Review

MOLECULAR METABOLISM

Estrogen receptor subcellular localization and cardiometabolism

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ABSTRACT

Background: In addition to their crucial role in reproduction, estrogens are key regulators of energy and glucose homeostasis and they also exert several cardiovascular protective effects. These beneficial actions are mainly mediated by estrogen receptor alpha (ERα), which is widely expressed in metabolic and vascular tissues. As a member of the nuclear receptor superfamily, ERα was primarily considered as a transcription factor that controls gene expression through the activation of its two activation functions (ERαAF-1 and ERαAF-2). However, besides these nuclear actions, a pool of ERα is localized in the vicinity of the plasma membrane, where it mediates rapid signaling effects called membrane-initiated steroid signals (MISS) that have been well described *in vitro*, especially in endothelial cells.

Scope of the review: This review aims to summarize our current knowledge of the mechanisms of nuclear vs membrane ERα activation that contribute to the cardiometabolic protection conferred by estrogens. Indeed, new transgenic mouse models (affecting either DNA binding, activation functions or membrane localization), together with the use of novel pharmacological tools that electively activate membrane ERα effects recently allowed to begin to unravel the different modes of ERα signaling *in vivo*.

Conclusion: Altogether, available data demonstrate the prominent role of ERα nuclear effects, and, more specifically, of ERαAF-2, in the preventive effects of estrogens against obesity, diabetes, and atheroma. However, membrane ERα signaling selectively mediates some of the estrogen endothelial/vascular effects (NO release, reendothelialization) and could also contribute to the regulation of energy balance, insulin sensitivity, and glucose metabolism. Such a dissection of ERα biological functions related to its subcellular localization will help to understand the mechanism of action of "old" ER modulators and to design new ones with an optimized benefit/risk profile.

Keywords Estrogen receptors; Genomic effects; Membrane-initiated steroid signals; Energy balance; Glucose homeostasis; Cardiovascular system

1. STEROID HORMONE RECEPTORS: FROM SUBCELLULAR LOCALIZATION TO SIGNALING PATHWAYS

In a classical view of steroid receptor activation, the hormone binds to its cognate receptor in the cytoplasm, leading to dimerization and nuclear translocation. Then, this complex interacts with specific DNA sequences in target genes, providing the basis of the initial "two-step mechanism" of hormone action [1]. Accordingly, steroid receptors were initially viewed as primarily localized in the cytoplasm as monomers bound to heat shock proteins (HSPs). Steroid binding alters receptor conformation and triggers release from the HSPs, thereby allowing receptor dimerization and translocation in the nucleus where these dimers bind to specific DNA sequences and recruit numerous co-factors to regulate gene transcription [2,3]. This scheme perfectly applies to glucocorticoid and androgen receptors, as well as to estrogen receptors (ER) [4], although in many cells and tissues, unliganted ER have been mainly characterized as monomers primarily located in the nucleus [5,6].

Importantly, numerous steroid and non-steroid nuclear receptors have now been reported to be also present at the plasma membrane, even though their physiological functions *in vivo* remained completely unknown until recently. In addition to nucleus and plasma membrane, steroid receptors have been also visualized at the level of the mitochondria, endoplasmic reticulum and Golgi [6,7]. Interestingly, extranuclear actions of steroid receptor pools have been also described in plants, contributing to flowering and fertility regulation through the activation of tyrosine kinase receptors expressed at the plasma membrane [8]. The existence of a pool of steroid receptors at the plasma membrane suggests that these receptors have evolved to mediate extranuclear membrane-initiated signaling in addition to their role of transcription factor in the nucleus. Over the past few decades, more and more proteins have been identified for their ability to perform two or more distinct and relevant biochemical or biophysical functions that cannot be explained by gene fusions, multiple RNA splice variants, or pleiotropic effects [9]. We will see that ER should be considered as

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moonlighting proteins that switch between nuclear and membrane functions after undergoing post-translational modifications. According to the tissue-specific expression and subcellular localization of these receptors, the membrane and nuclear pools play their specific roles in rapid signaling and regulation of transcription, respectively, but can also probably interact in a still poorly recognized way.

Estrogen actions essentially result from the activation of two molecular targets, the estrogen receptors alpha (ERα) and beta (ERβ), which are encoded by two distinct genes (*ESR1* and *ESR2*, respectively) [10,11]. However, beside these two well recognized ER, two other G-protein-coupled membrane receptors (GPCR) have been reported to be activated by 17β-estradiol (E2), namely the G-protein-coupled receptor 30 (GPR30) and Gq-mER, this latter being identified mainly thanks to a pharmacological compound [12]. A paragraph will be devoted to ERβ and these two GPCRs, but the present review will be mostly focused on ERα, as a consequence of its prominent role in vascular and metabolic physiology and pathophysiology. We will attempt to summarize our current knowledge on the role of ERα subcellular localization, eliciting either nuclear or MISS effects, with a focus on their respective involvements in the vascular and metabolic actions of estrogens. Indeed, as described below, recent transgenic mouse models (targeting ERα activation functions or membrane addressing elements), together with the availability of new pharmacological compounds that selectively induce ERα MISS effects, recently provided significant new insights into the understanding of ERα signaling *in vivo*.

2. ERα IS THE MAIN MEDIATOR OF METABOLIC AND VASCULAR EFFECTS OF ESTROGENS

2.1. Evidence for the metabolic and vascular protection conferred by estrogens

Classically considered as reproductive hormones, estrogens have been recognized to also influence numerous physiological or pathophysiological processes. Among them, both clinical and experimental data demonstrate that estrogens elicit numerous beneficial actions on energy and glucose homeostasis. After menopause, estrogen deficiency favors visceral fat deposition, insulin resistance, and beta-cell dysfunction, leading to significantly increased risk of type 2 diabetes [13,14]. Conversely, in the main randomized trials, hormonal replacement therapy has been shown to reduce the incidence of type 2 diabetes in post-menopausal women [15–17]. Accordingly, in animal models from rodents to monkeys, bilateral ovariectomy impairs energy balance, insulin sensitivity, and glucose tolerance, while exogenous estrogen administration restores this protection [18]. Finally, the abolition of estrogen synthesis in subjects bearing inactivating genetic mutations of aromatase leads to obesity, visceral adiposity, insulin resistance and impaired glucose tolerance [19,20]. Genetically engineered mice models allowed to phenocopy these clinical observations, since aromatase gene invalidation similarly favors several features of the metabolic syndrome in both males and females [21].

Clinical and preclinical studies also provided considerable evidence that estrogens modulate cardiovascular physiology and responses to various situations [22]. The first decade after menopause is accompanied by an increase in blood pressure and has been associated with a higher risk of cardiovascular events such as myocardial infarction and stroke [23]. Even if the Women's Health Initiative (WHI) [24,25] did not initially confirm the expected protective action of estrogens against coronary heart disease and questioned their overall benefits, posthoc analysis suggested that the incidence of coronary artery events was reduced in women who initiated estrogen therapy soon after the onset of menopause, in contrast to the neutral or even increased risk observed in more aged women [26,27]. Experimental data demonstrate the major protective actions of estrogens on arteries. In particular, chronic E2 administration strongly prevents lipid deposition in mouse models of atherosclerosis, namely apolipoprotein E–deficient (*ApoE*$^{-/-}$) [28,29] and low-density lipoprotein receptor-deficient (LDLr$^{-/-}$) [30] mice. In addition, in several experimental models, ovariectomy exacerbates, whereas estrogen replacement attenuates, the course of hypertension [31,32]. E2 also increases basal nitric oxide (NO) production [33], accelerates reendothelialization [30,34] and prevents medial as well as neointimal hyperplasia after vessel injury [35]. Finally, E2 plays a crucial role in the ability of resistance arteries to remodel in response to a chronic increase in blood flow, which is necessary to optimize tissue perfusion [36,37].

Estrogens also exert protective effects in various animal models of myocardial and brain ischemia [38,39]. Enhancement of post-ischemic cerebral reperfusion, limitation of endothelial dysfunction, anti-inflammatory, and anti-apoptotic effects could participate to the neuroprotective action. Chronic and acute E2 treatment improves functional recovery after cardiac ischemia/reperfusion injury [40,41] and reduces cardiac necrosis [42]. E2 is also beneficial in a model of ischemic rabbit hindlimbs by favoring angiogenesis and perfusion [43]. However, the most impressive prevention of tissue necrosis elicited by E2 probably occurs at the level of the skin. Indeed, in a mouse model of skin ischemia, mimicking the surgery of skin flaps, we reported that E2 reduced up to 10-fold the necrosis as compared to untreated ovariectomized mice [44]. Altogether, it is now well accepted that estrogens elicit numerous beneficial actions on whole-body metabolism as well as on the vascular system.

2.2. Key role of ERα in vascular and metabolic protection

The crucial involvement of ERα in the metabolic and vascular protection conferred by endogenous estrogens was first suggested by a case report describing a man with a mutation in *ESR1* gene that led to a premature and severe metabolic syndrome associated to arterial dysfunctions [45]. A few years later, the first mouse model of ERα gene invalidation resulted in a similar phenotype characterized by accelerated weight gain, visceral adiposity, insulin resistance and glucose intolerance in both males and females [46,47]. However, these mice, generated by inserting a neomycin resistance cassette into *ESR1* exon 2 and thus named *ERα-Neo-KO* mice, were later demonstrated to still express a truncated 55 kDa ERα mutant form, resulting from a non-natural alternative splicing [48]. This ERα isoform, lacking a major part of the B domain and thus probably the functional AF-1 (see below for detailed ERα molecular structure), was sufficient to mediate the effects of E2 on endothelial NO production [48], on post-injury medial hyperplasia [49], as well as on endothelial healing [50]. Nevertheless, using a second mouse model characterized by a complete deletion of ERα (*ERα*$^{-/-}$) [51], our group demonstrated that ERα, but not ERβ, is absolutely necessary for E2 effect on reendothelialization [34] and on endothelial NO production [33]. Then, Pare et al. reported the same essential role of ERα for the protective action of E2 against medial hyperplasia in response to vascular injury [52].

Along with pharmacological approaches using selective ERα or ERβ agonists [53], studies in transgenic mouse models indicate that ERα is absolutely required for almost all the beneficial vascular actions of E2 [54]. The same conclusion applies to the metabolic influence of estrogens since ERα-deficient male and female mice (both *ERα-Neo-KO* and *ERα*$^{-/-}$ models) spontaneously develop a severe dysmetabolic phenotype associated with impaired energy expenditure and locomotion [46,47,55]. Moreover, we observed that ERα deletion (in *ERα*$^{-/-}$ males and females) exacerbates all the metabolic disorders (obesity, insulin

resistance and hyperglycemia) induced by a high-fat diet feeding, and totally abrogates the metabolic protection conferred by exogenous estrogen administration [55,56]. Importantly, the overall metabolic protection exerted by estrogens results from their specific actions on different ERα-expressing tissues including the central nervous system, white and brown adipose tissues, skeletal muscles, the liver, and the endocrine pancreas, as reviewed elsewhere [14,57–60].

3. CONTRIBUTION OF ERα NUCLEAR EFFECTS TO METABOLIC AND VASCULAR PROTECTION

As already mentioned, the conventional view of ERα signaling following estrogen binding successively implies dimerization and nuclear translocation, followed by dimer interactions with specific DNA sequences in target genes (Figure 1). Alternatively, ER can also modulate gene expression without direct DNA binding, but through interaction with other transcription factors, such as AP1 or SP1, a mechanism known as tethered steroid signaling. Thus, the multiple ERE distribution throughout the genome and these tethered interactions with other transcription factors contribute to the complex estrogen effects on gene expression (Figure 1).

From immunochemical studies performed on various cell types and tissues, it has been proposed for a long time that ERα, either complexed with a ligand or not, is essentially a nuclear protein [5]. This preferential nuclear location, along with the characterization of hormone-sensitive breast cancer according to positive ERα nuclear staining, have certainly contributed to the dogma that ERα is nothing more than a nuclear receptor that regulates gene transcription. As summarized hereafter, the conformational changes leading to the activation of ERα activation functions (ERαAF-1 and ERαAF-2) allow the recruitment of transcriptional coregulators and of the RNA polymerase II complex that finally regulate the transcription of a wide range of genes [61]. We will then detail how the more recent mouse models provided very interesting new insights into the specific role of ERα nuclear signaling in vascular and metabolic protective effects of estrogens.

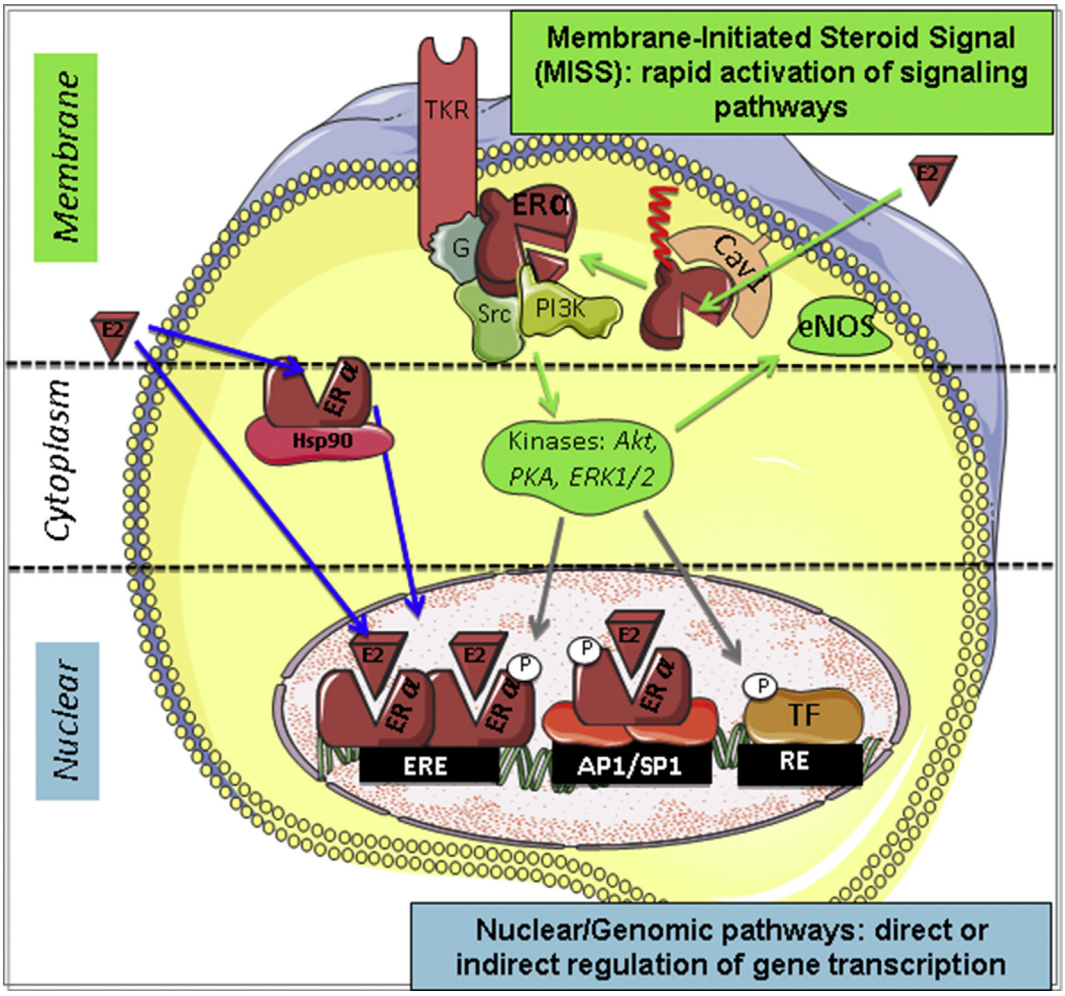

Figure 1: ERα subcellular localization allows the activation of both nuclear and membrane-initiated signaling pathways. This schematic representation first indicates the mechanistically distinct molecular pathways used by ERα to regulate gene transcription. The classical pathway (blue arrows) sequentially includes ligand (17β-estradiol, E2) activation of cytosolic ERα bound to heat shock protein 90 (Hsp90), ERα dimerization, and direct DNA binding to estrogen response elements (ERE). The tethered pathway (middle) depends on protein–protein interaction with other transcription factors following ERα ligand activation. In that case, gene regulation relies on indirect DNA binding on AP1/SP1 sites (through such interactions with other transcription factors). The membrane-initiated signal (MISS) pathway, also known as nongenomic pathway (green arrows), is mediated by a small pool of ERα localized close to the plasma membrane through ERα posttranslational modifications such as palmitoylation of Cys447 (human) or Cys551 (mouse) and direct interaction with caveolin-1. Upon E2 activation, membrane ERα interacts with protein kinases (Src and PI3K) or G-coupled protein αi (Gαi), leading to distinct signaling cascades (Akt, PKA, ERK1/2), and activation of endothelial NO synthase (eNOS). Grey arrows indicate the possible impact of the MISS pathway on nuclear/genomic pathways.

3.1. ERα molecular structure and role as a transcription factor

As a member of the nuclear receptor superfamily, ERα is characterized by a modular organization subdivided into six domains (A to F, see Figure 2) with a high degree of homology with ERβ in the C and E domains (97 and 60%, respectively), while the other domains are more divergent [62–64].

As a phylogenetic signature of the nuclear receptor superfamily, the central C region of ERα includes the DNA Binding Domain (DBD). The compact globular structure of the C domain involves two zinc fingers. The α-helix of the first zinc finger module controls DNA binding by lodging directly into the major groove of DNA. ERα specifically recognizes DNA sequences characterized by a 13 base-pair consensus (GGTCAnnnTGACC), termed estrogen-response elements (EREs). The binding of an ERα dimer to such an inverted palindrome implies that the two monomers are arranged in a symmetrical face-to-face way.

The D domain is a hinge region between the C and E domains [63], which is involved in ERα conformational changes upon ligand and DNA binding, and in protein–protein interactions [65]. The D domain plays a crucial role in the interactions between the receptor and other transcription factors, namely Fos/Jun or SP1, allowing indirect binding to DNA. This ERα region also comprises nuclear localization signals (NLS), which cooperate to promote receptor translocation from the cytoplasm to the nucleus [66]. Finally, the D domain is submitted to numerous post-translational modifications (including phosphorylation, acetylation, methylation, ubiquitination or sumoylation) that modulate the activity of the receptor [67].

Identified as the ligand-binding domain (LBD), the E domain comprises three layers of twelve α-helices, including helix H12, and two β-sheets that overall form a hydrophobic pocket allowing ligand binding [68]. The E domain contains a ligand-dependent activation function, named AF-2 involving the amino acids 538 to 552 of H12 [69]. The E domain (LBD) is the main dimerization interface of the receptor upon hormone binding, although unliganded LBD can form stable dimers that are further stabilized by ligand binding.

The N-terminal sequence includes two distinct domains called A and B [63]. In unliganted human ERα, the A domain interacts with the C-terminal region, competing with H12 and corepressors for an identical binding site [70]. The B domain bears another activation function named AF-1 that can mediate transcriptional activity when isolated from the rest of the protein, in a hormone-independent manner (i.e. in the absence of E2) [71]. However, AF-1 and AF-2 both contribute to the transcription of target genes *in vitro* by recruiting transcriptional coregulators, demonstrating that, in the entire protein, ERαAF-1 is also involved in ligand-dependent activation. About 300 coregulators (coactivators or corepressors) of nuclear receptors have been identified [72], which generally exhibit intrinsic enzymatic activities involved at various transcriptional steps. Among them, 17 to more than a hundred have been reported to be recruited by ERα complexes [73,74].

Interestingly, N-terminal serine residues (104/106 or 118) in the B domain can be phosphorylated by several intracellular kinases downstream of growth factor receptors [75], contributing to the recruitment of various coactivators [76,77]. The importance of AF1 is further supported by the existence, beside the "classic" and most

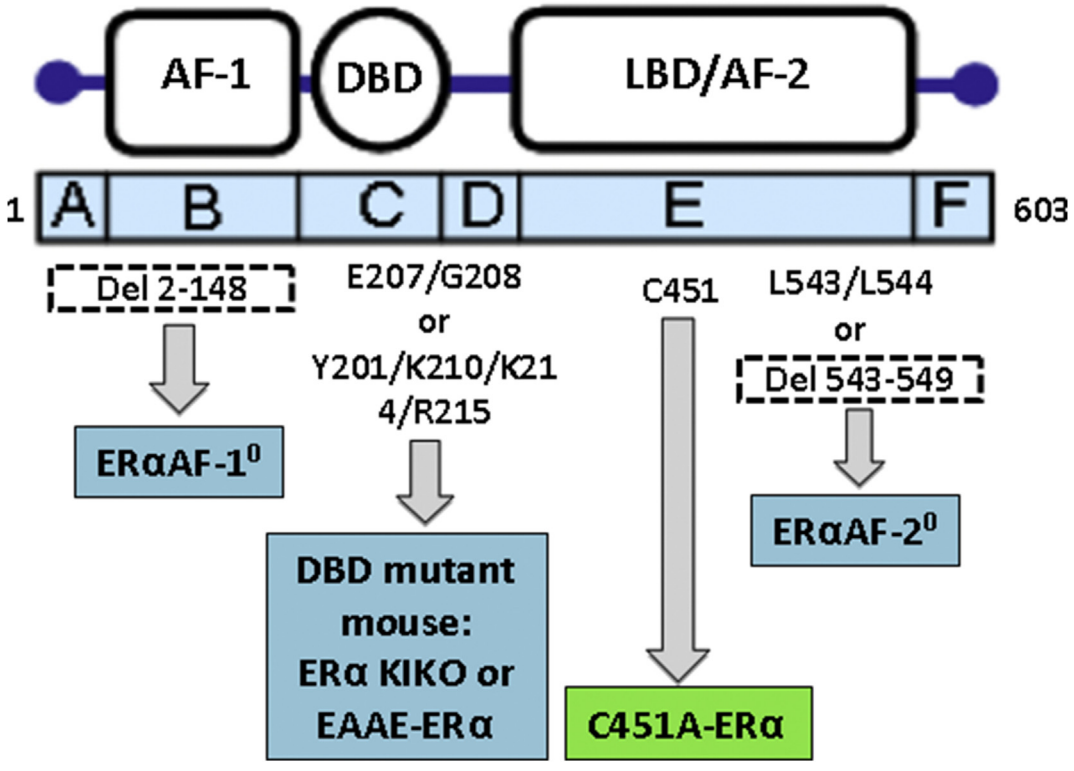

Figure 2: ERα modular structure and molecular strategy used for the generation of ERα mutant mice targeting either nuclear or MISS signaling pathways. The full length 66 kDa ERα can be subdivided into six domains (A to F), including a DNA binding domain (DBD), a ligand binding domain (LBD), and two transcriptional activation functions, respectively named AF-1 and AF-2. For a more detailed description of ERα molecular structure, please refer to the text and the following references [80] [89] [82] [92] [94]. Below ERα linear structure are indicated the strategies that have been held (targeted amino acids for point mutations or deletions) to inactivate specific ERα functions and generate the main mouse models available to study the respective roles of nuclear ERα (*ERαAF-1⁰*, *ERαAF-2⁰*, *ERα KIKO/EAAE-ERα*, leading to full or partial inactivation of genomic actions) and MISS ERα (*C451A-ERα*).

abundant full-length 66 kDa ERα (ERα66), of another ERα isoform of 46 kDa (ERα46) which lacks the A/B domain and consequently AF-1. This isoform is expressed in various cell types, in particular endothelial cells [78], but also in most breast tumors [79]. It can be generated by either alternative splicing, proteolysis or translation through an internal ribosome entry site (IRES) [79], but its physiological role is still unknown.

3.2. Mouse models with altered ERα DNA binding or impaired nuclear localization

The first experimental strategy dedicated to the study of ERα nuclear effects *in vivo* consisted in the inactivation of the DNA binding [80–82]. To differentiate the direct interactions of ERα with ERE from indirect interactions at AP1 sites through tethering with Fos/Jun dimers [83], the DBD was first targeted by specific mutations that altered its DNA sequence selectivity [84].

A first mouse model was obtained by mutating the residues 207 and 208, two amino acids previously demonstrated to govern DNA sequence selectivity into the mouse ERα sequence (Figure 2). This mutated allele (E207A/G208A, or AA) was then introduced onto the $ER\alpha^{-/-}$ background to generate $ER\alpha^{-/AA}$ mice, known as the KIKO mouse model [80]. In this model, Park et al. suggested a substantial role for the ERE-independent non-canonical pathway. Indeed, the AA mutant allele was found to normalize energy expenditure and locomotor activity, and thus to rescue in a large part the dysmetabolic phenotype of $ER\alpha^{-/-}$ mice [85]. However, further investigations revealed that this mouse model retains DNA-binding activity [86].

Another transgenic mouse model has been described by Ahlbory–Dieker et al., consisting of four amino acid exchanges in the DNA recognition helix (Y201E, K210A, K214A, R215E), and hence named EAAE (Figure 2) [82]. In contrast to the KIKO model, this mutated ERα lacks its ability to induce the expression of ERE reporter gene, and to bind to other HRE (Hormone Response Elements) motifs [86]. Sharing several phenotypic features with $ER\alpha^{-/-}$ mice (impaired uterine growth and mammary gland development, hemorrhagic ovaries), the EAAE-ERα DBD mutant mouse demonstrated that ERα DNA-binding is required for biological and transcriptional processes in reproductive organs. In addition, the modulation of gene expression by E2 in the liver was nearly abolished in this model, and thus reported to be also dependent on an intact ERα DBD *in vivo* [82].

Then, a distinct experimental strategy has been developed, targeting the ERα D-domain to prevent the nuclear localization of the receptor as it contains nuclear localization signals (NLS). As previously indicated, this domain is mainly known as a flexible linker between the DBD and the LBD, but it also contributes to tethered-mediated transcriptional regulation. Combining point mutations in the NLS and incorporation of a nuclear export signal (NES) in the D-domain, Burns et al. recently proposed the *H2NES ERα* mutant as another model devoid of ERα nuclear actions [87]. *In vitro* studies demonstrated that *H2NES ERα* mutant does not, or very transiently, localize into the nucleus. This mutant thus lacks the ability to regulate E2-dependent gene expression but still mediates ERα MISS effects (MAPK activation) when transfected in HeLa cells [87]. The *H2NES ERα* mutant mice have been very recently generated and preliminary observations suggest that their phenotype is similar to that of $ER\alpha^{-/-}$ mice including infertility and features of the metabolic syndrome [88]. However, the complete description of this *H2NES ERα* mouse is now mandatory to determine to which extent it fully abrogates the nuclear, transcriptional actions and retains the MISS effects involved in particular in the endothelial actions of estrogens (see below).

3.3. Dispensable role of ERαAF-1 in vascular and metabolic effects of estrogens

To explore the role of ERαAF-1 *in vivo*, a mouse model specifically deficient in this activation function (named $ER\alpha AF\text{-}1^0$) has been generated by deleting the amino acids 2–147, resulting in the expression of a short 49 kDa ERα isoform, deficient in most of the AB domain (Figure 2). The effects of E2 on the reproductive tract are dependent on ERαAF-1 activation while this activation function is dispensable for several vascular protective actions of E2, namely acceleration of endothelial healing, increased NO production, and prevention of atheroma [89], contrasting with the complete abolition of these beneficial actions in $ER\alpha^{-/-}$ mice (30). Interestingly, as mentioned above, E2 vasculoprotective effects are conserved in *ERα-Neo-KO* mice, the first model of *ERα* deletion developed by Korach et al., which expresses a residual 55 kDa ERα isoform probably devoid of functional AF-1 [48,50]. Unfortunately, *ERα-Neo-KO* mice cannot be used reliably as a model of $ER\alpha AF\text{-}1^0$, because, although expressed in most animal tissues, including uterus [90] and vessels [48], the leakage leading to the expression of this truncated mutant ERα appears to be inconstant.

Similarly, in contrast to $ER\alpha^{-/-}$ mice that spontaneously developed an obese and dysmetabolic phenotype, $ER\alpha AF\text{-}1^0$ mice were similar to their wild-type littermates in terms of body weight, adiposity, insulin sensitivity, and glucose tolerance when maintained on a chow diet or submitted to a high-fat diet (HFD), at least until 7 months of age [55]. Interestingly, the prevention of bone demineralization by E2 was also maintained in $ER\alpha AF\text{-}1^0$ mice [91]. Altogether, these observations indicate that, although ERαAF-1 is essential for the proliferative effects of E2 on the reproductive targets (uterus and breast), its activation is not required for estrogen-mediated vascular, metabolic, and cortical bone protection.

3.4. ERαAF-2 is absolutely required for the vascular and metabolic protection conferred by estrogens

To study the contribution of ERαAF-2, the murine ERα helix 12 of the LBD (also known as AF-2) has been targeted using knock-in strategies resulting in the deletion of 7 amino acids (543–549, $ER\alpha AF\text{-}2^0$ mice) [92] or in two point mutations (L543A and L544A, *ERαAF2-KI* mice) [93] (Figure 2). Both models demonstrate the essential role of ERαAF-2 in the effects of estrogens on the reproductive tract [92], as illustrated by the abolition of the transcriptional response to acute E2 administration in the uterus of $ER\alpha AF\text{-}2^0$ females [93,94]. Unlike $ER\alpha AF\text{-}1^0$ mice, prevention of bone demineralization by E2 is also abrogated in these mutant mice [91]. Furthermore, ERαAF-2 mediates the actions of estrogens on energy balance and glucose homeostasis since, $ER\alpha AF\text{-}2^0$ males and females maintained on a chow diet were found to develop severe obesity and metabolic disorders, as previously reported in $ER\alpha^{-/-}$ mice. The dysmetabolic features were also exacerbated in HFD-fed $ER\alpha AF\text{-}2^0$ mice with a severe insulin resistant status as compared to wild-type and $ER\alpha AF\text{-}1^0$ mice [55]. Furthermore, ERαAF-2 deletion abrogated the regulation by E2 of the expression of key genes involved in adipose tissue and liver metabolism and E2 failed to protect ovariectomized $ER\alpha AF\text{-}2^0$ females from HFD-induced obesity and hyperglycemia [55].

Nuclear ERα signaling is also necessary for the atheroprotective effect of estrogens since E2 failed to prevent fatty streak development in $ER\alpha\text{-}AF2^0\ LDLr^{-/-}$ mice at the level of the aortic sinus [92]. Accordingly, we also reported the potent atheroprotective effect of estetrol (E4), a fetal estrogen with an unusual profile of ERα activation, i.e. activating nuclear ERα but not membrane ERα [95]. E4 is also able to prevent neointimal hyperplasia following endovascular femoral

artery injury [35]. Altogether, these data indicate that nuclear ERα activation is sufficient to mediate both atheroprotection and prevention of post-injury smooth muscle cell proliferation. Of note, whereas these actions of E2 were similarly abrogated in *ERαAF-2^0* mice and in *ERα$^{-/-}$* mice, the acceleration of endothelial repair in response to E2 persisted in the former but not in the latter [92]. This result showed that the ERαAF-2^0 mutant is sufficient to mediate this vascular effect, and thus probably to elicit MISS effects in response to E2.

Finally, it is clear that further investigations are now needed to fully understand the interplay between the two ERαAFs *in vivo*. Indeed, combining pharmacologic and transgenic experimental approaches, we recently demonstrated that selective ERαAF1 activation by tamoxifen, a selective ER modulator that acts as an ERαAF1 agonist/ERαAF2 antagonist, is sufficient to elicit part of the metabolic (prevention of HFD-induced adiposity, insulin resistance, hyperglycemia and liver steatosis) and vascular (prevention of atheroma) protection, contrasting with the specific requirement of ERαAF2 in these E2 actions [96,97]. Altogether, these observations thus demonstrate that the two ERαAFs can separately and independently mediate the prevention of metabolic disorders and atheroma, suggesting a redundancy of ERαAF1 and AF2 in these beneficial actions.

4. ERα-MEDIATED CARDIOVASCULAR AND METABOLIC ACTIONS: INVOLVEMENT OF MEMBRANE-INITIATED STEROID SIGNALS

Over the last two last decades, it has become obvious that the biological functions of ERα could not be restricted to the nuclear actions of the receptor. Indeed, following post-translational modifications, a fraction of ERα has been identified to be associated with the plasma membrane where it can activate membrane-initiated steroid signaling (MISS) (Figure 1). These rapid actions of estrogenshave been well characterized *in vitro* in a variety of cell types [78,98,99]. The generation of new pharmacological compounds and animal models recently allowed the exploration of the role of these ERα MISS effects *in vivo*, as summarized below.

4.1. ERα post-translational modification leading to membrane localization

Using CHO cells transfected with either ERα or ERβ, *in vitro* studies revealed that only 2–3% of these receptors were localized to the plasma membrane [100]. Importantly, *via* post-translational modifications, endothelial nitric oxide synthase (eNOS) is targeted to caveolae, a subset of lipid rafts that compartmentalize signal transduction molecules at the plasma membrane, [101]. In membranes isolated from caveolae, E2 activates eNOS to a similar extent than acetylcholine [102]. Furthermore, in endothelial cells, direct interactions between ERα and G proteins are required for the activation of kinase cascades and the phosphorylation of eNOS Ser-1177 in response to E2 [103–105].

ERα membrane localization depends on a palmitoylation site located in the ERα LBD (Cys-447 in human and Cys-451 in mouse) [106,107]. The reversible S-palmitoylation of these specific residues dynamically regulates the intracellular traffic of ERα and favors its localization at the plasma membrane in association with caveolin-1. Other post-translational modifications are involved in membrane ERα signaling, at least in cancer cell lines [108]. As an example, methylation of Arg-260 in the ERα DBD allows direct interactions with the PI3K p85 subunit and with c-Src in MCF-7 breast cancer cells [67].

4.2. Demonstration of ERα MISS effects in endothelial cells

The first observation that suggested the hypothesis of ERα-mediated membrane effects was the identification of very rapid intracellular events in response to E2 (increased AMPc synthesis measured in the seconds to minutes following exposure to E2) as opposed to the time needed for transcriptional effects [109]. These rapid effects have been successively named “extra-nuclear”, “non-nuclear", or “non-genomic", but “membrane initiated steroid signaling” (MISS) is now a widely accepted designation that encompasses all steroid signaling events initiated within the membrane compartment: changes in second messengers such as AMPc, calcium mobilization, or activation of various kinase cascades including MAPKs and PI3K/Akt pathways. Most of these MISS actions have been described in cancer cell lines [6,110,111] and in the endothelium [78]. In summary, ERα MISS includes the activation of different rapid signaling pathways initiated at the plasma membrane, however, which can lead to the activation of transcription factors able to mediate more sustained effects.

As detailed thereafter, ERα MISS have been demonstrated to exert a prominent influence on endothelial functions *in vivo*. First, endothelial cells are well recognized to exert several vasculoprotective actions (vasorelaxation, prevention of platelet activation/aggregation, protection from leukocyte-endothelium adhesion/interaction) through the synthesis and the rapid diffusion of NO. Endothelial cells express eNOS, the enzyme that converts arginine into citrulline and NO, the activity of which is mainly stimulated *in vivo* by the shear stress exerted by the blood flow but can also be acutely enhanced by several agonists such as acetylcholine [112]. Estrogens also rapidly (within seconds) stimulate eNOS activity through ERα signaling [113,114], and this mechanism is thought to explain the short-term vasodilative properties of estrogens reported in humans [115]. The full-length ERα (66 kDa) has been identified as the main ERα isoform associated with MISS signaling in endothelial cells [54,104,116] although the contribution of other receptors to these extra-nuclear estrogen actions has been also suggested. As previously mentioned, some studies in human endothelial cell lines revealed the expression of the ERα46 isoform that colocalizes with caveolin-1 and mediates E2-induced MISS effects [117,118]. Finally, ERβ overexpression in transfected cell lines is also able to elicit MISS signaling in a similar extent than ERα [102].

4.3. Selective ERα MISS activation with pharmacological tools

Initially, membrane impermeable ER ligands, typically conjugated with bovine serum albumin (BSA), have been useful to characterize MISS actions and to exclude the involvement of nuclear ER [119]. For instance, E2-BSA and E2 similarly caused a rapid increase in intracellular calcium and eNOS activation in endothelial cells [120,121]. Then, estrogen dendrimer conjugates (EDC) were generated by J. Katzenellenbogen’s group by coupling ethinylestradiol molecules with a large and positively charged poly (amido)amine (PAMAM) dendrimer [122]. EDC is considered as an interesting tool to interrogate MISS estrogen actions *in vivo* since it retains the ability to activate ER at the plasma membrane but cannot reach the nucleus due to its high molecular size and chemical properties [50,123]. *In vitro*, EDC enhances the proliferation and the migration of endothelial cells, and also stimulates eNOS enzymatic activity [50]. Moreover, EDC administration to ovariectomized mice reproduces some of the ERα-dependent vascular beneficial effects of E2 such as NO-dependent vasorelaxation and endothelial healing [50]. Indeed, in a model of carotid artery injury, EDC treatment accelerates reendothelialization in normocholesterolemic mice and prevents neointima formation in hypercholesterolemic *ApoE$^{-/-}$* mice [50,94].

Since NO is a well-recognized vasculoprotective mediator and guardian of arterial integrity [124], and since E2 stimulates endothelial NO production through ERαMISS activation [94], it was tempting to speculate that this pathway would exert an atheroprotective action, at least in part, through enhanced NO production. However, EDC failed to protect *ApoE*$^{-/-}$ female mice from atheroma depots [102], although in this experimental schema using a Western diet, E2, considered as a positive control, only induced a 20% reduction in lesion size. Noteworthy, we previously reported that the atheroprotective effect of exogenous E2 was not altered by NOS inhibition [125]. Maeda's group subsequently refined this conclusion using hypercholesterolemic *eNOS*$^{-/-}$ mice, showing that the lack of local endothelial NO after blood pressure normalization did not contribute to the E2 atheroprotective effect [126].

In contrast to E2, EDC also failed to prevent increased adiposity and glucose intolerance in ovariectomized female mice fed a Western diet [102]. E2 and EDC, however, exert a similar protection against the constitution of hepatic steatosis in response to the Western diet, along with the down-regulation of hepatic genes involved in lipid synthesis [102]. Interestingly, EDC was also very useful to demonstrate that ERα MISS effects favor insulin synthesis [127], preserve beta-cells from lipotoxicity [128], and contribute to pancreatic islets survival [129]. Finally, further demonstrating the essential role of ERα nuclear activation in the proliferative actions of estrogens on uterus and breast cancer cells, EDC has been reported to be devoid of these classical undesired effects [50].

More recently, Katzenellenbogen's group designed "pathway preferential estrogens" (PaPEs), novel chemical compounds that allow the preferential activation of ER extranuclear pathways and are thus able to exert favorable tissue-selective actions. Estrogenic components of PaPEs are characterized by their reduced binding affinity for ER and therefore preferentially activate the extranuclear ERα signaling pathways over the transcriptional nuclear response [130]. PaPE-1 has been demonstrated to activate mTOR and MAPK signaling pathways and thereby to influence the expression of a set of genes involved in metabolism through mechanisms that appeared independent of ERα DNA binding. When administered to ovariectomized female mice, PaPE-1 reduced plasma triglycerides and prevented body weight gain as well as fat deposition. On the arterial side, PAPE-1 enhanced endothelial healing following vascular injury in the same way as E2 or EDC. Importantly, as previously also reported with EDC, PaPEs did not exert any proliferative effects on uterine and breast tissues, nor on breast cancer cell lines *in vitro* [130].

To summarize, the selective activation of ERα MISS with these new pharmacological approaches recently provided evidence that extranuclear ERα signaling is sufficient to mediate some of the endothelial and metabolic beneficial actions of estrogens. However, these chemical tools do not allow exploring the contribution of ERα MISS in physiological responses or addressing the complex interactions between MISS and nuclear effects.

4.4. Mouse models with ERα activation restricted to membrane-induced pathway

As previously mentioned, *ERαAF-2*^{0} mice are devoid of ERα nuclear actions but this *ERαAF-2*^{0} mutant retains the ability to respond to EDC and thus to mediate MISS signaling, as EDC still accelerates endothelial repair *in ERαAF-2*^{0} mice [94]. However, E2 administration failed to prevent *ERαAF-2*^{0} mutant mice from obesity and metabolic disorders. This suggests a minor, if any, contribution of membrane-initiated effects in the metabolic protection conferred by estrogens, at least in the absence of functional nuclear ERα and thus of direct transcriptional regulation [55].

To dissociate MISS from nuclear effects, Levin's group generated the membrane only ERα (MOER) mouse which only express the ERα E domain, adorned with multiple palmitoylation sites from a neuromodulin-derived peptide to enforce its localization at the plasma membrane, in an *ERα*$^{-/-}$ background [131]. The MOER mouse phenotype is very similar to that of *ERα*$^{-/-}$ mice since MOER mice are totally infertile and characterized by severe abnormalities of their reproductive tract [131]. Moreover, MOER females are prone to weight gain and accumulation of visceral fat as compared to their wild-type littermates, demonstrating that the ERα E domain is not sufficient to rescue most of the physiological actions of estrogens including metabolic protection [132]. However, although inefficient in *ERα*$^{-/-}$ mice, exposure to propyl-pyrazole-triol (PPT), a selective ERα agonist, similarly influenced the expression of many genes involved in lipid synthesis in the liver and decreased hepatic lipid contents (cholesterol, triglycerides, fatty acids) in wild type and MOER mice [133]. These latter data thus suggest that, when localized at the plasma membrane, ERα E domain could be able to regulate some metabolic responses, at least in the liver, through mechanism independent from the nuclear ERα pathway [133]. In a similar way, extranuclear ERα signaling has been reported to suppress lipogenic genes in pancreatic islets [59,128]. Noteworthy, the vascular phenotype of the MOER mice has not been investigated, nor has their metabolic response to nutritional challenges such as HFD or Western diet feeding.

4.5. Murine models with abrogated ERα membrane addressing

To address the physiological roles of ERα MISS *in vivo*, we [94] and Levin's group [134] generated mouse models characterized by a single point mutation targeting the palmitoylation site of ERα (*C451A-ERα*, corresponding to the human C447A residue) (Figure 2). From *in vitro* studies in HeLa cancer cells, this point mutation was predicted to result in a membrane-specific loss of function of ERα *in vivo* [106,107,135]. Indeed, as already mentioned, the non-palmitoylable C447A ERα mutant failed to interact with caveolin-1 and to localize to the plasma membrane and, therefore, lost its ability to induce MISS effects in response to E2 [106].

C451A-ERα female mice are infertile and characterized by ovarian abnormalities including hemorrhagic cystic follicles [94]. Furthermore, part of the beneficial endothelial actions of estrogens (eNOS phosphorylation, vasorelaxation, acceleration of endothelial healing) were also abrogated in this mouse model, contrasting with maintained uterine responses (gene expression and endometrial epithelial proliferation) to acute or to more long-term exposure to E2 [94]. Altogether, these data thus demonstrate the genetic segregation of MISS versus nuclear ERα actions in the *C451A-ERα* mouse, which represents an interesting model to study respective tissue-specific roles *in vivo*. In addition, besides its significant contribution to fertility and vascular estrogen effects, ERα palmitoylation was also recently reported to play a role in E2-mediated bone protection [136,137].

The *C451A-ERα* mice developed by Levin's group, also called NOER (nuclear only estrogen receptor) mice, were recently reported to exhibit increased weight gain and visceral fat accumulation as compared to their wild-type littermates [132]. Since both MOER and NOER mice seems to be prone to fat excess, the authors proposed that membrane and nuclear ERα signaling pathways interact to prevent adiposity. However, *in vitro* experiments led them to conclude that extranuclear ERα signaling specifically mediates the inhibition of lipid synthesis in mature adipocytes [132]. Our preliminary data also support a role for ERα MISS in the metabolic protection exerted by endogenous estrogens. Indeed, we recently observed that *C451A-ERα* females are prone to rapidly become obese and insulin resistant when fed with a

HFD, although in a lesser extent than *ERα*$^{-/-}$ and *ERαAF-2*^{0} mice (unpublished data). Altogether, the available data highlight the physiological importance of interactions between membrane and nuclear ERα signaling to preserve energy and glucose homeostasis. In contrast, E2 still protects hypercholesterolemic *LDLR*$^{-/-}$ *C451A-ERα* female mice from atheroma constitution, suggesting the dispensable role of ERα MISS in this vascular protective action of estrogens (unpublished data).

5. INVOLVEMENT OF OTHER ESTROGEN RECEPTORS IN THE CARDIOMETABOLIC EFFECTS OF ESTROGENS

5.1. Role of estrogen receptor β

After the cloning of ERβ in 1998, expectations were placed in this new receptor both in physiology and in medicine. When transfected in cell lines, ERβ classically interferes with gene expression but this receptor is also able to elicit MISS actions [102]. However, the physiological role of ERβ, including in female fertility, has been and remains controversial in several respects. The first *ERβ-KO* mouse models that were generated by the insertion of a neo cassette in exon 2 of *ESR2* exhibited widely different phenotypes [51,138]. Moreover, these two mouse mutants displayed alternative splicing transcripts [51,138]. Thus, another ERβ mutant mouse model (*ERβ*$^{-/-}$) has been more recently generated by deleting exon 2 through Cre/LoxP-mediated excision [139]. Both female and male *ERβ*$^{-/-}$ mice are sterile, but their development and homeostasis of the major body systems remain about normal [139]. This phenotype has been confirmed by Gustafsson's group that independently generated the same model [140]. However, despite the two in-frame stop codons introduced by splicing between exons 2 and 4, a residual ERβ protein was found in the prostate. The authors demonstrated that an in-frame LBD and C terminus were present in these ERβ$^{-/-}$ proteins and showed that ER tethering through AP-1, rather than binding to classical EREs, can mediate estrogen signaling in the mouse prostate [140].

ERβ has been reported to play a significant role in the central nervous system [141] and in the heart [142]. Indeed, cardiac fibroblast ERβ activation allows to prevent cardiac hypertrophy and fibrosis [53,143]. In contrast, ERβ does not appear to play a significant role in numerous vascular [30,33,34] and metabolic [56,144] actions of estrogens. However, ERβ could mediate specific effects of these hormones on pancreatic β cells since the ERβ selective agonist WAY200070 was found to enhance glucose-stimulated insulin secretion both in mouse and human islets [145]. Thus, although ERβ plays a significant role in reproduction [4,146], its contribution to the vascular and metabolic actions of E2 is probably minor. Importantly, a recent paper questioned the reliability of the antibodies used to recognize ERβ [147]. They performed a rigorous validation of 13 anti-ERβ antibodies and concluded that only one monoclonal antibody, that is rarely used, specifically targets ERβ in immunohistochemistry. Applying this antibody for protein expression profiling in 44 normal and 21 malignant human tissues, they detect ERβ protein only in testis, ovary, lymphoid cells, granulosa cell tumors, and a subset of malignant melanoma and thyroid cancers, showing a previous overestimation of the expression of ERβ.

5.2. Contribution of G-protein-coupled receptors

Finally, the seven-transmembrane G-protein-coupled receptor 30 (GPR30), localized in the plasma membrane or in other intracellular compartments (endoplasmic reticulum, Golgi complex), has been recognized in 2005 as a non-classical ER, also activated by the ER antagonists tamoxifen and fulvestrant (ICI 182,780) [148–150]. However, whereas wild-type endothelial cells display rapid signaling in response to E2, this is not the case of cells from *ERα/ERβ* double knock-out mice, despite unchanged GPR30 expression [151]. Thus, although GPR30 is expressed in endothelial cells, it is unlikely that this receptor significantly contribute to most of the endothelial effects of estrogens.

The influence of GPR30-mediated signaling on physiological and pathological processes *in vivo* remains elusive since the four GPR30-deficient mouse models display varying phenotypes [152]. However, none of them displays cycling or fertility abnormalities in females, questioning the biological relevance of GPR30 in reproduction. Thus, GPR30 could be rather viewed as a collaborator in non-nuclear functions of the classical ER in certain contexts [153].

The role for GPR30 as a regulator of energy homeostasis also remains to be elucidated since published studies provided discrepant results. The selective stimulation of GPR30 has been first reported to prevent vasoconstriction in both murine and human arteries and these vascular effects were abolished in GPR30-deficient (*GPR30*$^{-/-}$) mice, which were also characterized by visceral obesity [23]. Then, female but not male *GPR30*$^{-/-}$ mice were shown to exhibit reduced body growth with impaired skeletal development, increased blood pressure and vascular resistance as well as hyperglycemia resulting from altered insulin expression and release [154]. Moreover, Liu et al. suggested that estrogens could enhance pancreatic β-cell survival through GPR30-dependent mechanisms [129], and Davis et al. provided new data supporting a sexual dimorphism in GPR30 contribution to the development of postpubertal energy balance [155]. However, other studies reported no significant influence of GPR30 on body weight [129,156], or even showed that GPR30 activation promotes adipogenesis and therefore the development of obesity in female mice exposed to excess fat energy [157]. In summary, although some experimental data support GPR30 as a modulator of metabolism and cardiovascular function [150], the numerous inconsistencies that exist in the literature still challenge the biological relevance of this receptor for estrogen response *in vivo* [152,153].

Finally, estrogens can also signal via another distinct membrane ER named Gq-mER that can rapidly activate kinase pathways to have multiple downstream actions in central nervous system (CNS) neurons. Kelly et al. [12] have found that E2 increases the excitability (firing activity) of pro-opiomelanocortin (POMC) and dopamine neurons through the rapid inhibition of G-protein coupled, inwardly rectifying K + (GIRK) channels in hypothalamic neurons. These effects are mimicked by a selective ligand (STX) for Gq-mER with no binding to ERα, ERβ, or GPR30. These findings thus demonstrate the ability of E2 to induce the activation of signaling pathways through this plasma membrane Gq-mER that is, unfortunately, not cloned so far. Interestingly, as observed with E2, STX reduces food intake and body weight gain in ovariectomized female mice [12]. The same group identified that in the arcuate nucleus, numerous genes were significantly regulated by STX, indicating that this molecule not only controls neuronal excitability but also alters gene transcription via signal transduction cascades initiated by Gq-mER activation [158].

6. CONCLUSION AND PERSPECTIVES

Now recognized as the main actor of the beneficial cardiometabolic actions of estrogens, ERα should thus represent a pertinent target to prevent metabolic and vascular disorders. In the last decade, sustained experimental research efforts significantly improved the understanding of ERα-mediated mechanisms that contribute to these protective actions, as summarized in this review.

ERα nuclear effects play a prominent role through ERαAF2 activation which is absolutely required for the prevention of fat excess, diabetes and atheroma conferred by estrogens (Table 1). Importantly, selective activation of ERαAF1 with tamoxifen exerts a similar protective effect against HFD-induced metabolic disorders and against atheroma. As E2 is also able to protect *ERαAF1°* mice (that only harbor an ERαAF2 function) from atheroma and metabolic disorders these data reveal an apparent redundancy in the ability of the two ERαAFs to separately mediate such a vascular and metabolic protection. This contrasts with the requirement of both ERαAF1 and AF2 to promote breast cancer growth.

On the other hand, recent studies indicate that ERα MISS signaling selectively mediates some of the endothelial/vascular effects of estrogens (NO release, reendothelialization), and could also contribute to their beneficial actions on energy and glucose homeostasis (Table 1). Indeed, the selective activation of membrane ERα signaling pharmacological compounds is sufficient to mediate endothelial beneficial effects but also to prevent fat mass accumulation and the development of fatty liver diseases. Moreover, our unpublished observations in the *C451A-ERα* mouse model support a relevant role of ERα MISS effects in the beneficial metabolic actions of estrogens but not in their atheroprotective effect.

In conclusion, addressing the role of ERα subcellular localization and specific mode of activation provided new insights into the understanding of estrogen actions on energy balance and metabolic homeostasis, as well as on the vascular system. These findings first open the way to characterize the mechanism of action of already available ER ligands including natural estrogens and SERMs. Then, improving our knowledge of ERα-dependent signaling pathways, in particular according to the subcellular localization of the receptors, will be very helpful to design new selective modulators with the final ambition to preserve bone and cardiometabolic protection without the classical undesired effects of estrogens on the reproductive organs.

Table 1 — Summarized view of the respective role of nuclear and MISS ERα pathways in vascular and metabolic protection, according to the main studies performed in mutant mouse models (loss of function) or with pharmacological tools (selective activation).

	Genetically modified mice = Loss of function		Pharmacological tools = Selective activation	
	MISS	Nuclear	MISS	Nuclear
Mouse genotype	C451A-ERα	ERα-AF2^0	Wild-Type	
Treatment	Estradiol (E2)		EDC/PaPE-1	Estetrol (E4)
Vascular protection:				
Acceleration of endothelial healing	NO [94]	YES [92]	YES [50]/YES [130]	NO [95]
Increased endothelial NO production/vasodilation	NO [94]	YES [92]	YES [50]/YES [130]	NO [95]
Prevention of neointimal hyperplasia	*nd*	*nd*	NO [35]/nd	YES [35]
Prevention of atheroma	YES*	NO [92]	NO [102]/nd	YES [95]
Metabolic protection:				
Prevention of HFD-induced obesity	NO*	NO [55]	NO [102]/nd$^{\#}$	nd
Prevention of HFD-induced hyperglycemia	NO*	NO [55]	NO [102]/nd$^{\#}$	nd
Prevention of HFD-induced fatty liver	NO*	NO*	YES [102]/nd$^{\#}$	nd

nd: not determined. *: unpublished data. #: PaPE-1 reduces the increase in body weight and adipose stores observed after ovariectomy in mice fed a normal chow diet [130].

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CONFLICTS OF INTEREST

None declared.

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