British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01538.x
www.brjpharmacol.org

REVIEW

From *in vivo* gene targeting of oestrogen receptors to optimization of their modulation in menopause

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Keywords
oestradiol; atheroma; artery; uterus; menopause; SERM

Received
17 March 2011
Revised
4 May 2011
Accepted
17 May 2011

The ancestral status of oestrogen receptor (ER) in the family of the steroid receptors has probably contributed to the pleiotropic actions of oestrogens, and in particular, that of 17β-oestradiol (E2). Indeed, in addition to their well-described role in sexual development and reproduction, they influence most of the physiological processes. The pathophysiological counterpart of these actions includes prevention of osteoporosis, atheroma and type 2 diabetes, and also the promotion of uterus and breast cancer growth. Thus, the major challenge consists in uncoupling some beneficial actions from other deleterious ones, that is, selective ER modulation. Tamoxifen and raloxifene are already used, as they prevent the recurrence of breast cancer and mimic oestrogen action mainly on bone. Both E2 and tamoxifen exhibit a proliferative and, thus, a protumoural action on the endometrium. Activation of ERα and ERβ regulates target gene transcription (genomic action) through two independent activation functions, AF-1 and AF-2, but can also elicit rapid membrane-initiated steroid signals. In the present review, we attempted to summarize recent advances provided by the *in vivo* molecular 'dissection' of ERα, allowing the uncoupling of some of its actions and potentially paving the way to optimized selective ER modulators.

Abbreviations

AF, activation function; CEE, conjugated equine oestrogen; CHD, coronary heart disease; E2, 17β-oestradiol; ENISS, extranuclear initiated steroid signalling; ER, oestrogen receptor; HERS, Heart and Estrogen/Progestin Replacement Study; HT, hormone therapy; MPA, medroxyprogesterone acetate; SERM, selective ER modulator; VTE, venous thromboembolism; WHI, Women's Health Initiative

Introduction

After a century of implementing public health advances with vaccination and antibacterial therapies, life expectancy for women increased from 48 years in 1900 to more than 80 years nowadays (National Center for Health Statistics, 2004). Thus, facing menopause, which occurs on average at 52 years, perceived mainly as the menopausal symptoms (e.g. hot flushes) is a relatively recent challenge for women. Hormone therapy (HT) consisted initially of oral oestrogens derived from pregnant mares (Premarin®), also known as conjugated equine oestrogen (CEE), to replace endogenous oestrogen production that ceased at menopause. Oestrogens combined with a progestin have been used routinely for more than 50 years to ameliorate the menopausal symptoms, and, subsequently, to maintain bone density or prevent the rise of coronary heart disease (CHD) in women later in life. More generally, oestrogens and, in particular, 17β-oestradiol (E2), are implicated in a large number of physiological processes including the cardiovascular system, energetic metabolism,

and bone and central nervous systems, and the ambition of HT is to maintain physiologic homeostasis after menopause as close as possible to the premenopausal state.

Furthermore, oestrogens are also a potent stimulus for the growth of its target organs, such as the uterus, vagina and, often, oestrogen receptor (ER)-positive breast cancers. The link between oestrogens and breast cancer growth served as the incentive to develop anti-oestrogenic treatments, leading to an important therapeutic advance. ER antagonists, selective ER modulators (SERMs) and aromatase inhibitors were developed, but the later option also suppresses the beneficial actions of oestrogens.

Controversies about oestrogens and CHD: a question of timing

Although HT is recognized as the most efficient treatment of climacteric symptoms, its overall benefit was questioned since the publication of the Women's Health Initiative (WHI) (Rossouw *et al.*, 2002) in 2002, as this trial did not confirm the protective action of oestrogens against CHD. It was concluded to have an overall harm leading to a dramatic worldwide decrease in its use and concerns from clinicians and regulatory authorities (Clarkson and Appt, 2005; Dubey *et al.*, 2005; Rossouw *et al.*, 2007; Stevenson *et al.*, 2009). Indeed, epidemiological studies (Kalin and Zumoff, 1990; Dubey *et al.*, 2005) and the Nurses' Health Study (Grodstein *et al.*, 1996) suggested, and all animal models of early atheroma clearly demonstrated a vasculoprotective action of both endogenous and exogenous oestrogens (Holm *et al.*, 1999; Clarkson and Appt, 2005; Arnal *et al.*, 2007). However, the WHI trail reported that postmenopausal women receiving CEE with medroxyprogesterone acetate (MPA) had an increased frequency of CHD compared with women taking placebo (Rossouw *et al.*, 2002). Interestingly, the risk of CHD in hysterectomized postmenopausal women given CEE alone was quite similar to those receiving placebo (Anderson *et al.*, 2004). Altogether, these results underlined the deleterious effect of the association of oestrogens with the peculiar progestin MPA, associated to prevent the proliferative and thus, the protumoural action of oestrogens on the endometrium. (Clarkson and Appt, 2005; Dubey *et al.*, 2005; Rossouw *et al.*, 2007; Stevenson *et al.*, 2009). An analogous concern was demonstrated as early as 1997 by Miyagawa *et al.*, (1997). who showed that, whereas E2 prevented coronary endothelial dysfunction in ovariectomized rhesus monkeys, the combination of E2 plus MPA failed to exert this protective effect. Importantly, they also demonstrated that the prevention of vasospasm was fully observed with the association of E2 with progesterone.

It should be also underlined that in WHI, HT was prescribed, on average, 11 years after menopause, whereas HT in everyday life are given at the onset of menopause, at a time where more than 70% of the women suffer from climacteric symptoms. It is noteworthy that women of the WHI study who initiated HT closer to menopause tended to have reduced CHD risk compared with increased CHD risk among women more distant from menopause, showing that the outcomes of women under age 60 years or within 10 years of menopause closely resemble those from observational cohorts (Rossouw *et al.*, 2007; Stevenson *et al.*, 2009). Our understanding of the potential cellular targets and mechanisms of the vasculoprotective actions of oestrogens, as well as of the lack of action of oestrogens when administered after a period of hormonal deprivation, have been recently reviewed (Barrett-Connor, 2007; Rossouw *et al.*, 2007; Lenfant *et al.*, 2011)

The beneficial actions of oestrogens

The main reason of the HT at menopause is represented by a spectacular benefit on climacteric symptoms, as well as on quality of life. HT was introduced in the early 1930s to relieve hot flushes, night sweats, insomnia and dryness of the vagina, and, overall, to improve the quality of life for menopausal women. Over the past 70 years, it has continued to be the most effective therapy to reduce these symptoms. Nevertheless, after the publication of the WHI results, the impact of HT on health-related quality of life outcomes was questioned (Hays *et al.*, 2003; Brunner *et al.*, 2005). Although the study reported significant improvement in physical functioning and pain relief in women taking combined HT as compared with placebo, the differences were small. However, when the analyses were restricted to those women with menopausal symptoms, there were significant improvements in health-related quality of life related to emotional measures. Recent studies (Maki *et al.*, 2007; Welton *et al.*, 2008) undoubtedly confirm that HT is still the therapy of choice to maintain health, well-being and sexual enjoyment. Women randomized to HT experience reduced vasomotor symptoms and improved sleep. They also report fewer aching joints and muscles, less vaginal dryness and improved sexual enjoyment. Interestingly, the beneficial changes in sleep and sexual functioning are generally independent of the presence of baseline vasomotor symptoms. On the other hand, even though few observational studies have reported a positive effect of HT on depression in controlled trials, the depression component of the questionnaire showed no difference between HT and placebo groups. Altogether, these effects can now be factored into a woman's choice to use HT.

Besides the vasculoprotective actions of oestrogens when given early after menopause [detailed in several recent reviews (Rossouw *et al.*, 2007; Stevenson *et al.*, 2009; Lenfant *et al.*, 2011)], we will focus in the present paper on two major beneficial actions elicited by oestrogens: prevention of osteoporosis and of type 2 diabetes.

Osteoporosis affects more than 80 million people in the world and promotes fractures. Menopause-associated oestrogen deficiency causes accelerated bone loss and alterations in bone microarchitecture (Akhter *et al.*, 2007; Wehrli *et al.*, 2008). The beneficial effects of HT to prevent postmenopausal bone loss are indisputable. A meta-analysis of 57 randomized controlled trials (Wells *et al.*, 2002) reported that HT had a favourable effect at all sites (lumbar spine, forearm, femoral neck). The 2 year weighted differences in percentage changes in bone mineral density in controls and in women treated with HT averaged +6.8% at the spine and +4.1% at the femoral neck in favour of HT. The efficacy was independent of age or the duration of menopause. Moreover, in studies that

also reported on fracture, there was a trend towards a reduced incidence of vertebral and non-vertebral fractures. Ultimately, the WHI trial (Cauley *et al.*, 2003; Anderson *et al.*, 2004) has undoubtedly confirmed the beneficial effect of HT in reducing the risk of fracture. In hysterectomized women, oestrogen alone was also found to reduce the fracture risk to a similar extent as combined HT. Furthermore, it is important to outline that most of the women included in the WHI trial were at low risk for osteoporosis. The incidence of fracture was 10–15 times lower than that in most trials on the prevention of osteoporosis with bisphosphonates or raloxifene. The impact of HT on fracture rate is thus likely to have been less marked in the WHI population than among the general population of women who receive HT for the prevention and treatment of osteoporosis (Banks *et al.*, 2004). In a 5 year controlled study, combined HT given to early postmenopausal women was shown to be associated with a 70% reduction in the risk of non-vertebral fractures as compared with placebo (Komulainen *et al.*, 1998). Therefore, it has been suggested that HT provides the best protective effect against fractures among women at highest risk such as those who, for example, begin menopause with low bone mineral density and/or a high rate of postmenopausal bone loss.

A less expected observation was the unambiguous prevention of type 2 diabetes conferred by HT in menopausal women. It is well recognized that women before menopause are significantly more insulin sensitive than age-matched men (Nuutila *et al.*, 1995; Donahue *et al.*, 1997), and that menopause favours visceral fat deposition and insulin resistance, leading to a significant increase in type 2 diabetes risk (Wedisinghe and Perera, 2009). Thus, in addition to lifestyle westernization, especially the spreading of fat-enriched diet and sedentariness, menopause should be considered as a critical risk for metabolic syndrome and type 2 diabetes. As suggested by observational studies (de Lauzon-Guillain *et al.*, 2009), the main randomized placebo-controlled trials demonstrated that HT strongly reduces the incidence of type 2 diabetes in postmenopausal women. A 21 and 35% decrease in diabetes occurrence was reported in menopausal women receiving the association of CEE and MPA, in WHI and Heart and Estrogen/Progestin Replacement Study (HERS), respectively (Kanaya *et al.*, 2003; Margolis *et al.*, 2004). Although numerous studies in rodents also suggested an enhancing effect on insulin secretion and the preservation of pancreatic beta cells (Nadal *et al.*, 2009; Liu and Mauvais-Jarvis, 2010), corroborating data from clinical and experimental studies indicate that oestrogens mainly contribute to glucose homeostasis through their positive actions on body composition and insulin sensitivity (Louet *et al.*, 2004; Wedisinghe and Perera, 2009). For instance, during the follow-up of the WHI trials, fasting plasma glucose and insulin sensitivity index (homeostasis model assessment) were significantly improved by the administration of CEE + MPA or CEE alone in hysterectomized women (Margolis *et al.*, 2004; Bonds *et al.*, 2006). Finally, the crucial role of ERα in fat mass distribution and glucose homeostasis was first suggested by the unique clinical observation of a man bearing a mutation in the ERα gene who developed a premature and severe metabolic syndrome associated to vascular dysfunctions (Smith *et al.*, 1994). A few years later, ERα gene invalidation in mice led to a similar phenotype characterized by weight gain, visceral adiposity, insulin resistance and glucose tolerance in both males and females (Heine *et al.*, 2000; Cooke *et al.*, 2001), and we recently demonstrated the protective effect exerted by E2 against high-fat diet-induced insulin resistance, and glucose intolerance was totally abolished in ERα-deficient ($ER\alpha^{-/-}$) mice (Riant *et al.*, 2009). Altogether, these observations suggest that, in addition to the classical preventive/therapeutic intervention aiming to encourage physical activity and to reduce caloric intakes, the ERα pathway could represent an effective therapeutic target to fight against the epidemic worldwide progression of type 2 diabetes.

The deleterious actions of oestrogens

Both oral HT and oral contraceptives increase the risks of venous thrombosis and stroke (Herrington and Klein, 2001; Koh and Yoon, 2006), but the route of oestrogen administration might be a key determinant of these deleterious effects probably due to systemic hypercoagulability. Transdermally-administered oestrogen has less or no effect on circulating coagulation factors, as well as no effect on lipids and lipoproteins. This minimal effect on hepatic metabolism is attributable to the bypass of the portal circulation. Indeed, in the Estrogen and Thromboembolism Risk case/control study, transdermal oestrogen alone or combined with either progesterone/dydrogesterone or pregnane derivatives did not increase the risk of venous thromboembolism (VTE), which was not the case with the oral route (Canonico *et al.*, 2007). Furthermore, the type of progestin may also and again be a determinant factor since combinations with 19-norpregnanes were found to be associated with an increased VTE risk even when the oestrogen is given by the transdermal route (Canonico *et al.*, 2007). More recently, transdermal HT did not appear to increase the risk of stroke in another independent case/control study, especially with regimens using low doses of oestrogens (Renoux *et al.*, 2010). Thus, although these findings should be confirmed by randomized clinical trials, they strongly suggest that both the route of oestrogen administration and the type of progestin may be important determinants of the overall benefit–risk profile of HT.

However, the most feared effect of HT deals with the increase in cancer risk, not so much endometrial, but mainly breast cancer. It is well established that unopposed oestrogen therapy is associated with endometrial hyperplasia leading to an increased risk of endometrial cancer (Jordan, 2001). The stimulatory action of oestrogens in the uterus can be neutralized by combining the oestrogen with a progestin (such as progesterone or MPA). This combination was thus used by patients to prevent menopausal symptoms and osteoporosis, and was found to be safe in regard to endometrial cancer risk.

Importantly, inhibiting ER activity in breast cancer led to another important therapeutic success. More than one century ago, George Beaston was the first to report the benefit of oophorectomy in a young woman who had inoperable advanced metastatic breast cancer (Beatson, 1896). The identification of ER and the development of an ER assay by Jensen and Jacobson (Jensen *et al.*, 2010) allowed to demonstrate that the presence of the ER in a breast tumour increased the probability of endocrine ablation to be successful. The drug ICI-46474, known as tamoxifen (discovered in the anti-

fertility programme at Imperial Chemical Industries, ICI now AstraZeneca) was demonstrated by Craig Jordan (Jordan, 2003) to be efficient in the treatment and prevention of breast cancer by blocking oestrogen action at the level of the ER. Five years of adjuvant tamoxifen is known to reduce the local recurrence and distant metastatic disease by approximately 50% in patients whose breast cancer is ER-positive (2005). Adjuvant tamoxifen also reduces the risk of breast cancer mortality by approximately one third.

The comparison of the two arms of WHI (2002 and 2004) underlined the complex relationship between oestrogen and progestin not only in terms of CHD as previously mentioned, but also of breast cancer (Rossouw *et al.*, 2007; Stevenson *et al.*, 2009). Whereas CEE combined with MPA increased the incidence of breast cancer in non-hysterectomized women (Rossouw *et al.*, 2002), CEE alone unexpectedly decreased this risk in hysterectomized women (Anderson *et al.*, 2004). However, this latter result was questioned when more detailed analyses were conducted of overall hormone use, emphasizing cumulative hormone exposure as related to breast cancer risk (Anderson *et al.*, 2006). These contrasting findings underline that any effect of HT on breast cancer remains extremely controversial and emphasizes the necessity to further define the various actions of sex hormones. For instance, Horwitz (Horwitz, 2008) proposed the idea that systemic progestins have the ability to reawaken cancers that were presumed to be either non-existent or cured.

Altogether, according to the harmful action of at least some progestins, it is accepted that the strategy to develop new HT is to avoid the association of progestins to SERMs. Consequently, these SERMs should be devoid of endometrium action. For this purpose, a better understanding of the molecular mechanisms of ER activation is required to allow to the design of molecules, which would preserve the beneficial effects of oestrogens detailed above, while being devoid of any deleterious effects on the breast and endometrium (Jordan, 2001).

Molecular targets: ERs

The biological effects of E2 are mediated through binding and activation of intracellular receptors, the ERs α (ERα, NR3A1) and β (ERβ, NR3A2) (Ascenzi *et al.*, 2006). They belong to the nuclear receptor subfamily of ligand-inducible transcription factors whose members, based on structural and functional similarities, can be subdivided into six distinct regions termed A to F (Krust *et al.*, 1986; Tora *et al.*, 1989; McKenna and O'Malley, 2002; Ascenzi *et al.*, 2006) (Figure 1A). The C and E domains are responsible for DNA and ligand binding, respectively (DBD and LBD). Ligand-induced transcriptional activation by ER involves the action of two distinct activation functions (AFs), located in the N-terminal A/B (AF-1) and the C-terminal E (AF-2) domains (Tora *et al.*, 1989) (Figure 1A). Upon oestrogen binding, ER undergoes a conformational change that facilitates its recruitment to the promoter regions of target genes either directly through interaction with cognate DNA sequences (oestrogen responsive elements), or through protein/protein interaction with other transcriptional factors such as AP1 and SP1 (McKenna and O'Malley, 2002; Edwards, 2005; Ascenzi *et al.*, 2006). The subsequent recruitment of transcriptional coactivators via one or both AFs follows an ordered, cyclical and combinatorial process that leads to the activation of transcription of target genes (Metivier *et al.*, 2003). Transcriptional activation by the ER can thus be promoted through functional cooperation between both AFs or through each AF independently. The relative contribution exerted by AF-1 and AF-2 on the transcriptional activity of ERα varies in a promoter- and cell type-specific manner (Tora *et al.*, 1989; Berry *et al.*, 1990; Tzukerman *et al.*, 1994). Notably, it has been shown that the more a cell is differentiated, the more this cell mediates ERα signalling through its AF-1 in cell line models. In contrast, AF-2 is the only active AF in cells that have achieved their epithelial-mesenchymal transition (Merot *et al.*, 2004). Even though the mechanisms responsible for the differential sensitivity to both AFs still remain incompletely elucidated, tissue-dependent expression and/or activation of co-regulators is likely to play a key role (Smith and O'Malley, 2004). Moreover, several cell signalling pathways such as the MAPK (Kato *et al.*, 1995) or the Rho GTPase pathways (Huet *et al.*, 2009) can directly (*via* phosphorylation) or indirectly modulate the transcriptional activity of the receptor.

Besides these classic genomic actions, E2 also triggers many intracellular signalling pathways in a variety of cell types. For instance, E2 rapidly and transiently induces the activation of several kinases (MAPK, phosphatidylinositol 3-kinases or PKC), phosphatases and the adenylyl cyclase, as well as changes in calcium level (Kim and Bender, 2009). This 'extranuclear initiated steroid signalling' (ENISS) is mediated by a pool of intracellular receptors that are localized at the plasma membrane in caveolae rafts. Here, liganded ER initiates ENISS by transactivating receptor (growth factor receptors) and non-receptor (Src) tyrosine kinases, as well as G proteins, depending on cell context (Levin, 2005). The E domain of ERα appears to be sufficient for membrane localization and signalling (Kousteni *et al.*, 2001). Notably, palmitoylation of cysteine 447 appears important for membrane location via a physical interaction with caveolin-1 (Razandi *et al.*, 2003; Acconcia *et al.*, 2005). Finally, other proteins such as the adaptor protein MNAR or striatin were also shown to participate to membrane oestrogen action (Wong *et al.*, 2002; Lu *et al.*, 2004). Several recent reviews have been devoted to these ENISS actions in vascular cells (Levin, 2005; Mendelsohn and Karas, 2005; Kim and Bender, 2009; Simoncini, 2009) and, therefore, will not be detailed further.

As detailed later in this review, mouse models targeted for either ERα or ERβ allowed us and others to demonstrate that ERα is absolutely necessary for most of the beneficial actions of E2. Pharmacological approaches using receptor-specific agonists also support the importance of ERα (Bolego *et al.*, 2005). This does not exclude beneficial roles of ERβ. Induced expression of ERβ in ERα positive T47D (Strom *et al.*, 2004) or MCF7 (Paruthiyil *et al.*, 2004) breast cancer cells causes cell cycle arrest via the modulation of expression of important cell cycle regulators. Inhibition of tumour growth by ERβ might be mediated by an anti-angiogenic effect (Hartman *et al.*, 2006). However, at the molecular level, the mechanism of this antagonizing effect of ERβ on the function of ERα is still not well known, since ERα dominates ERβ in chromatin binding and that heterodimers are rare at common binding sites (Charn *et al.*, 2010).

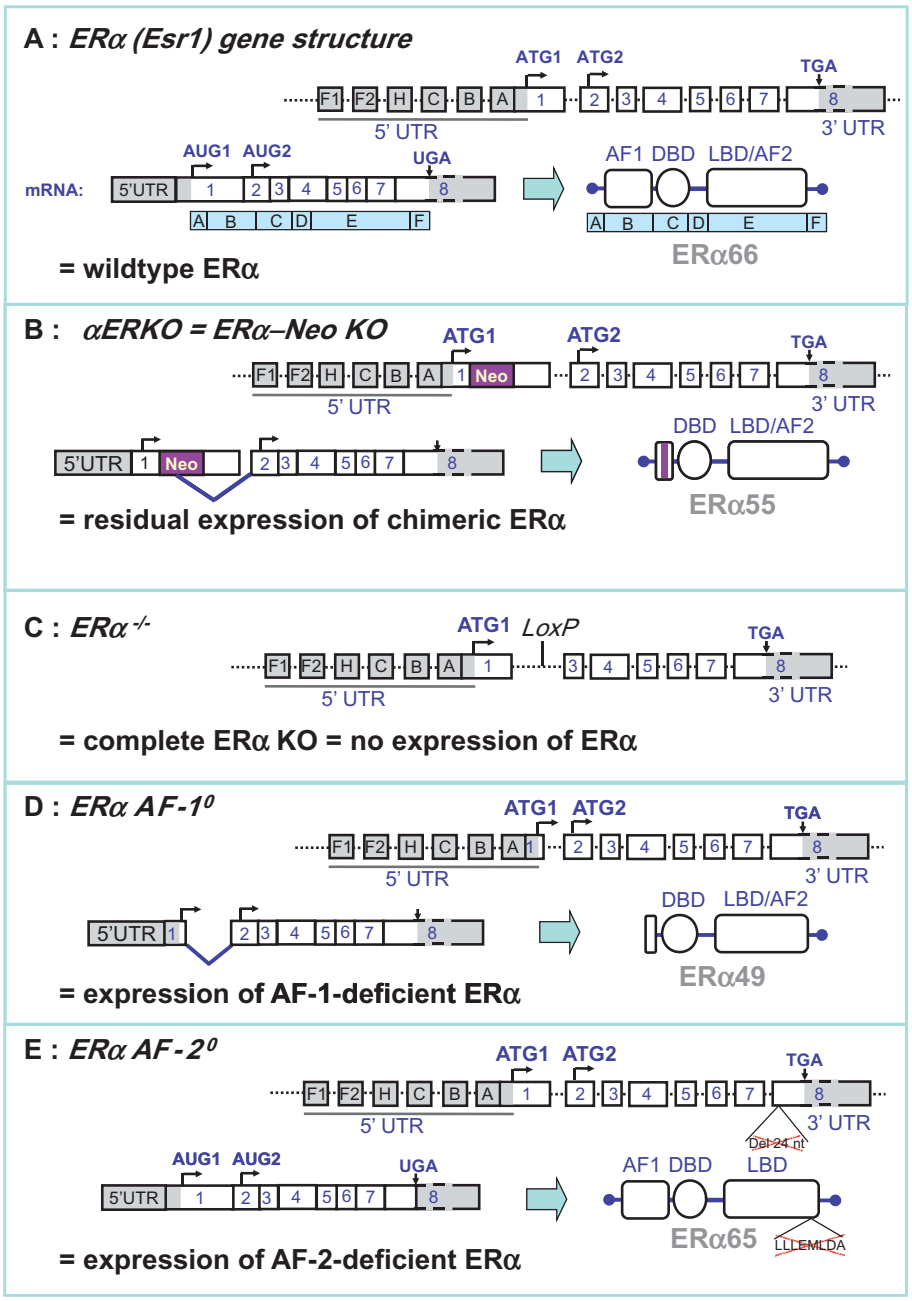

Figure 1

Schematic representation of the mouse ERα gene structure and the strategies of *Esr1* gene inactivation. (A) The *Esr1* gene encompasses eight coding exons and at least six non-coding 5′ exons. The full length 66 kDa ERα protein is composed of six domains (A to F), comprising a DNA-binding domain (DBD), a ligand-binding domain (LBD) and two activation functions (AF-1 and AF-2). The translation of the physiologically expressed 46 kDa isoform (not shown) is initiated at AUG2. This isoform lacks the entire A and B domains and hence, AF-1. (B) The first strategy of ERα gene targeting consisted in inserting a neomycin cassette in the first exon of the ERα gene (referred to as *αER-NeoKO*) (Lubahn *et al.*, 1993). The *αER-NeoKO* expresses at least two truncated ERα proteins, due to natural and non-natural splicing events, devoid of AF-1 function but with a functional AF-2. The splicing involving the neomycin cassette generates a chimeric 55 kDa isoform (Kos *et al.*, 2002; Pendaries *et al.*, 2002). The level of expression of these truncated isoforms is enough to mediate several actions of E2 in the vessel wall. (C) The second knockout approach (referred to as *ERα*$^{-/-}$ mice) consisted in introducing LoxP sites and then excising the second coding exon of *Esr1* gene coding for parts of the DBD (Dupont *et al.*, 2000). This strain does not allow the expression of any functional ERα. Accordingly, the vascular effects of E2 in that persisted *αER-NeoKO* were abolished in *ERα*$^{-/-}$, demonstrating that ERα appears to mediate most of the vascular effects of E2 (Pare *et al.*, 2002; Pendaries *et al.*, 2002). (D) *ERαAF-1°* mice consist in targeting the first exon of ERα gene coding for the A and B domains and thereby AF-1 (deletion corresponding to amino acids 2–148) (Billon-Gales *et al.*, 2009). The phenotype of *ERαAF-1°* mice is reminiscent to that *αER-NeoKO* mice, although the leakage and, thereby, the expression of chimeric 55 kDa isoform is highly variable (Kos *et al.*, 2002). (E) *ERαAF-2°* mice consist in deleting seven amino acids in the helix 12 and, thereby, AF-2 (deletion corresponding to amino acids 543–549) (ms submitted to publication).

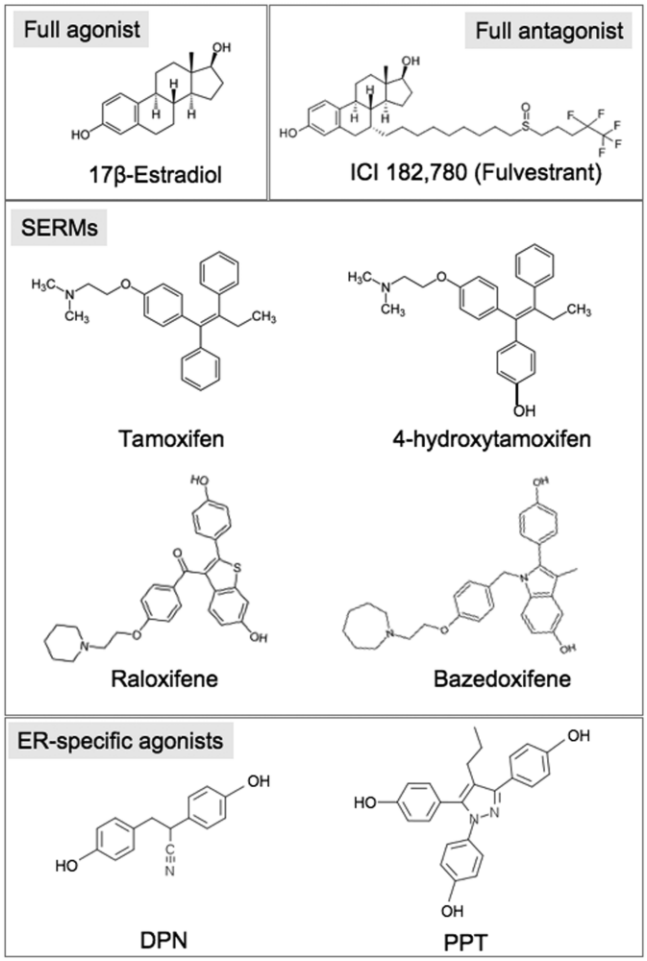

Figure 2
Structure of oestradiol, full antagonist, ER-specific agonists and SERMs.

Selective ER agonists and modulators

SERMs (Figure 2) are a group of drugs with heterogeneous structural chemical characteristics that are characterized as high-affinity ligands (in the subnanomolar concentration range) to ERs but differ from oestrogens by eliciting agonist or antagonist effects, depending on the target tissue (Katzenellenbogen and Katzenellenbogen, 2002). Selective ER modulation can be pursued because the conformational changes of ER induced by synthetic ligands are different from those induced by E2. In particular, the interaction of agonists with ERs results in the recruitment of co-activators, whereas antagonists favor recruitment of co-repressors.

A subclass of SERMs exhibits distinct binding affinity towards ERα or ERβ. The propyl pyrazole triol is a potent ERα agonist, which does not activate ERβ. This compound binds with high affinity and a 400-fold preference to ERα compared with ERβ (Kraichely *et al.*, 2000). In contrast, the compound diarylpropionitrile is a potency-selective agonist for ERβ with a more than 70-fold higher binding affinity for ERβ than ERα (Meyers *et al.*, 2001).

In addition to subtype-specific activation of ERs, other SERMs allow selective tissue-specific activation of ER. Tamoxifen (and its metabolite 4-hydroxytamoxifen) along with raloxifene are the two most widely studied partial ER agonists. Both exhibit anti-oestrogenic properties in ERα positive breast cancer. However, in the uterus, tamoxifen is associated with an increased risk of endometrial hyperplasia, which limits its use to women with breast cancer. As raloxifene does not increase the risk of endometrial cancer, its oestrogenic activity in bone combined with the added benefit of breast cancer prevention promoted raloxifene in the prevention and treatment of osteoporosis in postmenopausal women. However, both molecules increase the risks of VTE and fatal stroke and are devoid of benefit on hot flushes. Several third-generation SERMs are already undergoing tests in clinical trials and future studies will indicate which improvement they provide. Nonetheless, the analysis of the effect of these SERMs in ER-targeted mouse and cell culture models will allow improving our understanding of their mechanisms of action. In particular, the tissue-dependent anti-oestrogenic or oestrogenic activity of synthetic ER ligands is believed to be dependent on partial or full activation, or antagonism on AF-1 and AF-2, and the subsequent specific binding with co-regulators (Shang and Brown, 2002). These mechanisms will be approached and refined *in vivo* thanks to mice selectively inactivated for ER or one of the two specific AFs (see below).

Another way to selectively modulate ER action is to target ENISS effect. Recently, Chambliss *et al.* (2010) showed that an oestrogen-dendrimer conjugate, which activates membrane ERs without eliciting classic genomic effects, stimulates endothelial cell migration *in vitro* and accelerates re-endothelialization *in vivo*. Furthermore, they show that this vascular benefit of oestrogen-dendrimer conjugate *in vivo* occurs without stimulating the uterus or enhancing the growth of breast cancer xenografts. Taken together, these findings indicate that activation of ENISS regulates vascular events of physiological relevance and suggest that this approach could be helpful in particular to accelerate re-endothelialization after endovascular angioplasty. To which extent oestrogen-dendrimer conjugate does prevent atheroma remains to be determined.

Taken together, selective modulation of ERs could offer the possibility for uncoupling some beneficial actions of E2 from other deleterious ones. However, the molecular mechanisms underlying the tissue specificity of SERM effects are not yet completely elucidated and remain to be determined.

Lessons from ERs targeting in mice

Mouse models targeted for either ERα or ERβ were published in 1993 and 1998, respectively, but their respective roles remained elusive or even controversial until 2002 and even later. Why all these uncertainties?

The first mouse model of ERα gene disruption was generated by K. Korach's group, consisting in the insertion of the neomycin resistance gene in the first coding exon, thus named *ERα-NeoKO* mice (Lubahn *et al.*, 1993) (Figure 1B). As expected, these mice are unfertile when homozygous, and many of the actions of E2 on the reproductive targets were

abrogated. Studies of mice harbouring single gene targeting of either ERα or ERβ showed that E2 treatment of ovariectomized female mice inhibits the vascular injury-induced medial hyperplasia to the same extent than in wild-type mice. The authors suggested that ERα and ERβ are able to complement one another such that each receptor alone is sufficient to mediate the vascular protective effects of oestrogen. To test this hypothesis, they studied the effect of E2 on vascular injury in *ERα-NeoKO/ERβKO* mice; E2 no longer inhibited the increase in medial carotid area after injury, but still inhibited vascular smooth muscle cell proliferation after injury, raising the possibility of an unidentified third ER (Karas *et al.*, 2001).

However, at the same time, A Krust and P Chambon in Strasbourg generated a second mouse model of ERα gene disruption, consisting in floxing the second exon allowing the deletion of the C domain (Metzger *et al.*, 1995; Dupont *et al.*, 2000). These mice were demonstrated to be unambiguously and fully deficient in ERα, and only these mice can be considered as a real *ERα knockout* or *ERα–/–*. As expected, these mice are unfertile when homozygous and allowed to demonstrate that ERα is absolutely necessary to the beneficial actions of E2 on re-endothelialization (Brouchet *et al.*, 2001), on endothelial NO production (Darblade *et al.*, 2002) and on medial hyperplasia after vascular injury (Pare *et al.*, 2002).

We clarified the mechanisms accounting for the discrepancy between the two models of ERα gene targeting. We evidenced that *ERα-NeoKO* had a transcriptional leakage due to a non-natural alternative splicing of the ERα mRNA, resulting in the expression of a chimeric truncated 55 kDa isoform (Pendaries *et al.*, 2002). Such an ERα isoform, lacking a major part of the B domain and thus probably functional AF-1, was sufficient to mediate the E2 effect on endothelial NO production (Pendaries *et al.*, 2002) as well as on post-injury medial hyperplasia (Iafrati *et al.*, 1997). All these actions are fully abrogated in the second mouse model unambiguously and fully deficient in ERα (Dupont *et al.*, 2000; Brouchet *et al.*, 2001; Pare *et al.*, 2002; Pendaries *et al.*, 2002). Thus, along with pharmacological approaches using selective ERα or ERβ agonists (Bolego *et al.*, 2005), it is now clear that ERα, but not ERβ, is absolutely necessary for and, thereby, mediates most of the beneficial vascular actions of E2 (Arnal *et al.*, 2010). In addition, the discrepancies in phenotypes between the two mouse models of ERα gene disruption suggested that 'the AF-1 activation-function of ERα may be dispensable to mediate' some beneficial vascular actions of E2 (Pendaries *et al.*, 2002), opening a new field of research to uncouple the actions of ERα.

We recently directly explored the role of ERαAF-1 in the vascular actions of E2 *in vivo* using a mouse deficient in ERαAF-1 (named *ERαAF-1°*, Figure 1D). We found that ERα AF-1 is dispensable for several vasculoprotective actions of E2, whereas it is necessary for the reproductive actions of E2 (Billon-Gales *et al.*, 2009). In addition, ERαAF1 was dispensable for the atheroprotective action of E2 (Billon-Gales *et al.*, 2009). This last result probably explains why E2 prevented fatty streak in a fraction (4 of 14) of *ApoE$^{-/-}$ αER-NeoKO* mice (Lubahn *et al.*, 1993), since the leakage of the chimeric truncated 55 kDa ERα isoform was reported to be highly variable (Kos *et al.*, 2002). The respective roles of AF2 (thanks to *ERαAF-2°* mice, Figure 1E) and of ENISS in these actions should now be determined.

Several models of ERβ gene inactivation have also been generated and all concluded to have an important role in male and female reproduction. However, they also provided divergent phenotypes (Couse and Korach, 1999; Arias-Loza *et al.*, 2008; Zhao *et al.*, 2008), the more recent one (Antal *et al.*, 2008) lacking many of the previously described phenotypes, but the reasons for these discrepancies are still unclear.

Conclusions and perspectives

Various classes of oestrogens and SERMs have been described according to their molecular actions through ERα and ERβ. Due to the complexity of the mechanisms of action of ER, the *in vivo* effect of oestrogens and SERMs in various cell types and tissues cannot be predicted from *in vitro* studies. To date, the only SERMs currently available (tamoxifen, raloxifene, bazedoxifene) are characterized by an oestrogen-agonist effect on bone, as well as an oestrogen-antagonist effect on the breast, but are devoid of any positive effect on menopause symptoms and cardiovascular risk. Theoretically, it is conceivable to design a SERM (or a combination of molecules) devoid of the undesirable effects of E2 (mainly uterus and breast cancer), which would retain more desired effects of E2 (such as prevention of atheroma or type 2 diabetes) than do the SERMs presently available.

Hence, integrated mouse models allowing an '*in vivo* dissection' of ER and, in particular, of ERα, could represent an attractive way to conceive new tools to screen future SERMs in terms of beneficial and deleterious effects. For instance, as previously mentioned, the phenotype of *ERαAF-1°* mice (Billon-Gales *et al.*, 2009) suggests that SERMs stimulating ERα with minimal activation of ERα AF-1 could retain beneficial vascular actions while minimizing the sexual effects. The oestrogen-dendrimer conjugate, a selective activator of membrane ERα, could also provide vasculoprotection, although only acceleration of re-endothelialization was demonstrated so far (Chambliss *et al.*, 2010). Prevention of breast cancer, type 2 diabetes and cardiovascular diseases by novel SERMs thus represents the major challenge of the future treatment of menopause (Katzenellenbogen and Katzenellenbogen, 2002).

Acknowledgements

We are grateful to Professor F. Bayard and Dr J.C. Guery for their input in the work as well as to all the members of our team for their invaluable contributions over the years.

Sources of funding

The works performed in our team were supported in part by Université de Toulouse III, INSERM, Agence Nationale pour la Recherche (ANR), Fondation de France, Fondation Cœur et Artère, Conseil Régional Midi-Pyrénées, Société Française d'Athérosclérose, Société et Fédération Françaises de Cardiologie, and Laboratoires Pierre Fabre (CERPER).

Conflicts of interest

There are no conflicts of interest.

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