Inhibition of NF-κB transcriptional activity by the estrogen receptor

Insights into the molecular mechanism

Monique Quaedackers

Cover: Low power scanning electron microscope images, showing osteoporotic bone architecture of an 89 year old woman (top left) and normal bone architecture of a 30 year old woman (bottom left), by kind permission of Prof. Alan Boyde; overlays of pictures showing differential interference contrast (DIC), emission after excitation with 430 nm and emission after excitation with 490 nm, taken from unstimulated (top right) or estrogen-treated (bottom right) osteoblast-like cells expressing fluorescently labeled ERα and NF-κB (by Leon Tertoolen).

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Remming van NF-κB transcriptie activiteit door de oestrogeen receptor

Inzichten in het moleculaire mechanisme

(met een samenvatting in het Nederlands)

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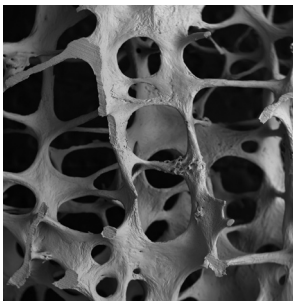
Chapter 1



General Introduction

1. BIOLOGY OF ESTROGENS

The steroid hormone estrogen plays a key role in the regulation of a wide variety of physiological processes. Although often referred to as the female sex hormone, due to its well known role in female reproduction, estrogen is now also recognized as an important hormone in male reproductive tissues. In addition, estrogen action extends beyond the reproductive system, having profound effects on tissues throughout the human body. Before menopause in women, estrogen is mainly produced by the ovaries and acts at distant organs via blood circulation. In men, estrogen is also produced by the gonads but this accounts only for a small amount of the circulating estrogen level, which is lower (about 3-fold) than in women. However, estrogen can also be produced and act locally at sites outside the gonads and these sources play an important physiological role in men and in postmenopausal women.

1.1. Target tissues and responses of estrogen

The existence of estrogen has already been recognized as early as 1905 (1). However, it was not until 1960 that experiments were performed leading to the hypothesis that estrogen exerts its effects by binding to a receptor (2, 3) and indeed a few years later the protein was identified (4, 5). Cloning of the estrogen receptor (ER) was reported by two research groups in the mid 1980s (6-8). For a decade it was thought that only one ER existed until a second ER was cloned, which was designated as ERβ (9), resulting in the first ER to be referred to as ERα. Tissues with measurable response to estrogen and detectable levels of one or both ER subtypes are considered as estrogen target tissues. Analysis of mRNA expression in rodents has revealed that ERα is predominantly expressed in uterus, testis, pituitary, liver, kidney, heart, and skeletal muscle, while the highest expression of ERβ was found in the ovary, prostate, lungs, bladder, gastrointestinal tract, central nervous system and hematopoeitic system. An equal level of expression for ERα and ERβ is observed in the mammary gland, epididymis, thyroid, adrenal, bone and various regions of the brain (10-12) (Table 1). Through the use of in situ hybridization it became evident that, although both receptors are coexpressed in one tissue, often the localization of ERα and ERβ is in the distinct cell types within the same tissue. The use of models in which estrogen signaling is impaired has led to the discovery of many biological responses of estrogen. These include mouse models involving targeted gene disruption, including ERα knock-out mice (ERKO or αERKO) (13), ERβ knock-out mice (BERKO or βERKO) (14) and the double knock-out (DERKO or αβERKO) (15). Studies with these receptor knockout mice have revealed a role for ER signaling in fertility, male and female sexual maturation, in the cardiovascular system, behavior and bone modeling and remodeling (16-20). The phenotypes of the ERKO and BERKO mice show that ERα and ERβ can have distinct or overlapping functions corresponding with the relative expression and cell type distribution of the receptors in the specific tissues (Table 1). In addition to receptor knock-outs, a mouse model was generated with disruption of the Cyp19 gene encoding cytochrome P450 aromatase, which is essential for estrogen biosynthesis (ArKO mice) (21). In the gonads cholesterol is conversed to testosterone via several steps, which can subsequently be conversed to estrogen by aromatase. In extragonadal tissues, such as adipose tissue, bone and brain, aromatase locally converses testosterone (delivered through the circulating system) to estrogen. In ArKO mice estrogen synthesis is completely blocked, which resulted in phenotypes similar to the receptor knock-outs including defects in fertility and bone homeostasis and effects on behavior, the vascular system and the brain (21-25). Furthermore, natural mutations of ERα and aromatase in humans were

observed to be associated with diseases related to ER signaling, such as breast cancer, and osteoporosis (26-29). Finally, in postmenopausal women it becomes evident that loss of estrogen signaling is associated with increased risk for impaired function of ER target tissues resulting in diseases such as osteoporosis and cardiovascular diseases (30, 31). During menopause circulating estrogen levels dramatically decline due to loss of ovarian steroid production and from then on estrogen synthesis can only take place in extragonadal tissues, which is dependent on the blood level of precursors, such as testosterone, that act as a substrate. Because in postmenopausal women testosterone levels are much lower (about 15-fold) than in men, estrogen levels in women can become insufficient at estrogen target sites (32, 33).

Table 1. Overview of estrogen target tissues with well described phenotypes in ER knock-out mice.

System		Tissue/cell type	ER expression	Affected Process	KO Model
Reproductive system	♀	Ovaries	ERβ (ERα)	Sex steroid production / Follicle development	α + β + αβ ERKO
		Uterus	ERα (ERβ)	Complete maturation	α + αβ ERKO
		Mammary gland	ERα + ERβ	Complete maturation	α + αβ ERKO
	♂	Testis	ERα (ERβ)	Sperm production and function	α + αβ ERKO
		Prostate	ERβ (ERα)	Growth promotion/ stimulatory	α + β + αβ ERKO *
Cardiovascular system		Heart	ERα (ERβ)	Protective effect in ischemic injury	α + β ERKO
		Vascular smooth muscle/endothial cells	ERα + ERβ	Protection against vascular injury	αβ ERKO
		Liver	ERα	Lipid profile / protection against atherosclerosis	αERKO
Skeletal system		Osteoblast/Osteocyte Osteoclast/ Chondrocyte	ERα + ERβ	Bone modeling / remodeling	α + β + αβ ERKO
Immune system		Bone marrow	ERβ	Differentiation of hematopoetic cells	β ERKO
Other		Pituitary	ERα	Gonadotropin synthesis and secretion	α + αβ ERKO

♀ : female; ♂ : male ; ER expression : expression of receptor subtypes is depicted for mouse adult tissues, receptor in brackets means expression is observed but minor to other subtype; KO model : the models in which a clear phenotype is observed (phenotypes may differ between α, β or αβ ERKO models and between males and females); * conflicting results between different laboratories

2. MOLECULAR MECHANISM OF ER ACTION

The two human ER proteins, ERα and ERβ, are two different subtypes expressed from separate genes on chromosome 6 and 14, respectively (34, 35). ERs are type I receptors belonging to the superfamily of nuclear receptor (NRs), which is the largest known group of eukaryotic transcription factors. Type I receptors can be activated upon binding of a steroid hormone and are thus called steroid receptors (36). According to conserved sequence and function, NRs can be divided into six domains labeled A to F (Fig. 1A). The central C-domain is the most highly conserved and encodes two zinc finger motifs enabling DNA binding. The carboxy-terminal E-domain is less well conserved and mediates ligand binding, receptor dimerization, nuclear localization and contains activation function 2 (AF-2). The amino-terminal A/B domain, which may contain an AF-1, is very poorly conserved. Poor conservation is also observed for domain D, which separates the DNA binding and ligand binding domain (DBD and LBD, respectively) functioning as a hinge region. The function of domain F is largely unknown and is absent in some receptors (37, 38). The functional domains of ERα and ERβ are conserved to the same degree as NRs in general, high homology in the DBD (96%), moderate in the LBD (58%) and low in AF-1 (20%) (Fig. 1B) (9, 39).

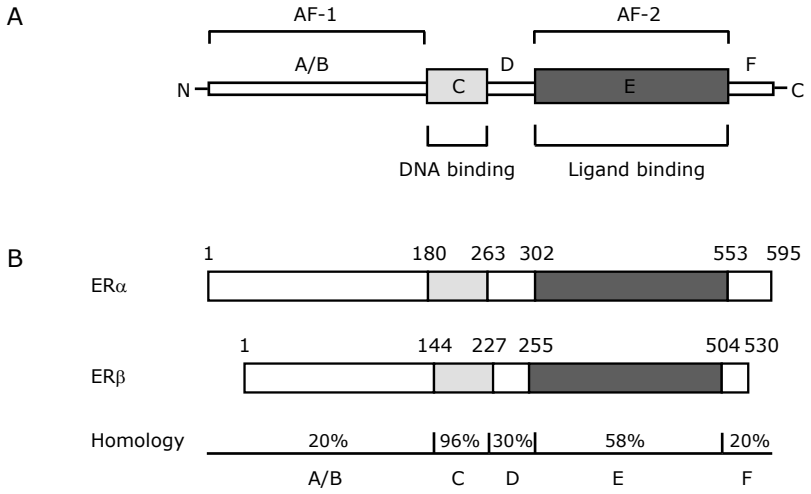


Figure 1. Nuclear receptor structure can be organized into six domains according to conserved sequence and function.
A, Schematic overview of nuclear receptor domains. **B,** Organization of ERα and ERβ, with amino acid numbers corresponding to the start and end of each domain and homology between the two subtypes.

2.1. Regulation of gene transcription by the estrogen receptor

In the absence of hormone, ER is present in an inactive form and predominantly located in the nucleus. Upon hormone binding, the receptor undergoes a conformational change that enhances receptor dimerization and enables recognition of specific DNA elements in the promoter region of target genes. Both ERα and ERβ bind to sequences known as estrogen response element (ERE), which are palindromic inverted repeats (40, 41). Once bound to DNA, due to the conformational change the activated receptor is capable of recruiting

the basal transcription machinery as well as other coregulatory proteins necessary for enhancing the expression of target genes (Fig. 2A) (42-45). These coregulatory proteins, that do not bind DNA directly, are collectively called coactivators because they promote gene transcription.

Induction of eukaryotic gene transcription involves several steps, starting with the formation of a stable preinitiation complex (PIC) on the promoter. The first step of PIC formation is binding of the TATA-box by the general transcription factor (GTF) TFIID, a complex consisting of TATA-binding protein (TBP) and TBP-associated factors (TAFs). TFIID enables binding of TFIIA and TFIIB followed by recruitment of RNA polymerase II (Pol II) and other GTFs resulting in full PIC assembly. The next step necessary to start transcription is the phosphorylation of the carboxy-terminal domain (CTD) of Pol II mediated by TFIIH, which is associated with several kinases (46, 47). Recruitment of the basal transcription machinery by ER can occur through direct interactions with several GTFs but also through interaction with the TRAP (thyroid receptor-associated protein)/mediator complex that acts by bridging ER to the GTFs (48, 49).

In addition to recruitment of the basal transcription machinery, ER is capable of enhancing gene transcription by recruitment of multi-protein complexes that locally modify the chromatin structure. Among these complexes is the SWI/SNF complex that is capable of chromatin remodeling in an ATP-dependent manner resulting in chromatin that is more accessible to GTFs (50-53). The second class of chromatin modifying factors recruited by ER is the group of enzymes that modify lysine or arginine residues of histone tails, including histone acetyltransferases (HATs) and histone methyltransferases (HMTs). Histone acetylation and methylation changes the chromatin structure diminishing the condensation state, which facilitates gene transcription. Complexes recruited by ER containing HAT activity include the p300/CBP (cAMP response element binding protein (CREB)-binding protein) and pCAF (p300/CBP-associated factor) complex (54-58), while CARM (coactivator-associated arginine methyltransferase) and PRMT (protein arginine methyltransferase) complexes contain HMT activity (59). ER can interact with HAT complexes directly but also indirectly by interacting with members of the p160 family of coactivators, which include steroid receptor coactivator 1 (SRC-1) (60, 61), SRC-2 (previously named TIF2/GRIP-1) (62, 63) and SRC-3 (previously named ACTR/pCIP/RAC3/ TRAM-1/AIB1) (57, 64-67). Besides acting as bridging factors these coactivators have also been shown to contain histone acetyltransferase activity (64, 68-70).

Recent studies using chromatin immunoprecipitation (ChIP) assays provide evidence for a model in which regulation of gene transcription by ER involves ordered and cyclical recruitment of the GTFs and coactivators described above. In this model coactivators do not bind ER at the same time, but a primary coactivator binds facilitating the recruitment of a secondary coactivator. This model assumes that transcription activation is the result of sequential and stochastic recruitment of specific protein complexes from a large array of factors with overlapping function (71, 72).

2.2. The selective estrogen receptor modulator (SERM) concept

Interaction with coactivators can occur through two activation domains of ER, AF-1 and AF-2, which can act independently or synergistically depending on cell type and promoter context. Coactivator interaction through AF-2, which is part of the LBD, is fully dependent on ligand binding. Crystal structure studies revealed that the LBD of NRs is composed of 12 helices of which helix 5, 6, 9 and 10 form a tightly packed core, with a hydrophobic cavity that serves as the ligand binding pocket, surrounded by helix 2, 3, 4, 7, 8 and 11. It

appeared that helix 1 does not take part of this "sandwich" and the orientation of helix 12 (H12) varies depending on the type of ligand occupying the binding pocket (73, 74). The sequence of H12 is highly conserved among NRs and mutation analysis revealed that this helix plays an essential role in ligand-dependent activation of transcription (75-77). When the LBD is occupied by an agonist, such as 17 β-estradiol (E2) for ER, the position of H12 is rearranged and packs against the core of the LBD forming a lid over the ligand-binding cavity. In this position H12 together with residues in H3, 4 and 5 create a coactivator-binding groove which can interact with so-called NR-boxes (73, 74). The NR-box is a conserved motif with a consensus sequence (LXXLL, where L is a leucine and X is any amino acid) that forms a α-helix (78, 79). Multiple NR-boxes have been observed in all members of the p160 family of coactivators and in many other coactivators that bind to NRs in a ligand-dependent manner, including CBP and p300.

Interaction of coactivators with the poorly conserved AF-1 can occur independently of ligand-binding and it is thought not to involve docking of LXXLL motifs. Although the structural basis for the interaction between AF-1 and coactivators is relatively unknown, a role in enhancing AF-1-mediated transactivation is acknowledged for the phosphorylation of several residues the A/B-domain of ERα (80). It is proposed that these phosphorylation events may change the secondary structure of AF-1 indirectly stabilizing the interaction with coactivators. This assumption is in accordance with the induced-fit model, in which AF-1 interacts with a coactivator through weak electrostatic interactions and upon conformational changes in the interaction surface the complex is stabilized (81, 82).

In addition to the natural agonist E2, a variety of other estrogenic ligands bind the ER-LBD including synthetic compounds that bind ER with high affinity but confer antiestrogenic effects (83, 84). These antiestrogens can be full antagonists, such as ICI 164, 384 and ICI 182,780, or partial agonists/antagonist, such as tamoxifen and raloxifene. Crystal structures of liganded ER-LBDs revealed that antiestrogen action was mediated by disruption of the coactivator-binding surface caused by the bulky side chain of the compounds. Due to antiestrogen binding H12 does not close the ligand binding pocket, but instead H12 is disordered (for ICI) or adopts a position that mimics binding of the coactivator (for tamoxifen and raloxifene) (73, 74, 85-87). In addition to blocking AF-2, the ICI compounds also block the action of AF-1 and modify other properties of the receptor, such as enhancing receptor protein degradation (88). In the case of tamoxifen or raloxifene binding, AF-1 function remains intact and therefore ER is still capable of enhancing transcriptional activity (89). Because, AF-1 activity of ERβ is in general very weak, tamoxifen and raloxifene are capable of completely blocking ERβ transcriptional activity (90). Recent studies revealed that in the presence of tamoxifen ERα specifically interacts with the molecules SMRT (silencing mediator for retinoid and thyroid hormone receptor) and NCoR (nuclear receptor corepressor) that act as corepressors by recruiting HDACs (histone deacetylases) opposing the effect of HATs (91, 92). Therefore, depending on cell type specific relative expression of ER subtypes, coactivators and corepressors, tamoxifen and raloxifene are capable of exhibiting agonist or antagonist activity (93). The molecular mechanisms of antiestrogen action have provided insight into the understanding of the tissues selective effects of tamoxifen and raloxifene that became evident from their clinical use. Tamoxifen is widely used as a drug to treat ER positive breast cancer due to its antiestrogenic effect in breast tissue (94). Moreover, tamoxifen appeared to have protective effects on bone and cardiovascular system acting as an agonist (95, 96). Unfortunately, tamoxifen increases the risk for endometrium cancer due to estrogen-like activities in this tissue (97). Raloxifene is used to treat osteoporosis in postmenopausal women due to agonist effects in bone, while opposing estrogen action in the uterus and breast thereby preventing cancer (98-101). Due to tissue-selective

agonist or antagonist effects tamoxifen and raloxifene are called selective estrogen receptor modulators (SERMs).

2.3. Alternative modes of ER action

In addition to regulation gene transcription via direct binding to EREs, ER can modulate the expression of genes by modifying the activity of other transcription factors (Figure 2B). Estrogen-responsiveness of several genes is linked to the presence of AP-1 sites or specific GC-rich sequences present in their promoters to which ER is tethered through binding the Fos/Jun or Sp1 proteins, respectively (102, 103). In these cases, ER is capable of activating gene transcription by indirectly binding to DNA. Furthermore, some genes that are regulated by nuclear factor-κB (NF-κB) have been shown to be inhibited in the presence of ER (104-106). Interference of ER with NF-κB transcriptional activity, the subject of this thesis, will be discussed in section 4.

Besides hormone-dependent activation of the receptor, there is much evidence that ERs can be activated in the absence of hormone upon phosphorylation of the receptor. Several growth factors, such as epidermal growth factor (EGF), insulin-like growth factor I (IGF-I) and transforming growth factor β (TGFβ), can mimic E2 by inducing the activity of kinases, such as mitogen-activated protein kinase (MAPK), that target the A/B domain of both ERs (Fig. 2C) (80, 107).

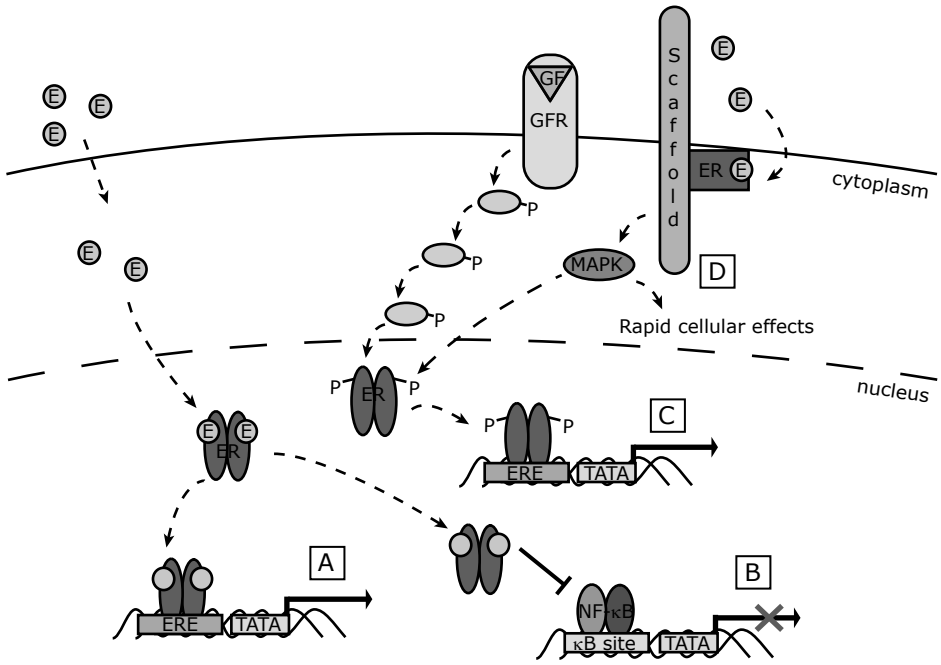


Figure 2. Mechanisms of estrogen receptor signaling.
A, Classical ERE pathway. **B,** ERE-independent pathway. **C,** Ligand-independent pathway. **D,** Non-genomic pathway. Additional abbreviations: E, 17β-estradiol; κB site, NF-κB binding site; GF, growth factor; GFR, growth factor receptor; P, phosphorylated amino acid residue. For detailed description see text. For simplification the basal transcription machinery and coactivator complexes are not shown.

Finally, E2 can induce very rapid effects via membrane-associated or cytoplasmic ERs through a less well characterized mechanism. This mode of action involves the modulation of signaling pathways in the cytoplasm, such as the MAPK pathway. Because gene expression is not directly modulated, these effects of E2 are referred to as the non-genomic or non-nuclear pathway (Figure 2D) (108, 109).

3. THE NF-κB PATHWAY

NF-κB was first described in 1986 by Sen and Baltimore, who identified it as a B cell nuclear factor that bound to an enhancer in the gene encoding the immunoglobulin κ light chain (110). From then on, it became clear that NF-κB is an evolutionary conserved and ubiquitously expressed protein capable of transducing various signals from outside the cell into a specific cellular response by regulating the expression of a variety of genes. Moreover, it is now recognized that NF-κB regulates many processes vital for normal physiology, while disturbance of NF-κB signaling is involved in various diseases.

3.1. The mammalian family of NF-κB/Rel and IκB proteins

NF-κB is a dimeric transcription factor formed between the subunits encoded by five different NF-κB/Rel genes, NF-κB1, NF-κB2, RelA, RelB and c-Rel. NF-κB1 and NF-κB2 encode a precursor protein, p105 and p100, respectively, containing ankyrin repeats in their carboxy (C)-terminus (111, 112). Cotranslational and continuous processing of p105 gives rise to the p50 subunit, which corresponds to the amino-terminal half of the precursor protein (113-115). The p52 subunit can be cleaved from the amino (N)-terminus of p100 through a tightly regulated posttranslational mechanism (116, 117). The other subunits RelA (p65), RelB and c-Rel are not processed during or after translation and contain a transactivation domain (TAD) at their C-terminus. At the N-terminus all subunits contain a Rel homology domain (RHD), which is a highly conserved sequence of 300 amino acids involved in dimerization, nuclear localization, DNA binding and interaction with members of the NF-κB inhibitor (IκB) family (111, 112). The most abundant NF-κB complex is a heterodimer between the p65 and p50 subunits, although heterodimers of p65/c-Rel, c-Rel/p50, RelB/p50 and RelB/p52 and homodimers of p65 and c-Rel are also capable of activating gene transcription. On the other hand p50 and p52 homodimers are known as repressors of gene transcription, since these NF-κB molecules cannot confer transcriptional activity due to lack of TADs (Fig. 3A) (111, 112).

The IκB family consists of seven members, including IκBα, IκBβ, IκBε , IκBγ, Bcl-3 and the precursor proteins p105 and p100. These members all contain a domain with multiple ankyrin repeats that mediates the interaction with the RHD of NF-κB subunits thereby inhibiting NF-κB activity (111, 112, 118). Crystal structure studies revealed that upon NF-κB binding IκB proteins mask the nuclear localization signal (NLS) of p65, while the NLS of p50 remains exposed, allowing translocation to the nucleus. However, due to nuclear export signals (NES) in both p65 and IκB, the complex is shuttled back into the cytoplasm, resulting in a steady-state localization in the cytosol (119-124). In addition to preventing nuclear translocation, IκBs inhibit NF-κB activity by blocking DNA binding. Bcl-3 is included in the IκB family, because it also contains an ankyrin repeat domain. However when Bcl-3 forms a complex with homodimers of p50 or p52 , the complex is located in the nucleus and capable of activating transcription due to the presence of a TAD in Bcl-3 (Fig. 3B) (125, 126).

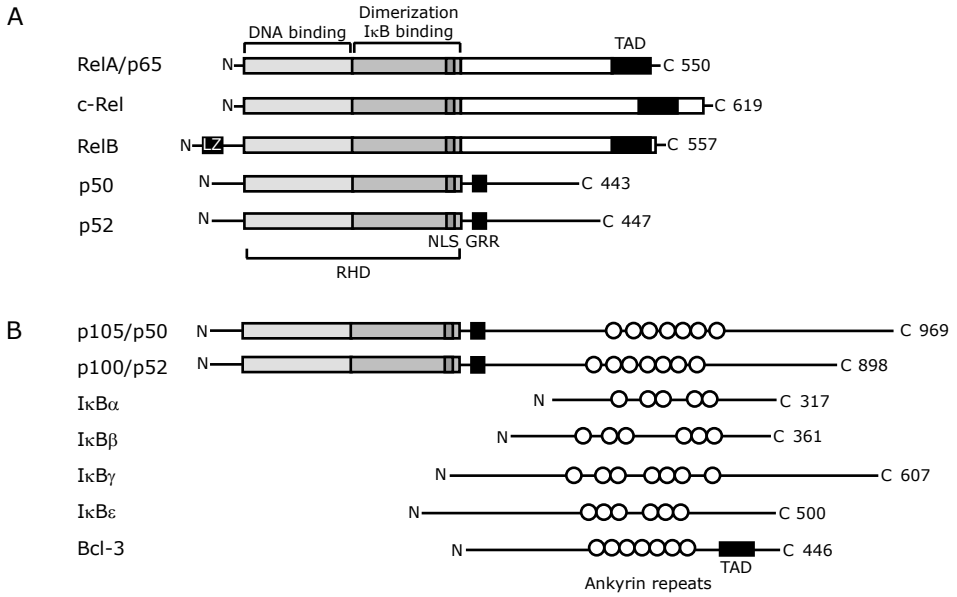


Figure 3. Mammalian NF-κB and IκB family members.
A, The NF-κB family consists of five members, which all contain a highly conserved Rel homology domain at their N-terminus. **B,** The IκB family consists of seven members, which are characterized by the presence of an ankyrin repeat domain. Additional abbreviations: LZ, leucine zipper (necessary for transactivation by RelB); GRR, glycine rich regions (necessary for processing of p100 to p52 and p105 to p50).

3.2. NF-κB signal transduction cascade

In unstimulated cells NF-κB is retained in an inactive state in the cytoplasm due to its association with IκB. Activation of NF-κB can be achieved upon exposure of cells to a variety of stimuli and although acting through many different pathways, their action converges at the degradation of IκB. This results in liberation of the NF-κB dimer, which can now rapidly translocate to the nucleus where it can regulate transcription of target genes. The NF-κB pathway can be considered as a two-step process, activation taking place in the cytoplasm and regulation of gene transcription taking place in the nucleus (127, 128). Activation can occur in two distinct ways, called the classical (or canonical) and the alternative (or non-canonical) pathway (Fig. 4).

The classical pathway can be activated by bacterial or viral infections and by several pro-inflammatory cytokines, such as tumor necrosis factor (TNF)α and interleukin (IL)-1 (129). Cytokines, viral and bacterial products act as ligands by binding to specific membrane-bound receptor, which subsequently triggers a series of events ultimately resulting in recruitment and activation of a specific IκB kinase (IKK) complex (127). This complex is composed of two catalytic subunits, the kinases IKKα and IKKβ, and the regulatory subunits IKKγ (also referred to as NEMO) and ELKS (130). Upon IKK activation, specific serine residues in the N-terminus of IκBs associated with NF-κB are phosphorylated, mainly carried out by IKKβ. Phosphorylation targets the IκBs for poly-ubiquitination and subsequent recognition and degradation by the 26S proteasome liberating the NF-κB molecule (Fig. 4A) (127).

Signals activating the alternative pathway are certain members of TNF superfamily, including lymphotoxin β (LTB), B-cell activating factor (BAFF) and CD40 (131-133). These stimuli

trigger the activation of a specific kinase, NIK (NF-κB inducing kinase), that phosphorylates IKKα homodimers (116). Activated IKKα phosphorylates p100 (in a complex with RelB) leading to ubiquitination and proteasomal degradation of the C-terminus of p100 resulting in the release of p52/RelB dimers (Fig. 4B) (117, 134).
Furthermore, DNA damage and UV radiation are known as stimuli of the NF-κB pathway. Although the exact mechanism of action is less well understood, it appears that the responses to DNA damage and irradiation involves IKK mediated degradation of IκB as well (127, 135).

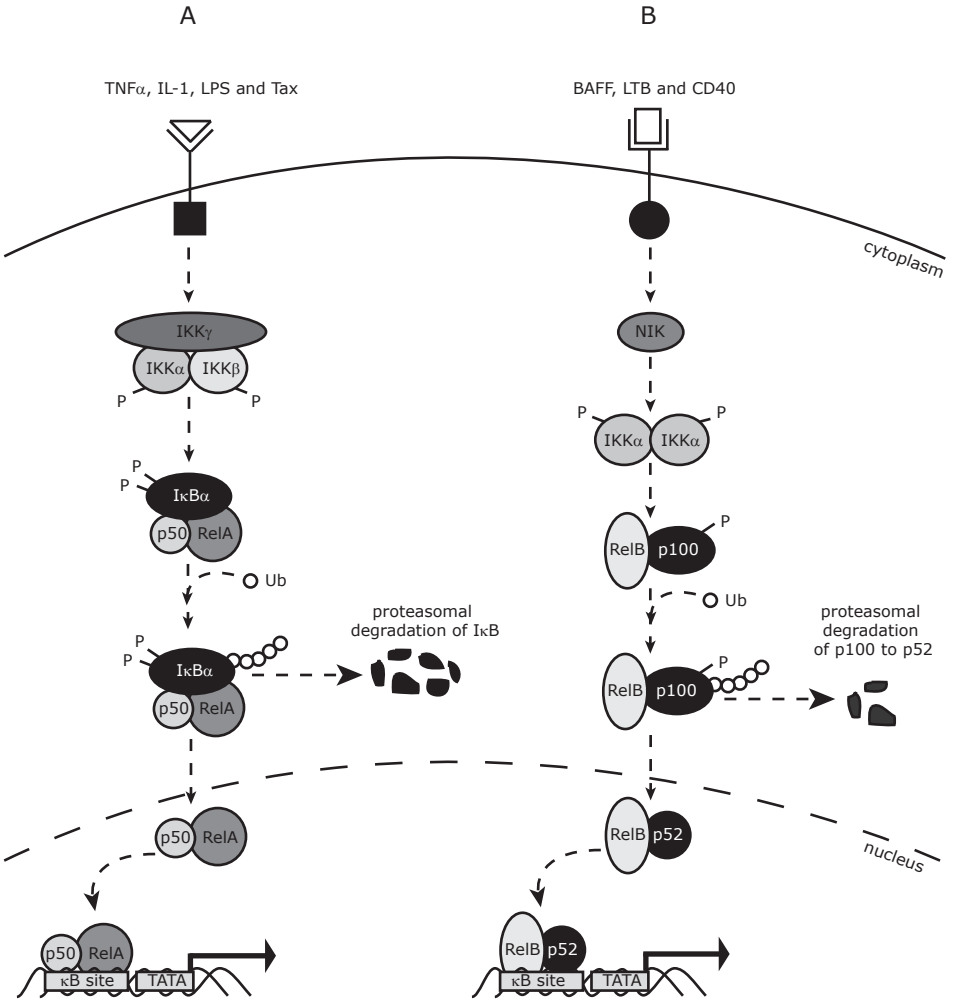


Figure 4. Two separate signaling pathways lead to activation of NF-κB.
A, The classical pathway can be induced by proinflammatory cytokines (TNFα and IL-1) and bacterial (LPS) or viral (Tax) products. **B,** The alternative pathway can be stimulated by certain members of the TNF-family. Additional abbreviations: LPS, lipopolysaccharide; Ub, ubiquitin. For detailed description see text.

Following liberation from IκB and translocation to the nucleus, NF-κB is able to recognize and interact with specific NF-κB binding sites (κB sites) with the consensus sequence GGGRNNYYCC (where N is any base, R is a purine and Y is a pyrimidine), which are present in promoter and enhancer regions of target genes. Upon DNA binding, NF-κB dimers facilitate the recruitment of the basal transcriptional machinery and coactivators to the promoter in order to enhance gene transcription. NF-κB is known to interact through its TAD with both GTFs (TBP and TFIIB) as well as coactivators (CBP, p300 and SRC1) (128). Recent studies revealed that posttranslational modifications of the NF-κB dimer and the histones surrounding the promoter play an important role in obtaining a maximal transcriptional response. In particular RelA has been shown to be a target of phosphorylation, both in the cytoplasm and the nucleus, mediated by various protein kinases induced by various stimuli, including LPS, TNF α and IL-1. Phosphorylation has so far been shown to mediated by protein kinase C-ξ (PKCξ), the catalytic subunit of protein kinase A (PKA_c) A, mitogen- and stress-activated kinase-1 (MSK1), casein kinase II (CKII), ribosomal subunit kinase-1 (RSK1), glycogen-synthase kinase-3β (GSK3β) and phosphatidylinositol 3-kinase (PI3K)/AKT (128). Phosphorylation of serine (S)276 and S311 residues in the RHD of RelA is necessary for enhancing the interaction with p300/CBP, the efficiency of DNA-binding and the displacement of HDAC complexes from the chromatin (136). On the other hand, phosphorylation of the TAD at S529 or S536 is thought to enhance interaction with components of the basal transcriptional machinery. Many NF-κB inducing stimuli are also found to stimulate phosphorylation of serine 10 in the tail of histone H3 and this has been shown to be necessary for the transcription of several NF-κB target genes (137-139). In addition to phosphorylation, NF-κB and histones are also modified by acetylation in a stimulus-dependent manner mediated by several HATs, including CBP/p300 and pCAF. The acetylation status of both RelA and p50 has been shown to affect transcriptional potency and DNA-binding affinity (140-144). The acetylation state of NF-κB is shown to be reversible due to HDAC-mediated deacetylation, which is believed to be important for the termination of the NF-κB response, which will be described below. The stimulus-induced acetylation of the histone tails of H3 and H4 is also associated with increased expression of NF-κB target genes (145, 146). Hyperacetylation of histones arround the promoter results in remodeling of the chromatin structure enabling easy access of NF-κB and the transcriptional machinery, while deacetylation by HDACs reverses this process and inhibits gene expression.

The negative feedback pathway that shuts down NF-κB activity involves the de novo synthesis of IκBα. The gene encoding IκBα is regulated itself by NF-κB and upon synthesis it binds to nuclear NF-κB exporting it back to the cytosol. However, because acetylated NF-κB has low binding affinity for IκB, it first needs to be deacetylated in order to allow efficient IκB binding and termination of the NF-κB response (140).

Taken together, activation of the IKK complex, degradation of IκB, nuclear translocation and modification of NF-κB and histone tails, which are all under control of specific stimuli together define the final transcriptional response of NF-κB target genes. A recent study revealed that the sequence of the κB site does not only determine which NF-κB family members can effectively bind, but more importantly it affects which coactivators are recruited to the promoter, probably caused by a sequence-induced change in dimer configuration (147, 148). Therefore, both the stimulus as well as the promoter sequence determines the response of a specific target gene.

3.3. NF-κB target genes and physiological responses

NF-κB plays its most important role in the immune system by controlling both innate and adaptive immune responses. Rapid activation of NF-κB activity, induced by intruding pathogens, is very crucial in the first-line defense against the invasion carried out by cells of the innate immune system. Viral and bacterial products are recognized by membrane-bound Toll-like receptors that trigger activation of the NF-κB pathway, resulting in enhanced expression of a variety of genes (149). These include genes coding for pro-inflammatory cytokines, chemokines and adhesion molecules that are necessary to recruit and activate other cells of the immune system to the infection site (111). The innate immune-response also activates antigen-presenting cells, which together with pro-inflammatory cytokines stimulate T cells of the adaptive immune system. Furthermore, NF-κB regulates adaptive (or acquired) immune responses by controlling B- and T-cell development, proliferation, activation and function (150, 151). Many of the essential functions of NF-κB in the immune system were revealed by the use of gene-knockout mice that are deficient in one or more members of the NF-κB family, IκB family, IKK complex or other components of the signaling pathway (152). Moreover, these knockout models and other genetic models gave insight into actions of NF-κB outside the immune system. It became clear that NF-κB is also involved in regulating development and physiology of the mammary gland, skin and tissues of the skeletal and central nervous system (an updated list of published phenotypes can be found at http://people.bu.edu/gilmore/nf-kb/genek/index.html). In summary, the target genes of NF-κB encompass genes involved in immune and inflammatory responses (cytokines and their receptors; chemokines and their receptors; cell adhesion molecules and their receptors; chemoattractant molecules; inflammatory enzymes; antibacterial peptides), genes involved in cell proliferation and survival (growth factors and regulators; apoptotic and antiapoptotic genes) and genes involved in cell differentiation and cell migration (angiogenic factors; matrix-degrading proteases) (153) (an update list of published NF-κB target genes can be found at http://people.bu.edu/gilmore/nf-kb/target/index.html).
Regulation NF-κB target genes is essential in many processes of normal physiology, however, aberrant NF-κB activity has been implicated in various processes of pathology associated with autoimmune and inflammatory diseases, neurological disorders, diabetes and cancer (154-157).

4. CROSS-TALK BETWEEN ER AND NF-κB

4.1. Cross-talk between nuclear receptors and NF-κB

Glucocorticoids (GCs) are the first steroid hormones for which it is described that they have the ability to interfere with the NF-κB signal transduction pathway. In normal physiology GC action, controlled by the hypothalamic-pituitary-adrenal (HPA) axis, is essential in modulating inflammatory and immune responses by providing a negative feedback-loop. The potency of GCs as immune suppressor and anti-inflammatory agent was already recognized in the 1950 by Hench and colleagues, when they described to have beneficial effects in the treatment of rheumatoid arthritis, which even led to receiving the Nobel Prize in Medicine or Physiology (158). Currently, GCs are widely used as a standard treatment for a variety of autoimmune and inflammatory diseases (e.g. rheumatoid arthritis, inflammatory bowel diseases and systemic lupus erythematosus) and locally applied against psoriasis, eczema, and asthma. The inhibitory effect of GCs on the immune system has largely been attributed to the down regulation of expression of proinflammatory genes, which are the key regulators of the

immune and inflammatory response. Basic research on the cellular effect of GCs revealed that they act by binding to the glucocorticoid receptor (GR) that functions as a ligand-dependent transcription factors regulating expression of genes containing glucocorticoid response elements (GREs) in their promoter regions. It appeared that the majority of proinflammatory genes repressed by GCs do not contain GREs in their promoters. However, NF-κB was recognized as the main regulator of proinflammatory genes and this led to the proposal of a mechanism for GC-mediated downregulation involving negative cross-talk between GR and NF-κB. From then on several research groups provided evidence for such a mechanism (159). Up till now, cross-talk with NF-κB has also been described for other steroid receptors (ER, progesterone receptor and androgen receptor) and also other members of the nuclear receptor family (peroxisome proliferator-activated receptor (PPAR)α, PPARγ, liver X receptor and vitamin D receptor) (160-168).

4.2. Estrogen action in the skeletal system

The skeletal system is the most well-known and best understood system in which cross-talk between the ER and NF-κB pathway is recognized as physiologically relevant. Knock-out models of mice lacking genes involved in the estrogen signaling pathway (ERKO, BERKO, DERKO, ArKO) or NF-κB pathway (double knock-out of both NFκB1 and NFκB2 genes, single knock-out of IKKα or TNF-receptor-associated factor (TRAF)6 revealed that both separate signaling pathways are essential in normal bone physiology (22, 152, 169). Initially, estrogen was thought to affect the skeletal system only indirectly by regulation the secretion of system hormones that regulate calcium homeostasis. However, it is now generally accepted that estrogen acts directly at the organ level mediated by ERs expressed in all cells types involved in bone remodeling (as described in part 1.). Maintenance of the adult skeleton involves continuous remodeling, which is necessary to replace old or damaged bone with new bone. Remodeling is a tightly regulated process and occurs at restricted sites accomplished by so-called basic multicellular units (BMUs). A remodeling cycle begins with the recruitment of osteoclasts (OCs) that locally start resorption of the bone matrix and, after termination of this process, the cavity is filled up gradually with new bone by osteoblasts (OBs) (170, 171). The major action of estrogen on the skeletal system is the preservation of bone mass and this is largely attributed to inhibition of bone remodeling (e.g. the activation of new BMUs), suppression of bone resorption and stimulation of bone formation (172). The discovery of several members of the TNF ligand and receptor family greatly contributed to the understanding of the molecular mechanism of estrogen action in bone. The first factor to be identified independently by three different groups was the TNF receptor-like molecule termed osteoprotegerin (OPG) that inhibited bone resorption due to decreased osteoclast formation(173-175). Unlike other TNF receptors, OPG is a secreted soluble receptor due to the lack of a transmembrane domain. The second molecule to be identified was the TNF-like ligand for OPG (OPGL), which was also identified by several other groups and therefore also termed osteoclast differentiation factor (ODF), TNF-related activation-induced cytokine (TRANCE) and receptor activator of NF-κB ligand (RANKL), of which the latter is preferentially used (176-179). RANKL is expressed on the cell surface by stromal/osteoblast lineage cells and is therefore a membrane-bound ligand. It appeared that RANKL is the key molecule necessary for osteoclast development. RANKL binds its cognate receptor RANK that is expressed on the cell surface of the osteoclast lineage (180). Only direct cell-to-cell contact between cells of both the osteoblast and osteoclast lineage allows interaction between RANK and RANKL, providing an elegant system in which bone resorption by osteoclasts can only be achieved in the presence of osteoblasts that subsequently refill the

bone cavity. Therefore OPG can be seen as the decoy receptor for RANKL that neutralizes the biological activity of RANKL preventing RANK-RANKL interactions and thus inhibits osteoclast activity (Fig. 5)(181).
Estrogen has been shown to suppress RANKL and increase OPG production in osteoblastic cells thereby functioning as an inhibitor of BMU activation (182-184). RANK-RANKL interaction is not sufficient for osteoclast activation and needs additional stimulation with macrophage-colony-stimulating factor (M-CSF) (185). Furthermore, proinflammatory cytokines such as IL-1, IL-6, TNFα and granulocyte/macrophage-colony-stimulating factor (GM-CSF) are also known as stimulators of bone resorption and can exert their effect in several ways; 1) by inducing the osteoblastic expression of RANKL, 2) by direct activation of the osteoclast independent of RANKL, or 3) by increasing the life span of the osteoclast. Therefore, estrogen can also act as an inhibitor of bone resorption by suppressing the production of bone-resorbing cytokines (Fig. 5) (186).

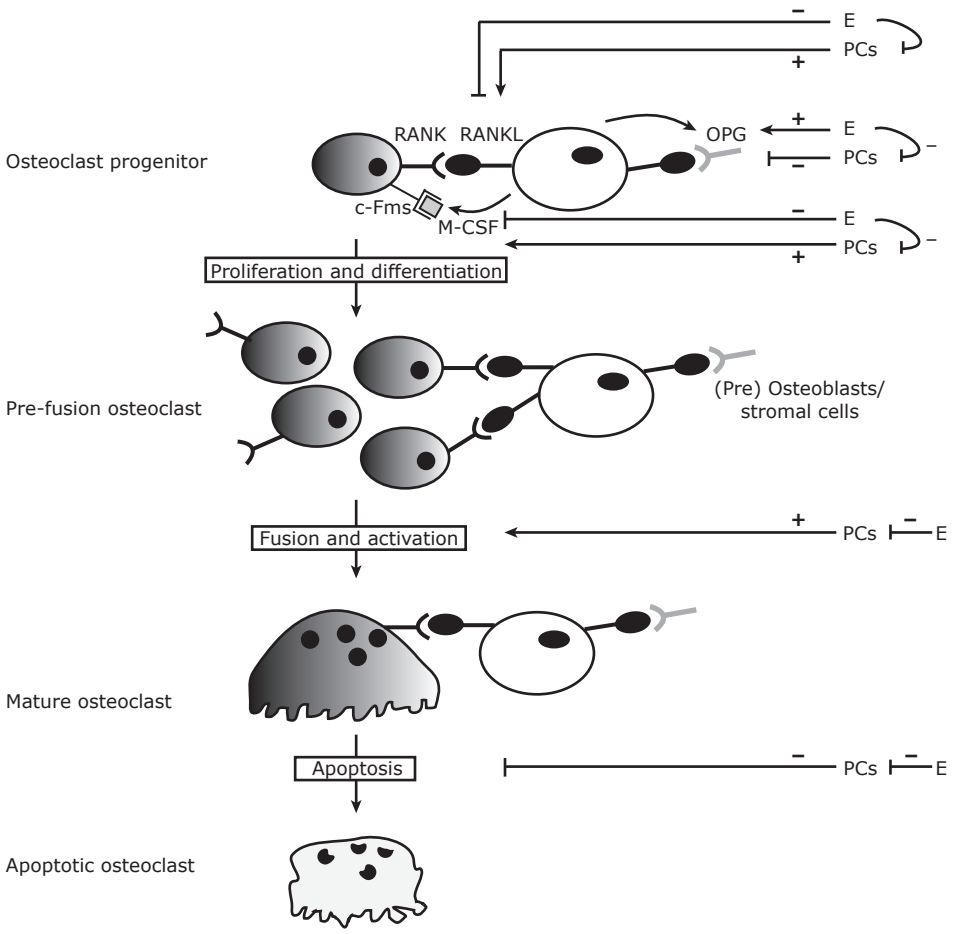


Figure 5. Model for the inhibitory effect of estrogen on bone resorption.
Schematic representation of osteoclast differentiation and function mediated by the interaction with osteoblasts/stromal cells. Stimulatory (+) or inhibitory (-) effects of estrogen (E) or bone-resorbing proinflammatory cytokines (PCs) are depicted. Additional abbreviation: c-Fms, receptor for M-CSF.

Since the expression of many of these inducers of bone resorption is regulated by NF-κB, it is proposed that estrogen downregulates gene expression by interfering with the NF-κB signaling pathway. In 1994, Ray and coworkers showed for the first time that IL-6 could be down-regulated by estrogen without direct binding of ER to the IL-6 promoter, while the presence of an element containing NF-κB binding sites was essential (187).

5. OUTLINE OF THIS THESIS

At the start of this PhD project, several important advances had been made in the preceding years by members of the group in relation to a better understanding of the molecular basis for the anti-inflammatory actions of glucocorticoids. They showed that negative cross-talk between GR and NF-κB represents an important mode of action through which glucocorticoids downregulate the expression of genes involved in inflammation. The aim of this thesis is to get more insight into the mechanism of cross-talk between ER and NF-κB, which is considered to play an important role in the protective effects of estrogen on bone.
In **Chapter 2**, the potency of ERα and ERβ to inhibit NF-κB transcriptional activity, in the human osteoblast-like U2-OS cell line, is studied. The effects of the natural ligand E2, the SERM tamoxifen and the pure antagonist ICI on ER-mediated NF-κB transrepression were compared and the involvement of several functional domains of ER in mediating repression is studied. Furthermore, effects of these ligands on NF-κB DNA binding and the expression level of IκB were assessed in order to obtain more insight into the molecular mechanism underlying repression. In **Chapter 3**, the involvement of coactivators and corepressors in ER-mediated NF-κB transrepression is investigated. These results provide details on the mechanism of SERM-mediated inhibition of NF-κB activity. In order to investigate whether cross-talk between ER and NF-κB possibly involves direct protein-protein interactions we performed co-immunoprecipitation assays and fluorescent resonance energy transfer (FRET) analysis (**Chapter 4**). Moreover, this study reveals cellular localization of ER and the NF-κB subunits p50 and p65 under conditions of functional cross-talk. In **Chapter 5**, microarray analysis studies reveal to what extent endogenous NF-κB regulated genes can be downregulated by ER and the expression profiles in the presence of E2, SERMs and ICI are compared. In **Chapter 6**, the results are summarized and discussed in relation to the present view on and relevance of cross-talk between ER and NF-κB.

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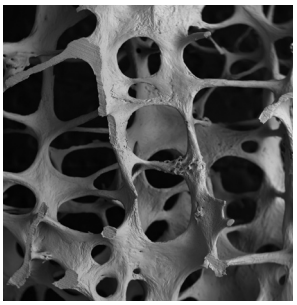
Chapter 2



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4-Hydroxytamoxifen Transrepresses Nuclear Factor-κB Activity in Human Osteoblastic U2-OS Cells through Estrogen Receptor (ER) α, and Not through ERβ

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Estrogens are important mediators of bone homeostasis, and postmenopausal estrogen replacement therapy is extensively used to prevent osteoporosis. The biological effects of estrogen are mediated by receptors belonging to the superfamily of steroid/thyroid nuclear receptors, estrogen receptor (ER)α and ERβ. ERα, not only transactivates target genes in a hormone-specific fashion, but it can also neutralize other transcriptional activators, such as nuclear factor-κB (NF-κB), causing repression of their target genes. A major mechanism by which estrogens prevent osteoporosis seems to be repression of transcription of NF-κB target genes, such as the osteoclast-activating cytokines interleukin-6 and interleukin-1. To study the capacity of both ERs in repression of NF-κB signaling in bone cells, we first carried out transient transfections with ERα or ERβ of the human osteoblastic U2-OS cell line, in which endogenous NF-κB was stimulated by tumor necrosis factor α. Repression by ERα was already observed without 17β-estradiol, whereas addition of the ligand increased repression to 90%. ERβ, however, was able to repress NF-κB activity only in the presence of ligand. Because it is known that some antiestrogens can also display tissue-specific agonistic properties, 4-hydroxytamoxifen was tested for its capacity in repressing NF-κB activity and was found to be active (albeit less efficient than 17β-estradiol) and, interestingly, only with ERα. The pure antagonist ICI 164,384 was incapable of repressing through any ER subtypes. Deletion analysis and the use of receptor ERα/ERβ-chimeras showed that the A/B domain, containing activation function-1, is essential for this suppressive action. Next, we developed stable transfectants of the human osteoblastic U2-OS cell line containing ERα or ERβ in combination with an NF-κB luciferase reporter construct. In these cell lines, repression of NF-κB activity was only mediated through ERα and not through ERβ. These findings offer new insights into the specific role of both ER subtypes in bone homeostasis and could eventually help in developing more specific medical intervention strategies for osteoporosis.

INTRODUCTION

During adult life, bone tissue is continuously remodeled, which is needed to replace damaged or aged bone. Bone remodeling is a dynamic process involving bone formation by osteoblasts and bone resorption by osteoclasts. To maintain a constant bone mass, osteoblastic and osteoclastic activities are closely coordinated in a homeostatic system (1, 2). Disturbances of this balance are responsible for a variety of skeletal diseases, *e.g.* osteoporosis. Osteoporosis is associated with accelerated bone loss caused by increased bone resorption relative to bone formation. Estrogen deficiency is the main cause of osteoporosis in postmenopausal women and is contributing to bone loss in aging men (3). An effective treatment to prevent the development of osteoporosis is estrogen replacement therapy (ERT) (4). The beneficial effect of estrogen as regulator of bone homeostasis is well established; however, the precise molecular mechanisms involved in the action of estrogen in bone are still unclear.

The major action of estrogen on the skeleton *in vivo* is the inhibition of bone resorption. Bone resorption is inhibited indirectly by suppressing the production of bone-resorbing cytokines in osteoblasts. These cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)α, enhance osteoclast differentiation and activity. Therefore, estrogen deficiency leads to an increase of bone resorbing cytokines in blood and bone marrow; and this, in turn, leads to increased bone resorption (5).

The biological effects of estrogen are mediated by the estrogen receptor (ER), a ligand-dependent transcription factor belonging to the superfamily of steroid/thyroid nuclear receptors. ER exists as two subtypes, ERα (6) and ERβ (7, 8), which share a well-conserved modular structure composed of six functional domains, labeled A–F. The C domain, the DNA-binding domain, is nearly completely conserved between ERα and ERβ (± 96% homology); whereas the ligand binding domain, E domain, is clearly less conserved (± 58% homology). The N-terminal A/B region is the least conserved between the two receptors (± 20% homology) (7, 8). A separate gene encodes each subtype. Differences in splicing or initiation of translation cause the existence of ER isoforms. For the human ERβ, two major isoforms are known, one protein of 530 and one of 485 amino acid residues (9). After ligand binding, the ER undergoes a conformational change, displacing the inhibitory heat-shock protein complex and permitting the receptor to form a dimer. In this form, the ER is capable of binding specific DNA sequences, termed estrogen response elements (EREs), located in the regulatory region of target genes. After DNA binding, the receptor is able to interact with basal transcription factors and/or coregulatory proteins to regulate target gene transcription (10). The ER interacts with the transcriptional complex through two distinct activation functions (AFs), AF-1 in the N-terminal A/B domain and AF-2 in the ligand-binding domain (11).

Recent studies have shown alternative pathways through which ER also participates in regulating target gene transcription. These include pathways in which ER interacts indirectly with non-classical ERE target gene promoters, *e.g.* by binding other DNA-bound transcription factors, such as AP-1 and Sp1 (12-15). In addition, other studies have shown that ER can act as a transcriptional repressor, by inhibiting the activity of transcription factors such as nuclear factor-κB (NF-κB). NF-κB is present, in an inactive form, in the cytoplasm associated with an inhibitory protein, IκB. A number of agents, including inflammatory cytokines, cause translocation of NF-κB to the nucleus through phosphorylation and subsequent degradation of IκB. Because NF-κB-binding sites have been identified in the promoters of genes encoding bone-resorbing cytokines, such as IL-6, the hypothesis has been put forward that estrogen is capable of inhibiting cytokine production in osteoblasts by repressing NF-κB activity (16-19). A similar mechanism has been described extensively for the repression of inflammatory responses, involving cytokines, by the glucocorticoid receptor (20). Depending on the cell

type, repression may involve direct protein-protein interactions but may also involve steroid-induced stabilization of IκBα (21, 22).
Besides regulating bone homeostasis, estrogen is an essential regulatory hormone in female and male reproduction systems, in the central nervous system and in the cardiovascular system. Consequently, estrogen deficiency in postmenopausal women can cause undesirable symptoms and other diseases besides osteoporosis. Thus, exposure to ERT has more beneficial effects besides maintaining bone mass, *e.g.* the prevention of cardiovascular diseases (23), improvement of cognitive functions, and prevention of Alzheimer's disease (24). Unfortunately, ERT is associated with side effects, including an increased risk for breast and uterine cancer (25). An important alternative to ERT for the prevention of osteoporosis is the use of selective ER modulators (SERMs). SERMs are compounds that bind with high affinity to ER and (depending on the tissue type) display estrogen agonistic or antagonistic activities. Tamoxifen [a triphenylethylene (26)] and raloxifene [a benzothiophene (27)] have been reported to have tissue-specific responses and are thus termed SERMs.
To study the molecular mechanism by which estrogen and SERMs maintain bone homeostasis through the ER, we investigated the effect of several estrogenic compounds on NF-κB activity in a human osteoblastic cell type, U2-OS. Because no functional ER was detectable in this cell line, we developed U2-OS clones that express physiological levels of ERα or ERβ. In the present study, we show that both 17β-estradiol (E2) and 4-hydroxytamoxifen (OH-T) selectively transrepress NF-κB activity in osteoblasts through ERα and not through ERβ. These findings provide new insights into the possible molecular mechanism of estrogen action in bone tissue.

RESULTS

ERα and ERβ repress TNFα-induced NF-κB activity in osteoblastic and nonosteoblastic cells.

To determine whether estrogen is capable of repressing NF-κB activity in osteoblasts, we studied the effect of E2 on TNFα-induced NF-κB activity in human osteoblastic U2-OS cells. These cells were chosen because they are relatively easy to transfect and showed a superior TNFα inducible NF-κB activity, compared with ROS 17/2.8, SaOs, and MG63 cells (data not shown). A luciferase-reporter construct, 4xNFκB(HIV)tkluc, was used to measure the induction of NF-κB activity. This construct contains four copies of a NF-κB -binding sequence derived from the HIV LTR placed in front of the thymidine kinase promoter and luciferase. Cells were transiently transfected with this reporter in combination with an expression vector encoding human ERα or ERβ. Cotransfection of ERα resulted in repression of the TNFα-induced transcriptional activity of NF-κB, already in the absence of E2 (70%; $P < 0.001$), whereas addition of E2 resulted in enhanced repression (90%). However, cotransfection of ERβ without ligand resulted in up-regulation of TNFα-induced NF-κB activity ($P < 0.05$), whereas addition of E2 gave considerable repression (60%) of NF-κB activity (Fig. 1A).
The same experiment was performed in human 293 embryonal kidney cells. In this case, the induced activity of the NF-κB-reporter construct by TNFα was not influenced by cotransfection of unliganded ERα or ERβ. Addition of E2 resulted in repression of NF-κB activity, both with ERα and with ERβ, 60% and 40%, respectively (Fig. 1B).
These results indicate that both ERα and ERβ can act as transcriptional repressors of TNFα-induced NF-κB activity on a NF-κB reporter construct after E2 stimulation in human osteoblastic U2-OS cells and nonosteoblastic 293 cells. The observation that ERα, and not ERβ, can act as a transcriptional repressor in the absence of hormone in U2-OS cells, and not in 293 cells, suggests that hormone-independent repression is receptor- and cell type-specific.

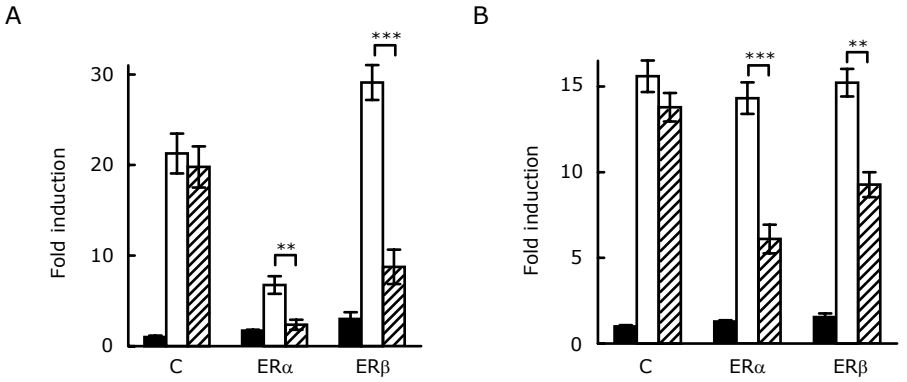

Figure 1. Repression of TNFα-induced NF-κB activity by E2 through human ERα and human ERβ in U2-OS and 293 cells.
U2-OS (**A**) and 293 (**B**) cells were transiently transfected with 4xNF-κB(HIV)tkluc reporter plasmid in combination with empty expression vector (control, C) or expression vector encoding ERα or ERβ. Sixteen hours after transfection, cells were treated with vehicle (0.1% ethanol = no treatment; *black bars*), with 250 U/ml TNFα plus vehicle (*white bars*), or with TNFα + 10^{-8} M E2 (*hatched bars*). After 24 h, cells were harvested and assayed for luciferase activity. Luciferase values were corrected for transfection efficiency by measuring β-galactosidase activity of cotransfected PDM-LacZ construct. Values are represented as the induction of luciferase activity evoked by ER over untreated cells transfected with empty expression vector. *Bars*, Means ± SEM of four independent experiments; **, $P < 0.01$; ***, $P < 0.001$ (by Student's *t* test).

OH-T-ligated ERα represses NF-κB activity in osteoblastic cells

Tamoxifen was initially developed as a drug to treat breast cancer (26) because it acts as an antiestrogen in breast tissue. However, tamoxifen is also known to have beneficial effects on bone density and serum lipids in postmenopausal women by acting as estrogen agonist (38). Because of its tissue-selective responses, tamoxifen can be designated a SERM.

To study the estrogen agonistic effect of tamoxifen in bone cells, we were interested in learning whether OH-T, the main metabolite of tamoxifen, was capable of repressing NF-κB activity in U2-OS cells, similar to E2. For this, U2-OS cells were transiently transfected with 4xNF-κB(HIV)tkluc in combination with an expression vector containing ERα or ERβ. Cells were cotreated for 24 h with TNFα and increasing concentrations of E2, OH-T, or ICI. E2 (10^{-8} M) caused about 70% decrease in TNFα-induced reporter activity, both through ERα and ERβ (Fig. 2, A and B, respectively). OH-T functioned as estrogen agonist in repressing NF-κB activity but only through ERα and not through ERβ (Fig. 2). However, OH-T (10^{-8} M) was less effective (40%) in repressing NF-κB, compared with E2 (Fig. 2A). The pure antagonist ICI did not show estrogen agonistic activity. However, a dose-dependent increase in NF-κB activity was observed in the presence of ICI in combination with ERα and ERβ (Fig. 2). Remarkably, also OH-T showed this increase with ERβ (Fig. 2B). Similar experiments were performed in 293 cells. Although, in these cells, E2 repressed TNFα-induced NF-κB activity through both receptor subtypes, OH-T and ICI had no effect on NF-κB activity (data not shown). These results indicate that OH-T is a selective estrogen agonist in an osteoblastic cell type and selectively transrepresses NF-κB activity through ERα.

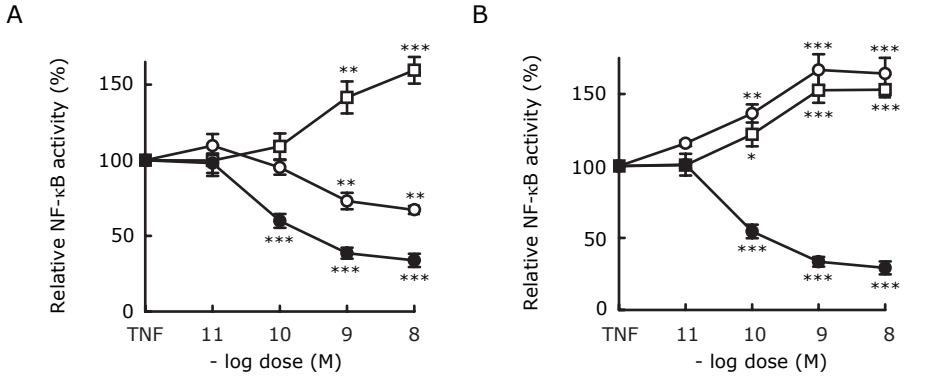

Figure 2. Dose response curves of the repression of NF-κB activity by different ER ligands through human ERα and ERβ in U2-OS cells.
Cells were transiently transfected with 4xNF-κB(HIV)tkluc reporter plasmid in combination with expression vector encoding ERα (**A**) or ERβ (**B**). Cells were treated with 250 U/ml TNFα plus vehicle or in combination with increasing doses (10^{-11} - 10^{-8} M) of E2 (●), OH-T (○), or ICI (□) and were assayed, after 24 h, as described in Fig. 1. The results are expressed relative to luciferase activity caused by TNFα in the absence of ERligand (100%). Values represent the means ± SEM of four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by one-way ANOVA and LSD for differences between TNFα treatment alone *vs.* TNFα plus ER ligand).

Identification of ER domains involved in the transrepression of NF-κB

Because OH-T transrepresses NF-κB activity selectively through ERα, we were interested in the involvement of the different domains of ERα involved in this repression. To determine this, deletion mutation constructs of mERα were cotransfected with the NF-κBluc reporter in a transient transfection assay in U2-OS cells. For mERα, the same ligand-independent and ligand-dependent effects on NF-κB activity were observed, compared with human ERα (Fig. 3A). Thus, it was clear that hERα and mERα have comparable actions in this cell and promoter context. The construct mERα121–599, which lacks part of the A/B domain, showed no hormone-independent repression, whereas E2 could still efficiently repress NF-κB activity. Strikingly, deletion of this part of the A/B domain abolished the repressive action of OH-T on NF-κB (Fig. 3A). These results suggest that the A/B domain is essential for ligand-independent and OH-T-induced repression of NF-κB but not for repression by E2. In addition, there was no significant increase in NF-κB activity caused by ICI with mERα121-599, indicating that the A/B domain is involved in this action of ICI. The deletion construct mERα1–399, which lacks part of the ligand-binding domain, resulted in a receptor that could repress NF-κB at least equally effective as wild-type ERα in the absence of ligand (Fig. 3A). This confirms the observation that the A/B domain is responsible for repression of NF-κB activity without activation of ERα by ligand. Addition of hormone did not further repress or induce NF-κB activity through mERα1–399 (Fig. 3A), which is explained by the fact that the ligand is not able to bind the shortened receptor.
In a control experiment, mERα and mutants of mERα were cotransfected with a luciferase reporter construct, 3xEREtata-Luc, to determine their ability to activate transcription from a classical ERE (Fig. 3B). Already, in the absence of ligand, mERα showed considerable transcriptional activity, but this was further enhanced by E2. OH-T also enhanced the ERα activity but much less effectively, as compared with E2. The antiestrogen ICI was not capable of enhancing basal activity of the receptor (Fig. 3B). ICI showed no significant effect on the

background activity of the receptor, suggesting that hormone-independent activity is not attributable to residual E2 in the charcoal-treated serum. With ERα121–599, lacking the A/B domain, no transcriptional activity was measured in the absence of ligand, whereas addition of E2 still stimulated transcription, and addition of OH-T or ICI had no further effect (Fig. 3B). Thus, the observed high basal transcriptional activity of wild-type ERα is caused by the A/B domain, and further induction by OH-T is also dependent on this domain. However, E2 can still induce transcription in the absence of A/B domain but not as strongly as wild-type ERα. The mutant ERα1–399, lacking AF-2, was already very active in the absence of ligand, and addition of hormone had no further effect (Fig. 3B).
Combining the effects of mutant ERs on activity of the NF-κBluc reporter and the EREluc reporter, we can summarize the following. Wild-type ERα is already active without ligand activation, both in ERE transactivation and repression of NF-κB activity, and this depends on the A/B domain. The natural ligand E2 can enhance basal activity of the receptor, leading to increased ERE transactivation and stronger NF-κB repression, and this is only partly dependent on the A/B domain. The synthetic compound OH-T mimics the estrogen effect, but less effectively, and this phenomenon is completely dependent on the A/B domain.

Comparison between the A/B domain of ERα and ERβ in repressing NF-κB activity
To further determine the importance of the A/B domain of ERα in NF-κB repression, chimeric constructs of hERα and hERβ were used. Chimeric human ERα/ERβ contains the A/B domain of ERα fused to C, D, E, and F domains of ERβ; and chimeric human ERβ/ERα contains the A/B domain of ERβ fused to C, D, E, and F domains of ERα (28). In transient transfection assays, the different receptor constructs of ER were cotransfected with the NF-κBluc reporter in U2-OS cells, and cells were treated with TNFα in combination with several ligands (Fig. 4A). Replacement of the A/B region of ERβ with the A/B region of ERα (ERα/ERβ) resulted in a receptor that was at least equally potent as wild-type ERα in repressing NF-κB without ligand. However, replacement of the A/B region of ERα with the A/B region of ERβ (ERβ/ERα) abolished the ligand-independent repression by ERα. Thus, ligand-independent actions of ERα and ERβ were interchanged by switching their A/B domain. However, exchanging the A/B domains did not simply switch the effects caused by OH-T and ICI, suggesting that other factors besides the A/B domain are also involved in their agonist/antagonist action. All receptor constructs were capable of repressing NF-κB activity mediated by E2 because this action is not dependent on the A/B domain, as shown in Fig. 3A.
As a control, hERα and ERβ chimeras were cotransfected with 3xERE-tata-Luc, to determine their ability to activate transcription from a classical ERE. ERα showed low transcriptional activity in the absence of ligand, but this was strongly induced by E2. In addition, OH-T and ICI induced basal transcription of ERα but much less strongly, compared with E2 (Fig. 4B). These results indicate that OH-T and ICI show some agonistic activity with ERα in this promoter and cell context. ERβ was transcriptionally active after addition of E2, but significantly less than ERα, and ERβ was not activated by OH-T and ICI. Replacement of the A/B domain of ERβ with the A/B domain of ERα (ERα/ERβ) resulted in a receptor that showed relatively high basal transcriptional activity and a weak induction after E2 stimulation. In addition, OH-T could further induce basal transcriptional activity, whereas ICI could not. Replacement of the A/B domain of ERα with the A/B domain of ERβ (ERβ/ERα) resulted in low basal activity, which was strongly induced by E2 and not influenced by OH-T and ICI.
In summary, these findings support the idea that the A/B domain of ERα is important for ligand-independent transcriptional activity, both in NF-κB repression and ERE induction. Moreover, the A/B domain of ERα is important for agonistic activation by OH-T.

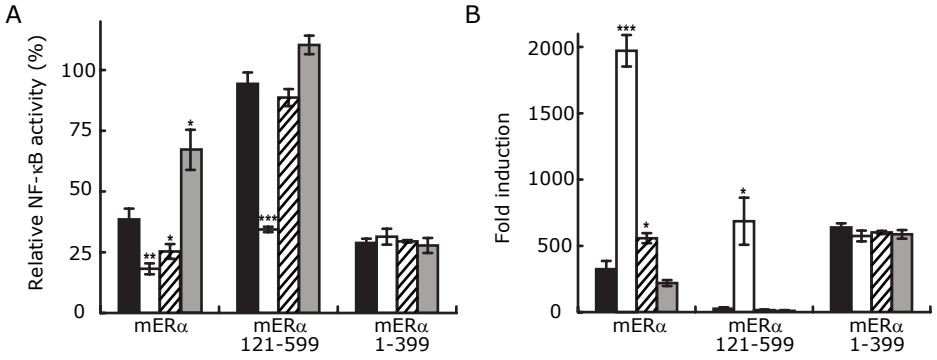

Figure 3. **Requirement of the A/B domain of mERα in repressing NF-κB activity.**
A, U2-OS cells were transiently transfected with 4xNFκB(HIV)tkluc reporter construct in combination with empty expression vector or expression vectors encoding mERα, mERα121–599, or mERα 1–399. Cells were treated with 250 U/ml TNFα plus vehicle (*black bars*) or in combination with 10^{-8} M E2 (*white bars*), OH-T (*hatched bars*), or ICI (*grey bars*) and assayed after 24 h as described in Fig. 1. Values are expressed relative to luciferase activity of cells transfected with empty expression vector treated with TNFα (100%). **B,** U2-OS cells were transiently transfected with 3xEREtata- Luc reporter construct in combination with empty expression vector or expression vectors encoding mERα, mERα 121–599, or mERα 1–399. Cells were left untreated (*black bars*) or treated with 10^{-8} M E2 (*white bars*), OH-T (*hatched bars*), or ICI (*grey bars*) and assayed after 24 h as described in Fig. 1. Values are expressed as the induction of luciferase activity evoked by ER and treatment over untreated cells transfected with empty expression vector. *Bars*, Mean ± SEM of three independent experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by Student's *t* test for cells treated with TNFα alone *vs*. TNFα in combination with (anti)estrogen or untreated *vs*. treated with (anti)estrogen).

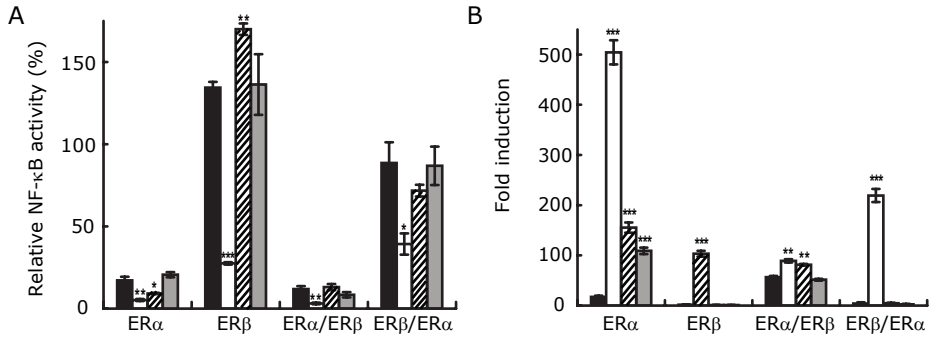

Figure 4. Requirement of the A/B domain of human ERα in repressing NF-κB activity.
A, U2-OS cells were transiently transfected with 4xNF-κB(HIV)tkluc reporter construct in combination with empty expression vector or expression vectors encoding hERα, hERβ, hERα/β, or hERβ/α. Cells were treated with TNFα alone (*black bars*) or in combination with 10^{-8} M E2 (*white bars*), OH-T (*hatched bars*), or ICI (*grey bars*) and assayed after 24 h as described in Fig. 1. Values are expressed relative to luciferase activity of cells transfected with empty expression vector treated with TNFa (100%). **B,** U2-OS cells were transiently transfected with 3xERE-tata-Luc reporter construct in combination with empty expression vector or expression vectors encoding hERα, hERβ, hERα/β, or hERβ/α. Cells were left untreated (*black bars*) or treated with 10^{-8} M E2 (*white bars*), OH-T (*hatched bars*), or ICI (grey *bars*) and assayed after 24 h as described in Fig. 1. Values are expressed as the induction of luciferase activity evoked by ER and treatment over untreated cells transfected with empty expression vector. *Bars*, Mean 6 SEM of three independent experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by Student's *t* test for cells treated with TNFα alone *vs*. TNFα in combination with (anti)estrogen or untreated *vs*. treated with (anti)estrogen).

Development of U2-OS cells stably expressing ER and reporter genes

A potential drawback of transient transfection assays is that, because of overexpression, physiologically irrelevant responses can be obtained. To verify our observations in a more natural situation, we developed clones of U2-OS that are stably transfected with an expression vector encoding hERα or hERβ. Several ERα and ERβ clones were screened for expression of ER mRNA using RT-PCR analysis. Stable clones with ERα and ERβ were found to express significant amounts of ERα and ERβ mRNA, respectively (data not shown). Transient transfection of the luciferase reporter construct 3xERE-tata-Luc was used to determine whether the expressed ER in the stable clones was capable of transcriptional activation. In all clones, a dose-dependent up-regulation of ERE-luciferase activity was observed after treatment with increasing concentrations of E2, with EC_{50} values roughly 10-fold lower in ERα-, compared with ERβ-expressing, clones (data not shown). We selected one representative ERα and one ERβ clone to determine the level of expressed ER protein and the binding affinity of E2 for ERα and ERβ, respectively. In the ERα clone, the level of ER expressed was 238 (±18) fmol/mg protein, with a K_d value of 5×10^{-11} M, which is in the same range as T47D cells expressing ER endogenously (39). In the ERβ clone, the level of ER was 296 (±64) fmol/mg, with a K_d value of 3×10^{-10} M. These results indicate that E2 has a higher affinity for ERα, compared with ERβ, in this cellular context.

Next, we developed clones of U2-OS stably expressing 3xERE-tata-Luc in addition to either ERα or ERβ. In these lines, half-maximal ERE induction is reached at about 10^{-11} M E2^{-10} M E2 for ERβ cells (data not shown), consistent with the differences in receptor-binding activity.

NF-κB repression is not mediated by ERβ in U2-OS cells expressing physiological levels of endogenous ER

We also isolated stably transfected U2-OS lines expressing the 4xNF-κB(HIV)tkluc reporter gene in addition to either ERα or ERβ. In these cell lines, the integrity of ERE-mediated transcriptional response was determined by transient transfection of an ERE-tata-LacZ construct. Both ERα- (Fig. 5A) and ERβ-expressing (Fig. 5B) cell lines showed significant (15- to 20-fold) ERE-dependent transcriptional activation. In the ERα-expressing line, both E2 and OH-T effectively transrepress NF-κB reporter gene activity (Fig. 5C). Half-maximal NF-κB repression was observed at similar concentrations of ligand, compared with transcriptional activity of the ERE construct (Fig. 5A). Surprisingly, NF-κB activity was only slightly (15%) repressed by E2 in cells stably expressing ERβ (Fig. 5D). The observation that ERβ strongly (70%) represses NF-κB activity after E2 addition in transient transfection assays (see Fig. 2), and not in cells stably expressing ERβ, could be caused by the fact that the receptor is often more highly expressed in cells that are transiently transfected. This was confirmed by the fact that, when the reporter gene was transiently induced in several clones expressing ERα or ERβ only, again only ERα-mediated repression of a 4xNF-κB(HIV)tkluc reporter gene was observed (data not shown).

To further investigate this point, wild-type U2-OS cells were transiently transfected with an increasing amount of expression vector encoding ERα or ERβ, ranging from 0.01–1.33 μg/well, in combination with 4xNF-κB(HIV)tkluc. Cells were treated for 24 h with TNFα alone or in combination with E2 (10^{-8} M). As shown in Fig. 6, it became clear that E2-ligated ERα could efficiently repress NF-κB activity already at much lower amounts of transfected expression vector than ERβ. At 0.033 μg/well NF-κB, activity is repressed by 60% by ERα (Fig. 6A), whereas no significant repression is observed with ERβ (Fig. 6B). At 0.33 μg/well, used in transient transfection assays described above, both receptors efficiently repress NF-κB when

activated by E2. In addition, the hormone-independent repression by ERα is increased with increasing amounts of transfected receptor, whereas it is not observed with ERβ. These results indicate that NF-κB repression by ERα and ERβ is dependent on the expression level of the receptor. The observation that ERα, and not ERβ, is capable of repressing NF-κB activity in stable U2-OS-ER clones suggests that the expression level of ±200–300 fmol/mg protein is at the point where ERα (but not ERβ) already efficiently represses NF-κB.

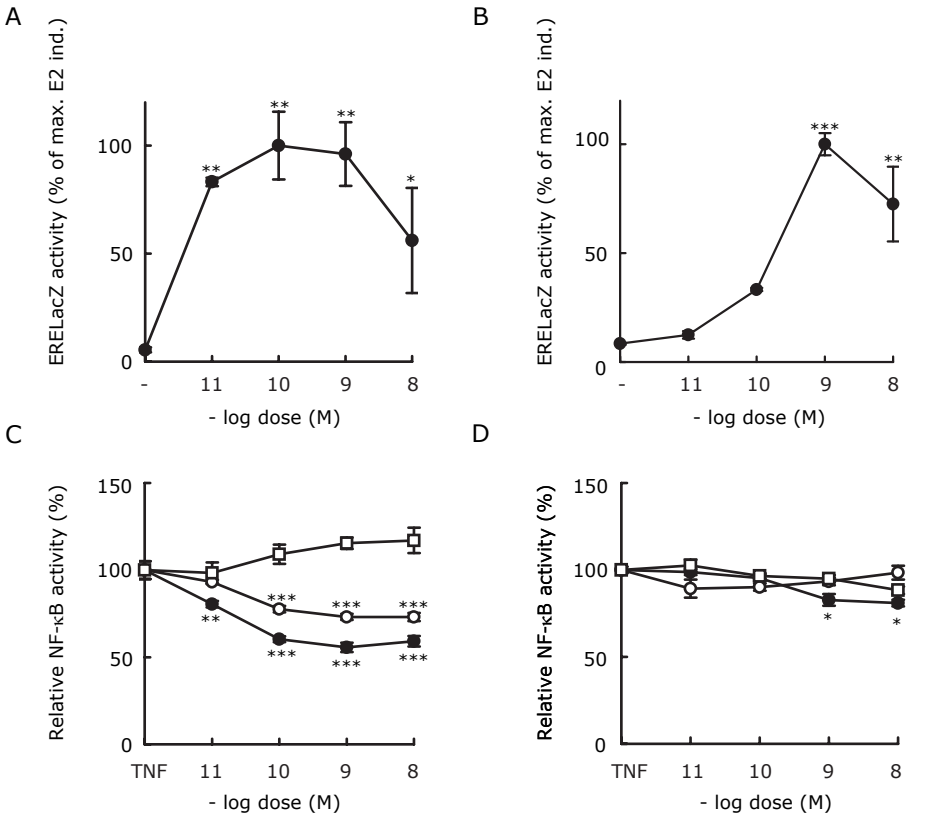

Figure 5. Activation of transient ERE-LacZ reporter and repression of stable NF-κBluc reporter in stable U2-OS/ER-NFκBluc cells.

U2-OS/ERα-NFκBluc (**A**) and U2-OS/ERβ-NFκBluc (**B**) cells were transiently transfected with 3xERE-tata-LacZ reporter construct and treated with vehicle or with increasing doses (10^{-11} – 10^{-8} M) of E2. After 24 h, cells were harvested and assayed for β-galactosidase activity. The results are expressed relative to the maximal β-galactosidase activity caused by E2 (100%). U2-OS/ERα-NFκBluc (**C**) and U2-OS/ERβ-NFκBluc (**D**) were treated with 250 U/ml TNFα plus vehicle or with TNFα in combination with increasing doses (10^{-11} – 10^{-8} M) of E2 (●), OH-T (○), or ICI (□). After 24 h, cells were harvested and assayed for luciferase activity. The results are expressed relative to luciferase activity caused by TNFα in absence of ERligand (100%). Values represent the means ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by one-way ANOVA and LSD for differences between no-treatment *vs.* estrogen or TNFα treatment alone *vs.*TNFα plus ER ligand).

Repression of TNFα-induced NF-κB activity is not caused by induction of IκB and does not lead to decreased NF-κB DNA-binding activity

Repression of NF-κB-regulated genes through the ER could be explained through different mechanisms. One of these mechanisms is the formation of an inactive complex by direct protein-protein interaction of the receptor and NF-κB, which has been described for the glucocorticoid receptor (18, 40). Glucocorticoids (41, 42) and estrogens (43) have also been found to repress NF-κB activity through increased expression of IκBα protein.

To determine whether estrogen has indirect effects on NF-κB activity through modulating IκBα levels, we analyzed IκBα expression in protein extracts from U2-OS cells, by Western blotting. Normal U2-OS cells or stable ERα- or ERβ cells were stimulated for 6 h with various combinations of estrogenic compounds and TNFα, as indicated (Fig. 7; A, B, and C, respectively). Stimulation with TNFα leads to a decrease in the level of IκB protein (compare lanes 1 and 2). Stimulation with E2, OH-T, or ICI alone did not influence the expression of IκB (compare lane 1 with lanes 3, 4, and 5). In addition, the TNFα-induced degradation of IκB was not influenced by E2, OH-T, or ICI (compare lane 2 with lanes 6, 7, and 8). There was no difference in the expression pattern between cells without ER (Fig. 7A) or clones stably expressing ERα (Fig. 7B) or ERβ (Fig. 7C). These results indicate that E2, OH-T, and ICI are not able to modulate IκB levels in U2-OS cells. Thus, the observed transrepression of NF-κB activity by E2 or OH-T cannot be ascribed to changes in IκB protein levels.

To study the mechanism of expression further, electrophoretic mobility shift assays were performed to study NF-κB DNA-binding activity. Nuclear extracts from TNFα-treated and untreated cells were incubated with a radiolabeled probe containing the κB-binding site from the HIV LTR. Specific binding activity was observed in extracts from TNFα-treated U2-OS cells. This TNFα-induced binding activity was not significantly influenced in wild-type cells (compare lane 3 with lane 5) and was reduced only slightly in cells stably expressing ER, upon additional incubation with E2 (compare lane 7 with lane 9, and lane 11 with lane 13).

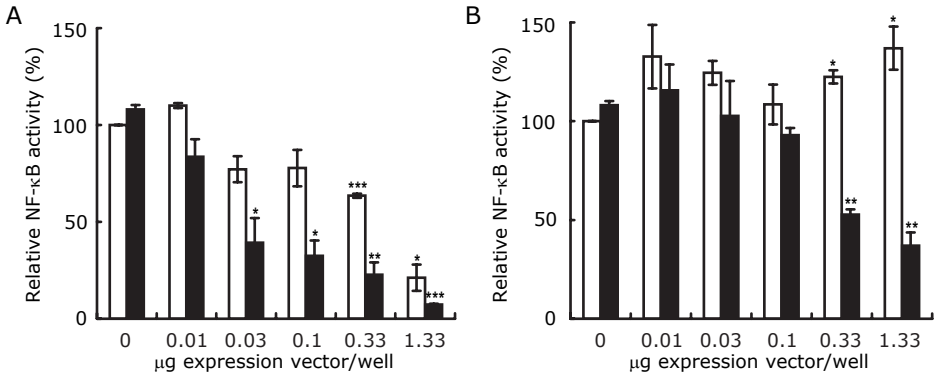

Figure 6. Repression of NF-κB activity is dependent on the amount of transfected ER-expression vector.

U2-OS cells were transiently transfected with 4xNF-κB(HIV)tkluc reporter plasmid in combination with empty expression vector (0 μg point) or increasing amounts (0.01–1.33 μg) of expression vector encoding ERα (**A**) or ERβ (**B**). Sixteen hours after transfection, cells were treated with vehicle (0.1% ethanol = no treatment), with 250 U/ml TNFα plus vehicle (*white bars*), or with TNFα + 10^{-8} M E2 (*black bars*). After 24 h, cells were harvested as described in Fig. 1. The results are expressed relative to luciferase activity of cells transfected with empty expression vector treated with TNFα (100%). Values represent the means ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by Student's *t* test for cells transfected with empty expression vector *vs.* expression vector encoding ER).

This again suggests that, in U2-OS cells, E2 does not induce significant amounts of IκB-like proteins, because this would have led to formation of an inactive complex not capable of DNA binding, as in the control situation where cells are not treated with TNFα.

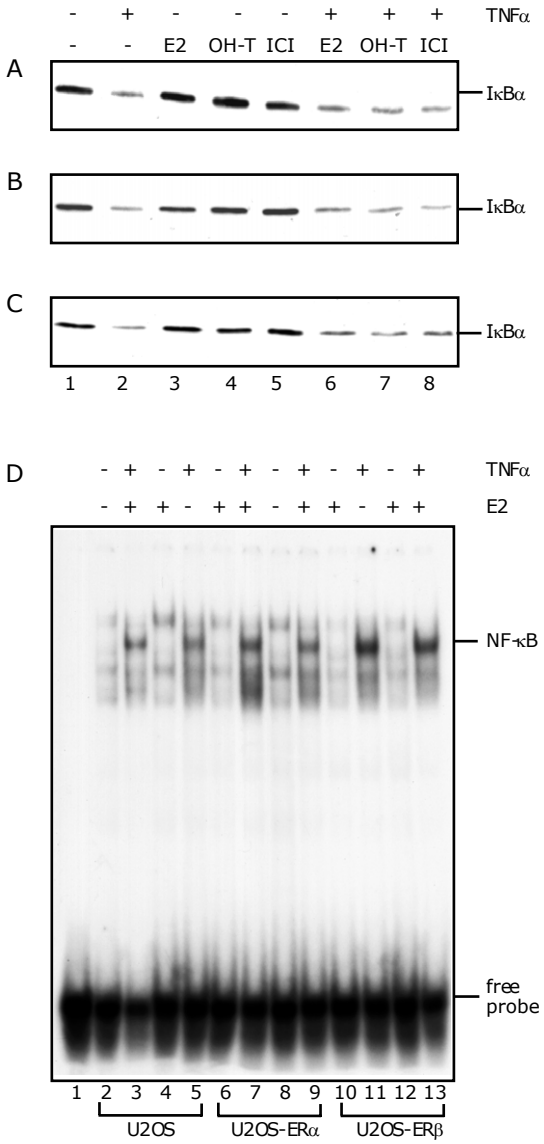

Figure 7. Expression of IκBα protein in U2-OS cells and binding activity of NF-κB to DNA.
IκBα protein expression of wild-type (**A**), ERα-expressing (**B**), or ERβ-expressing (**C**) U2-OS cells. Cells were treated with TNFα in the absence or presence of E2, OH-T, or ICI for 6 h. Western blots of whole-cell extracts were immunostained with a polyclonal antibody to IκBα. **D,** Wild-type and ERα- or ERβ-expressing cells were treated with TNFα or E2 alone or with TNFα plus E2. Nuclear extracts were analyzed by electrophoretic mobility shift assay with ^{32}P-labeled probe containing the κB site from the HIV LTR.

DISCUSSION

In the present study, we show that ERα, and not ERβ, is the major ER through which transcription of NF-κB-regulated genes is inhibited in osteoblastic cells. ERβ only repressed NF-κB activity at relatively high expression levels and only when activated by a full agonist. In contrast, ERα already repressed NF-κB activity in the absence of ligand. Both the agonist E2 and, in different contexts, the antagonist OH-T were capable of enhancing this repressive effect. Our results also show that important domains involved in the regulation of NF-κB activity are found in the AB and E regions of the two receptor subtypes. Differences in the non-conserved AB region were found to determine much of the differences observed between repression through ERα and ERβ.

It has been observed before that, in some cell types, glucocorticoids and estrogens (28, 41, 42) lead to enhancement of IκBα expression, thereby decreasing NF-κB activity. This effect is cell type-specific and contributes only in part to glucocorticoid-mediated NF-κB repression (21, 22). Although estrogens seem able to induce IκB under certain conditions (43), our results clearly show that this mechanism is not involved in NF-κB repression in U2-OS cells. Evidence for an alternative model of repression does point to a direct physical interaction between ERα and NF-κB, involving the ER DBD (18, 44). The mechanism of repression in U2-OS may involve formation of an inactive ER/NF-κB complex that seems to be to labile to resist the conditions used in our gel shift assays. Others obtained inconsistent results using the IL-6 promoter in gel shift assays, with respect to the ability of estrogens to repress NF-κB-binding activity (44-46). However, because in some studies a repressive effect on DNA binding has been found, the formation of a labile complex, at present, seems the most attractive model.

Our data suggest that, in addition to the ER DBD (18, 44), the AB and E domains may contain additional sites involved in repression. Interestingly, major functional domains in both the AB and E regions of ERs are the regions essential for transcriptional activation, AF-1 and AF-2, respectively. Our results, showing a high correlation between transcriptional activation and transcriptional repression, are consistent with an additional role of these domains in transcriptional repression. First of all, the role of AF-1 in repression is supported by the fact that OH-T-mediated repression is highly dependent on the presence of the AB domain of ERα. In contrast to ERβ, the AB domain of ERα contains a strong and independent AF (28). AF-1 cannot be activated by pure antiestrogens like ICI (11, 47-49), which also is unable to induce repression. Estrogens are potent AF-2 activators and repress NF-κB activity even in the absence of the AB domain of ERα.

Strikingly, a dose-dependent activation of NF-κB activity by ICI was observed, both through ERα and ERβ. This could involve a decrease in expression of ER protein levels, which has been described to occur upon ICI treatment (50, 51). It could also involve specific ER conformation-dependent effects on NF-κB activity, because NF-κB activation also occurred through ERβ without addition of ligand, compared with cells that did not express ER; and also, OH-T dose-dependently increased NF-κB activity through this receptor, suggesting that the effect occurs in the absence of repression through ERα AF1. This is reminiscent of antagonist-induced activation of AP1 activity, which also occurs in the absence of ERα AF-1 (52). Our results show that different ligands have very different effects on ER-mediated NF-κB repression. Both ERα and ERβ can bind a large number of compounds, and each compound induces distinct conformational changes within the ER structure (53-58), and this affects the presentation of the receptor surfaces to the transcriptional machinery. This, in turn, may alter the interactions of AF-1 and AF-2 with transcriptional coactivators/corepressors and thus affect regulation of gene transcription, not only with respect to transcriptional activation

but also repression. Clearly, it is of interest to decipher the molecular determinants involved in ER-mediated NF-κB suppression and to find ways to pharmacologically modulate the repressive function of the receptor, which may lead to novel drugs to alter bone physiology. The fact that ERα and ERβ modulate transcription of NF-κB-regulated genes in a different manner suggests that the two ER subtypes have different effects on cytokine production in osteoblasts and, consequently, bone formation. Analysis of the effects on bone in female ERα knock-out mice has revealed that disruption of ERα results in increased bone resorption (59). In contrast, in adult female ERβ knock-out mice, the bone mineral content is increased (60). The physiological importance of ERα in humans was illustrated when a 28-yr-old male with estrogen resistance was reported. The patient was found to contain a point mutation in his ERα gene, and he suffered from increased bone turnover and osteopenia, indicating that ERα is important for normal bone remodeling in humans (61).

Interestingly, in osteoblastic SV-HFO cells, ERα was expressed at a constant level, whereas ERβ expression gradually increased concomitantly with differentiation of the cell (62). We would expect, in the light of our current results, that osteoclast development would be inhibited through ERα mediated inhibition of production of cytokines. When the osteoblast reaches the latest phase of development, ERβ is highly expressed (62), and heterodimerization with ERα (63, 64) could lead to diminished repression of NF-κB activity and to an increase of osteoclast-activating cytokines. In this speculative model, the coupling of formation and resorption of bone, through the distinct actions of ERα and ERβ, are needed to maintain a constant bone mass. This could mean that diseases like osteoporosis, in which bone homeostasis is disrupted, could be treated by selectively inhibiting or activating one of the receptor subtypes to restore the balance of bone formation and resorption. Ligands that are selective agonists or antagonists of ERα or ERβ may therefore be helpful therapeutic agents.

MATERIALS AND METHODS

Chemicals and reagents

The steroid E2 was obtained from Sigma (St. Louis, MO). OH-T and ICI 164,384 (ICI) were kind gifts from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Recombinant human TNFα was obtained from Roche Molecular Biochemicals (Mannheim, Germany).

Cell culture

Human osteosarcoma osteoblastic U2-OS cells and human embryonal kidney 293 cells were obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium (DF; Life Technologies, Inc., Gaithersburg, MD), buffered with bicarbonate and supplemented with 7.5% FCS (Integro, Linz, Austria). Cells were cultured at 37 C in a 7.5% CO2 humidified atmosphere.

Plasmids

The expression vector encoding human ERα (pSG5-HEGO) was a kind gift of Dr. P. Chambon (Strasbourg, France). Chimeric human ERα/ERβ contains the A/B domain of ERα fused with C, D, E, and F domains of ERβ, whereas chimeric human ERβ/ERα contains the A/B domain of ERβ fused with C, D, E, and F domains of ERα (28) and were kind gifts of Dr. B. S. Katzenellenbogen. Mouse ERα (mERα) (pMT2MOR) and mutants ERα1–339 and ERα121–599 (29) were kindly provided by Dr. M. G. Parker (London, UK). The estrogen-responsive reporter plasmid 3xERE-tata-Luc, which contains three copies of a consensus ERE oligonucleotide and a TATA box in front of the luciferase complementary DNA (cDNA), has been described before (30). The luciferase reporter construct 4xNF-κB (HIV) tkluc, which contains four copies of a NF-κB-binding sequence from the HIV long-terminal repeat (LTR) placed in front of the thymidine kinase promoter coupled to luciferase, was described previously (31). The estrogen-responsive reporter

gene plasmid 3xERE-TATA-β-Galactosidase was a kind gift of Dr. J. G. Lemmen (Utrecht, The Netherlands). This construct contains three copies of a consensus ERE containing oligonucleotide and a TATA box in front of the β-galactosidase cDNA in pUC18.

Transient transfection and luciferase assay

U2-OS cells were cultured in 12-well plates and 293 cells in 24-well plates. Both cell lines were cultured in phenol red-free DF medium containing 0.2% BSA, 10 μg/ml transferrin, and 30 nm selenite supplemented with 5% dextran-coated charcoal (DCC) FCS. DCC-FCS was prepared by treatment of FCS with DCC to remove steroids, as described previously (32). Cells were transfected using the calcium-phosphate precipitation method (33). U2-OS cells were transfected with a total amount of 3.33 μg DNA/well, consisting of a mixture of 1.0 μg luciferase-reporter plasmid, 1.0 μg PDM-LacZ plasmid, 0.33 μg of the indicated ER expression plasmid, and 1.0 μg pBluescript SK2. 293 cells were transfected with a total amount of 1.6 μg DNA/well, consisting of a mixture of 0.6 μg luciferase-reporter plasmid, 0.6 μg PDM-LacZ plasmid, 0.2 μg of the indicated ER expression plasmid, and 0.2 μg pBluescript SK2. After 16 h, the medium was refreshed; and, when indicated, antihormones and/or TNFα was added to the medium (1:1000). Cells were harvested 24 h later and were assayed for luciferase activity using the Luclite luciferase-reporter gene assay kit (Packard Instruments, Meriden, CT), according to the manufacturer's protocol, in a Topcount liquid scintillation counter (Packard Instruments). Values were corrected for transfection efficiency by measuring β-galactosidase activity (34). Luciferase activity in stable transfectants was assayed in a similar fashion. EC50 values of reporter gene induction were determined as described before (30).

Establishment of stable transfectants of U2-OS cells

To obtain stable hERα or hERβ transfectants of U2-OS, cells were grown in 6-well plates, until approximately 50% confluency, before they were transfected with the calcium-phosphate coprecipitation method. A total amount of 10 μg DNA/well was used, consisting of a mixture of 8 μg PSG5 based-expression vector encoding either human ERα or ERβ and 2 μg of a selection plasmid encoding a neomycin-resistance gene. After 16 h, the medium was refreshed; and, 24 h later, cells were trypsinized and replated in the presence of geneticin (G418; 200 μg/ml). After 10 days, surviving colonies were isolated and established as stable cell lines. Stable U2-OS/ER cells were cultured under prolonged G418 selection. Reporter genes (4xNF-κB(HIV)tkluc or 3xERE-tata-Luc) were transfected similarly using a selection plasmid encoding a hygromycin-resistance gene. Stable double transfectants were grown under prolonged
G418 (200 μg/ml) and hygromycin (50 μg/ml) selection.

RT-PCR

Clones of U2-OS cells, stably transfected with an expression vector encoding hERα or hERβ, were cultured in 100-mm dishes, and total RNA was isolated using the acid-phenol method (35). Five micrograms of RNA was treated with 14 U RQ1 deoxyribonuclease (Promega Corp., Madison, WI) for 30 min at 37 °C. One microgram of total RNA was incubated at 65 °C for 3 min. After cooling, RNA was incubated for 90 min at 37 °C with 200 U Superscript Reverse Transcriptase (Life Technologies, Inc.), Superscript buffer, 100 ng oligo (dT), 1 x 10 mm dithiothreitol, and 500 μm of each deoxynucleotide triphosphate. One fifth of the first-strand product was added to a PCR amplification mixture containing 1 x Goldstar reaction buffer, 1.5 mm MgCl2, 200 μm of each deoxynucleotide triphosphate, 0.5 U Goldstar *Taq* Polymerase (Eurogentec, Seraing, Belgium), 100 ng forward primer, and 100 ng reverse primer. For PCR of hERα, forward primer 5'-GACAAGGGAAGTATGGCTATGGA-3' and reverse primer 5'-TTCATCATTCCCACTTCGTAGC-3' were used, corresponding to bp positions 799–822 and 1047–1026, respectively. For PCR of hERβ, forward primer 5'-TAGTGGTCCATCGCCAGTTAT-3' and reverse primer 5'-GGGAGCCACACTTCACCAT-3' were used corresponding to bp positions 125–146 and 518–499, respectively. All samples were positive, using primers for β-actin. Mixtures were overlaid with mineral oil, and amplification was carried out for 39 cycles in a Perkin-Elmer Corp. (Wellesley, MA) DNA thermal cycler. Each cycle consisted of 1 min of denaturation at 96 °C, 1 min of annealing at 55 °C, and 1 min of extension at 72 °C. The PCR reaction products were separated on 1.2% agarose gels containing ethidium bromide, to visualize the 393-bp (hERβ-primers) and 248 bp (hERα-primers) PCR product.

Steroid-binding assay

Specific E2 binding in stably transfected U2-OS/ER cells was measured by performing a saturation ligand-binding experiment. For this, U2-OS cells were grown in 6-well plates, for 2 days, until approximately 80% confluency, in phenol red-free medium containing 5% DF-DCC. Cells were rinsed with PBS, and intact monolayers were incubated with increasing concentrations of ^{3}H-E2 (Amersham Pharmacia Biotech, Little Chalfont, UK) with or without 200-fold excess unlabeled E2 in serum and phenol red-free medium. After 1 h, a medium sample was counted in a liquid scintillation counter. Then, cells were rinsed two times with PBS and incubated for 1 h with 0.5 M NaOH. Cell lysates were counted in a liquid scintillation counter and estimated for protein concentration [Bio-Rad Laboratories, Inc. (Philadelphia, PA) protein assay]. The dissociation constant (K_d) and the number of receptor sites (B_{max}) were obtained using Scatchard analysis (36).

Western blotting analysis

For isolation of whole-cell extracts, U2-OS cells were cultured in 100-mm dishes, treated as described, and harvested in buffer containing 50 mM Tris (pH 7.4), 50 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin at 4 °C. Subsequently, cells were centrifuged for 15 min at 4 °C, and protein concentration of the supernatant was determined by the Bio-Rad Laboratories, Inc. protein assay according to the manufacturer's protocol. Twenty-five micrograms of extract was separated on SDS-PAGE gels and transferred to Immobilon (Millipore Corp., Bedford, MA). Blots were immunostained with a polyclonal antibody against IκBα (catalog no. 06–494, Upstate Biotechnology, Inc., Lake Placid, NY) according to the manufacturer's protocol. Immunoreactive bands were visualized after incubation with a peroxidase-conjugated second antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech).

Gel shift assay

Gel shift assays were carried out as described before (31). Briefly, nuclear extracts of U2-OS cells were incubated with [^{32}P]deoxycycidine triphosphate-labeled double-stranded oligonucleotides containing the κB site from the HIV LTR and subsequently were run on nondenaturing polyacrylamide gels. Gels were dried and processed for autoradiography. Specificity of binding was assessed by competition with 100-fold molar excess of unlabeled oligonucleotide probe.

Statistical analysis

Data are represented as mean values ± sem from at least three independent experiments. An unpaired Student's *t* test was used to compare differences between mean values of two different treatments. Data for dose response studies were analyzed for statistical significance using one-way ANOVA. When the F test for the ANOVA reached statistical significance, differences between specific mean values were assessed by least-significant-difference (LSD) test (37). Differences of $P < 0.05$ were accepted as statistically significant.

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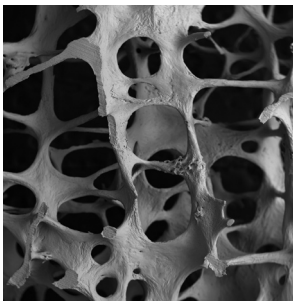
Chapter 3



Selective Estrogen Receptor Modulator and Antagonist Induced Transrepression of NF-κB Activity Mediated by Estrogen Receptor α

Submitted

Selective Estrogen Receptor Modulator and Antagonist Induced Transrepression of NF-κB Activity Mediated by Estrogen Receptor α

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Estrogen, but also the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene, are recognized as hormones that contribute to maintenance of bone mass. An important mode of action by which estrogen preserves bone is by suppressing the production of cytokines and this is attributed to inhibition of nuclear factor-κB (NF-κB)-regulated gene transcription through the estrogen receptor (ER). Here we addressed mechanisms of cross-talk between ER and NF-κB involving coregulators and compared the effects of 17β-estradiol (E2) and SERMs. Our results indicate that ERα-mediated NF-κB transrepression occurs without increased corepressor recruitment, since the histone deacetylase inhibitor Trichostatin A does not relieve transrepression. In order to determine the role of coactivator recruitment by ERα in cross-talk with NF-κB we generated mutants with impaired transcriptional activity due to loss of interaction with coactivators. Mutation of aspartate 351 to glycine in the ligand binding domain of ERα abolished SERM-induced transcriptional activity, while repression of NF-κB activity is enhanced. Combined mutation of activation function (AF)-1 (serine 118 to arginine) and AF-2 (leucines 539 and 540 to alanine) of ERα (ERα-AFmut) resulted in complete loss of transcriptional activity, while repression of NF-κB activity was retained. ERα-AFmut was capable of repressing NF-κB activity, not only upon stimulation with E2, tamoxifen and raloxifene, but also with the full antagonist ICI 182,780. These findings provide for the first time insight into the molecular mechanism of SERM-mediated inhibition of NF-κB activity at the transcriptional level and show that SERMs can behave as potent NF-κB repressors independent of coregulator recruitment.

INTRODUCTION

Estrogen has originally been identified as a sex hormone regulating the functions of the reproductive system. However, it is now generally accepted that estrogen also mediates important physiological processes in non-reproductive systems such as the skeletal, cardiovascular and central nervous system. This becomes evident in postmenopausal women where the decline in circulating estrogens is associated with increased risks for developing diseases such as osteoporosis, coronary heart disease and dementia (1-3). Estrogen deficiency in postmenopausal women is known to result in accelerated bone loss increasing the risk for the development of osteoporosis and treatment with estrogens reverses this process (4). Moreover, the selective estrogen receptor modulators (SERMs), tamoxifen and raloxifene, mimic the effect of estrogen by maintaining bone mass and thus decreasing the risk of osteoporotic fractures (5-10). SERMs are synthetic estrogen receptor (ER) ligands with specific estrogen agonist or antagonist activities depending on the tissue type. Besides bone, tamoxifen has estrogen-like properties in the cardiovascular system and, unfortunately, also in endometrium causing an increased risk for endometrial cancer (5, 11). Due to antagonistic activity in breast, tamoxifen is used to treat ER-positive breast cancers (5). Raloxifene is used to prevent osteoporosis, but also has beneficial effects on the cardiovascular system, without increasing the risk for breast and endometrial cancer (12, 13).

Estrogen and SERMs exert their effects by binding to ER, a ligand-inducible nuclear transcription factor. There are two receptor subtypes, ERα and ERβ, sharing a well conserved three domain structure containing a central DNA binding domain (DBD), an N-terminal activation function (AF)-1 and a C-terminal ligand binding domain (LBD) containing AF-2 (14, 15). Binding of the ligand induces a conformational change in the LBD and enhances receptor dimerization and interaction with estrogen response elements (EREs) in target promoters. Once bound to DNA, ER induces gene transcription by recruiting multiprotein complexes that remodel chromatin and enable the formation of a stable preinitation complex containing RNA polymerase II (16-18). Transcriptional activation by ER is achieved through its ability to interact directly with coactivators and general transcription factors, which occurs through AF-1 and AF-2 (19-21). Coactivators enhance transcriptional activity by recruiting general transcription factors and other proteins with chromatin modifying activities, such as histone acyltransferases (HATs), histone methyltransferases and ATP-dependent chromatin remodeling complexes, while some coactivators harbor enzymatic activity themselves (16, 17).

Partial agonist/antagonist effects by tamoxifen and raloxifene are attributed to the structural distortion of the LBD that prevents coactivator binding through AF-2, while AF-1 activity remains intact (22-24). Tamoxifen and raloxifene-induced transcription through AF-1 is mainly observed with ERα, because AF-1 activity of ERβ is relatively weak (25, 26). Moreover, upon tamoxifen and raloxifene binding, ERα is capable of specifically binding several corepressor molecules, which inhibit gene expression by recruiting histone deacetylases (HDACs) (17, 27, 28). Therefore, depending on cell type-specific expression of coregulators (coactivators and corepressors) and relative expression of ERα and ERβ, tamoxifen and raloxifene can display either agonist or antagonist effects. The pure antagonists ICI 164,384 and ICI 182,780 inhibit ER transcriptional activity independent of the cellular context due to the ability to completely block coactivator binding (29). Moreover, both ICI compounds strongly induce breakdown of the receptor, which contributes to their antagonist activity (30, 31).

In addition to regulating gene expression by direct binding to EREs, ER and also other steroid receptors are capable of modulating gene transcription without direct DNA binding

by modifying the activity of other transcription factors, such as activator protein-1 (AP-1), Sp1 and nuclear factor-κB (NF-κB) (32-36). NF-κB is the main regulator of genes involved in inflammatory responses including cytokines, cytokine receptors, chemokines and adhesion molecules (37, 38). The protective effect of estrogen on bone is attributed to the down regulation of osteoclast-activating cytokines produced by osteoblasts and this is thought to be due to inhibition of NF-κB activity mediated by ER (4, 39, 40). NF-κB exists as a homo- or heterodimer formed between the members of the NF-κB family of proteins, including p65 (RelA), RelB, c-Rel, p50 and p52. The prototypical form of NF-κB is the p50/p65 heterodimer and in unstimulated cells it is present in an inactive form in the cytoplasm due to association with the inhibitor of NF-κB (IκB). Upon various stimuli, such as cytokines and growth factors, degradation of IκB by the proteasome-pathway is induced, resulting in release of NF-κB enabling nuclear translocation and transcriptional activation of target genes (41).
The molecular mechanism of cross-talk between ER and NF-κB has been shown to occur at various levels in the NF-κB signalling pathway and appears to be stimulus- and cell type-dependent (34). In breast cancer cell lines inhibition of NF-κB activity by estrogen has been attributed to an increase in IκB protein levels, retaining NF-κB in the cytoplasm and thereby preventing it from mediating transcription (42, 43). In ERα-expressing coronary smooth muscle and hepatoma HepG2 cells, it was demonstrated that ER inhibited NF-κB activity by competing for a limited amount of coactivators, such as p300 and CBP (cAMP response element binding protein (CREB)-binding protein) (44, 45). In other cell types, including osteoblasts and endometrial cells, inhibition of NF-κB activity is attributed to a direct mechanism involving a protein-protein interaction between ER and NF-κB (46, 47). This direct interaction can result in inhibition of NF-κB transcriptional activity by preventing NF-κB binding to target gene promoters and/or by interfering with the NF-κB transcriptional complex present on the promoter. So far, only for the glucocorticoid receptor (GR) mechanisms have been proposed as to how a nuclear receptor can interfere with NF-κB transcriptional activity at the promoter level. In one model it is suggested that GR blocks RelA recruitment of the kinase P-TEFb, thereby diminishing phosphorylation of the RNA polymerase II C-terminal domain (48, 49). In another model, it was proposed that activated GR inhibits NF-κB transcriptional activity by acting as a direct inhibitor of CBP-associated histone acetylation and by recruiting HDAC2 to the NF-κB transcriptional complex (50, 51).
Inhibition of NF-κB activity by the natural ligand 17β-estradiol (E2) is intensively studied and has led to mechanistic models described above, as recently reviewed by Kalaitzidis *et al* (34). On the other hand, only a few studies reported SERM-induced repression of NF-κB activity and whether the mechanisms involved are similar or different is unknown (52-54). Since tissue-specific responses by SERMs on ERE-mediated transcription are attributed to receptor conformation-dependent interaction or repulsion of specific coregulator complexes, we were interested in determining the involvement of coregulators in SERM-induced NF-κB transrepression in osteoblast-like U2-OS cells. In this study we show that inhibiting corepressor-mediated HDAC activity (by TSA) enhances SERM-induced repression of NF-κB. Furthermore, a specific ERα mutant that fails to induce SERM-mediated ERE transcription, displays enhanced repression of NF-κB activity. Similar results were obtained with a second ERα mutant that completely lacks transcriptional activity due to combined mutations in AF-1 and AF-2 (ERα-AFmut). Interestingly, this mutant gained the capacity to repress NF-κB activity with the full antagonist ICI 182,780. These results show that recruitment of coactivators or corepressor is not necessary for transrepression of NF-κB activity by ERα upon SERM binding, which is consistent with the mechanism of repression induced by E2.

RESULTS

ERα-mediated repression of NF-κB activity is independent of increased HDAC activity

We showed before that human estrogen receptor α (ERα) is capable of repressing TNFα-induced NF-κB activity in an osteoblastic U2-OS cell line stably expressing the receptor and a NF-κB responsive luciferase reporter gene (U2OS-ERα-NFκBluc). Repression was observed both upon treatment of cells with 17β-estradiol (E2) and the SERM 4-hydroxytamoxifen (OH-T) (53). Since ERα specifically recruits HDAC complexes upon SERM binding (17, 27, 28), we were interested in determining whether this plays a role in repression of NF-κB activity induced by SERMs. In this situation it is assumed that ER interacts with DNA-bound NF-κB and interferes with transcriptional activation by recruiting HDACs to the promoter, as shown previously for inhibition of NF-κB activity by GR (50, 51). We used the U2OS-ERα-NFκBluc cell line in order to investigate the effect of the HDAC inhibitor Trichostatin A (TSA) on the activity of the integrated NF-κB reporter. TNFα strongly induced luciferase activity of the NF-κB reporter (~140 fold) and this was induced further by TSA in a concentration-dependent fashion (Fig. 1A). We can conclude from this that HDACs are involved in regulation of TNFα-induced NF-κB reporter activity by repressing the response of the reporter. Next, U2OS-ERα-NFκBluc cells were treated with TNFα alone or with TNFα + TSA and the effect of increasing concentrations of E2, OH-T, raloxifene (Ral) or ICI 182,780 (ICI) on NF-κB reporter activity was analyzed. In order to compare the relative effects of these hormones on reporter activity induced by TNFα alone or in the presence of TSA, luciferase activity without ligand was set at 100% for both conditions. It appeared that TNFα-induced NF-κB activity was repressed by E2 in the presence of TSA equally potently as in absence of TSA. Strikingly, OH-T became a stronger repressor in the presence of TSA. Moreover, Ral could not significantly repress TNFα-induced NF-κB activity but in the presence of TSA, repression was observed (Fig. 1B). As observed before (53), the full antagonist ICI increased NF-κB activity in a dose-dependent manner; this was not changed in the presence of TSA. The observation that TSA only enhances OH-T and Ral-mediated repression is in agreement with the ability of ERα to recruit corepressors only in the presence of OH-T and Ral and not in the presence of E2 or in the absence of ligand. Taken together our findings show that ERα-mediated NF-κB transrepression is not relieved by treatment with the HDAC inhibitor TSA, but even enhanced in the case of SERMS, which makes it unlikely that HDACs play an important role in this process.

Repression of NF-κB activity by SERMS is not dependent on transcriptional activity by ERα.

Next, we wished to determine the need for coactivator recruitment and thus transcriptional activity by ERα in ERα-mediated NF-κB transrepression. This model for ER-NF-κB cross-talk assumes that ER inhibits NF-κB transcriptional activity by sharing coactivators that are used by both transcription factors. Inhibition can occur either indirectly because the availability of these coactivators is limited ("squelching") or directly because ER interacts with DNA-bound NF-κB and also with coactivators in the transcriptional complex. Previously, we showed that OH-T-induced ERE-transactivation and NF-κB-transrepression in U2-OS cells was dependent on the presence of the A/B domain, containing AF-1, of ERα (53). Since ERα transcriptional activity induced by OH-T is enabled by coactivator recruitment via AF-1 (22, 24), we were interested in determining whether this is similar for SERM-induced transrepression of NF-κB activity. Therefore we changed aspartate to glycine at position 351 (ERα-D351G), a mutation that results in loss of transcriptional activity induced by SERMs (55, 56). In order to

determine the effect of this mutation upon transcriptional activity in the U2-OS cell line, we transfected cells with expression vectors for wild type ERα or ERα-D351G in combination with the 3xERE-luciferase reporter (Fig. 2A). As expected, OH-T-induced transcriptional activity was almost completely lost with ERα-D351 compared to wild type ERα, while E2-induced transcriptional activity was not affected. Furthermore, transcriptional activity induced by Ral was also abolished upon mutating D351. Next, cells were cotransfected with ER expression vectors and the 4xNF-κB-luciferase reporter to analyze the effect on repression of TNFα-induced NF-κB transcriptional activity. It appeared that ERα-D351G resulted in a receptor that efficiently repressed NF-κB activity, not only upon E2 stimulation, but also by OH-T and Ral (Fig. 2B). By comparing fold repression it became clear that E2, OH-T and Ral became more effective in repressing NF-κB activity upon mutating D351 (Fig. 2C). ICI remained a full antagonist by reversing ligand-independent NF-κB repression. Therefore, we can conclude that under these experimental conditions abolishing SERM-induced transcriptional activity does not result in loss of NF-κB transrepression, but in fact enhances repression by ERα.

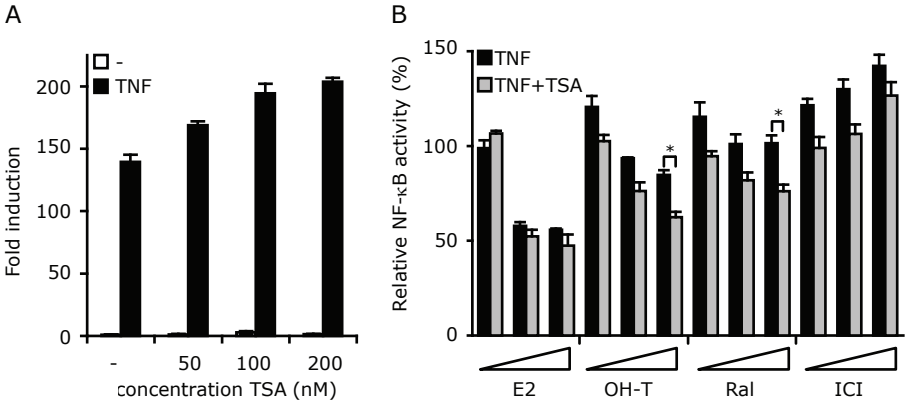

Figure 1. Inhibition of HDAC activity does not effect E2 induced repression of NF-κB activity by ERα, but enhances SERM induced repression.
A, U2OS-ERα-NFκBluc cells, stably expressing ERα and 4xNF-κB-tk-Luc reporter, were pretreated with increasing concentrations of TSA (50, 100 and 200 nM) or vehicle (0.1% ethanol, -) for 1h and next treated with or without TNFα (2.5 ng/ml) in combination with TSA or vehicle. After 24 h cell were harvested and assayed for luciferase activity. Results are expressed as fold induction of luciferase activity induced by TNFα (with or without TSA) over cells treated with vehicle alone. **B,** U2OS-ERα-NFκBluc cells were pretreated with TSA (100 nM) or vehicle (0.1% ethanol, -) for 1h and subsequently treated with TNFα (2.5 ng/ml) in combination with TSA or vehicle and increasing concentrations of E2, OH-T, Ral or ICI (10^{-14}, 10^{-10} and 10^{-6} M). After 24 h cells were harvested and assayed for luciferase activity. The effects of hormones in absence of TSA are expressed relative to luciferase activity induced by TNFα alone (set at 100%) and the effects of hormones in presence of TSA are expressed relative to luciferase activity induced by TNFα in combination with TSA (set at 100%). In both experiments values are presented as the average ± SEM of three independent experiments assayed in duplicate. Statistically significant differences between treatments with hormones in absence of TSA and in presence of TSA are indicated (*, $P<0.05$).

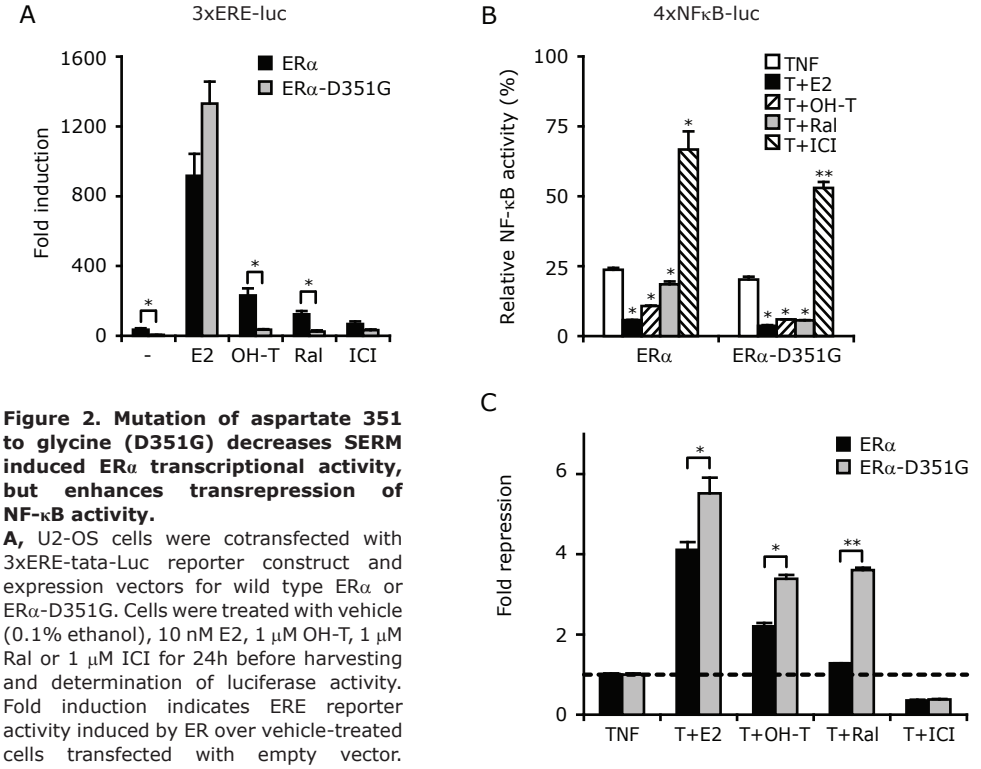

Figure 2. Mutation of aspartate 351 to glycine (D351G) decreases SERM induced ERα transcriptional activity, but enhances transrepression of NF-κB activity.
A, U2-OS cells were cotransfected with 3xERE-tata-Luc reporter construct and expression vectors for wild type ERα or ERα-D351G. Cells were treated with vehicle (0.1% ethanol), 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI for 24h before harvesting and determination of luciferase activity. Fold induction indicates ERE reporter activity induced by ER over vehicle-treated cells transfected with empty vector. **B,** U2-OS cells were cotransfected with 4xNF-κB-tk-Luc reporter construct and expression vectors for wild type ERα or ERα-D351G. Cells were treated with 2.5 ng/ml TNFα in combination with vehicle (0.1% ethanol), 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI for 24h before harvesting and determination of luciferase activity. Results are expressed relative to NF-κB activity induced by TNFα in control cells transfected with empty vector (100%). In both experiments values are presented as the average ± SEM of three independent experiments assayed in triplicate. **C,** Data from B are depicted as fold repression by ERα or ERα-D351G. Repression by hormones is expressed relative to reporter activity in presence of ER without hormone (ERα and ERα-D351G were both set at 1). Statistically significant differences between wild type ERα and ERα-D351G (in A and C) and between TNFα and TNFα+hormone in the presence of ER (in B) are indicated (*, P<0.05; **, P<0.001).

A transcriptionally inactive ERα mutant is still capable of repressing NF-κB activity through E2 and OH-T and gains repressive activity through Ral and ICI

To investigate the effect of complete loss of ERα transcriptional activity on transrepression of NF-κB activity induced by E2 and SERMs in U2-OS cells, we generated point mutations in both AF-1 and AF-2, since we observed that these domains act synergistically in E2-induced transcriptional activity of ERα in U2-OS cells (53). In AF-1 serine 118 was mutated to arginine (S118R) and in AF-2 leucines 539 and 540 in helix 12 were mutated to alanines (L539/540A). These mutations were described previously to result in disruption of the interaction with co-activators and to abolish transcriptional activity (57-59). We compared the ability to activate transcription from the ERE luciferase reporter between ERα and ERα-S118R+L539/540A (ERα-AFmut) in transient transfection assays in U2-OS. The ERα-AFmut protein indeed failed to activate transcription from the ERE reporter upon stimulation with

either E2 or SERMs (Fig. 3A). Next, ERα-AFmut was tested for its capacity to modulate TNFα-induced NF-κB activity. Similar to wild type ERα, we observed ligand-independent repression of NF-κB activity by ERα-AFmut, which could be repressed further upon stimulation with E2, although less strongly compared to wild type. As observed above for the ERα-D351G mutant (Fig. 2), NF-κB transrepression by OH-T was retained by ERα-AFmut. Strikingly, in contrast to wild type ERα, Ral and ICI became strong repressors of NF-κB activity through ERα-AFmut (Fig. 3B and 3C). Taken together, our findings with the SERM-occupied ERα-D351G mutant and with the E2- or SERM-occupied ERα-AFmut protein indicate that transactivation and transrepression can be uncoupled and therefore argue against a role for coactivators in ERα-mediated transrepression of NF-κB.

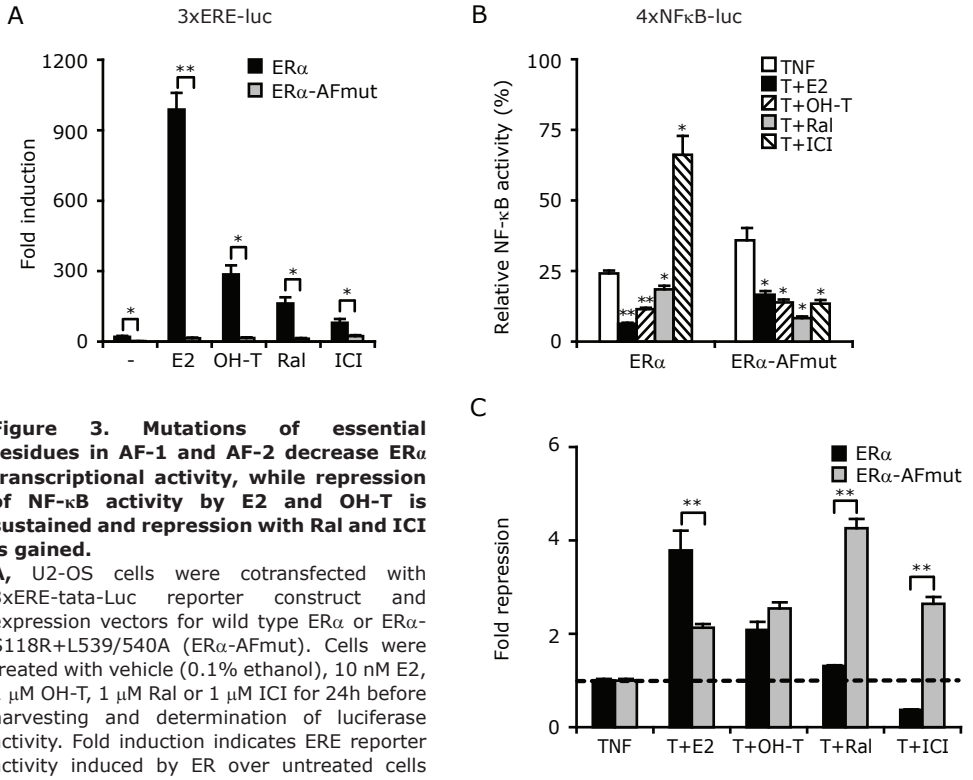

Figure 3. Mutations of essential residues in AF-1 and AF-2 decrease ERα transcriptional activity, while repression of NF-κB activity by E2 and OH-T is sustained and repression with Ral and ICI is gained.

A, U2-OS cells were cotransfected with 3xERE-tata-Luc reporter construct and expression vectors for wild type ERα or ERα-S118R+L539/540A (ERα-AFmut). Cells were treated with vehicle (0.1% ethanol), 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI for 24h before harvesting and determination of luciferase activity. Fold induction indicates ERE reporter activity induced by ER over untreated cells transfected with empty vector. **B,** U2-OS cells were cotransfected with 4xNF-κB-tk-Luc reporter construct and expression vectors for wild type ERα or ERα-AFmut. Cells were treated with 2.5 ng/ml TNFα in combination with vehicle (0.1% ethanol), 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI for 24h before harvesting and determination of luciferase activity. Results are expressed relative to NF-κB activity induced by TNFα in control cells transfected with empty vector (100%). In both experiments values are presented as the average ± SEM of four independent experiments assayed in triplicate. **C,** Data from B is depicted as fold repression by ERα or ERα-AFmut. Repression by hormones is expressed relative to reporter activity in presence of ER without hormone (ERα and ERα-AFmut were both set at 1). Statistically significant differences between wild type ERα and ERα-AFmut (in A and C) and between TNFα and TNFα+hormone in the presence of ER (in B) are indicated (*, P<0.05; **, P<0.001).

A transcriptionally inactive ERβ mutant completely loses the ability to repress NF-κB activity.

We and others observed before that ERβ is capable of repressing NF-κB activity in the presence of E2, but not with SERMs (53, 60, 61). In order to determine whether NF-κB repression by ERβ can also occur independently of transcriptional activity we generated the AF-2 mutant of ERβ-L490/491A (homologous to L539/540 in ERα). In U2-OS cells wild type ERβ can only activate transcription from the ERE reporter upon E2 treatment. In contrast to ERα, no ERβ activity can be observed without ligand or upon addition of OH-T and Ral. This can be attributed to an inactive AF-1 of ERβ in U2-OS cells, which we described before (53). Indeed, mutation of only AF-2 was sufficient to completely abolish the capacity of ERβ to activate transcription from the ERE reporter (Fig. 4A). Cotransfection of ERβ or ERβ-L490/491A with the NF-κB reporter revealed that E2-induced NF-κB transrepression was also lost upon mutation of AF-2. Wild type ERβ did not significantly inhibit TNFα-induced NF-κB activity without ligand, while OH-T, Ral and ICI induced NF-κB activity. Cotransfection of ERβ-L490/491A did not result in modulation of TNFα-induced activity, either in the absence of ligand or after addition of E2 or ICI. Only OH-T and Ral gave an effect through ERβ-L490/491A by inducing NFκB activity (Fig. 4B). Western blot analysis demonstrated that loss of transactivation and transrepression by mutant ERβ is not due to loss of protein expression (Fig. 4C). These results show that, in contrast to ERα, transrepression of NF-κB activity by ERβ upon E2 stimulation is not retained upon mutating AF-2. Furthermore, unlike ERα, repressive activity by ERβ in the presence of SERMs or the antiestrogen ICI is not gained upon mutating AF-2, indicating that ERα and ERβ repress NF-κB activity through distinct mechanisms.

Transrepression of NF-κB activity by ERα and ERα-S118R+L539/540A in U2-OS cell lines stably expressing the receptor and the NF-κB luciferase reporter

To study repression of TNFα-induced NF-κB activity by wild type ERα and the mutant ERα-S118R+L539/540A in a more physiological situation, we generated stable transfectants in U2-OS cells. In addition to the ER expression vector, the NF-κB luciferase reporter was also present in a stable fashion. Clones were screened for stable expression of the receptor by analyzing the effect of E2 stimulation on TNFα-induced activity of the integrated NF-κB luciferase reporter. Next, clones showing efficient E2-mediated NF-κB transrepression were compared by Western blot analysis. From multiple positive clones a representative cell line was selected stably expressing wild type ERα (U2OS-ERα) and a cell line expressing ERα-S118R+L539/540A (U2OS-ERαAFmut). The stable cell lines were selected to have expression levels similar to MCF-7 cells, which express endogenous ERα. As described by us before in a different U2-OS cell line stably expressing ERα (Fig. 1B) (53), repression of TNFα-induced activity was observed after stimulation with E2 and OH-T. Ral did not have a significant effect on NF-κB activity, while stimulation with ICI resulted in increased NF-κB activity (Fig. 5A). In the U2OS-ERαAFmut cell line repression of TNFα-induced NF-κB activity was observed upon stimulation with all ligands (Fig. 5B), which is in agreement with the results obtained in the transient transfections (Fig. 3). These results show that the repressive characteristics effects of the ERα-AFmut protein were sustained when the receptor was endogenously expressed at physiological levels.

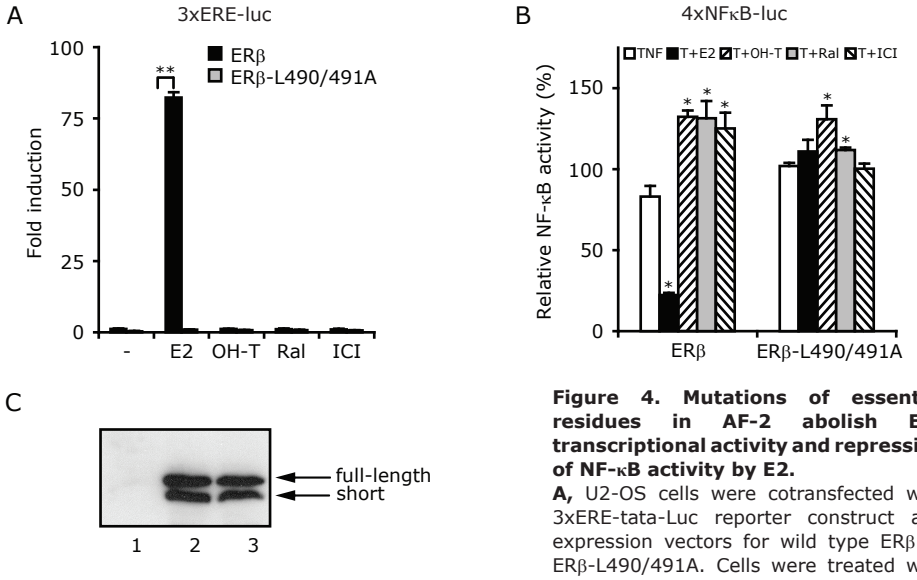

Figure 4. Mutations of essential residues in AF-2 abolish ERβ transcriptional activity and repression of NF-κB activity by E2.

A, U2-OS cells were cotransfected with 3xERE-tata-Luc reporter construct and expression vectors for wild type ERβ or ERβ-L490/491A. Cells were treated with vehicle (0.1% ethanol), 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI for 24h before harvesting and determination of luciferase activity. Fold induction indicates ERE reporter activity induced by ER over untreated cells transfected with empty vector. **B,** U2-OS cells were cotransfected with 4xNF-κB-tk-Luc reporter construct and expression vectors for wild type ERβ or ERβ-L490/491A. Cells were treated with 2.5 ng/ml TNFα in combination with vehicle (0.1% ethanol), 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI for 24h before harvesting and determination of luciferase activity. Results are expressed relative to NF-κB activity induced by TNFα in control cells transfected with empty vector (100%). In both experiments values are presented as the average ± SEM of four independent experiments assayed in triplicate. Statistically significant differences between wild type ERβ and ERβ-L490/491A (in A) and between TNFα and TNFα+hormone in the presence of ER (in B) are indicated (*, $P<0.05$; **, $P<0.001$). **C,** Western blot analysis of whole cell extracts of COS-1 cells transiently transfected with empty vector (lane 1), wild type ERβ (lane 2) or ERβ-L490/491A (lane 3) using an ERβ-specific antibody. The cDNA for ERβ encodes the full-length human protein (530 amino acid), which contains 45 additional amino acids in the N-terminus compared to the shorter version of ERβ (485 amino acids). However the full-length cDNA contains two start codons resulting in the expression of both the full-length and short protein.

Protein stability upon ligand stimulation of wild type ERα and ERα-S118R+L539/540A

Recent studies indicate that binding of coactivators to ERα is followed by the recruitment of factors involved in breakdown of the receptor by the ubiquitin-proteasome pathway (62-64). Based on these observations, it has been put forward that transcriptional activity of ERα and degradation of the receptor are processes that are mechanistically linked. In order to understand the newly gained ability of the ICI-occupied ERα-S118R+L539/540A mutant to repress NF-κB (Fig. 3), we were interested to see whether the stability of this receptor was changed compared to the wild type receptor. To investigate this, stable cell lines were treated with TNFα alone or in combination with E2, OH-T, Ral or ICI and wild type and mutant ER protein levels were determined by Western blot analysis. We observed an increase in receptor level of wild type ERα upon treatment with TNFα compared to basal levels of untreated cells. The proteasome inhibitor MG-132 completely abolished this effect (data not shown). MG-132 is also known as an NF-κB inhibitor since it prevents breakdown of IκB, which results in sequestration of NF-κB in the cytoplasm and inhibition of NF-κB activity. Therefore we can conclude that the stabilizing effect of TNFα on ER protein levels is dependent on the

activation of NF-κB, although the mechanism remains to be established. Treatment of cells with TNFα in combination with E2, Ral and ICI enhanced degradation of the ERα protein, while OH-T did not (Fig. 6A). The same effects on protein stability were observed when cells were treated with hormones without TNFα (data not shown), which is in agreement with previous publications (31). However, when U2OS-ERαAFmut cells were treated with TNFα alone or in combination with hormones we did not observe an effect on basal ER protein levels, showing that ERα-S118R+L539/540A is insensitive to stimulus-induced degradation by the ubiquitin-proteasome pathway. From these observations we conclude that enhanced protein stability could contribute to repression of NF-κB activity by ERα.

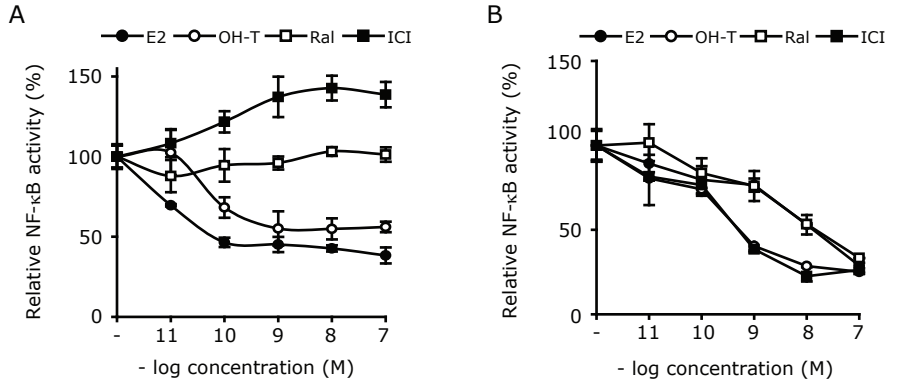

Figure 5. Transrepression of NF-κB activity in U2-OS cell lines stably expressing ERα or ERα-S118R+L539/540A.
U2-OS cell lines were generated stably expressing wild type ERα or ERα-S118R+L539/540A in combination with 4xNF-κB-tk-Luc reporter construct (U2OS-ERα and U2OS-ERαAFmut, respectively). U2OS-ERα (**A**) and U2OS-ERαAFmut (**B**) cells were treated with TNFα (2.5 ng/ml) and vehicle (0.1 % ethanol) or in combination with increasing concentrations of E2 (●), OH-T (○), Ral (□) or ICI (■) (10^{-11} to 10^{-7} M). After 24 h cell were harvested and assayed for luciferase activity. The effects of hormones are expressed relative to luciferase activity induced by TNFα alone (set at 100%) In both experiments values are presented as the average ± SD of a representative experiment assayed in triplicate. Experiments were repeated at least three times.

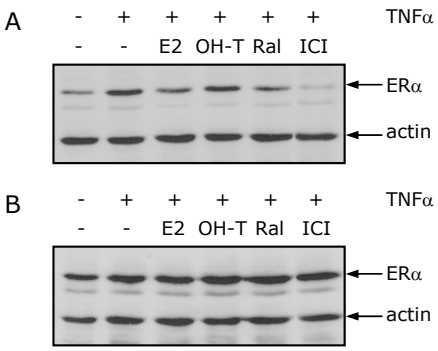

Figure 6. Stability of ER protein in U2OS-ERα and U2OS-ERαAFmut stable cell lines.
U2OS-ERα (**A**) and U2OS-ERαAFmut (**B**) cells were treated with vehicle (0.1% ethanol) or with 2.5 ng/ml TNFα in combination with vehicle, 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI. After 6 hours, whole cell extracts were harvested and ER protein expression levels were determined by Western blot analysis. Blots were incubated overnight with Ab-15 ERα-specific antibody and next for 2 h with Ab-5 actin-specific antibody, which served as internal control for equal loading of protein extracts. A representative blot of at least three experiments is shown.

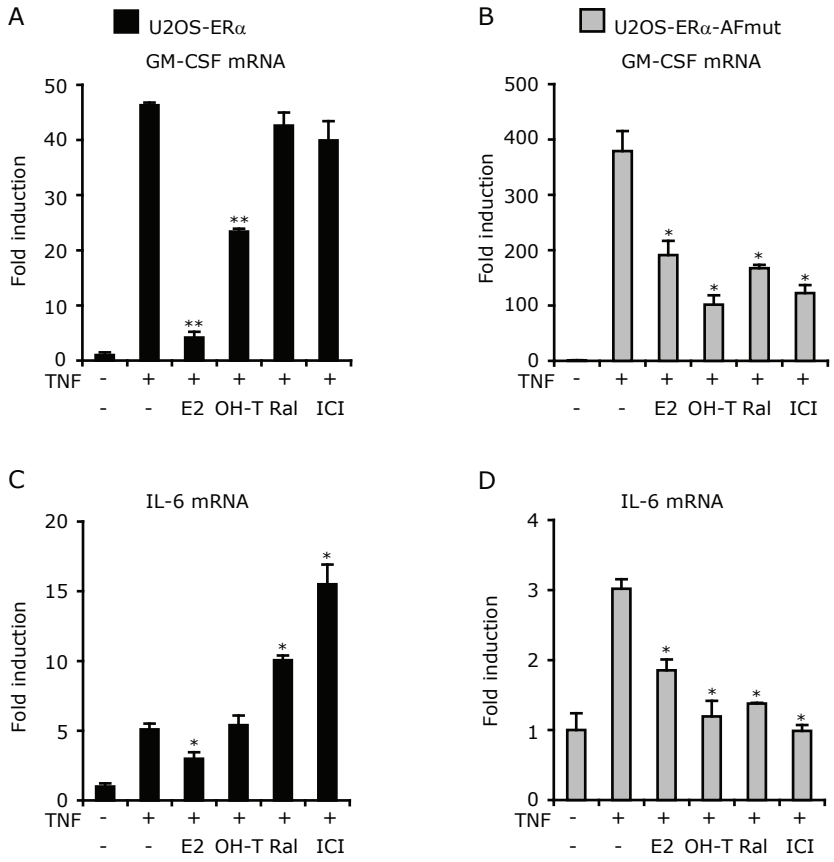

Figure 7. Repression of IL-6 and GM-CSF mRNA expression in U2OS-ERα and U2OS-ERαAFmut stable cell lines.

Quantitative real-time PCR on total RNA isolated from the U2OS-ERα and U2OS-ERαAFmut clones. U2OS-ERα (**A** and **C**) and U2OS-ERαAFmut (**B** and **D**) cells were treated with vehicle (0.1% ethanol) or with 2.5 ng/ml TNFα in combination with vehicle, 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI. After 8 hours, total RNA was isolated and relative expression of endogenous GM-CSF (**A** and **B**) and IL-6 (**C** and **D**) mRNA levels were determined by PCR. Values were normalized against the expression of the house keeping gene encoding human acidic ribosomal protein (hARP) determined for each sample. Fold induction indicates the mRNA expression level induced by TNFα (with or without hormones) over untreated cells. Bars represent the average ± SEM of three independent experiments assayed in duplicate. Statistically significant differences between TNFα and TNFα+hormone are indicated (*, $P<0.05$; **, $P<0.001$).

Wild type ERα and ERα-S118R+L539/540A inhibit the expression of endogenous TNFα-regulated target genes in U2-OS

Having established the effects of wild type ERα and the ERα-S118R+L539/540A mutant on the 4xNF-κB responsive reporter, we wanted to further explore the effects of wild type and mutant ER on the expression of physiologically relevant endogenous NF-κB regulated genes upon TNFα stimulation. In an earlier study, the expression of a number of cytokines, including interleukin (IL)-6 and granulocyte-macrophage colony stimulating factor (GM-CSF), in U2-OS

cells was described upon treatment with phorbol 12-myristate 13-acetate (PMA), which also activates the NF-κB pathway (68). To study ER actions in our model, we were interested in determining the effect of (anti)estrogens on the expression of IL-6 and GM-CSF in U2OS cells stably expressing ERα or the ERα-S118R+L539/540A mutant. Therefore quantitative RT-CR was performed on total RNA isolated from the isolated from the U2-OS-ERα and U2OS-ERαAFmut stable cell lines. Stimulation of U2-OS-ERα and U2OS-ERαAFmut cells with TNFα strongly induced GM-CSF expression (~50 and ~400 fold, respectively). Treatment of U2-OS-ERα cells with TNFα in combination with E2 efficiently (~90%) repressed GM-CSF expression, while OH-T repression was less efficient (~50%). Treatment of cells with Ral or ICI did not significantly change TNFα-induced expression of GM-CSF (Fig. 7A). E2 repressed GM-CSF expression in U2OS-ERαAFmut cells, albeit less strongly (~50%) than in U2-OS-ERα cells. However, repression by Tam was clearly enhanced and gained for Ral and ICI (~70%, 60% and 70%, respectively) (Fig. 7B). Induction of IL-6 expression by TNFα was weaker than GM-CSF in U2-OS-ERα (~5 fold) and U2OS-ERαAFmut cells (~3 fold). IL-6 expression in U2-OS-ERα cells was repressed by E2 (~40%), while OH-T had no effect and Ral and ICI increased expression. Repression of IL-6 expression by E2 (~40%), OH-T (~60%), Ral (~60%) and ICI (~70) in U2OS-ERαAFmut were similar to repression of GM-CSF. Collectively, these results show that the transcriptionally inactive ERα-AFmut protein is capable of repressing endogenous TNFα-regulated genes and even gains repressive activity through SERMs and the antagonist ICI.

DISCUSSION

So far, tissue selective effects of SERMs are mostly studied in the context of modulating the effects of ER on ERE mediated transcription. Transcriptional activation by the SERM-ER complex is determined to a great extent by the ligand-induced receptor conformation and subsequent recruitment of specific coactivator and corepressor complexes. Effects of SERMs in cross-talk of ER with other transcription factors are less well documented. Therefore, we were interested in studying the mechanism of SERM-induced repression of NF-κB activity in more detail and investigated the contribution of coactivator and corepressor activity in this crosstalk. ER-mediated NF-κB repression is described to occur through various mechanisms, which appear to be cell type-dependent. In this study we addressed the mechanisms of cross-talk that involve recruitment of coregulators.

First, we studied the model which implies that ER interacts directly with DNA-bound NF-κB and inhibits transcriptional activation by recruiting HDACs to the promoter. We show that HDACs are involved in the regulation of NF-κB transcriptional activity by actively repressing the response to TNFα, because activity is enhanced by the HDAC inhibitor TSA. It is described before that HDAC1 and HDAC2 can regulate NF-κB-induced transcription and that HDAC1 can interact directly with the NF-κB subunit p65 (RelA) (66). Adcock and coworkers previously demonstrated that the glucocorticoid receptor (GR) inhibits NF-κB-mediated gene expression by recruitment of HDAC2, which is relieved by TSA (50, 51). However, we show that repression of NF-κB activity by E2-stimulated ERα is not relieved in the presence of TSA, arguing against a role for recruitment of HDAC activity in E2-induced repression. Moreover, repression by OH-T and Ral is increased in the presence of TSA indicating that recruitment of HDAC activity diminishes the potential of ERα to repress NF-κB activity. OH-T and Ral induce a receptor conformation that enables ER to specifically interact with the corepressor molecules N-CoR and SMRT that mediate recruitment of a larger complex containing HDACs (27, 28). Thus, we can conclude from our data that inhibition of NF-κB-recruited HDAC

activity has no effect on ERα-mediated repression, while inhibition of ERα-recruited HDAC activity increases repression. Several earlier studies reported that OH-T and Ral did not repress NF-κB activity, while E2 was a potent repressor (44, 47, 67). In the light of our results, we propose that this can be attributed to cell type and tissue-specific expression or activity of corepressors/HDACs that prevent NF-κB transrepression.

Next, we were interested in determining the role of coactivator recruitment by ER in SERM-induced NF-κB inhibition. The model of cross-talk involving coactivator sharing assumes that ER can inhibit NF-κB activity either indirectly by competing for a limited amount of coactivators or directly by interacting with the NF-κB transcriptional complex on the promoter. In the coactivator-model, inhibition of NF-κB activity is dependent on the capacity of ER to interact with coactivators through intact AF-1 and/or AF-2 binding surfaces. Crystal structures of ERα-LBD revealed that, in contrast to E2, the bulky side chain of OH-T and Ral interacts with amino acid D351 through hydrogen bonds (22, 68). It is reported previously that D351 in the LBD is important in conferring estrogen/antiestrogenic properties by ERα after SERM binding (55, 69, 70). It is believed that after SERM binding, D351 influences the position of helix 12 in the LBD enabling AF-1 activity, while AF-2 activity is silenced. Mutating D351 to the uncharged amino acid glycine has been described to result in a receptor that loses SERM-induced transcriptional activity, while E2-induced activity is unchanged (55, 56). This effect is confirmed in this study in U2OS cells by analyzing ERα-D351G transcriptional activation of the 3xERE-tata-Luc reporter. It appeared that repression of TNFα-induced NF-κB activity by OH-T and Ral through ERα-D351G is not lost, but even enhanced. Moreover, while transactivation was unaltered compared to wild type ERα , NF-κB transrepression was enhanced by ERα-D351G upon E2 stimulation. These results show that loss of negative charge at position 351 results in loss of SERM-induced ERE-transactivation, while NF-κB transrepression is enhanced by both E2 and SERMs. From this we can conclude that while coactivator binding is required for transcriptional activation by SERMs, it is not necessary for repression of NF-κB activity.

The observation that transactivation and transrepression can occur independently of each other has been described before, for mouse ERα mutants that were incapable of activating transcription upon E2 stimulation (61). Therefore, we wanted to study the effect of disrupting both AF-1 and AF-2 coactivator interactions in cross-talk between NF-κB an ER upon E2 and SERMs in U2-OS. In AF-1 mutation S118R was generated because S118 is known to be the major site of E2-induced phosphorylation and necessary for enhancing AF-1 activity (71). Previous studies have shown that loss of phosphorylation, by mutating S118 to alanine or arginine, is associated with loss of transcriptional activity due to disruption of coactivator binding (57, 72). Furthermore, in AF-2 the hydrophobic pair of leucines at position 539 and L540 in helix 12 was mutated to alanines (L539/540A). These mutations are described to result in significant decrease in ERα transcriptional activity and this effect is attributed to loss of interaction between AF-2 and coactivators containing LXXLL-motifs (58, 59). Indeed, we observed that ERα-AFmut mediated ERE-transcription is completely abolished in U2-OS cells. In agreement with the results obtained for ERα-D351G, the capacity to repress TNFα-induced NF-κB transcriptional activity is retained by ERα-AFmut upon E2, OH-T and Ral stimulation despite loss of ERE-transcription. Strikingly, the full antagonist ICI became a strong inhibitor of NF-κB activity through ERα-AFmut. The repressive effects of ERα-AFmut on NF-κB activity were confirmed in a clone stably expressing the mutant receptor at physiological levels. Furthermore, we observed that, unlike wild type ERα, ERα-AFmut protein levels were not changed upon stimulation with (anti)estrogens. Like other members of the nuclear receptor family, ERα is targeted by the ubiquitin-proteasome pathway that mediates degradation of the receptor (73). It is known that upon ligand-binding the rate

of receptor degradation is changed (30, 74). Several proteins involved in mediating the ubiquitin-proteasome pathway have been described to interact with ERα through AF-2 (75-77). Moreover, mutations in AF-2 disrupting coactivator binding are reported to disrupt ligand-induced receptor breakdown (31, 62). Recently, it was also shown that mutation of S118 resulted in loss of E2-induced degradation of ERα (78). These results are in agreement with the observations that ERα-AFmut protein stability is unchanged upon ligand-binding. We propose that increased protein stability can contribute to the capacity of ERα to repress NF-κB activity, since the stable ERα-AFmut induces NF-κB transrepression through all types of ligands including the full antagonist ICI. For ERα-D351G it is described previously that the ligand-induced effects on protein stability were identical compared to wild type ERα (55, 56). Thus, ICI strongly reduced expression of ERα-D351G, which is consistent with our findings showing lack of transrepression of NF-κB activity upon ICI treatment.

In contrast to ERα, wild type ERβ is capable of repressing NF-κB activity only upon stimulation with E2 and not with SERMs. Moreover, the ERβ-AFmutant, with mutations L490/491A homologous to L539/540 of ERα, completely lost both the capacity to induce ERE-transcriptional activity and to repress NF-κB activity. These data suggest that coactivator binding by ERβ is necessary for NF-κB transrepression. Similar results were obtained in a previous study analyzing the repression of the TNF-responsive element of the human TNFα gene promoter by ERβ (60). In agreement with our observations, mutations in AF-2 of ERβ that disrupted coactivator binding also resulted in loss of both transactivation and transrepression. Therefore, we propose that the mechanism of inhibition of NF-κB activity mediated by ERβ involves the coactivator sharing model, which is in contrast to the model for ERα.

Concerning cross-talk between ERα and NF-κB in U2-OS cells, in this study we have excluded the mechanism of coactivator sharing and previously we excluded the mechanism of IκB induction (53). Therefore, in addition to previous studies (46, 53, 61, 79), we support the mechanism of cross-talk involving a direct interaction between ERα and NF-κB resulting in transcriptional interference. It has been shown before that the NF-κB subunits RelA (p65), NF-κB1 (p50) and c-Rel can interact directly with ERα *in vitro* (46, 79). Moreover, we have evidence that ERα interacts with p65 and p50 *in vivo* in U2-OS cells through co-immunoprecipitation and fluorescence energy transfer (FRET) (data submitted for publication). A model for transcriptional interference by GR on NF-κB transcriptional activity is proposed previously and it suggests that GR blocks RelA recruitment of the kinase P-TEFb, thereby diminishing phosphorylation of the RNA polymerase II C-terminal domain (48, 49). It will be interesting to study the mechanism of ERα-mediated inhibition of NF-κB transcriptional activity by using chromatin immunoprecipitation (ChIP), which enables determination of presence and modification status of factors involved in gene transcription *in vivo*. Preliminary data using ChIP show that ERα is present, together with NF-κB, on promoters of several cytokines and estrogen treatment results in diminished acetylation of histones (unpublished data).

The observation that the repressive properties of wild type ERα and ERα-AFmut are the same on the endogenous TNFα-regulated genes IL-6 and GM-CSF as on the 4xNF-κB-tk-Luc reporter emphasizes the physiological relevance of our observations. IL-6 and GM-CSF belong to a large group of cytokines and colony stimulating factors that are involved in inducing the development and activity of bone resorbing osteoclasts. Cells belonging to the stromal/osteoblast lineage produce these cytokines upon stimuli, such as TNF, and estrogen is known to suppress this production. The inhibitory action of estrogen on cytokine production is believed to be the main mechanism through which estrogen maintains bone mass and prevents osteoporosis in postmenopausal women (4).

In summary, we demonstrated that repression of NF-κB activity mediated by ERα upon E2 or SERM stimulation can occur independently of coactivator and corepressor recruitment in the osteoblast-like U2-OS cell line. Moreover, enhanced protein stability and inhibition of HDAC activity contribute to enhanced potential of a ligand to repress NF-κB activity through ERα. Transcriptional repression of NF-κB through ERβ is mechanistically different compared to ERα and involves coactivator recruitment. Consistent with SERM mediated ERE transactivation, we propose that depending on cell-type specific expression or activity of coregulators and relative expression of ERα and ERβ, SERMs can or can not mimic estrogen in its ability to inhibit NF-κB activity. These findings may contribute to the development of so-called "pathway selective" or "dissociating" ER ligands that selectively inhibit NF-κB activity, without activating transcription. These compounds could be used in the future to treat diseases with chronic NF-κB activity such as osteoporosis, inflammatory bowel disease and atherosclerosis (67).

MATERIALS AND METHODS

Chemicals

Recombinant human TNFα, E2 , OH-T and TSA were obtained from Sigma Chemical Co (St Louis, MO). Ral was provided by Organon International (Oss, The Netherlands). ICI was obtained from Tocris Cookson (Bristol, UK).

Cell culture

Human osteosarcoma osteoblastic U2-OS cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DF; Gibco, Invitrogen, Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS; Cambrex, Rockland, ME), 10 U/ml penicillin and 10 μg/ml streptomycin (Gibco, Invitrogen, Gaithersburg, MD). Cells were cultured at 37ºC in a 5% CO_2 humidified atmosphere.

Plasmids and Transient Transfection Assay

U2-OS cells were seeded in 24-well plates in DF medium without phenol red supplemented with 5% charcoal stripped FCS (Bodinco, Alkmaar, The Netherlands), 10 U/ml penicillin and 10 μg/ml streptomycin (Gibco, Invitrogen, Gaithersburg, MD). Cells were transfected with DNA constructs using the calcium-phosphate precipitation method, as described before (56). Cells were transfected with 0.1 μg of 4xNF-κB(HIV)-tk-Luc or 1.0 μg of 3xERE-tata-Luc reporter plasmid, 0.2 μg of SV_2-LacZ plasmid, 200 ng of ER expression plasmid or empty expression vector and pBluescript SK^- was added to obtain a total amount of 2 μg DNA/well. After 16 h, the medium was refreshed and when indicated hormones and/or TNFα were added to the medium (1:1000) to a final concentration of 10 nM (E2), 1 μM (OH-T, Ral and ICI) or 5 ng/ml (TNFα). Cells were harvested 24 h later and assayed for luciferase activity using the Steady-Glo Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's protocol, in a Topcount liquid scintillation counter (Packard Instruments, Meriden, CT). Luciferase values were corrected for transfection efficiency by measuring β-galactosidase activity of the constitutive expression plasmid SV_2-LacZ. Data are represented as mean values ± SEM from al least three independent experiments assayed in triplicate.

Site-directed Mutagenesis

Vectors encoding mutant ER were made by oligonucleotide site-directed mutagenesis, according to the manufacturer's protocol (Stratagene, La Jolla, CA). Expression vectors for wild type ERα and ERβ were used as a template in the PCR reaction using the following sets of complementary primers containing base substitutions in targeted triplet (underlined): forward primer 5'-CTGACCAACCTGGCAGGCAGGGAGCTGGTTCAC-3' and reverse primer 5'-GTGAACCAGCTCCCTGCCTGCCAGGTTGGTCAG-3' for generating ERα-D351G; forward primer 5'-CCGCCGCAGCTGAGGCCTTTCCTGCAG-3' and reverse primer 5'-CTGCAGGAAAGGCCTCAGCTGCGGCGG3' (S118R) and forward primer 5'-GTGCCCCTCTATGACGCGGCGCTGGAGATGCTGGAC-3' and reverse primer 5'-GTCCAGCATCTCCAGCGCCGCGTCATAGAGGGGCAC-3' (L539/540A) for generating ERα-S118R+L539/540A (ERα-AFmut); forward primer 5'-CCCAGTGTATGACGCGGCGCTGGAGATGCTGAATGC-3' and reverse

primer 5′-GCATTCAGCATCTCCAGCGCCGCGTCATACACTGG-G-3′ for generating ERβ-L490/491A. All mutant constructs were sequenced to ensure that point mutations were successfully generated and that no errors were introduced in the remainder of the coding sequence.

Generation of Stable Transfectants

The U2OS-ERα-NFκBluc cell line used in Fig.1 was described before. To obtain stable transfectants of U2-OS expressing wild type ERα and ERα-AFmut, cells were grown in 6-well plates and transfected, using calcium-phosphate precipitation, with 5 μg of the pSG5 expression vector encoding ER and the neomycin-resistance gene in combination with 5 μg of 4xNF-κB(HIV)-tk-Luc expressing the hygromycin-resistance gene. After 16 h the medium was refreshed and 24 h later cells were trypsinized and replated in the presence of geneticin (G418; 200 μg/ml). After 10 days surviving colonies were isolated and established as stable cell lines. Stable clones were cultured under prolonged G418 (200 μg/ml) and hygromycin (50 μg/ml) selection. The expression of ERα was determined by western blot analysis. Functionality of the protein was verified by determining transcriptional activity of the integrated 4xNF-κB(HIV)-tk-Luc reporter after stimulation with hormones.

Protein Isolation and Western Blotting Analysis

For isolation of whole cell extracts, U2-OS cells were cultured in 100mm dishes, treated as described and proteins were extracted by resuspending pelleted cells in hypertonic buffer (20 mM HEPES pH 8, 630mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 % NP-40, 1x proteinase inhibitor cocktail (Roche, Basel, Switzerland). The samples were freeze-thawed three times, incubated on ice for 30 min and cellular debris was pelleted by centrifugation. Protein concentration of the supernatant was determined by the BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's protocol. 25 μg of extract was separated on SDS-PAGE gels and transferred onto nitrocellulose membranes by electroblotting. After blocking, blots were probed with an antibody against ERα (Ab-15; Lab Vision, Fremont, CA) overnight and next for 2h with an antibody against actin (Ab-5; Lab Vision, Fremont, CA) according to the manufacturer's protocol. Immunoreactive bands were visualized after incubation with a peroxidase-conjugated second antibody and enhanced chemiluminesence.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated using Trizol (Invitrogen, Gaithersburg, MD) and purified with the RNeasy kit including a DNase treatment (Qiagen, Valencia, CA), following the manufacturer's instructions. In order to generate cDNA, one microgram total RNA was reverse transcribed with Superscript II (Invitrogen, Gaithersburg, MD) using poly(dT) oligonucleotides. Quantitative real-time PCR of diluted cDNA was carried out using a MyiQ I cycler (Bio-Rad, Hercules, CA) with for 40 cycles of two-step amplification. Each PCR amplification reaction consisted of 5 μl of diluted cDNA (1:10 for IL-6 and GM-CSF; 1:50 for hARP), 1xiQ SYBR green Supermix (Bio-Rad, Hercules, CA) and 0.5 μM of forward and reverse primers. Gene expression is normalized to human acidic ribosomal protein (hARP) and the fold change in expression is determined relative to control cells treated with vehicle. The following primers were used to detect the genes of interest: GM-CSF forward primer 5′-GGCCAGCCACTACAAGCAGCACT-3′ and reverse primer 5′-CAAAGGGGATGACAAGCAGAAAG-3′, IL-6 forward primer 5′-CAGACAGCCACTCACC-3′ and reverse primer 5′- TTCACCAGGCAAGTCTC-3′ and hARP forward primer 5′- CACCATTGAAATCCTGAGTGATGT -3′ and reverse primer 5′- TGACCAGCCCAAAGGAGAAG -3′.

Statistical Analysis

Data are presented as mean values ± SEM from al least three independent experiments. An unpaired Student's *t* test was used to compare differences between mean values of two different treatments. Differences with P values less than 0.05 were accepted as statistically significant.

ACKNOWLEDGEMENTS

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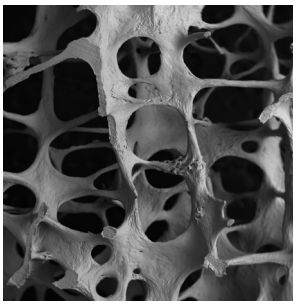
Chapter 4



Direct Interaction between Estrogen Receptor α and NF-κB in the Nucleus of Living Cells

Submitted

Direct Interaction between Estrogen Receptor α and NF-κB in the Nucleus of Living Cells.

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Inhibition of NF-κB transcriptional activity by steroid receptors is the basis for the antiinflammatory actions of steroid hormones and the molecular mechanism underlying this cross-talk is thought to involve direct protein-protein interactions. In this study, we show that estrogen receptor (ER)α and NF-κB interact *in vivo* by using fluorescence resonance energy transfer (FRET) and coimmunoprecipitation. U2-OS cells were used to study direct interactions between fluorescent fusion proteins of ERα and the NF-κB subunits p50 and p65. Interactions were observed only in the nucleus and maximal FRET signal was detected when ERα is coexpressed with both NF-κB subunits and cells were stimulated with estrogen. This is in agreement with the induction of nuclear colocalization of the proteins under this condition. Moreover, in a U2-OS clone stably expressing ERα, interaction with NF-κB was confirmed. A p65 deletion mutant lacking the Rel homology domain was strongly impaired in its interaction with ERα showing the importance of this domain. Taken together, these findings provide a strong basis for the direct protein-protein interaction model for cross-talk between ERα and NF-κB.

INTRODUCTION

The estrogen receptor (ER), like other members of the nuclear receptor family, is a ligand-inducible transcription factor capable of regulating gene expression in several ways. In the classical pathway, the ligand-activated receptor binds directly to specific hormone response elements in the promoters of target genes and recruits co-regulators to enhance transcription (1). In an alternative pathway, nuclear receptors modulate gene transcription without directly binding DNA by changing the activity of other transcription factors such as activator protein-1 (AP-1), Sp1 and nuclear factor-κB (NF-κB) (2-4). A variety of steroid hormones, including estrogens, glucocorticoids, androgens and progestins, are known to inhibit NF-κB activity upon binding to their cognate steroid receptor (5-7). NF-κB is well-known for its key role in tissues of the immune system, but it is also important in development and physiology of bone, skin, mammary gland and central nervous system. Although NF-κB activity is essential in the regulation of many processes in normal physiology, dysregulation is known to be involved in various diseases including inflammatory diseases, autoimmune diseases and cancer. Inhibiting the NF-κB pathway has proven to be an effective treatment for several of these diseases (8). Therefore, glucocorticoids are widely used to treat a variety of inflammatory diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease (IBD) (9). Furthermore, estrogen is used to prevent postmenopausal osteoporosis and also relieves symptoms in animal models of atherosclerosis, rheumatoid arthritis and IBD (10-13).

NF-κB is expressed as a homo- or heterodimer formed between the five members of the NF-κB protein family consisting of p65 (RelA), RelB, c-Rel, p50 and p52. Each protein contains a Rel homology domain (RHD) at the N-terminus which is responsible for DNA binding, dimerization, and association with the inhibitor of NF-κB (IκB). In most cell types NF-κB is present as a heterodimer between p50 and p65 and in unstimulated cells it remains inactive in the cytoplasm due to association with IκB. The classical pathway of activation involves a phosphorylation cascade and subsequent degradation of IκB, which can be induced by various signals including cytokines, growth factors and UV radiation. Once NF-κB becomes liberated from IκB it rapidly translocates to the nucleus where it can bind to specific NF-κB binding sites in the promoters of target genes to enhance transcription (14).

Although different theories have been proposed to explain the molecular mechanism of NF-κB transrepression, models involving direct protein-protein interactions between steroid receptors and NF-κB are most likely (4, 15). In this study, we used fluorescence resonance energy transfer (FRET) to investigate the interaction between ERα and homo- or heterodimers of p65 and p50 in living osteoblastic U2-OS cells. Interactions are observed only in the nucleus and are strongly enhanced by estrogen. Maximal interaction is observed when ERα is coexpressed with both p50 and p65 in the presence of E2. This is consistent with the observation that all three proteins are colocalized under this condition. Interaction between ERα and NF-κB is confirmed in cells stably expressing fluorescently-labeled ERα. A deletion mutant of p65 lacking the RHD, necessary for interaction with ERα *in vitro*, also loses its capacity to interact *in vivo*. Therefore our findings provide a solid basis for the direct interaction model for cross-talk between ERα and NF-κB.

RESULTS

Coimmunoprecipitation of ERα and NF-κB

The human osteoblast cell line U2-OS, was used to perform a luciferase reporter assay in order to analyze the effect of ERα on p65 and p50/p65 induced transcriptional activity. Cells were cotransfected with the NF-κB responsive reporter 4xNF-κB-Luc and an expression vector encoding p65 alone or in combination with an expression vector for p50. In both conditions NF-κB reporter activity was efficiently induced (~ 6 fold). Cotransfection of ERα resulted in 50% reduction of p65 and p50/p65 induced transcriptional activity. Upon stimulation with 17β-estradiol (E2), NF-κB transcriptional activity was almost completely abolished, reducing reporter activity to basal activity of the empty vector control (Fig.1A). Next, we investigated complex formation between ERα and p50/p65 in U2-OS cells by coimmunoprecipitation. In order to achieve this we used an expression construct encoding p65 with an N-terminal FLAG-tag, which does not interfere with the ability to activate transcription (16) and transrepression by ERα (data not shown). Immunoprecipitation of Flag-p65, from cell extracts coexpressing ERα and treated with E2, resulted in efficient pull down of ERα. When Flag-p65 and p50 were coexpressed, p50 was also coimmunoprecipitated showing that p50/p65 heterodimers are formed. Since the affinity between homodimers of p50 or p65 is weaker than between heterodimers, it can be assumed that when p50 and p65 are coexpressed, mainly the heterodimer will be present (17). When ERα was coexpressed with both Flag-p65 and p50, immunoprecipitation of Flag-p65 still resulted in efficient pull down of ERα after E2 stimulation (Fig.1B). Taken together, these results show that ERα interacts directly with p65, present as homodimer or heterodimer with p50, in extracts from mammalian U2-OS cells treated with E2.

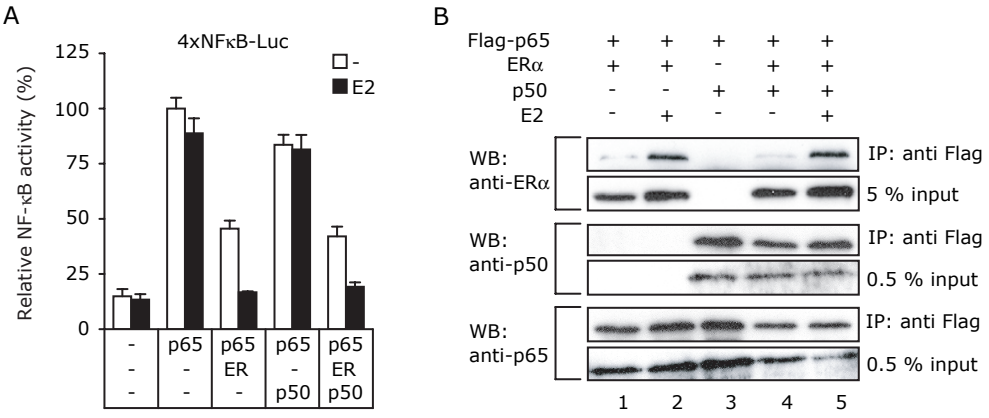

Figure 1. **ERα represses NF-κB transcriptional activity and directly interacts with the NF-κB subunit p65.**
A, The effect of ERα, in the absence and presence of E2, on p65 and p65/p50 induced transcriptional activity determined by luciferase reporter assay in U2-OS cells. NF-κB reporter activity induced by p65 was set at 100%. **B,** Coimmunoprecipitation assay in U2-OS cell extracts coexpressing Flag-p65, ERα and/or p50 in absence and presence of E2, as indicated (top). Flag-p65 was immunoprecipitated using M2 anti-Flag antibody (IP). Precipitated proteins and input levels were visualized by Western blot analysis (WB) using the indicated antibodies.

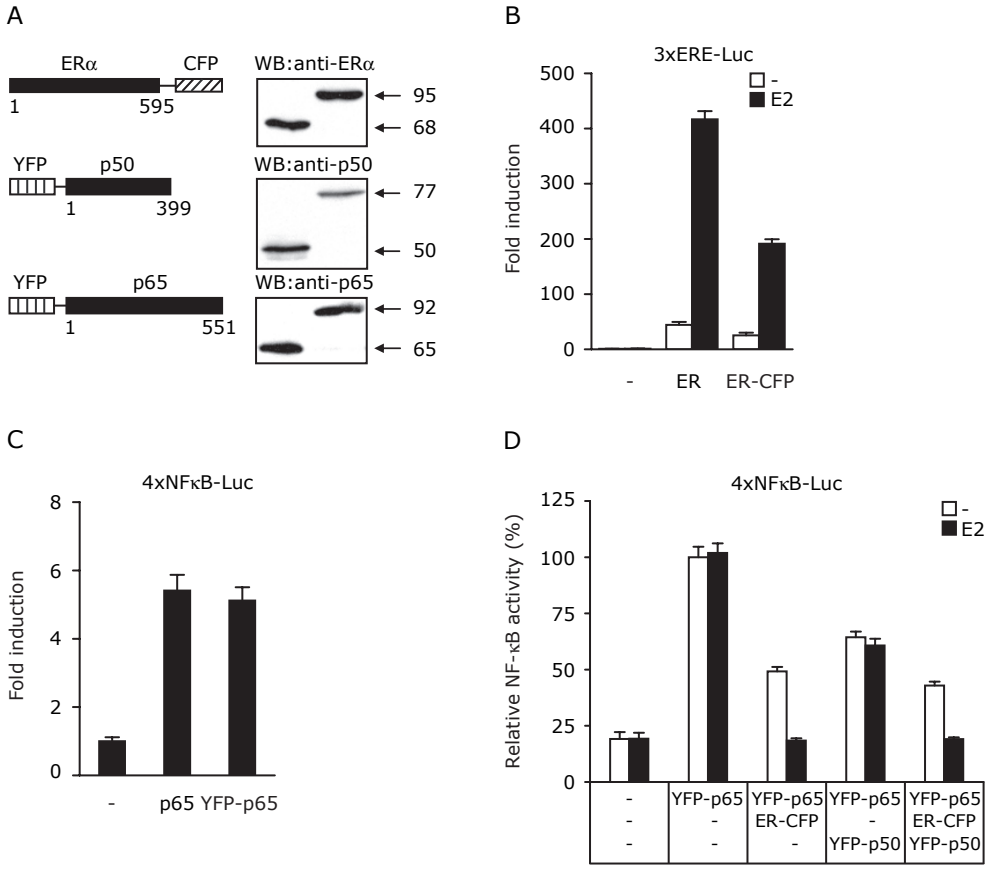

Figure 2. Expression and functional activity of fluorescent fusion proteins ER-CFP, YFP-p65 and YFP-p50 compared to wild type proteins.
A, Schematic representation and expression of fusion proteins in U2-OS cells. The expected size (kDa) is indicated on the right and verified using a molecular weight marker. **B,** Transcriptional activity of ER-CFP, in absence and presence of E2, compared to wild type ERα determined by luciferase reporter assay in U2-OS cells. Fold induction indicates ERE reporter activity induced by ER over untreated cells transfected with empty vector. **C,** Experiment performed the same as in B, for comparison of transcriptional activity of YFP-p65 and wild type p65 on NF-κB reporter. **D,** Same experiment as 1A, except ER-CFP, YFP-p65 and YFP-p50 were tested.

Expression and functionality of CFP and YFP fusion proteins of ERα and NF-κB

In addition to coimmunoprecipitation, we were interested in investigating direct protein-protein interactions between ERα and NF-κB in living cells by using Fluorescence Resonance Energy Transfer (FRET). Therefore, we generated fluorescent fusion constructs encoding ERα with cyan fluorescent protein fused to the C-terminus (ER-CFP) and p50 and p65 with yellow fluorescent protein fused to the N-terminus (YFP-p50 and YFP-p65, respectively). Expression of correct molecular weight of the fusion proteins was investigated by Western blot analysis of whole cell extracts from transiently transfected U2-OS cells (Fig.2A). To study the ability

of the fusion proteins to activate transcription compared to wild type proteins, ER-CFP or ERα was cotransfected with the estrogen responsive reporter 3xERE-Luc (Fig.2B) and YFP-p65 or p65 with 4xNF-κB-Luc (Fig.2C). ER-CFP strongly induced transcription of 3xERE-Luc after E2 stimulation, although less efficiently than with wild type ERα. This could be due to sterical hindrance of CFP with ER-coactivator interactions or increased protein turnover. YFP-p65 was equally potent in inducing transcription as wild type p65. Similar to wild type ER, cotransfection of ER-CFP resulted in 50% reduction of YFP-p65 transcriptional activity and E2 stimulation completely abolished transcriptional activity. Although, cotransfection of YFP-p50 resulted in reduced transcriptional activity (60% compared to YFP-p65 alone), it was still repressed to basal activity by E2-stimulated ER-CFP (Fig.2D). Therefore, we can conclude that the functional characteristics of the fusion proteins are comparable to wild type proteins and can be used for studying *in vivo* interactions by FRET analysis.

Colocalization of fluorescent fusion proteins

To examine the cellular expression and dynamic behavior of the fluorescent proteins we studied their localization upon transient transfection in U2-OS cells. When ER-CFP was coexpressed with YFP-p50, we observed that both proteins were exclusively present in the nucleus, with and without E2 stimulation (Fig.3A). Coexpression of ER-CFP with YFP-p65 revealed that ER-CFP remained localized in the nucleus, while YFP-p65 was present in both the cytosol and the nucleus, and this did not change upon E2 treatment (Fig.3B). However, when ER-CFP was coexpressed with both YFP-p65 and YFP-p50 we observed that upon E2 treatment, YFP fluorescence was almost completely translocated to the nucleus resulting in colocalization of all three proteins (Fig.3C). These observations enabled us to study the interaction between ERα, p50 and p65 in the nucleus of living U2-OS cells by FRET.

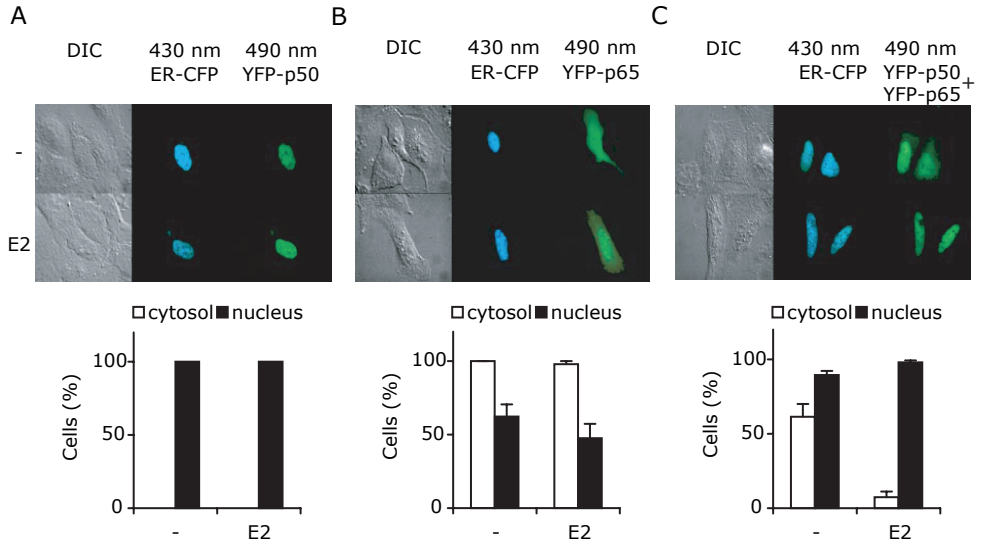

Figure 3. Colocalization of fluorescent proteins in U2-OS cells.
A, Representative images of a cell coexpressing ER-CFP (excitation 430 nm) and YFP-p50 (excitation 490 nm), before and after treatment with E2. The graph represents the percentage of cells positive for YFP expression in the cytosol (white bars) and YFP expression in the nucleus (black bars). Total number of YFP positive cells analyzed was set at 100%. **B,** The same is depicted as in A, for a cell coexpressing ER-CFP and YFP-p65. **C,** The same is depicted as in A, for cells coexpressing ER-CFP, YFP-p50 and YFP-p65.

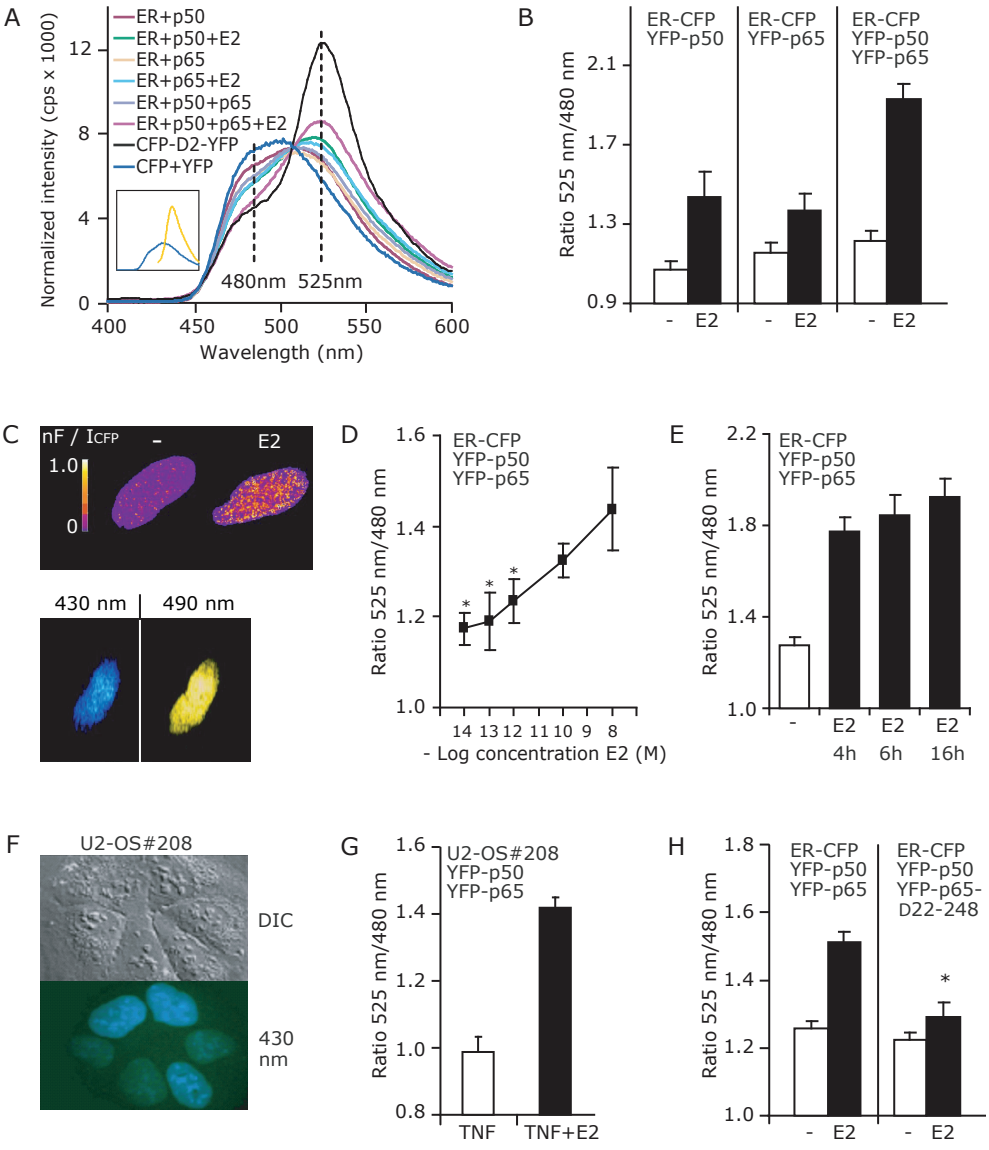

Figure 4. FRET analysis of the interaction between ERα and the NF-κB subunits p50 and p65. **A,** Emission spectra, after excitation with 430 nm, of U2-OS cells coexpressing ER-CFP in combination with YFP-p50 and/or YFP-p65 and treated overnight (16 h) with or without 10^{-8} M E2. Negative control (CFP/YFP): dark blue line; positive control (CFP-D2-YFP): black line. Insert: separate CFP spectrum (excitation 430 nm) and YFP spectrum (excitation 490 nm) emitted by the same cell. **B,** Graphical representation of the protein interactions between ER-CFP and YFP-p50 or YFP-p65 alone or in combination, with or without E2, expressed as $R_{525/480}$. **C,** FRET images depicting FRET-to-donor fluorescence ratio (nF/ICFP) of U2-OS cells coexpressing ER-CFP, YFP-p50 and YFP-p65, treated with or without E2 after excitation with 430 nm of ER-CFP (top). False-colored pictures of a U2-OS cell coexpressing ER-CFP, YFP-p50 and YFP-p65, treated overnight with E2, after excitation with 430 nm of ER-CFP and after excitation with 490 nm of

Interaction between ERα and NF-κB in living U2-OS cells

We applied the method of spectral analysis to detect transfer of energy from the excited donor fluorophore (CFP) to the acceptor fluorophore (YFP), which occurs if the two fluorophores are in very close vicinity of each other (less than 60Å) (18). The quantitative parameter for donor/acceptor interaction is depicted by the ratio between emission intensities at 525nm and 480nm ($R_{525/480}$) of the spectra obtained from cells excited with 430 nm. Increased efficiency of energy transfer between CFP- and YFP-fusion proteins, leads to decreased donor emission intensity at 480 nm and a subsequent increase in acceptor intensity at 525 nm and thus an increased $R_{525/480}$. As a negative control, cells were transfected with equal amounts of expression vectors encoding CFP and YFP alone, which did not result in a FRET signal ($R_{525/480}$=0.84). As a positive control cells were transfected with an expression construct encoding the fusion protein CFP-D2-YFP (19) resulting in a strong FRET signal ($R_{525/480}$=3.44) (Fig.4A).

To study interactions between ERα and NF-κB, U2-OS cells were transfected with ER-CFP in combination with YFP-p50 or YFP-p65 alone or in combination. In all three transfection conditions, $R_{525/480}$ was increased in absence of ligand, compared to the negative control, and this was induced further upon stimulation with E2. It appeared that maximal FRET signal was observed when ER-CFP was coexpressed with both YFP-p50 and YFP-p65 after stimulation with E2 (Fig.4A and 4B). We used a second method to quantify FRET by determining the FRET-to-donor fluorescence ratio (nF/I_{CFP}) as described by Xia *et al* (20). Upon E2 treatment nF/I_{CFP} was strongly enhanced and the signal was only detected in the nucleus (Fig.4C, top). This is consistent with colocalization of the interacting proteins in the nucleus (Fig.4C, bottom). Moreover, it was determined that the interaction induced by E2 is dose-dependent (Fig.4D), which is consistent with our observations of functional reporter assays in U2-OS (21). In addition to overnight (16 h) E2 incubation, the condition used in reporter assays, interaction between ERα and NF-κB was observed already after 4h and longer incubations resulted only in a minor increase in $R_{525/480}$ (Fig.4E).

Because the use of transiently transfected cells can lead to complications arising from overexpression, we isolated an U2-OS clone stably expressing ER-CFP (Fig.4F), which retained functional characteristics (Fig.5). It appeared that when cells were cultured in charcoal-stripped serum, necessary for studying hormone effects, the expression of ER-CFP was reduced beneath levels required for spectral analysis. This effect is also observed in U2-OS clones stably expressing wild type ERα (data not shown). This problem was overcome by performing incubations in combination with TNFα, which results in higher ERα expression. Upon transient coexpression of YFP-p50 and YFP-p65 we were able to detect E2-induced interaction with stably expressed ER-CFP (Fig.4G).

YFP-p50 and YFP-P65 (bottom). **D,** Dose-dependent increase in $R_{525/480}$ of cells coexpressing ER-CFP, YFP-p50 and YFP-p65 after E2 treatment (ranging from 10^{-8} M to 10^{-14} M). **E,** Time-dependent increase of $R_{525/480}$ in cells coexpressing ER-CFP, YFP-p50 and YFP-p65 after E2 treatment (4h, 6h or 16h). **F,** Images of the U2-OS clone stably expressing ER-CFP (U2-OS#208). Upper panel: differential interference contrast (DIC) image. Lower panel: Emission after excitation with 430 nm. **G,** Interaction between stably expressed ER-CFP and transiently coexpressed YFP-p50 and YFP-p65 in U2-OS#208 upon treatment with 5 ng/ml TNFα, with or without 10^{-8} M E2. **H,** The interaction between ER-CFP, YFP-p50 and YFP-p65 is greatly reduced when YFP-p65Δ22-248 is coexpressed instead of wild type YFP-p65. In all graphs values were significantly different (P<0.05) from control values of unstimulated cells, unless indicated (*) as determined by two-tailed Student's t-Test.Localization of FRET in nuclei of U2-OS cells.

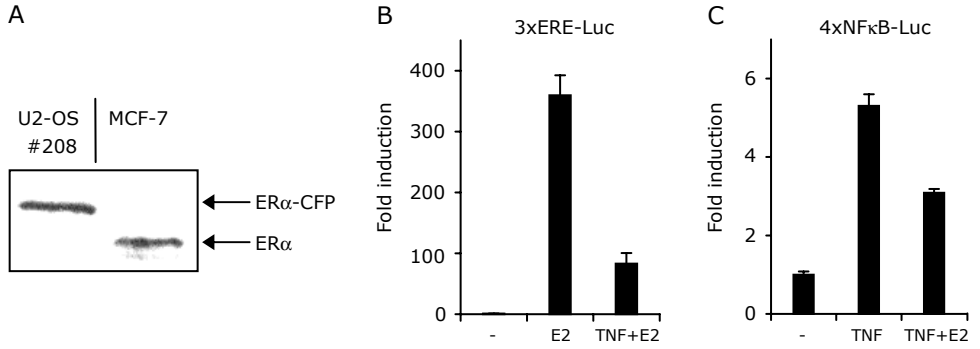

Figure 5. Characterization of U2-OS clone stably expressing ER-CFP (U2-OS#208).
A, Comparison of expression level of ER-CFP in U2-OS#208 with the expression level of ERα endogenously expressed in the breast cancer cell line MCF-7. The expected size of the proteins is indicated on the left and was verified using a molecular weight marker. **B,** E2-induced transcriptional activity of stably expressed ER-CFP and repression by TNFα determined by luciferase reporter assay in U2-OS#208. Fold induction indicates luciferase activity of the transiently transfected ERE reporter induced by the stimuli over untreated cells. **C,** Repression of TNFα-induced NF-κB activity by stably expressed ER-CFP, upon E2 treatment, determined by luciferase reporter assay in U2-OS#208. Fold induction indicates luciferase activity of the transiently transfected NF-κB reporter induced by stimuli over untreated cells.

In a previous study we showed that the Rel homology domain (RHD) of p65 is essential for *in vitro* interaction with the glucocorticoid receptor (22). Moreover, it has been reported that the RHD of p65 was sufficient for the *in vitro* interaction with ER (23). Therefore, we generated a deletion construct of YFP-p65, lacking amino acids 22 to 248 comprising a large part of the RHD (YFP-p65Δ22-248). Expression and functionality of p65Δ22-248 has been published previously (22) and fusion of YFP to the N-terminus did not change its functionality (Fig. 6A-C). Coexpression of YFP-p65Δ22-248 instead of wild type YFP-p65 resulted in a strong reduction of the E2-induced interaction between ERα and NF-κB in our FRET analysis (Fig.4H). Moreover, the effect of estrogen on colocalization was not observed with the RHD deletion mutant YFP-p65Δ22-248 (Fig. 6D).
Taken together, our FRET analysis shows that ERα directly interacts with NF-κB in living U2-OS cells. This interaction is observed in the nucleus and is strongly enhanced in the presence of E2, which is in agreement with functional data obtained from reporter assays in U2-OS.

DISCUSSION

Repression of NF-κB transcriptional activity by ER has been described to occur in a variety of cell types, including osteoblasts, macrophages, hepatoma cells, breast cancer cells and cardiac myocytes. Moreover, in human diseases and in several animal models for specific diseases, estrogen has antiinflammatory activity which is attributed to ER-mediated inhibition of NF-κB activity (4). Furthermore, it has been established that estrogen plays an important role in bone physiology by maintaining bone mass, which is thought to be due to downregulation of NF-κB mediated cytokine production. This becomes evident in postmenopausal women where estrogen deficiency results in rapid bone loss and consequently a higher risk for developing osteoporosis, which appears to be associated with increased levels of circulating proinflammatory cytokines (10).

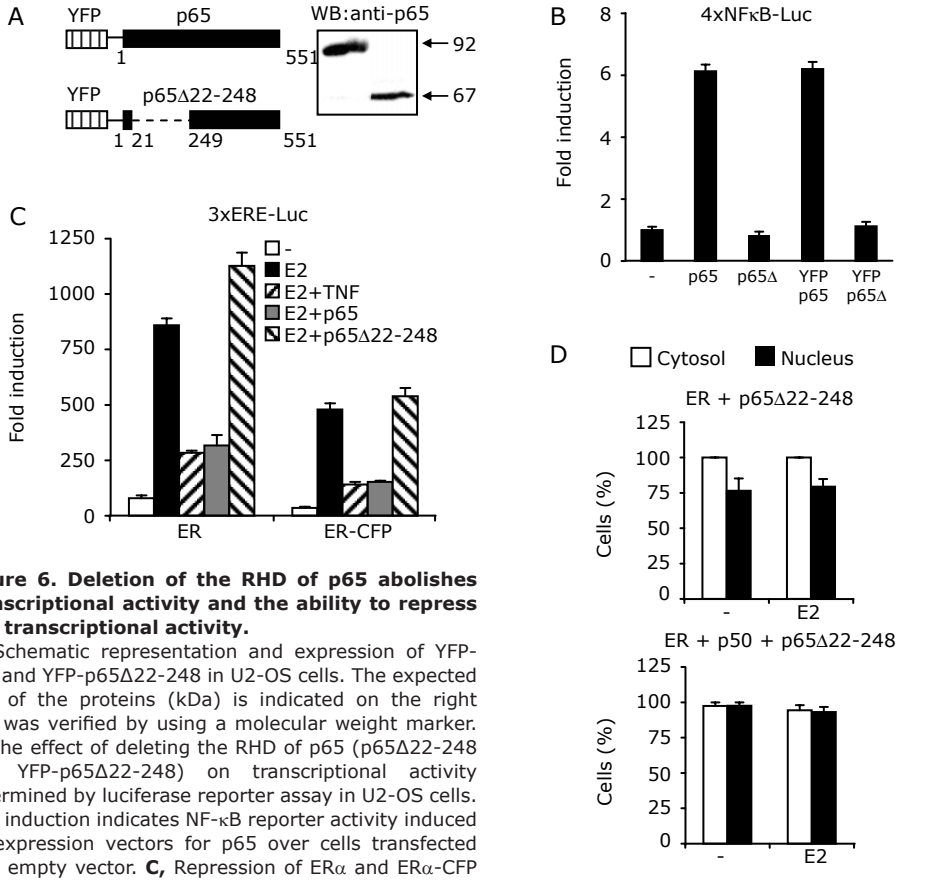

Figure 6. Deletion of the RHD of p65 abolishes transcriptional activity and the ability to repress ERα transcriptional activity.
A, Schematic representation and expression of YFP-p65 and YFP-p65Δ22-248 in U2-OS cells. The expected size of the proteins (kDa) is indicated on the right and was verified by using a molecular weight marker. **B,** The effect of deleting the RHD of p65 (p65Δ22-248 and YFP-p65Δ22-248) on transcriptional activity determined by luciferase reporter assay in U2-OS cells. Fold induction indicates NF-κB reporter activity induced by expression vectors for p65 over cells transfected with empty vector. **C,** Repression of ERα and ERα-CFP transcriptional activity by p65 analyzed by luciferase reporter assay in U2-OS cells. Cells were cotransfected with empty vector and left untreated (white bars) or treated with E2 alone (black bars) or in combination with 5 ng/ml TNFα (hatched bars) or cells were co-transfected with expression vectors for p65 or YFP-p65 (grey bar) or with p65Δ22-248 or YFP-p65Δ22-248 (double hatched bar) and treated with E2. Fold induction indicates ERE reporter activity induced by ER over untreated cells transfected with only empty vectors. **D,** Localization of YFP fusion proteins in U2-OS cells coexpressing ER-CFP and YFP-p65Δ22-248 alone (left) or in combination with YFP-p50 (right), before and after treatment with E2. Graphs represent the percentage of cells positive for YFP expression in the cytosol (grey bars) and YFP expression in the nucleus (black bars). Total number of YFP positive cells analyzed was set at 100%.

To explain the molecular basis by which ER and other steroid receptors, mediate repression of NF-κB activity, both indirect and direct mechanisms have been proposed. It appears that the mechanisms involved vary between studies and depend on promoter context, cell type and stimuli that were investigated. Indirect effects include competition for a limited amount of coactivators shared by both pathways (24) and induction of IκB expression levels (25). In this study, we use U2-OS cells to investigate ER/NF-κB cross-talk. Since previous work has shown that this cross-talk is not the result of indirect mechanisms (21, 23), it must be the result of a direct mechanism such as inhibition of NF-κB DNA binding or interference with components of the NF-κB transcriptional complex (4, 15, 26, 27). These direct mechanisms are believed to be the result of physical interaction between NF-κB and ER. Although, it has

been found that ERα can directly bind to p65, p50 and c-Rel *in vitro* via GST pull down (23, 28), the only *in vivo* evidence for an interaction between ERα and NF-κB was seen in the use of a fusion protein between c-Rel and the ligand binding domain of ERα that is functionally inhibited by estrogen, presumably through an intramolecular interaction (28). Here, we report that ERα is coimmunoprecipiated with p65 when expressed as either a homodimer or a heterodimer with p50 from E2 treated U2-OS cell extracts showing that ERα can interact with NF-κB *in vivo*. This is in agreement with data obtained from reporter assays showing a functional interaction between ERα and NF-κB in U2-OS cells. Moreover, we determined *in vivo* interaction between ERα and the NF-κB subunits p50 and p65 in living U2-OS cells by using FRET. It has been shown before that FRET is an effective method for detecting ligand-induced interactions between ERα and other proteins including the transcription factor Sp1 and the coactivators SRC1 and PBP (29, 30). In this study, we measured FRET by applying spectral analysis, which is a quantitative and extremely sensitive method. However, several precautions must be taken into account to avoid misinterpretation of results. Most importantly, it is necessary to establish equal expression levels of interacting donor and acceptor proteins. Under all circumstances this was carefully controlled and confirmed by measuring the ratio of CFP and YFP emission intensities for every individual single cell. We also confirmed that expression and transcriptional properties of the fluorescent fusion proteins ER-CFP, YFP-p65 and YFP-p50 were comparable to wild type proteins in U2-OS cells. Using FRET we observed maximal interaction when ER-CFP was coexpressed with both YFP-p65 and YFP-p50 and cells were treated with E2. This is in agreement with the observation that all three proteins are colocalized only under this condition, indicating that this is the result of interaction between ERα and NF-κB. In contrast, when the RHD deletion mutant YFP-p65Δ22-248 is coexpressed with ERα-CFP and YFP-p50 and cells are treated with E2, we do not observe colocalization and the FRET signal is strongly reduced. From this we can conclude that the RHD of p65 is required for the *in vivo* interaction between ERα and NF-κB, which is consistent with *in vitro* data.

We generated a stable U2-OS cell line expressing ER-CFP at a level equal to endogenously expressed ERα in breast cancer cells. In this cell line we were able to verify the interaction with YFP-p50 and YFP-p65. It appeared that fluorescence intensity levels of ER-CFP transiently expressed in U2-OS were only about 3-fold higher than of the stable U2-OS clone. Therefore we can conclude that the observed interaction between ERα and NF-κB, expressed both in a transient and stable fashion, are occurring at physiological expression levels and are not due to overexpression.

In summary, we demonstrate for the first time that ERα and NF-κB interact *in vivo* by using coimmunoprecipitation and FRET analysis. We show that the interaction is observed in the nucleus upon estrogen treatment in living U2-OS cells expressing ERα at physiological levels. Using ERα as a model, these data provide a strong basis for the protein-protein interaction model for cross-talk between steroid receptors and NF-κB. In addition, our observations enable *in vivo* testing of newly developed ER ligands for their ability to induce interaction between ERα and NF-κB. This could be an important contribution to the development of estrogen-like compounds with antiinflammatory properties for the treatment of diseases, such as osteoporosis, without side effects observed with current estrogen therapy (13, 31).

MATERIALS AND METHODS

Transfection and Reporter Assays

U2-OS cells were cultured and transiently transfected with DNA constructs, using calcium-phosphate precipitation, as described previously (21). For studying NF-κB transcriptional activity, cells were cotransfected with 100 ng 4xNF-κB(HIV)-tk-Luc and expression vectors encoding p50 or p65 (10 ng and 2.5 ng, respectively) or empty expression vectors. Repressive effects of ERα on NF-κB activity were assessed by cotransfecting 50 ng ERα expression vector. ERα transcriptional activity was analyzed by cotransfecting 1.0 μg 3xERE-tata-Luc and 200 ng ERα or empty expression vector. Each transfection also contained 200 ng SV_2-LacZ plasmid as an internal reference for transfection efficiency; pBluescript SK^- was added to obtain a total amount of 2 μg DNA/well. After 16 h, cells were treated with vehicle (0,1% EtOH), 10 nM E2 and/or 5 ng/ml TNFα. Cells were harvested 24 h later and assayed for luciferase and β-galactosidase activity. Data are represented as mean values ± SEM from al least three independent experiments assayed in triplicate. For FRET analysis cells were transfected as described above, with 60 ng ER-CFP in combination with 70 ng YFP-p50 alone, 75 ng YFP-p65 alone or 40 ng YFP-p50 together with 50 ng YFP-p65. pBluescript SK^- was added to obtain a total amount of 3.3 μg DNA/well. Methods to obtain stable transfectants of U2-OS expressing ER-CFP were described previously (21).

Plasmids

Plasmids used were described before (21, 22), except expression vectors encoding fluorescent fusion proteins. To create an expression vector containing enhanced cyan fluorescent protein (ECFP) fused to the C-terminus of human ERα, PCR was performed using the forward primer 5′-GGAATTCATGGTGAGCAAGGGCGAGGAG-3′ and the reverse primer 5′-TCCCTCGAGTTAGATATCCTTGTACAGCTCGTCCATGCC-3′ to generate the ECFP fragment with an *Eco*RI (5′) and a *Xho*I (3′) restriction site (underlined in primer sequence). After digestion of this fragment with *Eco*RI and *Xho*I restriction enzymes it was cloned into the modified mammalian expression vector PSG5(neo) containing a multiple cloning sequence and the neomycin resistance gene (gift of Dr. E. Sonneveld, Amsterdam, The Netherlands). An ERα fragment with a *Spe*I (5′) and an *Eco*RI (3′) restriction site was made by using the forward primer 5′-AAACTAGTATGACCATGACCCTCCACACCAAAG-3′ and the reverse primer 5′-AAGAATTCGACTGTGGCAGGGAAACCCTCTG-3′ and after digestion it was cloned into PSG5(neo)- ECFP. Expression vectors for human p50 and p65 with enhanced yellow fluorescent protein (EYFP) fused to the N-terminus, were made by made by cutting the EYFP PCR fragment with *Eco*RI and *Eco*RV (forward primer 5′-GGAATTCCACCATGGTGAGCAAGGGC-3′ and reverse primer 5′-TCCCCCGGGTTAGATATCCTTGTACAGCTCGTCCATGCC-3′) and cloning into PSG5(neo). pBluescript SK^- containing p50 was cut with *Eco*RI and after filling-in with the Klenow enzyme, the p50 fragment was cut with *Not*I and cloned into PSG5(neo)-EYFP. PGEX-3X containing p65 was partially cut with *Sma*I and cloned into PSG5(neo)-EYFP. The expression vector for YFP-p65Δ22-248 was made partially cutting PGEX-3X containing p65Δ22-248 with *Sma*I and cloning into PSG5(neo)-EYFP.

Coimmunoprecipitation and Western Blotting

Cells were transiently transfected with a 1:5 mixture of DNA and linear polyethylenimine MW 25K (L-PEI; Polysciences, Warrington, PA). This mixture consisted of serum free medium with 2.5 μg pFlag-CMV-2-p65 (16), 5μg pSG5-ERα and 2.5 μg CMV-4 p50 or empty vectors, as indicated, and 52.5 μl L-PEI (1 mg/ml) and was incubated at room temperature for 15 minutes before adding to the cells. After two days cells were treated with 10 nM E2 for 3 h before harvesting. Whole cell extracts were made by resuspending pelleted cells in hypertonic buffer (20 mM HEPES pH 8, 630mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 % NP-40, 1x proteinase inhibitor cocktail). Samples were freeze-thawed three times, incubated on ice for 30 min and cellular debris was removed by centrifugation. Supernatants were diluted ten times in IP buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) and E2 was added to a final concentration of 10 nM. After preclearing with protein G-agarose, extracts were incubated for 5h at 4°C with 4 μg of M2 anti-Flag antibody (Sigma, St. Louis, MO) preabsorbed to protein G-agarose. Immunoprecipitates were washed four times with wash buffer (24 mM Tris-HCl pH 7.5, 2mM KCl, 163mM NaCl) and the pellets were boiled in Laemmli sample buffer. Proteins were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies against p65, p50 (sc-109 and sc-114, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) or ERα (Ab-15; Lab Vision, Fremont, CA) according to manufacturer's protocols. After incubation with peroxidase-conjugated secondary antibody, immunoreactive bands were visualized using enhanced chemiluminesence.

FRET Analysis

A Leica DMR upright microscope (Leica Microsystems, Wetzlar, Germany) was used, equipped with a fluorescence system, a 63x/0.9 U-V-I long distance water-immersion objective and a temperature-controlled specimen holder at 33°C. Cells were kept in DMEM without phenol-red, supplemented with 10 mM HEPES (pH 7.4). As an excitation source, a SPEX Fluorolog (Spex Industries, Edison, NJ) was used. The light from the excitation monochromator was coupled into the microscope via an UV fiber optic cable (Schott, Mainz, Germany). The emission fluorescence from the microscope was collected (via another fiber optic cable) and fed back into the emission monochromator of the SPEX Fluorolog. Spectral analysis of CFP and the FRET (430 nm excitation) was performed with a >455 nm dichroic mirror (Chroma Technology, Rockingham, VT). Separate YFP spectra (excitation 490 nm) were collected with a dichroic mirror >510 nm. Spectral data were recorded with an integration time of 0.5 second/nm, 1 nm step resolution, 8 nm slit width. Emission wavelength ratios ($R_{525/480}$) of a single experiment are averages of 10-20 individual single cell spectra. Each experiment was repeated at least two times. For every single cell FRET spectrum (excitation 430 nm), a separate spectrum of YFP (excitation 490 nm) from the same cell was collected as a control for expression levels of CFP and YFP constructs. The molar extinction coefficients, $\varepsilon_{CFP}(430)$, $\varepsilon_{YFP}(490)$ are respectively, 32.5 and 55.3 x 10^3 $M^{-1}cm^{-1}$. As a consequence only cells with intensity ratios CFP/YFP within 1.6–2.2 were taken into account in order to avoid the formation of low donor/acceptor pairs (R<1.6) and to avoid unspecific bleed-through of 430 nm excited YFP at ratios R> 2.2. FRET images of Supplementary Figure S1A were captured with an Image Intensifier (Delft Electronic Products, Roden, The Netherlands) coupled to a progressive scan CCD camera CV-M10RS (JAI, Copenhagen, Denmark) with a frame capture board AG5 (Scion, Frederick, MD). The images were processed with a program (written in the Pascal macro language of Scion Image) based on the algorithms of Xia *et al* (20).

Cellular Localization of Fluorescent Proteins

Images of YFP fluorescence were collected with a Leica NT confocal system (Leica Microsystems, Mainz, Germany) with a 40x objective (same detection photomultiplier settings in all experiments). Maximum projections of Z-series (10 slices) were made. For each experimental condition, 100-120 cells were counted. Values are expressed as percentage of fluorescence present in either cytosol or nucleus of the total number of cells found in one image.

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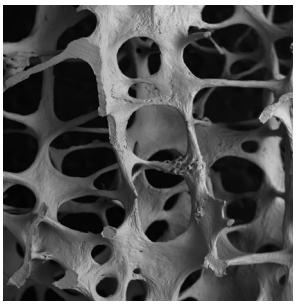
Chapter 5



Transcriptional Profiling of TNFα-Induced Genes Repressed by Estrogen Receptor α in U2-OS Cells

In preparation

Transcriptional Profiling of TNFα-Induced Genes Repressed by Estrogen Receptor α in U2-OS Cells

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The estrogen receptor (ER) is not only capable of regulating gene expression through direct DNA binding, but also by modulating the activity of other transcription factors, such as NF-κB. In this study we focused on cross-talk of ERα with the tumor necrosis factor-α (TNFα) pathway, in order to investigate transcriptional interference with NF-κB. Inhibition of NF-κB transcriptional activity by ER is particularly relevant in bone physiology, where ER opposes NF-κB-induced expression of cytokines that promote bone resorption. Through microarray analysis we show that in U2-OS cells stably expressing ERα, expression of a large group (40%) of TNFα-induced genes is inhibited upon stimulation with an estrogenic ligand. We compared the effect of four different ligands: the natural agonist 17β-estradiol (E2), two selective estrogen receptor modulators, 4-hydroxytamoxifen and raloxifene, and the antagonist ICI 182,780. It became clear that E2 was the most potent repressor of these four ligands. The function of the genes selectively regulated by E2 could largely be assigned to immune and inflammatory responses and appeared to be mainly regulated by NF-κB. Therefore we propose that ERα-mediated transcriptional interference through tethering to the regulatory region of NF-κB target genes is an important mode for inhibiting TNFα-induced gene expression in U2-OS cells.

INTRODUCTION

Regulation of eukaryotic gene transcription is accomplished through the formation of multiprotein complexes that determine the rate and amplitude of mRNA synthesis. An essential step in the process of gene regulation is the binding of specific transcription factors to their cognate consensus binding site, which is part of the regulatory region proximal to target gene promoters (1). A sequence-specific transcription factor can act via three classes of response elements: simple, composite and tethering. Gene regulation mediated by a simple response element requires only the binding of a single transcription factor, while composite response elements contains additional binding sites for other transcription factors that can modulate each others activity. Via a tethering response element the transcription factor is recruited to the element without direct DNA binding via protein-protein interactions with a different DNA-bound transcription factor (2).

The estrogen receptor (ER) belongs to the nuclear receptor (NR) super family, which are hormone-inducible transcription factors recognizing specific hormone responsive elements (HRE) in target promoter regions. According to the conserved NR structure, ER can be divided in three functional domains consisting of a N-terminal domain containing activation function (AF)-1, a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) containing AF-2 (3). There are two different ER subtypes, termed ERα and ERβ (4, 5)**,** that are both capable of modulating gene transcription upon binding of various hormones, including the natural estrogen 17β-estradiol (E2), synthetic selective estrogen receptor modulators (SERMs) and synthetic estrogen agonists (6). Upon hormone binding, ER undergoes a conformational change that subsequently enables the receptor to bind DNA and to recruit coregulatory molecules (coactivators and corepressors) and the basal transcription machinery through AF-1 and/or AF-2 (7, 8). Gene regulation by ER can be accomplished through direct DNA binding via estrogen response elements (EREs) or through indirect tethering to DNA via interaction with other transcription factors, such as Sp1, AP-1 and NF-κB (9-14).

The strength of the response and positive or negative regulation of gene expression induced by ER depends on various factors, including 1) the composition of the *cis*-regulatory region; 2) ER subtype; 2) nature of the bound ligand (determining receptor conformation and therefore coregulator recruitment); 3) availability of coregulator molecules (which is cell-type dependent) (1, 15).

The use of microarray technology enables to investigate global genomic responses under a given condition due to the availability of expression levels of a large group of genes (almost) completely covering the whole transcriptome. Recently, several studies using microarray analysis reported the identification of global gene expression profiles resulting from treatment with estrogenic compounds of various cell lines expressing ER (16-23). These expression profiles form the basis for further research necessary to understand the complex gene regulatory network that is involved in ER-mediated transcription. These profiles include genes directly regulated by ER through EREs or indirectly via interference or cooperation with other transcription factors.

The aim of this study was to focus on cross-talk of ER with a different signal transduction pathway, the tumor necrosis factor-α (TNFα) pathway, in order to investigate transcriptional interference with transcription factor NF-κB.

TNF is a pro-inflammatory cytokine that plays a central role in mediating immune and inflammatory responses. Although TNF signaling is essential in host defense against invasions by pathogens, it is also involved in a variety of human diseases, including diabetes, cancer, osteoporosis, rheumatoid arthritis and inflammatory bowel disease (24-27). Blocking TNF-

signaling has been proven to be an effective way to treat several of these diseases. TNF elicits its function via interaction with its cognate membrane receptor belonging to TNF receptor family, which subsequently activates several signaling pathways that control a network of genes involved in immune and inflammatory responses and cell proliferation, differentiation and apoptosis. A major signal transduction pathway induced by TNF involves the activation of the transcription factor nuclear factor κB (NF-κB) (28, 29).
In several (mouse models of) human diseases, such as osteoporosis, rheumatoid arthritis and inflammatory bowel disease, estrogens are shown to inhibit inflammatory activity resulting in prevention or relieve of pathogenesis (30-32). The anti-inflammatory properties of estrogen are attributed to ER-mediated inhibition of NF-κB transcriptional activity, which is thought to be due to tethering of ER to NF-κB target genes trough protein-protein interactions (13).
Here we show through microarray analysis that, upon stimulation with an estrogenic ligand of U2-OS cells stably expressing ERα, the expression of a large group (40%) of the TNFα-activated genes is inhibited. We compared the effect of four different ligands: the natural agonist 17β-estradiol (E2), two selective estrogen receptor modulators (SERMs), 4-hydroxytamoxifen (OH-T) and raloxifene (Ral), and the antagonist ICI 182,780 (ICI). It became clear that E2 was the most potent repressor of the four ligands that were used. We determined whether there were overlapping gene sets between the four ligands and out of all possible combinations there were only two sets that comprised a large group of genes: the set downregulated only by E2 and the set downregulated by all four compounds. The function of the genes selectively regulated by E2 could largely be assigned to immune and inflammatory responses. The group of genes repressed by all four ligands largely represented other functions than the E2-set and these could not be directly linked to inflammation.

RESULTS AND DISCUSSION

Identification of genes upregulated by TNFα in U2-OS cell using microarray analysis

To identify TNFα-activated genes that can be repressed by estrogenic compounds, we used a U2-OS cell line stably expressing ERα (U2OS-ERα), which was used previously to study inhibition of TNFα-induced NF-κB activity through ERα (33). U2OS-ERα cells were treated with vehicle or 5ng/ml TNFα and after 8 h total RNA was isolated in order to generate labeled cRNA for hybridization with HG-U133A 2.0 GeneChips (Affymetrix). These microarrays enable analysis of the expressions level of 18.400 transcripts and variants, including 14.500 well-characterized human genes. By using Rosetta Luminator software, we identified 283 known and 106 unknown genes that were significantly upregulated by TNFα (P value ≤0.05 and fold change ≥1.75; supplementary file "TNFup.xls" is available upon request). In order to determine the physiological relevance of the genes in this list, we made use of the "Web-based Gene Set Analysis Toolkit" (Webgestalt; http://genereg.ornl.gov/webgestalt). The GO-tree module (GOTM) enabled us to identify Gene Ontology (GO) categories with enriched gene numbers in the TNFup-set compared to a reference set (e.g. the list of genes on the array). A GO categorie is enriched if the observed number of genes in this category (from the TNFup list) exceeds the expected number of genes. GOTM reports only those enrichments that are statistically significant as determined by the hypergeometric test (P value ≤0.01; ≥ 2 genes / category). GO terms are organized into three independent main categories: biological process, molecular function and cellular component and P values are calculated for each of the categories separately. The output of the GOTM analysis is a GoTree, which is an expandable tree with a node for each category. All 283 known genes were used to build the GoTree and this resulted in a tree consisting of 1240 GO categories. However,

only 123 categories were identified by GOTM as significantly enriched (supplementary file "enriched_GOcategories.doc" is available upon request). The GOTree can be visualized as a Directed Acyclic Graph (DAG), which is shown here for the main category "molecular function" in Figure 1 (DAG for all three main categories is available upon request). From the 283 known genes of the TNFup list, 244 genes were identified as significantly enriched in the main category "biological process", 194 genes in "molecular function" and 77 in "cellular component", which make up 86%, 69% and 27%, respectively of the TNFup set. In total these enrichments represent 261 different genes, which make up 92% of the TNFup gene set.

An alternative way to study the TNFup gene list with Webgestalt is using the module that organizes the gene list based on biochemical pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG). The output of this analysis is a KEGG table that shows all pathways in which at least 2 genes, involved in a certain pathway, are present in the TNFup set (supplementary file "KEGG_TNFup.doc is available upon request). Again the hypergeometric test is used to determine statistical significance of enrichment of individual pathways (P value ≤0.05). Twelve KEGG pathways were determined as significantly enriched in the TNFup list and contained a total number of 115 genes (Table 1). Since some genes were present in several pathways, the total number of different genes was 59 (Table 2). KEGG biochemical pathways are organized in categories according to the KEGG orthology (orthology for TNFup enriched pathway is available upon request). Among the twelve enriched pathways were six signal transduction pathways, including the Toll-like receptor, Jak-STAT, B cell receptor, TGFβ, T cell receptor and adipocytokine signaling pathways (3, 5, 7, 8, 11 and 12, respectively, in Table 1). Although, the TNFα signal transduction pathway is not included in the KEGG orthology, four of the six enriched pathways, however, involve induction of the NF-κB pathway, which is the result of TNFα stimulation. Furthermore, also the apoptosis pathway involves NF-κB signaling and for all other pathways many of the enriched genes involved have been described previously to be upregulated by TNFα mediated by activation of NF-κB through microarray analysis (34-37) or promoter analysis (summary of published NF-κB target genes can be found at http://people.bu.edu/gilmore/nf-kb/target/index.html). The KEGG pathway "cytokine-cytokine receptor interaction" contained the largest set of genes present in the TNFup set and is shown in more detail in Figure 2 as a KEGG Map (an overview of all KEGG pathways is available upon request). Taken together, upon stimulation of U2OS-ERα cells with TNFα of a large group of genes is upregulated that comprises a group of biologically relevant genes as determined through GO annotation (92%) and KEGG pathway analysis (20%).

Table 1. Significantly enriched KEGG pathways associated with the TNFup gene set.

#	KEGG pathway	Gene number	Enrichment
1	Cytokine-cytokine receptor interaction	28	O=28;E=6.4;R=4.4;P=4.0E-11
2	Focal adhesion	13	O=13;E=6.1;R=2.1;P=0.008
3	Toll-like receptor signaling pathway	11	O=11;E=2.5;R=4.4;P=3.7E-05
4	Apoptosis	10	O=10;E=2.6;R=3.8;P=0.0003
5	Jak-STAT signaling pathway	10	O=10;E=3.9; R=2.6;P=0.006
6	Ribosome	8	O=8;E=2.3; R=3.5;P=0.002
7	B cell receptor signaling pathway	6	O=6;E=1.8; R=3.3;P=0.009
8	TGF-β signaling pathway	6	O=6;E=2.2; R=2.7;P=0.03
9	ECM-receptor interaction	6	O=6;E=2.2; R=2.7;P=0.02
10	Hematopoietic cell lineage	6	O=6;E=2.4; R=2.5;P=0.03
11	T cell receptor signaling pathway	6	O=6;E=2.6; R=2.3;P=0.05
12	Adipocytokine signaling pathway	5	O=5;E=1.9; R=2.7;P=0.04

O is the observed gene number in the KEGG pathway; E is the expected gene number in the KEGG pathway (Expected number of genes in a specific KEGG pathway for an interesting gene set=Total number of genes in the KEGG pathway for the reference set * Total number of genes in the interesting set / Total number of genes in the reference set); R is the ration of enrichment for the KEGG pathway (R=O/E); P is the p value indicating the significance of enrichment calculated from Hypergeometric test. Gene information is presented in Table 2.

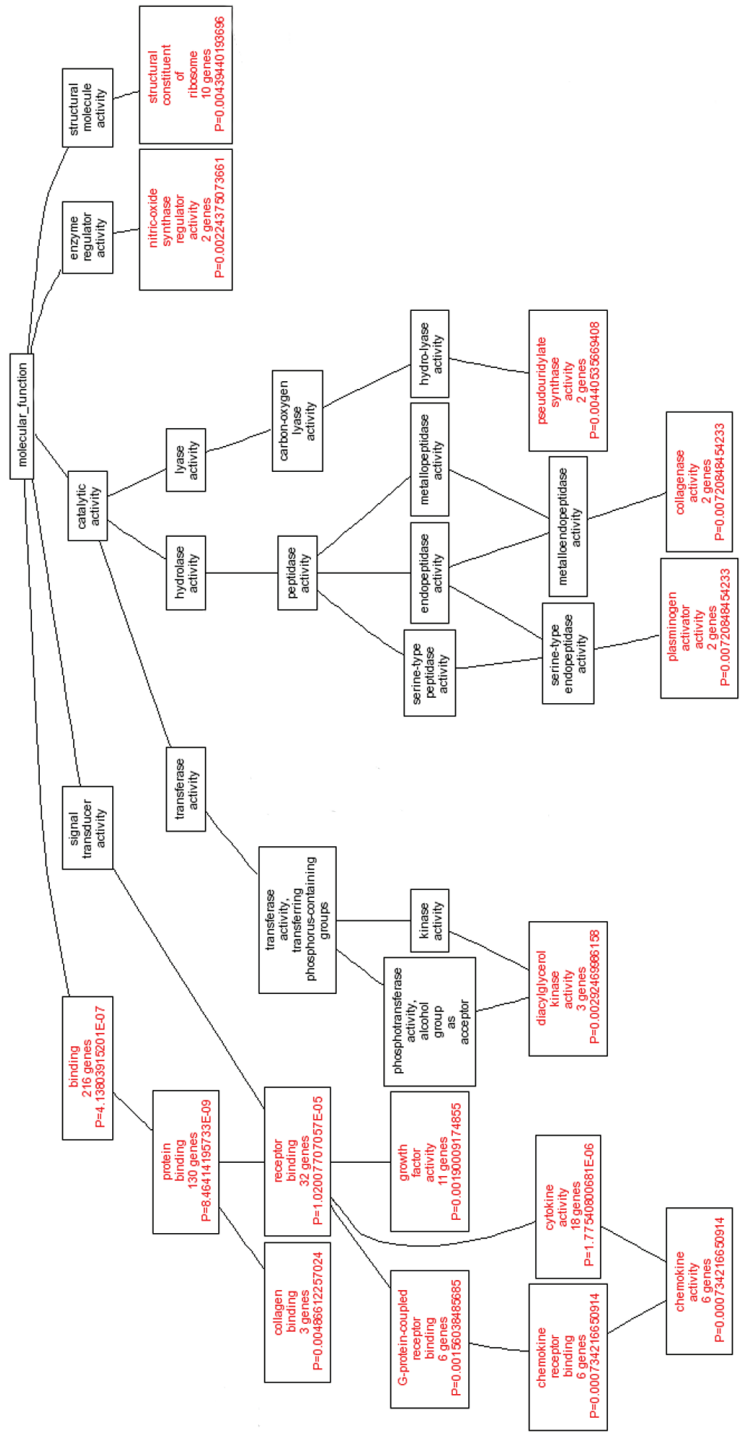

Figure 1. Enriched Directed Acyclic Graph (DAG) for the main category "molecular function".
Each GO category is a node in the graph. Categories in red are enriched and show the name of the GO category, number of genes in the category and the p value indicating the significance of enrichment. Categories in black are non-enriched parents.

Table 2. Genes present in the enriched KEGG pathways associated with the TNFup set.

Entrez Gene ID	Gene Symbol	Gene Name	Fold change
329	BIRC2	baculoviral IAP repeat-containing 2	2.5
330	BIRC3	baculoviral IAP repeat-containing 3	80.2
355	FAS	Fas (TNF receptor superfamily, member 6)	1.8
637	BID	BH3 interacting domain death agonist	4.2
650	BMP2	bone morphogenetic protein 2	10.7
653	BMP5	bone morphogenetic protein 5	2.0
958	CD40	CD40 antigen (TNF receptor superfamily member 5)	2.2
960	CD44	CD44 antigen (homing function and Indian blood group system)	3.4
970	TNFSF7	tumor necrosis factor (ligand) superfamily, member 7	4.2
1435	CSF1	colony stimulating factor 1 (macrophage)	7.4
1437	CSF2	colony stimulating factor 2 (granulocyte-macrophage)	12.3
1634	DCN	decorin	3.6
1896	EDA	ectodysplasin A	2.4
1956	EGFR	epidermal growth factor receptor	3.4
3460	IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)	2.0
3566	IL4R	interleukin 4 receptor	3.6
3572	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	4.8
3575	IL7R	interleukin 7 receptor	6.4
3576	IL8	interleukin 8	100.0
3600	IL15	interleukin 15	1.8
3604	TNFRSF9	tumor necrosis factor receptor superfamily, member 9	39.4
3624	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)	5.0
3626	INHBC	inhibin, beta C	2.6
3627	CXCL10	chemokine (C-X-C motif) ligand 10	7.0
3673	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	2.8
3693	ITGB5	integrin, beta 5	2.0
3914	LAMB3	laminin, beta 3	2.8
3918	LAMC2	laminin, gamma 2	4.7
3976	LIF	leukemia inhibitory factor (cholinergic differentiation factor)	2.1
4050	LTB	lymphotoxin beta (TNF superfamily, member 3)	19.5
4233	MET	met proto-oncogene (hepatocyte growth factor receptor)	2.8
4609	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	3.4
4790	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	4.3
4791	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	8.1
4792	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	9.9
4794	NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	10.4
5293	PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide	3.8
5606	MAP2K3	mitogen-activated protein kinase kinase 3	3.0
5880	RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	2.1
6157	RPL27A	ribosomal protein L27a	4.9
6168	RPL37A	ribosomal protein L37a	5.2
6169	RPL38	ribosomal protein L38	3.3
6181	RPLP2	ribosomal protein, large, P2	15.1
6204	RPS10	ribosomal protein S10	4.1
6205	RPS11	ribosomal protein S11	8.0
6223	RPS19	ribosomal protein S19	15.0
6224	RPS20	ribosomal protein S20	3.2
6347	CCL2	chemokine (C-C motif) ligand 2	100.0
6351	CCL4	chemokine (C-C motif) ligand 4	32.0
6354	CCL7	chemokine (C-C motif) ligand 7	14.8
6374	CXCL5	chemokine (C-X-C motif) ligand 5	6.2
7424	VEGFC	vascular endothelial growth factor C	5.0
8471	IRS4	insulin receptor substrate 4	2.6
8795	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	2.7
8837	CFLAR	CASP8 and FADD-like apoptosis regulator	1.9
9641	IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	7.0
11009	IL24	interleukin 24	12.0
50509	COL5A3	collagen, type V, alpha 3	2.2
57396	CLK4	CDC-like kinase 4	1.8

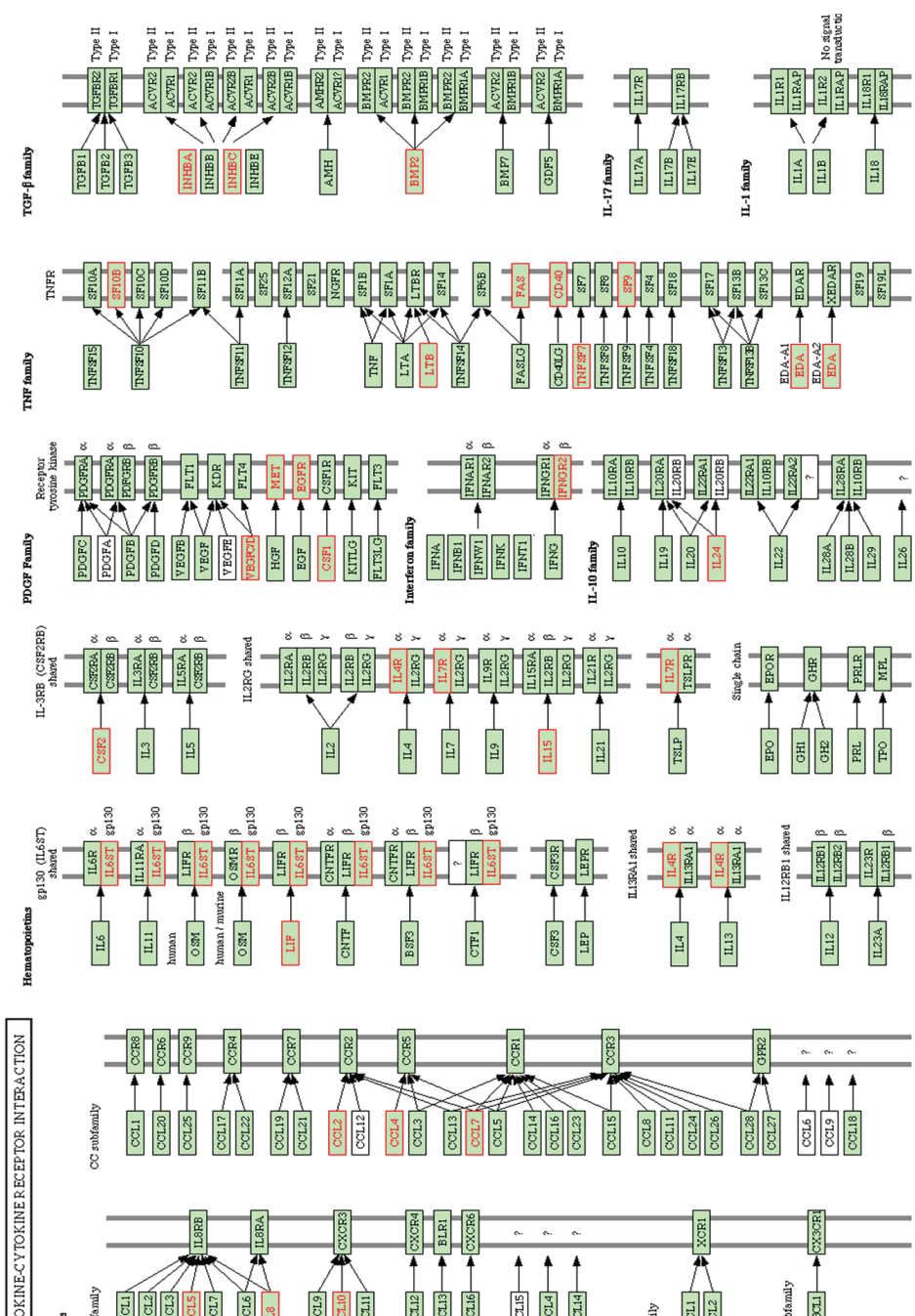

Figure 2. KEGG Map for the KEGG pathway "cytokine-cytokine receptor interaction".
This pathway contains the largest set of genes present in the TNFup set, which are highlighted in red in the Map.

Downregulation of TNFα-upregulated genes upon stimulation of U2OS-ERα cells with estrogenic compounds

The aim of this study was to identify genes upregulated by TNFα that are repressed by ERα in the presence of an estrogenic compound. We chose to determine the effect of the agonist 17β-estradiol (E2), the partial agonist/antagonists 4-hydroxytamoxifen (OH-T) and raloxifene (Ral), and the full antagonist ICI 182,780 (ICI) on the expression of TNFα-upregulated genes in U2OS-ERα cells. Therefore, cells were pre-incubated for 1 h with 10 nM E2, 1 μm OHT, Ral or ICI and subsequent stimulated for 8h in combination with TNFα. By using Rosetta Luminator we determined that, out of the 389 genes upregulated by TNFα, 121 genes (83 known and 38 unknown) were downregulated by E2 (P value ≤0.05 and fold change ≥1.75). For OH-T, 78 genes (47 known and 31 unknown) and for Ral only 72 genes (44 known and 28 unknown) were repressed. Surprisingly, we observed a group of 82 genes (45 known and 37 unknown) that were repressed by ICI. These four groups made up a total of 153 different genes (103 known and 50 unknown), which is 40% of the complete TNFup set (separate and combined list of downregulated genes are available upon request).

To obtain more insight in the set of downregulated genes we assessed the number of overlapping genes when comparing the lists for all four compounds. The results are visualized in a Venn diagram showing all possible overlaps and the number of genes included in each separate overlap (Figure 3).

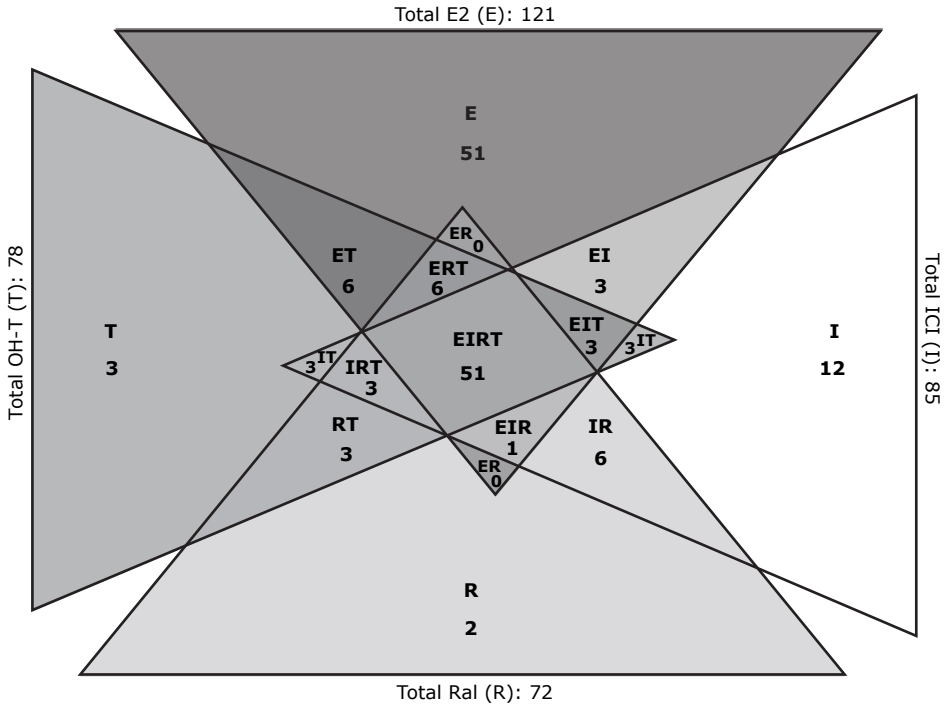

Figure 3. Venn diagram showing all possible overlaps and the number of overlapping genes. Data was obtained by comparing the separate lists for TNFα-induced genes downregulated by E2, OH-T, Ral or ICI.

Out of 15 different sets, it became evident that there were two lists that comprised a large group of genes, the set downregulated only by E2 (list E: 51 genes) and the set downregulated by all four compounds (list EIRT: 51 genes), while the other lists contained much less genes (ranging from 0 to 12 genes) (all separate lists are available upon request). Out of the 42 known genes in list E, 40 genes were present in the set of TNF-upregulated genes that were identified as significantly enriched in the three main GO categories. Next, we performed another GOtree analysis on the E-list and EIRT-list in order to identify enriched GO categories for each list. To classify the 40 genes into functional groups, we chose the following categories: immune response, peptidase activity, growth factor activity, apoptosis, signal transduction, cell adhesion, transcription factor activity and transport (Table 3). Although several genes were present in more than one category, each gene was assigned to only one category. For the EIRT-list, out of the 30 known genes 29 genes were present in the set of GO-enriched TNF-upregulated genes. To classify the 29 genes into functional groups, we assigned the genes, if possible, into the same categories as for the E-list or to the following two additional categories: protein biosynthesis and regulation of protein metabolism (Table 4). It became evident that there was only a small overlap of genes present in the same GO category from both lists (overlapping categories: cell adhesion, transport and apoptosis), indicating that most genes downregulated only by E2 have a different biological function when compared to the genes downregulated by all four compounds. On the other hand, we determined for two other overlapping groups that most of the genes did have the same function. All five known genes (bone morphogenetic protein 2, colony stimulating factor 2, neuregulin 1, laminin gamma 2 and endothelial cell-specific molecule 1) that were downregulated by both E2 and OH-T (list ET), could be assigned to the enriched categories of the E-list. For the known genes downregulated by E2, OH-T and Ral (list ERT), three out of four genes were assigned to the categories of the E-list (epidermal growth factor receptor, PDZ and LIM domain 4 and interleukin 7 receptor).

From these results we can conclude that upon stimulation of U2OS-ERα cells with TNFα about 31% of the upregulated genes are repressed by E2. In the presence of OH-T, Ral and ICI 20%, 19% and 21 %, respectively, of the TNFα-upregulated genes was repressed. We identified a group of 51 genes that was downregulated by all four compounds (E2, OH-T, Ral and ICI), which made up 42%, 65%, 71% and 62%, respectively, of the total number of genes for each compound. The number of genes repressed only by E2 also made up 42% of the complete list for E2. However, for OH-T, Ral and ICI this was 4%, 3% and 15%, respectively. Thus, from this analysis it became clear that E2 is capable of selectively repressing a large set of genes in contrast to OH-T, Ral and ICI. Furthermore, only for OH-T and Ral we identified sets of genes, which were also downregulated by E2, with almost all genes having a similar functions as for the genes downregulated by E2 only.

In a recent study employing microarray analysis, the TNFα-mediated expression profile was determined in primary human endothelial cells. Moreover, it was shown that TNFα-induced expression of virtually all genes was dependent on NF-κB, which was determined by impairments NF-κB signaling (34). Our list of TNFα-induced genes downregulated only by E2 contained nine genes that were also induced in endothelial cells by TNFα, including IL-8, inhibin βA, lymphotoxin β, sequestosome 1, plasminogen activator (urokinase), baculoviral IAP repeat-containing 3, TNFα-induced protein 3, TNF receptor-associated factor 1 and CD69 antigen, which are all dependent on NF-κB signaling (34) (Table 3). In a similar manner, two other research groups identified direct downstream target genes of NF-κB upon stimulation with TNFα of Hela cells, endothelial cells or keratinocytes (35-37). Some of the NF-κB target genes described above were also found in these study, while also additional targets were found of which several were present in our list of E2-downregulated genes, including

Table 3. Classification of genes downregulated only by E2 (from the GO enriched TNFup list).

ID	Symbol	Name	FC	TNFα/NF-κB (ref)
Immune response				
958	CD40	CD40 antigen (TNF receptor superfamily member 5)	-2.1	? /YES (38)
3576	IL8	interleukin 8	-2.2	YES/YES (34-37, 39)
3624	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)	-2.0	YES/YES (34, 36)
4050	LTB	lymphotoxin beta (TNF superfamily, member 3)	-1.7	YES/YES (34, 37, 40)
4233	MET	met proto-oncogene (hepatocyte growth factor receptor)	-1.8	? /?
5806	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	-1.8	YES/YES (41)
6351	CCL4	chemokine (C-C motif) ligand 4	-10.8	* YES/? (42)
8061	FOSL1	FOS-like antigen 1	-1.8	? /?
8878	SQSTM1	sequestosome 1	-1.8	YES/YES (34, 36)
Peptidase activity				
4314	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	-3.4	? /YES (43)
4318	MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	-3.6	YES/YES (37, 44-46)
4319	MMP10	matrix metallopeptidase 10 (stromelysin 2)	-3.1	YES/YES (37, 47)
4322	MMP13	matrix metallopeptidase 13 (collagenase 3)	-8.0	YES/YES (37, 47)
5328	PLAU	plasminogen activator, urokinase	-4.9	YES/YES (34, 35, 48)
Growth factor activity				
653	BMP5	bone morphogenetic protein 5	-2.2	? /?
1839	HBEGF	heparin-binding EGF-like growth factor	-3.5	YES/? (49)
7424	VEGFC	vascular endothelial growth factor C	-2.8	YES/YES (36, 37, 50)
Apoptosis				
330	BIRC3	baculoviral IAP repeat-containing 3	-1.8	YES/YES (34-36)
7128	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	-1.8	YES/YES (34-37)
7185	TRAF1	TNF receptor-associated factor 1	-1.8	YES/YES (34-37, 51)
8837	CFLAR	CASP8 and FADD-like apoptosis regulator	-2.0	YES/YES (36, 37)
Signal transduction				
969	CD69	CD69 antigen (p60, early T-cell activation antigen)	-4.4	YES/YES (34, 52)
3431	SP110	SP110 nuclear body protein	-1.8	YES/YES (36)
5606	MAP2K3	mitogen-activated protein kinase kinase 3	-2.5	? /?
6447	SGNE1	secretory granule, neuroendocrine protein 1 (7B2 protein)	-2.6	? /?
26499	PLEK2	pleckstrin 2	-2.0	? /?
79412	KREMEN2	kringle containing transmembrane protein 2	-1.7	? /?
Cell adhesion				
960	CD44	CD44 antigen (homing function and Indian blood group system)	-1.9	YES/? (53)
3673	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	-3.5	? /?
3914	LAMB3	laminin, beta 3	-2.0	YES /? (54)
23037	PDZK3	PDZ domain containing 3	-3.0	? /?
Transcription factor activity				
687	KLF9	Kruppel-like factor 9	-3.0	? /?
5201	PFDN1	prefoldin 1	-1.9	? /?
Transport				
7126	TNFAIP1	tumor necrosis factor, alpha-induced protein 1 (endothelial)	-2.2	YES/? (55)
9554	SEC22L1	SEC22 vesicle trafficking protein-like 1 (S. cerevisiae)	-1.9	? /?
9645	MICAL2	microtubule associated monoxygenase, calponin and LIM domain containing 2	-1.9	? /?
23327	NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like	-2.1	? /?
Other				
9469	CHST3	carbohydrate (chondroitin 6) sulfotransferase 3	-1.7	? /?
9945	GFPT2	glutamine-fructose-6-phosphate transaminase 2	-4.1	YES/YES (35)
10938	EHD1	EH-domain containing 1	-2.3	YES/? (56)

ID: identification number according to Locus Link ; FC: fold change (p-value ≤ 0.05);

TNF/NF-κB (ref): Cited in the literature as TNFα induced gene/ cited in the literature as NF-κB target gene (reference);

*: other NF-κB-activating stimulus than TNFα;?: no information available

Table 4. Classification of genes downregulated by E2, OH-T, Ral and ICI (from the GO enriched TNFup list).

ID	Symbol	Name	FC E2	FC ICI	FC Ral	FC OH-T
Protein biosynthesis						
6157	RPL27A	ribosomal protein L27a	-3.7	-3.1	-4.0	-3.9
6168	RPL37A	ribosomal protein L37a	-4.5	-4.1	-5.2	-3.7
6169	RPL38	ribosomal protein L38	-3.4	-3.3	-4.4	-3.4
6181	RPLP2	ribosomal protein, large, P2	-3.9	-19.0	-4.6	-4.4
6204	RPS10	ribosomal protein S10	-4.1	-4.5	-4.8	-3.8
6205	RPS11	ribosomal protein S11	-5.7	-3.2	-9.5	-9.1
6223	RPS19	ribosomal protein S19	-7.6	-10.9	-8.3	-9.3
6224	RPS20	ribosomal protein S20	-3.4	-3.1	-3.3	-3.0
1936	EEF1D	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	-3.9	-3.2	-3.2	-2.8
1984	EIF5A	eukaryotic translation initiation factor 5A	-2.8	-2.2	-2.9	-2.9
9669	EIF5B	eukaryotic translation initiation factor 5B	-2.3	-2.3	-2.5	-2.4
Regulation of protein metabolism						
382	ARF6	ADP-ribosylation factor 6	-2.8	-2.8	-2.9	-2.9
6418	SET	SET translocation (myeloid leukemia-associated)	-2.4	-1.8	-2.1	-2.0
6710	SPTB	spectrin, beta, erythrocytic (includes spherocytosis, clinical type I)	-6.5	-5.2	-6.5	-6.0
Cell adhesion						
1834	DSPP	dentin sialophosphoprotein	-2.0	-2.9	-1.8	-2.4
3693	ITGB5	integrin, beta 5	-3.0	-3.6	-2.9	-3.4
Transport						
7295	TXN	thioredoxin	-3.3	-3.0	-3.4	-3.4
8992	ATP6V0E	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e	-4.1	-3.7	-4.0	-3.7
Apoptosis						
4170	MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	-2.9	-3.4	-2.5	-2.8
Other						
384	ARG2	arginase, type II	-3.0	-2.6	-2.1	-2.6
811	CALR	calreticulin	-5.9	-6.4	-11.9	-6.3
988	CDC5L	CDC5 cell division cycle 5-like (S. pombe)	-2.6	-2.4	-3.0	-2.4
2348	FOLR1	folate receptor 1 (adult)	-2.4	-2.5	-2.9	-2.8
3014	H2AFX	H2A histone family, member X	-2.1	-2.8	-2.5	-2.5
3626	INHBC	inhibin, beta C	-2.8	-3.6	-3.5	-2.6
6613	SUMO2	SMT3 suppressor of mif two 3 homolog 2 (yeast)	-1.9	-2.7	-2.8	-2.1
7531	YWHAE	tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, epsilon polypeptide	-3.9	-2.8	-4.1	-3.5
55884	WSB2	WD repeat and SOCS box-containing 2	-3.5	-3.2	-3.2	-2.0
80204	FBXO11	F-box protein 11	-2.1	-2.2	-2.1	-1.9

metallopeptidase 9, matrix metallopeptidase 10, metallopeptidase 13, vascular endothelial growth factor C, SP110 nuclear body protein, CASP8 and FADD-like apoptosis regulator, and glutamine-fructose-6-phosphate transaminase 2 (35-37) (Table 3). Furthermore, classical analysis of promoters of individual genes provided evidence for transcriptional regulation by NF-κB for many separate genes, which applies to CD40 antigen (38), pentraxin-related gene (41) and matrix metallopeptidase 3 (43). Some of the genes that were designated as NF-κB target genes trough the microarray studies mentioned above, were also previously identified as such by classical promoter analysis (references are provided in Table 3). In addition, if a gene is previously described to be induced by TNFα or a different stimulus that activates the NF-κB pathway, it is likely that NF-κB is involved in regulation of the gene. This is the case for chemokine (C-C motif) ligand 4 (42), heparin-binding EGF-like growth factor (49), CD44 antigen (53), laminin β3 (54), tumor necrosis factor α-induced protein 1 (55) and EH-domain containing 1 (56) (Table 3). Taken together, out of the 40 genes in our list of TNFα-induced genes and only downregulated by E2, 19 genes are previously characterized as NF-κB target genes and five additional genes were previously shown to be induced by TNFα,

while chemokine (C-C motif) ligand 4 was induced by lipopolysaccharide (LPS). Therefore, we propose that for these 25 genes downregulation by E2 is mediated by transcriptional interference of ERα with NF-κB. For the remaining 15 genes further (functional) promoter analysis needs to be carried out in order to determine the presence of (functional) NF-κB binding sites. Furthermore, TNFα also induces activation of a second transcription factor, activator protein 1 (AP-1). Composite promoter elements may contain both NF-κB and AP-1 sites, which can cooperatively mediate transactivation of these target genes. *Cis*-regulatory regions of the genes encoding pentraxin related gene (41), matrix metallopeptidase 9 (44, 45) and CD69 antigen (52) have been described to contain AP-1 sites in addition to NF-κB binding sites, while the regulatory region for CD44 antigen contains only AP-1 sites (53). Thus, we suggest that inhibition of AP-1 transcriptional activity may mediate or contribute to inhibition of TNFα-induced gene expression. This has been previously described for ER-mediated repression of Tax-activated transcription of the TNFα gene. This study provides evidence that ER inhibits activity of the TNFα promoter by interacting with a complex consisting of NF-κB and de AP-1 subunit c-Jun (57).

Next, we wanted to determine wether the genes that are downregulated by all four ligands, representing the second largest group of the Venn diagram (Figure 3, list EIRT), are also previously described to be regulated by TNFα in an NF-κB-dependent manner. However, so far for none of the genes information could be found and thus further promoter analysis needs to be carried out to determine whether NF-κB is a player in the regulation of the expression of this group of genes.

Furthermore, out of the five known genes that were downregulated by both E2 and OH-T (list ET), four genes [bone morphogenetic protein 2 (34, 37, 58), colony stimulating factor 2 (34, 37, 59), neuregulin 1 (36), laminin gamma 2 (36)] have been previously described to be regulated by NF-κB upon TNFα stimulation. For the known genes downregulated by E2, OH-T and Ral (list ERT), two out of four genes are previously identified as NF-κB target genes [epidermal growth factor receptor (60) and interleukin 7 receptor (35, 36)]. For the genes downregulated by both OH-T and Ral, one out of three genes [nuclear receptor interacting protein 1 (34)] was found to be induced by TNFα, which was fully dependent on NF-κB. Out of the two known genes downregulated by both ICI and OH-T, one gene was identified as a TNFα-induced NF-κB target gene [chemokine (C-X-C motif) ligand 10 (36)]; one of the two known genes downregulated by both ICI and Ral was found to be regulated by NF-κB [transglutaminase 2 (61)]; and one out of the eight known genes downregulated by ICI was identified previously as an NF-κB-dependent TNFα-upregulated gene [ninjurin 1 (37)].

For the remaining genes in the lists described above and the remaining gene lists in the Venn diagram, further (functional) promoter analysis is necessary to determine whether or not (functional) NF-κB binding sites are present in the promoter regions of these genes.

In order to start confirmation our results, we performed quantitative RT-PCR (Q-PCR) on two representative genes, interleukin 8 (IL-8) and GM-CSF. Our microarray analysis revealed that TNFα-upregulated IL-8 expression was selectively repressed by E2, and is therefore representative for the E2 list of the Venn diagram. Q-PCR analysis of IL-8 mRNA revealed that expression was strongly induced by TNFα (~270 fold) and in combination with E2 expression was inhibited (~60%), while cotreatment with OH-T, Ral and ICI did not result in significant inhibition of gene expression (Fig. 4A). TNFα-induced GM-CSF expression was repressed by both E2 and OH-T in our array study representing the ET-list of the Venn-diagram. GM-CSF mRNA expression was also induced by TNFα (~35 fold) and inhibited in combination with E2 or OH-T (95% and 50%, respectively), while Ral and ICI did not have an effect (Fig. 4B). Taken together, the expression patterns of IL-8 and GM-CSF, obtained by Q-PCR, are in agreement with the microarray data. Additional Q-PCR analysis is necessary

to confirm expression patterns of genes in other lists of our Venn-diagram.

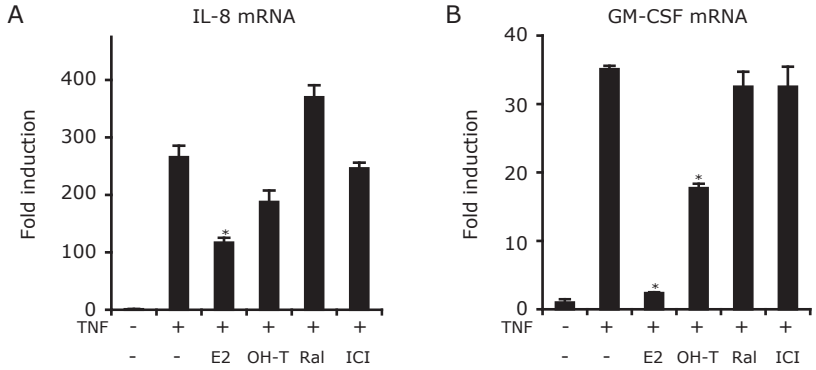

Figure 4. Quantitative RT-PCR analysis of IL-8 and GM-CSF expression in U2OS-ERα.
Determination of relative IL-8 (**A**) or GM-CSF (**B**) mRNA expression levels upon pre-incubation for 1 h with vehicle (-), 10 nM E2, 1 μm OH-T, Ral or ICI and subsequent stimulation for 8h in combination with TNFα. Values were normalized against the expression of the house keeping gene encoding human acidic ribosomal protein (hARP) determined for each sample. Fold induction indicates the mRNA expression level induced by TNFα (with or without hormones) over untreated cells. Bars represent the average ± SEM of two independent experiments assayed in duplicate. Statistically significant differences between TNFα and TNFα+hormone are indicated (*, P<0.05).

CONCLUSION

In U2-OS cells stably expressing ERα, expression of a large group (40%) of TNFα-induced genes is inhibited upon stimulation with estrogenic ligands, including E2, OH-T, Ral and ICI. From this group of inhibited genes most of the genes (30%) were selectively downregulated by E2. Many of the E2-selective genes appeared to be mainly regulated by NF-κB and therefore we propose that ERα-mediated transcriptional interference through tethering to the regulatory region of NF-κB target genes is an important mode for inhibiting TNFα-induced gene expression in U2-OS cells.

MATERIALS AND METHODS

Chemicals

Recombinant human TNFα, E2 and OH-T were obtained from Sigma Chemical Co (St Louis, MO). Ral was provided by Organon International (Oss, The Netherlands). ICI was obtained from Tocris Cookson (Bristol, UK).

Cell culture

The U2-OS (human osteosarcoma) cell line stably transfected with human ERα (U2OS-ERα) was described before (33). Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DF; Gibco, Invitrogen, Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS; Cambrex, Rockland, ME), 10 U/ml penicillin and 10 μg/ml streptomycin (Gibco, Invitrogen, Gaithersburg, MD). Cells were cultured at 37ºC in a 5% CO_2 humidified atmosphere.

Microarray experiments and data analysis

U2OS-ERα cells were seeded in 150 mm plates in DF medium, without phenol red and supplemented with 7.5% charcoal-stripped FCS (Bodinco, Alkmaar, The Netherlands), at least 3 days before treatment with hormones. Cells were pre-incubated for 1 h with vehicle (0.1% ethanol), 10 nM E2, 1 μM OH-T, 1 μM Ral or 1 μM ICI (two separate plates for each incubation). Next, cells were stimulated for another 8 h with hormones (or vehicle) in combination with or without 5 ng/ml TNFα. Two independent experiments were performed resulting in twenty separate samples. Total RNA was isolated using Trizol Reagent (Invitrogen, Gaithersburg, MD) and purified with the RNeasy kit including a DNase treatment (Qiagen, Valencia, CA), following the manufacturer's instructions. Total RNA of each sample was used to generate biotin-labeled cRNA for hybridization with Affymetix human HG-U133A 2.0 GeneChips according to Affymetrix (Santa Clara, CA) protocols and was performed by ServiceXS (Leiden, Netherlands). These microarrays contain probe sets representing 18.400 transcripts and variants, including 14.500 well-characterized human genes. The chips were scanned and analyzed using MicroArray Suite 5.0 software (Affymetrix). Data from individual arrays were then analyzed using GeneChip Operating Software (GCOS; Affymetrix) in order to select the genes, on the arrays of the samples treated with TNFα alone, that were marked as present (P) by GCOS. The raw signal intensities were imported into the gene expression analysis software Rosetta Luminator (Rosetta, Seattle, WA) in order to build ratio's between TNFα-treated and untreated samples and between TNFα-treated and TNFα+hormone-treated samples. For each analysis gene lists were generated according to the following parameters: P value ≤0.05 and fold change ≥1.75 (up with TNFα and down with hormones). These lists were next analyzed with Rosetta Luminator in order to determine overlapping genes between different hormone treatments. Biological relevance of known genes present in the generated lists was determined by identifying enrichments of Gene Ontolgy (GO) categories and of biochemical pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG). This was done by using the GoTree and KEGG modules of the "Web-based Gene Set Analysis Toolkit" (Webgestalt; http://genereg.ornl.gov/webgestalt). Statistical significance of enrichments was determined by Webgestalt using the hypergeometric test, which is described in detail in the manual available on the website.

Quantitative Real-Time RT-PCR

cDNA was synthesized with Superscript II (Invitrogen, Gaithersburg, MD) using poly(dT) oligonucleotides. Quantitative real-time PCR of diluted cDNA was carried out using iQ SYBR green Supermix on a MyiQ I cycler (Bio-Rad, Hercules, CA) with 40 cycles of two-step amplification. For detection of GM-CSF the forward primer was 5'-GGCCAGCCACTACAAGCAGCACT-3' and the reverse primer 5'-CAAAGGGGATGACAAGCAGAAAG-3', for IL-8 the forward primer was 5'-CAGACAGCCACTCACC-3' and the reverse primer 5'-TTCACCAGGCAAGTCTC-3' and for hARP the forward primer was 5'-CACCATTGAAATCCTGAGTGATGT-3' and the reverse primer 5'-TGACCAGCCCAAAGGAGAAG-3'. Gene expression was normalized to human acidic ribosomal protein (hARP) and the fold change in expression was determined relative to control cells treated with vehicle. An unpaired Student's *t* test was used to compare differences between mean values of two different treatments. Differences with P values less than 0.05 were accepted as statistically significant.

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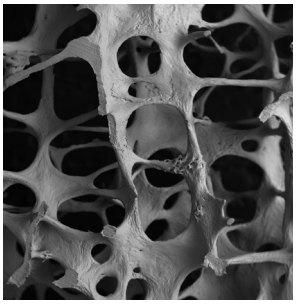
Chapter 6



Summarizing Discussion

1. COMPLEXITY OF GENE REGULATORY NETWORKS AS THE BASIS FOR ORGANISMAL COMPLEXITY

The start of my PhD period coincided with a major achievement for the study of human biology, namely the publication of the human genome sequence by two separate research groups (1, 2). The availability of the sequence marked the beginning of a challenging task to proceed from sequence to function for the complete genome. Predictions of the total number of protein-encoding genes have been made long before the availability of the whole genome sequence and were as high as 140.000 genes. Upon availability of the draft sequence a new calculation of both research groups resulted in an estimation of around 35.000 genes and presently the number is thought to be even lower. This relatively low number of genes in the human genome was found surprising and is even more puzzling considering the fact that the genome of a grain of rice encodes twice as much genes (3, 4). Up till now, whole genome sequencing of almost 200 organisms (including microbes, plants and animals) is completed and comparative genome analysis revealed a common theme that increased morphological, physiological and behavioral complexity cannot be explained by an increase in gene number. One mechanism through which a relatively small gene number can result in increased complexity is alternative splicing, which results in different RNA species being transcribed from one gene. However, recently Levine and Tjian have suggested that the molecular basis for increased organismal complexity is more likely to be due to increased complexity in the regulation of gene expression (5). Therefore, the hypothesis that increased complexity of organisms arises from a greater diversity in protein function, is now challenged. According to Levine and Tjian, two mechanisms can account for increased complexity at the genetic level. One mechanism requires a more elaborate organization of *cis*-regulatory DNA, manifested by an increased number and increased complexity of the regulatory sequences (enhancers, silencers, promoters). The second mechanism involves an increase in the complexity of the multiprotein transcriptional complexes that regulate gene expression. These proteins include sequence-specific transcription factors, general transcription factors and proteins involved in chromatin remodeling and modification. The likelihood of this hypothesis is reflected by the organization of the genome in coding and non-coding sequences and the percentage of genes encoding transcription factors. In the case of the human genome, less than 2% of the genome encodes proteins while 10% of all expressed genes are proteins involved in regulation of gene transcription and furthermore a third of the human genome sequence is estimated to regulate of the chromosomal structure and gene expression. In contrast, 27% of the genome of the nematode *Caenorhabditis elegans* (a lower metazoan) encodes proteins and only about 3% of the 20.000 genes encode proteins acting as transcription factors (6). Thus, there is only one transcription factor for every 40 genes in *C. elegans*, but one for every ten in humans. According to the hypothesis the complexity of an organism would then correlate with an increase in both the absolute number transcription factors and the ratio per genome. Moreover, a greater complexity in the *cis*-regulatory sequences enables an increase in the number of possible combinations of transcriptional regulators resulting in an enormous increase in the complexity of the gene regulatory network.

In the light of the hypothesis of Levine and Tjian, understanding the molecular basis of convergence of separate signal transduction pathways at the transcriptional level will contribute to the understanding of how complex gene regulatory networks can arise and how they act at the molecular level. The results presented in this thesis deal with the convergence of estrogen signaling mediated via the estrogen receptor (ER) and TNFα signaling mediated by NF-κB. We like to propose that cross-talk between the transcription factors ER and NF-κB can be considered as an example of increased complexity in gene regulation in a higher organism.

2. THE MOLECULAR BASIS FOR CROSS-TALK BETWEEN ER AND NF-κB

Many studies have reported cross-talk between ER and NF-κB, which was described to occur in various cell types, in the presence of a variety of stimuli and analyzed on several different target genes/promoter elements. However, the models that have been proposed to explain ER-mediated inhibition of NF-κB activity appeared to differ and were shown to involve both direct as well as indirect mechanisms. Moreover, these mechanisms for repression of NF-κB activity have been investigated not only for ER, but also for other steroid receptors and other members of the nuclear receptor family and reveal overlapping modes of action (7-9).

2.1. Indirect mechanisms

2.1.A. IκB induction model

In the first indirect model, repression of NF-κB activity by NRs is suggested to result from induced IκB levels, which may arise from direct regulation of the gene encoding IκB or indirect regulation of IκB synthesis. Enhanced IκB levels would result in increased sequestering of NF-κB in the cytoplasm and thus in inhibition of NF-κB transcriptional activity.
In the case of ER, only indirect effects on IκB synthesis have been described involving the ability to modulate upstream signaling events of the NF-κB pathway and subsequent induction of IκB expression. These effect were found to occur in brain tissue, breast cancer cells and endothelial cells (10-13). It was shown that estrogen treatment may result in decreased IκB phosphorylation (10) and diminished processing of p105 (11), which might be due to inhibition of IKK activity (12).

2.1.B. Cofactor competition or squelching model

Several coactivators are known to interact with both nuclear receptors (NR) as well as several subunits of NF-κB and therefore it has been proposed that repression of both NR- and NF-κB-dependent gene transcription is the result of competition for common coactivators, which are present in limiting amounts. Coactivators that are described to enhance transcriptional activity of members from both the NF-κB and NR family of transcription factor include CBP, p300, p/CAF, SRC-1 and SRC-3 (14-19). It was suggested that overexpression of one of these common coactivators could relieve the mutual repression. Although there are a few reports providing evidence that may point in the direction of the cofactor-competition model (20, 21), there is a vast amount of arguments and evidence that contradict such a model (as recently reviewed for ER by Kalaitzidis *et al.* and for the glucocorticoid receptor (GR) by De Bosscher *et al.*) (7, 8), making it very unlikely that this competition plays an important role in cross-talk between NRs and NF-κB.

2.1.C. Non-genomic model: cytoplasmic sequestration of NF-κB

Recently, one group reported a third indirect model for cross-talk between ER and NF-κB involving a non-genomic effect. The authors show that 17β-estradiol prevents inflammatory gene transcription in macrophages by inhibiting NF-κB intracellular transport resulting in cytoplasmic sequestration. In addition, they show that the effect of E2 on NF-κB is selectively mediated by the intracellular receptor ERα, and not ERβ, through a non-genomic signaling pathway that involves the activation of phosphatidyl-inositol 3-kinase (22).

2.2. Direct mechanism: protein-protein interaction model

Ray *et al.* were the first to show that ERα is capable of modulating gene expression, without directly binding DNA, through a mechanism involving interference with NF-κB activity (23). They suggested that activation of the IL-6 promoter may be repressed by ERα because of interference with the transactivation functions of NF-κB involving protein-protein interactions, which was subsequently supported by *in vitro* data showing a direct interaction between ERα and the NF-κB subunits p50, p65 and c-Rel (24-26). Although, the protein-protein interaction model is acknowledged as an important mechanism for repression of NF-κB activity for both ER and GR, the subsequent steps that result in inhibition of NF-κB transcriptional activity are poorly understood.

2.2.A. Inhibition of DNA binding by NF-κB

It has been suggested that upon formation of a complex between ER (or GR) with NF-κB, the DNA binding capacity of NF-κB is impaired. Indeed several studies reported reduced DNA binding activity of several NF-κB subunits *in vitro* in the presence of recombinant ERα or ERβ (25, 27).

2.2.B. Interference with transcriptional activity of DNA-bound NF-κB

Many groups did not observe loss of DNA binding affinity by NF-κB, upon ER activation, and therefore a model is proposed in which ER interacts with DNA-bound NF-κB and subsequently interferes with the ability of NF-κB to activate transcription. So far, only one group was able to show that ERα is indeed present in a complex with NF-κB on the response element of the TNFα gene through gel-shift experiments with nuclear extracts from U2OS cells stably expressing ERα (28). Furthermore, the use of mammalian two-hybrid assays, performed in HepG2 liver cells, provided strong evidence for the formation of a DNA-bound complex between ERα and NF-κB, which was synergistically enhanced in the presence of the coactivator CBP (29). In this study it was suggested that, due to cooperative binding ("sharing") of CBP by NF-κB and ERα, a transcriptionally inactive complex is formed. This model could explain the observation that overexpression of p300 or CBP partially relieves ERα-mediated repression of NF-κB activity in smooth muscle or HepG2 liver cells, respectively (21, 29). However, it should be noted that overexpression of coactivators in the cell generally leads to an increase in absolute gene expression levels of NF-κB driven promoters and this increase is in my opinion misinterpreted as relief of repression in the presence of ER, because relative transrepression (p65 *vs.* p65+ER or p65+coactivator *vs*. p65+coactivator+ER) remains unaffected. Moreover, McKay *et al.* showed for GR that the coactivator CBP can serve as an adapter bringing the NF-κB subunit p65 and GR into a specific physical association on the DNA (30). This is also a more likely explanation for the effect observed in the mammalian two-hybrid assay, where CBP synergistically enhanced the formation of DNA-bound ER/NF-κB complexes (29). In another study, it was shown through a mammalian one-hybrid assay, performed in Hela cells, that ERα and ERβ interact with the DNA-bound p50/p65 heterodimer and can disrupts transactivation capacity of the complex (31).

3. NEW EVIDENCE FOR THE PROTEIN-PROTEIN INTERACTION MODEL

As described above, the repressive effect of ER on NF-κB activity can be explained through various mechanisms, which appear to be cell type- and context-dependent. In this thesis we used the osteoblast-like U2-OS cells to study inhibition of TNFα-induced NF-κB activity mediated by ERα or ERβ. Our results show that ERα is more potent than ERβ in repressing NF-κB activity and provide strong evidence for the protein-protein interaction model for cross-talk between ERα and NF-κB.

Ad 2.1.A. IκB induction model

In **Chapter 2**, we show that IκBα levels are not changed in U2-OS cells stably expressing ERα or ERβ upon stimulation with various estrogenic compounds, in the presence or absence of TNFα. Previously, similar results were shown for U2-OS cells transiently transfected with ERα and stimulated with E2 in combination with IL-1β (24). Therefore, we conclude that modulation of IκB levels is not involved in ER/NF-κB cross-talk in U2OS cells.

Ad 2.1.B. Squelching model

In **Chapter 3**, ERα point mutations are described that are transcriptionally inactive due to disruption of interaction with coactivators. These mutants are therefore not capable of competing with NF-κB for the limited pool of coactivators and would lose the ability to inhibit NF-κB activity according to the squelching model. However, we show that these ERα mutants can still inhibit NF-κB demonstrating that activation of transcription and repression of transcription are two separate functions and thereby excluding the squelching model in U2-OS cells. On the contrary, the transcriptionally inactive ERβ mutant is no longer capable of transrepressing NF-κB, which keeps the possibility open for the squelching model for cross-talk mediated by ERβ.

Ad 2.1.C. NF-κB sequestration model

Chapter 4, reveals that cross-talk between ERα and the NF-κB molecules p50 and p65 is a nuclear event in U2-OS cells, because upon E2 stimulation all three proteins co-localize in the nucleus. We show that nuclear localization is not prevented, but even enhanced in the presence of E2, thereby excluding the non-genomic model of sequestration of NF-κB in the cytoplasm in U2-OS cells.

Ad 2.2: Protein-protein interaction model

In **Chapter 4**, we provide evidence that ERα can interact with NF-κB *in vivo* as determined by coimmunoprecipitation and fluorescence resonance energy transfer (FRET) in U2-OS cells. Interactions were enhanced in the presence of E2, which was not observed in previously published studies on *in vitro* interaction between ER and NF-κB (24-26). Consistent with nuclear localization of ERα and NF-κB upon E2 stimulation, FRET interaction is only observed in the nucleus under this condition. However, the question remains how the interaction between ERα and NF-κB can subsequently result in inhibition of NF-κB transcriptional activity.

Ad 2.2.A. Inhibition of DNA binding by NF-κB

We performed gel shift experiments with nuclear extracts of U2-OS cells stably expressing ERα or ERβ and stimulated with TNFα with or without E2 (**Chapter 2**). We observed only a slight reduction of the DNA binding capacity of TNFα induced NF-κB, when cells were treated with E2.

Ad 2.2.B. Interference with transcriptional activity of DNA-bound NF-κB

Again our ERα point mutants exclude the absolute requirement of interaction with coactivators in order to confer transrepression of NF-κB activity, while for ERβ coactivator recruitment may be essential.

3.1. Preliminary evidence for in vivo presence of ERα on endogenous NF-κB regulated promoters

In order to study the mechanism of ERα-mediated inhibition of NF-κB transcriptional activity in more detail, we used chromatin immunoprecipitation (ChIP), which enables determination of presence and modification status of factors involved in gene transcription *in vivo*. First, we assessed the effect of stimulation with E2 of U2-OS cells stably expressing ERα, on the acetylation status of histones in the promoter regions of the TNFα-inducible genes GM-CSF and IL-8, which contain one or more NF-κB binding sites (Figure 1A and 1B). Stimulation with TNFα induced acetylation of both promoters and was enhanced even further in the presence of the HDAC inhibitor TSA. TNFα-induced acetylation was completely reversed in the presence of E2, showing that transcriptional activity of the promoter is inhibited. As a control the NF-κB inhibitor parthenolide was used and it appeared that E2 was at least as effective as parthenolide in inhibiting TNFα-induced NF-κB activity (Figure 1C and 1D). Next, we determined that NF-κB was recruited to these promoters upon stimulation with TNFα and observed diminished presence on the promoters after co-stimulation with E2 (Figure 1E and 1F). In the presence of TNFα, ERα was not detected, while in the presence of both TNFα and E2 we observed recruitment of ERα to these promoters (Figure 1G and 1H). From these preliminary data, we can conclude that ERα is tethered to promoters containing NF-κB binding sites upon estrogen treatment resulting in diminished acetylation of histones.

We propose that ERα, upon interacting with NF-κB, may interfere with NF-κB transcriptional activity resulting in inhibition of histone acetylation in several ways:
1) binding of ERα changes the DNA or protein binding properties of NF-κB, and consequently blocks interaction with DNA or proteins necessary for activation of transcription; 2) binding of ERα repulses or excludes proteins from the NF-κB transcriptional complex necessary for activation of transcription; 3) binding of ERα interferes with catalytic activities of enzymes present in the NF-κB transcriptional complex necessary for activation of transcription; 4) binding or ERα changes post-translational modifications (phosphorylation, acetylation) of NF-κB, which inhibits optimal transcriptional activity. The proteins mentioned here as necessary for activation of transcription include chromatin modifying enzymes (HATs, methyl transferases, kinases etc.), ATP-dependent nucleosome remodeling complexes (*e.g.* SWI/SNF complexes) and the basal transcription machinery (general transcription factors and RNA polymerase II). These effects mediated by ERα can be the result of sterical hindrance, conformational changes in NF-κB or competition for binding sites in NF-κB necessary for interacting with proteins or DNA.

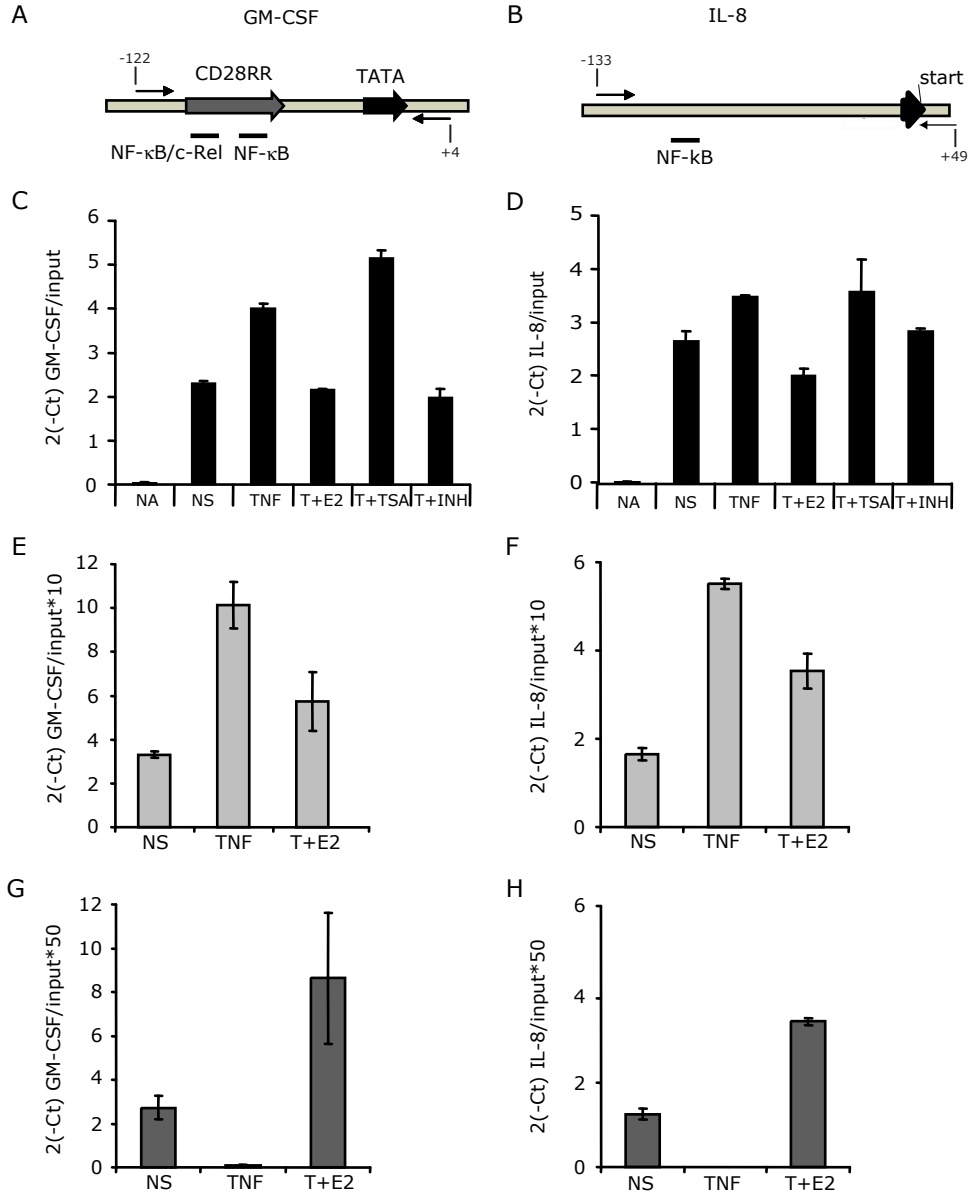


Figure 1. Chromatin immunoprecipitation (ChIP) analysis reveals inhibition of H3 acetylation, diminished presence of NF-κB and recruitment of ERα at NF-κB regulated promoters upon estrogen treatment of U2OS-ERα cells.

Structure of the PCR-amplified region (indicated by arrows), containing NF-κB binding sites, of the proximal promoter of GM-CSF (**A**) and IL-8 (**B**). ChIP experiments were performed on chromatin prepared from U2-OS cells stably expressing ERα and treated as indicated for 4h using antibodies against acetylated H3 (**C** and **D**), against NF-κB (**E** and **F**) or against ERα (**G** and **H**). The amount of precipitated GM-CSF promoter (**C, E** and **G**) or IL-8 promoter (**D, F** and **H**) are depicted as $2^{-(Ct)}$ corrected for the amount of input. Abbreviations: NA, no antibody; NS, no stimulus; TSA, trichostatin A; INH, NF-κB inhibitor parthenolide; Ct, value at crossing point with preset threshold, as determined by quantitative real time PCR.

4. SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS)

4.1. Effects of SERMs on NF-κB activity

SERMs are ligands for ER that are capable of conferring both antagonistic as well as agonistic activities depending on the target tissue. So far, the molecular basis for tissue-selective actions of SERMs is mainly studied in the context of the ability of ER to activate gene transcription. This led to the hypothesis that antiestrogenic effects of SERMs are due to the capacity to block ER transcriptional activity, while estrogen-like effects related to their capacity to mediate (partial) transactivation. However, we propose that tissue-selective effects by SERMs can also be the result of modulating the repressive effect of ER on NF-κB transcriptional activity. In this way, blocking ER-mediated NF-κB transrepression causes antiestrogenic effects, while mediating NF-κB transrepression results in estrogen-like effects.

In **Chapter 2**, we show that the prototypical SERM, tamoxifen, is indeed capable of mediating NF-κB transrepression in U2-OS cells, although less strongly than E2, and repression was mediated by ERα and not through ERβ. In addition, we used the SERM raloxifene and observed a minor but significant repression of NF-κB activity through ERα in transient transfection assays, but not in cells stably expressing the receptor **(Chapter 3)**. However, upon inhibiton of HDAC activity, NF-κB transrepression by raloxifene was observed and repression by tamoxifen was enhanced in cells stably expressing ERα **(Chapter 3)**. Furthermore, we showed that upon mutating specific residues in the AF-domains of ERα involved in coactivator interaction, tamoxifen and raloxifene became strong repressors of NF-κB activity **(Chapter 3)**. Taken together, tamoxifen and raloxifene are capable of blocking or mediating NF-κB transrepression dependent on ER-subtype, coactivator interaction and corepressor activity. These observations support our proposal of tissue-selective effects of SERMs through modulation of ER-mediated NF-κB transrepression. Relative ER subtype expression and coregulator expression are known tissue-dependent factors and are also known to determine tissue-selectivity by SERMs in the context of modulating of gene activation.

4.2. SERMs as safer alternatives for hormone replacement therapy

Estrogen deficiency during menopause in women is associated with various symptoms, including hot flashes and vaginal dryness. Furthermore, postmenopausal women have an increased risk for developing several diseases such as osteoporosis and cardiovascular diseases. The prescription of hormone replacement therapy (HRT; combined estrogen and progesterone therapy) is considered an effective way not only to relieve symptoms associated with menopause, but also to prevent postmenopausal diseases. However, the recent results of the Women's Health Initiative (WHI) raised concern and doubts about the use of HRT since, in addition to known harmful effects such as an increased risk for breast cancer, unexpected harmful effects were observed such as an increased incidence in stroke (32, 33). The official medical guidelines for treating postmenopausal women with HRT have been drastically changed on the basis of these studies. But, critical review of these data and taking into account the vast amount of evidence that was already available, resulted in the conclusion by many researchers and clinicians that HRT should remain an important tool in preventing diseases and maintaining quality of life (34, 35). More importantly, regarding the prevention of osteoporosis, the WHI was the first large, placebo-controlled, randomized clinical study that demonstrated that estrogen decreases the risk of hip fractures in

postmenopausal women and therefore providing strong evidence for the beneficial effects of estrogen on bone.
The discovery of the tissue-selective biological activities of tamoxifen and raloxifene has led to the proposal that it may be possible to develop a perfect SERM that opposes estrogen action in reproductive tissues but exerts beneficial estrogen-like effects in non-reproductive tissues. These SERMs would provide a safer alternative to traditional hormone therapy resulting in improvement of quality of life of postmenopausal women without harmful side effects. We believe that it is important that these ligands have the property to inhibit NF-κB activity and will not only prevent osteoporosis, but also other diseases associated with chronically enhanced NF-κB activity known to occur frequently in postmenopausal women. In **Chapter 3**, we show that transcriptionally inactive ERα mutants are still capable of mediating NF-κB transrepression upon ligand binding. At this point, it is important to determine the conformation of these ERα mutants and to test new or existing ligands that induce this specific conformation in wildtype ERα. Such a ligand would enable dissociation of the transactivating and transrepressing capacity of ERα. This could lead to a safer SERM due to the lack of transactivating capacity and therefore completely blocking estrogen action in reproductive tissue, while still capable of mediating NF-κB transrepression necessary for beneficial effects in non-reproductive tissues. Moreover, there are numerous inflammatory and autoimmune diseases associated with high NF-κB activity, such as inflammatory bowel disease and atherosclerosis, where treatment with estrogen seems a useful therapy and thus a dissociating ligand would be a safer treatment. Recently, researchers at the pharmaceutical company Wyeth reported the identification of a non-steroidal ligand for the estrogen receptor, WAY-169916, that selectively inhibited NF-κB transcriptional activity mediating potent anti-inflammatory activity in a rat model for inflammatory bowel disease and in two models for rheumatoid arthritis (36, 37). This pathway-selective ligand did not stimulate uterine proliferation, which indicates lack of classical ER-mediated activation of gene transcription. These results show that it is indeed possible to selectively target ER-mediated effects through specific ligands.

5. ERα-MEDIATED TRANSREPRESSION OF ENDOGENOUS TNFα-REGULATED GENES

In **Chapter 5**, we show through microarray analysis that ~40% of the TNFα-induced genes could be downregulated upon stimulation of U2-OS cells, stably expressing ERα, with an estrogenic ligand. This raises the question which factors determine whether a TNFα-upreglated gene can be downregulated by ERα upon stimulation with a specific ligand. A substantial part of the downregulated genes has already been established to be mainly regulated by NF-κB and therefore we focus here on this transcription factor. Recently, Luecke and Yamamoto showed that in A549 human lung carcinoma cells, expression of both IL-8 and IκBα mRNA is enhanced by TNFα, but IL-8 expression is selectively inhibited by GR in the presence of dexamethasone. They determined that RelA, p50 and GR were recruited to the NF-κB binding site (κB site) in the promoter region of both IL-8 and GM-CSF. It appeared that the regulatory complex recruited to the proximal promoter of IL-8 contained the kinase P-TEFb, which was absent in the complex at the proximal promoter of the IκBα gene (38). P-TEFb enhances RNA polymerase II (Pol II) elongation by phosphorylating the C-terminal domain of Pol II. In the presence of dexamethasone, GR inhibits RelA activity at the IL8 gene by interfering with Pol II CTD phoshorylation, which is not observed at the IκBα gene (39). Therefore, they suggested a model for GR-mediated inhibition of NF-κB transcriptional activity in which GR blocks recruitment of P-TEFb by RelA to the IL-8 gene, wich subsequently results

in decreased phosphorylation of the Pol II CTD. In contrast, transcription of the IκBα gene does not require recruitment of P-TEFb to the promoter and thereby GR-mediated repression cannot occur. Therefore, it can be postulated that the difference in the sequence of the κB site in the IL-8 and IκBα promoter region leads to recruitment of transcriptional complexes of different composition and therefore to differential regulation by GR due to competition with a promoter-specific NF-κB coregulator. In a recent study, Baltimore and coworkers provide evidence that the primary sequence of the κB site indeed affects which coactivators will be efficiently recruited to the DNA bound NF-κB dimer (40). They suggest a model in which the sequence of the κB site contains information that is interpreted by the bound NF-κB dimer, subsequently resulting in a change in the configuration of the dimer determining which coactivators will form functional interactions with NF-κB. In an earlier study, Baltimore and coworkers also reported that a particular NF-κB target gene requires the binding of specific members of the NF-κB family for functional activation (41). Therefore, we propose that NF-κB dimer specificity and the composition of the recruited coregulator complex are determinants for susceptibility of a specific NF-κB target gene to downregulation by ERα.
Furthermore, comparison of the expression profiles resulting from stimulation with E2, tamoxifen, raloxifene or ICI, revealed distinct effects of all four ligands on TNFα-upregulated gene expression. Figure 2, summarizes the percentage of genes in the Venn diagram that is selectively inhibited by only one ligand or inhibited by more than one ligand. We suggest that this ligand-specificity may arise from the unique conformational change in ER that is induced by the different ligands used (42-44). This distinct ER conformation may or may not enable interference with the NF-κB transcriptional complex.
Finally, the expression profiles of the different ligands that we obtained by our microarray analysis may serve as a comparative tool to test newly developed ERα ligands in order to determine their relative potency to inhibit NF-κB transcriptional activity.

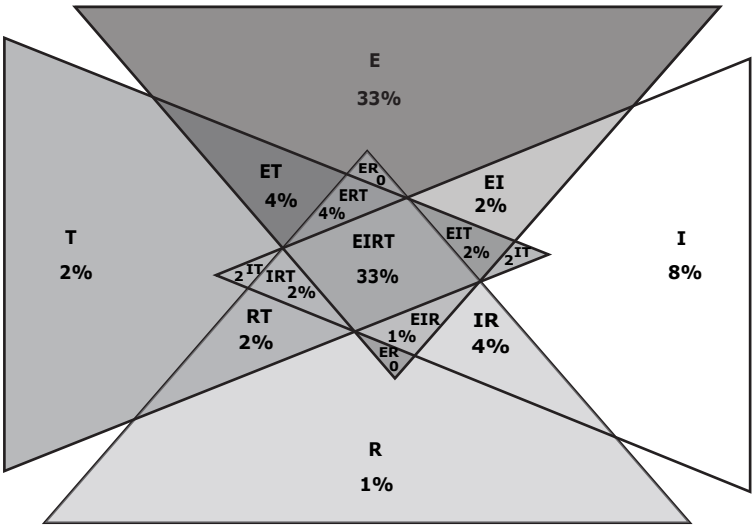


Figure 2. Venn diagram showing all possible overlaps and the percentage of overlapping genes.
Numbers represent the percentages out of the total number of all downregulated genes by ERα in the presence of an estrogenic ligand. Overlapping gene sets were obtained by comparing the separate lists for TNFα-induced genes downregulated by E2, OH-T, Ral or ICI.

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Samenvatting

ACHTERGROND

Oestrogeen wordt vaak uitsluitend beschouwd als het vrouwelijke geslachtshormoon en dankt zijn naam aan de belangrijke rol die het hormoon speelt in de regulatie van de oestrus (menstruatiecyclus). Hoewel oestrogeen inderdaad essentieel is bij de ontwikkeling en het functioneren van de vrouwelijke geslachtskenmerken, doet deze benaming tekort aan de diversiteit van functies die het hormoon vervult bij zowel de vrouw als de man.

Tijdens de vruchtbare periode van de vrouw wordt oestrogeen voornamelijk geproduceerd door de eierstokken (gonaden). Oestrogeen wordt ook in de gonaden van de man gemaakt, maar dit draagt alleen voor een klein deel bij aan de totale hoeveelheid oestrogeen in het bloed die overigens veel lager is dan bij vrouwen. Bij de man is de belangrijkste bron van oestrogeen afkomstig van andere weefsels, zoals bot, bloedvaten en hersenen, waar testosteron (het mannelijke geslachtshormoon) kan worden omgezet in oestrogeen. In deze zogenaamde extra-gonadale weefsels wordt oestrogeen dus lokaal geproduceerd en heeft het ook een lokale werking (paracrine of intracrine hormoon werking). Tijdens de overgang (climacterium of menopauze) houden de eierstokken op met het produceren van oestrogeen, waardoor de hoeveelheid oestrogeen in bloed na de overgang (postmenopauzaal) dramatisch gedaald is. Bij postmenopauzale vrouwen is de bron van oestrogeen dan, net als bij mannen, afkomstig van de lokale productie in andere weefsels. Omdat de lokale oestrogeenproductie afhankelijk is van de hoeveelheid testosteron in het bloed en omdat deze bij vrouwen lager is dan bij mannen, is er bij postmenopauzale vrouwen sprake van een tekort aan oestrogeen (oestrogeendeficiëntie). Het verlies van vruchtbaarheid bij vrouwen en de daarop volgende oestrogeendeficiëntie gaat gepaard met een verhoogd risico op het krijgen van bepaalde ziektes, zoals botontkalking (osteoporose), hart- en vaatziekten en de ziekte van Alzheimer. Het verhoogde risico op deze ziektes na de menopauze weerspiegelt de belangrijke rol die oestrogeen speelt bij het goed functioneren van de betrokken weefsels.

De gemiddelde levensverwachting van vrouwen in veel westerse landen is inmiddels gestegen boven de 80 jaar en wordt verwacht te blijven toenemen, terwijl de gemiddelde leeftijd waarop de menopauze optreedt (51 jaar) onveranderd is gebleven. Dit betekent dat de vrouw bijna een derde van haar leven doorbrengt na de menopauze, met alle bijkomende gezondheidsrisico's. Het is al heel lang bekend dat het aanvullen van het oestrogeentekort bij postmenopauzale vrouwen, hormonale substitutietherapie genoemd (ofwel HRT naar het Engelse "hormone replacement therapy"), heel effectief is om overgangsklachten, zoals opvliegers, te verminderen. HRT bestaat uit een combinatie van zowel oestrogeen als progesteron (een tweede hormoon betrokken bij regulatie van de menstruatiecyclus), omdat het geven van alleen oestrogeen de kans op het krijgen van baarmoederkanker verhoogt. De resultaten van de Amerikaanse studie "Women's Health Initiative" (WHI) die vanaf 2002 gepubliceerd zijn hebben vastgesteld dat osteoporose en bijbehorende botbreuken aanzienlijk minder voorkomen bij vrouwen die HRT kregen. Bovendien bleek uit de studie dat na hormoonsubstitutie deze vrouwen minder risico hadden op het krijgen van dikke darmkanker. Ondanks deze positieve resultaten is deze studie vroegtijdig afgebroken omdat onder met HRT behandelde vrouwen bepaalde ziektes vaker voorkwamen, waaronder het krijgen van een beroerte en borstkanker (dit laatste was overigens al langer bekend).

Ondanks de negatieve effecten die gepaard gaan met HRT, heeft de "International Menopause Society" geadviseerd aan medische behandelaars dat HRT een belangrijke therapie blijft om de kwaliteit van leven van postmenopauzale vrouwen te verbeteren, maar wel kritisch moet worden toegepast rekening houdend met onder andere erfelijke aanleg voor bepaalde ziektes.

Als veiliger alternatief kunnen postmenopauzale vrouwen behandeld worden met een relatief nieuwe medicatie, de zogenaamde SERMs ("Selective Estrogen Receptor Modulators"). Dit zijn stoffen die in bepaalde organen de effecten van oestrogeen kunnen nabootsen (agonistisch of oestrogeen effect), terwijl ze in andere organen de werking van oestrogeen remmen (antagonistisch of anti-oestrogeen effect). De eerste stof waarvan werd erkend dat het de eigenschappen van een SERM heeft, is tamoxifen dat oorspronkelijk is ontdekt en toegepast voor de behandeling van borstkankerpatiënten door de anti-oestrogene werking op borstweefsel. Later bleek dat tamoxifen een agonistisch effect heeft op bot door te beschermen tegen osteoporose, maar het bleek tevens een ongewenst agonistisch effect op het slijmvlies van de baarmoeder te hebben en daardoor de kans op kanker te vergroten. Raloxifene is een tweede SERM die wordt toegepast in de kliniek vanwege agonistische werking op bot, maar zonder het verhoogde risico op borst- en baarmoederkanker door anti-oestrogene werking in deze weefsels.

De verontrustende resultaten van de WHI studie benadrukken hoe belangrijk het is om een veiliger alternatief voor de hedendaagse HRT te vinden. Het uiteindelijke doel zal zijn om een "ideale" SERM te ontwikkelen die alleen de voordelen heeft van HRT (zoals verminderen van overgangsklachten, beschermen tegen osteoporose, verminderd risico op hart- en vaatziekten) zonder het verhoogde risico op borst- en baarmoederkanker.

DOEL VAN DIT ONDERZOEK

Om in staat te zijn veiligere medicatie te ontwikkelen ten behoeve van HRT is het essentieel om beter te begrijpen welke moleculaire mechanismen ten grondslag liggen aan de regulatie van diverse fysiologische processen door oestrogeen. Weefsels die gevoelig zijn voor oestrogeen worden gekenmerkt door de expressie van de zogenaamde oestrogeen receptor (hierna ER genoemd naar de Engelse benaming "estrogen receptor"). ER behoort tot de familie van nucleaire receptoren, die fungeren als transcriptiefactoren na binding van een ligand (ofwel hormoon). Transcriptiefactoren zijn eiwitten die in staat zijn om de expressie te reguleren van specifieke genen in het DNA (die de code bevatten voor een bepaald eiwit). Oestrogeen oefent zijn fysiologisch effect op een weefsel uit door te binden aan de oestrogeen receptor, die hierdoor in staat is om specifieke elementen in het DNA te herkennen en te binden. Na DNA binding rekruteert ER een complex van eiwitten, dat nodig is voor de activering van transcriptie van het betreffende doelwit gen. Behalve door direct aan DNA te binden, is ER ook in staat om genexpressie te reguleren door de activiteit van andere transcriptiefactoren te moduleren. Het onderzoek beschreven in dit proefschrift heeft zich gericht op het mechanisme waarmee ER in staat is om te interfereren met de transcriptie activiteit van NF-κB. NF-κB is een van de belangrijkste transcriptiefactoren die betrokken is bij de activatering van genen die een rol spelen in het immuunsysteem en bij ontstekingsreacties, zoals cytokines en cytokine receptoren. Het is bekend dat oestrogeen de productie van bepaalde cytokines kan remmen en dit proces wordt onder andere belangrijke geacht voor de botbeschermende werking van oestrogeen.

De beschermende werking van oestrogeen op bot bestaat voornamelijk uit het remmen van botafbraak. Bot is een dynamisch levend weefsel waar gedurende het hele leven een actief proces plaatsvindt om oud of beschadigd bot te vervangen. Dit proces bestaat uit een cyclus die begint met botafbraak, uitgevoerd door gespecialiseerde cellen (osteoclasten), gevolgd door botopbouw, uitgevoerd door andere cellen (osteoblasten). Om een constante botmassa te behouden is het is heel belangrijk dat de activiteiten van osteoclasten en osteoblasten in evenwicht zijn. Oestrogeen speelt een zeer belangrijke rol in het in stand houden van dit evenwicht en dus in het behoud van botmassa. Daarom kan oestrogeendeficiëntie leiden tot een verstoring van dit evenwicht met als gevolg dat het bot sneller wordt afgebroken dan opgebouwd. Wanneer het botmassaverlies zo snel verloopt dat de botdichtheid beneden een bepaalde grens komt, neemt het risico op botbreuken sterk toe en dan spreekt met van osteoporose. Een belangrijke manier waarop oestrogeen botafbraak remt is door het remmen van de productie van cytokines (door onder andere osteoblasten) die de activiteit van osteoclasten kunnen induceren. Omdat de expressie van cytokines door NF-κB wordt gereguleerd gaat men uit van de stelling dat oestrogeen cytokineproductie remt door remming van de activiteit van NF-κB en hiervoor zijn in 1994 voor het eerst bewijzen gepubliceerd. Doel van dit onderzoek was om het mechanisme van remming van NF-κB activiteit door ER te ontrafelen en om de werking van oestrogeen en SERMs te vergelijken.

RESULTATEN

In **hoofdstuk 2** worden de effecten beschreven van de twee voorkomende oestrogeen receptoren, ERα en ERβ, op de activiteit van NF-κB. Dit werd gedaan met behulp van luciferase reporter assays, waarbij U2-OS cellen worden getransfecteerd met een expressieconstruct dat codeert voor ER en een NF-κB responsief luciferase reporterconstruct. U2-OS cellen zijn osteoblast-achtige cellen afkomstig van een menselijke tumor in bot (osteosarcoom). Het bleek dat ERα NF-κB activiteit al remde in afwezigheid van een ligand en dit wordt versterkt door zowel het natuurlijke ligand 17β-estradiol (E2) als door de SERM tamoxifen. ERβ remde de activiteit van NF-κB alleen in aanwezigheid E2. Door expressieconstructen te gebruiken van een getrunceerde ER of hybride vormen tussen ERα en ERβ, werd aangetoond dat het N-terminale domein van ER belangrijk is voor de specifieke remmende effecten van ERα.

In **hoofdstuk 3** wordt dieper ingegaan op het moleculaire mechanisme waarmee ER de transcriptie activiteit van NF-κB remt. Er is gesuggereerd dat remming van NF-κB activiteit door ER het gevolg is van een indirect mechanisme waarbij beide transcriptiefactoren concurreren voor een beperkte hoeveelheid aan coactivatoren, die voor beide essentieel zijn voor de activering van transcriptie. Er werd gebruik gemaakt van ER mutanten die niet meer in staat zijn om transcriptie te activeren omdat de mutaties gemaakt zijn in de domeinen die belangrijk zijn voor de interactie met coactivatoren. Het bleek dat de ERα mutanten nog steeds in staat waren om NF-κB activiteit te remmen. Bovendien werd er in tegenstelling tot wildtype ERα sterke remming geobserveerd in aanwezigheid van de SERM raloxifene en het anti-oestrogeen ICI. Terwijl de ERβ mutant volledig de capaciteit verliest om NF-κB activiteit te remmen. Daarom kon worden geconcludeerd dat E2 en SERMs sterke NF-κB remmers kunnen zijn via ERα, onafhankelijk van de mogelijkheid om coactivatoren te rekruteren en dus te transactiveren.

In **hoofdstuk 4** wordt aangetoond dat ERα direct kan interacteren met de NF-κB moleculen p50 en p65. Hierbij werd gebruik gemaakt van "fluorescence resonance energy transfer"

(FRET) die het mogelijk maakt om in levende cellen interacties tussen eiwitten te bestuderen. De te bestuderen eiwitten worden gefuseerd met een cyaan of geel fluorescerend eiwit en door het cyaan fusie-eiwit aan te stralen wordt er licht uitgezonden dat vervolgens het geel fusie-eiwit kan aanstralen, maar alleen als ze zeer dicht bij elkaar in de buurt zijn (dus als ze interacteren). Als er een interactie plaats vindt tussen beide eiwitten kan de golflengte worden gedetecteerd die het aangestraalde geel fusie-eiwit uitzendt en bij geen interactie wordt de golflengte gedetecteerd die het aangestraalde cyaan fusie-eiwit uitzendt. Er werd aangetoond dat een maximaal FRET signaal wordt gedetecteerd in de kern van de cel als ER samen met zowel p50 als p65 tot expressie komt en de cellen worden gestimuleerd met E2. Deze bevindingen vormen een sterke basis voor het model van NF-κB remming door ER, waarbij wordt uitgegaan van een directe interactie tussen ER en NF-κB.

In **hoofdstuk 5** worden de resultaten beschreven van de microarray-analyse uitgevoerd met U2-OS cellen die ERα tot expressie brengen en gestimuleerd werden met E2, tamoxifen, raloxifene of ICI. Door middel van microarray-analyse is het mogelijk om onder een gegeven conditie tegelijkertijd het expressieniveau te bepalen van een groot aantal genen, een zogenaamd globaal expressieprofiel. In deze studie zijn de expressieprofielen van de vier verschillende liganden vergeleken en is bepaald welke genen, die door tumor necrosis factor α (TNFα) worden geactiveerd, kunnen worden geremd door ERα in de aanwezigheid van een ligand. De bijna vierhonderd TNFα-geïnduceerde genen bleken grotendeels qua functie te kunnen worden ingedeeld in biologisch relevante processen, zoals onder andere cytokine signaaltransductie. Het bleek dat ongeveer 40% van alle genen die door TNFα werden geïnduceerd, in aanwezigheid van een van de ER liganden, werd geremd. Van deze groep werden de meeste genen (30%) selectief door E2 geremd, waarvan bleek dat een groot aantal NF-κB doelwit genen zijn. Dit bevestigt dat remming van TNFα-geïnduceerde genen plaatsvindt via het mechanisme van remming van NF-κB transcriptie activiteit.

In **hoofdstuk 6** worden de tot nu toe voorgestelde modellen beschreven die verantwoordelijk kunnen zijn voor het remmende effect van oestrogeen op NF-κB activiteit. Vervolgens worden de modellen bediscussieerd in het licht van de resultaten beschreven in dit proefschrift en uiteengezet welke nieuwe inzichten er zijn verkregen met betrekking tot het directe interactie model.

ALGEMENE CONCLUSIE

Het beschreven onderzoek verschaft inzicht in de moleculaire werkingsmechanismen waarmee oestrogenen fysiologische processen kunnen aansturen via regulatie van genexpressie. Deze inzichten kunnen bijdragen aan het ontwikkelen van verbeterde SERMs ten behoeve van een veiligere HRT voor de behandeling van postmenopauzale vrouwen, maar ook bij de behandeling van ziektes die gerelateerd zijn aan overactieve NF-κB activiteit, zoals reumatoïde artritis (een vorm van chronische ontstekingsreuma).

INTERESSANTE WEBSITES

Informatie over "Women's Health Initiative": http://www.whi.org/
Website van de "International Menopause Society": http://www.imsociety.org/

Dankwoord

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Curriculum Vitae

Monique Elisabeth Quaedackers werd geboren op 2 mei 1978 te Heerlen. In 1996 behaalde zij haar VWO diploma op het Rombouts College te Brunssum, en ging zij Biologie studeren aan de Universiteit Utrecht. Tijdens haar studie liep zij onderzoeksstages bij het Hubrecht Laboratorium (onder begeleiding van Dr. P. T. van der Saag, Dr. B. van der Burg en Dr. S. Wissink) en bij het Genomics laboratorium van de afdeling Fysiologische Chemie, UMC Utrecht (onder begeleiding van Dr. F.C.P Holstege en Prof. Dr. P.C. van der Vliet). In januari 2001 werd het doctoraal eindexamen behaald (*cum laude*). Vanaf februari 2001 tot februari 2006 was zij werkzaam als onderzoeker in opleiding op het Hubrecht Laboratorium (Nederlands Instituut voor Ontwikkelingsbiologie) te Utrecht, in dienst van de Koninklijke Nederlandse Academie van Wetenschappen. In deze periode werd het in dit proefschrift beschreven onderzoek verricht onder begeleiding van Dr. P. T. van der Saag. Vanaf juni 2006 zal zij werkzaam zijn als wetenschappelijk onderzoeker bij het transplantatielaboratorium van de afdeling Inwendige Geneeskunde, Erasmus Medisch Centrum, Rotterdam.

Monique Elisabeth Quaedackers was born on the 2nd of May, 1978 in Heerlen. She graduated with a secondary school diploma from the Rombouts College, Brunssum in 1996, and studied Biology at the University of Utrecht. During this study she carried out research projects at the Hubrecht Laboratory (under supervision of Dr. P. T. van der Saag, Dr. B. van der Burg en Dr. S. Wissink) and the Genomics Laboratory, department of Physiological Chemistry, UMC Utrecht (under supervision of Dr. F.C.P Holstege and Prof. Dr. P.C. van der Vliet). She graduated in January 2001 (cum laude). From February 2001 till February 2006 she was employed as a PhD student at the Hubrecht Laboratory (Netherlands Institute for Developmental Biology) in Utrecht, an institute of the Royal Netherlands Academy of Arts and Sciences. During this period the research described in this thesis was conducted under supervision of Dr. P. T. van der Saag. From June 2006 she will be employed as a scientific researcher at the transplantation laboratory of the department of Internal Medicine, Erasmus Medical Centre, Rotterdam.

List of Publications

Quaedackers M. E., van den Brink C. E., Wissink S., Schreurs R. H., Gustafsson J. Å., van der Saag P. T. & van der Burg B. (2001) 4-hydroxytamoxifen transrepresses nuclear factor-κB activity in human osteoblastic U2-OS cells through estrogen receptor (ER) α, and not through ERβ. *Endocrinology* 142, 1156-1166.

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Quaedackers M. E., Zeinstra L. M., van den Brink C. E., Kalkhoven E. & van der Saag P. T. Selective estrogen receptor modulator and antagonist induced transrepression of NF-κB activity mediated by estrogen receptor α. *Submitted*

Quaedackers M. E., van den Brink C. E., van der Saag P. T. & Tertoolen L. G. J. Direct interaction between estrogen receptor α and NF-κB in the nucleus of living cells. *Submitted*

Quaedackers M. E., Zeinstra L. M., van den Brink C. E. & van der Saag P. T. Transcriptional profiling of TNFα-induced genes repressed by estrogen receptor α in U2-OS cells. *In preparation*