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Sex Hormones and Vascular Function

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1. Introduction

The relationship between sex hormones and cardiovascular function and disease has long been recognized. As early as the 1950s, researchers concluded that although levels of cholesterol played a major role in the development of cardiovascular disease (CVD), other factors, including gender and hormones, played a role as well. (Anonymous, 1958). Since that time, despite extensive research focusing on the effects of estrogen on vascular function, the relationship remains poorly understood. Furthermore, clinical treatment of postmenopausal women with hormone replacement therapy (HRT) continues to be controversial due to conflicting findings in clinical trials.

Until the 1990s, extensive observational data suggested that HRT was cardioprotective. However, results from the Heart and Estrogen/Progestin Replacement Study (HERS-I and –II) did not confirm a protective effect of HRT on the heart. (Hulley et al 1996; Grady et al 2002). Later, data from the Women's Health Initiative (WHI) reported an increase in coronary heart disease (CHD) risk in women treated with combined estrogen-progestin compared to placebo, while the WHI unopposed-estrogen arm showed no increase in CHD events (Roussow et al, 2002; Anderson et al, 2004). Since release of the WHI, follow-up analyses have shown that the timing of initiation of HRT makes a difference in outcomes. These analyses showed that younger postmenopausal women who initiate therapy at the time of menopause are not at increased risk of CHD events compared to women who initiate therapy at a later age.

In this chapter, we will discuss the pathophysiologic effects of sex hormones on the vasculature, describe both clinical and basic research that has led us to our current understanding, and conclude with future perspectives on avenues of investigation that may lead to innovative treatments for postmenopausal women.

2. Physiology of estrogen actions

2.1 Estrogen metabolism

Estrogen is a steroid hormone that is produced by aromatization of androgen precursors, specifically androstenedione. (Speroff and Fritz, 2005) Estrogens are synthesized primarily in the ovary, with minor contribution from adipose, skin, muscle, and endometrial tissue. In premenopausal women, the primary form of estrogen is 17β-estradiol, often simply referred to as estradiol or E2 (for the two hydroxyl groups located on the basic estrogen ring

structure). (Speroff and Fritz, 2005) Estradiol is the form of estrogen used in most pre-clinical studies, and will be abbreviated as 'E2' in this chapter. Clinical studies, particularly studies of HRT, have employed a variety of naturally occurring or synthetic estrogens, which will be identified specifically in the text. Other forms of estrogen include estrone and estriol. Estrone, like estradiol, is produced by aromatization of androstenedione, and is the primary estrogen in postmenopausal women. Estriol is a peripheral metabolite of estrone and estradiol and is not secreted by the ovary. Estriol is the dominant form of estrogen in pregnant women. (Speroff and Fritz, 2005)
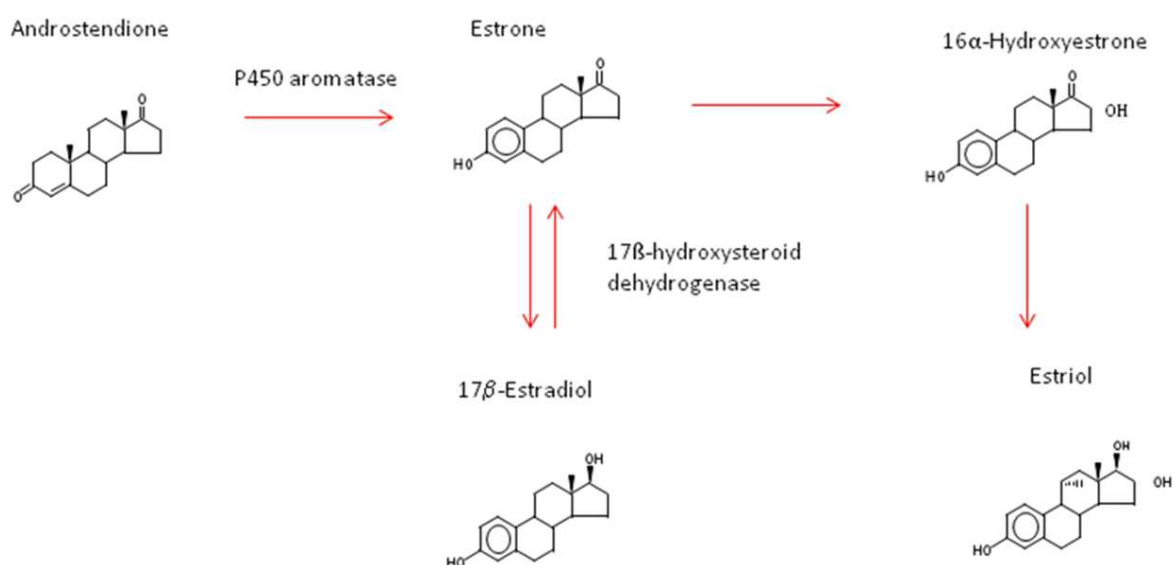

Fig. 1. Estrogen Metabolism (modified from Science Slides Suite © 2010)

The majority of circulating estrogens are bound to carrier proteins, including albumin and sex hormone-binding globulin (SHBG). Albumin binds 30 percent of circulating estrogen and SHBG binds another 69 percent. (Speroff and Fritz, 2005) Only the remaining 1 percent of estrogen that is not protein bound is physiologically active.

2.2 Estrogen receptors

Estrogens act on specific estrogen receptors (ERs) that are differentially expressed in various tissues. There are at least three, and possibly four distinct estrogen receptors. Two of these are the classic ERs: ERα and ER-β. Other ERs include the more recently discovered G protein-coupled receptor (GPER, GPR30, and a putative receptor (ER-X), that has been studied mainly in brain. (Miller et al, 2008)

ERα and ERβ are members of the nuclear steroid hormone receptor superfamily and function as ligand-activated transcription factors. (Speroff and Fritz, 2005) They are expressed in the vasculature and play a role in mediating/modulating responses to vascular injury. Once an estrogen ligand binds to its receptor, the receptor undergoes a conformational change that leads to downstream events in the nucleus, activating or inactivating transcription factors that lead to alterations in gene expression. The conformational plasticity of the ERs is a major reason that estrogen is able to have a variety of agonist/antagonist effects in a given cell or tissue. (Speroff and Fritz, 2005) ERα and ERβ play a pivotal role in vascular remodeling in response to vascular injury.

GPER (GPR30) is an intracellular transmembrane ER that initiates many rapid non-genomic signaling events, including intracellular calcium mobilization and synthesis of phosphatidyl-inositol 3,4,5-triphosphate in the nucleus of multiple cell types. (Revankar et al, 2005). GPER has been indentified in human internal mammary arteries, saphenous veins, and contributes to vasorelaxation in arteries, although this mechanism remains to be fully understood (Haas et al, 2009)

Estrogen acts on various cell types through both genomic and non–genomic mechanisms. Genomic effects occur when estrogen binds to ERs in target tissue cell nuclei, resulting in changes in gene expression. Multiple genes in both the nuclear and mitochondrial genomes are regulated by ERα and ER-β. (O'Lone et al, 2007) In aortic smooth muscle cells and endothelial cells of wild-type ovariectomized mice, E2 treatment resulted in both up- and down-regulation of multiple genes involved in mitochondrial fuction. ERα upregulated four clusters of genes, while ERβ downregulated a different set of mitochondrial genes. E2 also stimulates oxidative phosphorylation and inhibits production of superoxide and other reactive oxygen species in mitochondria. (O'Lone et al, 2007) It is hypothesized that this mechanism decreases the rate of accumulation of mitochondrial DNA mutations over a lifespan, and therefore protects against age-related disease. This notion is relevant to the development of CVD and timing of HRT initiation. (O'Lone et al, 2007)

Estrogen can also trigger non-genomic events by binding to targets other than nuclear receptors, eg., cell membrane ERs. (Kelly and Levin, 2001) Non-genomic effects, such as direct activation of intracellular signaling pathways, can be rapid and do not require changes in gene expression, although the long-term consequences include altered transcription of targeted genes.

3. Physiology of progesterone actions

3.1 Progesterone metabolism and progesterone receptors

Progesterone is a steroid hormone that is synthesized from the precursor pregnenolone (a cholesterol metabolite) by 3β-hydroxysteroid dehydrogenase in the ovaries and adrenal glands. (Figure 2)
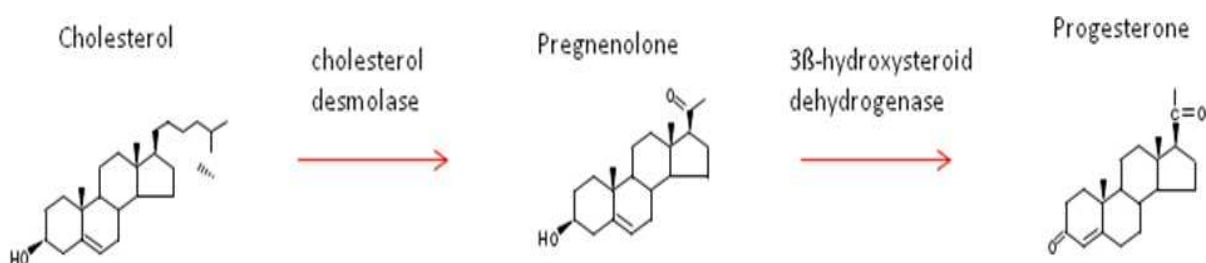

Fig. 2. Progesterone Metabolism ((modified from Science Slides Suite © 2010))

Progesterone acts on two major progesterone receptors (PRs), PR-A and PR-B. (Speroff and Fritz, 2005) While the role of ERs in vascular physiology and pathophysiology is well studied, the literature on PRs is limited and most of what is known about their biological function is derived from studies of reproductive tissues. PR-A and PR-B can form homodimers (AA and BB) or heterodimers (AB) upon binding to a progestin. Downstream effects include protein phosphorylation and modulation of gene transcription. (Speroff and Fritz, 2005) Based on in-vitro studies of endometrium and breast tissue, PR-B is a positive

regulator of progesterone-responsive genes, whereas PR-A activation inhibits PR-B activity. Like estrogen, progesterone has both genomic and non-genomic effects, binding both nuclear and cell membrane receptors.

4. Physiologic effects of sex hormones on the vasculature

4.1 Estrogen effects on vascular reactivity

E2 has rapid non-genomic actions on the arterial wall, resulting in vasodilation. Administration of E2 to ovariectomized ewes results in rapid uterine vasodilation, leading to a rise in uterine blood flow within 30 to 45 min (Killam et al, 1973). This rise in uterine blood flow is partially mediated by ER activation and release of nitric oxide (NO), as shown by local infusion of the nonselective ER blocker ICI 182,780 or the NO synthase blocker L-NAME, respectively, into the main uterine artery of nonpregnant ewes (Van Buren et al, 1992) A better understanding of the mechanism(s) by which E2 increases NO production in the vasculature comes from in vitro experiments with cultured endothelial cells. E2 stimulates eNOS activity via an ERα-mediated process in endothelial cells (Chen et al, 1999). ERα and ERβ are present on the endothelial cell membrane and are expressed in a wide range of blood vessels from different vascular beds and species (Andersson et al, 2001). The ERα and eNOS proteins are organized into a functional signaling module in caveolae located on endothelial cell membranes (Chambliss et al, 2000). The role of the ERβ in vasodilation is less clear, but studies from ERβ knockout mice have shown an inhibitory role of ERβ in ERα-mediated NO relaxation (Petterson et al, 2000).

GPER is a seven-transmembrane G protein-coupled ER that has only recently been shown to play a role in the vasculature (Haas et al, 2007). Isoflavones, natural estrogenic compounds (phytoestrogens) found in soy products, e.g. genistein and dadein, and selective ER modulators (SERMs), e.g. tamoxifen and raloxifene, bind to GPER (Filardo et al, 2000). Selective stimulation of GPER by intravenous infusion of the GPER agonist G-1 results in an acute reduction in blood pressure in rats (Haas et al, 2009). G-1 relaxes ex vivo rat and human arteries via an endothelium-dependent and L-NAME-sensitive mechanism (Haas et al, 2009). It is uncertain whether E_2-induced relaxing responses are mediated via GPER or whether crosstalk between ERα/ERβ and GPER exists. Selective GPER antagonists like G-15 (Dennis et al, 2009) might unravel a role for GPER-dependent vasorelaxation upon E2 signaling in the vasculature.

E2 results in vasorelaxation even in the absence of a functional endothelium (Jiang et al. 1991), due primarily to Ca^{2+}-antagonistic effects in smooth muscle cells. E2 inhibits voltage-dependent calcium inward currents on smooth muscle cells, but not on endothelial cells (Shan et al, 1994; Kitazawa et al, 1997). This leads to a reduction in intracellular Ca^{2+} concentration and lower Ca^{2+}-calmodulin-dependent myosin light chain phosphorylation and contraction (Somlyo and Somlyo, 1994). In addition to these Ca^{2+}-antagonistic effects on smooth muscle cells, a variety of endothelium-independent mechanisms have been proposed to account for E_2-induced vasodilation. E2 has been reported to increase cAMP and cGMP levels in the vasculature, thus suggesting a cyclic nucleotide-dependent mechanism of relaxation (Kuehl et al, 1974). For instance, in the porcine coronary artery, E2 causes relaxation via protein kinase G activation and cAMP-dependent opening of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) (Rosenfeld et al, 2000). In human coronary artery smooth muscle cells, E2 has been shown to open BK_{Ca}

channels by stimulating neuronal NOS via a signal pathway involving PI3-kinase and Akt (Han et al, 2007).

In summary, E2 at pharmacological concentrations causes vasorelaxation via a combination of endothelium-dependent, ER-mediated actions and contraction-modulating effects at the level of the smooth muscle cell. The E_2-induced relaxing profile in a specific vascular bed depends on the species, gender, expression patterns and degree of dimerization and crosstalk between the ER subtypes.

4.2 Sex hormone effects on blood pressure
4.2.1 Estrogen effects on blood pressure

Endogenous E_2 lowers BP. Observational studies have demonstrated that BP is lower when E_2 levels peak during the luteal phase than when they are at their nadir during the follicular phase of the menstrual cycle (Dunne et al, 1991; Karpanou et al, 1993; Chapman et al, 1997). Menopause is associated with a significant increase in BP in cross-sectional studies (Staessen et al, 1998). In a prospective study of BP in premenopausal, perimenopausal, and postmenopausal women, an age-independent 4-5 mmHg increase in systolic BP was found in postmenopausal women (Staessen et al, 1997). Further, BP is reduced when endogenous E_2 levels are elevated during pregnancy (Siamopoulos et al, 1996). Data on the BP effects of estrogen replacement therapy (ERT) in menopausal women have been inconsistent, with reports of BP neutral (PEPI Trial Writing Group, 1995), BP lowering (Mercuro et al, 1997; Mercuro et al, 1998; Cagnacci et al, 1999; Seely et al, 1999; Butkevich et al, 2000) and BP elevating effects (Anderson et al, 2004; Wassertheil-Smoller et al, 2000). In the Postmenopausal Estrogen/Progestin interventions (PEPI) trial, which enrolled 875 healthy normotensive early postmenopausal women, assignment to conjugated equine estrogens (CEE), 0.625 mg/d ± a progestin did not impact systolic or diastolic BP when compared with placebo controls (PEPI Trial Writing Group, 1995). In contrast, when transdermal E_2 was administered at physiologic doses to healthy postmenopausal women in two studies that evaluated ambulatory BP, active treatment significantly lowered nocturnal systolic, diastolic and mean BP by 3-7 mmHg compared with placebo (Cagnacci et al, 1999; Seely et al, 1999). The observational study component of the WHI (WHI-OS) collected data on risk factors for CVD, including BP, from 98,705 women aged 50-79 yr, the largest multiethnic, best characterized cohort of postmenopausal women ever studied (Wassertheil-Smoller et al, 2000). WHI-OS found that current HRT use was associated with a 25% greater likelihood of having hypertension compared with past use or no prior use. Further, among 5310 postmenopausal women randomized to CEE (0.625 mg/d) alone compared to a placebo group as part of the randomized controlled trial component of WHI, there was a 1.1-mmHg increase from baseline in systolic BP that persisted throughout the 6.8 yr of follow up (Anderson et al, 2004). There was no difference in diastolic BP between treatment groups.

4.2.2 Progestin effects on blood pressure

Similar to estrogens, the effects of progestins on BP are dependent on the type of progestin. Natural progesterone has been associated with BP lowering or neutral effects. Higher levels of progesterone correlate with lower systolic but not diastolic BP during the second and third trimesters of pregnancy (Kristiansson et al, 2001). In a crossover study of 15 postmenopausal women assigned to placebo or transdermal E_2 ± intravaginal progesterone,

addition of progesterone did not affect the nocturnal BP lowering seen with E2 compared with placebo (Seely et al, 1999). Similarly, medroxyprogesterone acetate (MPA) appears to have BP neutral or lowering effects. In a double-blind, crossover study of postmenopausal women assigned to CEE and placebo or increasing doses of MPA, there was a dose-dependent decrease in ambulatory daytime diastolic and mean BPs for women assigned to the progestin compared with placebo (Harvey et al, 2001). In contrast, most studies of synthetic progestins for contraception or hormone therapy have revealed a BP-elevating effect. Oral contraceptives in particular appear to precipitate or accelerate hypertension (Rosenthal et al, 2000).

4.3 Estrogen effects on lipoproteins

E2 also affects serum lipoprotein levels and the interaction of lipoproteins with cellular elements in the vessel wall. E2 has been shown to protect against atherosclerotic lesion formation in multiple animal models. In primate models, E2 results in up to a 66 percent decrease in aortic atherosclerotic plaque size. (Bjarnson et al, 1997) Mouse models have been widely used to study atherosclerosis because of the ability to easily inactivate targeted genes coding for apolipoprotein E (*Apoe*) and the LDL receptor (*Ldlr*) which lead to spontaneous development of atherosclerosis. E2 prevents both initiation and progression of atherosclerotic plaque development in these models. Using subcutaneous implanted E2-releasing pellets to achieve physiologic serum levels, atherosclerotic plaques did not progress beyond the fatty streak stage in apolipoprotein E-deficient mice. (Elhage et al, 1997) Similarly, E2 has been shown to reduce atherosclerotic lesion size in male *Apoe-/-* mice. (Tse et al, 1999) Treatment of minimally-oxidized LDL with E2 leads to decreased cytotoxicity in cultured endothelial cells. (Negre-Salvayre et al, 1993) E2 also inhibits LDL oxidation and decreases formation of cholesterol esters. (Huber et al, 1990)

E2 effects on lipoproteins and atherosclerosis are mediated by both ERα and ERβ. When E2-treated mice that were deficient in ApoE alone were compared to mice that were deficient in both ApoE and ERα, E2 reduced atherosclerotic plaque size in *Apoe-/-* mice by 80%. This effect was not seen in the *Apoe-/- , ERα-/-* mice, indicating that ERα plays a critical role in prevention of aterhosclerosis in this model. (Hodgin et al, 2001)

Clinical studies have shown reductions in serum lipoproteins following oral estrogen replacement therapy. One study randomized women to treatment with CEE (Premarin 0.625 mg) daily versus placebo. (Walsh et al, 1991) The estrogen treatment group had a 15% reduction in serum concentrations of LDL cholesterol and a 16% increase in high density lipoprotein (HDL) cholesterol. Triglyceride levels increased by 24%. These results were consistent across the age spectrum; even women in their 8th decade of life showed similar changes in serum cholesterol levels. Oral estrogens facilitate postprandial clearance of atherogenic lipoproteins and increase serum HDL levels, specifically HDL_2, which may play a major role in reduction of atherogenesis. Oral CEEs appear to increase HDL levels to a greater degree than oral E2. Triglyceride levels are increased with administration of both oral CEE and oral E2, though to a lesser extent by oral E2.

Transdermal E2 formulations also decrease LDL levels, but to a lesser extent than oral preparations. (Stevenson et al 2009) Transdermal estrogens have not been shown to alter postprandial lipoprotein clearance or circulating HDL levels, but may lower triglyceride levels. (Godsland et al, 2001)

4.4 Estrogen effects on C-reactive protein

C-reactive protein (CRP) is an acute phase reactant that has been shown to be both a marker and a mediator of vascular disease. There is an E2-dependent sexual dimorphism in expression of human CRP in experimental models, i.e., the transgenic mouse expressing human CRP (CRPtg) (Szalai et al 1997, 1998, 2002) and in some human populations (Yamada et al, 2001). E2 treatment of male CRPtg can lower baseline CRP levels and removal of E2 can restore its high baseline expression. (Szalai et al, 1998) In postmenopausal women, oral CEE increases baseline CRP levels, but low dose oral or transdermal E2 supplementation does not affect CRP. (Cushman et al, 1999; Vongpatanasin et al, 2003; Lakoski et al, 2005; Mosca et al, 2004) This HRT-induced CRP increase occurs without a significant change in IL-6 or TNF-α, major regulators of CRP under inflammatory conditions, suggesting that the effects of menopausal hormones on CRP do not reflect a generalized inflammatory state. (Vongpatanasin et al, 2003; Mosca et al, 2004) Data from the WHI and the Women's Health Study have demonstrated that CRP predicts CVD risk in post-menopausal women independent of HRT. (Kurtz et al, 2011) HRT use had less predictive value than CRP levels in these studies. Thus, the clinical significance of hormone-related changes in circulating CRP levels remains uncertain.

5. Estrogen effects on inflammation and vascular pathology

5.1 Estrogen modulates pro-inflammatory mediator expression after vascular injury

Inflammation plays a critical role in the pathogenesis of atherosclerosis and subsequent CVD. (Hansson et al, 2005) The process is initiated by activation of endothelial cells due to deposition of lipoproteins, pressure overload, and/or hyperglycemia, leading to increased expression of adhesion molecules (including selectin, vascular cell adhesion molecule 1 [VCAM-1], and intercellular adhesion molecule 1 [ICAM-1]). These molecules cause circulating leukocytes to bind to vascular endothelial cells and release pro-inflammatory cytokines and growth factors. The bound leukocytes then infiltrate the vascular smooth muscle cells layer, leading to a cascade of cytokine secretion, further contributing to the local inflammatory environment within the vessel.

Based on extensive studies using the rat carotid injury model, E2 has been shown to be a negative modulator of injury-induced vascular inflammation and neointima formation. (Bakir et al, 2000; Miller et al, 2004; Xing et al, 2004)   There is a sexual dimorphism in the response to vascular injury, with males demonstrating increased neointima formation compared to females. (Chen et al 1996, 1998; Levine et al 1996; Miller et al, 2004) This sexual dimorphism is E2-dependent, based on evidence that physiologic levels of circulating E2 (40-60 pg/ml) decrease neointima formation in both male and female gonadectomized animals. Furthermore, addition of MPA, the progestin used in the Women's Health Initiative, opposes the effects of E2 on injury-induced vascular inflammation and neointima formation. (Levine et al, 1996)

E2 modulates the vascular response to injury by reducing local expression of inflammatory mediators and influx of leukocytes into balloon-injured carotid arteries of ovariectomized rats. (Miller et al, 2004; Xing et al, 2004) In particular, E2 decreases expression of cytokine-induced neutrophil chemoattractant (CINC-2β), a chemoattractant for neutrophils and monocyte chemoattractant protein (MCP-1) in injured arteries. (Xing et al, 2004) This results in significant reductions in influx of these inflammatory leukocyte subtypes, limiting the injury response. (Figure 3)
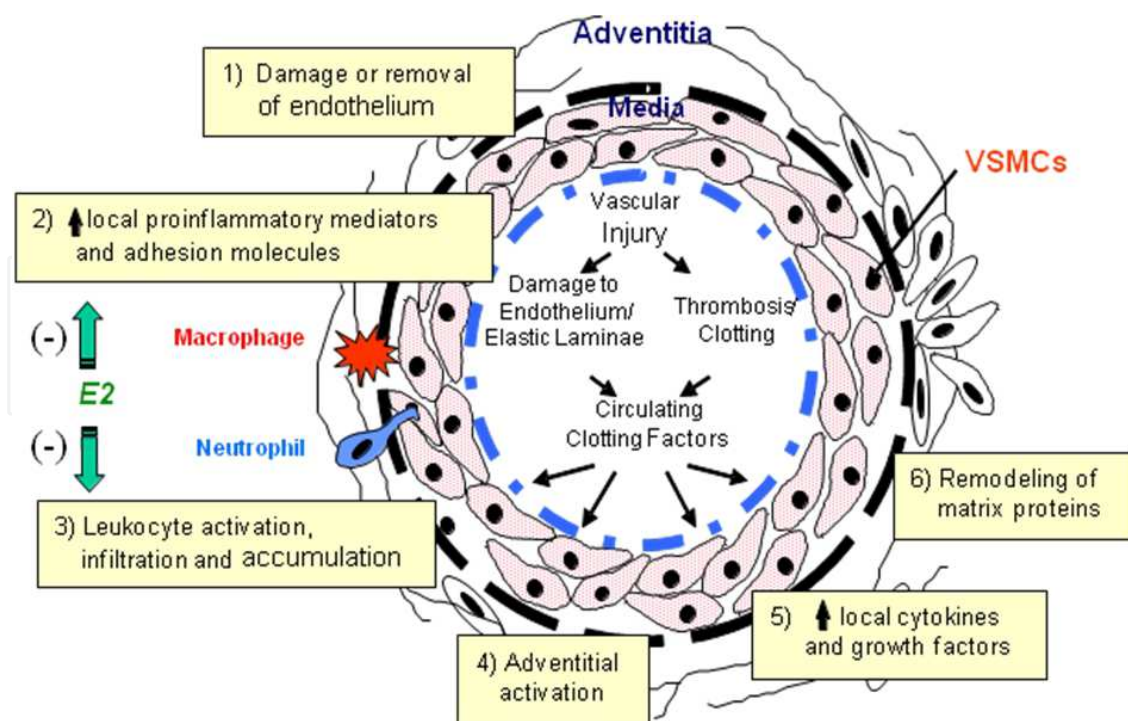
Fig. 3. E2 effects on the early vascular injury response. (*Adapted from Xing et al, 2009*)

5.2 The role of C-reactive protein in E2 modulation of vascular inflammation

E2 also exerts an anti-inflammatory and vasoprotective effect in injured arteries of CRP transgenic (CRPtg) mice. CRPtg mice carry a transgene containing the entire human *CRP* gene and its promoters while the mouse supplies all the required *trans*-acting factors. (Hage et al, 2008) Since in CRPtg mice, human CRP increases several hundred-fold during an acute phase response, analogous to the human condition, this model is convenient for the *in vivo* study of the biologic activities, including vascular effects, of human CRP. Using CRPtg mice, we and others have established that human CRP is a pathogenic mediator of cardiovascular disease. (Danenberg et al, 2003; Paul et al, 2004; Zhang et al, 2010; Nagai et al, 2011; Takahashi et al, 2010)

Using the carotid ligation model of acute vascular injury, we showed that young ovariectomized CRPtg mice develop twofold greater neointima formation than control non transgenic (NTG) mice and that there are extensive deposits of human CRP in the neointima of injured vessels of these animals in the absence of an increase in blood levels of the protein. (Kumar et al, 1997; Hage et al, 2010; Wang et al, 2005; Xing et al, 2008) These findings suggest that local expression of human CRP may exacerbate the adverse remodeling seen after acute arterial injury in the CRPtg model. To test the hypothesis that E2 can inhibit the vascular injury response attributed to human CRP, we treated ovariectomized CRPtg and control NTG mice with E2 prior to carotid ligation and observed that E2-treated CRPtg mice had a significant, ~85%, reduction in neointima formation compared to vehicle-treated CRPtg mice. The E2 effect was directionally similar but somewhat smaller in magnitude in control NTG mice. (Wang et al, 2005) Since the exaggerated vascular injury response in CRPtg mice is mediated by immunoglobulin G Fc receptors (FcγRs) on macrophages, (Xing et al, 2008) and since E2, via its interaction with ERs, can reduce inflammatory cytokine release from activated human macrophages by decreasing expression

of the excitatory FcγRs on these cells, (Kramer et al, 2004, 2007) it is plausible that the vasoprotective effects of E2 against CRP-mediated vascular injury response in female mice are regulated by its modulation of macrophage phenotype in order to express less activating receptors (FcγRI and FcγRIII) and more inhibitory receptors (FcγRIIb).

5.3 Estrogen receptors and vascular inflammation
Studies in ERα- and ERβ-deficient mice and in rats treated with pharmacologic antagonists of ERs have provided evidence that both ER subtypes contribute to the vasoprotective effects of E2 in the setting of acute injury. (Mori et al, 2000; Brouchet et al, 2001; Karas et al, 1999; Geraldes et al, 2003; Xing et al, 2007). The ER subtypes contribute to vasoprotection in a cell-specific manner. In porcine endothelial and vascular smooth muscle cells, E2 acts through inhibition of PDGF-BB-induced p38 and p42/44 mitogen-activated protein kinase (MAPK) phosphorylation to stimulate migration and proliferation. (Geraldes et al, 2003) Down-regulation of ERβ, but not ERα, prevented the effects of E2 on smooth muscle cell migration and proliferation. In contrast, in porcine endothelial cells, down-regulation of ERα prevented E2-induced p38 and p42/44 MAPK activation, while down-regulation of ERβ had no effect.

Administration of the ERβ selective agonist DPN has been shown to result in dose-dependednt attenuation of neointima formation induced by injury of the mouse femoral artery. (Krom et al, 2007) The ERα selective agonist PPT prevented neointima formation at low but not high concentrations in this study. In a subsequent study, MPP, an ERα selective antagonist, blocked the inhibitory effect of PPT on neointima formation, but did not block the effects of E2 or DPN. (Harrington et al, 2003) This suggests that E2 acts through a selective ERβ pathway to attenuate neointima formation following restenosis in this mouse model.

The TNF-α-stimulated vascular smooth muscle cell has been used as an in-vitro model of the vascular injury response in order to examine cellular/molecular mechanisms of E2-induced vasoprotection. (Xing et al, 2007) E2 has been shown to attenuate TNF-α induced expression of pro-inflammatory mediators in rat aortic smooth muscle cells through ERβ. (Xing et al, 2007) In this model, DPN reduced TNF-α-induced expression of the neutrophil cytokine CINC-2β in a dose-dependent fashion, while PPT had no effect. The non-selective ER antagonist ICI-182,780 blocked the anti-inflammatory effects of both DPN and E2. Furthermore, both DPN and E2 reduced neutrophil chemotactic activity in TNF-α-treated rat aortic SMCs.

5.4 The role of aging in loss of estrogen-induced vasoprotection
In order to reconcile laboratory findings that E2 provides vascular protection with clinical trial results indicating harmful effects of E2 on the cardiovascular system, studies have been done in models comparing young versus aged animals. Results from one study showed opposing effects of E2 based on age: E2 increased neointima formation in balloon-injured carotid arteries of aged (+75%) versus young (10-12 weeks; -40%) ovariectomized rats. (Miller et al, 2007) The attenuating effect of E2 on inflammatory mediator expression and neutrophil and monocyte infiltration was lost in the injured arteries of aged rats. ERα and ERβ expression was similar in both the young and aged animals. This laboratory evidence was the first of its kind to show that E2 exacerbates the vascular response to injury in aged animals. This seminal finding indicates that the protective effect of E2 is impaired following long periods of hormone deprivation, supporting the timing hypothesis. (Pinna et al, 2008)

The potential role of age-related alterations in ER signaling in these processes remains poorly understood and warrants further study.

6. Reproductive aging, sex hormones, and women's health

Reproductive aging is a function of decreased production of sex hormones by the ovaries. As a woman enters her fifth decade of life, depletion of remaining ovarian follicles occurs. Estrogen production by the ovaries begins to decrease, and women experience progressive loss of menstrual cyclicity. When total depletion of follicles occurs, menses cease, and the woman enters menopause, defined as the absence of menses for a 12-month period. The average age of menopause in the United States is 51 years. (Speroff and Fritz, 2005)   Due to increasing lifespan, women can now expect to spend a significant portion of their lives in the postmenopausal state. This prolonged hypoestrogenism may have important consequences for quality of life, as well as various other health parameters, including cardiovascular function, bone health, and cognitive function.

6.1 Age- and sex-specific trends

CHD is rare in premenopausal women, but the incidence of myocardial infarction rises dramatically after menopause. Furthermore, women with premature ovarian failure or early natural menopause (≤ 44 years) have an associated increase in the risk of CVD. (Hu et al, 1999) (Mondul et al 2005) These observations led to the belief that menopause itself is a risk factor for CHD. However, the relationship between menopause, age and CHD is complex and it is not clear that menopause per se is a risk factor for CHD. It is important to recognize that women who develop CHD after menopause have more CHD risk factors (dyslipidemia, family history, hypertension, tobacco use, and diabetes mellitus) compared to postmenopausal women who remain free of disease. Currently available data from randomized controlled trials, e.g., WHI and HERS, do not indicate that HRT is useful in the primary or secondary prevention of CHD. However, a growing body of evidence suggests that initiation of treatment with different HRT preparations in the perimenopausal period may have beneficial effects on the vasculature that may delay the progression of CVD and prevent ischemic events.

6.2 Types and routes of administration of postmenopausal estrogen replacement therapy

ERT is only indicated for the treatment of moderate to severe menopausal symptoms, specifically vasomotor symptoms (eg. hot flushes). Contraindications to ERT include known CHD, breast cancer, a previous venous thromboembolic event or stroke, active liver disease, or high risk for these conditions. ERT should be initiated as close to the time of menopause as possible, typically beginning in the late forties to early fifties. Initiation of therapy beyond age 59 is controversial due to increased risk of CHD events. Most physicians now agree that the benefit of estrogen treatment in healthy, early menopausal women, using the lowest dose and shortest duration of therapy, outweigh the risks of treatment.

Systemic ERT can be given orally or non-orally in the form of transdermal patches and topical creams, gels, and mists. Estrogen can also be given vaginally via tablets, topical formulations and vaginal rings. However, vaginal therapy is only indicated for the treatment of vaginal atrophy and not for systemic/vasomotor symptoms. Assuming that equivalent doses of replacement estrogen are given, the different routes of administration

are equivalent in ameliorating menopausal symptoms. Among the oral estrogens, E2 is considered to be the most potent estrogen and estrone is reported to be 50-70% less active. Estriol is the least potent of the three estrogens, with a potency one-tenth that of E2.

While both oral and transdermal estrogens are absorbed systemically, oral estrogens are unique in that they undergo the "first-pass effect" in the liver. Intestinal absorption of estrogens leads to high concentrations of hormone in the portal vein, stimulating hepatic production of thyroxine-binding globulin, corticosteroid-binding globulin, SHBG, triglycerides, HDL, triglycerides, and clotting factors. Transdermal administration of estrogen does not have this effect and there is no resulting increased hepatic production of the above proteins.

Multiple oral estrogen preparations are available, including CEE, E2, esterified estrogen, and conjugated synthetic estrogens. (Table 1)

Estrogen Replacement Therapy					
Drug	**Company**	**Dose**	**Company**	**Drug**	**Dose**
Oral Therapy			*Transdermal Therapy*		
Estradiol			**Estradiol**		
Estrace	Warner Chilcot	0.5, 1, 2 mg	*Patches*		
Gynodiol	Firlding	0.5, 1, 1.5, 2 mg	Alora	Watson	0.025, 0.05, 0.075, 0.1 mg/d
Conjugated equine estrogens			Climara	Berlax	0.025, 0.05, 0.06, 0.075, 0.1 mg/d
Premarin	Wyeth-Ayerst	0.3, 0.45, 0.625, 0.9, 1.25 mg	Esclim	Women First	0.025, 0.0375, 0.05, 0.075, 0.1 mg/d
Esterified Estrogens			Estraderm	Novartis	0.05, 0.1 mg/d
Menest	Monarch	0.3, 0.625, 1.25, 2.5 mg	Vivelle	Novartis	0.025, 0.0375, 0.05, 0.075, 0.1 mg/d
Ogen	Pharmacia	0.75, 1.5, 3 mg	Menostar	Bayer	0.014 mg/d
Ortho-Est	Women First Healthcase	0.5, 1.5, mg	*Gel*		
Conjugated synthetic estrogens			EstroGel	Solvay	0.75 mg/pump
Cenestin	Elan	0.3, 0.45, 0.625, 0.9, 1.25 mg	*Emulsions*		
Enjuvia	Elan	0.625, 1.25 mg	Estrasorb	Novavox	0.025 mg/pouch
Vaginal Therapy			Divigel	Upsher-Smith	0.25, 0.5, 1 mg/pouch
Estradiol			Elestrin	Kenwood	0.52 mg/pump

Rings			Spray		
Estring	Pharmacia	0.0075 mg/day	EvaMist	KV pharmaceutical	1.5 mg/spray
Femring	Warner-Chilcott	0.05 mg/day			
	Tablet				
Vagifem	Novo Nordisk	0.025 mg/tablet			
Cream					
	Estradiol				
Estrace	Warner-Chilcot	0.1 mg/gram			
	Conjugated equine estrogen				
Premarin	Wyeth-Ayerst	0.625 mg/gram			

Table 1. Available Estrogen Formulations (*Modified from Martin and Barbieri, 2011*)

CEE is one of the most commonly used preparations and, derived from mare urine, is composed of up to 10 different estrogenic compounds, predominantly the sodium sulfated conjugates of estrone. (Lyman GW 1982) The metabolism of CEE is a complex and still poorly understood process which occurs in the liver. After oral ingestion of CEEs, the compounds are rapidly absorbed by the gastrointestinal tract, then may become conjugated by hepatocytes or excreted in the feces. (Pan CC, 1985) Following oral intake of CEEs, mean serum estrone levels (152 pg/mL) are far higher than estradiol levels (31 pg/mL). (Powers MS et al 1985) However, the estrone component is largely inactive because it is albumin-bound. The clinical response to CEE is hypothesized to be mediated through a mechanism involving conversion of circulating, bound estrone to E2 in the liver. (Barnes RB et al 1987)

E2 is another commonly prescribed oral form of postmenopausal ERT. Native E2 is poorly absorbed; therefore it is manufactured in micronized, sulfated, and esterified forms to improve absorption. (Krantz JC et al 1958) Similar to metabolism of CEE, the majority of E2 is converted to estrone. However, following oral administration of E2, mean circulating levels of estrone (200 pg/mL) and E2 (50 pg/mL) are higher compared to serum levels following ingestion of CEE when equivalent doses are given. (Lobo RA and Cassidenti DL 1992) E2 also induces hepatic production of proteins, but this effect is much less than that of CEEs. (Maschak CA et al 1982).

Esterified estrogens result in serum E2 and estrone levels similar to those seen with CEEs. Synthetic conjugated estrogens are derived from plant sources. They are similar but not identical to CEEs and contain fewer molecular forms of estrogen. (Lobo et al, 2000)

6.3 Types and routes of administration of progesterone replacement therapy

Progesterone is indicated in addition to estrogen as part of a HRT regimen in postmenopausal women with an intact uterus (who have not undergone hysterectomy). Progesterone opposes the effects of estrogen on the endometrial lining and prevents development of endometrial hyperplasia and malignancy which occur in women treated with unopposed estrogen. Both natural and synthetic progestins are available. (Table 2)

Progesterone Formulations			
Generic Name	**Brand Name**	**Company**	**Dose**
Oral Therapy			
Micronized Progesterone	Prometrium	Abbott	100, 200 mg
Medroxyprogesterone Acetate	Provera	Pfizer	2.5, 5, 10 mg
Norethindrone acetate	Aygestin	Teva Pharmaceuticals	2.5, 5, 10 mg
Vaginal Therapy			
Progesterone Cream	Crinone	Columbia Laboratories	90 mg/applicator
Progesterone Gel	Prochieve	Columbia Laboratories	90 mg/applicator
Intrauterine Device (IUD)			
Levonorgestrel IUD	Mirena	Bayer	52 mg/5 yrs
Combination Estrogen-Progesterone Formulations			
Oral Therapy			
CEE/medroxyprogesterone	Prempro	Wyeth	0.3/1.5, 0.45/1.5, 0.625/2.5, 0.625/5 mg
Estradiol/norgestimate	Prefest	Duramed	1/0.9 mg
Estradiol/norethindonr acetate	Activella	Novo Nordisk	1/0.5 mg
Ethinyl estradiol/norethindrone	FemHRT	Warner-Chilcot	5 mcg/1mg
Estradiol/drosperinone	Angeliq	Berlex	1/0.5 mg
Transdermal Patches			
Estradiol/norethindrone	Combi-Patch	Novartis	0.05/0.14, 0.05/0.25 mg
Estradiol/levonorgestrel	Climara Pro	Berlex	0.045/0.015 mg

Table 2. Available Progesterone Preparations. (*Modified from Martin and Barbieri, 2011*)

Micronized progesterone is the major natural progesterone available, and while it has been less well studied than MPA, it appears to be similar in efficacy and is widely prescribed. Synthetic progestins, including MPA, norgestrel, and norethindrone acetate, appear to increase hepatic lipase activity and attenuate the beneficial effects of estrogen on HDL levels (Stevenson 2009), while natural progesterone appears to have no adverse effect on HDL. MPA also opposes the NO-dependent vasodilator effect of E_2, while natural progesterone was found to have no effect. (Williams AK et al 1994)

7. Clinical research in hormone replacement therapy

7.1 Observational studies

The slope of the age-related rise in incidence of CVD in women increases in the post-menopausal period, suggesting that withdrawal of ovarian hormones, particularly E2, has an adverse effect on cardiovascular health. (Lloyd-Jones et al, 2010) This increase is thought

to be a consequence of the loss of the multiple protective effects of E2 on the vascular system. Multiple observational studies have suggested that HRT may protect against CVD in postmenopausal women. In a meta-analysis of 16 prospective observational trials, the relative risk of CVD for postmenopausal women who ever used any form of estrogen vs. those who had never used estrogen was 0.70 (95% CI, 0.63-0.77). (Grodstein et al, 1995) The relative risk in current users, calculated from 6 prospective studies, was even more impressive at 0.55 (95% CI, 0.44-0.70). In the Nurses' Health Study, which followed more than 70,000 postmenopausal women for 20 years, the risk for major coronary events was lower among current users of HRT compared with never-users (multi-variate adjusted relative risk, 0.61 [95% CI, 0.52-0.71]). (Grodstein et al, 2000) Buoyed by observational studies suggesting that HRT, including various E2 preparations with or without a progestin (most commonly a synthetic one), reduced CVD risk by ~50%. (Psaty et al, 1994) HRT use increased dramatically during the 2 decades prior to the publication of the WHI. It is estimated that annual hormone therapy prescriptions increased from 58 million in 1995 to 90 million in 1999, representing ~15 million women per year. (Hersh et al, 2004) This rate remained stable until 2002, but after the publication of WHI and other randomized controlled trials that showed no benefit of HRT, fell sharply by 66% in a single year. Many of the studies that prompted the upswing in postmenopausal HRT suffered from the limitations of discordance of the two treatment groups: women who take HRT are on average better educated, have higher incomes and better access to health care and are healthier even before starting therapy. (Barrett-Connor et al, 1989; Matthews et al, 1996) In a meta-analysis that adjusted for socioeconomic status and other risk factors, HRT was not associated with CVD risk reduction. (Humphrey et al, 2002)

7.2 Clinical trials

Publication of the estrogen plus progestin clinical trial component of the Women's Health Initiative (WHI) (Rossouw et al., 2002; Manson et al., 2003) initially sounded a death knell for hormone use in post-menopausal women. This placebo-controlled trial of HRT (CEE 0.625 mg/day plus MPA 2.5 mg/day) in 16,608 post-menopausal women found significant increases in the risk of CHD, stroke, venous thromboembolism and invasive breast cancer in the HRT group. The reductions in colorectal cancer and hip fracture seen with HRT did not balance these increased CVD and cancer risks, and publication of the WHI results stimulated consensus panels to recommend against the use of HRT for chronic disease prevention in post-menopausal women (Mosca et al., 2004). Based on the widely publicized findings of harm in the estrogen plus progestin (E+P) trial of the WHI and a major secondary prevention study that used the same hormone regimen, the Heart and Estrogen/Progestin Replacement Study (HERS) (Hulley et al., 1998; Grady et al., 2002), prescribing of HRT fell drastically. (Hersh et al., 2004) Transdermal hormone preparations were less affected, and transvaginal and low-dose preparations gained somewhat, reflecting caution in the use of the full-dose oral regimens that had been used in WHI and HERS.

Attempts to explain the unanticipated deleterious effects of HRT gave consideration to whether the formulation, dose and route of administration of HRT might play a role (Dubey et al., 2004; Turgeon et al., 2004; Phillips & Langer, 2005). In particular, the progestin MPA was identified as having potential deleterious effects on the vasculature. Pre-clinical studies had shown that MPA negates the vasoprotective and anti-inflammatory effects of E2 in the setting of acute vascular injury (Levine et al., 1996; Oparil et al., 1997; Xing et al., 2004; Miller

et al., 2004) and in vitro studies found that MPA signals differently from native progesterone in endothelial cells (Simoncini et al., 2004). The surprising outcomes of the estrogen-alone (EA) component of WHI (WHI SC, 2004) added further evidence that MPA might be a problem and that unopposed estrogen benefits younger post-menopausal women. This trial, which was stopped early, showed no significant effect of unopposed CEE on the primary CHD outcome and a surprising tendency for benefit in the primary safety (invasive breast cancer) outcome.

7.3 The timing hypothesis

The advanced age (63 years in WHI, 67 years in HERS) and long period of hormone deprivation prior to starting HRT may account for deleterious outcomes of hormone treatment in WHI and HERS. Based on a review of pre-clinical studies, as well as observational studies and clinical trials in women, including those with intermediate end-points and CVD outcomes, the "timing hypothesis" was developed (Phillips & Langer, 2005). The timing hypothesis states that the effects of HRT on the vasculature are dependent on the time of initiation of treatment. The timing hypothesis predicts that HRT initiated at the time of or prior to menopause should produce a decrease in CHD over time, while HRT begun years after menopause should produce an increase in CHD events shortly after therapy is begun, followed by later benefit. This hypothesis attributes the complex CHD responses to HRT in human trials to a combination of early erosion/rupture of 'vulnerable' coronary plaque, which is made worse by HRT; long-term reduction in plaque formation, which is improved by HRT; and modulation of the vasoprotective actions of estrogens by systemic progestins.

Indirect support for the timing hypothesis has come from the report of final results from the EA trial in WHI, which included detailed analyses of primary and secondary coronary outcomes and subgroup analyses of participants by age and years since hysterectomy with no menopausal hormone therapy (Hsia J, et al). During the active intervention period, 201 coronary events were confirmed among women assigned to CEE compared with 217 events among women assigned to placebo (HR=0.95%; 95% CI 0.79-1.16). Among women aged 50-59 years at baseline, the HR for the primary outcome (nonfatal myocardial infarction or coronary death) was 0.63 (95% CI 0.36-1.08). In that younger age group, coronary revascularization was less frequent among women assigned to CEE (HR=0.55; 95% CI 0.35-0.86), as were several composite outcomes. Further analyses of the E+P arm of the WHI demonstrated a non-significant trend towards cardioprotection in women who began HRT less than 10 years after menopause (HR = 0.89; 95% CI 0.5-1.5), while women who initiated HRT more than 20 years after menopause had a significantly elevated risk of coronary events (HR = 1.71; 95% CI, 1.1-2.5). (Manson et al, 2003) When the EA and E+P arms from the WHI were combined, a similar trend was seen. (Rossouw et al, 2007)

Another consideration in the relationship between HRT and CVD risk is the duration of HRT. A recent post-hoc analysis of the E+P trial in WHI showed that in women less than 10 years since menopause, HRT resulted in a slightly lower event-free survival rate during the first 5 years of therapy compared to placebo; however, at 6 years, the two curves crossed each other and showed a non-significant trend towards a higher rate of event-free survival in the group using HRT. (Toh et al, 2010) Further analysis of women less than 10 years since menopause in the WHI E+P arm showed an increased risk in the first 2 years of HRT, followed by a decreased risk in the next 2 years, and an overall risk reduction over 8 years.

(Toh et al, 2010). Similar results were seen in the EA arm of the WHI, with a significant decrease in CHD risk after 6 years of CEE alone compared to placebo. (Harman et al, 2011) Among women in the EA arm of the WHI followed up over 10.7 years, there was no difference in CHD risk in those using CEE for a median of 5.9 years compared to placebo at the end of the active treatment period, or overall. (LaCroix et al, 2011)   In the post-intervention follow-up period, the annualized rate for CHD in the EA arm was 0.64% compared to 0.67% in the placebo group (HR 0.97, 95% CI, 0.75-1.25). Health outcomes, including CHD, were more favorable for younger women compared to older women (P=0.05 for interaction). These findings support the current clinical recommendations to treat postmenopausal women with HRT for the "shortest possible duration" and may lead to more individualized management.

The WHI was limited by use of only one type of ERT (CEE), by inclusion of women who initiated HRT late after many years of ovarian hormone deprivation, and by exclusion of women who were experiencing menopausal symptoms. Ongoing clinical trials are addressing these deficiencies by examining the timing hypothesis in perimenopausal women. The Kronos Early Estrogen Prevention Study (KEEPS) is a prospective, randomized, double-blind study of 900 healthy perimenopausalwomen aged 45-54 with menopausal symptoms. (Harman et al., 2005) The main hypotheses are 1) HRT initiated early in menopause (before development of atherosclerotic lesions) will prevent progression of atherosclerotic lesions, and 2) both oral CEE and transdermal E2 will be similarly efficacious. Participants were randomized to oral CEE and a placebo patch, oral placebo and a transdermal patch containing E_2, or placebo in both pill and patch. The primary endpoints of KEEPS are carotid intimal medial thickness by ultrasound and the progression of coronary calcium by electron beam tomography, surrogates for CVD. Another ongoing prospective, randomized, controlled trial, the Early versus Late Intervention Trial with Estradiol (ELITE) randomized 643 women who were less than 6 years or more than 10 years since menopause to receive oral E2 versus placebo. (clinicaltrials.gov NCT00114517; Hodis and Mack, 2011) The primary endpoint is rate of change of carotid artery intima-media thickness. These two prospective studies will provide much-needed information regarding the timing hypothesis and use of HRT in reducing CVD risk.

7.4 Oral versus transdermal HRT

To date, few studies have examined the difference in CHD outcomes between postmenopausal women treated with oral versus transdermal therapy. The one existing trial in the literature suggests no difference in CHD outcomes with regard to route. (Clarke SC 2002) This study examined transdermal E2 with or without transdermal norethindrone acetate, and found similar CHD outcomes to the WHI. The literature indicates that dose may be more important than route.

8. Conclusions

Importantly, cellular and molecular studies are urgently needed to elucidate the differential effects of HRT and its components on young, healthy arteries and on older, diseased arteries. Emerging evidence suggests that HRT administered to young healthy women has anti-inflammatory and vasodilator effects that tend to lower blood pressure and slow the progression of atherosclerotic lesions, while the same HRT preparation administered to

older women, particularly those with established vascular disease, has a proinflammatory effect, perhaps leading to atherosclerotic plaque instability and neovascularization (Störk et al., 2004; Mendelsohn & Karas, 2005). The mechanisms of these altered vascular responses are not fully understood, but may relate to age-related deterioration in ER expression and signaling. Recent studies of the effects of HRT on blood pressure and vascular function support the age-dependence of the action of HRT on the vasculature. Beneficial effects of HRT appear to be realized only in younger, perimenopausal women in whom hormone response systems remain intact. However, further study of the timing, dose, duration, and route of administration of HRT in postmenopausal women may be informative.

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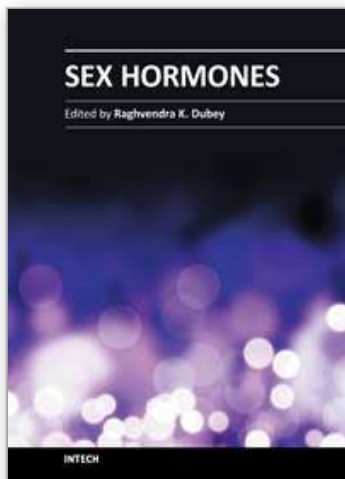
Sex Hormones

Edited by Prof. Raghvendra Dubey

Sex Hormones not only regulate reproductive function, but they also play a prominent role in the biology and physiology of several organs/tissues and in the pathophysiology of several diseases. During the last two decades, the information on the mechanisms of action of sex hormones, such as estrogens and androgens, has rapidly evolved from the conventional nuclear receptor dependent mechanisms to include additional non-nuclear, non-genomic and receptor-independent mechanisms. This highlights the need to update the current knowledge on sex hormones and their mode of action. Increasing evidence that exogenous/epigenetic factors can influence sex hormone production and action highlights the need to update our knowledge on the mechanisms involved. This book provides a systematic and updated overview of the male/female sex-hormones and their impact in the biology and physiology of various organs. Additionally, the book discusses their positive and negative association with the pathophysiology of various diseases (e.g. osteoporosis, cardiovascular-disease, hypogonadism, reproduction, cancer) and their therapeutic potential.

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