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E10. Interpreting the results of the women's health initiative within the context of our current understanding of oestrogen and progesterone action

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1. Introduction

When initially designed, the stated objective of the Women's Health Initiative (WHI) was to evaluate the impact of exogenous "oestrogens" and "progestins" on cardiovascular health, osteoporosis and other conditions associated with long-term deprivation of oestrogen in menopausal women [1]. It remains to be determined whether or not the completed studies have provided definitive answers as to the impact of oestrogens and progestins on the health of menopausal women. However, what has become clear in the interim, between the initiation and completion of the study, is that the WHI was not an evaluation of oestrogens and progestins *per se,* but of a selected preparation of conjugated equine oestrogens administered with or without medroxyprogesterone acetate. There is now overwhelming evidence to suggest that different oestrogens (and progestins) are not recognised in the same way in all cells. Thus, it is incorrect to assume that similar outcomes, be they positive or negative, will be observed in patients treated with different estrogen (ET) or different estrogen/progestin combinations (HT) regimens other than those studied in the WHI. This extremely important issue has not been considered adequately by either the media or the medical community who have, based on the findings of the WHI, condemned ET/HT in general. It is worth noting that the Food and Drug Administration (FDA) has given specific guidance on this issue affirming in an important decision in 1996 that not all oestrogens are biologically equivalent. This direction was provided following a scientific discussion that concluded that a drug containing most of the high affinity oestrogens present in Premarin (conjugated equine oestrogens) could not be approved as a generic of the innovator drug as it was not biologically equivalent. Specifically, they [the FDA] contended that since all oestrogens were not functionally the same, it was not possible to exclude any component of Premarin and assume that biological equivalence could be assured. So why then, given that the FDA has dissuaded the grouping of oestrogens into a single functional category, has the WHI had such an impact on all ET/HT therapies? One obvious answer is that there has been a problem incorporating the recent advances in our understanding of the molecular pharmacology of oestrogens and progestins into medical practice. Consequently, one of the aims of this Review is to detail the important advances in our understanding of oestrogen and progesterone action that have occurred over the past few years and how these advances should impact upon modern menopausal medicine. This discussion will also serve the purpose of outlining what the future holds in this area of pharmaceutical exploration.

2. Oestrogen action

The first oestrogen-containing medicines were introduced into the clinic in 1947 for the treatment of the climacteric symptoms associated with menopause. At that time, the belief was that these hormones functioned as cofactors for a transhydrogenase that was involved in the generation of cellular nicotinamide adenine dinucleotide phosphate (NADPH) [2]. However, the discovery in the late 1950s by Jensen and colleagues of a high affinity oestrogen receptor (ER) in oestrogen

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responsive tissues and the subsequent demonstration by O'Malley and coworkers that this functioned as a ligand-dependent transcription factor laid to rest the transhydrogenase hypothesis [2–5]. It was these seminal discoveries that marked the beginning of the modern era of oestrogen (and progestin) pharmacology, a field of endeavour which in recent years has yielded a detailed, though probably not complete, understanding of the molecular determinants of ER pharmacology.

3. The oestrogen receptors are versatile regulators of transcription and cell signalling

The biological actions of oestrogen are mediated by two genetically distinct oestrogen receptors, ERα and ERβ, which share similar, though distinct, cellular expression profiles [6]. The second of these two receptors (ERβ), identified in 1996, has been shown to be functionally distinct from the previously described ERα [7]. In the absence of hormone, these receptors are maintained in a quiescent state in target cell nuclei through their association with heat-shock protein containing inhibitory complexes. Upon binding an agonist, they undergo a conformational change which facilitates their displacement from this inhibitory complex [8–10]. This results in the formation of ERα/ERβ heterodimers in cells expressing both receptor subtypes and homodimeric complexes in cells expressing a single subtype [9]. Whereas both receptor subtypes are capable of activating transcription, ERα appears to be a more robust activator than ERβ [9]. Not surprisingly, therefore, in cells where both receptors are expressed, it has been shown that ERβ moderates ER agonist activity [11]. Thus, the cellular response to oestrogens and anti-oestrogens depends on the relative expression level of both receptor subtypes in cells. That each ER subtype plays a different role in oestrogen action is supported by (a) the distinct phenotypes observed in mice in which either or both subtypes have been genetically disrupted [12] and (b) the observation that ERβ specific agonists function as effectively as prednisone in animal models of inflammatory bowel disease and rheumatoid arthritis, but do not manifest agonist activity in the reproductive tract, bone or mammary gland [13].

When functioning as regulators of transcription, the activated ERs can interact with target gene promoters (a) directly by binding to specific, high affinity Oestrogen Response Elements (EREs) within the regulatory regions of target genes or (b) indirectly through protein–protein interactions with transcription factors, like AP1 or SP1, which are prebound to target genes [14] (Fig. 1). The DNA bound receptor is then capable of positively or negatively regulating transcription. Of late, it has become clear that ER can also function as a transcriptional repressor by inhibiting the activity of transcription factors such as NFκB [15]. This inhibition results as a consequence of the physical interaction of the agonist-bound ER with the p65 subunit of activated NFκB. Although the precise mechanism by which this inhibitory activity is achieved has yet to be determined, it appears that this function of ER explains the anti-inflammatory actions of oestrogens observed in the brain and cardiovascular system [16]. This inhibitory function of ER may also play a role in mediating the anti-resorbtive actions of oestrogens in bone, though the latter conclusion remains controversial [17]. Finally, it has been shown *in vitro* that oestrogens acting through ERα or ERβ can also participate in non-genomic, extra-nuclear signalling events [18]. Although the field has not reached consensus as to the mechanism or physiological relevance of these latter activities, it is clear that there are responses to oestrogens that occur in the cytoplasm, or at the cell membrane of target cells, that do not impact upon gene expression directly. Among these observed responses, the activation of mitogen activated protein kinase (MAPK), regulation of calcium transients and activation of endothelial nitric oxide synthase (eNOS) in caveoli are the most intriguing [19–21]. The activation of MAPK appears to occur as a result of cSrc activation by a complex containing ligand-activated ER and the coregulator MNAR (modulator of non-genomic activity of oestrogen receptor). This pathway is of particular interest since MAPK itself can directly phosphorylate and potentiate ER transcriptional activity. Thus, it is possible that MAPK activation and other rapid non-genomic responses to oestrogens may provide a priming mechanism by which the cell can amplify hormonal responses. How ER, which is found predominantly in the nucleus, is relocalised to the cytoplasm or cell membrane to enable its participation in non-genomic signalling events is currently unknown.

4. Oestrogen receptor pharmacology

The demonstration that ER can function as a ligand-activated regulator of transcription allowed a simple model of oestrogen and anti-oestrogen pharmacology to emerge. In this model, agonists such as oestradiol, were proposed to function as molecular switches that bound and converted the receptor from an inactive to an active form. When corrected for affinity, therefore, all agonists were considered to be qualitatively the same and, by inference, receptor affinity was believed to be the major predictor of biological activity. It was also inferred by this model that antagonists functioned by competitively inhibiting agonist binding, freezing the receptor in an inactive state [22]. It has now become clear that this model does not adequately describe the actions of known ER ligands. Of note, was the observation in humans that the "anti-oestrogen" tamoxifen, while

capable of opposing ER action in the breast, could function as an estrogenic ligand in bone and in the uterus [23–26]. These important studies led to the reclassification of tamoxifen as a Selective Oestrogen Receptor Modulator (SERM), a compound whose relative agonist/antagonist activities can differ between cells [27]. A second study that contributed significantly to our understanding of ER pharmacology was that which demonstrated that receptor affinity was a poor predictor of biological activity [28,29]. Together these and other compelling studies indicate that the same ER-ligand complex is not recognized in the same manner in all cells and that factors other than the receptor and its ligand were important in regulating ER pharmacology. A resolution to this conundrum came in 1995 with the discovery of the first nuclear receptor co-factor, SRC-1 [30]. This protein interacts with agonist-activated ER and potentiates its transcriptional activity. To date, a large number of additional factors have been identified which have been shown to interact with ER and modulate its activity in target cells [31]. Functionally, there are two main classes of receptor interacting proteins, coactivators which enhance, and corepressors that repress, transcription activity [32]. Coactivators function by nucleating multiprotein complexes at target gene promoters, which by catalysing histone acetylation facilitate chromatin decondensation and enhanced transcription [33]. Corepressors function in reverse by recruiting enzymes that enable histone deacetylation, local condensation of chromatin and dampening of transcriptional activity [33]. With regard to the ER pharmacology, the most important finding was that the functional activities of different coactivators are not the same and that the absolute and relative expression level of these proteins can differ between cells. Similarly, it appears that cell-dependent differences in corepressor expression levels and activity also exist [32]. Reflecting these observations, it is now generally accepted that the transcriptional activity of an ER-ligand complex in a given cell depends on what coactivators and corepressors are available [32–34]. However, a further embellishment of this model was needed to explain how chemically similar agonists can manifest different activities within the same cell. We now know that the overall structure of the ER-ligand complex is not the same in the presence of different ligands [10,35,36]. Specifically, using a series of different biochemical and structural techniques, it has been shown that different ligands induce distinct structural alterations in ER [37–39]. In addition to classical ligands, the sequence of the DNA to which the ER is tethered is also a key modulator of receptor structure [40]. The significance of these differences in conformational states was revealed when it was demonstrated that conformation influences the recruitment of cofactors to receptors [36,41,42]. Thus, the cellular response to a given ligand is a reflection of the conformation of the ER-ligand complex and its ability to find an appropriate cofactor. We now know that additional complexity is engendered by cell signalling pathways, which through their action on receptors directly or through cofactors can impact upon ER transcriptional activity [43]. Indeed, so powerful are these alternate pathways that they can lead to the activation of the ER in the absence of a classical ligand [44,45]. Under normal physiological conditions, these alternate pathways of activation are probably involved in the fine-tuning of ER action [46,47]. However, it is clear that in certain pathological conditions they can dominate and even obviate the need for a ligand [48].

In summary, the molecular pharmacology of ER is extremely complex and is dictated by many factors. The most important of which are (a) the expression level of each of the two receptor subtypes in cells, (b) the impact which ligands have on receptor structure, (c) the relative and absolute expression level of cofactors, and (d) the activity of signalling processes that can interface with and modulate the activity of the component parts of the ER signalling pathway. Within the context of this new information, it is easy to appreciate why structurally different oestrogens can have dramatically different biological activities.

5. Progestin action

The essential elements of the progesterone signalling pathway are similar to what has been described for ER. The progesterone receptor exists in two forms in cells, hPR-A and hPR-B, both derived from the same gene by alternate use of two distinct promoters [49]. The B-form of the receptor differs from hPR-A by an amino-terminal extension of 164 amino acids [49]. However, this small difference is sufficient to generate two molecules with completely different biological activities [50]. In most environments, hPR-B functions as a ligand-dependent activator of transcription whereas hPR-A displays minimal transcriptional activity on Progesterone Response Element (PRE)-containing genes [50,51]. Instead, it appears from studies performed both *in vitro* and *in vivo,* that hPR-A functions as a modulator of hPR-B transcriptional activity [52]. This occurs as a consequence of hPR-A's ability to (a) form heterodimers with hPR-B and dampen its activity and (b) function as a transdominant inhibitor by interfering with the activities of coactivators associated with hPR-B [50,53]. The latter mechanism has been shown to explain how progestins, acting through hPR-A, can inhibit agonist-activated ER [54]. Specifically, hPR-A can interfere with the ability of ER to assemble coactivator complexes on target gene promoters. Because hPR-A and hPR-B are mechanistically different, it has been difficult to come up with a unifying model to describe PR pharmacology.

Indeed, it has been more instructive to consider them as "different" receptors that just happen to be regulated by the same class of hormones. For instance, it has been noted that any ligand that binds to hPR-A and facilitates its displacement from inhibitory heat-shock proteins is capable of opposing ER signalling in cells where both receptors are expressed [54]. For this reason, it is not surprising that all known PR agonists and antagonists have been shown to be equally effective at opposing ER action *in vitro* and *in vivo* [54–56]. The actions of progestins that are manifest in tissues such as the mammary gland appear to require hPR-B [52]. In this case, agonists can be distinguished from antagonists although it remains to be determined if the currently available progestins differ in their ability to regulate hPR-B function. The molecular pharmacology of PR has not been as well-defined as that for ER. Regardless, we know enough to predict that, as with ER, it will be possible to develop Selective Progesterone Receptor Modulators (SPRMs), whose relative agonist/antagonist activity will be manifest in a cell-selective manner. There are now reports in the literature of new progestins which display SPRM activity [57]. Confirmation of this activity in humans will change the face of HT removing some of the liabilities believed to be associated with currently prescribed progestins. To date, PR has been underexplored as a drug discovery target. However, as the specific regulators of progestin and antiprogestin pharmacology are defined, it is likely that this will change and new improved pharmaceuticals will emerge.

6. The future of ER and PR pharmacology

In the aftermath of the WHI, there has been a tremendous amount of discussion as to the future of ET/HT and the role of ER and PR as therapeutic targets in menopausal women. However, even a cursory consideration of the mechanism of action of these two receptors reveals that the existing pharmaceuticals are relatively primitive and that by using mechanism-based approaches for new drug discovery, improved therapeutics should emerge. Indeed, this has already started to happen with the appearance of the SERM, raloxifene, on the scene. This will soon be followed by bazedoxifene and lazofoxifene, two third-generation SERMs [58–60].

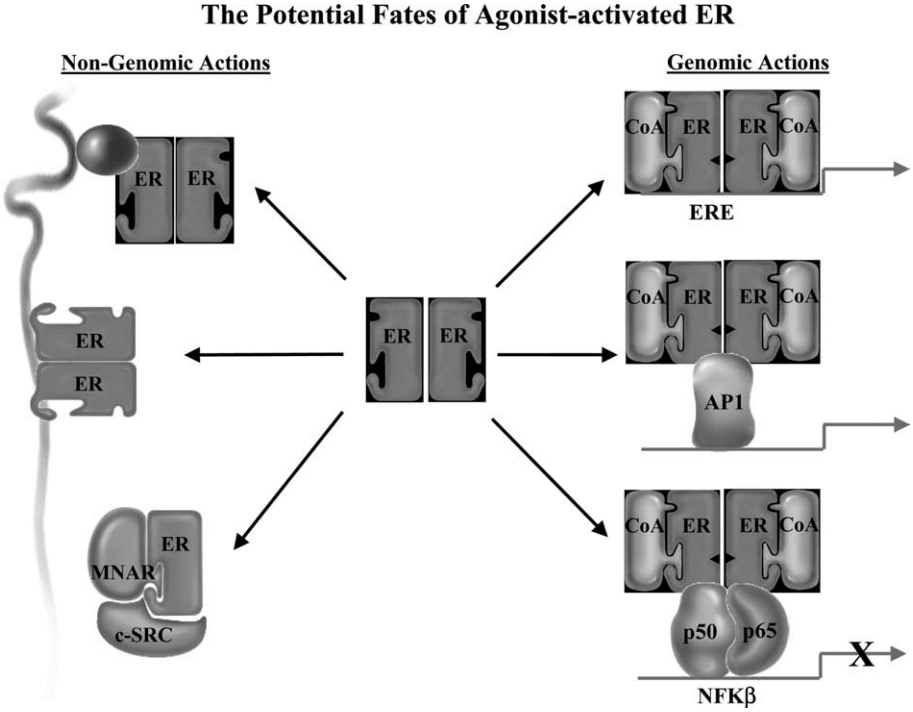

Fig. 1. *The oestrogen receptor (ER) signal transduction pathways*. Upon binding an agonist, ER undergoes agonist-specific conformational changes and forms dimeric complexes which then participate in several different signalling pathways, depending on the cell type. Within the nucleus, the receptor dimers recruit cofactors (coactivators or corepressors) and can interact with DNA directly through Oestrogen Response Elements (EREs) or indirectly through prebound transcription factors, such as AP1, to affect the transcription rate. Interestingly, although ER inhibits Nuclear Factor κ B (NFκB) activity, there is reason to believe that coactivators may be required. The non-genomic actions of ER occur in the cytoplasm or in caveoli at the cell membrane. Data suggest that ER can activate mitogen-activated protein kinase (MAPK) with one possible route occurring through the mediation of cSrc and MNAR (modular of non-genomic activity of oestrogen receptor) [42]. Finally ER, associated with the cell membrane, may be responsible, e.g., for rapid oestrogen-induced calcium transients [63,64]. Whether the membrane receptors represent subtypes of ER different than the nuclear and cytoplasmic receptors remains to be determined [19,64]. For activated progesterone receptor (PR), the overall scheme of fates and pathways is similar.

Unfortunately, none of these SERMs can treat the vasomotor symptoms associated with menopause [61]. However, although difficult to understand from a mechanistic point of view, there is reason to believe that combinations of a classical oestrogen with a SERM may bring satisfactory relief from vasomotor symptoms with a lower side-effect profile than currently approved medicines [62]. Obviously, the next advance in this field will be the development of truly specific SERMs and SPRMs. This, we believe, will be accomplished by defining the specific coactivators involved in a given process and screening for compounds that enhance specific ER-coactivator interaction.

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