*Review Article:*

# Rapid Signalling Mechanisms in the Cardiovasculo-Protective Effects of Estrogen.

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**ABSTRACT**

In modern society, cardiovascular disease remains the biggest single threat to life, being responsible for approximately 1/3 of worldwide deaths. Male prevalence is significantly higher than that of women until after menopause, when the prevalence of CVD increases in females until, over the age of 80, it eventually exceeds that of men. Because of the coincidence of CVD prevalence increasing after menopause, the role of estrogen in the cardiovascular system has been intensively researched during the past 2 decades *in vitro*, *in vivo* and in observational studies. Most of these studies suggested that endogenous estrogen confers cardiovascular protective and anti-inflammatory effects. However, clinical studies of the cardioprotective effects of hormone replacement therapies (HRT) not only failed to produce proof of protective effects, but also revealed the potential harm estrogen could cause. The "critical window of hormone therapy" hypothesis affirms that the moment of its administration is essential for positive treatment outcomes, pre-menopause (3-5 years before menopause) and immediate post menopause being thought to be the most appropriate time for intervention. Since many of the cardioprotective effects of estrogen signaling are mediated by effects on the vasculature, this review aims to discuss the effects of estrogen on vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) with a focus on the role of estrogen receptors (ERα, ERβ and GPER) in triggering the more recently discovered rapid or membrane delimited (non-genomic) signaling cascades that are vital for regulating the vascular tone, preventing hypertension and other cardiovascular diseases.

**Abbreviations.**

CVD: Cardiovascular disease; HRT: hormone replacement therapy; SMC: smooth muscle cells; RAAS: renin angiotensin aldosterone system; Akt: PKB (protein kinase B); PI3K: Phosphoinositide 3-kinase; HSP90: heat shock protein 90; c-Src: proto-oncogene tyrosine-protein kinase Src; eNOS: endothelial nitric oxide synthase; ERK: Extracellular signal regulated kinase; MAPK1: Mitogen-activated protein kinase 1; NO: nitric oxide; iNOS: inducible nitric oxide synthase; CAM: Calmodulin; HUVEC: human umbilical vein endothelial cells; UAECs: uterine artery endothelial cells; ECs: endothelial cells; VSMCs: vascular smooth muscle cells; E2BSA: estradiol conjugated to

bovine serum albumin; Grb2: Growth factor receptor-bound protein 2; GPCR: G protein coupled receptor; Cos7: fibroblast-like cell line; EGFR: epidermal growth factor receptor; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; IP3: inositol trisphosphate; DG: diacylglycerol; PIP2: phosphatidyl inositol 4,5 bisphosphate; SD: Sprague Dawley rats; ET-1: endothelin 1; VDCC: voltage dependent calcium channel; MAGUK: membrane associated guanylate kinase; PCAs: porcine coronary arteries; PCASMCs: porcine coronary artery smooth muscle cells.

## 1. Introduction.

Estrogens are a class of steroid hormones that are mainly synthesized by the ovaries, adrenals and by the placenta in pregnancy. The main estrogens are 17β-estradiol (E2) (the most potent and main circulating one); estrone (E1) and estriol (E3). There are a number of extragonadal sites where low quantities of E2 are produced. These remain the only endogenous estrogen source in postmenopausal and ovariectomized women. These sites include the vascular endothelium, aortic SMCs, adipose, brain, and bone tissues (see for instance [1]). Extragonadal E2 acts as a paracrine or autocrine modulator in its origin tissue, so for instance, would have a paracrine action in the vasculature [2]. Besides their role in developing the primary and secondary sexual characteristics of women, multiple studies have shown that they can also protect the cardiovascular system of pre-menopausal women against disease (see [3]).

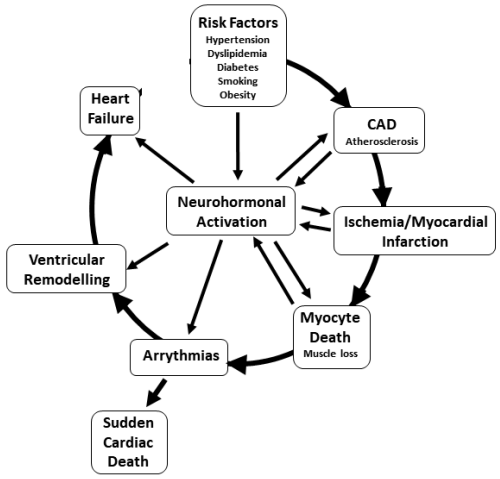


**Figure 1. The Cardiovascular Disease Continuum.**

In the search for the mechanisms by which estrogens exert their genomic and rapid non-genomic actions in the vasculature, 3 main types of estrogen receptors (ER) were found to exist in both vascular smooth muscle cells (VSMC) and endothelial cells (EC): ERα, ERβ and GPER1 (G-protein coupled estrogen receptor 1; formerly GPR30: G-protein coupled receptor 30), (see also [4-7]). By binding these receptors, estrogen can induce vasodilation, reduce inflammation, act as a potent antioxidant, and alter gene expression. Furthermore, estrogens are well-known for their actions in reducing plasma cholesterol levels and its deposition in the wall of arteries [8].

By inducing vascular relaxation, E2 is thought to be able to mitigate against hypertension (see [9, 10]. However, it is a complicated picture because contrary to this is the possibility that long-term exposure to estrogen in the form of oral contraceptive or HRT medications may increase hypertension due to the buildup of superoxide radicals [11, 12], but this may be more prevalent with synthetic estrogens rather than natural estradiol [13]. Hypertension is an elevation of systolic and/or diastolic blood pressure. It is a major risk factor in the initiation and progression of cardiovascular disease ie the “cardiovascular disease continuum” [14], as shown in figure 1 and is also associated with renin-angiotensin-aldosterone system (RAAS) activation. It can be aggravated (or initiated) by other risk factors such as obesity, smoking, male gender, lack of physical activity, stress, old age, excess salt in the diet and by estrogen loss due to menopause. High pressure damages the endothelium of the arteries thus promoting the development of atherosclerosis. Furthermore, it increases ventricular afterload (force the left ventricle has to generate to expel blood into the aorta), leading to cardiac hypertrophy and demand ischemia. Low resistance, high flow vascular systems such as the brain and kidney, are

the tissues that are most affected by hypertension. Increased pressure in a low resistance microcirculation, exposes it to even higher pressures and pressure pulsatility compared to other organs. This damages the microcirculation and can lead to end organ damage, renal failure and stroke. Therefore, interrupting this vicious cycle, can substantially decrease the morbidity and mortality associated with it.

The effects of estrogen in reducing hypertension and other risk factors have been researched *in vivo*, *in vitro* and in observational studies. Overall experimental research has suggested that estrogen is protective of both the heart and the vasculature. On the other hand, comprehensive clinical research on hormone replacement therapy (HRT) in post-menopausal women has failed to provide strong evidence for protection and even revealed the potential harm it can cause to the patients. The "theory of timing and opportunity" explained that whether estrogen administration is beneficial or deleterious, depends greatly on the stage of menopause [6, 15].

## 2. Estrogen receptors.

Estrogen signaling in target organs depends on estrogen receptors (ERα, ERβ and GPER). These receptors can either work synergistically or antagonistically, but they exert similar actions in ECs and VSMCs. By activating these receptors, E2 can trigger both genomic and non-genomic actions [6, 16]. Although the role of these receptors has been intensively studied in arteries, less is known regarding their role in the venous system.

### 2.1 Estrogen receptor alpha (α) and beta (β).

The gene for human ERα is encoded on chromosome 6. It has 595 amino acids that are arranged in 5 different domains and its molecular size is 66kDa. ERα is found both in association with the cell membrane, allowing fast, 'non-genomic' estrogen signaling and in the nucleus, mediating estrogen's longer term, 'genomic' actions [4, 5, 16]. However, as discussed below, the more rapid signaling from the plasma membrane does impinge on changes in gene transcriptional regulation via intermediate kinase mediated signaling and via co-operation with nuclear ER and therefore 'non-genomic' signaling may be more accurately referred to as 'membrane delimited' signaling (see [6]). Also, other intracellular localisations of ER have been described, such as mitochondria, endoplasmic reticulum and Golgi [17] [18]. Localization of ERα in endothelial cells (ECs) and VSMCs has been extensively investigated (see [16, 19].

ERα does not contain a typical trans-membrane domain, but palmitoylation (covalent attachment of palmitic acid to an amino acid residue) at $Cys^{477}$ regulates membrane trafficking and localization to the plasma membrane, where it is concentrated in caveolae associated with caveolin-1 [16, 20, 21]. $Cys^{477}$ is contained within a 9 aa palmitoylation motif which is critical for full palmitoylation of ER$\alpha$. In addition, methylation of $Arg^{260}$ in the DNA binding domain by Protein Arginine Methyl Transferase 1 (PRMT1) plays a role in exclusion of ER from the nucleus and cytoplasmic localization and trafficking. Furthermore, in breast cancer cells $Arg^{260}$ methylation triggers interaction with the p85 subunit of phosphatidyl inositol-3-kinase (PI3K) and c-Src tyrosine kinase (c-Src) and recruitment of Focal Adhesion Kinase (FAK) [22]. Approximately 5-10% is found in the PM but it is also found in the endoplasmic reticulum and mitochondria (see [6]). It has also been shown that in ECs ER$\alpha$ exists as a 46 kDa N-terminal truncated splice variant, ER46, that also complexes with caveolin-1, Akt, HSP90, PI3K, Src-TK and endothelial Nitric Oxide Synthase (eNOS) in caveolae (see Figure 2) [23]. Li et al showed that ER46 is more efficient at generating Nitric Oxide (NO) from eNOS than the full length ER66, but that ER66 is more efficient at triggering genomic ER signaling. Also, interestingly antibody accessibility of the C-terminus of ER46 in intact cells suggests that it may at least partially span the caveolar PM. Furthermore, ER46 is activated efficiently by membrane impermeant E2, therefore the ligand binding domain is extracellular [24].

ERβ in humans is a 530-amino acid protein that has a molecular size of 54 kDa and its gene is encoded on chromosome 14. Just like ERα it has 5 domains all having distinct functions. Fewer investigations were made on ERβ because it was not cloned until 1996 [16]. Also, the relative amounts of ERα and ERβ varies depending on the cell type. In an early study of ERα and ERβ mRNA distribution, it was shown that ERα mRNA was expressed in all tissues and ERβ mRNA mainly in reproductive tract except for mammary and testes. Hypothalamus and lung were high for ERα and ERβ but ERβ was downregulated in ERα knockouts, suggesting that ERβ expression was dependent on ERα [25]. ERs are expressed in both ECs and VSMCs and E2 affects blood vessel structure as well as function [26] and ERα knockout mice express a vascular as well as metabolic phenotype [27]. The differences between ERα and ERβ expression are complex and appear to depend on the vascular bed as well as sex and disease state. Ma et al [28] compared the relaxation responses of male and female rat aortic VSMCs to ERα, ERβ and GPER agonists against constrictors AngII, PE and KCl and examined the intracellular distribution of the different receptors as well as mRNA and protein levels. AngII and PE caused less contraction in female VSMCs, although in the absence of agonist, this may be due to genomic effects unless the cells themselves pro0duce intrinsic E2 which acts in an autocrine fashion. The ERα agonist PPT caused similar relaxation to E2, suggesting a predominance of the ERα mediated effect. The ERβ agonist DPN caused mainly relaxation to PE and KCl, suggesting an ERβ-$Ca^{2+}$ channel interaction. The ERα/ERβ antagonist ICI 182, 780 (which is a GPER partial agonist) did not reduce VSMC contraction, suggesting little role for GPER, although they didn't directly test a GPER agonist (ie G-1). ERα and ERβ expression were higher in females, in keeping with the smaller contraction in female VSMCs and were largely expressed in the nucleus. Given that this was an analysis of the acute effects on VSMC contraction, there may have been sensitivity issues with the immune detection in other compartments. GPER expression was similar in males and females and mainly non-nuclear in localization. However, although PPT increased the nuclear localization of ERα, DPN had no effect on ERβ localization and ICI did not increase GPER localization to the cell surface. These results suggest a predominance of ERα mediated responses in VSMCs, at least from the aorta. However, VSMC contraction/relaxation in vitro may not be very representative of the intact artery.

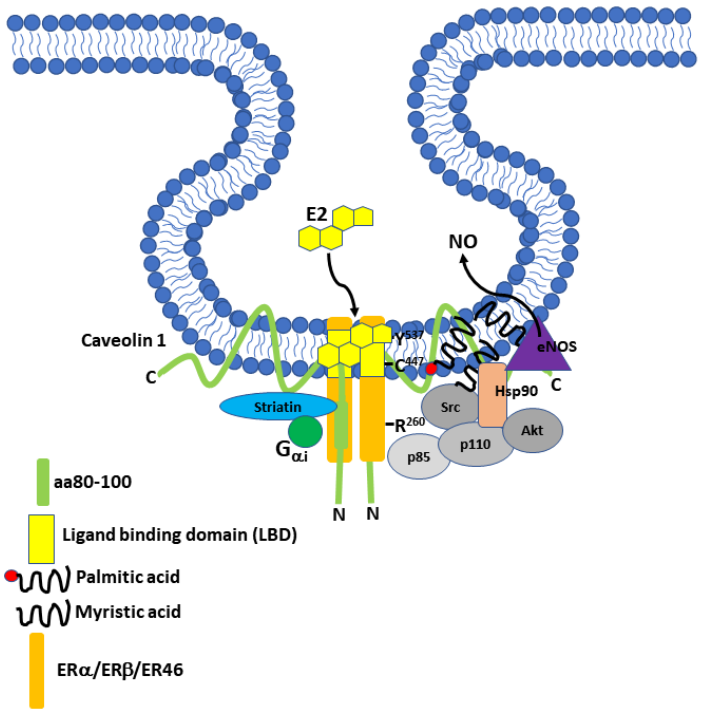


**Figure 2. The Estrogen Receptor Caveolar Membrane Complex.**

ERα and ERβ are highly homologous and have approximately 56% homology in the ligand binding domain and 95% in the DNA binding domain [29]. ERβ distribution and role varies within different vessels with different cell types. For example, in embryonic ovine intra-pulmonary arteries it is mainly associated with non-caveolar sites of the plasma membrane of the endothelial cells and it was found to be the main eNOS activator compared to ERα.

On the other hand, ERα is the predominant activator of eNOS in adult models [30], but again may depend on the vascular bed as it was similar in UAECs [31]. Similar to ERα, ERβ can also activate PI3K, Akt, and the Extracellular Signal Regulated Kinase 1 and 2 (ERK1/2) pathways [32]. In the proximity of caveolae ER$\alpha$ and ER$\beta$ signaling are essentially the same ie leading to activation of G$\alpha$ and G$\beta\gamma$, increases in $Ca^{2+}$ and cAMP and activation of c-Src, PI3K and the distal kinases Akt and ERKs (see for instance [33]). As mentioned above, in VSMCs, ERβ is primarily found in the nucleus but can also be found in lower quantities at the plasma membrane, associated with caveolin-1 [20].

**2.2 GPER1 (GPCR30/GPER).**

GPER1/GPR30 was discovered as an orphan G protein coupled receptor with 7 transmembrane helices [34, 35]. Its gene is located on chromosome 7p22, a region that is implicated in arterial hypertension and familial hyperaldosteronism (FH), although the role of GPER in hypertension is complex (see for instance [7, 36-38]). FH is associated with several genes including AT1, CYP11b2, MEN1 and PKRAR1B, RBaK, PMS2 and GNA12 on the 7p22 locus [39] and a later study identified a comprehensive gene list associated with FHII on the 7p22 locus, but the mutations remain elusive [40] and this did not include polymorphisms in the GPER gene. However, this does not preclude a possible role for GPER loss-of-function in other forms of primary hypertension. Recently an association was found between lower GPER levels and hypertension in post-menopausal but not pre-menopausal women [41]. However, the complexity of the role of GPER appears to be due in part to the fact that it is disputed whether or not GPER also functions as an aldosterone receptor [42-44]. Nevertheless, female wild type mice are protected against AngII-induced hypertension whereas as GPER knockout mice are not. Furthermore, this was associated with increased oxidative stress as evidenced by increased ROS, NADP/NADPH and NOX4. The GPER agonist G-1, the ARB losartan and NOX4 siRNA decreased NOX4 mRNA and protein and blocked the effects of AngII. The G-1 protective effects were also blocked by adenylate cyclase inhibition and mimicked by phosphodiesterase inhibition, highlighting the importance of the cAMP pathway downstream of GPER in the protective effects [45]. The involvement of NOX4 is a surprising finding given that the main generator of vascular ROS (superoxide, $O_2^-$) in response to AngII is NOX1/2 rather than NOX4 and NOX4 is protective as it generates $H_2O_2$ rather than superoxide which is pro-relaxant probably by activating eNOS, SR CICR and BKca channel activation [46, 47]. GPER has also been reported to protect against high salt-induced hypertension in ovariectomized rats [48].

Structurally, GPER is not related to any of the classical estrogen receptors. E2's affinity for ERα and β is ten times higher than that for GPER. However, cells that express both GPER and ERα such as coronary vessel cells (either VSMCs or ECs), may respond to coordinated signaling of the two receptors [49]. Evidence for functional crosstalk between ER$\alpha$ and GPER was obtained in porcine coronary artery where acute NO-dependent vasodilation was observed in response to ER$\alpha$ selective agonism but blocked when E2 activated all three receptors simultaneously. However, these observations are complicated by the fact that ER$\alpha$-selective agonists such as PPT may also activate GPER [50, 51].

When a GPER1 specific agonist was administered intracellularly in VSMCs of arterial vessels, the vasodilatory response was faster compared to that resulted from external administration. Also the use of differentially permeable E2 derivatives have suggested that membrane permeability is a necessity for rapid signaling by GPER, suggesting the intracellular GPER is the main functional pool [52]. This agrees with the fact that most GPER is localized intracellularly. Studies using fluorescent GFP-tagged GPER appeared to confirm this as high levels were found in the endoplasmic reticulum and Golgi but not the PM [53]. However, although it is now generally accepted that GPER1 is predominantly located intracellularly in vascular cells [36, 54] it is not clear whether or to what extent intracellular signaling contributes to its function. Estrogen binding to GPER induces calcium mobilization

possibly via EGFR transactivation [55] rather than via $IP_3$-mediated action, so the relative contributions of the two mechanisms and the localization of the effect are debated [6]. Furthermore, PLC$\gamma$ docks with EGFR and is activated by tyrosine phosphorylation [56], which may link the two effects. Also, GPER appears to be constitutively endocytosed and to have a high rate of turnover and therefore this may explain the apparent lack of PM located GPER and its accumulation in endosomes [57]. This may be important in view of the growing recognition of endosome signaling in its own right, so it is possible that GPER signals from this compartment rather than simply transits through it. Therefore, the high levels in the endoplasmic reticulum and Golgi may simply reflect high rates of synthesis and trafficking through these compartments during protein synthesis and post-translational processing [6]. Thus, evidence for a direct intracellular signaling role of GPER is somewhat sketchy. However, there is evidence for rapid G-protein-dependent signaling in ER$\alpha$ and ER$\beta$ negative or inhibited cells suggesting a direct role for GPER which is independent of ER$\alpha$ an or ER$\beta$ [58] (and see [35]).

The actions of GPER activation varies with each type of vascular bed. For example, GPER selective activation in mesenteric arteries leads to endothelium-dependent vasorelaxation contributed to by signaling cascades activated in both VSMCs and ECs [36]. Likewise, porcine coronary artery relaxation induced by the GPER selective agonist G-1 is endothelium-dependent via eNOS activation [59]. However, other studies suggest that vasorelaxation in aorta and coronary arteries in response to G-1 can occur in an endothelium-independent manner [60-62]. In these studies eNOS inhibition by L-NG-nitroarginine methyl ester (L-NAME) did not effect the vasodilatory response. Furthermore, Yu et al [61] suggest that the endothelium independent effect of G-1 in coronary VSMCs is mediated via a large conductance $Ca^{2+}$-activated potassium (BK) channel (see also [6, 7]). Therefore, there are endothelium-dependent and -independent effects of GPER signaling.

Extensive analysis of GPER knockout mice show relatively subtle phenotypes compared to the extensive and reproducible differences seen in ER$\alpha$ knockout mice (see [63]). For instance, an age-dependent effect on blood pressure was observed in female mice, since an increase in blood pressure was observed in female GPER knockout mice only after 9 months of age [64] which likely relates to the age-dependent decrease in endogenous estrogen and/or ER$\alpha$ and ER$\beta$. Interestingly, in humans a mutant allele P16L which confers a single nucleotide polymorphism of GPER producing a hypofunctional phenotype is associated with significantly greater systolic blood pressure in female but not male carriers [65, 66]. G-1 reduces atherosclerotic lesion size in ovariectomized mice and lesion size is increased in the aortas of intact and ovariectomized female GPER knockout mice [67]. However, there is some evidence that GPER co-operates with ER$\alpha$/ER$\beta$ in a larger signaling complex. Also, ER$\alpha$ and ER$\beta$ can exist as homodimers or heterodimers in the PM of endothelial cells [68]. The importance of the constituents of the ER receptor complex and their role in the protective actions of estrogen will be discussed further throughout this review.

### 3. Estrogen and the regulation of vascular tone.

Cardiovascular disease in general is associated with the loss of estrogen and ERs with aging, especially post-menopause and therefore estrogen-dependent responses are considered to be a mechanism of protection against CVD and cancer. Given that estrogen receptor isoforms have differing binding affinities for E2 [69] and isoform distribution changes during and after menopause, this combined with decreased levels of circulating E2 may contribute to the relative loss of estrogen protective responses. This leads to hypertension, endothelial dysfunction and high circulating levels of cholesterol. To understand the causes of increased CVD prevalence in post-menopausal women, it is essential to comprehend the mechanism underlying E2 actions in pre-menopausal women [70].

Vascular tone represents the level of blood vessel constriction in relation to its maximum diameter when dilated. Both the endothelium and the vascular smooth muscle in the vessel take part in maintaining the vascular tone. Furthermore, extrinsic and intrinsic factors also determine the extent to which a vessel relaxes/constricts. Extrinsic factors (such as circulating hormones; sympathetic nervous system) can alter the systemic (peripheral) resistance of the vasculature, thus modifying arterial pressure, while intrinsic factors (such as autocrine and paracrine mediators: CO, NO, histamine, neuropeptides etc and sheer stress via eNOS/NO) are primarily involved in maintaining/altering local blood flow within an organ. E2 is primarily an example of an extrinsic factor ie circulating gonadal E2 mainly responsible for its effects. However, autocrine regulation in hippocampal neurons by locally produced E2 [71], the regulation of luteinizing hormone (LH) production in ovarian follicle granulosa cells by local estrogens [72] and autocrine regulation of cell proliferation in estrogen-dependent breast cancer cells via ER$\alpha$ [73] are examples of intrinsic actions.

Furthermore, the conversion of androgens such as testosterone to estrogen by estrogen synthase (aromatase) is an important source of E2 in local tissues and is particularly important in males (see [74] [75]). Aromatase is found in vascular tissue, in both ECs and VSMCs and is widely distributed in extragonadal sites such as bone, brain, adipose tissue (including perivascular adipose tissue) and blood vessels [76, 77] and is important in the cardiovascular system including coronary arteries [78]. The use of aromatase inhibitors in women to treat breast cancer comes with significantly increased cardiovascular disease risk [79]. Perivascular adipose tissue (PVAT) plays an important protective role in the maintenance of function of normal blood vessels and dysregulation in disease such as central obesity it becomes proinflammatory and contributes to atherosclerosis [80, 81]. Local production of estrogen by PVAT plays a role in its homeostatic protective function (see for instance [82]). PVAT produces PVAT-derived relaxing factors (PVRFs) which activate VSMC ATP-dependent $K^+$ channels [83] and voltage dependent (Kv) $K^+$ channels such as KCNQ (Kv7) [84]. Because (PVAT-derived) E2 acts in a similar manner on VSMC relaxation and growth inhibition, it perhaps could be considered a PVRF (see for instance [82, 85]).

**3.1 Endothelial cells and NO production.**

NO is a molecule synthesized by the vascular endothelium from L-arginine via eNOS and is considered to be an intrinsic vasodilating factor. Vascular relaxation is only one of the multiple roles of NO and it is achieved through the activation of soluble guanylate cyclase (sGC), an enzyme that catalyzes the reaction which forms cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G (PKG). PKG can induce relaxation by triggering a decrease in intracellular $Ca^{2+}$ by stimulating both the $Ca^{2+}$ plasma membrane pumps and the sarcoplasmic reticulum $Ca^{2+}$ uptake pumps [86] as well as via phosphorylation of MYPT1 which antagonizes the actions of Rho kinase and activation of myosin light chain phosphatase (MLCP) [87] and phosphorylation of $K^+$ channels which induce hyperpolarization [88].

NO also inhibits VSMC proliferation and is well known for its antiplatelet, antithrombotic and anti-inflammatory actions. However, NO can also inhibit cytochrome C oxidase (CcOX), in complex IV of the mitochondrial electron transport chain, in competition with oxygen, and therefore cellular respiration. However, this is highly dependent on the respiratory state of the cell, the prevailing oxygen concentration and electron flux (reducing equivalents) [89-91], for instance under hypoxic conditions, with an increasing proportion of CcOX in the reduced state. This is an adaptive response and thus under low [$O_2$] conditions the resulting increased bioavailability of NO locally can activate sGC resulting in vasodilatation and therefore the local supply of $O_2$ [89]. Under certain conditions (ie hypoxia, or at least when [NO] exceeds [$O_2$] and reduction of CcOX occurs) this could (in theory) lead to peroxynitrite ($ONOO^-$) radical formation (a strong oxidant species formed from the reaction of NO and superoxide-$O_2^-$) due to increased $O_2^-$ generation. This is due to the leak of electrons from complexes I and III when $O_2$ is not

completely reduced to $H_2O$ [90]. It has been proposed that this may be a mechanism associated with vascular aging and disease [92]. However, this idea remains controversial, because other oxidoreductase enzyme systems in ECs and VSMCs may, in fact, contribute more to $O_2^-$ generation, such as PM NADPH oxidases (NOXs) and uncoupled eNOS, particularly under normoxic conditions and in response to GPCR agonists and stretch. Notwithstanding the source of vascular $O_2^-$ and $ONOO^-$ formation, there is evidence that E2 reduces the formation of these by inducing the transcription of SOD1 and SOD2 (a genomic effect) [93] and/or has direct radical scavenging properties [94] as well as reducing production of the pro-inflammatory cytokines TNF$\alpha$ and IL-1$\beta$ [95, 96] and therefore plays a major role preventing vascular aging and disease. This may underlie not only the cardioprotective effects of GPER activation against I/R injury (ie by G-1) [97], but also the inhibitory effects of ER$\alpha$ and GPER activation on VSMC proliferation post-injury [98, 99]. However, both pro- and anti-inflammatory effects of E2 have been reported (see for instance [96]). Interestingly, this may be dependent on relative changes in the levels of ER$\alpha$ versus ER$\beta$, since an age-related increase in ER$\beta$ is associated with a pro-inflammatory profile of E2 [96].

E2 initiates the production of NO by activating eNOS, both in a $Ca^{2+}$ independent and a $Ca^{2+}$-dependent manner via ER$\alpha$, ER46 or ER$\beta$, depending on the vascular bed. E2 can promote NO production in two ways: via Mitogen Activated Portein Kinase (MAPK-ERK1/2) and via PI3K/Akt, but how these mechanisms occur independently of $Ca^{2+}$ increase, is still not completely understood. Russell et al [100] demonstrated that E2 facilitates Hsp90 association with eNOS (an important association because Hsp90 acts as a scaffold and aids the phosphorylation of eNOS by Akt), thus reducing $Ca^{2+}$ requirements of eNOS. By using geldanamycin, a drug that binds Hsp90's ATP binding region and inhibits its action, NO could not be released under the stimulation of E2, showing the importance of Hsp90 in E2's action on eNOS.

### 3.2 PI3K/Akt/eNOS pathway.

After it was demonstrated that Akt, the downstream target of PI3K, phosphorylates eNOS and potentiates $Ca^{2+}$ and calmodulin association [101], Haynes et al [102] used human umbilical vein endothelial cells (HUVECs) and EA hy926 cells to evaluate E2's ability to activate Akt by binding cellular membrane receptors. The cells were incubated with either E2 alone, LY294002-an inhibitor of PI3K, ICI182,780-a non-specific ER antagonist or ionomycin-an ionophore used to raise intracellular levels of $Ca^{2+}$. When E2 was administered there was a 4-fold increase in NO. Neither LY294002 nor ICI182,780 affected the basal control levels of NO (tonic levels of NO produced by the endothelium) but abrogated the E2 induced NO increase. This shows that E2 can significantly increase NO release in ECs via an ER-mediated pathway in a PI3-kinase-dependent manner. By using a non-selective ER antagonist, activation of all estrogen receptors was inhibited. Therefore, this study cannot indicate the specific receptor that produced the vasodilatory actions, but we can presume that it is either ERα or ERβ (as depicted in figure 3) because of the Akt-PI3K association with ER46 and due to the ability of ERβ to also trigger the activation of this pathway. A functional signaling complex containing c-Src, PI3K and ER46 co-localised with Caveolin-1 in ECs has been demonstrated [24, 103-105]. Membrane localization requires palmitoylation of ER46 at $Cys^{447}$ and also lipid modification of c-Src. ER$\alpha$ membrane localization also requires palmitoylation at $Cys^{415}$ [106]. E2 was reported to stimulate proliferation via activation of PI3K via ER$\alpha$ but not ER$\beta$ in breast cancer cells [107], although this was secondary to increased PI3K p85 subunit expression. Nevertheless, rapid activation of eNOS in EA Hy926 cells was PI3K- and ER-dependent [108]. Activation was blocked by ICI 182,780 which would appear to implicate ER$\alpha$ coupling to PI3K. However, as mentioned above, ICI 182,780 also activates GPER.

Nevertheless, a direct association between ERα rapid, membrane delimited signaling and PI3K has been demonstrated in ECs [109, 110] and breast cancer cells [111] and ERα binds directly to p85 [109] (see also [6]). A more definitive association must surely come from individual knockout studies ? Indeed, using this approach it was shown that ERα mediates endothelial NO production and anti-atherosclerotic effects [30, 67, 112]. Likewise, ERα rapid signaling is required for E2 induced proliferation and migration of ECs [113], as shown using a triple (KRR) mutant of ERα which is specifically defective in rapid signaling but leaves the ERα genomic signaling function intact. PI3K-dependent Akt phosphorylation was also defective in the KRR mutant ECs. Reduced PI3K activation has also been observed in ERβ knockout hearts [114] and hypothalamic neurons [115]. It therefore seems reasonable to assume that bot ERα and ERβ can activate PI3K in a similar, direct, membrane delimited manner. In contrast, GPER appears to activate PI3K indirectly, via transactivation of the EGFR (see for instance [49]).

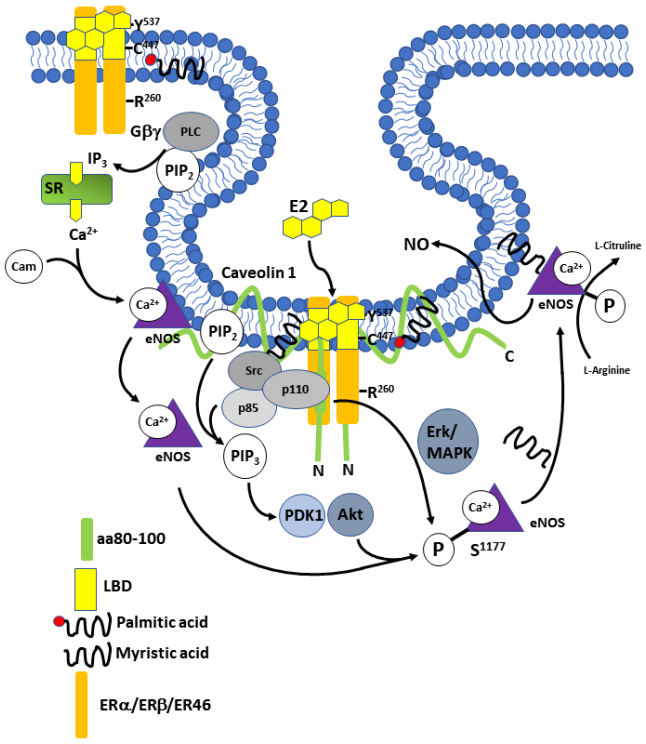


**Figure 3. Modes of Estrogen-Dependent eNOS Activation.**

eNOS is largely regulated by cytosolic $Ca^{2+}$ and calmodulin. Estrogen binding to membrane ER in various cell types induces $Ca^{2+}$ mobilization with rapid kinetics. Particularly in neuronal cells this can be mediated by several types of PM voltage operated or store operated $Ca^{2+}$ channels (see [116]). Indeed, activation of PM receptors by E2 results in the formation of $IP_3$ and cAMP and therefore stimulates $Ca^{2+}$ release from the endoplasmic reticulum and also E2 induces $Ca^{2+}$ transients in HUVECs [117], although in ECs this is probably via store operated as thy don't have voltage operated channels. Interestingly, Rybalchenko et al [116] showed that ERβ can induce RyR2-dependent $Ca^{2+}$ release from the endoplasmic reticulum by direct interaction in the absence of ligand (E2) binding in HT-22 neuronal cells. However, the situation in vascular cells is less clear, but RyR2/3 in ECs could possibly amplify $IP_3$-induced release via CICR. The CAM complex is formed by $Ca^{2+}$ binding to calmodulin and aids the activation of eNOS by dissociating it from the caveolin 1 complex [118], as depicted in figure 3. However, in another study, cytosolic levels of $Ca^{2+}$ were measured by fluorometric assay in response to E2 and histamine administration. The experiment demonstrated the expected intracellular $Ca^{2+}$ increase but estradiol alone did not promote HUVEC $Ca^{2+}$ fluxes at any concentration although it still rapidly (10 min) induced an increase in NO release. Thus, in E2 (alone) mediated NO release, a rise in intracellular $Ca^{2+}$ is not required even though E2 can elicit a $Ca^{2+}$ transient [117]. Despite this, there is some evidence that by phosphorylating eNOS, Akt can activate the enzyme at much lower $Ca^{2+}$ and calmodulin concentrations. This is thought to be caused by a faster electron flux that passes across the eNOS reductase domain and due to a lower rate of calmodulin dissociation from eNOS at low $Ca^{2+}$ levels [119], similar to the effect of Hsp90.

The extent to which ER and GPER signaling is $IP_3/Ca^{2+}$-dependent may depend on the relative distribution of the receptors in ECs and VSMCs and also differences between vascular beds. For instance, vasodilation induced by

selective GPER agonists (ie G-1) in different vascular beds ie female mesenteric resistance arteries requires endothelial NO and smooth muscle cAMP [120]. In rat carotid GPER induced vasodilation is endothelium-dependent rather than endothelium independent and VSMC-driven [121]. In coronary arteries the data is conflicting because G-1-dependent relaxation has been shown to be endothelial NO-dependent in porcine coronary arteries because it was abolished by L-NAME or endothelial denudation [59]. However, in other studies relaxation to G-1 still occurred in endothelium denuded aorta or coronary arteries [60, 61]. GPER activation does increase $Ca^{2+}$ intracellular mobilization, but in some cells this may be due to EGFR transactivation rather than a PLCβ-$IP_3$ -mediated mechanism [53], because EGFR can activate PLCγ-$IP_3$. However, this was in fibroblasts where GPER is localised to endoplasmic reticulum and Golgi rather than the PM. In vascular cells GPER activation can lead to endothelium-dependent and endothelium independent mechanisms, as mentioned above, therefore again, responses may differ between different vessels and different vascular beds. As mentioned previously, the VSMC-relaxing effect of GPER activation may be mediated via a $Ca^{2+}$-activated large conductance $K^+$ channel (BKca) [61], therefore presumably involves $Ca^{2+}$ mobilization or changes in BKca $Ca^{2+}$ sensitivity, for instance via PKG-dependent phosphorylation. Given that increased intracellular $Ca^{2+}$ induces VSMC contraction, this perhaps suggests a localized $Ca^{2+}$ pool at the PM which regulates BKca. Indeed, others suggest that E2 induces VSMC relaxation by interfering with $Ca^{2+}$ mobilisation and entry [122-124]. Furthermore, activation of $K^+$ channels would lead to membrane hyperpolarization and therefore relaxation via feedback inhibition of VOCC (see [3, 125]). In female rat mesenteric microvessels ER (mainly ERα) directly mediates decreased VSMC $Ca^{2+}$ entry via endothelium- and $K^+$ channel-independent mechanisms [124]. Interestingly, some studies have shown effects of GPER activation on $Ca^{2+}$ handling in cardiomyocytes ie inhibition of $Ca^{2+}$ influx and decreased myofilament sensitivity to $Ca^{2+}$ [36]. Cardiomyocyte specific KO of GPER in mice leads to profound adverse cardiac remodeling and diastolic dysfunction in both male and female mice, with sex-based differences in gene expression profiles [126]. Also, ERβ or GPER activation opens L- and R-type voltage gated $Ca^{2+}$ channels in hypothalamic neurons [127-129] and GPER leads to $IP_3$ generation in breast tumour cells [130, 131], so although GPER effects may be $Ca^{2+}$-dependent, the mechanism may be different in different cell types.

Rapid signaling by ERα and ERβ mainly involves kinase activation at the membrane via PI3K, ERK (MAPK) and eNOS activation in ECs and impinges on proliferation and migration as well as vasodilation [132]. ERα also activates Gαi and Gβγ [133] which is also involved in eNOS and ERK activation [132] seemingly via Gαi induced activation of c-Src [105]. This pathway contributes to the stimulatory effect of ERα activation on EC proliferation and migration [134, 135]. However, ERα activation in human and rat ECs modulates intracellular $Ca^{2+}$ and causes a rapid increase in intracellular $Ca^{2+}$ which is blocked by the ERα antagonist ICI 182,780 [136, 137]. This effect is presumably mediated via Gβγ-dependent $IP_3$ -mediated $Ca^{2+}$ release.

ERα and ERβ signaling is similar in VSMCs where activation rapidly inhibits proliferation. However, in the case of VSMCs the balance appears to be in favour of phosphatase rather than kinase activation ie ERα activation stimulates increased activation as well as expression (ie genomic effect) of the phosphatases MKP-1, PTEN, PP2A and SHP-1 which inhibits PI3K and ERK activation and reduces proliferation and migration [6, 138-140]. ERα membrane recruitment and activation is dependent on an interaction with the scaffold protein striatin. In transgenic mice where this interaction is inhibited [141] or overexpressing an ERα trafficking inhibitory peptide [135] the E2-depedent inhibition of VSMC proliferation and migration is lost.

**3.3 MAPK/eNOS pathway.**

The MAPKs are serine threonine kinases and one of their subcategories is represented by the extracellular-signal regulated kinases (ERKs). ERKs can be activated by the Ras-Raf-MEK cascade. E2 binding to ERα or ERβ leads to

GTP loading of Ras and activation. Ras then recruits Raf kinase which activates MEK1 (MAPK/ERK kinase or mitogen-activated protein kinase kinase (MAPKK)). In turn, MEK1 will phosphorylate ERK 1/2. To investigate whether E2 activates this pathway, Chen et al [142] analyzed how 17βestradiol affects the phosphorylation of ERK1/2 in steroid-starved UAECs. Treatment for 10 mins with physiological concentrations of estradiol (10 nM-1 μM) caused the rapid phosphorylation of ERK1/2 up to 6-fold the basal level after 5 minutes and the maximal response was then maintained for up to 60 min. Furthermore, to show that the ERK1/2 upstream activator Raf-1 is also activated by E2 in ECs an immunocomplex kinase assay was carried out. The assay revealed an ordered activation of Raf-1, MEK1, and ERK2, with a 30% (above control) increase in Raf-1 activity after administration of 10 nM E2 (10 min). When pretreated with a MEK1 and MEK2 inhibitor (PD98059), E2 treated UAECs showed reduced eNOS activity, demonstrating that the MAPK pathway has a high importance in mediating E2 induced eNOS activity. Results using E2-BSA, a membrane impermeable estradiol conjugate, to test whether the ER involved in eNOS activation is a membrane receptor (i.e. non-genomic rapid signaling) were essentially the same. However, a possible caveat of using 17β- estradiol-BSA is that it was reported to be susceptible to contamination with 17β-estradiol and as a consequence it could produce inaccurate results [143].

The upstream activation of Ras by E2 binding is less well documented, but it is thought that activation of c-Src (Figure 2) is one of the initiating steps of this signaling cascade. E2-induced MAPK activation was abrogated by treatment of HUVECs with a c-Src selective inhibitor [144]. GTP bound Ras can also bind and activate PI3K thus further potentiating the action of E2 [103]. However, these 2 studies did not investigate the interaction between ERα and Gαi, a mechanism that is fundamental for both c-Src and ERK activation. Also the Gβγ subunit facilitates ERα interaction with Gαi. This mechanism is independent of GPCR activation [133] (and see [6]). Gαi and Gβγ directly interact with ERα via two regions-aa251-260 and aa271-595 respectively, which leads to eNOS activation. Furthermore, E2 induces the release of Gαi and Gβγ without GTP binding to Gαi. Disruption of the Gαi interaction with ER by mutation of these regions or using blocking peptides, or of Gβγ to the β-adrenergic receptor kinase (βARK) blocked the non-genomic response to E2 and also downstream c-Src and ERK activation in Cos7 cells (another fibroblast cell line). In ECs disruption of ERα and Gαi interaction blocked E2-induced eNOS activation and also attenuated monocyte adhesion [133]. Other studies in Cos-7 cells expressing ERs have shown that overexpression of ERβ in the membrane caveolae also induces rapid eNOS activation, independently of ERα, which suggests that ERβ can also mediate the rapid effects of E2 on eNOS [145]. The ERβ signaling complex is similar ie involving Gαi, Gβγ, c-Src and PI3K.

**3.4 Involvement of GPER (GPR30) in Endothelium-Dependent and -Independent Vasodilation.**

The constriction and relaxation of smooth muscle cells is mainly managed by paracrine or autocrine factors, by hormones and by stretch (myogenic response). VSMCs can also respond to changes in load by tonic and phasic contractions. To initiate contraction, VSMCs require myosin and actin cross-bridging and an increase in cytosolic $Ca^{2+}$. $Ca^{2+}$ increases in response to stimuli such as endothelin-1 and angiotensin II and forms a complex with Calmodulin (CAM). This complex can activate Myosin Light Chain Kinase (MLCK) which will phosphorylate the light myosin chain. The increase in cytosolic $Ca^{2+}$ is due to both its release from the sarcoplasmic reticulum and because of it entering the cell from the extracellular space via $Ca^{2+}$ channels. Voltage gated (L-type) $Ca^{2+}$ channels open in response to agonists or stretch which cause depolarization of the membrane. Furthermore, they can also open when they are phosphorylated by protein kinase C (PKC). PKC activation occurs as a result of agonist binding to GPCRs that are coupled to a heterotrimeric G protein. The Gβγ subunit stimulates phospholipase C activity which catalyzes the formation of $IP_3$ and diacylglycerol (DAG) from the membrane phospholipid $PIP_2$. By binding

sarcoplasmic reticulum $IP_3$ receptors, $IP_3$ triggers $Ca^{2+}$ release into cytosol. The existing $Ca^{2+}$ and DAG then activate PKC which further aids the increase of intracellular $Ca^{2+}$ by phosphorylation of the L-type $Ca^{2+}$ channel [26].

GPER has been shown to trigger the activation of rapid signaling pathways when bound by E2. Confocal microscopy studies localized GPER primarily to the endoplasmic reticulum, although it was presumed to be localized at the plasma membrane. One explanation for this apparent discrepancy might be that after agonist stimulation, or during receptor biogenesis, GPCRs traffic between the endoplasmic reticulum and the plasma membrane [52, 146] (and see section 2.2 above). Also, possible, although not substantiated, is that GPCRs can traffic between the PM and endosomes and receptors can signal from endosomes in complexes similar to caveolae. GPER activation separately leads to both increases in cAMP and also triggers c-Src with opposing effects on relaxation or contraction ie c-Src is involved in EGFR transactivation via the activation of matrix metalloproteinases (MMPs) which will then lead to the MAPK and PI3K cascade activation and vasoconstriction in VSMCs. However, GPER activation in ECs leads to E2- induced NO release and relaxation. Also, GPER-dependent cAMP activation in VSMCs leads to vasorelaxation in coronary arteries. How do these conflicting pathways co-exist ?

GPER activation can lead to both relaxation or contraction of coronary arteries depending on the specific conditions. This apparent contradiction may be due in part to direct GPER-dependent relaxation in ET-1 pre-constricted porcine coronary arteries (ie EC-dependent via eNOS ?) compared to GPER-dependent EGFR transactivation in VSMCs causing constriction [147]. In this scenario the GPER agonist G-1 caused relaxation of ET-1 pre-constricted arteries which was blocked by the GPER antagonist G36. However, G-1 pre-treatment enhanced the ET-1 dependent constriction (ie if given before ET-1). This was blocked by the EGFR antagonist AG4178 or inhibition of c-Src. Also, this enhanced relaxation, suggesting that these two mechanisms are in opposition in PCASMCs. Furthermore, the G-1 enhanced ET-1-induced constriction was blocked by the ERK inhibitor PD98059, which also enhanced relaxation. However, since ERK is also involved in eNOS activation, the net effect of ERK inhibition on vascular tone will depend on the experimental conditions, such as viability of the EC *in vitro*, the choice of pre-constrictor agent and perhaps more importantly the dose of pre-constrictor used. Also, intriguingly inhibition of $G\beta\gamma$ with gallein inhibited G-1 enhanced constriction and potentiated relaxation [147]. However, although this study didn't directly test whether the G-1-induced relaxation was $G\alpha$ or cAMP-dependent, previous work from the same group reported GPER-dependent coronary artery relaxation was due to cAMP/PKA-dependent phosphorylation and activation of myosin light chain phosphatase (MLCP) in VSMCs, which was in itself $G\alpha s$-dependent rather than $G\alpha i$ [148].

These apparently opposing responses are likely dependent on the relative distribution of GPER between ECs and VSMCs. For instance, if EGFR activation of c-Src and ERK/MAPK causes constriction in VSMCs, how does this then relate to eNOS-dependent NO production in ECs ? The rapid effects of E2 on VSMCs which induce vasodilation also occurs in endothelium-denuded vessels of rabbit and human coronary arteries that were treated with ET-1. This implies that the relaxation caused by E2 in VSMCs is independent of E2's actions on the endothelium [149].

A surprising finding with possible clinical significance was that ICI182,780 an antagonist of ER, (and other antagonists such as tamoxifen, raloxifene and phytoestrogens) are agonists for GPER [150]. This finding highlights the importance of taking into consideration all 3 types of receptors in experiments studying both ECs and VSMC, something that was not done in the experiments above. For example, by administering ICI182,780, GPER stimulated pathways would be activated and classical ERs pathways inhibited.

**3.5 Contradictory effects of GPER1 activation.**

Before the discovery of GPER, it was thought that only ERα and ERβ are involved in VSMC relaxation. In contrast, knockout mice with no classical ERs, still had vasodilatory responses to E2 administration. GPER was found to be expressed at high levels in arterial VSMCs, the arteries of humans with coronary atherosclerosis [151] and in the arteries of hypertensive mRen2 Lewis rats [60]. Furthermore, by injecting G-1, a GPER agonist, in rats with normal blood pressure, a marked reduction in MABP was noticed within 2 minutes after infusion. By measuring changes in the lumen diameter of pre- constricted rat mesenteric arteries over time, after the administration of the same agonist, acute dilation was recorded. Interestingly, the effect of G-1 in human internal mammary arteries were greater than those of E2, with G-1 exerting a stronger relaxant response. In contrast to G-1, 17β-estradiol only dilated murine aortas, but not murine carotid arteries [36], which suggests how different and varied the distribution of estrogen receptors is throughout the vasculature. Additionally, GPER knockout mice, had increased blood pressure due to increased peripheral vascular resistance as manifested by increased media to lumen ration in resistance arteries as well as hyperglycaemia, reduced glucose tolerance and skeletal/growth defects in female mice, emphasising the important metabolic roles of GPER [64].

In contrast to expectations, Kurt and Buyukafsar [152] found that when isolated perfused rat kidney arteries were treated with G-1, in the absence of vasoconstrictors, they experienced a substantial vasoconstriction. E2 treatment had similar but weaker effects, while PPT, the ERα agonist had no effects over the perfusion pressure and neither did DPN, the ERβ agonist, showing that the mechanism is or can be independent of the classic estrogen receptors. Furthermore, G-1 vasoconstriction was not modified by endothelium denudation, showing that none of the endothelial factors are able to compensate for the vasoconstriction caused by GPER1 activation. To clarify that GPER was responsible for the actions, G15, its antagonist was used in the same preparation after G-1 treatment and it significantly abrogated the vasoconstrictor response. ERK1/2 inhibitors also blocked E2 and G-1 effects, suggesting the involvement of this pathway in VSMCs constriction. Interestingly, in the same arteries that were pre-constricted using different factors, G-1 acted as a vasodilator. This shows that the effects may be different in vessels isolated from different vascular beds and that the results depend on experimental set-up (ie whether they possess pre-existing tone or have been pre-constricted with agonists). The mechanism proposed by Yu et al, [147] could explain this contradiction and some of the conflicting results obtained from HRT studies and the question that arises from the study by Kurt and Buyukafsar [152] as to why endothelium denuded renal arteries that were not treated with ET-1, but only with G-1 still had vasoconstricting effects. Yu et al showed that G-1 mediated relaxation in PGF2$\alpha$ pre-constricted porcine coronary arteries (PCAs) and porcine coronary artery smooth muscle cells (PCASMCs) is cAMP and PKA-dependent via decreased phosphorylation of the MLCP regulatory subunit MYPT1 [153]. This occurred in both aortic rings and PCASMCs via downregulation of RhoA/Rho kinase activity and thus phosphorylation of MLC. Furthermore, the resulting PKA activation was A-kinase anchoring protein (AKAP)-dependent [148]. They further showed that this occurs via Epac/Rap1-mediated inhibition of RhoA/Rho kinase in *parallel* with PKA-dependent phosphorylation of vasodilator stimulated phosphoprotein (VASP) which also inhibits Rho kinase. In a further investigation of the upstream signalling the same group showed that GPER activation by G-1 in PCAs and PCASMCs occurs via G$\alpha$s activation by forming a plasma membrane complex with AKAP and the membrane-associated guanylate kinase (MAGUK) SAP97 [154]. Interestingly, these relaxant effects of GPER activation also occurred in endothelium denuded coronary arteries, so were direct effects on CASMCs and endothelium-independent. Perhaps, the final piece in the jigsaw is the observation that whereas G-1 caused dose-dependent relaxation in ET-1 pre-constricted arteries, it enhanced ET-1 contraction when given before ET-1. Inhibition of G$\beta\gamma$ with gallein, EGFR with AG1478 or Src with phosphatase-2 blocked the constrictor effects of G-1 and enhanced the relaxant effects. Also, inhibition of ERK1/2 with PD98059

did the same. So GPER causes EGFR transactivation which causes ERK-dependent constriction via Gβγ -dependent Src activation [147]. It could also be that there are other mechanisms that remain to be discovered or GPER plays different roles in different vascular tissues or GPER distribution between ECs and VSMCs has different effects, but these studies go a long way to explaining the paradoxical effects of GPER activation.

Despite the finding that GPER can produce vasoconstriction, studies on rat aorta, human mammary arteries and rat and porcine coronary arteries (lacking or having intact endothelium) concluded that its actions are predominantly vasodilating [36, 60, 121, 148] ie if arteries have pre-existing vascular tone as they are likely to have *in vivo*. As mentioned above, one of the main ways to produce vascular contractions is by phosphorylating myosin light chain (MLC) by MLCK [26]. The finding that cAMP decreases this phosphorylation [155] opened new horizons for investigating whether there could be a link between E2 and cAMP's actions. As mentioned above, studies on porcine endothelium-denuded arteries, pre-constricted with PGF2α and then G-1 treated showed a significant concentration dependent increase in cAMP production compared to the control group and tension studies also confirmed G-1-dependent relaxation. PKA is a well-known target of cAMP and a very small concentration of G-1 raised PKA activity two-fold compared with the control, whilst G36 (GPER antagonist) completely blocked this effect. One of PKA's targets is RhoA, which is inhibited by PKA phosphorylation. The Rho family of small GTPases are involved in multiple cellular mechanisms, one being switching off MLCP to allow for smooth muscle contraction. Inhibiting G-1 action using a PKI, decreases the phosphorylation of RhoA, whilst a PKA agonist in absence of G-1, produces similar effects to those of G-1 alone. This demonstrates that RhoA inactivation can be induced by G-1. In support of this general scheme, it was reported that cAMP/PKA also mediates relaxation in adult New Zealand white rabbit femoral arteries downstream of activation of GPER with G-1 [156]. The probable mechanisms of GPER mediated vasodilation and constriction are outlined schematically in figure 4 panels A and B (and see [148]).

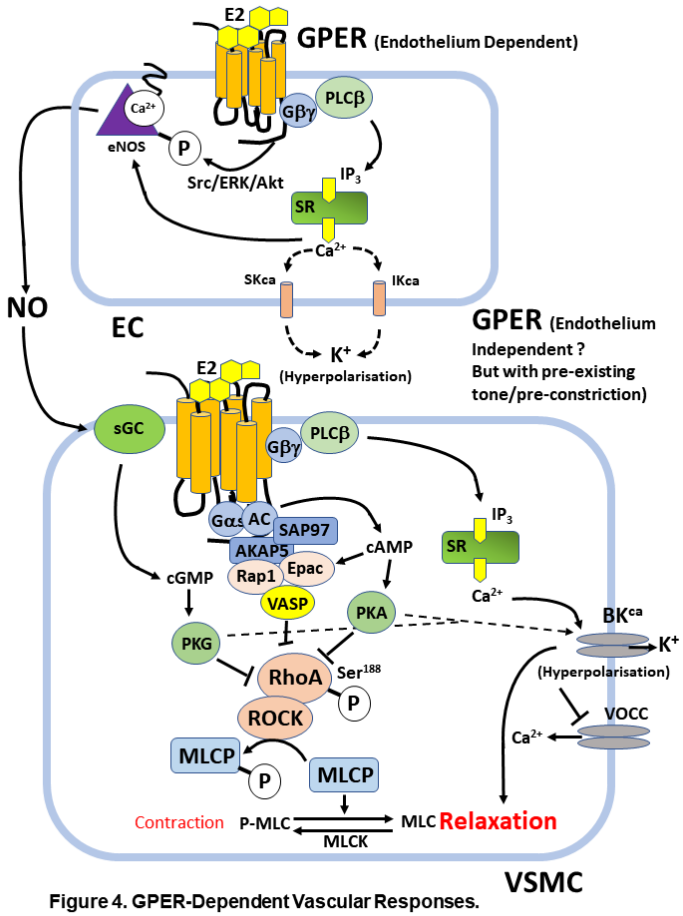


**Figure 4. GPER-Dependent Vascular Responses.**
**Panel A:** Endothelium-dependent and –independent modes of GPER-dependent relaxation in the presence of pre-existing vascular tone or pre-constriction.

### 3.6 BKca activation and VSMCs relaxation.

BKca channels (large conductance calcium and voltage dependent $K^+$ channels) are one of the main $K^+$ channels present in VSMCs of human coronary and other arteries and have an important role in regulating membrane potential, in response to depolarization by vasoconstrictor molecules or stretch by causing an opposing hyperpolarization (see [157] and refs). Patch-clamped cultured human coronary smooth muscle cells, were treated with 17β-estradiol to study whether estrogen could activate these channels and therefore induce relaxation by increasing potassium efflux and closing VDCCs. The results indicated a concentration and time dependent stimulation of these channels, with maximal effects noticed 30

minutes after E2 administration, that persisted for 100 minutes on average [86]. Although in this study these were supraphysiological concentrations of E2 (5 μM), physiological concentrations of E2 (100 pM-1 nM) also activate BKca [158].

Yu et al, [61] showed increased channel opening by E2 treatment. Furthermore, in the same cells (E2 treated) this activation was blocked by the highly selective BKca channel blocker, iberiotoxin, supporting the conclusion that E2 opens BKca channels. It is well accepted that PKG depends on cGMP to activate myosin light-chain phosphatase (MLCP) which then leads to smooth muscle relaxation. Usually cGMP increases in response to sGC activation by endothelial NO. However, E2 stimulated VSMCs showed increased levels of cGMP and increased PKG activity, which meant that there is an association between cGMP, PKG and BKca channels. This was independent of NO since there were no endothelial cells present. Additionally, inhibition of PKG reversed the effect of E2 on BKca activity which suggests that E2 activation of BKca is PKG-dependent [125]. Despite this finding, the exact mechanism through which E2 activates cGMP and PKG in the absence of NO is unclear. However, it is also likely that increased NO production in ECs in response to E2 also contributes to the cGMP-mediated BKca activation. Also, differences between different arterial beds in BKca β-subunit expression regulates BKca $Ca^{2+}$ sensitivity and therefore the magnitude of the response to changes in intracellular $Ca^{2+}$ [157]. Also, E2 affects β-subunit expression in arterial smooth muscle [159] and mouse uterus [160] and induction of BKca subunit expression was ERβ-dependent in neuronal cells [161] and possibly myometrium (see also [162]). Although these are genomic effects, since BKca subunit expression declines with age, it is relevant for HRT studies.

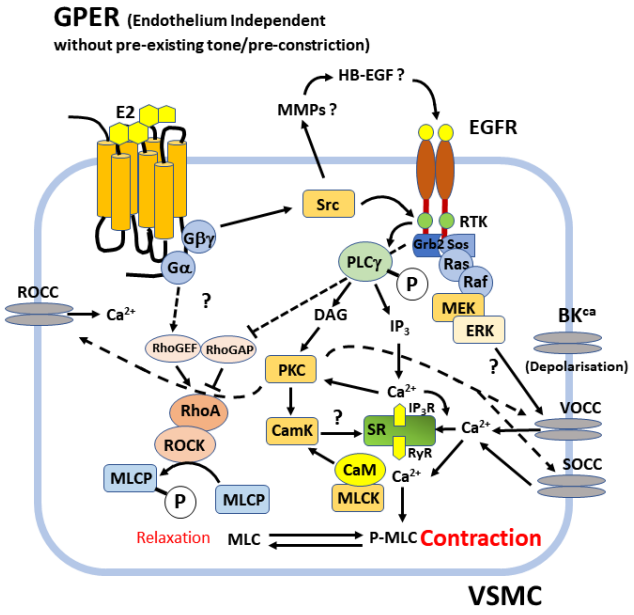


**Figure 4. GPER-Dependent Vascular Responses.**
**Panel B:** Endothelium–independent GPER-dependent constriction in the absence of pre-existing vascular tone or pre-constriction via EGFR transactivation.

More recent findings showed cAMP-dependent cross-crosstalk with PKG activation in VSMCs, which was GPER1-dependent. This is partly due to endothelial NO-dependent activation of sGC in VSMCs downstream of GPER and crosstalk with GPER-dependent AC activation in VSMCs themselves, which then potentiates PKG [120]. Similarly, Keung et al [163] showed that administration of cAMP and cGMP antagonists to porcine coronary arteries pre-treated with 8-Br-cGMP (an activator of PKG) or estradiol, inhibited relaxation of the vessels. In this study PKA inhibition had no effect on estradiol treated arteries. However, other studies suggest that G-1 induced coronary artery vasodilation is due to Epac/Rap1 and PKA-dependent inhibition of RhoA/Rho kinase and activation of MLCP [153]. Administration of both AC and GC inhibitors also abolished vasorelaxation in mesenteric arteries but only partially reduced vasodilation individually [120]. Also, cAMP is a partial agonist for PKG [164]. So, the contribution of GPER to artery relaxation is complex due to the expression of GPER on both endothelial cells and VSMCs which are differently coupled to downstream signaling and may therefore produce direct or indirect effects on VSMCs. Furthermore, and as mentioned above, Yu et al showed that GPER induced relaxation involves recruitment and activation of Gαs and the guanylate kinase SAP97 which could also link cAMP and cGMP-dependent signaling [154].

## 4. Other E2 actions important in maintaining vascular health.

### 4.1 ERs in VSMC proliferation.

Although membrane ERα and ERβ do not mediate the relaxing effects of E2 upon VSMCs, they are required for rapid estrogen-induced inhibition of VSMC proliferation, a mechanism that is thought to stop the progression of atherosclerosis and restenosis. The mechanism through which E2 inhibits VSMC proliferation is not known in detail. However, protein phosphatase 2A (PP2A) activation is thought to play a role, due to its importance in regulation of cell metabolism and cell cycle [135].

The importance of these 2 receptors in the protection from vascular injury was also studied in knockout mice with either no ERα or ERβ. Mice deficient in ERβ were shown to still retain vascular protective mechanisms to an extent, when E2 was administered [165]. On the other hand, in ERα knockout mice, no inhibition of VSMCs proliferation was observed in response to vascular injury, showing the importance of ERα in this mechanism of action [98]. However, these studies did not investigate whether the rapid non-genomic mechanism is sufficient for the inhibitory effects on VSMC proliferation. It might be that both nuclear and non-genomic actions are required and that there is a link between the two that remains to be investigated. Discovering the pathway would open new perspectives in targeting specific molecules to create remedies for atherosclerosis and restenosis. However, a caveat to this approach is that promoting apoptosis of VSMCs could also lead to the weakening and/or rupture of an already existing atherosclerotic plaque [70], but may be a useful approach to treat restenosis following balloon catheterisation and stenting. Interestingly, EGFR transactivation by GPCRs such as AT1R and ET1R are associated with CVD and VSMC proliferation [166], so potentially EGFR transactivation by GPER could potentially contribute to some of the detrimental effects of HRT, although there is no direct evidence for this.

### 4.2 ERs in EC proliferation and monocyte adhesion.

Even after the discovery of rapid estrogen signaling, it was thought that EC proliferation and migration is only regulated by genomic E2 signaling and no credit was attributed to rapid signaling. Using disruptive proteins in mouse aorta, the association between ERs and the molecule striatin (vital for rapid signaling) was inhibited together with EC proliferation and migration [167]. However, the disruption of striatin association with ERs was nonselective, therefore this could have also disrupted the interaction between striatin and other proteins involved in the cell cycle. Another study carried out by Lu et al [113] on a KRR mutant ERα cell line (triple mutation in receptor that makes it dysfunctional), which confers more specific results than the previous study, also found that rapid signaling of E2 through ERα is indispensable for EC proliferation. In addition, *in vivo* animal studies showed that both ERα and GPER knockout mice experienced higher levels of vascular inflammation after injury and higher levels of monocyte adhesion to ECs, compared to the wildtype mice [168]. Furthermore, GPER knockout mice had a significant increase in abdominal fat [36], which is consistent with the observation that E2 can lower the circulating levels of LDL [169]. Knowing that excessive monocyte adhesion and high LDL levels are associated with atherosclerosis and that re-reendothelialization is a crucial step in vascular repair, it is intuitive to deduce that 17β-estradiol is vasculo-protective. However, despite the vasculo-protective properties of E2, the results of HRT studies are inconsistent.

## 5. Hormone replacement therapy studies (HRT).

Since experimental studies have had such success in demonstrating that E2 has vasculo-protective actions, the use of estrogen after menopause or after ovariectomy, was thought to be essential for preventing or stopping the progression of CVDs, as well as managing menopausal symptoms. However, one of the first, large-scale clinical

| Trial | Prevention Type | Treatment | Target Group | Benefit/No Benefit | Ref |
|---|---|---|---|---|---|
| 1998 HERS 1 (4.1 years) | Secondary prevention of CVD | CEE + MPA | 2763 postmenopausal women >55 with CVD history | No benefit with increased risk of CHD | [162] |
| 2000 ERA (3.2 years) | Secondary prevention of CVD | CEE + MPA | 309 postmenopausal women >55 with CHD history | No benefit | [163] |
| 2001 WEST (2.8 years) | Secondary prevention of CVD | E2 | 664 postmenopausal women >55 with history of stroke | No benefit with increased vascular events | [164] |
| 2001 PHOREA (48 weeks) | Secondary prevention of CVD | E2 and gestodene | 321 postmenopausal women >55 with atherosclerosis | No benefit | [166] |
| 2001 EPAT (2 years) | Primary prevention of CVD | E2 + statins | 199 postmenopausal women with high LDL-C | Slowed atherosclerosis progression if in early menopause | [167] |
| 2002 ESPRIT (2 years) | Secondary prevention of CVD | E2 | 1017 postmenopausal women 55-69 after an MI | No benefit with increased vaginal bleeding | [168] |
| 2002 HERS 2 (6.8 years) | Secondary prevention of CVD | CEE + MPA | 2321 postmenopausal women (survivors of 1998 trial) | No benefit with increased risk of ventricular arrythmias | [169] |
| 2006 EAGER (42 months) | Secondary prevention of CVD | Either E2 or E2+MPA | 83 postmenopausal women after coronary bypass surgery | No benefit with increased risk of CAD in the remaining healthy coronary vessels | [170] |
| 2007 WISDOM (10 years) | Primary prevention of CVD | CEE or CEE + MPA | 5694 healthy postmenopausal women 50-79 | | [165] |
| 2012 DOPS (16 years) | Primary prevention of CVD | E2 + norethistero ne | 1006 Healthy postmenopausal women 45-58 | Significantly reduced risk of mortality HF or MI | [174] |
| 2013 ELITE (5 years) | Primary prevention of CVD and cognitive decline | E2 or E2 + progesterone | 643 Healthy postmenopausal women 55-65 | Slowed atherosclerosis progression if in early menopause | [176] |
| 2019 KEEPS (4 years) | Primary prevention of CVD (carotid intima-media thickness) and coronary calcium | CEE or E2 + progesterone | 720 Healthy early postmenopausal women 42-58 | No benefit. Trend towards reduced coronary calcium and improved bone mineral density | [177] |

**Table 1. Clinical trials summary showing the association of HRT and CVD in postmenopausal women.**

trials, HERS, showed that conjugated equine estrogens (CEE) plus medroxyprogesterone acetate (MPA) administration had no benefits and, in most cases, it increased the prevalence of coronary heart disease in the first year of treatment, despite the fact that reductions in plasma LDL were recorded [170-172].

CEE is extracted from the urine of pregnant mares and it is a mixture of saturated (such as E1, E3) and unsaturated estrogens (such as equilin) but does not contain E2. Therefore, one of the reasons for the inconsistent results, might be that CEE is less effective than E2, but this does not explain the increased vascular events seen in HRT studies. Furthermore, MPA and gestodene are progestin medications that can antagonize the protective effects of CEE when administered together. This could explain the deleterious effects of this type of hormone therapy. The WISDOM [173] and PHOREA [174] studies compared the effects of CEE alone, CEE+MPA and CEE+gestodene respectively and discovered that although CEE alone can be beneficial as a primary prevention for CHD because it lowers LDL levels, it increased the risk of breast cancer [173]. On the other hand, transdermally administered E2 in combination with statins (a class of drugs that lower cholesterol levels) were proven to have some beneficial effects which include decreased plasma LDL levels, vasorelaxation and reduced vascular inflammation [175]. The ESPRIT study showed no benefit [176] and no benefit was shown in older women with coronary disease treated for 6.8 years with estrogen plus progestin and in fact increased the rates of venous thromboembolism and biliary tract surgery and trends in other disease outcomes were also not favorable [177]. The EAGAR trial showed slowed atherosclerosis progression in coronary saphenous vein bypass grafts but accelerated disease progression in non-bypassed native coronary arteries [178], so the effects are mixed and somewhat inconclusive.

Another reason for the discrepancy between experimental studies and the clinical trials might be that in most of the studies women had pre-existing CVDs. E2 can cause plaque instability and rupture due to inhibition of VSMC proliferation (see section 4.1) which can lead to myocardial infarction or stroke. Accordingly, the only postmenopausal women in the studies that experienced vasodilation were those that were healthy [173]. Moreover, for the same reason, the timing of the therapy (before or after menopause) is critical because as more years pass by, the CVD complications in women become more severe and irreversible. Hypertension, which is caused in part by E2 loss after menopause, also accelerates the progression of atherosclerosis. In the studies summarized in Table 1, women were on average 67 years old, which suggests that they were already postmenopausal for more than 5 years. The 'critical window hypothesis' [179] is based on both the observation of these studies and animal studies which suggested that the protective effects of E2, were more significant if the

therapy was initiated before the onset of atherosclerosis. This indicates that estrogen therapy is more likely to be successful when used as primary prevention rather than secondary prevention, since defective arteries are not as responsive to E2 stimulation as healthy arteries are [179].

The issue of the 'timing hypothesis' [180] was also addressed by a Danish study published in 2012 which was a 16 year follow-up of healthy women aged 45-58 who were recently postmenopausal or had perimenopausal symptoms in combination with recorded postmenopausal serum FSH values [181, 182]. The participants were randomized such that approximately half the women received treatment consisting of triphasic estradiol and norethisterone and women who had undergone hysterectomy (but with recorded FSH values) received 2mg estradiol per day. The control group received no treatment. Intervention was stopped after 11 years due to reports of adverse outcomes in other trials (see table 1) but the participants were followed up for an additional 5 years (16 years in total) or achieving the combined end point. The main outcome measure was a composite of death, heart failure and/or myocardial infarction. Interestingly, after 10 years of intervention 16 women in the treatment group experienced the primary endpoint compared to 33 in the control group ($p=0.015$). Therefore, the women receiving HRT early after menopause had a significantly reduced risk of mortality, heart failure or MI and without any increased risk of cancer, venous thrombosis or stroke.

It is important to relate these protective effects to possible mechanisms. The authors allude to the fact that, as mentioned above, other studies have shown a beneficial effect of 17-β-estradiol and ethisterone on lipid metabolism, reducing LDL levels, improving endothelial function and reducing carotid intima media thickness [170, 175, 183]. It is important to again point out that these protective effects are only seen in otherwise healthy women in the absence of overt CVD. It is also reiterated that certain gestogens such as MPA compared to natural progesterone blunt the positive effects of estrogen. An additional strength of the Danish study is the successful randomization of healthy postmenopausal women and the long-term follow-up, things not achieved in many other trials. Other trials that address the 'timing hypothesis' include the ELITE [184] and KRONOS/KEEPS [185] studies. Although the findings in these studies with respect to primary and secondary endpoints of CVD and osteoporosis were mixed and somewhat inconclusive, these were only short-term follow-ups (approximately 4 years). It is not the purpose of this review to assess these trials in depth, but rather to focus on possible vascular mechanisms. A systematic review of these clinical trials is covered elsewhere (see for instance [186]).

## 6. Conclusions.

Cardiovascular disease is still the number one killer worldwide, being more frequent in post- menopausal women than in pre-menopausal women, which lead to the belief that estradiol (E2) loss, is partly responsible for this. Figure 5 describes some of the events linked to vascular ageing and E2 loss.

Experimental studies demonstrated that endogenous E2 has vascular protective effects, the best described being that of mediating arterial vasodilation, thus preventing hypertension and the progression of atherosclerosis. By binding to its receptors, E2, activates multiple signaling pathways in both VSMCs and ECs. The PI3K/Akt/eNOS and MAPK/eNOS pathways are activated by the classical receptors ERα and ERβ in endothelial cells and lead to an increased production of NO. Additionally, GPER has been found to mediate most of E2's rapid signaling mechanisms responsible for VSMC relaxation. This occurs by activating BKca channels and initiating the cAMP/PKA/RhoA pathway. However, the results of the studies varied within different vascular beds, with GPER mediating vasoconstriction in rat kidney arteries via the transactivation of EGFR and ERK1/2. Rapid E2 signaling is also necessary for VSMC apoptosis, EC proliferation, inhibition of monocyte adhesion and decreasing LDL levels, mechanisms that are vital for vascular healing and preventing atherosclerosis. Furthermore, estrogen is also

involved in genomic signaling (e.g. via MAPK-mitogen activated protein kinase), which was not discussed in this review, but also plays an important role in its protective effects.

Studying ER signaling mechanisms and subsequently discovering therapies to treat these complications or at least prevent them is harder than originally thought, not least because most antagonists for either ERα or ERβ are agonists for GPER. Although HRT studies showed increased risk of CV events, they also opened new doors for further research, since it was observed that these results are influenced by different factors such as: type of hormone treatment, timing and duration of HRT administration, patient's health, estrogen receptors levels, their sensitivity to E2 and the changes in the structure of the vascular wall.

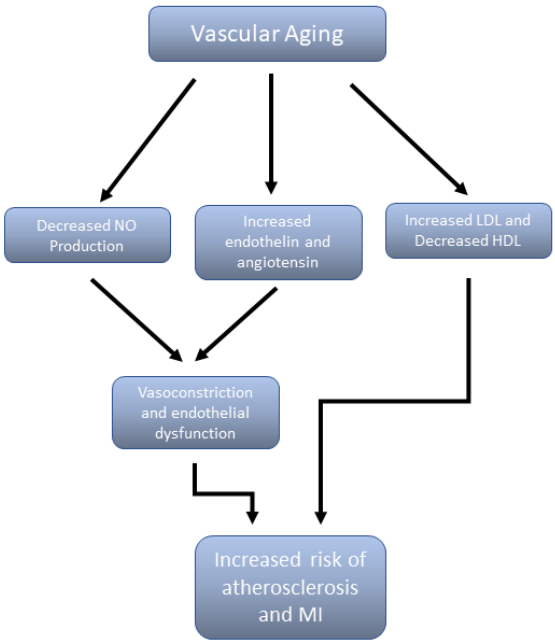


**Figure 5. Events linked to vascular aging and E2 loss.**

In conclusion, in order to fully exploit E2's protective actions and get better clinical trial results, future research should be more focused on characterizing the receptors' function and distribution in both arteries and veins. This could lead to the discovery of new signaling pathways that are responsible for the deleterious effects observed in HRT, which would make their specific inhibition possible. So could E2 be deleterious in post-menopausal women based on our current understanding of the signalling described in this review ? For instance, as we mentioned above GPER can induce EGFR transactivation which, in general, contributes to CVD and it is possible that GPER could be more pro-contractile in post-menopausal women in addition to the fact that EC dysfunction impairs the beneficial actions of E2 via ERα and ERβ in general. This could be relevant given that the relative and regional expression of the three receptors varies with age and with hormonal status. Another question is whether or not menopausal EC dysfunction is really irreversible. If E2 and PG alter expression of their own receptors, there may be negative and/or positive feedback. One problem is that menopause is almost only seen in humans, so longitudinal studies in animal models are difficult to achieve. Nevertheless the protective role of E2 in general is not disputed and there is hope for the future.

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**Figure Legends.**

**Figure 1.**

The Cardiovascular Disease Continuum.

**Figure 2.**

The Estrogen Receptor Complex. ERα, ERβ or ER46 is complexed with Gαi, c-Src TK, PI3K, Akt, Hsp90 and eNOS in caveolae, where it assembled on caveolin 1 with striatin. In this depiction ER is shown assembled on caveolin 1 and spanning the membrane. There is evidence that ER presents an ectodomain, although it is not clear whether the C-terminal ligand binding domain is extracellular or in the membrane, because E2 is lipid soluble and can cross the membrane. ER assembles on the aa80-100 region of the caveolin 1 N-terminus. ER is palmitylated on $Cys^{447}$ which anchors it to the membrane. The recruitment of Src TK and PI3K can occur on the methylated $Arg^{260}$. However, phosphorylation of $Tyr^{537}$ has also been reported to recruit Src TK via its SH2 group, although this would probably occur in a model where ER is tethered to the inside of the membrane rather than spanning the membrane. Src TK is also myristylated on $Gly^{2}$. In endothelial cells estrogen (E2) binding to this complex rapidly initiates a signaling cascade leading to the activation of eNOS and NO production. p85 and p110 are regulatory subunits of PI3K. Rapid signaling is essential for inhibiting VSMC proliferation, inducing vasorelaxation and endothelial cell proliferation and migration. Adapted from various sources, including [104].

**Figure 3.**

Production of NO after E2 binding. Gβγ activation of phospholipase C generates $IP_3$ and diacylglycerol from membrane PIP2. $IP_3$ causes $Ca^{2+}$ release from the endoplasmic reticulum which binds to calmodulin (Cam) which then binds to eNOS and causes eNOS to dissociate from caveolin 1 and come off the membrane. Activation of PI3K leads to the conversion of PIP2 to PIP3 which activates PDK1 and Akt. Along with MAPK (Erk1/2) activation this phosphorylates eNOS on $Ser^{1177}$ and translocates it back to the plasma membrane following myristylation, where it catalyses the formation of NO and L-citrulline from L-arginine. NO release induces VSMC relaxation. Adapted from [187].

**Figure 4.**

GPER-dependent signaling events in vascular endothelial and smooth muscle cells (VSMCs). **Panel A:** Endothelium-dependent (NO-dependent) and -independent modes of GPER-dependent relaxation in the presence of pre-existing vascular tone or pre-treatment with GPCR vasoconstrictors (Ang II or ET-1) or PGF2α. GPER agonists such as G-1 or E2 cause vasorelaxation when administered *after* pre-constriction. Note in this diagram, for simplicity, GPER is depicted as being in the PM, but in fact may signal intracellularly, ie from the endosomal membrane. **Panel B:** Endothelium-independent GPER-dependent vasoconstriction occurs in the absence of pre-existing vascular tone or when GPER agonists (G-1 or E2) are administered *before* vasoconstrictor agonists and probably involves Epidermal Growth Factor Receptor (EGFR) transactivation.

**Figure 5.**

Events linked to vascular aging and E2 loss.

**Table 1.**

Clinical trials summary showing the association of HRT and CVD in post-menopausal women. CEE: conjugated equine estrogens; MPA: medroxyprogesterone acetate; HERS: Heart and Estrogen/Progestin Replacement Study;

ERA: Estrogen Replacement and Atherosclerosis; WEST: Women's Estrogen for Stroke Trial; PHOREA: Postmenopausal Hormone Replacement Against Atherosclerosis; ESPRIT: Estrogen in the Prevention of Reinfarction Trial; EAGAR: Estrogen and Graft Atherosclerosis Research; WISDOM: Women's International Study of long Duration Oestrogen after Menopause; EPAT: Estrogen in the Prevention of Atherosclerosis Trial; DOPS: Danish Osteoporosis Prevention Study; ELITE: Early versus Late Intervention Trial with Estradiol; KEEPS: Kronos Early Estrogen Prevention Study.