Published in final edited form as:

Cardiovasc Hematol Agents Med Chem. 2010 January ; 8(1): 29–46.

Potential Approaches to Enhance the Effects of Estrogen on Senescent Blood Vessels and Postmenopausal Cardiovascular Disease

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Abstract

Cardiovascular disease (CVD) is more common in postmenopausal than premenopausal women, suggesting vascular protective effects of estrogen. Vascular estrogen receptors ER α , ER β and a transmembrane estrogen-binding protein GPR30 have been described. Also, experimental studies have demonstrated vasodilator effects of estrogen on the endothelium, vascular smooth muscle and extracellular matrix. However, randomized clinical trials have not supported vascular benefits of menopausal hormone therapy (MHT), possibly due to the subjects' advanced age and age-related changes in estrogen synthesis and metabolic pathways, the vascular ERs number, distribution and integrity, and the post-ER vascular signaling pathways. Current MHT includes natural estrogens such as conjugated equine estrogen, as well as synthetic and semi-synthetic estrogens. New estrogenic formulations and hormone combinations have been developed. Phytoestrogens is being promoted as an alternative MHT. Specific ER modulators (SERMs), and selective agonists for ER α such as PPT, ER β such as DPN, and GPR30 such as G1 are being evaluated. In order to enhance the vascular effectiveness of MHT, its type, dose, route of administration and timing may need to be customized depending on the subject's age and pre-existing CVD. Also, the potential interaction of estrogen with progesterone and testosterone on vascular function may need to be considered in order to maximize the vascular benefits of MHT on senescent blood vessels and postmenopausal CVD.

Keywords

sex hormones; progesterone; testosterone; phytoestrogens; estrogen receptor; endothelium; vascular smooth muscle; hypertension

INTRODUCTION

Cardiovascular disease (CVD) such as coronary artery disease (CAD) and hypertension (HTN) is less common in pre-menopausal women (Pre-MW) than in men of the same age [1]. Also, the risk of CVD is greater in Post-MW than Pre-MW, partly due to decreased plasma levels of estrogen during menopause [2]. Earlier observational studies, such as the Nurses' Health Study (NHS), suggested that estrogen therapy in Post-MW reduced the risk of CVD by 35 to 50% [3,4]. Also, a meta-analysis of these observational studies has shown ~33% less fatal CVD among estrogen users compared to nonusers [5].

Experimental evidence also suggests beneficial effects of estrogen on the vasculature. Estrogen receptor ER α , ER β , and GPR30 have been identified in blood vessels of human and animal models. Estrogen causes vasodilation via an effect on endothelial cells (EC), vascular smooth muscle (VSM) and extracellular matrix (ECM) [1,4,6]. However, randomized clinical trials (RCT), such as the Heart and Estrogen/progestin Replacement Study (HERS) and the Women Health Initiative (WHI), showed increased CVD and cerebrovascular events among MHT users. The discordant findings between experimental studies and RCTs made it important to investigate possible changes in the effects of estrogen on the cardiovascular system during menopause. Factors related to the time of starting MHT, pre-existing CVD, and the estrogen form used have been suggested [4]. Also, age-related changes in estrogen synthesis and metabolism, the vascular ER amount, types, tissue distribution, integrity, and post-ER signaling pathways may have contributed to the unanticipated results of RCTs.

This review will discuss reports in the PubMed database for the reasons that the beneficial vascular effects of estrogen in experimental studies did not materialize in RCTs. Factors related to the chemical properties of estrogenic compounds, the MHT form, dose, route of administration, and timing will be described (Table 1). Factors related to the vascular ERs and post-ER signaling pathways in ECs, VSM and ECM, and vessel architecture will be discussed. The potential interaction of estrogen with other sex hormones such as progesterone and testosterone on the vasculature will be highlighted. The discussion of these factors should help to identify potential approaches to maximize the effects of conventional MHT and to develop alternative MHT with greater efficacy and specificity in postmenopausal CVD.

Estrogen Synthesis and Metabolism

The lack of benefits of MHT in postmenopausal CVD could be partly related to age-related changes in estrogen synthesis and metabolism (Table 1). Endogenous natural estrogens, including estradiol (E2), estrone (E1) and estriol (E3), have 4 rings A, B, C, D, a hydroxyl group at C3, and either a hydroxyl or a ketone group at C17 (Fig. 1) [7]. The phenolic A ring promotes selective high-affinity binding to ER. Synthesis of estrogens starts with cholesterol binding to lipoprotein receptors on steroidogenic cells, and its transfer by sterol carrier protein-2 to steroid synthesis sites ([8,9]. Because steroid hormones are derived from cholesterol, they have similar chemical structures, and may affect E2 binding and actions on the vascular ERs. For example, androstenedione, a precursor of E2 and testosterone, is used by athletes for its body-building properties, and its estrogenic properties may need to be further examined.

In Pre-MW, E2 is mainly synthesized in the ovaries, while E1 and E3 are formed in the liver from E2 or in peripheral tissues from androstenedione [9]. In Post-MW, androstenedione, testosterone and E1 are the major precursors of estrogen in peripheral tissues [10]. Polymorphisms in the genes coding for steroidogenic enzymes may influence estrogen production, and should help to design a more individualized MHT approach in Post-MW [11].

Most of circulating natural estrogens bind strongly but reversibly to sex hormone-binding globulin (SHBG) in a non-saturable and non-stoichiometric manner. A small 2-3% fraction of natural estrogen is unbound and active and distribute rapidly and extensively due to their small size and lipophilic nature. E2 half-life is ~3 hours and exists in a dynamic equilibrium of metabolic inter-conversions with E1 and E3 [7]. Age-related changes in the estrogen volume of distribution and its protein bound/free form could affect the levels of endogenous and exogenous hormone in Post-MW.

Estrogens are metabolized to less active metabolites that are excreted in urine and feces. Estrogen metabolism includes oxidation (hydroxylation) by cytochrome P450s (CYPs),

glucuronidation by UDP-glucuronosyltransferase, sulfation by sulfotransferase, and O-methylation by catechol O-methyltransferase (COMT). E2 metabolism occurs mainly in the liver, where it first undergoes hydroxylation by CYPs into 2-(OH)E2 (80%) and 4-(OH)E2 (20%), then inactivated by COMT [10,12]. E2 metabolism varies depending on the stage of menstrual cycle, menopausal status, ethnic background and genetic polymorphisms [13]. Also, certain drugs, environmental factors and cigarette smoke may induce or inhibit the enzymes that metabolize estrogens, and thus alter their clearance [9,10]. Age-related changes in E2 metabolism may also alter the effectiveness of administered estrogen on the vasculature.

Type of Estrogen

The lack of benefits of MHT in postmenopausal CVD could be related to the type, form or dose of hormone used (Table 1). Current MHT includes natural estrogens such as conjugated equine estrogen, as well as synthetic and semi-synthetic estrogens (Table 2). Estrogens are available in oral, transdermal, parenteral and topical forms (Table 3). The bioavailability of oral estrogen is usually low due to first-pass hepatic metabolism. Ethinyl substitution at C17 position to produce ethinyl E2 minimizes the first-pass metabolism. Also, the transdermal route bypasses the liver and portal circulation, and minimizes hepatic effects of estrogens on protein synthesis, lipoprotein profiles and triglyceride levels. Transdermal estrogen may also have less risk of thrombotic effects and gynecologic cancers than oral estrogen [14]. The type of estrogen administered (CEE vs E2) and the route of administration (oral vs transdermal) may partly explain the unanticipated lack of vascular benefits of MHT in HERS and WHI RCTs.

Phytoestrogens—Phytoestrogens are polyphenolic non-steroidal compounds with estrogenic activity, and are found in foods and plants particularly soybeans, red clover, and wheat grains (Table 2) [15]. Interestingly, large amount of food-containing phytoestrogens is consumed in cultures with lower rate of menopausal symptoms, osteoporosis, cancer and CVD [16]. Phytoestrogens include flavonoids (flavones, flavonols and flavanones), isoflavonoids (isoflavones and coumestanes), stilbenoids (stilbenes such as resveratrol in red wine and peanuts), and lignans (secoisolariciresinol and matairesinol in flaxseed, whole grain bread, vegetables and tea) (Table 2) [15,17]. Secoisolariciresinol is converted to enterodiol and then to enterolactone, and matairesinol is converted directly to enterolactone by intestinal microflora. The enterolignans enterodiol and enterolactone are phytoestrogens with structural similarity to natural estrogens. The key structural elements for the phytoestrogenic effects include the phenolic ring(s), low molecular weight and optimal hydroxylation patterns. Soybean foods, soybean protein extract and red-clover extract have limited effects on menopausal symptoms. On the other hand, soybean isoflavone extracts may reduce hot flashes [18]. Also, isoflavones stimulate enhance NO-mediated vasodilation and promote antithrombotic and antiatherogenic effects. A meta-analysis of 38 controlled human studies of soy consumption has shown decreased levels of LDL and triglycerides and increased HDL. In contrast, a 12-month RCT in 202 Post-MW aged 60-75 years demonstrated that consumption of soy protein containing 99 mg of isoflavones per day had no effect on blood pressure or endothelial function. Also, no RCT has examined the effects of phytoestrogens on clinical end points of CVD such as myocardial infarction. Thus phytoestrogens may not be used as a primary preventive measure of CVD, but when used to treat menopausal symptoms they may have additional benefits by reducing risk factors of atherosclerosis such as hyperlipidemia and hypertension [19]. Also, while phytoestrogens have weaker affinity to ERs than E2, they are more stable and have longer duration of action, and potential cumulative undesirable effects [20]. Further analysis of structure and activity of phytoestrogens, and adequately-powered RCTs may further define their potential vascular benefits and usefulness as alternative MHT.

Specific Estrogen Receptor Modulators (SERMs)—SERMs such as raloxifene and tamoxifen are non-steroidal compounds that bind ERs with high affinity (Table 2) [21]. SERMs

have a wide range of activity from purely estrogenic, purely anti-estrogenic, to combined partial estrogenic in some tissues and anti-estrogenic or no effect in other tissues [22]. The agonist/antagonist activity and tissue selectivity may be related to the ratio of co-activator/co-repressor proteins in the target cells and the ER conformation induced by drug binding. This in turn determines how strongly the drug/receptor complex recruits co-activators, resulting in agonism, relative to co-repressors, resulting in antagonism. For example, raloxifene is an ER agonist in bone and serum lipids, but an ER antagonist in endometrial and breast tissue [23, 24]. The Raloxifene Use for The Heart (RUTH) RCT in Post-MW with CVD or at increased risk for CVD demonstrated that treatment with raloxifene for a median of 5.6 years reduced the risk of invasive breast cancer but did not change the incidence of coronary events [25]. A recent study in post-MW women (mean age 67.5 years) showed that raloxifene had no effect on the risk of primary coronary events, but was associated with increased risk of fatal stroke and venous thromboembolism [26]. However, as in the WHI study, the advanced age of the participants could have affected the estrogenic response. In ovariectomized (OVX) rats, tamoxifen may protect against myocardial ischemia-reperfusion injury due to its antioxidant properties [27]. Also, raloxifene induces rapid endothelium-dependent relaxation of coronary artery [28,29] and endothelium-independent relaxation of rat renal and pulmonary artery and porcine coronary artery through inhibition of Ca^{2+} influx via voltage-gated channels [30,31].

Specific ER Agonists—Recently developed triarylpyrazoles such as propylpyrazole trisphenol (PPT) are 400-fold more potent on $\text{ER}\alpha$ than $\text{ER}\beta$ [32,33]. PPT increases flow-mediated relaxation in small mesenteric arteries from females, but not males [34]. Diarylpropionitrile (DPN) is a potent $\text{ER}\beta$ agonist with a 30- to 70-fold selectivity over $\text{ER}\alpha$ [35]. Studies using selective ER agonists suggest that $\text{ER}\alpha$ mediates most of the vascular actions of estrogen [36]. However, DPN, a selective $\text{ER}\beta$ agonist, also induces acute NO-dependent vasodilation [6,37]. While the currently available SERMs and selective ER agonists show limited vascular benefits, new compounds with improved vascular effects and ER selectivity may provide alternative MHT to decrease the risk of CVD in Post-MW.

VASCULAR ESTROGEN RECEPTORS (ERs): The lack of benefits of MHT in RCTs could be related to changes in the amount, distribution, and structural integrity of the vascular ERs (Table 1). Estrogenic compounds bind to ERs with high affinity and specificity. Two nuclear ERs, $\text{ER}\alpha$ and $\text{ER}\beta$, have been cloned: [38]. $\text{ER}\alpha$ and $\text{ER}\beta$ are abundant in the female reproductive system and mammary gland, and also in the immune, skeletal, central nervous system and cardiovascular system [39,40]. $\text{ER}\alpha$ and $\text{ER}\beta$ have been localized in ECs and VSM [41,42]. The expression of vascular ERs varies with gender and the vascular bed studied. In human VSM from the coronary artery, iliac artery, aorta, and saphenous vein, both $\text{ER}\alpha$ and $\text{ER}\beta$ mRNA are expressed and the expression of $\text{ER}\beta$ is greater in females than males [43]. In rats, $\text{ER}\alpha$ is found mainly in the uterine vasculature, whereas $\text{ER}\beta$ is more abundant in ECs and VSM from the aorta, tail and uterine arteries [44]. In many cell types, $\text{ER}\alpha$ and $\text{ER}\beta$ form either homodimers or heterodimers.

$\text{ER}\alpha$ and $\text{ER}\beta$ are members of the nuclear receptor superfamily, in which one gene may result in the expression of multiple receptor proteins [45]. Such diverse response could be due to multiple sites for initiation of translation of the ER mRNA, alternative RNA splicing leading to multiple mRNA isoforms for each ER, as well as epigenetic changes and methylation of the genes encoding ERs [36,46].

The full-length human $\text{ER}\alpha$ mRNA encodes 595 amino acids, and the full-length human $\text{ER}\beta$ mRNA encodes 530 amino acids [47]. Human $\text{ER}\alpha$ and $\text{ER}\beta$ have 44% homology in amino acid sequence and share a common domain structure: A/B, C, D, E and F domains (Fig. 2) [38]. The A/B region is involved in protein-protein interactions and transcriptional activation of target-gene expression. This region harbors an activation function 1 (AF-1), located toward

the N-terminal end, that is ligand-independent and shows promoter and cell-specific activity [48]. Human ER α and ER β have <20% amino acid identity in the AF-1 region, which may contribute to ER-specific actions on target genes [38,49,50]. The central C-domain or DNA-binding domain (DBD) contains 4 cysteines arranged in 2 zinc fingers, is involved in specific DNA binding and ER dimerization, and is highly conserved between ER α and ER β and shares 95% amino acid identity, and therefore recognizes similar DNA sequences and regulates similar target genes [48]. The D-domain works as a flexible hinge between the DBD and ligand-binding domain (LBD). The hinge domain, is not well-conserved between ER α and ER β (30%), and promotes the association of ER with heat shock protein 90 (HSP90) and nuclear localization of ER [38,51]. The E-domain is the LBD, and ER α and ER β share ~55% amino acid identity in this region. The LBD contains a ligand-dependent AF-2 that is important for ligand binding and receptor dimerization. The F-domain has <20% amino acid homology between the two ERs, and the function of this domain is unclear. The two ERs have similar affinities for E2 and bind the same DNA response elements [38,40,47].

Polymorphisms in ER genes may affect ER properties and thereby the outcome of MHT. Polymorphisms of ER α at positions c.454–397 T>C (PvuII) and c.454–351 A>G (XbaI) may be associated with the severity of CAD in Post-MW [52]. Post-MW with the ER α IVS1-397 polymorphism C/C genotype (recessive) have higher HDL and apolipoprotein A-1 levels and greater forearm blood flow, brachial artery diameter, and endothelium-dependent dilation compared to those with the dominant phenotype [53]. In Post-MW with established CAD and being treated with estrogen alone or estrogen plus progesterone, a 2 times greater levels of HDL levels were seen in women with the ER α IVS1-401 polymorphism C/C genotype as compared to those without the polymorphism [54].

Several ER splice variants or isoforms have been described. Most ER α variants differ in their 5'-untranslated region, not in the coding sequence. ER α isoforms have not been identified in tissues and their function *in vivo* is unclear. However, they are important research tools as they heterodimerize with the full-length ER α and repress AF-1-mediated activity. They also localize in the plasma membrane and thereby may help to elucidate the mechanisms of non-genomic estrogen signaling [55]. Multiple ER β isoforms exist as a result of either alternative splicing of the last coding exon 8, deletion of one or more coding exons, or alternative usage of untranslated exons in the 5' region [56]. Among them, 5 full-length transcripts ER β 1-5 have been reported in human [40]. Whether ER isoforms are distributed differently in the vasculature and whether they change with age is an important area for future investigation.

One mechanism for downregulation of gene expression is methylation of the CpG island, a cytosine and guanine rich area in the promoter region, resulting in permanent inactivation of gene transcription [57]. Age-related increase in ER promoter methylation is a possible epigenetic mechanism contributing to atherosclerosis and vascular aging [58]. Methylation of ER genes increases with passage of cultured ECs and VSM and may be responsible for the decreased ER responsiveness and vascular genomic effects of E2 with cell aging [59,60].

Although E2 levels and release patterns change with aging, little is known about the age-related changes in ERs. Studies have shown no significant difference in ER expression in aorta of aging and adult OVX spontaneously hypertensive rats (SHR) [61]. However, age-related changes in ER have been found in other tissues. ER α protein was detected in the retina of young females, but not in the eyes of Post-MW [62]. Also, the number of ER β mRNA positive cells in the brain is less in old compared with young and middle-aged female rats, and this expression pattern is not altered by estrogen replacement [63].

Estrogenic Structure Activity Relationship—The lack of vascular benefits of MHT in Post-MW could be due to changes in the E2/ER binding. There are five structural features that

are important for binding to ER including: 1) H-bonding ability of a phenolic ring, mimicking the C3-OH in the A ring, 2) H-bond donor mimicking the C17 β -O, and an O-O distance equivalent to that between C3-OH and C17 β -OH, 3) hydrophobicity, 4) precise steric hydrophobic centers mimicking steric C7 α and C11 β substituents, and 5) a ring structure [64,65].

The 3D structure of ER α and ER β LBD is very similar and reflects their high sequence identity. The LBD has twelve helices (H1-H12), folded into a three-layered anti-parallel α -helical sandwich comprising a central core layer of three α -helices (H5-6, H9, and H10) sandwiched between two additional layers of α -helices (H1-4) and (H7, H8, and H11). This helical arrangement creates a 'wedge-shaped' molecular scaffold that maintains a sizeable ligand-binding cavity into which the hormone binds. The ligand binding sites of ER α and ER β are nearly identical; only two residues are different: Leu384/Met421 in ER α correspond to Met336/Ile373 in ER β . Also, the ligand binding cavity of ER β is smaller (<20%) than that of ER α and this influence the selective affinity and pharmacology of ligands and ERs [38,50,52].

The binding of E2 with ERs typically involves non-covalent H-bonding and hydrophobic interactions), for which the contribution of the phenolic ring is very important. Strong estrogens also have an additional OH group within a certain distance from the phenolic ring [66]. Mutational studies indicated that Cys residues Cys381, 417, 447 and 530 in ER α LBD, and Cys334, 369, 399, and 481 in ER β LBD play a role in ligand binding. In particular, Cys530 builds up the primary site for E2 binding, whereas Cys381 and Cys417 are involved in the recognition of non-steroidal anti-estrogens [67]. Interestingly, E2 binds 'upside-down' to ER β compared to ER α [47,66,68]. Age-related mutations in the ligand binding domain or binding cavity of the ER may decrease the beneficial effects of MHT in postmenopausal CVD.

It is important to note that the volume of the ER binding pocket is about twice that of the E2 molecule. Therefore, there are large unoccupied cavities opposite the C7 α and C11 β positions of E2. These cavities allow steric groups of certain sizes to fit. While the rigid protein architecture around the A-ring pocket imposes an absolute requirement for effective ER ligands, the remainder of the binding cavity is quite accommodating. Particularly, the distal end or D-ring binding site of the cavity is quite flexible and permits a variety of ligand-binding modalities. The discrepancy between the volume of the ER binding cavity and the size of its cognate ligand suggests that the LBD have evolved sufficiently so that it can discriminate between E2 and other steroids. In addition, the sub-optimal architecture in certain regions of the LBD suggests that unknown endogenous ER modulators remain to be identified [66,69].

Tamoxifen has similar binding affinities for human ER α and ER β , but its binding affinity is only 3-4% of E2 and 7-10% of ICI-182,780. Raloxifene has similar binding affinity for ER β as tamoxifen, 16-fold higher affinity for ER α than tamoxifen, and comparable affinity as ICI-182,780. Therefore, raloxifene has preferential affinity for ER α over ER β . The phytoestrogen genistein has high affinity for ER β , almost identical to that of E2, although its affinity for ER α is only 6% of that. Coumestrol has very high binding affinity for human ER α and slightly higher affinity for ER β . Daidzein has very weak binding affinity for both ER α and ER β , but its relative affinity for ER β is higher than that for ER α . Metabolites such as C-3 sulfated estrogens (E1-3-sulfate and E2-3-sulfate) have little affinity for ER α and ER β , while 16 α -OH-E1 has a higher affinity than E1 [7]. Further elucidation of the estrogen-ER interactions should permit the rational design of drugs with selective estrogenic activity particularly in aging women.

GPR30—GPR30, a protein structurally unrelated to ER α or ER β , binds E2 with high affinity, and may mediate its nongenomic effects [70,71]. GPR30 is located on chromosome 7p22 [72]. It has an extracellular N terminal, seven transmembrane α helices, 3 exo-loops involved

in ligand binding, 3 or 4 cyto-loops involved in G protein subunit binding, and a C terminal linked to the membrane through lipid addition, and also involved in binding G protein subunits. GPR30 is widely distributed in the brain and peripheral tissues and is expressed in human mammary artery and saphenous vein [73]. GPR30 has been localized in the endoplasmic reticulum [70], the plasma membrane [71], and its subcellular localization may depend on the cell type studied and the GPR30-tag used for analysis [74]. Activation of GPR30 by estrogen results in intracellular Ca^{2+} mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus [70]. GPR30 may also function in conjunction with ER α to assemble a signal complex essential for rapid estrogen signaling [75]. The ER antagonists tamoxifen and ICI 187,280 may act as GPR30 agonists [70]. The physiological role of GPR30 as an ER is unclear. In contrast to previously published reports [71], Levin and co-workers could not demonstrate cAMP or ERK activation in GPR30-positive and ER-negative breast cancer cells [76]. Also, in ER-positive, GPR30-positive MCF-7 cells, nongenomic E2 responses were blocked by ICI 187,280 and were dependent on ER. Silencing of GPR30 function in these cells had no effect on E2-induced cAMP elevation and ERK activation [77]. Thus, although GPR30 has been found in the vasculature, its role needs further investigation.

ER POST-RECEPTOR SIGNALING PATHWAYS: Age-related decrease in the vascular effects of estrogen may be due to downregulation of post-ER signaling mechanisms. Estrogen has multiple vascular effects including alteration of serum lipid concentrations, coagulation and fibrinolysis, antioxidant properties, and the production of vasoactive molecules. The vascular effects of the estrogen-ER interaction are regulated via both genomic and non-genomic pathways [1,36].

Genomic Effects of Estrogen—Estrogen via ERs regulates transcriptional processes that involve nuclear translocation, binding to specific response elements and regulation of target gene expression [78]. Estrogen affects genes regulating vascular tone, as well as the response to vascular injury and atherosclerosis [79]. Estrogen increases the expression of genes for vasodilatory enzymes such as NOS and prostacyclin synthase. In the absence of estrogen, ER exists as an inactivated monomer bound with HSP90 (Fig 3). Upon binding to estrogen, ER undergoes conformational change resulting in dissociation of HSP90 and formation of a homo- or heterodimer with high affinity for estrogen and DNA [80].

Estrogen-activated ER can bind directly to estrogen response elements (ERE) in the promoters of target genes or interact with other transcription factor complexes like Fos/Jun or SP-1 and influence transcription of genes whose promoters do not harbor EREs [81,82]. Estrogen-dependent activation recruits a variety of coregulators to ER in a complex that alters chromatin structure and facilitates recruitment of the RNA polymerase II transcriptional machinery. Estrogen-independent pathways may also activate ERs [40]. For example, growth factor-induced activation of kinases may phosphorylate and activate ERs or associated coregulators in the absence of estrogen [83].

Nongenomic effects of Estrogen—Nongenomic effects occur too quickly to be mediated by gene transcription, are independent of protein synthesis, and typically involve modulation of membrane bound and cytoplasmic regulatory proteins [78]. Nongenomic effects of estrogen include vasodilation, activation of kinases and phosphatases and changes in ion fluxes across membranes [75,78]. Whether the nongenomic effects involve the nuclear ERs or distinct membrane associated receptors is unclear [36,40]. In several cell types, ERs associate with caveolae and other signaling molecules to trigger G protein-coupled receptor-mediated second messengers and intracellular pathways including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI₃K)/Akt, and activation of ion channels [75,84].

GPR30 is thought to promote rapid estrogen actions [70,85]. Estrogen binding to GPR30 results in the dissociation of Gα-GTPase from the heterotrimeric Gαβγ complex. Dissociated Gβγ-subunit may activate membrane associated matrix metalloproteinases (MMPs) with subsequent transactivation of epidermal growth factor receptor (EGFR) through the cleavage and release of pro-heparan-bound EGF from the cell surface and transient activation of MAPK [86,87]. This is supported by reports that E2 induces the phosphorylation of p38 and p42/44 MAPK (ERK-1/2) as well as proliferation and migration of porcine aortic endothelial cells [88].

Estrogen and the Endothelium

Estrogen affects vascular reactivity and endothelial cell (EC) function [79,89]. Human umbilical vein ECs (HUVECs) express both ERα and ERβ, E2 potentiates endothelium-dependent flow-mediated vasodilatation in the brachial artery of Post-MW [90,91]. Also, endothelium-dependent vascular relaxation is greater in female than male SHR [92]. Selective ERα agonists improve EC dysfunction in blood vessels of OVX female SHR [93]. Also, E2-induced vascular relaxation and NO production are greater in mice expressing only ERα [94, 95]. E2 administration in OVX female mice causes rapid arterial dilation of elastic and muscular arteries, and ER-mediated NO production. E2 causes rapid activation of MAPK/ERK and PI₃K activity in these arteries, and inhibiting either kinase prevented E2-induced vasodilatation. E2-induced Kinase activation and vasodilator responses are absent in both ERα and ERβ knockout mice, implicating both ER subtypes in mediating E2 actions. Thus E2 modulation of arterial tone *in vivo* may involve plasma membrane ER and rapid signaling [96].

In vitro studies also suggest beneficial effects of estrogen on ECs. In small arteries isolated from healthy Post-MW not receiving MHT, the morphology and function of the endothelium are impaired, and these impairments are improved by treating the vessels with E2 [91]. Also, E2 induces the phosphorylation and activation of MAPK, and proliferation of ECs [97]. Estrogen-induced endothelium-dependent vasodilation involves increased release of relaxing factors such as NO, PGI₂ and endothelium-derived hyperpolarizing factor (EDHF), and decreased contracting factors such as endothelin-1 (ET-1) and thromboxane A2 (TXA2) [98].

Estrogen and NO—E2-mediated vasodilation is partly due to increased endothelial eNOS expression and activity [99]. Studies in humans have shown a correlation between NO production and E2 levels [100]. Increased eNOS expression has been demonstrated in the uterine vasculature during pregnancy, suggesting that endothelial-derived NO is involved in the vasorelaxant actions of E2 [101]. Also, NO release is greater in arteries of females than males, and estrogen may mediate the gender differences in NO production [100].

Experimental studies have demonstrated gender differences in NO production. The inhibitory effect of the NOS inhibitor N-Nitro-L-arginine methyl Ester (L-NAME) on acetylcholine (ACh)-induced relaxation is greater in mesenteric artery of female than male rats [102]. Also, in basilar arteries from OVX female rabbit, E2 treatment increases the response to NO in VSM cells [103]. Studies on aortic rings from wild-type mice with trauma hemorrhage have shown increased ET-1 induced vasoconstriction, and treatment with E2 and DPN (ERβ agonist), but not PPT (ERα agonist), counteracted the vasoconstriction [104]. It was concluded that ERβ-mediated NO production attenuates ET-1 mediated vasoconstriction, particularly during trauma hemorrhage. E2 also increases eNOS mRNA expression in cultured ECs [99].

Estrogen has antioxidant properties and increases NO bioavailability [105]. E2 decreases the expression of NADPH oxidase and the generation of superoxide and peroxynitrite. In OVX female rats, the increased blood pressure is associated with low plasma antioxidant levels and increased plasma lipoperoxides and vascular free radicals, and E2 replacement prevents these

effects. Also, the amount of vascular superoxide is less in blood vessels of female compared with male rats [98].

Estrogen and Prostacyclin (PGI₂)—Estrogen may induce EC release of PGI₂, an inhibitor of platelet aggregation and a vasodilator. PGI₂ is produced from free arachidonic acid through the catalytic activity of cyclooxygenase-1 (COX-1) and COX-2. Estrogen may modulate cross-talk between NOS and COX pathways where estrogen-induced NO-mediated vasodilation may cause a decrease in the COX-mediated component [106]. In Post-MW, COX-2 plays a specific role in the rapid E2-induced potentiation of cholinergic vasodilation [107]. Also, in arteries from OVX female monkeys with induced atherosclerosis, the amount of PGI₂ is inversely related to plaque size, and arteries treated with E2 show increased PGI₂ production [108]. E2 stimulates urinary excretion of COX-2-derived PGI₂ metabolites in ER β but not ER α deficient mice, indicating that ER α mediates the effects of estrogen on PGI₂ production [109]. Also, COX inhibitors such as indomethacin inhibit a significant portion of endothelium-dependent vascular relaxation, and gender differences in indomethacin-sensitive vascular relaxation may involve differences in COX products [110]. Other studies have shown that indomethacin does not affect E2-induced relaxation in endothelium-intact coronary artery [111]. Also, in OVX female rats, administration of E2 was associated with COX-2 upregulation in the uterus, but its down-regulation in the vena cava [112]. E2 also caused upregulation of COX-2 in human uterine microvascular ECs, but not in dermal microvascular ECs, suggesting that COX modulation by E2 may be tissue specific [113]. Studies in cultured ECs demonstrated a more positive relation between E2, COX and PGI₂. E2 causes rapid PGI₂ synthesis in ovine fetal pulmonary artery ECs via a Ca²⁺-dependent pathway [114]. PGI₂ production by HUVECs is stimulated by serum from Post-MW treated with phytoestrogens [115]. Also, in cultured HUVECs raloxifene increases PGI₂ synthesis by increasing the expression/activity of COX-1 and -2 [116].

Estrogen and Endothelium-Derived Hyperpolarizing Factor (EDHF)—ECs also release EDHFs that cause relaxation of VSM. E2-ER stimulation increases the production of EDHF, which activates K⁺ channels, causes hyperpolarization, inhibits Ca²⁺ influx and causes VSM relaxation. Ach-induced hyperpolarization and relaxation of mesenteric arteries are less in intact male and OVX female than intact female rats, and the differences are eliminated by K⁺ channel blockers. In mesenteric arteries isolated from OVX female rats, E2 increases EDHF release [117]. Ach-induced vascular hyperpolarization and relaxation is improved in E2-replaced OVX female rats, confirming that E2-deficient states attenuate vascular relaxation by EDHF [118]. Phytoestrogens also induce vascular relaxation through production of EDHF [119].

Estrogen and Endothelium-Derived Contracting factors—ECs release contracting factors such as ET-1. ET-1 stimulates ET_AR and ET_{B2}R in VSM to cause vasoconstriction. ET-1 induces greater contraction in mesenteric arteries of male deoxycorticosterone acetate (DOCA)-salt hypertensive rats than those of females. Ovariectomy is associated with increased ET-1 and ET_{B2}R mRNA in mesenteric arteries, and E2 replacement reverses these effects. Also, the ET_BR agonist IRL-1620 induces less vasoconstriction in mesenteric arteries of intact than OVX females, and E2 supplement decreases IRL-1620-induced vasoconstriction in OVX females, suggesting that E2 attenuates ET-1/ET_BR expression and their vascular responses in DOCA-salt hypertensive rats [120]. ET-1 release is also less in ECs of female than male SHR. Prolonged E2 treatment of ECs inhibits ET-1 production in response to serum, tumor necrosis factor- α , transforming growth factor β 1, and AngII [121,122].

Other endothelium-derived contracting factors such as AngII and TXA₂ may be modulated by estrogen. The Angiotensin Converting Enzyme (ACE) Insertion/Deletion(I/D) polymorphism may be involved in EC dysfunction in Post-MW [123]. Also, basal release of TXA₂ from

platelets is greater in raloxifene- compared to E2-treated OVX pigs. Raloxifene treatment, compared to E2, increases the production of contractile and proaggregatory prostanoids from venous ECs and platelets. If these differences also occur in humans, they may contribute to the thrombotic risk with SERMs compared to natural estrogen [124].

Estrogen and Mechanisms of VSM Contraction

Estrogen causes relaxation of endothelium-denuded vascular segments, suggesting direct effects on VSM [1]. The lack of benefits of MHT in postmenopausal CVD could be due to age-related decrease in the inhibitory effects of E2 on VSM contraction (Table 1). VSM contraction is triggered by increases in $[Ca^{2+}]_i$ due to Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} entry from the extracellular space. Activation of myosin light chain kinase (MLC) contributes to VSM contraction. Also, PKC and Rho-kinase increase the myofilament force sensitivity to $[Ca^{2+}]_i$ and MLC phosphorylation, and enhance VSM contraction [125].

Estrogen and VSM $[Ca^{2+}]_i$ —We have shown that contraction to the α -adrenergic agonist phenylephrine (PHE) is less in aortic strips of intact female than intact male or OVX female Sprague-Dawley rats, suggesting a role of E2 in the reduced vascular response in females [126]. In rat aortic strips incubated in Ca^{2+} -free solution, PHE-induced contraction, a measure of Ca^{2+} release from the intracellular stores, is not different between intact and gonadectomized male and female rats, indicating lack of sex differences in the Ca^{2+} release mechanism. Contraction to membrane depolarization by high KCl, which stimulates Ca^{2+} entry through voltage-gated channels, and the PHE- and KCl-induced $^{45}Ca^{2+}$ influx are less in aortic strips of intact female than intact male or OVX female rats, suggesting an effect of E2 on the expression/permeability of Ca^{2+} channels [126]. The gender differences in vascular contraction may involve direct effects of E2 on VSM. In endothelium-denuded porcine coronary artery and coronary VSMCs, E2 inhibits prostaglandin $F2\alpha$ ($PGF2\alpha$) and KCl-induced contraction and $[Ca^{2+}]_i$, suggesting E2-induced inhibition of Ca^{2+} entry mechanism of coronary VSM contraction [127,128]. E2 also attenuates voltage-dependent Ca^{2+} current in A7r5 VSMCs [129].

If E2 promotes vascular protection, then its inhibitory effects on VSM contraction should be enhanced in CVD such as HTN. In VSMCs of male WKY, PHE causes an initial followed by maintained increase in $[Ca^{2+}]_i$. PHE-induced maintained $[Ca^{2+}]_i$ is greater in SHR than WKY, and the % reduction of PHE-induced $[Ca^{2+}]_i$ in females compared with males is greater in SHR than WKY. These sex differences are related to endogenous E2 because they are eliminated in OVX female rats, and restored in E2-replaced OVX females. E2 also caused greater reduction of PHE- and KCl-induced $[Ca^{2+}]_i$ in VSM cells of OVX female SHR than WKY, suggesting enhanced vascular protective effects of E2 in genetic HTN [130].

Estrogen and Protein Kinase C (PKC)—PKC is a family of Ca^{2+} -dependent and Ca^{2+} -independent isoforms expressed in VSM. During cell activation, PKC activates a cascade of protein kinases that ultimately interact with the VSM contractile myofilaments and enhance contraction [125]. The decreased VSM contraction in female compared with male WKY rats is associated with decreased expression/activity of vascular α -, δ -, and ζ -PKC. E2 replacement in OVX females causes reduction in PKC activity that is greater in SHR than WKY. Thus the sex differences in VSM contraction and PKC activity are possibly mediated by E2 and are enhanced in genetic HTN [131].

Estrogen and Rho-Kinase—In VSM, Rho-kinase inhibits MLC phosphatase and enhances the myofilament force sensitivity to $[Ca^{2+}]_i$. Rho-kinase is upregulated in CVD and may play a role in the pathogenesis of coronary arteriosclerosis and vasospasm [132]. E2 may inhibit Rho-Kinase expression/activity. The vasodilator response to the Rho-kinase inhibitor Y-27632

is similar in OVX female and male rats, and E2 treatment of OVX rats normalizes the vasodilator effects of Y-27632 to those observed in intact females [133]. Long-term inhibition of Rho-kinase *in vivo* causes regression of coronary arteriosclerosis. Also, E2 decreases Rho-kinase expression in cultured human coronary VSM cells [132].

Estrogen and Extracellular Matrix (ECM)—Vascular remodeling occurs during all stages of atherosclerotic progression, and MMPs, a family of zinc-binding proteolytic enzymes, are involved in these processes [134]. MMP activity increases in CVD and cancer, and MMP-induced ECM degradation within the atherosclerotic plaque promotes plaque instability and cardiovascular events. Changes in the levels of MMP-2, -9 and -10 in women receiving MHT may contribute to the potential risk of cardiovascular events and cancer [135]. Also, E2 enhances the release of MMP-2 from human VSMCs [136].

Aging and the Vascular Architecture

The reduced vascular effects of E2 after menopause could be due to age-related structural changes in the blood vessel architecture (Table 1). Vascular aging is associated with changes in the mechanical properties of the vascular wall. Arterial compliance, defined as change in arterial diameter for a given change in pressure, decreases with age [137]. Elastin fibers determine the strength of the vascular wall at lower pressures and collagen fibers bear most of the strength at higher pressures [138]. During vascular aging, there is progressive arterial stiffening and arteriosclerosis due to increased collagen, cross-linking of collagen, elastin fracture, decreased elastin, and calcification. Also, with aging there is impaired endothelial-mediated vasodilation, decreased NO and increased ET-1 production. This favors a procoagulant state and promotes VSM growth. Age-related EC dysfunction and excess deposition of oxidized lipids also promote vascular inflammation and atherosclerosis. Atherosclerosis leads to thick and stiff arterial wall, calcification and plaque formation, and increases the risk of cardiovascular events [138]. Arteries of Post-MW have some degree of atherosclerosis that could impede the vasodilator effects of E2.

The Sex Hormone Environment

The lack of vascular benefits of MHT in postmenopausal CVD could be due to age-related interaction between exogenous E2 and endogenous sex hormones or their precursors (Table 1). Other sex hormones may modify the vascular actions of estrogen, and could have direct effects on the vasculature. The ratio between circulating levels of free E2, free testosterone and SHBG may be more predictive of carotid intimal thickening than the level of any of these hormones alone [139]. Also, the biosynthesis of gonadal steroid hormones allows the inter-conversion of hormone precursors and metabolites (Fig. 1). Administration of an aromatase inhibitor to young men results in decreased endothelial vasodilator function, suggesting that conversion of testosterone to E2 may regulate the peripheral circulation in men [140]. Both aromatase and 5- α -reductase are found in many tissues including blood vessels, and therefore the circulating levels of estrogen and androgens may not reflect their local tissue levels [141]. With the growing use of aromatase and 5- α -reductase inhibitors e.g. in women with history of breast cancer, cardiovascular side effects are predicted [142].

Role of Progesterone—Progesterone is produced by the gonads, adrenal cortex, and the placenta. Like E2, progesterone is synthesized from pregnenolone (Fig. 1). Progesterone receptors have been identified in ECs and VSM of humans, mice, rats, rabbits and primates [143]. Similar to E2, progesterone has anti-atherosclerotic effects, decreases LDL, and increases HDL. Progesterone causes pulmonary vasodilation via endothelium-dependant and -independent pathways. It stimulates eNOS expression, NO production and NO-mediated relaxation in rat aorta and ovine uterine artery. It also causes non-genomic activation of COX and increases vascular PGI₂ production. It inhibits VSM proliferation/migration and facilitates

the inhibitory effects of estrogen. Progesterone also causes rapid relaxation of agonist- or KCl-induced contraction in endothelium-denuded porcine coronary artery [127].

However, progesterone produces less vasorelaxation than estrogen, and may even antagonize the vasoprotective effects of estrogen. Progesterone counteracts E2-induced NO production and vascular relaxation in canine coronary artery. In porcine coronary artery rings, progesterone-induced reduction in NO production is blocked by E2 [144]. Progesterone antagonizes the anti-oxidant effects of E2, and enhances NADPH oxidase expression/activity and the production of reactive oxygen species in OVX mice [145]. Progesterone also promotes upregulation of vascular AT₁R [146,147]. Progestins diminish the anti-inflammatory effects of E2 and its attenuation of ischemic brain injury [148]. Therefore, more research is needed to determine the benefits vs. risk of combined estrogen/progestins in postmenopausal CVD.

Role of Androgens—Androgens may play a role in determining the cardiovascular risk in Post-MW. Androgens are produced in the testis, adrenal glands and ovaries. In men, androgens control the development of sexual characteristics. In women, androgens maintain bone mass and libido. Dehydroepiandrosterone (DHEA), a precursor of sex steroids, and its sulfate ester (DHEAS) are abundant in the human circulation [149]. DHEA and androstenedione do not have significant biological activity, but are converted to testosterone. Testosterone is converted to dihydrotestosterone (DHT), which has higher binding affinity to androgen receptor. Testosterone is also aromatized mainly in adipose tissue to E2.

The observations that men have higher BP than women, and CVD develops at an earlier age in men than in women have led to the suggestion that androgens promote CVD in men [150]. However, serum testosterone levels are lower in men with chronic CVD than in healthy age-matched men [150,151]. Although this suggests that androgens may not mediate CVD, the downregulation of androgen synthesis may be a protective compensatory mechanism that occurs in response to CVD [150]. Experimental studies suggest that androgens may mediate CVD in males. Adult male SHR have higher BP than females, and castration of male rats is associated with reduction in BP to the levels found in females. Also, testosterone treatment of OVX female rats increases BP [152]. Sex differences in BP have also been demonstrated in Dahl salt-sensitive and DOCA-salt treated rats [152,153].

Although testosterone is thought to exert harmful cardiovascular effects, some studies suggest that it may be beneficial. Low circulating testosterone levels in men are positively correlated with risk factors for CAD [154]. Testosterone may have beneficial effects on plasma lipid profile and against atheroma formation [155]. Acute intra-coronary or intravenous infusion of testosterone rapidly improves myocardial ischemia in men [156,157]. Also, testosterone causes direct vasodilation and decreases Ca²⁺ influx in porcine coronary artery [127].

Serum testosterone level is markedly lower in women than in men, but its level after menopause is unclear [152]. Serial measurements of sex hormones in Post-MW for 10 years after cessation of cycling showed age-related increase in serum testosterone and androstenedione and decrease in E2 and dihydrotestosterone [158]. In cross-sectional studies of women in the Rancho Bernardo cohort, serum testosterone decreased immediately after menopause, but then increased with age, reaching Pre-MW levels at 70-79 years of age. Also, in women with surgical menopause serum testosterone levels did not increase with age, but were 40-50% lower than in women with natural menopause [159]. These data suggest that natural postmenopause is a relatively hyperandrogenic state [152,160].

Lessons from MHT RCTs

The results of HERS and WHI RCTs challenged the concept that MHT has cardiovascular benefits. The unexpected outcome of RCTs could be related to the participants' age, the time

of starting MHT, preexisting CVD, socioeconomic status, type of estrogen, and amount of progestins in MHT. In the NHS, which showed protective effects of MHT, ~80% of women initiated MHT within 2 years of menopause [161]. In contrast, in WHI and HERS women were on average 63 and 67 years of age, ~10 years postmenopausal. Even younger healthy participants in WHI aged 50-59 had been menopausal ~6 years before using MHT. Further, the WHI did not indicate the subjects' age at menopause, which may be indicative of the age-related changes in cardiovascular status [4]. Experimental studies have shown that estrogen reduces early atherogenesis but augments it in later stages [162], supporting the concept that the timing of MHT may be essential for successful outcome in postmenopausal CVD.

It is important to note that the participants in HERS had CVD at baseline. Also, while WHI was a primary prevention RCT in "healthy" women, 36% of the women that received MHT had HTN, 49% were current or past smokers, and 34% were obese, suggesting that they may have had an active atherosclerosis process. Because atherosclerosis and vascular remodeling advance with age, a delay in MHT by even a few years may influence the outcome [4]. Early MHT administration caused a 70% protection in OVX primates on an atherosclerotic diet, whereas delay in therapy until after development of moderate atherosclerosis resulted in only 50% protection, suggesting that MHT may only afford primary prevention. In primates that had received an atherosclerotic diet for 2 years before initiating MHT, MHT did not protect against atherosclerosis [163]. Also, administration of E2 before and during, but not 7 days after, balloon injury resulted in inhibition of neointima formation in rats [164]. Furthermore, delayed administration of E2 failed to prevent neointima formation in rabbits [165]. Thus, a 6-year delay in MHT may be sufficient to reduce its protective effects.

The subjects' socioeconomic status may have also affected the MHT results. MHT users are often more educated, and have healthier lifestyle and fewer cardiovascular risk factors. A meta-analysis showed that the previously observed reduced risk for CAD among MHT users was lost when the analysis included socioeconomic status [4].

Although the levels of E2 diminish dramatically after menopause, the levels of E1 remain unchanged. Thus the type of estrogen in MHT needs to be carefully examined. HERS and WHI used CEE, a mixture extracted from the urine of pregnant horses. The main active ingredients in CEE include sodium estrone sulfate, sodium equilin sulfate, and sodium 17 α -dihydroequilenin. Thus contrary to the nomenclature, CEE does not actually replace E2. Also, compared with E2, estrogens in CEE may have different binding affinity, selectivity, ER agonistic properties and metabolic products [166]. Because both ER-dependent and -independent mechanisms mediate the cardiovascular actions of E2, CEE and other estrogens may not mimic the cardiovascular protective effects of E2, and may even induce deleterious effects. In human aortic VSMCs, CEE is less potent than E2 in inhibiting mitogen-induced VSM growth and MAPK activity [166]. Because abnormal VSMC growth plays a role in CHD, lack of antiproliferative effects of CEE may explain the negative outcome of HERS and WHI. Also, in a primate model of balloon injury, administration of CEE had no effect on intimal hyperplasia [167]. In contrast, in the Estrogen in the Prevention of Atherosclerosis Trial (EPAT), administration of E2 to Post-MW without CVD reduced the progression of intimal thickening [2]. These data suggest that the use of estrogens other than E2 may contribute to the lack of protective actions of MHT.

In women with intact uterus, estrogen is given in combination with a progestin in order to reduce the risk of endometrial cancer. While the negative findings of HERS and the E2+MPA arm of WHI may be caused by MPA, in the E2 alone arm of WHI, no protective effects were observed even though lipids were favorably changed. Also, the NHS demonstrated a similar risk reduction for CHD among women taking CEE alone or CEE+MPA. However, there was an increase in stroke risk in women taking CEE+MPA versus women never using MHT

[168]. In the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial, CEE caused beneficial effects on LDL and HDL levels that were attenuated by MPA [4]. Also, CEE-induced brachial artery dilatation is attenuated by MPA. However, other studies did not find any attenuation of CEE-induced dilatation by MPA or micronized progesterone [169,170].

Also, experimental studies did not demonstrate consistent effects of progestins. In cynomolgus monkeys, chronic administration of E2 or E2+progesterone had similar anti-atherosclerotic effects [4]. In postmenopausal monkeys a 72% reduction in coronary artery atherosclerosis was observed, and these anti-atherosclerotic effects were not demonstrated in monkeys receiving CEE+MPA [171]. Also, in menopausal cynomolgus monkeys MPA appears to abrogate the vascular benefits of estrogen [172]. Treatment of surgically postmenopausal OVX female monkeys with CEE alone, but not in combination with MPA, inhibits aortic connective tissue remodeling after plasma lipid lowering [173]. Furthermore, MPA antagonizes the inhibitory effects of CEE on coronary artery atherosclerosis [174]. Ach caused vasoconstrictor responses in estrogen-deficient monkeys not receiving MHT, but a vasodilatory response in monkeys treated with estrogen alone, and co-administration of MPA reduced the beneficial effect of estrogen by 50% [172]. MPA abrogated the ability of E2 to attenuate balloon injury-induced intimal thickening in rats, a process independent of lipids [175]. Also, progesterone and MPA inhibit mitogen-induced proliferation of VSM cells in culture. These data suggest that MPA may block the protective actions of E2 on vascular cells. In contrast, in rabbits the protective actions of CEE or E2 on atherosclerosis were not prevented by MPA, or other progestins such as norethindrone acetate and hydroxyprogesterone caproate [4]. Thus, it may be difficult to conclude that MPA was responsible for the lack of protective effects of MHT observed in RCTs [4].

Another important distinction between E2 and CEE is the route of administration. Oral, but not transdermal estrogen increases C-reactive protein and IL-6 levels [176,177]. Because CEE is given orally, whereas E2 is often administered transdermally, the difference in their effects may be related in part to the route of administration.

Data from new clinical trials suggest that starting MHT within a few years of menopause may reduce atherosclerosis progression, CHD events and total mortality. The Kronos Early Estrogen Prevention Study (KEEPS) would investigate the effects of MHT on carotid intima-media thickness (a measure of atherosclerosis) and the accrual of coronary calcium in early menopausal women aged 42-58 years at 6 to 36 months postmenopause [178]. Participants are given either low dose oral CEE or weekly transdermal E2 (both in combination with cyclic oral, micronized progesterone) or placebo 12 days per month. The Early versus Late Intervention Trial with Estradiol (ELITE) will randomize women, either less than 6 years (early) or more than 10 years (late) postmenopausal, to receive oral E2 for 2-5 years, and the primary endpoint will also be change in carotid intima-media thickness. Because the early-start subjects of ELITE are roughly comparable with the KEEPS subjects, and because the studies' primary endpoint is the same, pooling of results of the two studies may clarify possible differences in atherosclerosis effects between oral E2 and oral CEE, and between oral and transdermal E2 [36,162].

Perspective

While MHT reduces vasomotor menopausal symptoms such as hot flashes and night sweats, its usefulness in postmenopausal CVD remains unclear. Variables in the design of HERS and WHI may have contributed to the unanticipated results. The failure of the beneficial effects of estrogen on vasculature to materialize in RCTs is also likely due to age-related changes in ER amount, distribution, integrity, and downstream signaling mechanisms as well as structural changes in the blood vessel architecture. These changes may be attributed to age-related ER gene mutations or methylation. Investigation of the structure-activity relationship of estrogenic

compounds would enhance our understanding of the molecular interactions of estrogen and ER. Further characterization of currently available estrogens and the development of novel estrogenic compounds would provide more efficient and specific ER modulators. Also, the potential benefits/risks of multiple sex hormone therapy and modulators of progesterone and androgen receptors should be examined. As newer forms of MHT become available, and if used at the right dose, route of administration and timing depending on the subjects' age and preexisting cardiovascular condition, the beneficial vascular effects of estrogen could be translated into the outcome of MHT in post-menopausal CVD,

Acknowledgments

This work was supported by grants from The National Heart, Lung, and Blood Institute (HL-65998 and HL-70659), and The Eunice Kennedy Shriver National Institute of Child Health and Human Development (HD-60702).

List of Abbreviations

CVD	cardiovascular disease
E2	17 β -estradiol
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
HERS	Heart and Estrogen/progestin Replacement Study
HTN	hypertension
KEEPS	Kronos Early Estrogen Prevention Study
MAPK	mitogen-activated protein kinase
MHT	menopausal hormone therapy
MMP	matrix metalloproteinase
MPA	medroxyprogesterone acetate
NHS	Nurses' Health Study
NO	nitric oxide
OVX	ovariectomized
Post-MW	postmenopausal women
Pre-MW	premenopausal women
SHBG	sex hormone-binding globulin
SHR	spontaneously hypertensive rat
VSM	vascular smooth muscle
WHI	Women's Health Initiative

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Fig. 1.

Biosynthesis of sex hormones. In premenopausal women, the ovary is the principal source of circulating E2. First, cholesterol is transferred from the cytosol to the inner membrane of the mitochondrion, where it is converted to pregnenolone. Side chain cleavage by delta-4 or delta-5 isomerase leads to the formation of androstenedione. Androstenedione is either directly “aromatized” to E1, then converted to E2, or first converted to testosterone, then to E2 by aromatase. After menopause, circulating androstenedione, testosterone and E1 become the major precursor substrates of E2 production in peripheral estrogen target tissues.

Fig. 2.

Primary Structure of ERα, ERβ and GPR30. ERα and ERβ have five functional domains termed A/B, C, D, E and F. Domain A/B contains the activation function-1 (AF-1). Domain C is the DNA binding domain (DBD). Domain D is the hinge domain. Domain E contains the ligand binding cavity (LBD) and AF-2. Domain F contains co-factor recruitment regions. GPR30 is a G-coupled protein receptor that shares little homology with the classical ERs, and consists of an N-terminal domain, a 7- transmembrane domain and a C-terminal domain.

Fig. 3.

Estrogen mediated pathways of vascular relaxation. Estrogen binds to endothelial ER and activates phospholipase C (PLC), leading to the generation of inositol 1,4,5- triphosphate (IP $_3$). IP $_3$ stimulates Ca $^{2+}$ release from the endoplasmic reticulum, followed by influx of extracellular Ca $^{2+}$. Ca $^{2+}$ binds calmodulin (CAM) and promotes dissociation of eNOS from caveolin-1. Estrogen/ER-mediated activation of Akt or MAPK causes eNOS phosphorylation and translocation to the cell membrane where it undergoes myristoylation, palmitoylation and full activation. Activated eNOS transforms L-arginine to L-citrulline, stimulates NO production and promotes VSM relaxation. Estrogen also activates COX-1 to produce prostacyclin (PGI $_2$), and induces the release of EDHF. Estrogen may also bind to plasma membrane ER in VSM. Estrogen activates K $^+$ channels, leading to membrane hyperpolarization, inhibition of Ca $^{2+}$ influx, and inhibition of Ca $^{2+}$ -dependent MLC phosphorylation and VSM contraction. Estrogen may also inhibit PKC and Rho kinase Ca $^{2+}$ sensitization pathways of VSM contraction.

Table 1

Potential Causes of Decreased Estrogen Responses in Senescent Blood Vessels.

Potential Cause	Age-Related Change
Endogenous Estrogen Levels	↓
Ineffective MHT	Large Dose Route (Transdermal vs Oral) Delayed Timing after Menopause Long Duration
E2 Metabolism	↑ E2 Metabolism Inactive Metabolites
ER Amount	↓ Expression Promoter Methylation
ER Integrity	↓ Functional ER Degradation
ER subtypes ER α , ER β , GPR30	Inactive Isoforms Inaccessible Subcellular Distribution
ER Binding	↓ Binding Defective LBD
Post-ER signaling	↓ Signaling ↓ Genomic and Non-genomic Response
Endothelial Cell Signaling	- ↓ NO, PGI ₂ , EDHF - ↑ Reactive Oxygen Species - ↑ ET-1, TXA ₂
VSM Signaling	- ↑ Ca ²⁺ , PKC, Rho-kinase
Extracellular Matrix	Accumulation, Remodeling
Vascular Architecture	- Inflammation - Atherosclerosis
Sex Hormone Environment	Effect of Progesterone Effect of Testosterone

Table 2

Representative Agonists and Antagonists of Vascular Estrogen Receptors

ER Agonists	Phytoestrogens	SERMS	ER Antagonists
Natural Estradiol Estrone Estrilol Conjugated Conjugated equine estrogen (CEE) Semi-Synthetic Ethinylestradiol (ER α) Synthetic Diethylstilbestrol Dienestrol Fosfestrol Mestranol Quinestrol ER Subtype Specific Propylpyrazole trispherol (PPT) (ER α) Diarylpropionitrile (DPN) (ER β)	Isoflavones Genistein, Genistin (ER β) Daidzein, Daidzin Glycitein, Glycitin Formononetin Biochanin A (ER β) Coumestans Coumestrol 4-Methoxycoumestrol Flavonoids - Flavones Apigenin, Baicalein Chrysin, Norwogonin - Flavonols Fisetin, Kaempferol Quercetin - Flavanones Naringenin 8-Prenyl naringenin Pinocembrin Silybin, Taxifolin Stilbenes <i>trans</i> resveratrol Pterostilbene Lignans Matairesinol (Enterolactone) Secoisolariciresinol (Enterodiol)	Tamoxifen Raloxifene Idoxifene Bazedoxifene Clomifene Toremifene	MPP (ER α) RR-tetrahydrochrysene (ER β)

Table 3

Representative Estrogens and Estrogen-Progestins Used for MHT

Route	Compound	Commercial Name
ORAL Tablets	CEE	Premarin®
	CEE/MPA	Prempro™
	Estradiol - Drospirenone	Angeliq®
	Synthetic conjugated estrogens	Enjuvia™
	Norethindrone acetate/ethinyl estradiol	Femhrt®
	Estradiol/norgestimate	Prefest™
TRANSDERMAL	Estradiol	Alora®, Estraderm®, Menostar®, Vivelle®, Vivelle-Dot®
	Estradiol/Levonorgestrel	Climara Pro™
	Estradiol/norethindrone acetate	CombiPatch®
TOPICAL EMULSION	Estradiol	Estrasorb™
INJECTION	CEE	Premarin®
	Estradiol valerate	Delestrogen®
VAGINAL	CEE	Premarin®
	Estradiol acetate	Femring®