Estrogen Receptors α and β: Two Receptors of a Kind?

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Abstract: Ever since the discovery of estradiol and the elucidation of its chemical structure, there has been a great deal of interest in its mechanism of action and its potential therapeutic value. It is now well established that estrogens have many different functions in many different cell-types. With respect to the potential use of estrogens as therapeutics, there is an interest in controlling reproductive function, bone metabolism, cardiovascular disease, as well as in the prevention of hot flushes, mood changes and Alzheimer's disease. For over a decade, it was believed that estrogens signal through a a single estrogen receptor, now referred to as ERα, which belongs to a family of ligand-activated transcription factors. More recently, however, a second estrogen receptor ERβ was identified. The current review describes similarities as well as differences between these two distinct estrogen receptors. Both ERα and ERβ bind 17β-estradiol with high affinity and they bind to classical estrogen response elements in a similar if not identical fashion. However, there are also major differences between ERα and ERβ for instance with respect to their tissue distribution, the phenotype of the corresponding knock-out mice and their transcriptional activities. It is anticipated that a better understanding of these two receptors will eventually lead to more selective ways of modulating physiological processes which are influenced by estrogens. For this purpose, the development of ERα and ERβ specific ligands, both agonists as well as antagonists, will be of great importance.

Estrogen: A Renowned Hormone

Although the steroid hormone estrogen was already discovered decades ago, the complex regulation of its pleiotropic effects in physiology is only partially understood to date. Despite years of dedicated research, and despite the fact that especially during the last decade a great deal of progress has been made, we are just beginning to understand some of the diverse mechanisms by which estrogen modulates expression of target genes.

The earliest reports on estrogens describe effects of ovarian extracts on vaginal epithelium in studies performed by Allen and Doisy in the 1920's; the structure of the active compound was solved in 1932. The Allen-Doisy test is still used today as a very sensitive monitor of estrogenic activity. For reasons that will become clear below, nowadays, it is questionable whether this test is suitable for the detection of all compounds with estrogenic activity.

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In humans the three most important estrogenic hormones are estradiol, estrone and estriol. However, several natural products, the so-called phyto-estrogens, and a large number of synthetic compounds that have been developed were also shown to have estrogenic activity. In addition to agonists, estrogen antagonists have been developed for specific applications, most importantly the treatment of breast cancer. In the 1970s, the availability of radiolabeled estrogen revealed the existence of high affinity binding proteins in the cytoplasm of specific cell types. However, it took another two decades before a high affinity receptor for estradiol was cloned in 1986. In retrospect, the molecular cloning of the estrogen receptor has proven to be a prerequisite for understanding the basic mechanism of estrogen signalling at the molecular level.

Cloning of the Estrogen Receptor α, A Member of the Nuclear Receptor Gene Superfamily

A high affinity receptor for estrogen could be cloned due to the fact that it was abundantly expressed in the human breast cancer cell line MCF-7. This work was performed by purification of the estradiol-binding protein from MCF-7 cells. The strategy that was

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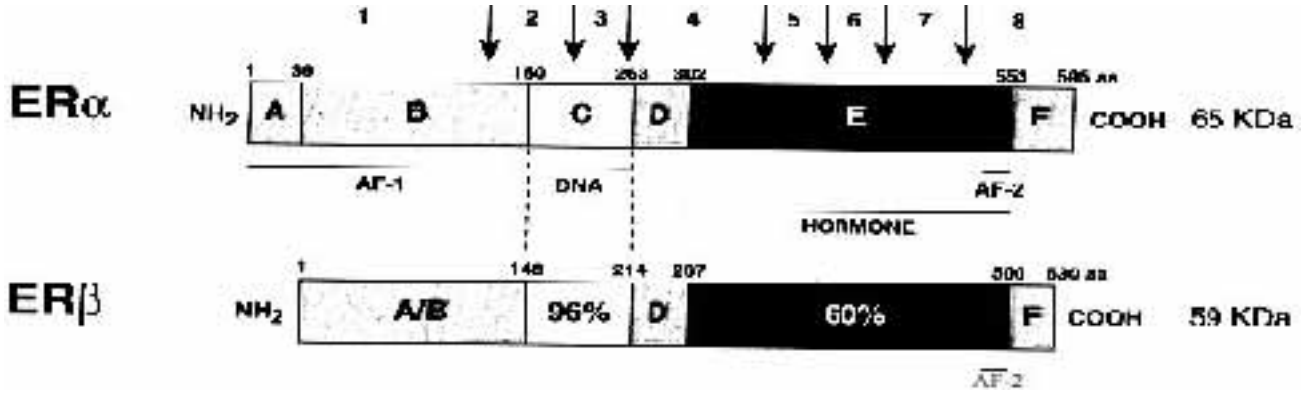

Fig. (1). Schematic representation of the full length human ERα and ERβ proteins. Arrows indicate exon boundaries for the ERα gene. See text for further details.

subsequently employed to clone the corresponding cDNA, was two-fold: (1) screening of a cDNA library with synthetic oligonucleotides corresponding to peptides obtained from purified receptor protein and (2) screening of a cDNA expression library with monoclonal antibodies raised against purified receptor protein [1-3]. This estrogen receptor (referred to as ERα throughout this paper) was very similar in structure to the glucocorticoid receptor (GR), the cloning of which had been reported just previously [4,5]. Once GR and ERα were identified, a large number of structurally related receptors were cloned subsequently. Some of these have known ligands, both steroids (testosterone, progesterone, aldosterone and glucocorticoids) as well as non-steroids such as vitamin A/retinol, thyroid hormone, vitamin D, and the insect moulding hormone ecdysone [6,7]. In addition, a number of related receptors without known ligands, so-called orphan receptors were identified [6,8]. Thus, ERα is a member of a family of genes which is commonly referred to as the nuclear receptor superfamily.

Members of this superfamily are structurally related and are thought to share their basic mechanism of action. Upon binding of a ligand, the conformation of the receptor changes into a transcriptionally active form. This then enables the receptor protein to bind to specific response elements in the promoter region of target genes, either as a dimer or as a monomer, and modulate the rate of transcription of these target genes. Therefore, this class of receptors is referred to as ligand-activated transcription factors. As such they represent a very direct way of regulating the expression of specific sets of genes, under the influence of low molecular weight compounds. It is not surprising, therefore, that these receptors have attracted the attention of the pharmaceutical industry.

Discovery of ERβ and Structural Comparison with ERα

Although a number of experimental data were inconsistent with the finding of only one estrogen receptor, there was a general acceptance in the field that a single receptor for estrogens, ERα, existed. Moreover, attempts to clone receptors closely related to ERα only revealed the identification of the orphan receptors ERRα and ERRβ which have been reported not to bind estrogens [9]. Therefore, the publication in 1996 of two papers on the cloning of a second high affinity estrogen receptor was surprising as well as exciting. The second human estrogen receptor, ERβ, was cloned as a result of our studies on the role of estrogens in the immune system and in the gonads [10]. Independently, a rat ERβ cDNA was isolated from prostate [11]. The existence of two distinct estrogen receptors indicated that estrogenic signalling was more complex than had been thought previously. In addition, it indicated that there might be therapeutic opportunities for compounds that would display selectivity in their interactions with ERα and ERβ.

An alignment of the protein sequences of human ERα (595 amino acids) [2] and human ERβ (530 amino acids) proteins [10,12] is shown in Fig. (**1**). Both receptors have the modular structure that is characteristic for members of the nuclear receptor superfamily with the different domains denoted A through F [7]. By far the best conserved is domain C (96%), the DNA-binding domain (DBD). All cystein residues which co-ordinate around the Zinc-atoms to form the Zinc fingers are fully conserved. In addition, there is complete conservation of the so-called P-box, the residues that are essential for the physical interaction with the DNA response element [13], as well as the D-box which is required for receptor dimerization [14]. As a consequence, ERα and ERβ bind to very similar if not identical DNA response elements [15,16].

The ligand-binding domain E (LBD) has approximately 60% amino acid identity which is, however, not evenly distributed. The crystal structure of the ERα LBD has recently been solved by several groups [17-19]. Fig. (**2**) shows a model of the overall structure of the ERα LBD which is primarily composed of α-helices and contains two very short β-pleated sheets. The picture clearly shows that all residues which are in close proximity with the ligand are conserved in ERα and ERβ. This presumably explains why, despite the relatively low degree of overall amino acid identity within the LBD, the affinity of ERα and ERβ for 17β-estradiol is indistinguishable (Fig. (**6**), see below).

The carboxyterminus of the LBD contains an important transcriptional activation function (AF-2), which is dependent on the binding of an agonist. AF-2 resides in helix 12 that is well conserved between both receptors (Fig. (**2a**)). As will be pointed out below, binding of an agonist results in a conformational change in the receptor and the activation of AF-2, which provides an interaction interface for transcriptional coactivators. Antagonists fail to induce this specific conformational change and are, therefore, incapable of activating AF-2.

A region which is strikingly dissimilar between ERα and ERβ encompasses amino acids 389 to 448 in human ERβ [12], This region corresponds to helix 9 in the crystal structure of ERα [18] (Fig. (**2a**)), and suggests that this helix, which is not located in the proximity of the ligand-binding pocket, is of mere structural importance.

The hinge region (domain D), as well as the carboxy-terminal F-domain are both not well conserved and somewhat shorter in length in ERβ compared with ERα. There is also hardly any conservation within the amino-terminal A/B domain, although a consensus sequence for MAPK-mediated serine phosphorylation is conserved and has been shown to be functional in mouse ERβ [20]. The A/B domain contains a second transcriptional activation function, AF-1, which functions ligand-independently although its activity may be blocked by full antagonists such as ICI 164384. In addition, the AF-1 activity can be modulated by growth factor induced phosphorylation pathways [21].

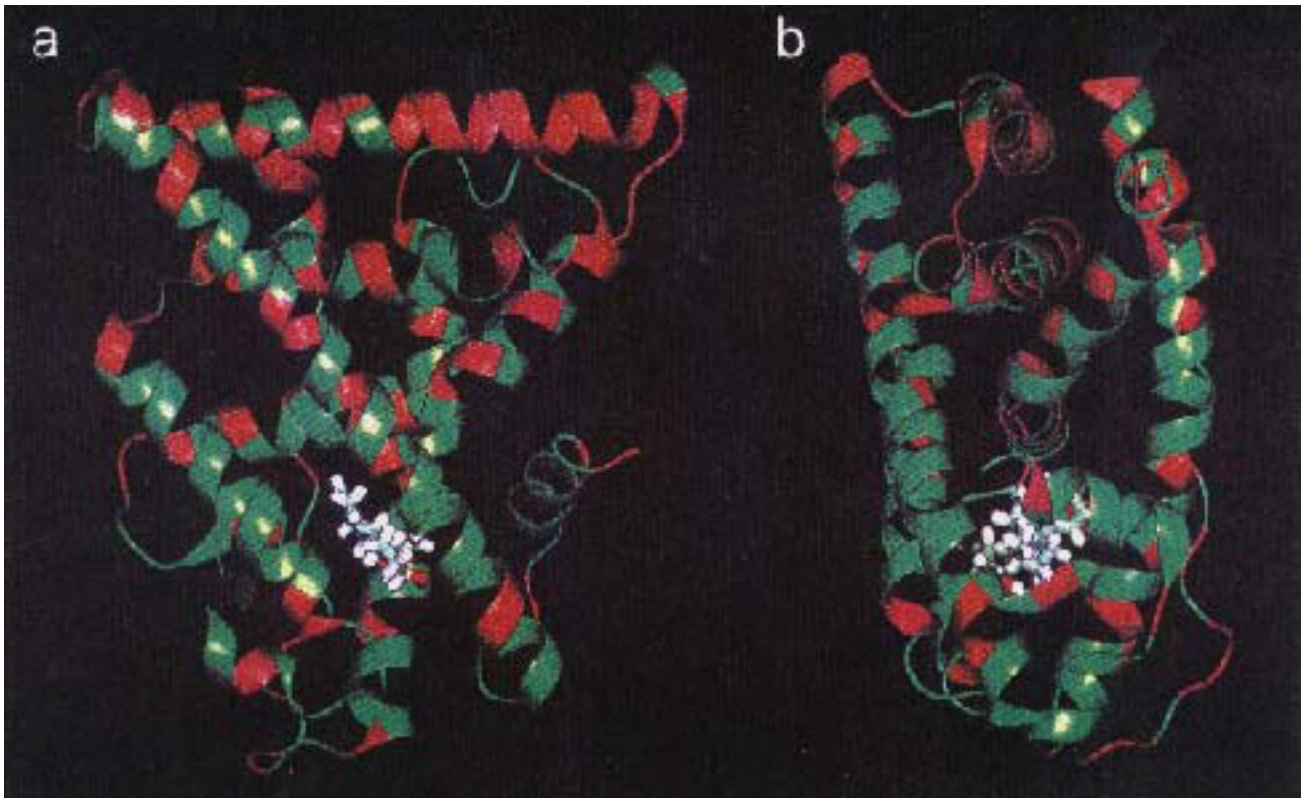

Fig. (2). Schematic representation of the crystal structure of the LBD of ERα liganded with 17-β estradiol. The model is based on data that have been published previously [17-19]. Two orthogonal views are shown in which amino acids identical between ERα and ERβ are shown in green whereas non-identical amino acids are indicated in red. It should be noted that in the majority of cases, non-identical residues are in fact similar residues. A). In this view, helix 9 is the top horizontal helix. The carboxy-terminal helix 12 is at the right hand side of the figure. B). Helix 12 is in the front whereas helix 9 is at the top center.

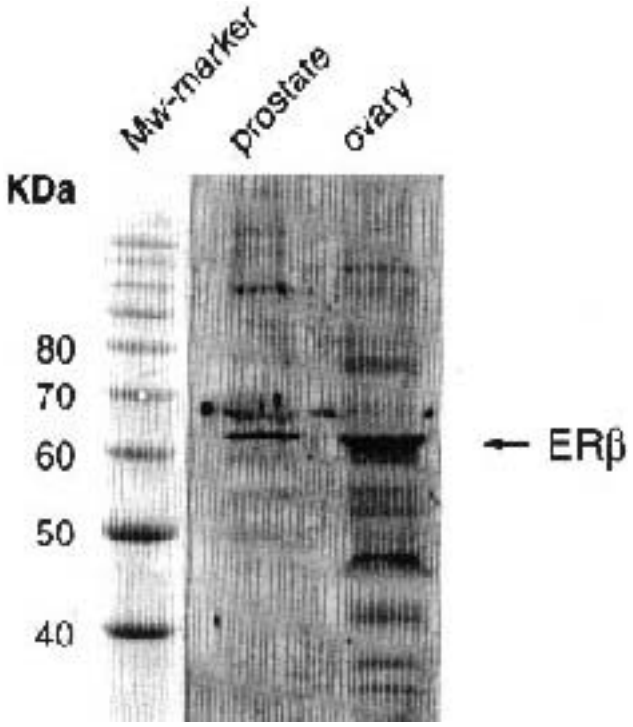

Fig. (3). Rat ERβ protein has a molecular weight of approximately 61 kDa. The figure shows a Western Blot analysis of ERβ protein in extracts of rat prostate and ovary, using ERβ specific antibody PAI-310 (Dianova, Hamburg, Germany).

As expected from the sequence divergence between ERα and ERβ in the A/B domain, the AF-1 functions of ERα and ERβ behave differently in their transcription activation potential [22].

There has been some debate with respect to the length of the ERβ A/B domain. Our initial publication reported that human ERβ was at least 477 amino acids and the subsequent analyses of 5' RACE PCR fragments as well as analyses of human genomic clones revealed that full-length hERβ is 530 amino acids [our unpublished results]. The latter sequence is in full agreement with that published by several other groups [23-25]. The A/B-domain in mouse ERβ is very similar in length and sequence and has an additional in-frame ATG codon 19 codons further upstream which is not present in human ERβ [25]. The A/B domain of rat ERβ (GenBank Accession # AJ002602) is very similar in length and sequence to mouse ERβ. The sequence in the original report describing rat ERβ lacks a single C-residue resulting in a frame-shift [11]. This mistake has caused some confusion with respect to the size of the full-length protein. Since Western blot data have been lacking until recently, the confusion sustained. Recently, a 60 kDa ERβ protein was detected in rat myocytes on immunoblot [26]. In addition, we have detected a single protein of approximately 61 kDa in rat ovary and prostate on Western blot (Fig. (**3**)). These data are in perfect agreement with the calculated molecular weight (61.33 kDa) of the 549 amino acid open reading frame of rat ERβ.

ERα and ERβ Splice Variants

Several ERβ splice variants have been reported. In rat ERβ an insertion of 54 nucleotides between exons 5 and 6 was found which potentially introduces 18 extra amino acids within the LBD [27]. Both wild-type mRNA and the splice variant are expressed at similar relative abundance in a large number of tissues. A similar splice variant occurs in the mouse [28]. However, we have been unable to detect a similar ERβ splice variant in human tissues (our unpublished results). The presence of the insertion within the LBD of rat ERβ has been described to affect ligand-binding [29]. It could also affect dimerization and the specificity of protein-protein interactions.

Two splice variants have been reported for human ERβ which have distinct carboxy-termini and, if translated, would lack helix 12 which has been shown to be crucial for AF-2 activity [23,30]. Specific RT-PCR reactions for these splice variants have shown that their tissue distribution is non-identical [23]. In our lab, three similar splice variants were found, which diverge from full-length ERβ at exactly the same position as the two reported variants [23,30]. Intriguingly, all five sequences are distinct from each other down-stream from the splice site. The putative truncated ERβ proteins encoded by these transcripts may interfere with normal estrogenic signaling by full-length ERα and ERβ as has been shown in cotransfection experiments [30].

Over the years, a large number of splice variants have been reported for ERα [31,32]. However, with very few exceptions [33] there have been no reports on the identification of the corresponding proteins. Thus, it remains to be established whether or not the existence of the various ERα and ERβ mRNA splice variants has any physiological significance.

Molecular Mechanisms Of Estrogen Signaling

Within the archetypal model of estrogen action the ligand diffuses freely across the cell membrane and binds to the receptor which then dissociates from cytoplasmic chaperones that keep it in an inactive state. Subsequently, the liganded receptor translocates to the nucleus and binds as a homodimer to a specific response element within the promoter region of the target gene and hence activates transcription. However, this model seems to be oversimplified and has been challenged by a number of observations: 1) there are now two known estrogen receptors, which may heterodimerize and create diversity in estrogen receptor complexes; 2) ERα and ERβ can function independently of their canonical response elements via direct association with other proteins; 3) a single ligand may exert opposite effects in different target tissues; 4) the liganded estrogen receptor recruits a number of co-regulators that affect the transcriptional response; 5) estrogen receptors may be activated by ligand-independent mechanisms. Fig. (**4**) gives a schematic overview of the different levels at which the signal transduction by estrogen receptors may be modulated.

Mixed Agonists/Antagonists

Especially the finding that several compounds display partial agonist/antagonist activity has challenged the conventional model of estrogen signaling. The anti-estrogen 4-hydroxytamoxifen (OHT), which has been used for the treatment of hormone responsive breast cancers for over 20 years, and is still being used, acts as an anti-estrogen in breast but has estrogenic activity in other tissues [34]. The related benzothiopene analogue raloxifene displays a similar mixed agonist/antagonist profile. Initial studies on the molecular mechanism of the mixed agonist/antagonist profile of OHT have indicated that the agonist activity relies on its ability to stabilize DNA binding and to activate AF-1 whereas its antagonist activity is due to competitive inhibition of estradiol-dependent activation of AF-2 [35,36]. These observations were confirmed and substantiated by the structural data that have been published recently [17-19]. The crystal structure of the 17β-estradiol (E2) liganded LBD of ERα showed that within the E2-receptor complex, helix 12 in the carboxy-terminal LBD folds over the ligand binding cavity to form, in conjunction with helices 3, 5, 6 and 11, the AF-2 domain. In the crystal structure of ERα complexed with the anti-estrogen raloxifene, helix 12 was rotated 130° and shifted 10 Å compared with the E2-liganded receptor, resulting in the disruption of AF-2 activity. Recently, the ERα LBD liganded with the agonist diethylstilbestrol (DES) was co-crystalized with a peptide derived from the nuclear receptor interaction box II of the coactivator GRIP1 [17]. In addition, this study presented the solved crystal structure of a OHT liganded LBD. It was shown that in the DES liganded complex the coactivator peptide interacts with a peptide binding groove formed by residues from helices 3,4,5 and 12 that together form the AF-2 surface. At the side of the coactivator peptide, three conserved leucine residues are critical for the interaction with the AF-2 domain of the receptor as had been shown by mutational analysis previously [37]. The exciting outcome of the structural studies on the OHT liganded LBD was that in this complex helix 12 acts as an intramolecular mimic of the coactivator helix and blocks the coactivator recognition groove. Together, these data indicate that the binding of anti-estrogens to the receptor has two distinct effects. First, helix 12 is prevented from positioning over the ligand binding pocket which disrupts the AF-2 interface, and, second, helix 12 mimics bound coactivator and blocks the coactivator interaction groove. Interestingly, the amino acid residues that comprise the cofactor interaction groove necessary for complete AF-2 function are fully conserved between ERα and ERβ [17,38]. Therefore, the AF-2 functions of ERα and ERβ may be very similar, if not identical. Accordingly, OHT acts as an antagonist on both receptors. In contrast, ERα and ERβ diverge in their amino-termini, indicating that the AF-1 functions may differ. In an extensive comparison of the AF-1 and AF-2 domains of ERα and ERβ, McInerney and coworkers showed that ERβ fails to display the agonistic activity of OHT in cells where ERα can be activated by OHT. AF-1 domain swapping between ERα and ERβ reverses the effect of OHT on the receptor and indicates that the agonistic effect of OHT depends on the AF-1 domain of ERα [22].

Transcriptional Co-regulators

Katzenellenbogen and coworkers proposed a tripartite receptor pharmacology model to explain the tissue and promoter specific effects of estrogen signaling [39]. This model describes the cell- and/or promoter-specific effector mechanisms that exist to transduce the ligand-induced signal to the basal transcriptional machinery. Although the nature of these effector mechanisms is largely unclear, the past years

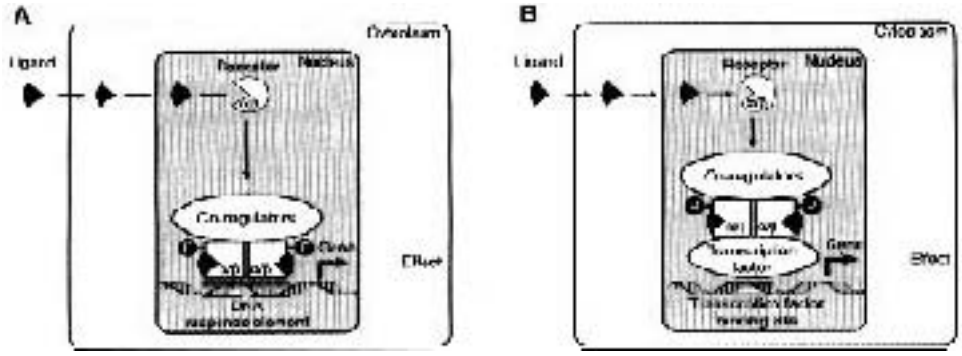

Fig. (4). Model for transcriptional activation by ERα and/or ERβ. A) In the classical pathway, the receptor dimerizes in a ligand-dependent fashion, binds to its specific DNA response element and recruits a complex of co-regulators that assist in activation of the target gene. Both homo- as well as heterodimers may be formed and phosphorylation events may modulate the activity of the receptor complex. B) In alternative pathways, the estrogen receptors function independently of their DNA-binding properties, but are tethered to the promoter region of the target gene by other transcription factors.

have seen rapid progress in the discovery of proteins that interact with ERα and/or ERβ and that may comprise (part of) the cell specific or promoter specific effector mechanism. These proteins are generally referred to as coactivators and corepressors and comprise a variety of nuclear proteins that assist in transcriptional processes by bridging between transcription factors and the basal transcription machinery (Fig. (**4**)). The notion that the nucleus contains proteins that can enhance or suppress nuclear receptor driven transcription was born out from the observation that overexpression of a nuclear receptor may suppress, or squelch, its own transcriptional activity or that of another nuclear receptor, supposedly by titration of the limiting amount of coactivator [40,41].

The first co-activator to be cloned was the steroid receptor co-activator 1 (SRC-1), which was isolated in a two-hybrid screen using the LBD of the progesterone receptor as a bait [42]. SRC-1 interacts with the LBDs of all steroid receptors characterized in this respect in a ligand-dependent fashion. Overexpression of SRC-1 can relieve the squelching of PR-mediated transcription by liganded ER, demonstrating that squelching indeed is due to titration of a rate limiting co-activator [42]. SRC-1 interacts with both AF-1 and AF-2 of the estrogen receptors and thereby increases the transcriptional synergy exhibited by the AF-1 and AF-2 domains. As ERα and ERβ differ in their AF-1 domains, it is conceivable that they interact differently with SRC-1. This notion is supported by the observation that SRC-1 enhances the ligand-independent transcriptional activity of ERβ, but not of ERα [20].

SRC-1 appears to be a member of a larger family of co-activators, which share a high level of homology. To date, SRC-2, also called TIF2, and SRC-3, also called AIB1/TRAM1/RAC3/ACTR have been described [43-46]. All these proteins contain an amino-terminal basic helix-loop-helix/Per-Arnt-Sim domain, a CREB Binding Protein (CBP) interaction domain, several nuclear receptor interaction domains and at least two intrinsic transcriptional activation domains that may explain part of their transcription enhancing effects [47,48]. In addition, it has been demonstrated that SRC-1 and SRC-3 exhibit histone acetyltransferase activity, indicating that they may be actively involved in remodeling of chromatin to an open conformation, which is believed to be a prerequisite for transcription as it allows the access of transcription factors to the DNA [43,49]. Apart from their intrinsic activities, the SRC family members serve as a platform for other accessory proteins such as CBP/P300 and form part of a ternary complex that altogether enhances the transcriptional activity of the target gene [50].

In addition to the SRC-like proteins, a plethora of co-activators and co-repressors with very diverse functions has been described. Among these are proteins that are homologues of the yeast SWI/SNF proteins that belong to the class of E3 ubiquitin-protein ligases, as well as an array of proteins that have no homology to any known protein [39,47,50]. Many of these proteins show little selectivity in their interactions with members of the nuclear receptor family. Sequence comparison and mutational analysis has identified a short sequence motif present in every cofactor described to date that is involved in the interaction with nuclear receptors [37]. This motif, LXXLL, is present in multiple copies in most of the coactivators. Studies on the mouse homologue of SRC-2 (GRIP1) have shown that different motifs are involved in the interaction with different nuclear receptors and may confer some specificity to the cofactor-nuclear receptor interaction. This selectivity is, for instance, seen for SRC-3, which enhances ERα mediated transcription but not ERβ stimulated transcription. Accordingly, the interaction between

SRC-3 and ERα is much higher compared with that between SRC-3 and ERβ [51]. Interestingly, the gene encoding SRC-3, *aib1*, is amplified or overexpressed in 74 percent of primary breast tumor species analyzed in this respect [52].Taken together, these results suggest that the combination ERα-SRC-3 may contribute to the development and progression of estrogen-dependent breast cancer.

Alternative Pathways

In addition to the classical pathway whereby ERα and ERβ bind as dimers directly to DNA response elements, there is evidence that they may act independent of their DNA-binding activities (Fig.(**4b**)). For instance, induction of the TGF-β3 promoter by raloxifene requires the presence of ERα but is independent of the DBD of the receptor [53]. Studies on the interleukin-6 (IL-6) promoter indicated that its activity can be repressed by 17β-estradiol. This repression is dependent on a functional estrogen receptor but is independent of the high affinity DNA binding property of the receptor [54]. Instead, ERα was shown to interact with the transcriptional activators NF-kappa B and C/EBPβ in a ligand-dependent manner, resulting in transrepression of the NF-kappa B and C/EBP β induced activity of the IL-6 promoter [55]. Whether or not ERβ exerts a similar transrepressive effect remains to be established. Indications for important differences between ERα and ERβ in ERE-independent transcriptional control were provided by other studies. In a study on the induction of the transcriptional activity of the RARα-1 gene it was shown that estrogen may activate the RARα-1 promoter via ERα but not via ERβ. However, anti-estrogens such as OHT activate the RARα-1 promoter via ERβ whereas they antagonize the transcriptional activity of ERα on this promoter. It was shown that the transcriptional effects of the ERα and ERβ on the RARα-1 promoter do not require direct binding of the receptors to the DNA. Instead, the ERβ mediated activation of the promoter by anti-estrogens seems to require binding of the transcription factor SP1 to the two natural SP1 sites contained within the RARα-1 promoter [56]. A similar reversed receptor pharmacology was observed on ligand activation of ERβ and ERα on AP1 sites [57,58]. These studies indicate that the pharmacology of certain ligands may be different on ERβ versus ERα mediated transcription. It is conceivable that the differential interaction potential of ERα and ERβ will provide opportunities for selective therapeutic intervention.

Besides from being active within the nucleus, several reports have suggested that ERα and ERβ may somehow associate with the plasma membrane and exert ligand induced, non-genomic effects. Estradiol rapidly induces several non-transcriptional responses, including the activation of MAP-kinases, the production of cAMP, the release of intracellular calcium, and the generation of inositol phosphates [59-62]. These rapid effects have been attributed to a membrane associated receptor with high affinity for E2. Recently, it was shown that the single transcripts derived from the ERα or ERβ genes encode both nuclear and membrane bound forms of these receptors [63]. The membrane bound receptors may induce signal transduction processes that are fundamentally distinct from those induced by the nuclear form of the receptor and hence provide mechanisms of cross-talk with growth factor induced pathways.

Ligand-independent Activation

In addition to ligand-dependent activation, both ERα and ERβ may be activated by ligand-independent mechanisms. It has been shown that phosphorylation of serine 118 in the AF-1 domain of ERα enhances the transcriptional activity of the AF-1 domain [21]. This ser118 is phosphorylated by mitogen-activated protein kinase (MAPK), which in turn may be activated by growth hormone signaling pathways triggered by the epidermal growth factor or insulin-like growth factor. Although the AF-1 domains of ERα and ERβ are quite divergent, the serine residue at position 118 in ERα is structurally and functionally conserved in ERβ [20,64]. In addition to the phosphorylation site in AF-1, both ERα and ERβ contain a tyrosine phosphorylation site in the c-terminus. Phosphorylation of this tyrosine induces a conformational change in the receptor that affects hormone binding and dimerization [65]. Apart from the phosphorylation sites at 118 and 537 (ERα), other targets for phosphorylation may exist, and have been implicated in the ligand-independent activation of the receptors. Together, these sites integrate signals from other signaling pathways such as the growth factor or dopaminergic pathways (Fig. (**4**)) [66].

Tissue Distribution of ERα and ERβ

Estrogens exert a variety of pleiotropic effects in target tissues as diverse as bone, brain, breast, blood vessel and the male and female gonads. Indeed, both estrogen receptors α and β are widely expressed in the body [67-71]. However, the tissue distribution of ERα and ERβ transcripts is quite different, suggesting that the two receptor subtypes exhibit distinct functions in different tissues. Figure **5a** gives an overview of the relative abundance of ERβ and ERα mRNA in various rat tissues as determined by RNAse protection analysis. These results show that the pituitary, vagina and uterus contain high levels of ERα mRNA, whereas the ERβ transcript is most abundant in the ovary and prostate. Transcripts for both receptors are expressed in the pituitary, hypothalamus, thymus, uterus and the

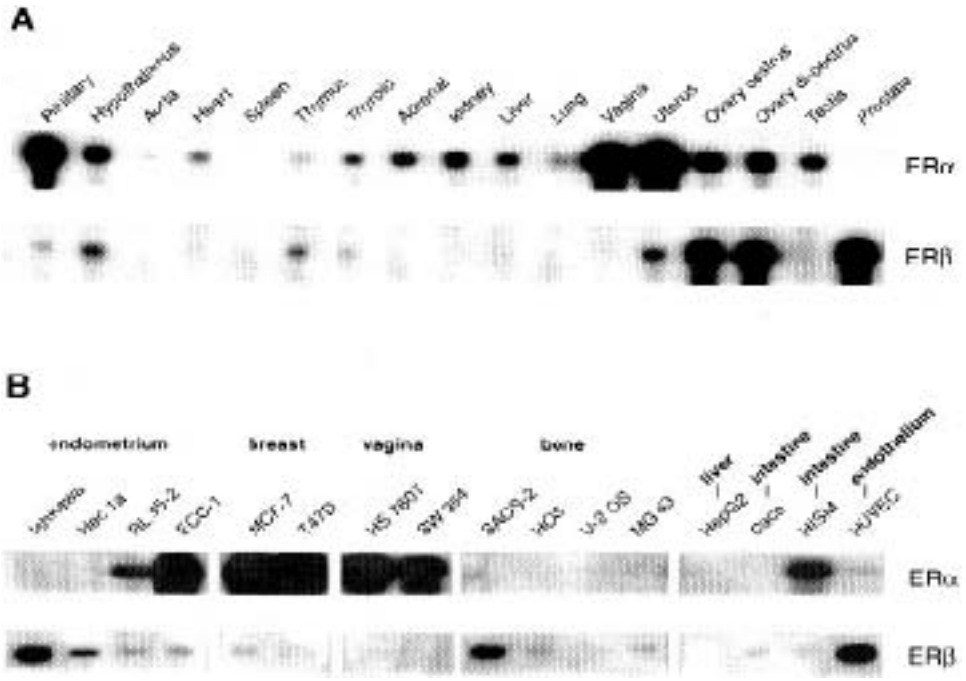

Fig. (5). Tissue distribution of ERα and ERβ transcripts. A) Expression of ERα and ERβ mRNA in various rat tissues as determined by RNAse protection analysis. B) RT-PCR analysis of ERα and ERβ mRNA expression in various human cell lines.

ovary. RT-PCR analysis, which is a more sensitive technique compared with RNAse protection, detects the ERα and ERβ transcripts in a variety of immortalized human cell lines that represent the various estrogen target tissues, or in carcinoma cell lines derived from these target tissues (Fig. (**5b**)). ERβ expression is relatively high in endometrial carcinoma Ishikawa cells and the osteosarcoma SAOS-2 cells. Consistent with the observation that ERα expression is high in primary samples from rat vagina, the RT-PCR shows an abundance of ERα transcripts in HS760T and SW954 vaginal epithelial cell lines (Fig. (**5b**)). In addition to the expression profile presented here, other published studies have indicated that ERβ expression is high in the epithelial cell layer of rat bladder, whereas ERα expression is very low in these cells [72].

From the data presented in Fig. (**5a**) it becomes clear that both ERα and ERβ are expressed in a variety of tissues. Techniques that allow the assessment of the expression profile at the single cell level have shown, however, that within a single tissue the expression of one of the receptor subtypes might be localized to a specialized cell type. We have performed *in situ* hybridization studies with specific ERα and ERβ probes. An example of these studies is presented in Fig. (**6**), which shows that within the rat vagina, ERα expression is exclusively restricted to the epithelial cell layer. Consistent with the RNAse protection assay data, *in situ* hybridization does not detect the ERβ transcript in the vagina. These results indicate that the reported effects of estrogen on the proliferation of the vagina epithelium can be completely attributed to ERα. At the same time, they stress that the discovery of ERβ urges a re-evaluation of the animal model systems that are currently being used to assess the estrogenic activity of novel compounds. For instance, the *in situ* hybridization data imply that the above-mentioned Allen-Doisy test, which is widely used as a monitor of estrogenic activity, is not suited for testing ERβ selective agonists.

In the ovary, the expression of ERα concentrates in the stromal compartment and theca cells whereas ERβ expression is restricted to the granulosa cells of developing follicles[11,71,73]. It is conceivable that the distinct localization of ERα and ERβ within a single tissue reflects the different functional requirements of cells within a tissue.

ERα and ERβ Expression in Target Tissues for Hormone Replacement Therapy

Postmenopausal women develop climacteric complaints, are subject to mood changes and are at an increased risk with respect to a number of diseases including osteoporosis, coronary heart disease and Alzheimer's disease. Hormone replacement therapy (HRT) can greatly reduce these risks [74-77]. However, one of the unwanted side effects of HRT is the

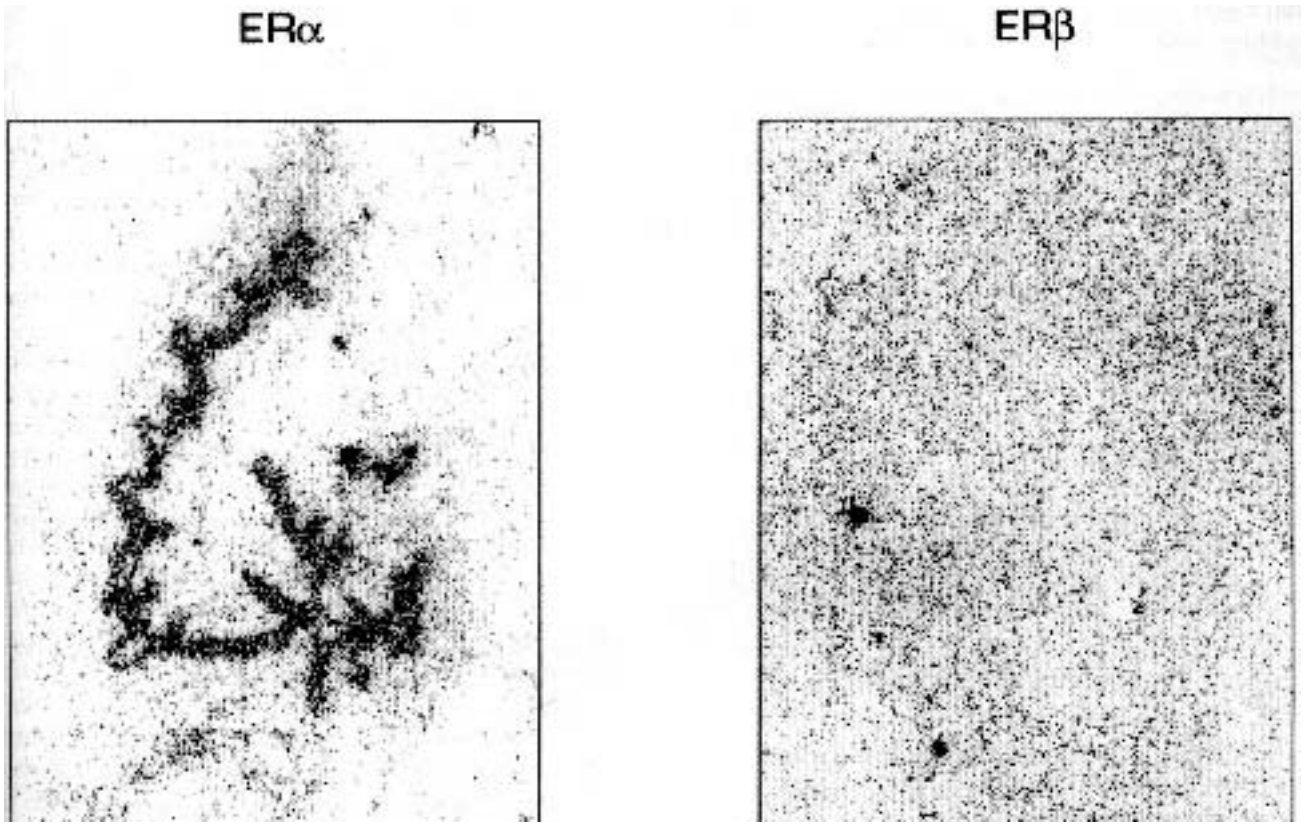

Fig. (6). *In situ* hybridization analysis of ERα and ERβ expression in rat vagina tissue sections. An antisense ERα ribonucleotide probe detects the ERα transcript in the epithelial cell layer (left panel). In contrast, no specific signal is observed in the hybridization with a specific antisense ERβ probe (right panel).

increased incidence of breast and endometrial cancers in long-term users of HRT [78,79]. The latter risks seriously impede the continuous use of HRT and emphasize the need for the development of selective estrogen receptor modulators (SERMs), which should have antagonistic properties or show no activity in breast and endometrium while retaining their protective (agonistic) effects on bone, the cardiovascular system and the brain [80]. The discovery of ERβ provided unique opportunities with respect to the development of SERMs as it became clear that the desired selectivity might be achieved by selectively targeting one of the two receptor subtypes. In this respect, the detailed tissue distribution of ERα and ERβ in the tissues that are a target for HRT is of great interest and has been the subject of a considerable number of studies.

Estrogen Receptors in the Brain

The important role of estrogen in the neuro-endocrine system and in the regulation of behavior and cognitive function has been recognized for a long time [81]. In addition, more recent data have indicated that estrogen depletion accelerates brain aging and presumably the expression of Alzheimer's disease [82]. It was, therefore, puzzling that some brain regions known to be the target of estrogenic activity apparently failed to express ERα [83,84]. It is now clear that these regions exclusively express ERβ, indicating that many of the functions of estrogen in the brain can be attributed to ERβ. In a detailed study of the distribution of ERα and ERβ transcripts in the rat brain, Shughrue and coworkers showed that ERα is the sole estrogen receptor expressed in the ventromedial hypothalamic nucleus and subfornical organ [85]. Regions in the brain that exclusively or predominantly express ERβ include nuclei in the basal forebrain, neocortex and hippocampus [85,86]. In addition, ERβ but not ERα is expressed in the supraoptic and paraventricular nuclei of the hypothalamus, suggesting that it may be primarily responsible for regulation of expression of the peptide hormone oxytocin, which is a well-known estrogen responsive gene [85,87]. Co-expression of both receptor subtypes was observed in the preoptic area indicating that both ERα and ERβ may be involved in the relief of hot flushes by estrogens [88]. The dominance of ERβ in the hippocampus, neocortex and nuclei of the basal forebrain may suggest that it plays an important role in mediating the beneficial effects of estrogen on learning and memory [88].

Estrogen Receptors in the Cardiovascular System and Bone

The mechanism of the beneficial effect of estrogen on the cardiovascular system is at least bipartial. On the one hand estrogens critically affect lipid metabolism in the liver [89]. On the other hand, estrogens may act directly on the blood vessel wall [90]. Accordingly, binding sites for radiolabeled estradiol were detected in the liver and in vascular endothelial and smooth muscle cells [91-94]. These observations were confirmed by studies that demonstrated the unique presence of ERα mRNA in liver tissue (Fig. (**5a**)) and the presence of both ERα and ERβ transcripts in vascular endothelial and smooth muscle cells [68]. In these cells the level of ERβ is much higher compared with that of ERα. Moreover, estrogen is still able to inhibit the vascular injury response in ERα knock-out mice, suggesting that ERβ is involved in the protective effects of estrogen on the cardiovascular system [95]. The latter notion is supported by the observation that expression of ERβ mRNA strongly increases after vascular injury [68,96].

Consistent with the reported effects of estrogen on bone physiology [97], estrogen receptors are expressed in osteoblasts (Fig. (**5b**)) [67,98] and chondrocytes [99]. In all of these cells, both receptors are present although their expression seems to be regulated differentially, with ERβ expression being constantly high during osteoblast differentiation while ERα expression starts at a low level and gradually increases [67].

ERα and ERβ Expression in Breast and Endometrium

Anti-estrogens are widely being used in the treatment of estrogen responsive breast cancer. However, long term treatment with tamoxifen may result in breast cancer cells that no longer respond to the anti-estrogen. Both estrogen receptors α and β were shown to be expressed in normal human breast as well as in some breast tumor species. In a study of ERα and ERβ mRNA levels in adjacent samples of normal breast tissue and matched primary tumors, a significantly higher ERα:ERβ ratio was detected in the tumor compared with the normal tissue. This difference derives from an upregulation of ERα expression in the tumor compartment, and, possibly, from a down-regulation of ERβ [100]. A similar observation of altered ERα and ERβ signaling pathways during tumorigenesis was observed in a comparative analysis of normal ovaries versus ovarian carcinomas [101]. In this study, ERα mRNA levels proved to be equal or slightly higher in ovarian carcinomas compared with normal ovaries. Conversely, a clear decrease in ERβ levels was observed in the tumor samples.

In situ hybridization showed that in the uterus, ERα expression localizes to the surface epithelium, the epithelial cells covering the endometrial glands and the stromal cells of the endometrium [71]. ERβ is very weakly expressed in the uterus as determined by RNAse protection analysis (Fig. (**5a**)). *In situ* hybridization analysis indicated that low level of ERβ expression is detected in the surface epithelium and endometrial glands [71]. The low expression of ERβ in the uterus indicates that ERα is responsible in mediating the uterotropic response upon estrogen treatment. Interestingly, studies with recombinant uterine tissues derived from ERα knockout and wild type animals have indicated that epithelial ERα is neither necessary nor sufficient for E2 induced cell proliferation. Instead, mitogenesis of epithelial cells may be mediated by a paracrine event originating from the ERα positive stromal cells [102].

ERα and ERβ Form Functional Heterodimers

Although ERα and ERβ do show a mutually exclusive expression pattern in some tissues, many cells contain both receptor subtypes. This raises the question whether the two receptors can heterodimerize, and whether such heterodimeric complexes would be functionally distinct from the two possible homodimeric complexes. This question is especially interesting in view of a recent observation that a dimeric nuclear receptor complex recruits a single co-activator [103]. Thus, heterodimers may expose a different interaction interface for cofactor recruitment compared with homodimers. Mammalian two-hybrid assays and *in vitro* interaction assays have shown that ERα and ERβ indeed can heterodimerize [24]. Interestingly, electrophoretic mobility shift assays indicate that ERα/ERβ heterodimers are preferentially formed over ERβ homodimers when both receptor subtypes are present within the same cell [104]. The affinity of the heterodimeric complex for a consensus ERE is identical to that of the ERα homodimeric complex. In contrast, ERβ homodimers show a reduced affinity for the target sequence, and in agreement with this observation, ERβ homodimers activate transcription of an ERE reporter gene to a lesser extent than ERα homodimers [104]. Within the same experiment, co-expression of ERα and ERβ under conditions where heterodimers are the predominant dimeric species, results in transcription of an ERE reporter gene to an intermediate level compared with that of either homodimer, suggesting that the heterodimer is transcriptionally active. In contrast, it has been shown that ERβcx, which is a c-terminally truncated form of ERβ, exhibits a dominant negative effect on ERα, but not on ERβ mediated transcription

[12]. Clearly, the distinct tissue distribution of ERα and ERβ and splice variants hereof, and their ability to heterodimerize generates a tremendous potential for functional diversity. In a very elegant study, Tremblay and coworkers [105] analyzed the individual contribution of ERα and ERβ within a heterodimeric complex to the transcriptional response by using mutant ERs with altered DNA binding specificities. This work showed that the AF-1 functions of both subunits in the heterodimer function independently with their specific properties being retained within the heterodimeric setting. Conversely, two functional AF-2 domains are required for transcriptional activation. This observation was in agreement with previous work that showed that receptors with deleted C-termini can heterodimerize to the wild type receptor but then function as dominant negative regulators [106]. Despite the requirement for two functional AF-2 domains in a dimeric complex, it seems that a single liganded subunit is sufficient to activate transcription, suggesting that AF-2 activation is not necessarily coupled to binding of ligand within the same subunit. Together, these studies provided evidence for different stochiometric requirements for AF-1 and AF-2 activities in dimeric receptor complexes.

Ligand Binding Specificity

The information that has come from crystal structures of LBDs of several members of the nuclear receptor gene family has suggested that the overall structures (twelve α-helices and two short β-sheets) are very similar [33,107,108]. However, the biology of these different receptors has also been characterized by a high degree of ligand-binding selectivity. For instance, the LBDs of the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) and those of the progesterone receptor (PR) and androgen receptor (AR) share approximately 60% amino acid sequence identity, yet their ligand specificity is very different.

The LBDs of ERα and ERβ share a similar degree of amino acid identity (60%) and, as a consequence, could have largely distinct ligand binding specificities. However, Fig. (**7**) shows that the affinity for 17β-estradiol is very similar for ERα and ERβ: Kd 0.16 nM for ERα, 0.12 nM for ERβ (pers. comm. W. Schoonen, Organon, The Netherlands). Others have reported a larger difference in Kd: 0.1 nM for human ERα, 0.4 nM for both rat ERβ and mouse ERβ [20,72]. This discrepancy could relate to species differences. the use of tritiated E2

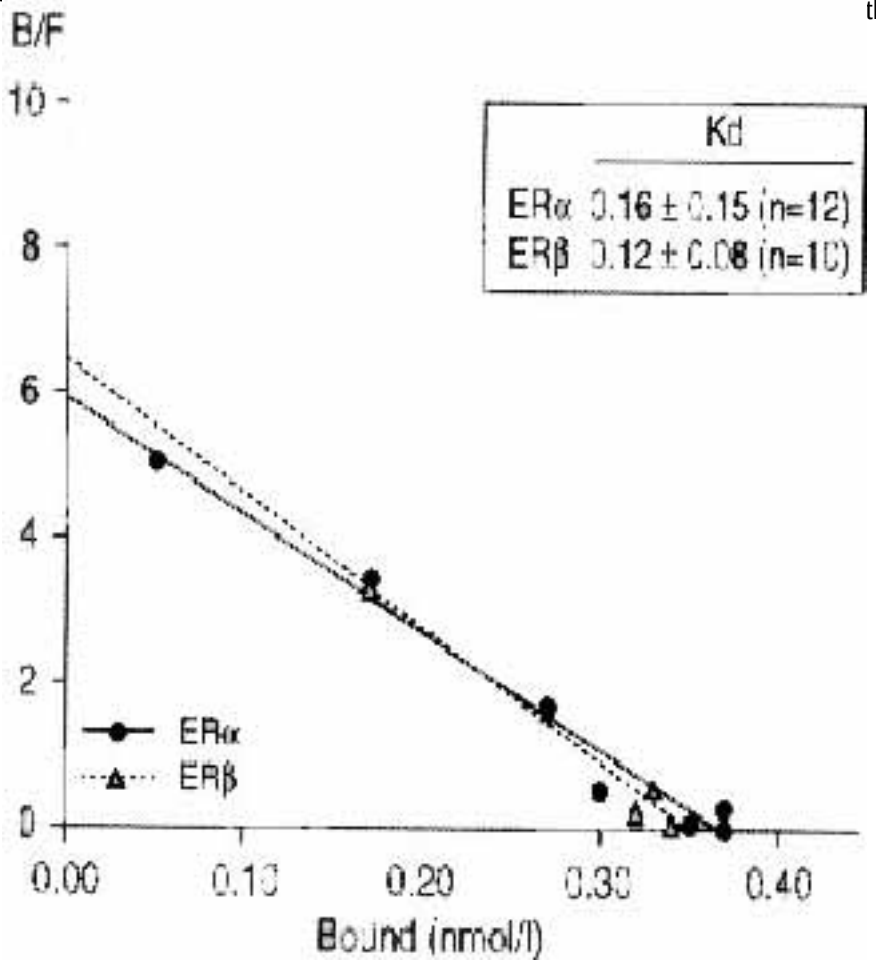

Fig. (7). ERα and ERβ have very similar binding affinities for 17β-estradiol. Scatchard analyses were performed on the human ERα and ERβ proteins expressed in CHO cells using tritiated 17β-estradiol.

versus 16α-iodinated E2, which contains a relatively large iodine atom, may differentially influence receptor binding for ERα and ERβ [72]. In transactivation experiments, it was shown that ERα and ERβ have very similar dose response curves for 17β-estradiol, although half-maximal activation is achieved for ERα at slightly lower hormone concentrations [88].

The first indication that the specificity of ERα and ERβ for certain ligands was non-identical came from saturation ligand binding analyses [72], although relatively small differences were observed. The non-steroidal agonists DES, genistein and coumestrol were shown to have a higher Relative Binding Affinity (RBA) for ERβ. The same holds for the antagonists OHT and ICI164384. In contrast, for 17α-estradiol a somewhat higher RBA for ERα was found.

Evidently, the affinity of receptors for ligands is determined by the LBD structure as a whole, however, within this domain very few residues of the ligand-binding pocket actually interact with the ligand. The recent publication of the crystal structure of the ERα LBD liganded with 17β-estradiol, DES and the antagonists OHT and raloxifene, revealed in detail which residues interact with these ligands [17,18]. Without exception the residues which have been shown to interact with either estradiol or raloxifene in the ERα crystal structure [18], are conserved in the ERβ LBD. In view of the 60% overall amino acid identity of the ERα and ERβ LBDs, it is striking to see the perfect conservation of the residues in the proximity of bound ligand. There are, however, a few residues in the proximity of the pockets that do differ: for instance Methionine 421 in ERα, corresponds to Isoleucine 373 in ERβ. These minor differences as well as the anticipated differences with respect to space within the ligand-binding pockets are suggestive for the possibility to screen for and develop receptor-specific ligands.

High Throughput Screening Assays for ERα and ERβ

We have generated stable Chinese Hamster Ovary (CHO) cell lines, which constitutively express ERα or ERβ. In addition, these cell lines carry an estrogen-responsive luciferase construct. These cell-based, so-called transactivation assays, which can be regarded very sensitive monitors of compounds with estrogenic activity, allow the screening of large numbers of compounds. These systems can also be used to identify antagonists by monitoring the ability of compounds to down-regulate E2-induced luciferase activity. A limited screening with ERα and ERβ assays has revealed that a number of compounds indeed have a certain degree of receptor-subtype specificity. Fig. (**8**) shows transactivation induced by E2 which is very similar for both receptors. However, the Organon steroid Org4094 is a much better agonist (over two orders of magnitude in transactivation) for ERα than it is for ERβ (Fig. (**8**)). Also, the well-known antagonist raloxifene has a higher antagonistic potency towards ERα. We have also tested several non-steroidal compounds such as phyto-estrogens, which are known to have estrogenic activity. Interestingly, many of them are better agonists for ERβ than for ERα [data not shown]. Transactivation data reported by others have also identified a compound that showed selectivity towards ERα. This compound, 17α-ethynyl-17β-estradiol, exhibits a 35-fold higher agonistic potency towards ERα as compared to ERβ [109].

Taken together, these data indicate that although the ligand-binding pockets of ERα and ERβ are very similar and as a consequence many compounds do not discriminate between the two in terms of potency, the ligand-binding cavities of ERα and ERβ are not identical. Moreover, the data obtained thus far already describe ligands that display receptor subtype specificity and it is anticipated therefore, that it may well be possible to develop ERα and ERβ specific agonists and antagonists.

ERα and ERβ Knockout Mice

The tissue distribution of ERα and ERβ shows considerable overlap, making it difficult to attribute a specific function to a specific receptor subtype. As yet, the precise contribution of ERα and ERβ in mediating estrogenic effects remains largely unclear. In this respect, the development of both ERα and ERβ knockout mice has been of extreme importance. The successful development of the ERα knockout (ERKO) mouse in 1993 was surprising, as it was thought that disruption of the ERα gene would result in embryonic lethality [110]. The discovery of ERβ, several years later, made clear that it may rescue part of the functions of ERα in the ERKO mice, although no significant upregulation of ERβ expression is seen in the ERKO animals [86]. ERKO females display elevated levels of luteinizing hormone, estradiol and testosterone, indicating that ERα is responsible for the negative feedback on gonadotropin release [111]. In addition, female ERKO mice are infertile and do not show an uterotropic response. Surprisingly, male ERKO mice also exhibit an infertile phenotype [110,112]. The latter observation was extended in a subsequent study that provided the mechanistic basis for the observed reduced fertility of male ERKO mice and showed that estrogen regulates the reabsorption of luminal fluid in the epididymis [113,114]. The importance of ERα in regulating reproductive function at multiple levels was furthermore stressed by the observation that female

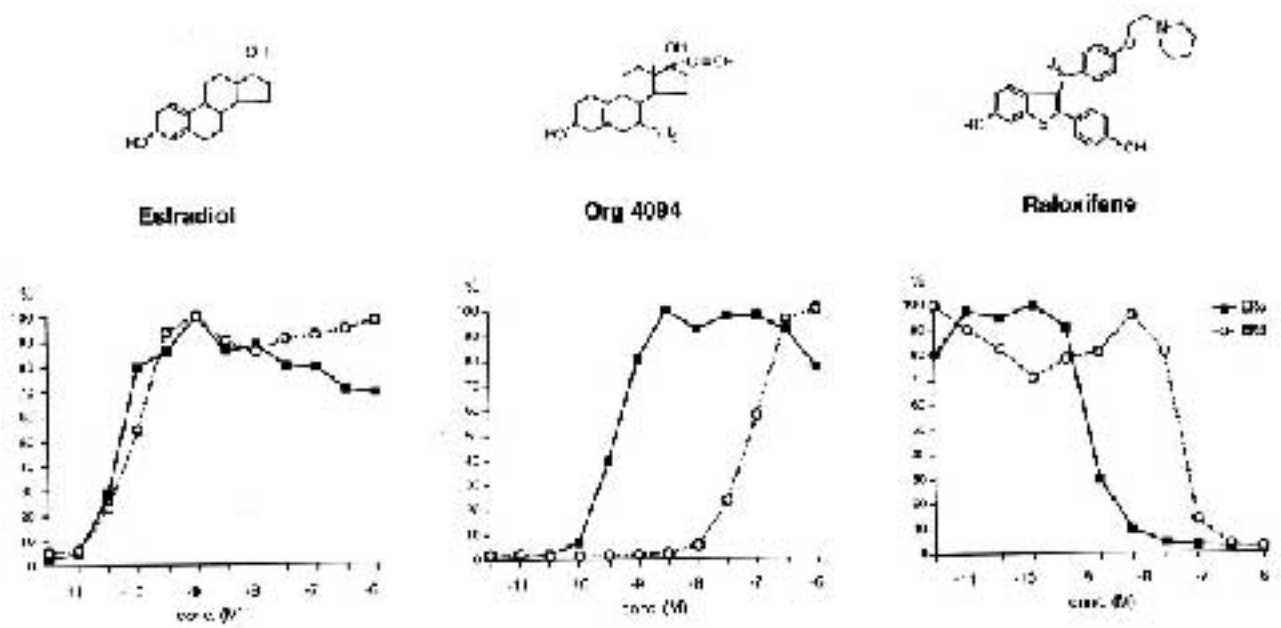

Fig. (8). Transactivation assays in CHO cells stably transfected with ERα or ERβ. Cells were incubated with increasing concentrations of 17β-estradiol (left panel), Organon steroid ORG4094 (middle panel) or an increasing concentration of raloxifene in combination with a fixed concentration of 10^{-10} M 17β-estradiol (right panel). The figure shows transactivation of a luciferase reporter gene placed under control of an ERE-containing promoter [10].

ERKO mice showed an absence of lordosis posture or receptivity [110]. Following the original publication on the phenotype of the ERKO mice, additional studies have addressed the effect of the disruption of the ERα gene on the bone maintenance and on the cardiovascular system. ERKO mice have a bone density that is 20 to 25 percent lower than in wild-type mice, clearly indicating that ERα contributes to bone maintenance [112]. Consistent with this observation is the finding that the bone mineral density was lowered in a man with a point mutation in the ERα gene that causes partial estrogen resistance [115]. With respect to the role of ERα in maintaining the normal cardiovascular function, it was shown that ERKO mice lack neovascularization and display a significant reduction in the production of nitric oxide in vascular endothelial cells [116,117]. The vascular injury response is still functional in ERKO mice, suggesting that also ERβ may be involved in the protective effects of estrogen on the blood vessel wall. However, this is based on indirect evidence and the involvement of other estrogen signaling mechanisms can not be excluded [95,96].

Recently, a mouse with a disrupted ERβ gene has been described [118]. These so-called BERKO mice develop normally and do not display gross abnormalities. However, the ovulation efficiency in these mice is lowered, resulting in a reduced fertility. In contrast to the ERKO mice, the ovaries of BERKO mice are histologically very similar to those of wild-type mice. These results are surprising, as ERβ is the predominant receptor subtype in the ovary and is highly expressed in granulosa cells. It was, therefore, expected that the BERKO mice would exhibit a more severe reproductive phenotype. To date, there have been no reports on the bone and cardiovascular phenotype of the BERKO mice.

Therapeutic Opportunities for ER-Subtype Selective Estrogens?

Although ERβ was discovered quite recently, it has been a drug target since estrogens were used to treat specific symptoms. Now that we know that estrogens signal through two distinct receptors, the question arises whether or not we can use this knowledge to develop compounds which would be more or equally efficacious than currently available drugs but which would lack side-effects. With respect to their binding of 17β-estradiol, it can be argued that ERα and ERβ are two receptors of a kind. However, an increasing number of papers describe important differences between the two estrogen receptors, which need to be further explored. At this point in time, the precise contributions of ERα and ERβ in mediating estrogenic effects is just beginning to become more clear. The tissue distribution of ERα and ERβ shows considerable overlap, which makes it complicated to attribute specific functions to either of these two receptor subtypes. However, the data on ERα and ERβ that exists to date

indicates that their mechanisms of action may differ considerably. It can be foreseen that a better understanding of the distinct roles of ERα and ERβ in normal physiology and in disease will be gained. This is likely to reveal more selective therapies for the treatment of cardiovascular disease, osteoporosis, menopausal hot flushes and mood changes and maybe also Alzheimer's disease. In addition, the regulation of fertility in males and females could benefit from knowledge regarding ERα and ERβ. Both experimental data obtained from ERα and ERβ knock-out mice as well as data obtained with receptor subtype-specific agonists and antagonists will further assist in revealing the significance of both receptors in normal physiology and disease.

Acknowledgements

We gratefully acknowledge Bart van Lith, Jan Polman, Karolien Makkink, Henri Klop, Willem Schoonen, Milou Kouwijzer, Joop van Orsouw, Rein Dijkema, and Wiebe Olijve for their contributions to this work.

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