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Topic 1.3

Function and mode of action of nuclear receptors: Estrogen, progesterone, and vitamin D*

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Abstract: Estrogens and progestins play important roles in regulating growth and differentiation of a wide range of cell types, in both reproductive and nonreproductive tissues. Not surprisingly, therefore, endocrine active substances that mimic the actions of these steroid hormones have been demonstrated to have untoward effects on the reproductive function of a variety of animals. Although direct links between exposure to endocrine active substances of this class and reproductive abnormalities in humans have been difficult to establish, the potential for harm by this class of chemicals warrants further investigation. This chapter summarizes our current understanding of the molecular pharmacology of the estrogen and progesterone receptors, ER and PR, beginning with a historical perspective of ER pharmacology and ending with a comparison of these receptors to the vitamin D receptor (VDR), a nonsteroid hormone nuclear receptor involved in regulating growth and development in nonreproductive tissues. Major topics discussed include receptor subtypes (or isoforms), receptor functional domains, ligand-binding characteristics, receptor structure, cofactor binding, effects of phosphorylation, and nonclassical modes of action. This discussion will demonstrate the need for developing novel screens for potential endocrine disruptors that incorporate our current understanding of nuclear receptor pharmacology.

INTRODUCTION: HISTORICAL MODELS OF ENDOCRINE RECEPTOR ACTION

The modern era of ovarian steroid hormone pharmacology began nearly 40 years ago when Elwood Jensen and colleagues demonstrated that radiolabeled estradiol was specifically retained in cells that exhibit a phenotypic response to this hormone [1]. This initial finding led to the discovery that the biological activity of these hormones is mediated by specific high-affinity receptors for estrogens and progestins, ER and PR respectively, which are expressed in target cells. It is now well established that these receptors are ligand-regulated transcription factors which, when activated, can facilitate alterations in gene expression and effect a phenotypic change in target cells.

Being a lipophilic molecule, estrogen passively diffuses across cell membranes and binds to the estrogen receptor (ER), located in the nucleus. When a ligand such as estrogen binds to ER, it allows the receptor to undergo a conformational change, bind to its response element within the promoter of a target gene and regulate transcription of that gene. Historically, it was thought that ligands control nuclear receptor activity much like a switch; binding to the receptor was thought to convert it from an "off"

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to an "on" state. With the identification of SERMs or "selective estrogen receptor modulators", compounds that display tissue-specific agonist/antagonist activities, it was realized that this on/off model of nuclear receptor action did not adequately describe the pharmacology of known ligands. Rather, it now appears that different ligands acting through the same receptor can manifest different activities in different cells; thus explaining why it has been difficult to establish a single predictive screen for potential endocrine disruptors.

ER subtypes

There are two subtypes of ER—ERα, which was first described in 1962, and the more recently discovered ERβ, which was first cloned in 1996 from rat prostate [1,2]. As members of the nuclear receptor superfamily, ERα and β contain the hallmark features of this family of transcription factors. These include a two zinc-finger motif DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD), which is composed of 12 alpha helical structures [3,4]. ERα and ERβ each also contain two activation functions, AF-1 in the N-terminus and AF-2 in the C-terminus, within the LBD (Fig. 1). Overall, the receptors are only 47 % identical; the most conserved region between them is the DBD, while the most divergent region is the N-terminal AF-1 [5]. ERα and β interact with endogenous estrogens and many antiestrogens with similar affinity, however, some phytoestrogens, such as genistein and coumestrol, appear to have stronger affinity for ERβ [6]. Both receptors bind estrogen response elements (EREs) in the promoters of target genes equally well, yet, due to their sequence differences, especially those in the N-terminus, they are not functionally equivalent [7]. When occupied by estradiol, the transcriptional activity of ERβ only reaches 20–60 % of the activity of ERα in a given tissue [8]. Molecular dissection of the divergent N-terminus of ERβ reveals that it may contain a repressor domain that dampens its activity; removal of the N-terminus renders the receptor more transcriptionally active than its wild-type counterpart ERβ [8]. Additionally, it has been found that the SERMs tamoxifen, raloxifene, GW7604, and idoxifene do not display any partial agonism on ERβ when analyzed on canonical ERE-containing genes as they do on ERα. Coexpression studies of ERα and β reveal that heterodimerization between the two receptor subtypes can occur, possibly explaining how ERβ can dampen ERα's transcriptional activity in response to physiological levels of estradiol [8]. It may also explain how the partial agonist activity of tamoxifen on ERα can be abolished in cells where both subtypes are expressed. Superimposed on their distinct molecular pharmacology, differences in the tissue distribution and expression levels of the two ER subtypes add complexity to the biological roles of es-

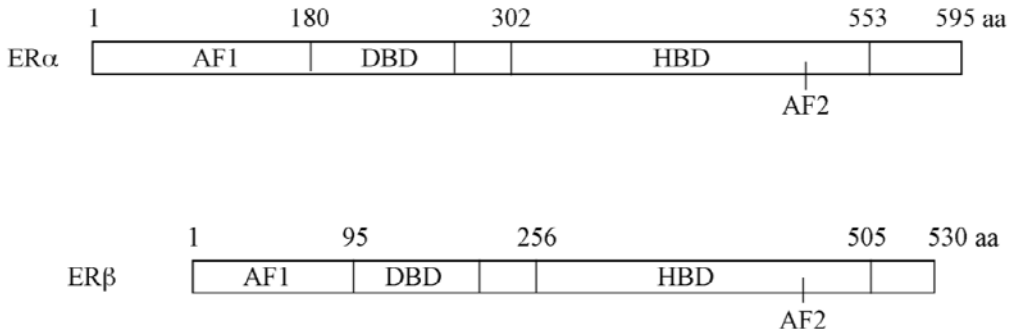

Fig. 1 Diagram of ERα and ERβ functional domains. These two subtypes of ER are 47 % identical overall, with ERα being slightly longer than ERβ (595 vs. 530 amino acids). The most conserved region between the two receptors is the DNA-binding domain (DBD), while the most divergent region is the amino-terminal activation function 1 (AF-1). Also shown are the hormone-binding domain (HBD) and the activation function 2 (AF-2), which resides within the HBD.

trogen. For instance, ERα is generally expressed at higher levels than ERβ and their expression patterns, while somewhat different, overlap in many target tissues such as the ovary, uterus, and testes [2,9]. The standardization of receptor quantitation techniques and their application to the evaluation of ERα and ERβ distribution would significantly help in studying estrogen action.

Molecular actions of ERα and β

In most tissue types, AF-2 is the dominant activation function for both ERα and β, and it operates in a ligand-dependent manner [10]. Upon ligand-binding, the p160 coactivators, SRC1, GRIP1, and ACTR are the principal coactivators that bind to AF-2 and each of these contains multiple copies of the binding motif, L*xx*LL (L = leucine, *x* = any residue) which is critical for their interaction with the AF-2 coactivator binding pocket. The p160 coactivators also serve as docking sites for the assembly of a large coactivator network, the function of which is to transmit information from the ER-ligand complex to the general transcription apparatus (GTA) and to promote transcriptional initiation [11–13]. A more detailed discussion of how coactivators coordinately regulate ER activity can be found elsewhere [14]. The AF-1 domain of ERα or β operates independently of ligand but contains sequences that allow the receptor to be controlled by other means, such as growth factor signaling. Studies with ERα show that EGF, IGF, and TGFα signaling can activate the receptor through phosphorylation of key residues in AF-1, such as serine 118 (in human ERα). Phosphorylation of these residues may then allow coactivators like SRC1 and CBP to be recruited to the AF-1 domain [15–17]. The AF-1 has also been found to bind to a variety of unique cofactors that do not interact with AF-2. These include the RNA coactivator, SRA (steroid receptor RNA coactivator), and p68 RNA helicase, a protein that preferentially binds to AF-1 when Ser 118 is phosphorylated [18,19]. Another AF-1-binding cofactor is the RNA-binding protein RTA (repressor of tamoxifen action), which represses the partial agonist activity of ER when bound by tamoxifen or other antiestrogens [20]. Differences in the expression levels of these non-AF-2 interacting cofactors suggest that communication between AF-1 and AF-2 may not occur in the same manner in all cells. When antiestrogens bind ER, corepressor proteins that impede transcriptional activation are recruited to the receptor. Corepressors like N-CoR and SMRT are thought to associate with a region encompassing the AF-2 domain while repressors like RTA, mentioned above, interact with the N-terminus [20,21]. These proteins facilitate the formation of a large corepressor complex on the receptor that opposes transcriptional activation, in part, through the enzymatic activity of histone deacetylases (HDACs) and chromatin remodeling factors [22–24]. Cofactors that bind agonist or antagonist-occupied ER have been found to cycle on and off ER target gene promoters in a highly regulated and dynamic pattern [25]. The availability of different coactivators and corepressors will help determine if a SERM acts as an ER agonist or antagonist in a given cell.

Molecular pharmacology of ER

In addition to the factors mentioned above, the differential activities of ER ligands are influenced by the specific ligand-induced conformation of the receptor [26]. Analysis of the crystal structures of several different ER-ligand complexes has been informative with regard to their agonist/antagonist actions. These structures have revealed that although narrow, the ligand-binding pocket of ER is flexible enough to accommodate a variety of ligands, some with bulky side chains and/or spatial requirements different than that of 17β estradiol (Fig. 2) [4,7]. Differences in ligand-binding characteristics will impact the structure of the coactivator-binding pocket, which is the primary region of contact between ER and the transcriptional apparatus. In an "agonist conformation", helix 12 of the LBD resides along the edge of the coactivator-binding pocket, allowing coactivators like GRIP1 to bind within the pocket using their L*xx*LL motifs [4] and activate transcription. However, when tamoxifen is bound, a conformational difference in the receptor causes helix 12 to wedge itself into the coactivator pocket through its own L*xx*LL-like region (aa540-544; LLEML). In this manner, helix 12 acts as a decoy coactivator

to block the transcriptional coactivation of tamoxifen-bound ER [4]. By exposing different surfaces on the receptor, small ligand-induced conformational differences can translate into significant pharmacological differences by offering new surfaces for cofactors to bind or by covering up surfaces that otherwise would be used in binding cofactors. Phage display screening has been used successfully to identify peptides that interact with unique surfaces on ER that are exposed in the presence of different ligands. By binding to sites that are important for protein–protein interactions, many of the identified peptides have been found to act as antagonists, modulating the pharmacology of the receptor when bound by a particular SERM [27,28]. These peptide antagonist studies have demonstrated how ligand-induced conformational differences in ER help determine the activity of SERMs. Cumulatively, the differential activity of SERMs depend on (a) which ER subtype(s) is present in a given cell type, (b) cofactor expression levels in that cell type, and (c) the specific ligand-induced conformation of the receptor.

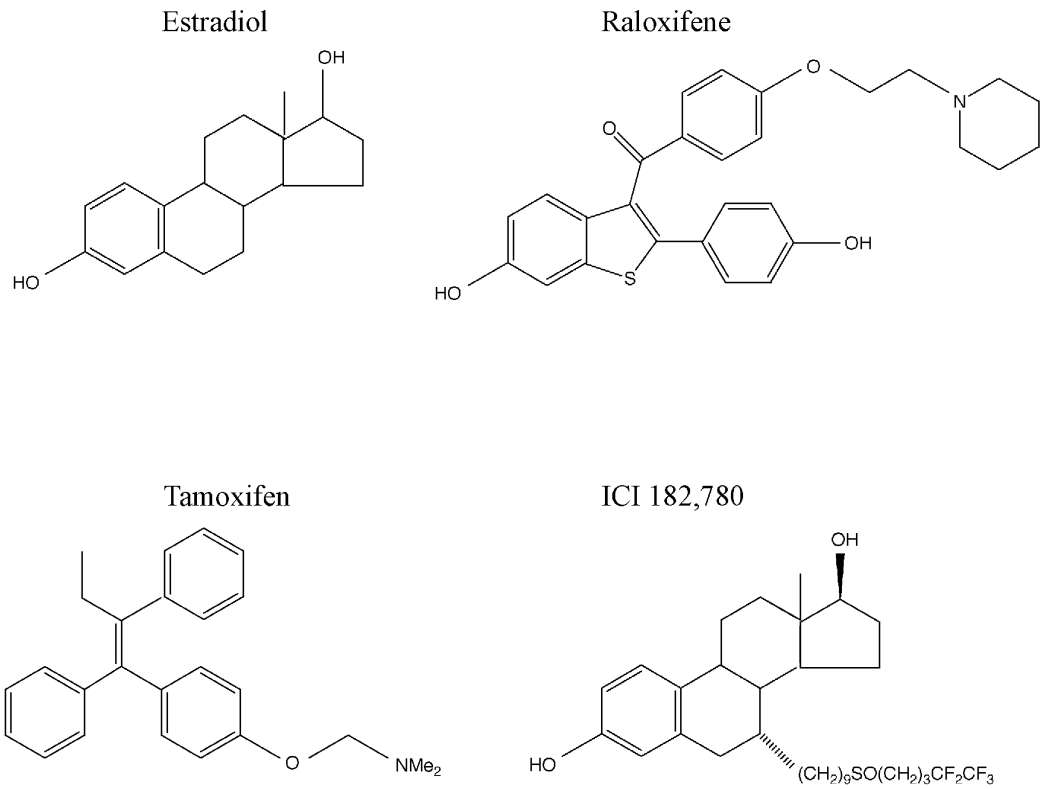

Fig. 2 ER ligands with different molecular activities. The natural ligand for the estrogen receptor, 17β estradiol is shown in the upper left-hand corner. Also depicted are the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene, which display tissue-specific agonist or antagonist activities and the pure ER antagonist, ICI 182,780.

Nonclassical mode of transcriptional regulation and nongenomic actions of ER

In addition to directly contacting promoters of target genes, ER can also affect transcription of genes through a "nonclassical" mode of action, by binding to and influencing the activities of other transcription factors, such as NFκB and AP-1 (Fig. 3). For example, by binding to AP-1, ERα and ERβ can enhance transcription of the collagenase gene, which contains an AP-1 responsive promoter [29]. All ligands tested, including the SERMs tamoxifen, raloxifene, and GW7604 allow ER to regulate

AP-1 activity in this manner. Therefore, nonclassical modes of transcriptional regulation may need to be considered when evaluating the actions of ER ligands. To add further to the complexity of ER action, there is increasing evidence that ERα may have roles that do not require its transcriptional activation, such as modifying the activity of enzymes and ion channels. For example, liganded ERα but not ERβ has been found to increase nitric oxide (NO) release from endothelial cells, which may prevent leukocyte accumulation in the vasculature [30,31]. Additionally, ERα has also been shown to interact with and modulate the activity of the tyrosine kinase, c-src, which is involved in signal transduction pathways of cell cycle progression, cell proliferation, differentiation, adhesion, and migration [32]. However, more studies are needed to understand the potential biological significance of nongenomic ER actions.

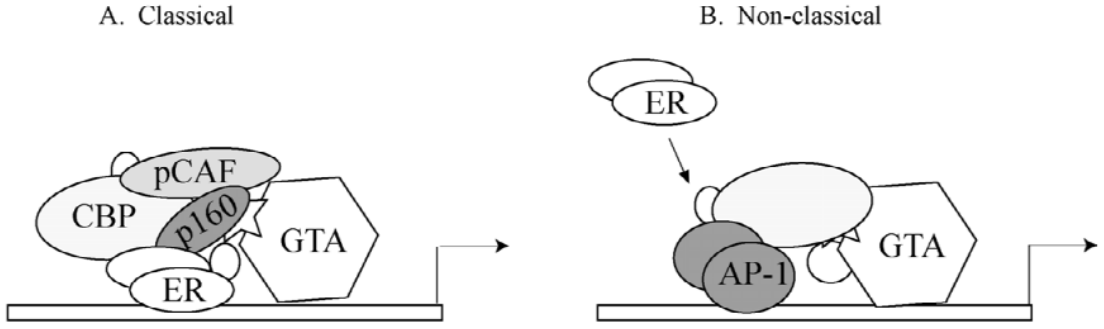

Fig. 3 Modes of ER action. (A) "Classical" mode of ER action occurs when ER directly binding to estrogen response elements (EREs) within the promoters of target genes. The coactivators pCAF and CBP, and a member of the p160 family of coactivators (SRC1, GRIP1, or ACTR) are tethered to ER and to the general transcription apparatus (GTA) to promote transcription. (B) "Nonclassical" mode of ER action occurs when ER regulates transcription in an indirect manner. ER binds to another transcription factors, such as AP-1, and either positively or negatively affects the activity of AP-1 on its target gene promoter.

PROGESTERONE RECEPTORS

Similar to ER, the human progesterone receptor (hPR) also exists in two forms, hPR-A and hPR-B (Fig. 4). Unlike ER, these two PR isoforms are derived from alternative start sites on the same gene. hPR-A and hPR-B are identical in amino acid sequence with the exception of an additional 164 aa on the N-terminus of hPR-B [33,34]. Despite their similarity, the two PR receptors have opposite transcriptional activities. hPR-B acts as a strong transcriptional activator, while hPR-A acts as only a weak activator or in many cases, a repressor of transcription [35–37]. Major target tissues of progesterone include those in the reproductive tract such as the ovary, uterus, and vagina, as well as the mammary gland, pituitary, and hypothalamus [38–42]. Progesterone is involved in maintaining female reproductive function and establishment and maintenance of pregnancy, making it a useful target for therapeutic intervention [43].

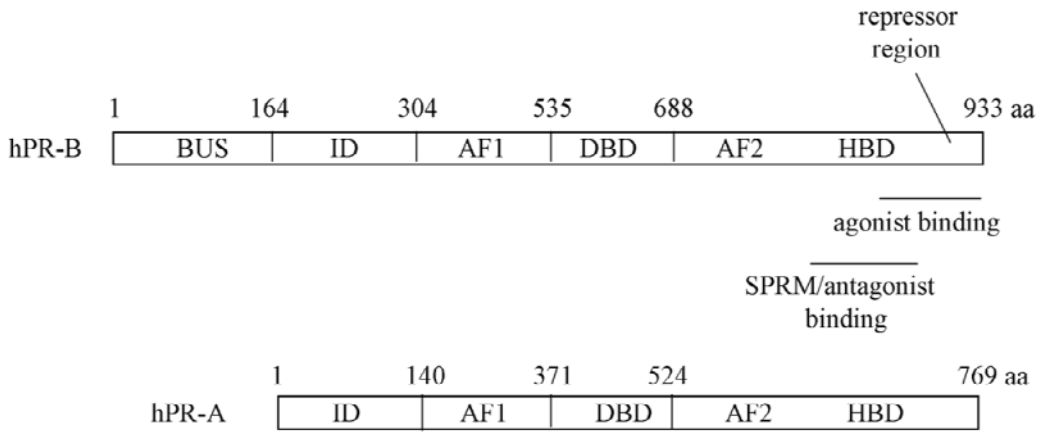

Fig. 4 Diagram of the two isoforms of human PR, hPR-B and hPR-A. These receptors are identical except for the 164 amino acid B-upstream sequence (BUS) on the N-terminus of hPR-B. The inhibitory domain (ID) confers a repressive function on hPR-A, causing this receptor to function as a repressor or weak activator of transcription. In hPR-B, the presence of the BUS overrides the function of the ID and allows hPR-B to function as a strong transcriptional activator. Also depicted on each receptor are two activation functions, AF-1 and AF-2, a DNA-binding domain (DBD), and hormone-binding domain (HBD). Residues located in the extreme C-terminus of each receptor (within the repressor region) are required for agonists to bind, whereas selective progesterone receptor modulators (SPRMs) and antagonists require residues more centrally located within the LBD of hPR-A or hPR-B.

PR domains

The primary sequences of PR-A and B contain the classic features of the nuclear receptor superfamily, but three additional features distinguish PR from other nuclear receptors (Fig. 4). These are (1) an amino-terminal inhibitory domain (ID), (2) a B upstream sequence or "BUS", which is only present in hPR-B, and (3) a small carboxy-terminal repressor region [36,44]. The ID, although present in both isoforms, functions as a repressor only within hPR-A. If the ID is removed, hPR-A turns into a strong transcriptional activator, similar to PR-B [36]. In the context of full-length hPR-B, however, the repressive influence of the ID is overcome by the BUS region, the N-terminal extension on PR-B that distinguishes it from PR-A. The BUS region allows hPR-B to function as an activator of transcription, most likely by imposing conformational changes that render the ID inactive [45]. Additionally, the BUS region may contain an additional AF between aa 54-154, called AF3, yet the relative contribution of this AF3 to the transcriptional activity of full-length hPR-B remains unclear [46]. The third feature of PR that distinguishes it from other nuclear receptors is its C-terminal repressor region. In an unliganded state, corepressor proteins are thought to bind to this region of the LBD and are only displaced by the binding of PR agonists. The binding of PR antagonists, however, does not displace these corepressors, and therefore they are able to contribute to the repression of the antagonist-bound receptor [47]. Like ER, PR activity is not only regulated by ligand binding, but it is also regulated by phosphorylation. To date, eight phosphorylation sites have been found on PR. Four of these sites, Ser 20, 81, 102, and 162 reside in the BUS region of hPR-B and the other four sites, Ser 190, 294, 345, and 400 are common to both hPR-A and B. The significance of phosphorylation of these residues is not entirely clear since only some of these residues appear to be required for PR activity [48]. Interestingly, some isoform preference for specific phosphorylation sites has been observed, and these sites may contribute to or reflect the different activities of hPR-A and B [49].

PR ligands

The physiological effects of progestins and antiprogestins can be difficult to interpret due to their potential to cross-react with other nuclear receptors, such as glucocorticoid, mineralocorticoid, and androgen receptors (GR, MR, and AR, respectively). The DBDs and LBDs of these receptors share a high degree of homology with PR, allowing ligands for one receptor to bind to and effect the activity of others. The noted cross-reactivity of ligands in the PR, GR, MR, and AR subfamily of receptors could confound the interpretation of results from whole animal studies set up to evaluate endocrine disruptors. In addition to cross-reactivity with other nuclear receptors, many PR ligands display agonist or antagonist effects in a cell- or tissue-specific manner. Analogous to SERMS, PR ligands with tissue-specific agonist/antagonist activities are now referred to as SPRMs for "selective progesterone receptor modulators". As with SERMs, SPRM action relies on the specific ligand-induced conformation of PR and the profile of cofactors available to modulate its activity (Fig. 5) [50–52]. In sum, the actions of PR ligands may be even more complex than those for ER because the actions of PR ligands depend on both their tissue-specific PR agonist or antagonist effects and their potential to cross-react with other nuclear receptors.

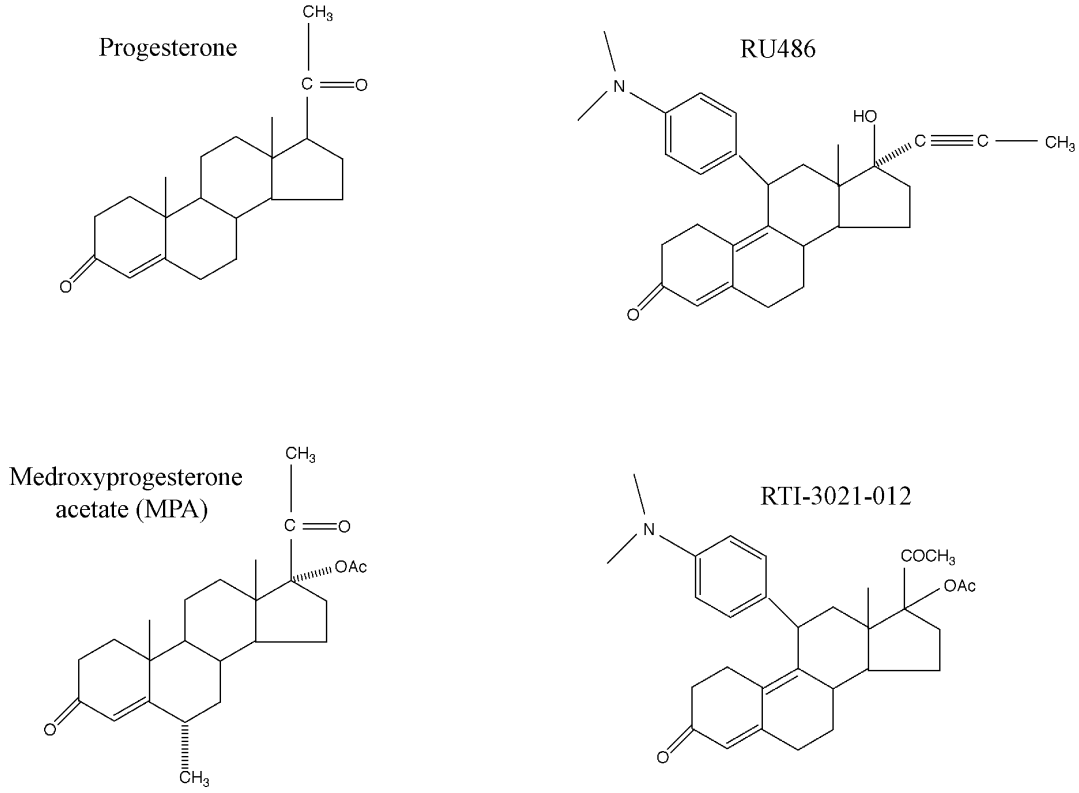

Fig. 5 PR ligands with different molecular activities. The natural ligand for PR, progesterone, is shown in the upper left-hand corner. Also shown are the clinically used PR agonist, medroxyprogesterone acetate (MPA), and the selective progesterone receptor modulators (SPRMs), RU486 and RTI-3021-012.

Antiestrogenic activities of PRs

The clinical uses of progestins rely primarily upon the ability of progestins to oppose ER activity [53,54]. The antiestrogenic effects of PR ligands can be accomplished through distinct processes regulated by either PR-A or B, the relative importance of which varies from cell to cell. For example, the estradiol-stimulated transcription of the lactoferrin gene is downregulated in mice when progesterone is administered. Studies with PR knock-out mice suggest that PR-B is responsible for this downregulation [38]. However, these studies also show that, in the lumen of the uterus, PR-A is responsible for opposing the estradiol-induced hyperproliferation of epithelial cells [38]. The antiestrogenic effects of PR may be accomplished by several different mechanisms. For example, PR-A or B may directly regulate the promoters of genes that are also regulated by ER. Alternatively, PR-A and B may regulate different genes whose protein products oppose the actions of those regulated by ER. Another possible mechanism by which PR could elicit its antiestrogenic effects is by interfering with the ability of ER to activate transcription. This nonclassical mode of PR action, termed "transrepression", has been observed in cell culture experiments for hPR-A but not hPR-B. When bound by either agonist or antagonist, hPR-A can inhibit or "transrepress" ER activity on ERE-containing reporter genes [35,55]. This PR-mediated transrepression occurs with both endogenously and exogenously expressed ERα and β in a variety of cell types, and the ID region within hPR-A seems to be important for this repressive function [36]. More studies are currently being performed in order to determine the importance of transrepression in the antiestrogenic activity of PR.

Nongenomic PR actions

Compelling evidence in support of nongenomic actions of PR has emerged recently. Specifically, it has been shown that both hPR A and B, in a ligand-dependent manner, can bind to the SH3 domain of the tyrosine kinase, c-src. The PR amino acid sequence contains an SH3 binding site in its AF-1 (aa 421-428, PPPPLPPR) [32]. Other nuclear receptors such as ER, GR, AR, and TR do not contain this SH3 binding site, although ER has been reported to bind the SH2 domain of the same protein, c-src. The interaction between PR and c-src appears to be rapid and transient and results in activation of c-src. However, the relative contributions of nongenomic actions to the biological function of PR remain to be determined.

VITAMIN D RECEPTORS

In contrast to ER and PR, the vitamin D receptor (VDR), a nonsteroid hormone nuclear receptor, does not form homodimers with itself, but by dimerizing with the retinoid X receptor (RXR) forms obligate heterodimers, enabling it to regulate transcription of target genes [56]. VDR's natural ligand, 1,25 dihydroxyvitamin D [1,25 $(OH)_2D_3$] is converted from its precursor, vitamin D, by two successive hydroxylations in the liver and kidney. When bound to VDR, the active ligand regulates transcription of genes involved in calcium and phosphate absorption and homeostasis. The main target tissues of VDR include the bone, where it functions in bone formation and remodeling, intestine, kidney, and skin [57]. In the intestine, however, a ligand for VDR other than 1,25 $(OH)_2D_3$ may also regulate its activity. Recently, it was discovered that VDR can bind lithocholic acid (LCA), a toxic bile acid that is produced in excess from continuous high-fat diets [58]. In the intestine, binding of LCA to VDR activates the receptor and enables it to upregulate expression of the p450 enzyme, cyp3A. By catabolizing LCA, cyp3A eliminates this toxic compound before it can accumulate in the colon. Adding to the biological function of VDR, recent research also indicates that VDR may also play a role in the immune, central nervous, muscle, and endocrine systems. On a molecular level, the 427 amino acid long VDR is the only one of its kind; no subtypes or isoforms of the receptor are known to exist. The DBD of VDR is located at the far amino terminus of the receptor and unlike ER and PR, VDR only contains one activation function,

AF-2, located within the LBD (Fig. 6). Several synthetic VDR ligands have been developed, and, similar to ER and PR ligands, many of these compounds display tissue-specific agonist/antagonist properties, making them selective vitamin D receptor modulators. Studies with deletion and mutant VDR proteins have revealed that the binding characteristics of agonists like 1,25 $(OH)_2D_3$ are different from those of mixed agonists/antagonists like ZK159222 (Fig. 6) and yet also different from the binding characteristics of antagonists, like ZK168281 [59]. Each of these three classes of VDR ligands can induce different conformational changes in the receptor, which alter the structure of the coactivator binding pocket and the positioning of helix 12 in the LBD. Similar to ER and PR, positioning of helix 12 regulates how well the liganded receptor will bind coactivator proteins. Additionally, the tissue-specific expression levels of coactivators and corepressors will help determine if a given VDR ligand will act as an agonist or antagonist. Regulation of VDR activity also depends on the phosphorylation state of the receptor. At least two serine residues within VDR are thought to be targets of phosphorylation. Ser 208 within the LBD represents the major phosphorylation site and contributes to the receptor's transactivation, while Ser 51 within the DBD may play a role in response element binding, nuclear localization, and transactivation [57]. Given VDR's important roles in growth, development, and differentiation, any chemicals that disrupt the receptor's normal activities could certainly impact the physiological well-being of an organism. However, the impact of interfering with VDR activity on endocrine function is not as clear as it is for ER or PR.

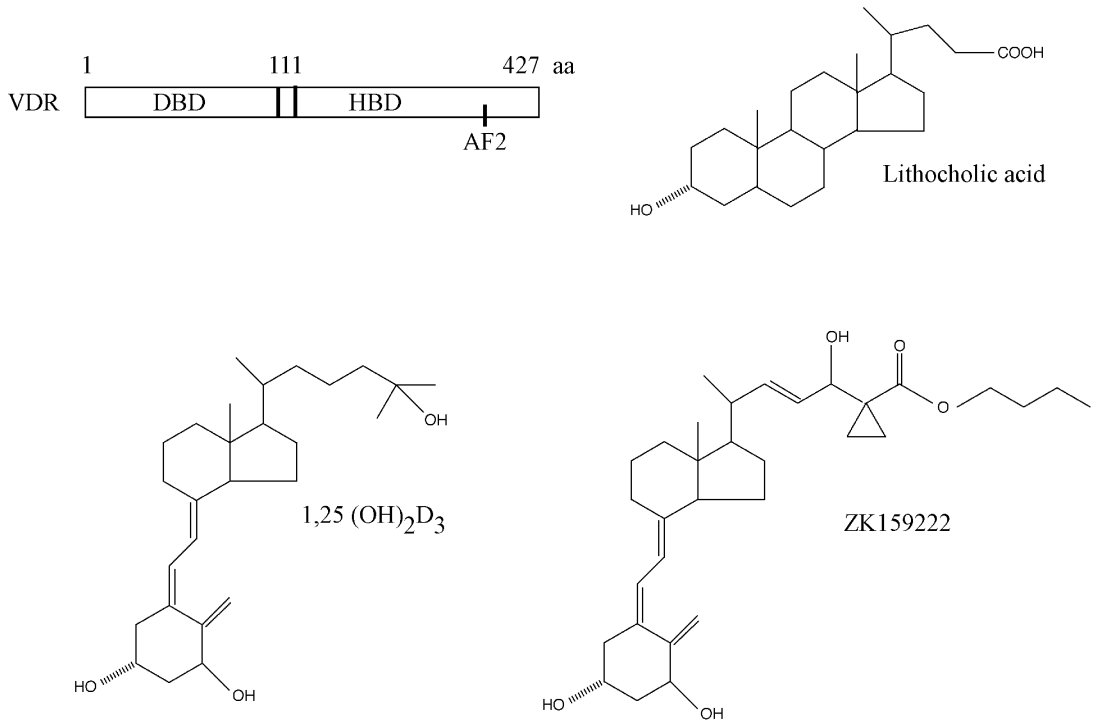

Fig. 6 (A) Diagram of the human VDR is shown in the upper left-hand corner. The natural VDR ligands, 1,25 $(OH)_2D_3$ and lithocholic acid are also shown. The synthetic VDR ligand, ZK159222, is shown in the bottom right-hand corner.

SCREENS FOR DETERMINING THE ROLE OF ER, PR, AND VDR IN ENDOCRINE DISRUPTION

The complexity of nuclear receptor pharmacology described in this topic illustrates the need for the development of screens that can accurately predict the effects of potentially hazardous compounds in different cell types. As we have discussed in this chapter, the ability of a compound to simply bind ER, PR, or VDR does not necessarily translate into its ability to affect the normal activities of the receptor. More accurately, the specific conformation of the receptor induced by binding of a particular ligand can help predict its effects. Screens that distinguish these various conformations can help determine the endocrine-disrupting potential of a new chemical. One such screen is the peptide-binding assay, which was recently developed through the isolation of ER-binding peptides from phage display analyses [27,28]. The binding of a SERM to ER changes the conformation of the receptor in such a way that each SERM/ER complex has a different profile of binding to various classes of peptides. The profile of peptide binding for a new or potentially hazardous compound could help categorize the compound as "tamoxifen-like" or "estradiol-like" depending on the similarities and differences in its peptide binding to those of known ER ligands. Another useful screen for predicting the effects of a potential endocrine active substance on ER, PR, or VDR activity is microarray analysis, which is covered in more detail in other topics. Given the sequencing of the entire human genome and advances in microarray chip design, thousands of genes from multiple tissue types can be classified according to their ability to be regulated by estrogens, progestins, or vitamin D. Comparison of these gene expression profiles to those induced by potentially hazardous chemicals can help predict their endocrine-disrupting abilities and should be a powerful tool for studying endocrine disruption in the future. However, these sorts of cell-based screening methods must be used in combination with other whole organism screens in order to take into account more global factors contributing to their endocrine-disrupting potential.

SUMMARY

This topic summarizes the molecular actions of the ligand-inducible transcription factors, estrogen, progesterone, and vitamin D receptors (ER, PR, and VDR). To begin with, two subtypes of ER, α and β, and two isoforms of PR, A and B, are responsible for responding to potential ligands of ER and PR. Their transcriptional regulatory function can occur through a direct mode, with the receptor binding to response elements within the promoters of target genes, or indirectly, through their effects on other transcription factors. The subtypes of ER and PR have different molecular activities, different expression patterns, and different expression levels throughout the body, which make it difficult to predict the effects of a given substance on ER or PR activity without taking into account which tissue is being considered. Although VDR only has one form, factors that determine its activity parallel many of those for ER and PR. To further complicate the actions of ER, PR, and VDR, the ligands that bind the receptors each do so in a slightly different manner. This imposes different conformational shapes on the receptor that ultimately affect the ability of coactivator and corepressor proteins to bind to and modulate the receptor's activity. The expression patterns and levels of coactivator and corepressor proteins as well as phosphorylation status of the receptor and its associated cofactors are also important regulators of the activities of ER, PR, and VDR. Therefore, the combination of many factors determines the potential endocrine-disrupting ability of a substance that acts through one of these receptors.

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