the dominant negative ER might be suppressing wild-type ER actions by recruiting repressor factors to this region of the receptor protein. Intriguingly, it is also the AF-2 region of ER that undergoes a marked shift in conformation in an ER–antiestrogen complex [52].

To investigate this possible recruitment of a repressor protein, we have utilized two-hybrid screening in yeast, from which we identified a protein, denoted Repressor of Estrogen receptor Activity (REA), that interacts preferentially with the dominant negative ER and with the antiestrogen-liganded ER [53]. This protein was found to enhance the potency of dominant negative ERs and antiestrogens as suppressors of ER activity. Interestingly, REA is an ER-selective coregulator. REA suppresses transcriptional activity of ERα and ERβ, but has no effect on the transcriptional activity of the progesterone receptor, retinoic acid receptor, and androgen or thyroid hormone receptors, or an unrelated transcriptional activator, Gal 4-VP16. Mapping studies showed that two regions of REA, encompassing amino acids 19–49 and 150–174, were required for repressive activity [53]. Further studies documented that REA interacts directly with ER in in vitro GST pull-down assays, that interaction was greater with the dominant negative ER than with the wild-type ER, and that the interaction with both receptors was greatly increased by hormone (estradiol or *trans*-hydroxytamoxifen) and was preferentially increased by antiestrogen. REA was found not to interfere with the ability of ER to bind to estrogen response element containing DNA. However, REA was found to compete with the coactivator steroid receptor coactivator 1 (SRC-1) for modulation of ER transcriptional activity. The coactivator SRC-1 enhanced the estradiol-mediated transcriptional activity of ER up to 4- to 5-fold, and coexpression of REA suppressed the enhancement of ER transcriptional activity by SRC-1 very effectively, and it did so in a concentration-dependent manner, implying mutual functional competition between these two coregulatory proteins [53].

REA represents an example of a protein that enhances the potency of two inhibitors of ER action, antiestrogens and dominant negative ERs, implying that interaction with REA may represent an important point of convergence in the mechanism of action pathway through which these two factors function. The activities of REA suggest that it may play an important role in determining the sensitivity of estrogen target cells, including breast cancer cells, to antiestrogens and estrogens. Ongoing studies are aimed at investigating this further.

2.4. The distinctive pharmacologies of estrogen and antiestrogen regulated genes mediated by estrogen receptor-α and estrogen receptor-β

Antiestrogens have long been thought to exert most of their beneficial effects in breast cancer by antagonizing the actions of estrogen. The antiestrogenic actions of these compounds in other target cells are likewise thought to be exerted predominantly by reversing the generally stimulatory effects of estrogen. In view of the fact that ERs are believed to exert most of their effects via changes in the regulation of gene expression, we examined the effects of antiestrogens on gene expression in breast cancer cells. Using differential display RNA methods and ER-containing MCF-7 breast cancer cells grown in the presence of tamoxifen, we consistently observed an ER target gene that was up-regulated in the presence of antiestrogens. This gene, quinone reductase (QR), showed reversed pharmacology, being markedly up-regulated by antiestrogen and suppressed by estrogen in breast cancer cells [54,55].

QR, by reducing quinones, decreases the generation of hydroxyl radicals and hence contributes to the antioxidant defense mechanisms in cells. The ability of

antiestrogens to increase QR activity, an activity that is reversed by estradiol and requires a functional estrogen receptor [54], suggests that the effectiveness of antiestrogens in anticancer drug therapy, as well as in prevention of breast cancer, may be due in part to the beneficial, antioxidant capabilities of tamoxifen and its stimulation of antioxidant detoxifying enzymes such as QR. Interestingly, antiestrogen stimulation of the QR gene is mediated by an electrophile response element, and not by typical estrogen response elements [54,55].

In comparing the activity of ERs complexed with estrogen and antiestrogen ligands via an estrogen response element, or an electrophile response element, we observed marked differences in ligand character at these different gene sites and with ERα or ERβ. Of interest, antiestrogens are stronger activators of the QR electrophile response element via ERβ than ERα. The response to ligands acting through estrogen receptors at the electrophile response element are quite different than the pattern observed at the estrogen response element. Estradiol is the best stimulator at the estrogen response element and tamoxifen and LY117018 showed weak agonistic activity at this gene site in the cell background examined [55]. The antiestrogens tamoxifen and LY117018 were completely inactive acting through ERβ and function as complete antagonists at estrogen response element-containing gene sites. In constrast, antiestrogens but not estrogen are stimulators of the quinone reductase 5′-flanking region and antiestrogen stimulation is greater via ERβ than ERα. Interestingly, antiestrogens show strong stimulatory activity at AP-1 sites also, as documented by Kushner and colleagues [56], highlighting the intriguing pharmacology of ligand receptor complexes at different gene sites. That ERβ is a more potent activator at electrophile response elements than is ERα suggests that the different levels of these two receptors in various estrogen target cells could impact importantly on the antioxidant potency of antiestrogens in different target cells. These findings may have broad implications regarding the potential beneficial effects of antiestrogens, since electrophile response elements mediate the transcriptional induction of numerous genes, including QR, which encode chemoprotective detoxification enzymes.

In contrast to the pattern of ligand regulation of the QR gene, most genes under estrogen receptor regulation appear to be up-regulated by estrogen, with this up-regulation being suppressed by antiestrogens. This more usual regulation was observed by us in differential gene regulation studies through which we identified estrogenic regulation of the sodium/hydrogen exchanger regulatory factor [57]. We identified the human homolog of the rabbit Na^+/H^+ exchanger regulatory factor (NHE-RF), a ~ 50 kilodalton protein also known as EBP50, that is also an ezrin–radixin–moesin binding phosphoprotein, as being under rapid and direct regulation by estrogen in ER-containing breast cancer cells. Stimulation by estrogen of NHE-RF RNA is rapid, being near maximal ($\sim$ six $\times$) by one h, and was not blocked by cycloheximide, indicating that it is a primary response. Stimulation is selective for estrogen ligands, with no stimulation by other classes of steroid hormones, and stimulation by estrogen is suppressed by the antiestrogens tamoxifen and ICI 182,780. Interestingly, NHE-RF/EBP50 is a PDZ domain-containing protein that is enriched in polarized epithelia where it is known to be localized in microvilli. NHE-RF/EBP50 regulates protein kinase A inhibition of the Na^+/H^+ exchanger, and may serve as a scaffold adaptor protein that contributes to the specificity of signal transduction events. These findings suggest that the early, known effects of estrogen on cell cytoarchitecture (e.g. increasing microvilli on breast cancer cells) and on some cell signaling pathways (e.g. those involving cAMP) may involve rapid estrogen-mediated changes in production of NHE-RF/EBP50. These observations of NHE-RF/EBP50 regulation by estrogen raise intriguing questions regarding the links between estrogens and cytoskeletal cell signaling, and cAMP-regulated events in breast cancer cells and in other polarized epithelia in which estrogen receptors function.

3. Conclusions

In the studies described above, we have illustrated that the intriguing biology of estrogens in their diverse target cells is determined by the structure of the ligand, the estrogen receptor subtype, the nature of the gene promoter responsive unit, and the character and balance of coactivators and corepressors present in different target cells. Our current ability to chemically modify the structure of estrogen receptor ligands, to better understand the functioning and tissue distribution of the two estrogen receptor subtypes, and the regulation and impact of coregulators such as REA in the actions of antiestrogens and other selective estrogen receptor modulators, suggests that ongoing estrogen receptor research should yield large dividends in terms of our being able to design ligands with optimized tissue selectivity that will be most effective for hormone replacement therapy and for the treatment and also, hopefully, the prevention of breast cancer and other hormone-dependent cancers.

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