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SYMPOSIUM REVIEW

Membrane oestrogen receptor α signalling to cell functions

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Conservation of steroid hormone action outside the nucleus occurs from plants that make brassinosteroids to higher metazoans (primates). In plants, steroid hormone action occurs when the brassinosteroids bind a membrane tyrosine kinase receptor. Ligated receptors for all sex steroids exist at the plasma membrane and rapidly signal through G proteins to second messengers including calcium, cAMP and cGMP, activating proximal and more distal kinases. These signal cascades impact many functions of steroid hormones, responsible for the biological actions of these molecules. Support also exists for membrane-localized receptors of other members of the steroid superfamily, responding to glucocorticoids, mineralocorticoids, thyroid hormone, and vitamin D. The nature of these receptors is in some cases unclear. Steroid receptors also exist in discrete cytoplasmic organelles, most notably the mitochondria, although the functions of these receptors are poorly understood. In this review, I highlight the essential elements of the membrane oestrogen receptor α, noting where conserved aspects exist for other steroid receptors.

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Rapid oestrogen signalling from the plasma membrane was first identified in Clara Szego's laboratory (Szego & Davis, 1967; Pietras & Szego, 1977). Her group showed that oestrogen activated calcium flux in the uterus upon injection of oestradiol in rodents within seconds. Furthermore, she identified a binding protein that responds to oestrogen at the plasma membrane of cells. It took approximately another twenty years to implicate classical oestrogen receptors (ER) as mediating the rapid actions of oestrogen (reviewed in Hammes & Levin, 2007). These results inspired subsequent investigators to define many key aspects of the nature and functions of extra-nuclear ER and other steroid receptors. ERα and ERβ, the two ER isoforms, have been found to localize in many cells to the plasma membrane and to cytoplasmic organelles including mitochondria (Hammes & Levin, 2007). However, little is known about the functions of mitochondrial ER. Extra-nuclear ER exist in virtually all organs in the body, and increasingly, important functions have been uncovered. Continuing work shows unanticipated, novel functions for ER, often resulting from hormone actions outside the nucleus.

Oestrogen receptors at the plasma membrane

Controversy dominated the identification of the membrane-localized binding protein for oestradiol. Using immunological detection, antibodies to classical ERα identified similar binding proteins at the membrane of several cells (Norfleet *et al.* 1999). Further studies using anti-sense oligonucleotides suggested that the membrane ER and classical nuclear ERα must be very similar. More definitive studies required isolating cells from combined ERα/ERβ knockout (KO) mice and showing these cells lack all oestrogen binding at the plasma membrane, and fail to respond to17-β-oestradiol (E2) in rapidly activating signal transduction (Pedram *et al.* 2006). Plasma membrane ER were also localized to caveolae rafts, upon trafficking to the cell surface (Kim *et al.* 1999; Razandi *et al.* 2002; Chambliss *et al.* 2002). Here, interactions with a variety of signal proteins propagated the proximal signals to kinase cascades that influenced cell biological actions.

Perhaps the final proof of identity of endogenous E2-binding proteins resulted from an unbiased isolation of

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membrane ER from a breast cancer cell line (Pedram *et al.* 2006). This was accomplished by affinity chromatography of membrane proteins that bind to oestrogen-conjugated beads (Pedram *et al.* 2006). The only oestrogen-binding protein identified by mass spectrometry was the classical ERα, and was found to be identical to the nuclear ERα from these cells. Further confirmation of the identity of membrane ER(s) resulted from siRNA knockdown of classical ERα and ERβ, resulting in the loss of all oestrogen binding at the plasma membrane (Pedram *et al.* 2006). Thus, both isoforms of ER are present at the cell surface of target cells.

Putative membrane ER

The orphan G-protein-coupled receptor 30 (GPR30) has been purported to act as an ER at the membrane or endoplasmic reticulum (Filardo *et al.* 2000; Revankar *et al.* 2005). However, GPR30 was not isolated from the affinity column strategy noted above, no rapid signalling by ER was found in endothelial cells from ERα/ERβ KO mice, and GPR30 siRNA had no effect on rapid signalling by E2 in MCF-7 breast cancer cells (Pedram *et al.* 2006). This is despite the fact that abundant GPR30 exists in both cell types. Most definitively, four different GPR30 KO mice show little phenotype and lack any reproductive or mammary gland disruption of structure or function (reviewed in Levin, 2009). By contrast, the ERα KO female mouse exhibits infertility, extreme atrophy of the uterus, haemorrhagic cysts in the ovary, and rudimentary development of the mammary glands (Lubahn *et al.* 1993). ERα KO mice are obese, and lack the hypothalamic/pituitary feedback regulation of LH secretion seen in wild-type mice. Importantly, GPR30 KO mice exhibit comparable functions in all these respects to wild-type mice. In recent cell-based studies, E2 fails to bind to GPR30, and a GPR30 chemical agonist, G1, does not simulate oestrogen actions in mammary gland or reproductive organs (Otto *et al.* 2008). These results clearly indicate that GPR30 is not an ER.

It is feasible there is a collaboration of GPR30 with membrane ERα in some cell types, but this has to yet to be convincingly shown *in vivo*. Recent work suggests GPR30 mediates the effects of E2 to acutely stimulate LHRH secretion from hypothalamic neurons (Noel *et al.* 2009). However, female ERα KO mice lack the negative feedback regulation of LH and FSH by E2, and there is no abnormality of cycling or fertility in female GPR30 KO mice, shown from intensive investigation. Thus, the importance of GPR30 and membrane ERα interactions in more subtle biological functions such as in the central nervous system remain to be established.

Plasma membrane ER trafficking

Approximately 5–10% of ERα traffics to the membrane of all target cells examined to date, and ERβ is also found at this site in cardiovascular cells. At the membrane of vascular endothelial cells, ERα and ERβ exist as both homodimers and heterodimers. The membrane heterodimer in endothelial cells probably mediates the rapid actions of oestrogen to induce vasodilatation *in vivo*, resulting from rapid activation of ERK and PI3K, leading to nitric oxide generation that affects smooth muscle tone (Guo *et al.* 2005). In breast cancer cells, it is mainly ERα that is found at the plasma membrane with scant ERβ present (Pedram *et al.* 2006).

What structures dictate trafficking of ER to the plasma membrane? Serine 522 was first identified to be necessary for ER translocation, as this residue promotes an interaction with the caveolin-1 protein (Razandi *et al.* 2003). Caveolin-1 is a required transporter of ERα to the caveolae rafts in the plasma membrane. In cells lacking caveolin-1, endogenous ERα is only found in the nucleus (Razandi *et al.* 2003). Marino and colleagues established that cysteine 447 in the E domain (steroid hormone binding domain) of human ERα undergoes palmitoylation, an acylation that promotes association of ER with caveolin-1 (Acconcia *et al.* 2004). The cysteine is part of a larger 9 amino acid palmitoylation motif, found in both ER isoforms (Pedram *et al.* 2007), and by mutational analysis, key residues in this motif are necessary for full palmitoylation of ER. Similar palmitoylation motifs were also found in both progesterone and androgen receptor E domains.

For all sex steroid receptors, palmitoylation is required for plasma membrane localization and rapid signalling through ERK and PI3 kinases to cell proliferation (Pedram *et al.* 2007). Cellular proteins that promote or cause palmitoylation may explain why only 5–10% of ER are found at the plasma membrane, despite 100% of ER containing the palmitoylation motif. We propose that the protein abundance of the palmitoylacyltransferase for ER, or other proteins that facilitate palmitoylation may limit receptor trafficking to the membrane. Defining the proteins that facilitate ER palmitoylation and the subsequent steps of trafficking between cytoplasmic organelles and the plasma membrane is an important focus for future work.

Rapid signalling by ER at the membrane

Membrane-localized ERα and ERβ associate with and activate Gα and G$\beta\gamma$ proteins leading to the generation of rapid signals (Razandi *et al.* 1999; Kumar *et al.* 2007). How membrane ER activates G proteins and proximal signalling has not been clarified. ER signalling takes place in proximity to the caveolae rafts, leading to calcium and cAMP generation, and the activation of proximal

kinases (Src, PI3K) and distal kinases (ERK, AKT). These and other signals lead to the post-translational phosphorylation of many proteins, modulating cell migration, survival, and proliferation. Signalling from the membrane also enhances the transcriptional effects of nuclear ERα by several mechanisms (Björnström & Sjöberg, 2005).

To better understand the impact of membrane ERα signalling in development, we generated a transgenic mouse lacking all cellular ERα except a knock-in of the E domain of this receptor targeted exclusively to the plasma membrane (Pedram *et al.* 2009*a*). This membrane-only ERα mouse (MOER) showed female reproductive tract and mammary gland development that was comparable to the complete ERα knockout mouse. Furthermore, the mice were obese and lacked normal regulation of the hypothalamic–pituitary–ovarian sex steroid regulatory axis, both comparable to the ERα KO mouse. The results strongly support the importance of the nuclear ERα for normal development and function of these organs. Interestingly, signal transduction through ERK and PI3K was comparable in wild-type and MOER mouse liver, yet completely absent in the ERα KO mouse (Pedram *et al.* 2009*a*). Thus, it is the membrane-localized ERα that is necessary and sufficient for rapid signal transduction. These studies do not rule out a possible collaboration between the membrane and nuclear ERα pools that facilitates normal development of traditional target organs. In this sense, loss of either membrane or nuclear ER may compromise the normal development or function of target organs. Also, potential differences between MOER and ERα KO mice in bone biology, cardiovascular responses to stress, and functions of the central nervous system or many other organs have not yet been determined.

Impact of rapid signalling by ER in the heart and vasculature

Membrane ER induces rapid signalling through p38β MAP kinase to rescue cardiomyocytes from simulated ischaemia/reperfusion injury (Kim *et al.* 2006). Recent work implicates membrane ERβ in preventing cardiac hypertrophy, both *in vitro* and *in vivo* (Pedram *et al.* 2005, 2008). E2/ERβ signals through PI3K, inducing the transcription of the modulatory calcineurin-interacting protein (MCIP) gene. This resulting protein binds the catalytic site of protein phosphatase 2B (calcineurin) in the cytoplasm, blocking the increased activity of calcineurin that is stimulated by hypertrophic factors including angiotensin II (Ang II) (Pedram *et al.* 2005). Loss of calcineurin activity prevents the dephosphorylation of the NFAT family of transcription factors, dephosphorylation being required for nuclear translocation of this family. By inhibiting NFAT dephosphorylation, E2 causes sequestration of the transcription factors in cytoplasm and prevents transcription of hypertrophic genes (Pedram *et al.* 2005). Also, E2/ERβ activates transcription of the natriuretic peptide genes (ANP, BNP) and the protein products inhibit hypertrophic signalling by Ang II through ERK MAP kinase in the cardiomyocyte. These effects of the sex steroid were seen in wild-type and ERα KO mice but not ERβ KO mice (Pedram *et al.* 2008). Also, E2/ERβ prevents the reversal of myosin heavy chain isoform formation and cardiac fibrosis induced by Ang II, important steps that prevent progression of the heart from hypertrophy to dilatation and heart failure. It is possible that an ERβ-specific agonist might prevent cardiac hypertrophy in post-menopausal women who have risk factors such as poorly controlled hypertension. An ERβ-selective agent would avoid the breast and uterine proliferative effects of E2 that are mediated through ERα.

DNA repair signalling in breast cancer

E2 and ERα perhaps in conjunction with progesterone and its receptor promote the development of breast cancer. When the DNA of normal or transformed breast epithelial cells is damaged, cell cycle checkpoints are induced and rapid assembly of repair complexes at chromatin occurs (Sancar *et al.* 2004). Cell cycle checkpoint induction allows sufficient time for repair of the DNA lesions before the occurrence of replication (S phase) or cell division (M phase). Signal transduction is critical for many of these responses to DNA damage and is mediated by the PI3K kinase family members ataxia-telangiectasia mutated (ATM) and ATM and Rad 3-related kinases (ATR). Mutation of ATR, ATM or genes that are downstream targets leads to increased malignancies (Kastan & Bartek, 2004). Endogenous inhibitors of these kinases may impede cell cycle checkpoint induction and delay/block DNA repair, leading to the acquisition of mutations that promote the development or progression of cancer.

E2 and membrane ERα were recently identified to block ATR and ATM signalling and the activation of downstream kinases and phosphatases (Pedram *et al.* 2009*b*). This occurs when E2 signals through membrane ERα and PI3K/AKT to phosphorylate the TopBP1 protein, a protein that is necessary for ATR activity. Increased ATR activity follows radiation or chemotherapy damage of breast cancer or normal mammary epithelial cells (Pedram *et al.* 2009*b*) and E2/ER-induced phosphorylation at the single AKT site of TopBP1 prevents the enhanced ATR: TopBP1 interactions that promote kinase activity. These functions were not found for progesterone or testosterone, indicating oestrogen/ER specificity.

E2/ERα also causes enhanced chromosomal breaks in the setting of γ radiation, probably resulting from the steroid receptor blocking the normal responses to DNA damage noted above. These effects of E2/ER are prevented by ICI182780 (Fulvestrant), an ER antagonist and adjuvant therapy for women with breast cancer. This may represent a novel function of this therapeutic agent. These results suggest a new mechanism for E2/ER promoting both the development and progressive biology of breast neoplasia.

Summary

Steroid hormone actions at the various intracellular pools lead to cell biological outcomes. Delineating the discrete actions of the various receptor pools and how they integrate to produce a final biological effect will help us understand the many mechanisms of steroid hormone action. This is especially relevant to organs that are not thought of as traditional targets but are affected by sex steroids. For instance, ERα KO or aromatase KO mice exhibit many features of the metabolic syndrome, including insulin resistance and obesity. Transcriptional actions, rapid signalling and mitochondrial effects of ER pools in liver, muscle, fat and the β cell of the pancreas probably contribute to this phenotype. In some instances, we will need to better define the receptor isoform sub-classes. As an example the roles of ERβ in prostate or breast cancer are poorly understood, in part because different length proteins exist in different compartments within the malignancy and may have different functions.

To understand these complicated functions, new *in vivo* models of selective receptor pool loss in specific organs must be created, and reagents targeted to modulate single receptor pools must be developed. Using such approaches, we may uncover additional novel functions of ERs that are entirely unanticipated, enlarging the scope of sex steroid receptor action beyond traditional endocrine functions such as reproduction.

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