INTRACELLULAR SIGNALING PATHWAYS: NONGENOMIC ACTIONS OF ESTROGENS AND LIGAND-INDEPENDENT ACTIVATION OF ESTROGEN RECEPTORS

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TABLE OF CONTENTS

1. ABSTRACT

Recognition of the complexity of estrogen and estrogen receptor (ER) signaling has substantially increased in the last several years. In their genomic role, estrogens enter the cell and bind to ERs which are members of a superfamily of ligand-regulated transcription factors. However, estrogens also exert non-genomic effects that occur independently of gene transcription. Typically, these relatively rapid events are initiated at the plasma membrane, and result in the activation of intracellular signaling pathways. Regulation of ER transcriptional activity is also complex. Not only do ligands regulate ER-dependent gene expression, but this receptor in the apparent absence of its estrogenic ligand can also be transcriptionally activated by a variety of intracellular signaling pathways. Recent evidence also extends the effects of these signaling pathways to regulating the activity of coactivators, proteins which bind to the ER and amplify its transcriptional activity. Taken together, it is clear that estrogens, ERs and intracellular signaling pathways are intimately linked and this review will explore the relationship between these components of the estrogen-ER signal transduction process.

2. INTRODUCTION

Estrogens are steroid hormones that play critical roles during development and reproduction, which also exert important biological effects in the cardiovascular and skeletal systems and the brain. Specific estrogenic responses have been recognized for decades [*e.g.* increase in uterine size (1,2)] and the cumulative efforts of many investigators led to the identification (3,4) and cloning of specific nuclear receptors for estrogens (5,6). Thereafter a general scheme of estrogen action emerged in which estrogens diffuse into the cell and bind to intracellular receptors resulting in changes in gene expression and cellular phenotype. However, this mode of estrogen action cannot account for a number of estrogenic responses [*e.g.* vascular dilation (7)], at least in part, because of the rapidity with which they occur. In another twist to estrogen receptor biology, there are a group of responses that appear to be estrogen-like [*e.g.* growth factor stimulation of progesterone receptor synthesis (8)] but arise independent of estrogens. Thus, while it is apparent that estrogens and their receptors play important roles in many aspects of physiology, it has become abundantly clear that many diverse mechanisms are utilized to achieve these responses. Many reviews have examined the current understanding of the molecular events through which steroids activate their receptors in their role as transcription factors. It is the purpose of this review to explore the non-traditional mechanisms through which estrogens and the estrogen receptor exert their biological effects.

3. CLASSICAL STEROID SIGNALING

The genomic effects of steroids are mediated by intracellular proteins that belong to the nuclear receptor

superfamily, which includes type I receptors for estrogens (ERα and ERβ), androgens (AR), glucocorticoids (GR), mineralocorticoids (MR), and progestins (PR), and type II receptors for thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR), and 9-*cis* retinoic acid (RXR). In addition, nearly 30 members of the nuclear receptor superfamily have been identified for which ligands do not exist or have yet to be identified, and these are commonly referred to as orphan receptors (9). In general, steroid (type I) receptors possess six structural domains labeled A through F that are often classified according to their functional properties (10). The A/B region is located in the N-terminus of these receptors and contains the activation function-1 (AF-1) domain. The centrally-located C region encompasses the DNA binding domain and the D region serves as a hinge between the amino- and carboxy-terminal halves of these receptors. The E region serves as the ligand-binding domain (LBD) and encompasses the activation function-2 (AF-2) domain. ERs are notable for their relatively large F domains. These are found at the extreme C-termini of these receptors, and have been shown to play a modulatory role in ERα transcriptional responses (10,11).

Genomic responses to steroids involve the synthesis of mRNAs over the time course of hours to days and are therefore sensitive to inhibitors of transcription such as α-amanitin and actinomycin D. Target gene expression is initiated when hormone enters the cell by diffusion and binds to its cognate receptor (10). In the absence of hormone, type I steroid receptors are complexed to heat shock proteins. Hormone binding induces a conformational change in the receptor, causing it to release heat shock proteins and bind as a homodimer to specific hormone-responsive elements in the promoters of target genes (10). The transcriptional activity of steroid receptors is derived from their ability to recruit coactivators to the promoter of target genes via their AF-1 and AF-2 domains.

Coactivators possess intrinsic enzymatic activities (histone acetyltransferase, ubiquitin ligase, and arginine methyltransferase) and their ability to link steroid receptors with the general transcription machinery is important for activation of receptor-dependent gene expression. To date, more than 40 nuclear receptor coactivators have been identified and the list continues to grow. The first such proteins identified were the steroid receptor coactivator (SRC)/p160 family of coactivators (12-15) which is composed of three members (SRC-1, SRC-2, and SRC-3) that have been referred to by various names (SRC-1/NcoA-1, SRC-2/TIF2/GRIP1/NcoA-2 and SRC-3/RAC3/p/CIP/ACTR/AIB1/TRAM-1). These coactivators possess multiple LXXLL (where L is leucine and X is any amino acid) signature motifs called nuclear receptor (NR) boxes that enable them to interact with the AF-2 domain of ligand-bound receptors. Crystallographic analyses of a GRIP1 NR box peptide bound to the ERα LBD illustrated the significance of these residues in mediating coactivator-receptor interactions (16). The LBDs of ERs are composed of 12 α-helices that in the ligand-occupied receptor are packed tightly together to form a hydrophobic groove on the surface of the receptor. The LXXLL motif and surrounding amino acids of the coactivator NR box form an amphipathic α-helix which make critical contacts with the residues of helices 3, 4, 5 and 12 that constitute the hydrophobic cleft of the LBD. The crystallographic structural data are consistent with earlier nuclear receptor-coactivator interaction models which were based on mutagenesis studies (17-19).

4. NONGENOMIC ESTROGEN SIGNALING

In contrast to these relatively well-characterized mechanisms for genomic steroid action, a growing body of evidence suggests that steroids have cellular effects which are not mediated by the transcriptional activation of their cognate nuclear receptors. According to the classical model of steroid hormone action, these lipophilic molecules must enter the cell to exert their biological effects. The model, however, cannot be reconciled with the increasing evidence which suggests that steroid hormones can also initiate signaling from the exterior of the cell, and may in fact use signal transduction pathways commonly employed by other extracellular signaling molecules such as growth factors. These rapid, steroid-mediated effects are referred to as nongenomic signaling responses and can be distinguished from those mediated by transcription. Nongenomic steroidogenic signaling is usually characterized by several criteria: 1) signaling responses occur within a time course (seconds to minutes) that is generally considered too rapid for transcriptional and translational events to take place; 2) responses are refractory to inhibitors of transcription (*e.g.* actinomycin D) and translation (*e.g.* cycloheximide) and 3) responses can be initiated by steroids conjugated to macromolecules [*e.g.* bovine serum albumin (BSA)] which are theoretically too large to enter cells.

Nongenomic effects have been reported for many classes of steroids (*e.g.* progestins, estrogens, androgens, mineralocorticoids, and glucocorticoids), as well as the secosteroids and thyroid hormones [reviewed in (20,21)]. Each of these classes of ligands exhibits broad and varied nongenomic responses which include modulations in the activity of signal transduction cascades, ion transport and neurotransmitter release (20,21). Moreover, these signaling events have been observed to take place in a number of cell types including those derived from bone, mammary gland, brain and ovary. Importantly, such responses have also been demonstrated in sperm, oocyte and neuronal cells, in which transcription is not thought to play a major role in mediating steroid action. There is considerable overlap in the nongenomic responses observed for the different classes of steroids. This review will focus on the expanding field of nongenomic estrogen signaling action, making occasional comparisons to other steroid hormones.

4.1. Nongenomic effects of estrogens on MAP kinase signaling pathways

To date, approximately 20 members of the mitogen-activated protein kinase (MAPK) family of proteins have been identified in mammals [reviewed in (22)]. These serine/threonine kinases exert their cellular effects by phosphorylating downstream targets such as

transcription factors. The MAPKs (*e.g.* Erks, p38, Jnk) are activated through phosphorylation by another family of proteins called MAP/Erk kinases (MEKs; *e.g.* MEK1 and MEK2), which themselves are phosphorylated and activated by MEK kinases (MEKKs; *e.g.* A-Raf, B-Raf, and Raf-1). The Rafs can be activated by various isoforms of ras. In addition, Raf-1 can be phosphorylated by numerous protein kinases, including the Src non-receptor tyrosine kinase, protein kinase C (PKC) and protein kinase B (PKB)/Akt (22). In general, this signaling cascade can be stimulated through extracellular stimuli such as growth factors, which are coupled to intracellular signaling via membrane receptors. Thus, it is clear that MAPKs can be influenced by multiple upstream factors.

Numerous studies have established that estradiol (E2) can rapidly activate extracellular-regulated kinases (Erk1/Erk2) and can influence c-Jun N-terminal kinase (Jnk) activity (23-30). In addition, E2-BSA conjugates also have been able to induce these nongenomic responses (30). The inability of E2-BSA to enter cells was demonstrated by the ability of E2 but not E2-BSA to stimulate ERE-dependent reporter activity (30). Moreover, this experiment indicated that the E2-BSA conjugates did not contain free, unconjugated E2, a potential problem noted to occur with some E2-BSA preparations (31). Collectively, these data suggest that E2 can stimulate MAPKs via a nongenomic mechanism(s).

Recent efforts have been directed toward identifying mechanistic steps involved in the steroidogenic activation of Erk1/Erk2. There is some indication that these mechanisms could involve the classical ERs. Several studies have demonstrated that E2 stimulates Src tyrosine kinase activity and promotes an interaction between ER (ERα or ERβ) and Src kinase both *in vitro* and in cells (23,26,32,33). This interaction takes place between the Src homology-2 (SH2) domain of Src kinase and the LBD of ERα (23,24,26,32,33). Moreover, mutation of tyrosine[537] to phenylalanine, located within the ERα LBD, abolishes the receptor's interaction with Src kinase and thus E2-dependent Src kinase activity. Although this is consistent with a previously established role for SH2 domains in mediating interaction with phosphotyrosine residues (22,33), it should be remembered that mutations in this region are likely to cause pertubations in the structure of the ligand binding domain. Similarly, androgen-induced Erk2 activity is achieved through interactions between a proline-rich region of AR and the SH3 domain of Src kinase (33). The interaction between Src kinase and ERα leads to activation of two Src substrates, Shc and ras GAP (GTPase activating protein)-associated p190, which in turn leads to activation of ras and theoretically activation of MEK kinases (23). Taken together, these results suggest that steroid hormones can stimulate Erk1/Erk2 activity by promoting interactions between classical nuclear receptors and Src tyrosine kinase.

Progestins can also stimulate the Src/Erk signaling pathway, but in contrast to androgens and estrogens which do so through their cognate receptors, progestin-dependent activation of Src/Erk apparently requires both PR and ER (26). Indeed, activation of Erk2 in T47D cells by the synthetic progestin R5020 can be inhibited by either antiprogestin (RU486) or antiestrogen (ICI 182,780) (26). Erk2 activation could not be achieved in R5020-treated T47D-Y cells, a T47D cell subtype that lacks PR (26). Moreover, R5020 could stimulate Erk2 activation in Cos-7 cells only when both PR and ER expression vectors were co-transfected but not when either of the expression vectors for these receptors was transfected alone (26). Although PR and Src kinase could be immunoprecipitated using an antibody to ER, ER but not Src was found in PR immunoprecipitates, which indicated that PR and Src kinase existed in mutually exclusive ER-containing complexes within cells. Therefore since ER, PR, and Src kinase do not appear to form a ternary complex in these cells, the mechanism whereby progestin stimulates Src/Erk signaling is presently unclear (26).

The role of the ER in mediating E2-stimulated Erk1/Erk2 activity in the brain cortex is less clear. It has been shown that Erk2 can be activated in E2-treated cortical tissue samples obtained from either wild type or ERα knockout (ERKO) mice (34). However, a partially ERβ-selective ligand (genistein) had no effect on Erk1 activation in wild type samples and ERβ mRNA expression levels are unchanged in ERKO samples (34). Taken together, these results suggest that neither ERα nor ERβ are required for E2-dependent activation of Erk1. However, this interpretation is complicated by the observation that the antiestrogen ICI 182,780 inhibited E2-dependent Erk2 activity in wild type but not the ERKO tissue cultures (34). Furthermore, an ERα-selective ligand (16α-iodo-17β-estradiol) inhibited basal Erk1 activation in wild type cortical samples (34). Therefore, further experiments are needed to clarify the roles that ERα and ERβ and/or potentially other factor(s) play in mediating E2-dependent Erk activation in the brain cortex.

Another member of the MAPK family, Jnk, can be influenced by treatment of cells with E2 (30). Jnk has been implicated as a factor that can induce apoptosis, in part by causing phosphorylation and thus inactivation of the antiapoptotic factors Bcl-2 and Bcl-xl. In contrast to the stimulatory effect that they exert on Erks, E2 and E2-BSA have been shown to inhibit Jnk activity and thus inhibit apoptosis in human breast cancer cells which have been stimulated with either ultraviolet irradiation or taxol (30). However, E2 treatment did not affect Jnk activity in cells that were not stimulated to undergo apoptosis, suggesting that the E2-stimulated pathway does not affect basal Jnk activity. As expected, the Erk1/Erk2 MAPKs were also activated in response to E2 treatment and are therefore likely to contribute to these E2-mediated anti-apoptotic effects. In contrast to Erks, which were activated within minutes of E2-BSA stimulation, activation of Jnk was observed following six hours of E2-BSA treatment. Parallel experiments were performed which demonstrate that E2-BSA did not significantly stimulate ERE reporter activity under the same experimental conditions, indicating that E2-BSA inhibition of Jnk likely occurred independently of transcriptional events. Estrogen treatment has also been shown to inhibit Jnk activity induced by

RANK ligand in the RAW 264.7 monocytic cell line, but this also required extended periods (24 hours) of hormone treatment (35).

Similar to Jnk-dependent signaling, p38 MAPK (also known as stress-activated protein kinase-2) induction has been correlated with increased apoptosis. Whereas E2 treatment of either MCF-7 or ZR-75-1 cells inhibits apoptosis by inhibiting Jnk activity (30), it has been reported that E2 treatment of HeLa cells stably-transfected with ERα promotes apoptosis by stimulating p38 MAPK (36). However, a subset of these ERα stable cell lines were resistant to E2-induced apoptosis, and upon further analysis it was revealed by gel mobility shift assays that the ER in these cell lines could not bind to EREs, suggesting that E2-induced apoptosis via p38 MAPK was related to classical genomic ER-mediated transcription.

4.2. Nongenomic Effects of Estrogens on Protein Kinase A

The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)-dependent signaling pathway can also be rapidly stimulated by E2 (37-40). Treatment with either E2 or conjugated E2-BSA promotes development of dopaminergic neurons, as determined by increased neurite outgrowth and arborization (37). These effects could be blocked by an inhibitor (H89) of PKA and the ability of antiestrogens to inhibit these responses seems to vary depending on cell type (37,38). Moreover, the E2-mediated increase in cAMP can be blocked by calcium chelators and therefore appears to require increases in intracellular calcium (38,39). Thus, the mechanisms by which E2 stimulates PKA may be due to crosstalk from other second messenger signaling pathway(s).

4.3. Nongenomic Effects of Estrogens on Protein Kinase C

Although the effects of estrogens on PKC activity have not been explored extensively, there is evidence to suggest that E2 can stimulate activity of this kinase signaling pathway. For example, in rat chondrocytes E2- and E2-BSA-dependent increases in alkaline phosphatase activity and proteoglycan sulfation have been linked to the ability of this steroid hormone to stimulate PKC activity (41). The increase in PKC activity could not be inhibited by the antiestrogen, ICI 182,780 (41). However, a G-protein inhibitor as well as a phosphatidylinositol-specific phospholipase C (PLC) inhibitor (U73122) attenuated PKC activity, which therefore appears to be related to a G-protein-dependent increase in PLC activity. In contrast, E2-dependent stimulation of PKC activity in guinea pig hypothalamic neurons desensitizes G-protein-coupled receptor (GPCR)-mediated events (42), which interestingly, may explain the rapid, negative feedback exerted by estrogens on the hypothalamic-pituitary axis *in vivo* (43). The mechanism by which E2 stimulated PKC in this latter case has not been identified.

4.4. Nongenomic Effects of Estrogens on Calcium

Calcium (Ca^{+2}) is a ubiquitous second-messenger which can influence several intracellular signaling pathways and conversely, these signaling pathways can influence Ca^{2+} levels [reviewed in (44)]. Normally, cytoplasmic Ca^{2+} levels are tightly controlled with relatively high levels found in the extracellular environment and in the endoplasmic reticulum (44). An appropriate stimulus can cause Ca^{2+} to be released from the endoplasmic reticulum and/or be taken up from the extracellular environment, resulting in a rise in cytoplasmic Ca^{2+} (44).

Although E2 stimulation can alter intracellular Ca^{2+}, no generalizations can be made about the mechanism(s) by which estrogens exert this response (28,39,45,46). In many cases these responses do not appear to be blocked by antiestrogens (39,46,47). Some E2-induced changes in cytoplasmic Ca^{2+} can be attenuated either by removing calcium from the extracellular medium or by subjecting cells to L-type Ca^{2+} channel blockers, which indicates that Ca^{2+} influxes through membrane Ca^{2+} channels can make a significant contribution to these cytoplasmic Ca^{2+} changes (39,45,48,49). However, an intracellular calcium chelator demonstrates that estrogen can also mobilize calcium from endoplasmic reticulum stores (28). These hormone effects on membrane calcium channels as well as the endoplasmic reticulum can be mediated through various signal transduction cascades which can involve receptor-tyrosine kinases and G-protein-coupled receptor stimulation of phospholipase C (44). For example, it has been demonstrated that PLC inhibitors (neomycin and U-73122) can attentuate E2-induced changes in Ca^{2+} (49), although in another study a PLC inhibitor had no effect, which might reflect cell type-specific differences in these mechanisms (48). However, the dynamics of Ca^{2+} signaling must also be considered when comparing these responses. Indeed, while it has been demonstrated that PLC inhibitors can block rapid (< 1 min.) increases in Ca^{2+} release, a second, presumably PLC-independent, wave (>5 min.) of Ca^{2+} release was also observed (49). Lastly, PKA and PKC are also thought to play roles in these E2-dependent Ca^{2+} responses (39,46). Thus, multiple signaling pathways can influence E2-dependent changes in cytosolic calcium and this likely depends on cellular context as well as other factors.

4.5. Nongenomic Effects of Estrogens on Endothelial Nitric Oxide Synthase (eNOS)

Endothelial nitric oxide synthase (eNOS) is an intracellular enzyme that converts L-arginine to L-citrulline and nitric oxide, a by-product which exerts vasodilatory as well as cardioprotective effects. Consequently, the induction of eNOS activity is typically determined by measuring the conversion of radiolabeled L-arginine to radioactive L-citrulline. A role for nongenomic responses is supported by the observation that E2 rapidly stimulates eNOS activity (50-55). Activation of eNOS by E2 was typically inhibited by ICI 182,780. Moreover, in the absence of transfected ER, E2 was unable to stimulate eNOS activity in Cos-7 and CHO cells (52,54), suggesting that E2 induction of eNOS is mediated through classical estrogen receptors.

The ability of E2 to stimulate eNOS was recently associated with ERα-mediated activation of the

phosphatidylinositol-3-OH (PI-3) kinase-Akt signaling pathway (53,54,56). Antiestrogens can block E2-mediated activation of PI-3 kinase (56) and this is consistent with the sensitivity of eNOS activation to these antihormones. Activation of PI-3 kinase results in an increase in intracellular, membrane-bound phosphoinositides. Akt via its N-terminal pleckstrin homology (PH) domain is recruited to these membrane-associated phospholipids and becomes phosphorylated by two additional kinases. The activated Akt is subsequently released from the membrane and exerts its cellular effects by phosphorylating downstream targets. It has been shown that ERα interacts directly with PI-3 kinase in a ligand-dependent manner both *in vivo* and *in vitro* via the p85α subunit of this kinase (56). This interaction is important for eNOS activation since E2 is unable to stimulate eNOS activity in p85-null fibroblasts (56). Moreover, an exogenously introduced dominant-negative Akt abolished E2 stimulation of eNOS activity (53). Thus, ERα via the PI-3 kinase/Akt signaling pathway can activate eNOS.

Interestingly, another step in the ERα-eNOS signaling pathway may involve the ability of ERα to be localized in caveolae (50,52). Caveolae are membrane invaginations, that are coated on the intracellullar surface with multiple proteins, the most important being caveolin-1 [reviewed in (57)]. Indeed, in the absence of caveolin-1 these invaginations do not form (57). Caveolin-1 possesses a 20 amino acid sequence called the caveolin scaffolding domain, which facilitates recruitment of several signaling molecules including ERα and PI-3 kinase (57). Thus these caveolae might function as a scaffold that can bring ERα and PI3-kinase together.

4.6. Putative Membrane Binding Proteins for Estrogens

One of the major controversies about the nongenomic effects of steroids has been the nature of the membrane steroid binding protein(s). Several mechanisms could possibly account for the nongenomic effects of steroid hormones. These include: 1) classical nuclear receptors located at the membrane; 2) estrogen binding proteins unrelated to the classical nuclear receptor; and 3) nonspecific steroid effects on the lipid bilayer.

There is some evidence to suggest that E2 binding to cells is mediated by the classical ERs (58-64). *In vitro* binding studies have demonstrated that E2 binds to the membrane of CHO cells transiently transfected with either ERα or ERβ expression vectors (59). These studies were conducted by isolating the nuclear and membrane fractions by centrifugation and subsequently incubating them with radiolabeled E2 and then a crosslinking reagent (59). Following SDS-PAGE and autoradiography of the E2-bound proteins, membrane estrogen receptors were detected although they were much less abundant than nuclear receptors (59). Although this methodology has a high potential for cross-contamination between the two fractions that may skew the relative amounts of membrane to nuclear ER, other approaches also suggest that classical nuclear receptors may be located on the plasma membrane. For example, microscopic analyses are commonly employed to visualize immunolabeled membranes of whole cells (58,59,62). In non-permeabilized GH₃/B6/F10 rat pituitary cells, membrane ERα could be detected by multiple enzymatically-labeled antibodies suggesting that all receptor domains were exposed to the extracellular environment (62). This raises interesting, unresolved, questions about the nature and topology of the putative membrane-bound ER relative to the plasma membrane. In addition, it is interesting to note that these ERα antibodies can also stimulate release of prolactin from these cells suggesting a link between these estrogen binding sites and a biological response (64).

It has also been proposed that protein(s) unrelated to the classical ER could mediate binding of E2 to the membrane. For example, in mouse macrophages where E2-induced Ca^{2+} fluctuations were not inhibited by antiestrogens, membrane binding sites could be labeled with fluorescently-tagged E2 (E2-BSA-FITC) but not with ER antibodies, despite labeling of intracellular ERα; ERβ was not detected in these cells (65). Surprisingly, confocal laser scanning microscopic analysis revealed that the E2-BSA-FITC was internalized into the cytoplasm over a short time course, suggesting that the putative E2 binding protein(s) could undergo a dynamic shift from the extracellular surface to the cytoplasm (65). Importantly, these internalized molecules did not colocalize with markers for acidic vesicles as would be the case if internalization occurred through the normal route of macrophagic phagocytosis. Moreover, non-specific molecules such as BSA and BSA-FITC were not internalized. The E2-induced alteration in Ca^{2+} as well as E2-BSA-FITC internalization was blocked by co-treating cells with pertussis toxin, indicating that this E2 binding protein(s) might be associated with G-protein(s). In addition to identifying an E2 binding protein which is unrelated to ER, these observations raise the interesting possibility that E2 entry into these cells could be regulated, as opposed to passive E2 diffusion.

Lastly, the possibility has been raised that nongenomic responses in part could be due to nonspecific steroid effects on the lipid bilayer. Alterations in membrane fluidity can be determined by first introducing a fluorophore into the plasma membrane and subsequently measuring hormone influences on membrane-dependent light scattering (66). However, these E2-induced effects on the plasma membrane have been observed to occur typically in the presence of micromolar concentrations of hormone while physiological estrogen concentrations are in the nanomolar range (66). Furthermore, these nonspecific effects have not been correlated with alterations in intracellular signaling. Moreover, it has been demonstrated that the 17α-estradiol stereoisomer has little to no effect in comparison to 17β-estradiol in mediating nongenomic effects (45,46,65,67), and general membrane effects are therefore not likely to contribute significantly to steroid-induced nongenomic activity.

5. LIGAND-INDEPENDENT ACTIVATION OF ESTROGEN RECEPTORS

The ability of steroids to stimulate various signal transduction pathways is interesting in view of the

association between cell signaling, receptor phosphorylation and activation of receptor transcriptional activity. In contrast to the relatively well-characterized steroid-dependent activation of transcription, several nuclear receptors (*e.g.* ER, AR, PR, RXR, RAR, and VDR) can be activated in the apparent absence of their cognate hormone by processes referred to as ligand-independent activation (68). In general, the ability of several intracellular signaling pathways (*e.g.* PKA, PKC, MAPK) to stimulate nuclear receptor-dependent transcription is thought to be due to the ability of these signaling pathways to target the receptors and their associated proteins (*e.g.* coactivators, corepressors, heat shock proteins), at least in part by phosphorylation-based mechanisms (68).

5.1. ER Phosphorylation

Nuclear receptors, including ERs, are phosphoproteins (69-72). To date, five *in vivo* phosphorylation sites (Ser^{104}, Ser^{106}, Ser^{118}, Ser^{167} and Tyr^{537}) have been mapped for human ERα (73-77). ERα is hyperphosphorylated in response to hormone, and while these studies suggest that Ser^{118} is the major estrogen-induced phosphorylation site (73,75,76) another study indicates that Ser^{167} is the predominant site phosphorylated in response to hormone (74). This discrepancy might be attributable to cell type-specific differences (COS-1 cells *vs.* MCF-7 cells) in ER phosphorylation or reflect differences in the techniques used to map these residues (73-76). Tyr^{537} is a basal phosphorylation site, which is not further altered upon stimulation with hormone (77), and the role it plays in receptor-mediated transcription is unclear. It has been suggested that phosphorylation of this residue, which is located in the LBD, contributes to the ability of the receptor to dimerize and bind DNA (78). In contrast, mutation of Tyr^{537} to various other amino acids either has little effect or renders the ERα mutants constitutively active, indicating that this residue need not be phosphorylated to attain maximal ERα transcriptional activity (79,80).

Research is ongoing to determine the mechanistic basis for hormone-induced phosphorylation of ERα, and several possible mechanisms have been examined by *in vitro* methods. Ser^{118} is part of a MAPK consensus sequence and the ability of MAPKs (Erk1/Erk2) to phosphorylate this residue in response to EGF stimulation has been observed (81). Somewhat surprisingly, the selective MAPK inhibitor, PD98059, was unable to block E2-induced receptor phosphorylation, measured as mobility upshift (slower migrating band, presumably due to phosphorylation) of ERα by SDS-PAGE, which suggested that E2-induced phosphorylation occurs independent of MAPK (82). Alternate factors that might contribute to E2-mediated phosphorylation of Ser^{118} were therefore explored. Based on studies demonstrating that cyclin-dependent kinase 7 (cdk7) mediated the ligand-dependent phosphorylation of the N-terminal domain of RARα, the ability of cdk7 to phosphorylate ERα was examined (83,84). Interestingly, in Cos-1 cells the combination of MAT1 and cdk7, two components of the general transcription factor TFIIH complex, phosphorylated Ser^{118} of ERα (83). Moreover, it was demonstrated that the ERα's AF-2 domain interacted with other subunits of

TFIIH, suggesting that ligand induces interaction with the TFIIH complex via the ERα LBD, thereby recruiting cdk7 to phosphorylate the AF-1 domain (83). This is consistent with the ability of the two AF domains to synergize in receptor-mediated transcription (85). The cell cycle regulator cyclin A/cdk2 has been shown to phosphorylate Ser^{104} and Ser^{106} *in vitro* and enhance E2-dependent activity in HeLa cells (86,87). Finally, another proposed E2-induced phosphorylation site (Ser^{167}) has been shown to be phosphorylated by casein kinase II *in vitro* (74). Thus, different signaling pathways can mediate phosphorylation on different residues within the AF-1 domain in response to hormone, and this may also contribute to the cell-specific activity of ERα. It is evident from recent studies that receptor phosphorylation also contributes to ERβ transcriptional activity (88,89).

5.2. Ligand-Independent Activation by Protein Kinase A

The ability of ERα to be phosphorylated in response to hormone is consistent with its ability to also be activated by several intracellular signaling pathways. Interest in these alternate signaling mechanisms began with earlier observations that a protein kinase A (PKA) activator 8-bromo-cAMP (8Br-cAMP) and a protein phosphatase inhibitor (okadaic acid) could stimulate the transcriptional activity of the A form of the chicken progesterone receptor (cPR_A) in the apparent absence of hormone (90,91). The neurotransmitter, dopamine, presumably through activating a PKA-dependent signaling pathway was subsequently found to stimulate both cPR_A- and ER-dependent transactivation (90,91). These results were surprising since the view at that time was that nuclear receptors were activated only in response to their cognate ligands. Like dopamine, cholera toxin (a G-protein activator) in combination with 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor), increases intracellular cAMP and thus activates PKA. These pharmacological agents when used to treat primary uterine cells in culture resulted in an increase in PR mRNA expression (92), a known ER target gene. As indicated by the ability of the antiestrogen, ICI 164,384, to block 8Br-cAMP-induced PR gene expression, this response was ER-dependent (92). Transient transfection studies indicated that cAMP activation of ERα does not require either Ser^{118} phosphorylation or the receptor's A/B domain (93). Indeed, an ERα deletion mutant lacking its entire amino terminus is capable of being phosphorylated to the same extent as the full-length receptor (73). Another study, which examined the ability of cholera toxin and IBMX to stimulate transcription of several synthetic ER target genes in CHO and 3T3 cells, revealed that activation also depends on cell and promoter context (94), which could be related to the ability of PKA-dependent pathways to regulate receptor dimerization and DNA binding (95). In addition, the ability of these cAMP/PKA-mediated responses to be blocked by cycloheximide, indicated that *de novo* synthesis of other cellular factor(s) might contribute to determining cell- and promoter-dependence (96,97).

The ability of the cAMP-dependent signaling pathway(s) to activate cPR_A in the absence of an

accompanying increase in receptor phosphorylation suggested that receptor-associated proteins might themselves be targets of phosphorylation and as a consequence, indirectly influence nuclear receptor-dependent transcription (98). Indeed, it has been demonstrated that 8Br-cAMP enhances phosphorylation of the SRC-1 coactivator on amino acids Thr[1179] and Ser[1185] and that mutating these residues to alanines (T1179A and S1185A) reduced coactivator-dependent enhancement of PR activity both for ligand-dependent and ligand-independent responses (99). These residues are thought to play a part in stabilizing SRC-1's interaction with another coactivator protein, p/CAF (99). Similarly, a PKA-dependent signaling pathway enhances the intrinsic transcriptional activity of another ER coactivator, CBP (100-103). The cell-cycle regulator, cyclin D1, has been demonstrated to enhance ER-dependent transcription, although it can also act as a corepressor of AR-dependent gene expression (104-107). Interestingly, the cAMP-mediated signaling pathway increases cyclin D1 association with ER, suggesting that the receptor's enhanced activity is a result of improved interaction with other coactivators (108). Thus, cAMP-mediated receptor-dependent transcription may depend on protein-protein interactions as well as modulating the intrinsic activity of coactivators.

5.3. Ligand-Independent Activation by MAP Kinases

Evidence that MAPK could stimulate ER transcriptional activity comes from observations that growth factors (e.g., EGF and IGF-1) can stimulate ER reporter gene expression in cultured cells (109-111). Importantly, these responses were blocked by the antiestrogen, ICI 164,384, demonstrating that they were ER-mediated (109-111). In contrast to the AF-2 domain of ERα, the AF-1 domain is considered to have constitutive activity and is strongly dependent on several of the mapped serine phosphorylation sites (73). Moreover, activation of the ERα by growth factors leads to phosphorylation of Ser[118] (81,109). Consequently, mutation of this residue to an alanine resulted in loss of EGF-induced activity (109). This phosphorylation was likely mediated by MAPK since overexpression of constitutively activated ras or MAPK kinase resulted in enhanced AF-1 activity (81,109). Conversely, a dominant negative form of MAPK kinase resulted in decreased activity (109). Taken together, ERα activation by EGF appears to be mediated through MAPK. However, another study suggests that EGF-mediated activation of ERα might also occur through phosphorylation of Ser[167] by pp90[rsk1] (112). The Src/Jnk signaling pathway also stimulates ERα AF-1 transcriptional activity, although this does not appear to require Ser[118] phosphorylation (113). Another report also demonstrates that the p38 and the Jnk MAP kinases stimulate ERα transcriptional activity independent of the known receptor phosphorylation sites (114). Thus, multiple factors mediate growth factor-dependent signaling to ERα.

Coactivators also play an important role in the ability of growth factors to activate ERα (115,116). The SRC/p160 family coactivators, AIB1 and GRIP1, can be phosphorylated by MAPK (Erk2) *in vitro* (115,116). Accordingly, AIB1 fused to the GAL4 DNA binding

domain stimulated GAL reporter activity when it was co-transfected with a constitutively active MAPK kinase, MEK1 (116). In addition, the p300 general coactivator could be co-immunoprecipitated along with AIB1 in a MEK1-dependent manner suggesting this signaling enhanced protein-protein interaction (116). Moreover, histone acetyltransferase (HAT) activity was detected in anti-AIB1-immunoprecipitated complexes prepared from cells expressing the active MEK1 (116). Similarly, the GRIP1 coactivator is phosphorylated by MAPK (Erk2) on Ser[736] *in vitro* and its EGF-induced intrinsic transcriptional activity is attenuated by mutating this residue to an alanine (115). Furthermore, the S736A mutant's ability to enhance the transcriptional activity of a GAL4 DNA binding domain-CBP coactivator fusion construct was substantially impaired relative to the wild type CBP counterpart (115). Taken together, these results suggest that MAPK phosphorylation of these p160 coactivator family members serve to enhance their interaction with p300/CBP and possibly other coactivators, and can therefore lead to indirect regulation of receptor-dependent transcription.

Some *in vivo* EGF responses have been attributed to this growth factor's ability to stimulate ER activity (8,110,117). In ERα knockout (ERKO) mice, the expression of PR in response to EGF is abolished (8). Importantly, EGF receptor expression, autophosphorylation and ability to stimulate c-Fos activity in response to EGF stimulation were not altered in ERKO compared to wild type mice, indicating that the EGF signaling pathway is intact (8). Recently, EGF-mediated activation of ERα was also found to be involved in the lordosis behavioral response in rodents (117).

5.4. Ligand-Independent Activation by Protein Kinase C

ERα-dependent transcriptional responses can also be influenced by agents that stimulate the intracellular PKC signaling pathway (118-121). Interestingly, the gonadotropin releasing hormone (GnRH) activation of ER reporter genes can be blocked by GF109203X, a PKC inhibitor (121). The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), which activates PKC, increases phosphorylation of the ERα A/B domain, and this is at least partially attributed to Ser[118] phosphorylation (73,76). However, TPA reduces ERα expression levels (120,122) and receptor binding to its estrogen response element (ERE) in vitro (123). The implications of these TPA-mediated effects relative to ER transcriptional activity are unknown. The ERα can be activated by TPA alone or in a synergistic manner with TPA plus E2, but ERα-dependent responses can also be inhibited by TPA in a promoter-dependent and cell type-dependent manner (118,122). In contrast to previously mentioned signaling pathways, the ability of PKC to modulate ERα-dependent transcription has received little attention and the significance of these effects on ERα activation remains to be determined.

6. PERSPECTIVES

In recent years, there has been a strong interest in characterizing non-genomic signaling by estrogens. Perhaps the biggest surprise has been the number of

different intracellular signaling pathways stimulated by estrogens, and the variety of different cell and tissue types that appear to respond to estrogenic stimuli from the extracellular compartment. The progress in defining estrogen-induced intracellular signaling pathways has been exciting and has revealed the broad potential of non-genomic steroid action to exert biological effects. Perhaps less clear at the present time is the mechanism(s) by which the estrogen signaling pathways are initiated at the plasma membrane. There is evidence both for and against the classical ERs being located at the plasma membrane in mediating these responses, and this is likely to remain an actively explored question in the years to come.

While it was initially surprising that the activities of ERs could be regulated by intracellular signaling pathways, the evidence supporting this mode of transcriptional regulation for ERα is increasing and ERβ studies are being actively pursued. At the present time, it is not clear whether ligand-independent ER signaling fulfills a role distinct from ligand-induced ER activity or whether cell signaling events merely augment estrogen-induced responses. Most studies indicate that ligand-independent signaling pathways do not serve any unique purpose, but rather seem to mimic steroid-induced responses. However, resolution of questions regarding the transcriptional targets of the ER induced by ligand-dependent versus ligand-independent signaling pathways awaits further experimentation, perhaps by microarray analyses of gene expression patterns. In view of the non-genomic ability of estrogens to induce the same signaling pathways that can activate the ER in a ligand-independent manner, it is possible that there will be significant overlap in the transcriptional targets of ER induced by ligand-dependent and ligand-independent signaling pathways. Perhaps a larger question at the present time is whether it is possible for estrogens to exert non-genomic and genomic responses in the same cell, and if so, whether these events are independent or influence each other. It seems likely that resolution of this question may have a significant impact on how estrogen action is viewed in a number of tissues, including the cardiovascular and skeletal systems, and may therefore provide novel and interesting pharmacological prospects.

7. ACKNOWLEDGMENTS

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