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Experimental models for evaluating non-genomic estrogen signaling

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ABSTRACT

Non-genomic effects of estrogen receptor α (ERα) signaling have been described for decades. However, the mechanisms and physiological processes resulting solely from non-genomic signaling are poorly understood. Challenges in studying these effects arise from the strongly nucleophilic tendencies of estrogen receptor, and many approaches to excluding ERα from the nucleus have been explored over the years. In this review, we discuss past strategies for studying ERα's non-genomic action and current models, specifically H2NES ERα, first described by Burns et al. (2011). In vitro and preliminary in vivo data from H2NES ERα and H2NES mice suggest a promising avenue for pinpointing specific non-genomic ERα action.

1. Introduction

1.1. Non-genomic estrogen signaling

Estrogen receptors play a crucial role in the maintenance of the female and male reproductive systems. They also bring about a wide range of effects in other tissues and organ systems. Known estrogen receptors include estrogen receptor α (ERα), estrogen receptor β (ERβ), and G protein-coupled estrogen receptor 1 (GPER1/GPR30). Investigators in the 1970's observed rapid estrogenic effects in uterine tissue and first proposed that these rapid actions could be modulated by estrogen receptors localized to the plasma membrane, where they also elicited signal transduction events. Elevation of uterine cAMP levels and eosinophilic infiltration [1], as well as calcium mobilization in endometrial cells following estrogen exposure [2], were the earliest observations of these rapid effects. In 1977, Pietras and Szego observed substantial binding of estrogen to the plasma membrane of endometrial and liver cells, and concluded the binding site was likely an estrogen receptor [3] due to the high affinity of 17β-estradiol (E2) to the binding site. Since then, non-genomic effects of estrogen receptor have been attributed to the increase of intracellular calcium concentration via activation of PLC*β* [4], activation of Gα and Gβγ proteins [5], regulation of potassium channels, activation of MAPK cascades, activation of lipid kinases such as phosphatidylinositol 3-kinase (PI3K), and adenylate cyclase [6].

Evidence for plasma membrane-localized estrogen receptor was introduced when Pedram and Levin isolated membrane-bound estrogen receptor from a breast cancer cell line, and with mass spectrometry confirmed its identity was identical to the *ESR1* gene product ERα [7]. However, attributing non-genomic signaling and estrogen action to the membrane localized ERα might be considered dubitable because ERα protein lacks known kinase or phosphatase motifs, thus it is unknown how E2 induces ERα-mediated non-genomic signal transduction events.

Non-genomic estrogen signaling is also carried out through GPER1, which was originally identified as the orphan G protein-coupled receptor 30 (GPR30) [8]. Of note is the fact that aldosterone binds GPR30 with higher affinity than estrogen [9], causing contention about whether GPER1/GPR30 should be considered an estrogen-specific receptor. Nonetheless, activation of GPER1/GPR30 elicits a variety of signal transduction pathways that execute estrogen's functions in vitro. Several different GPER1/GPR30 knock-out mouse models have been published, however results are variable and thus general conclusions are difficult to make [10–13]. One of the mutant mouse models used to report reproductive and estrogenic functionality and phenotypes showed no change in body weight, visceral adiposity, glucose tolerance, fertility, or normal estrogenic responses in the uterus and mammary gland of female mice, in contrast to the ERα knock-out mouse phenotypes [13].

In this review, we focus on previous and current efforts seeking to elucidate how ERα mediates non-genomic estrogen action.

1.2. ERα structure and mechanisms of action

Like other nuclear receptors, the structure of ERα is characterized by several motifs: the amino-terminal domain (A/B-domain), the DNA-binding domain (DBD; C-domain), the hinge region (D-domain), the

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ligand binding domain (LBD; E-domain) and the carboxy-terminal domain (F-domain) [14].

ERα's classical mechanism of signaling involves its localization to the nucleus where it directly binds to estrogen responsive DNA elements (ERE). This action results in changes in gene expression involving either stimulation or repression [15]. ERα's other mechanism of action in the nucleus involves tether-mediated signaling, in which it binds to other transcription factors such as c-Jun and Sp1, which in turn bind to AP-1 and Sp-1 DNA response elements to elicit changes in gene expression [16]. The third mechanism of ERα action is non-nuclear, non-genomic signaling in the cytoplasm of cells [17]. At least, the E-domain is involved in non-genomic signaling [18] but the involvement of other domains of ERα is still unclear.

2. Models of non-genomic estrogen action

Little is known about the precise physiological effects of non-genomic ERα signaling, and pinpointing these effects has proven to be complicated due to the difficulty in controlling for the strong nucleophilic nature of ERα. Observations of its non-genomic effects have been made by blocking RNA and protein synthesis for ERα-mediated gene expression, leading to the conclusion that non-genomic action can stimulate cAMP levels through adenylate cyclase activity [19]. Earlier pharmacological studies attempted to use E2 covalently conjugated with BSA (E2-BSA) to test for non-genomic E2 action, proposing that the E2-BSA complex could not enter the cells [20]. This approach was brought into question when Stevis et al. reported continuous leaching of free E2 from the E2-BSA conjugates and observation that E2-BSA stimulates sustained MAPK activity where free E2 does not activate under the same conditions. These results warned that biological activity of E2-BSA can lead to erroneous conclusions regarding the effects of E2 at the membrane [21]. Second generation approaches have employed estrogen-dendrimer conjugates (EDCs), where estradiol is confirmed to be covalently linked as another means to explore estrogen receptor signaling outside of the nucleus in both in vitro and in vivo models [22]. EDCs are multiple E2 molecules conjugated with polyamidoamine dendrimer macromolecules that are excluded from the nucleus due to their size and charge [23]. Utilization of EDCs has contributed to the findings that non-genomic ERα activates p44/42 MAPK (ERK1/2), Shc, and Src [23], stimulates vascular endothelial cell migration and proliferation, and protects against vascular injury without creating uterotrophic responses [22]. Additionally, use of EDCs in mice has shown that non-genomic ERα may prevent cortical bone loss post-ovariectomy [24] and reverse hepatic steatosis [25]. However, conclusions drawn from pharmacological studies in vivo to explore non-genomic ERα are limited by the fact that endogenous estrogen is present in non-ovariectomized animals and activates gene transcription.

Another method of studying non-genomic ERα action is the alteration of the receptor to create a mutant ERα that cannot localize to the membrane. Theoretically, any estrogenic effects seen in cells or animals with such mutation(s) are due to nuclear effects only, therefore loss of wild-type-associated phenotypes could be attributed to the loss of non-genomic action. Palmitoylation of cysteine 451 in the E-domain of ERα in mice (cysteine 447 in human ERα) causes the receptor to localize to the plasma membrane [26]. Taking advantage of this necessary modification, the C451A-ERα mutant mouse line was generated, in which C451A-ERα has an alanine instead of a cysteine at position 451 of ERα [27,28]. Alanine cannot be palmitoylated, thus the C451A-ERα cannot bind to the plasma membrane. This was confirmed in primary hepatocytes [27]. C451A-ERα was used to show E2-dependent carotid artery reendothelialization and endothelial NO synthase activation did not occur when ERα could not associate with the plasma membrane [27]. In C451A-ERα mice, uterine response to a 28-day exposure to E2 was normal as was the endometrial endothelial proliferative response to 24-h E2 exposure, however the ovaries were abnormal, with hemorrhagic and cystic follicles and no corpora lutea. Additionally, luteinizing hormone levels were significantly higher than normal [27].

The same point mutation in receptor position 451 was used by another group to create nuclear-only ERα mice (NOER), however these mice had differences in phenotype compared to C451A-ERα mice [28]. Pedram et al. observed that these mice had an abnormal uterine response to a 21-day E2 exposure [28]. These authors did not assess the acute response to E2, gene expression, or proliferation like Adlanmerini et al., making comparisons between the studies difficult. The contrasting phenotypes of these two mouse models, despite both models having the same mutation, might call into question the construction of the models. Indeed, hepatocytes in the C451A-ERα mouse showed a 55% reduction of membrane ERα [27], whereas in the NOER mouse, hepatocytes show no membrane ERα [28]. Pedram et al. postulated the incomplete reduction of membrane ERα to be the root of the inconsistent phenotypes of those mice [28]. While these nuclear-only ERα models are useful to study what happens when ERα cannot associate with the membrane, it is impossible to show the physiological function and signaling of membrane-associated ERα directly. To address this issue, a membrane only ERα mouse model (MOER) was generated by Pedram et al. [18]. MOER mice express a transgenic human ERα E-domain, which contains the palmitoylation site for localization to the plasma membrane, in an ERα knockout background. The uterus and vagina of MOER mice are atrophic, the ovaries have hemorrhagic cysts with no corpora luteum, mammary glands are underdeveloped, and there is increased visceral fat accumulation. All these effects are hallmark phenotypes of the ERα knockout mice [29]. E2 could activate ERK and PI3K in the liver cells isolated from MOER mice, in contrast to the liver cells isolated from ERα knockout mice. This mouse model, while effective in modeling effects of ERα at the membrane, is limited by the fact that only the E-domain of the receptor is present. Other domains of ERα that may play significant roles in protein interaction as part of cytoplasmic signaling are no longer present.

A more robust model was necessary to study the effects of non-genomic, non-nuclear ERα to account for its action in both the plasma membrane and the cytoplasm. The D-domain of ERα provided a novel opportunity to create a mutation excluding ERα from the nucleus. This domain is most commonly known as the hinge region because it is a flexible linker between the DBD and the LBD [30], but is also involved in tethered-mediated transcriptional regulation [16] and contains putative nuclear localization signals (NLS) [31]. It is also the site of several post-translational modifications including phosphorylation, acetylation, methylation, ubiquitination, and sumoylation [32–37].

Due to its NLS, the D-domain was targeted to prevent ERα localization to the nucleus. Earlier studies deleted this hinge domain and incorporated myristoylation and palmitoylation sequences to drive localization to the membrane [38]. This model demonstrated that nuclear ER genomic responses were lost but some rapid estrogenic effects were induced [38]. However, this approach may be problematic because the deletion of the D-domain may eliminate residues essential for normal ERα function. In a different approach, without deleting any functional domains, Burns et al. created the H2NES ERα mutant, which has point mutations in the NLS and an incorporated nuclear export signal (NES) sequence in the D-domain [39]. In vitro studies of H2NES ERα demonstrate that it is not localized to the nucleus even in the presence of ligand, or only very transiently localized in the nucleus, allowing observation of estrogenic effects mediated by membrane associated or cytoplasmic ERα, thus affirming that it is a useful model of non-nuclear ERα actions.

3. H2NES ERα

3.1. In vitro studies of H2NES

Burns et al. confirmed the putative nuclear localization sequences of ERα using the computational analysis tools LOCTree and Motif Scan. A bipartite NLS was observed in the D-domain. First, the H1 ERα mutant