Drug Targeting of Estrogen Receptor Signaling in the Cardiovascular System: Preclinical and Clinical Studies

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Abstract: Atherosclerosis and associated coronary heart disease events have lower prevalence in women than in men, especially during young adult years. Although multiple lines of evidence suggest that estrogens contribute to this difference, the efficacy of hormone replacement therapy for the prevention of cardiovascular disease in postmenopausal women is controversial. The protective action of estrogen in the cardiovascular system appears to be mediated indirectly by an effect on serum lipoprotein and triglyceride profiles and on the expression of coagulant and fibrinolytic proteins, and by a direct effect on the vessel wall itself. Estrogen has both rapid effects involving alteration of membrane ionic permeability and activation of membrane-bound enzymes and increases in endothelial cell nitric oxide synthase activity, as well as longer-term effects on gene expression that are mediated, at least in part, by the ligand-activated transcription factors, estrogen receptor and . Compounds with pure antiestrogenic activity and selective estrogen receptor modulators that regulate estrogen receptor function in a tissue-specific manner have been developed in an attempt to achieve the cardioprotective effects of estrogens while minimizing the undesirable risks associated with hormone replacement therapy (e.g., endometrial and breast cancer). In this review, we will discuss recent developments on the mechanisms of estrogen action in the cardiovascular system. The results of clinical trials testing the long-term efficacy of hormone replacement therapy for the treatment of cardiovascular disease will also be discussed.

Key Words: estrogens, SERM, cardiovascular system, animal models, clinical trials, endothelial cell, vascular smooth muscle cell.

1. INTRODUCTION

After the discovery of the estrogen receptor (ER) and the demonstration that it functions as a ligand-dependent transcription factor, a model of estrogen action based on genomic effects was suggested. According to this "classic pathway", ligand-activated ER interacts with estrogen-responsive elements (ERE) within the promoter of target genes, thus modulating their rate of transcription. However, the simplicity of this model has been challenged by the discovery of several selective ER modulators (SERMs) displaying both agonistic and antagonistic functions in different tissues. Additional complexity arose with the discovery of a novel ER isoform (ER), and the observation that different tissues display varying level of expression of both ER isoforms. ER and ER participate in multiprotein transcription factor complexes involving proteins of the superfamily of nuclear receptors and transcriptional coregulators. Importantly, the DNA-binding specificity of such transcriptional complexes is affected by their protein components.

The identification of alternative estrogen actions has been accumulating steadily over the past two decades. Typically, these novel actions do not directly implicate nuclear transcriptional events but are related to the interaction of estrogen with sites present at the plasma membrane or cytosolic locations. These alternative effects, incorrectly named nongenomic effects, range from the modulation of plasma membrane ion channel activity to the regulation of different intracellular signaling cascades and the control of gene expression [1,2]. Thus, it is currently accepted that the cellular responses to estrogens and SERMs in different pathophysiological situations are dependent on the cell type, ERE-promoter context, the relative level of expression of ER and ER , the level of agonists and antagonists, and the balance between "classic" and alternative mechanisms of estrogen action.

Both ER subtypes are coexpressed in many tissues and cell types, including cardiomyocytes, fibroblasts, mammary gland, and aorta [3-9]. Regarding cells involved in atherosclerosis, ER and ER are expressed in vascular smooth muscle cells (VSMCs) [10-16] endothelial cells (ECs) [16,17], monocytes, and B and T cells [18,19]. ER

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expression may prevail in epididymis, testis, pituitary gland, ovary, uterus, adrenal glands and heart, while ER is expressed at higher levels in vascular smooth muscle, prostate, bladder, lung, thymus and certain hypothalamic cells [20-22].

Epidemiological and animal studies have suggested a cardioprotective effect of estrogens [23-26] (see below). The mechanisms that have been proposed to contribute to these beneficial effects include reduced low density lipoprotein (LDL) and increased high-density lipoprotein (HDL) levels [27-34], vasodilation in response to altered production of endothelium-derived factors, such as an increase in nitric oxide (NO) and a decrease in endothelin-1 [35-39], and enhanced angiogenesis as a result of increased attachment, proliferation, and migration of ECs [40-42]. Estrogen can also regulate a number of VSMC functions relevant to atherosclerosis (e.g., contractility, proliferation, migration and production of extracellular matrix components) [23,41,43].

Studies over the past few years suggest that estrogens can reduce the accumulation of cholesteryl esters in macrophages [44]. Moreover, estrogen inhibits the expression of the proatherogenic factors monocyte chemoattractant protein-1 and vascular cell adhesion molecule-1 in cultured VSMCs and ECs [45-47]. Importantly, these inhibitory effects of estrogen correlated with reduced leukocyte adhesion and transendothelial migration in rabbits *in vivo* [47]. Using intravital microscopy in the rat mesenteric microcirculation, Alvarez *et al*. have shown that estrogen inhibits angiotensin II-induced leukocyte-EC interactions *in vivo* via rapid endothelial NO synthase (eNOS) and cyclooxygenase activation [48]. These authors have also provided compelling evidence that scarceness of estrogen reduces the levels of vasodilators and exposes the endothelium to the deleterious action of angiotensin II.

2. MECHANISMS OF ESTROGEN ACTION

2.1. The "Classic Pathway"

The ER is a ligand-activated transcription factor which belongs to the superfamily of nuclear receptors (class I). Two distinct ERs have been identified, ER and ER , which display different tissue distribution [25,49,50]. Both ER and ER bind 17 −estradiol (E2) with high affinity and have a similar, if not identical, manner of interaction with EREs in the promoter of target genes. However, the two ER subtypes display different transcriptional activity, and the corresponding knockout mice show significant phenotypic differences (see below). Estrogen-dependent ER-mediated gene activation has been demonstrated in VSMCs and ECs [25]. ERs can be activated via their interaction with growth factors in the absence of estrogen [51]. This estrogen-independent activation of ERs may occur by different intracellular pathways in vascular and nonvascular cells [52].

ERs have six functional domains (A/B, C, D, E and F) implicated in four separate functions: hormone binding, homo and heterodimerization, DNA binding, and transcriptional activation [53]. Although structurally and functionally distinct, ER and ER have a high degree of sequence homology in the DNA-binding domain

(approximately 96%) and in the hormone-binding region (approximately 53%). In contrast, their aminoterminal region is highly divergent [25,49].

In the absence of ligand, ERs heterodimerize with members of the family of heat shock proteins (HSPs) [16,54]. While HSPs keep the ER in a conformation that has high affinity for the hormone, ER/HRP complexes are sequestered in an inactive state in the nucleus of target cells. In the presence of hormone, ER undergoes a conformational change that promotes homodimerization and binding to EREs located within the regulatory regions of target genes, thus modulating their transcriptional rate [49,55-58]. The minimal ERE sufficient for specific binding is a palindromic sequence composed of two hexanucleotide half-sites separated by three nucleotides (5´-GGTCAnnnTGACC-3´), although the sequence of nucleotides immediately flanking this minimal ERE play an important role in determining the affinity of ER binding [59,60]. ER /ER heterodimers bind to EREs with similar specificity and affinity than that of the respective homodimers [61]. Fig. (**1B**) shows the ability of ER and ER to interact with a consensus ERE probe in electrophoretic mobility shift assays, and Fig. (**1C**) illustrates the ability of ER to confer reporter gene transactivation via an E2-mediated ERE-dependent mechanism. In addition to ERE-dependent transcriptional regulation, ER can also indirectly modulate gene expression through interaction with other DNA-bound transcription factors ('tethering' mechanism), such as AP-1, SP-1 and NF -B [62,63].

2.2. The Alternative Pathways

The alternative pathways for estrogen action involve interaction of the hormone with membrane and cytosol targets [1,64]. At the membrane level, estrogen can modulate the activity of ion channels [65,66] via binding to one of the components of the channel complex, thus resulting in changes in the electrical excitability of the cells. Estrogen can also generate intracellular signals, following its interaction either with putative membrane ER (mER) or cytosolic ER [64]. In that respect, reports describing the immunolocalization of ER to plasma membrane [67] postulated that the classical ER, or a closely related form, might also be present at the plasma membrane. However, this view has been challenged by several laboratories showing estrogen binding to plasma membranes lacking classical ER [68]. Moreover, the putative mER has to be an integral membrane protein, probably spanning the entire lipid bilayer and offering an extracellular binding site for estrogen. These requirements are not fitted by the classical ERs, which do not conform to a transmembrane molecular structure. Therefore, the mER might share some features of typical membrane receptors (e.g., G-protein coupled receptors, and epitopes present in classical ER). Alternatively, estrogen can interact with known membrane receptors such as the -adrenergic receptor [68] or other membrane proteins (see above). -adrenergic receptors have been characterized in vascular smooth muscle [69], although they are unlikely to mediate estrogen action in this tissue because activation of these receptors results in vasoconstriction.

Estrogen can also interact with ERs present in the cytosol eliciting rapid cellular responses. An excellent example of
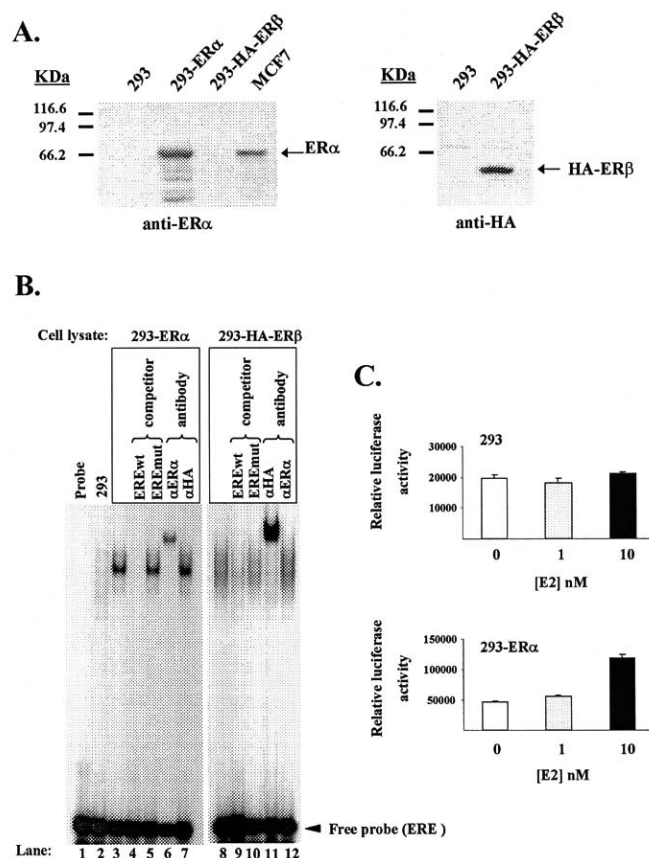
Fig. (1). Ectopically expressed ER binds to the consensus ERE sequence and activates transcription of reporter genes driven by the ERE site. 293 cells were transfected with expression vectors encoding human ER (293-ER) and hemaglutinin-tagged ER (293-HA-ER) using the calcium phosphate technique. A. Western blot analysis using the indicated antibodies. The parental 293 cells do not express ER. MCF7 cell lysate was used as a positive control for ER expression. B. Electrophoretic mobility shift assay using a radiolabelled probe spanning the consensus ERE sequence (5´-AGGTCAnnnTGACCT-3´). Lane 1 shows the electrophoretic mobility of the free ERE probe (no cell lysate added). The rest of binding reaction contained ERE probe and lysates from 293 (lane 2), 293-ER (lanes 3 to 7) or 293-HA-ER (lanes 8 to 12) cells. Addition of an excess of wild-type (lanes 4 and 9) but not mutated ERE (lanes 5 and 10) competed away the retarded band. Supershift experiments with specific antibodies demonstrated the presence of ER (lane 6) or HA-ER (lane 11) in the retarded nucleoprotein complexes of 293-ER and 293-HA-ER cells, respectively. C. Effect of E2 on the activation of a luciferase reporter gene under the transcriptional control of the ERE sequence. 293 cells were transiently cotransfected with 15µg of luciferase reporter gene and 5 µg of either control CMV-Neo-Bam vector (293) or CMV-ER (293-ER). The cells were maintained in phenol red-free DMEM supplemented with 10% charcoal/dextran-treated FBS. E2 was added to the culture medium at the indicated concentrations.

such alternative mechanism of action is the modulation of NO bioavailability in ECs. Rapid activation of eNOS by estrogen, increasing NO bio-availability, requires ER with an intact hormone binding domain [70]. This effect can not be reproduced by binding of estrogen to ER [71,72], although both ER and ER are expressed in vascular cells. The activation of NOS by estrogen involves the phosphatidylinositol 3-kinase (PI3K), a signaling cascade that appears to be triggered in the caveolae. These specialized structures form in ECs signal-transducing membrane microdomains which contain not only ER and eNOS, but also PI3K [73]. Binding of estrogen to the ER via a direct physical interaction with the regulatory p85 subunit of PI3K activates serine/threonine protein kinase B (also termed Akt) which, in turn, will phosphorylate and activate NOS. Estrogen- and raloxifene-mediated activation

of NOS can also be induced via the extracellular signal-regulated kinase (ERK) pathway [74]. Both the PI3K and ERK pathways are relevant for enhanced phosphorylation of NOS after brief treatment with estrogen and raloxifene. Estrogens can also modulate cell function via a mechanism related to their antioxidant properties which is independent of their binding either to classical or novel receptors. This property of estrogens might be relevant to their neuroprotective effect against -amyloid neurotoxicity [75], although such protective effect has not been reproduced in -amyloid-challenged vascular cells [76].

3. SERMS

SERMs are structurally diverse non-steroidal compounds that bind to ERs and produce estrogen agonist or antagonist

effects in a species- and tissue-specific manner. The family of SERMs include: triphenylethylenes (clomiphene, tamoxifen, droloxifene, idoxifene and toremifene), benzothiophenes (raloxifene and LY353381.HCl), benzopyrans (EM-800), chromans (levormeloxifene), naphthalenes (CP-336, 156), and pure antiestrogens (ICI182780 and EM-652). An exhaustive discussion on the mechanisms of action of SERMS and their application to clinical practice, including cardiovascular disease (CVD), can be found elsewhere [77-79].

## 4. LOCAL CONTROL OF VASCULAR TONE BY ESTROGENS

Estrogens modulate vascular tone at several different levels, from controlling the expression of circulating vasodilator and vasoconstrictor elements, to local regulation of VSMC excitability [25]. The main mechanism at the vessel wall level used by estrogens to induce vasodilatation is the increased bioavailability of NO (see above). Other endothelial-independent, estrogen-induced vasodilatory mechanisms also exist, including the inhibition of voltage-dependent Ca^{2+} channels [80] and rapid activation of potassium channels in the vascular smooth muscle [65,66,80,81]. Long-term control of channel function by estrogens due to changes in channel expression has also been seen in vascular and non-vascular cells [82-84].

5. CONTROL OF VASCULAR CELL PROLIFERATION BY ESTROGENS

At homeostasis, ECs and VSMCs display a very low proliferative activity. However, EC and VSMC proliferation is abundant during the vascular remodeling process that takes place during the pathogenesis of vascular obstructive disease [85]. E2 promotes the proliferation of ECs of different origin, including human umbilical vein ECs [42], porcine aortic ECs [41], and bovine retinal capillary ECs.

We found that E2 induced growth arrest in murine fibroblasts that were forced to overexpress ERα but not in parental cells (Fig. (**2**)). The effect of estrogens on VSMC proliferation is controversial. Several studies have reported
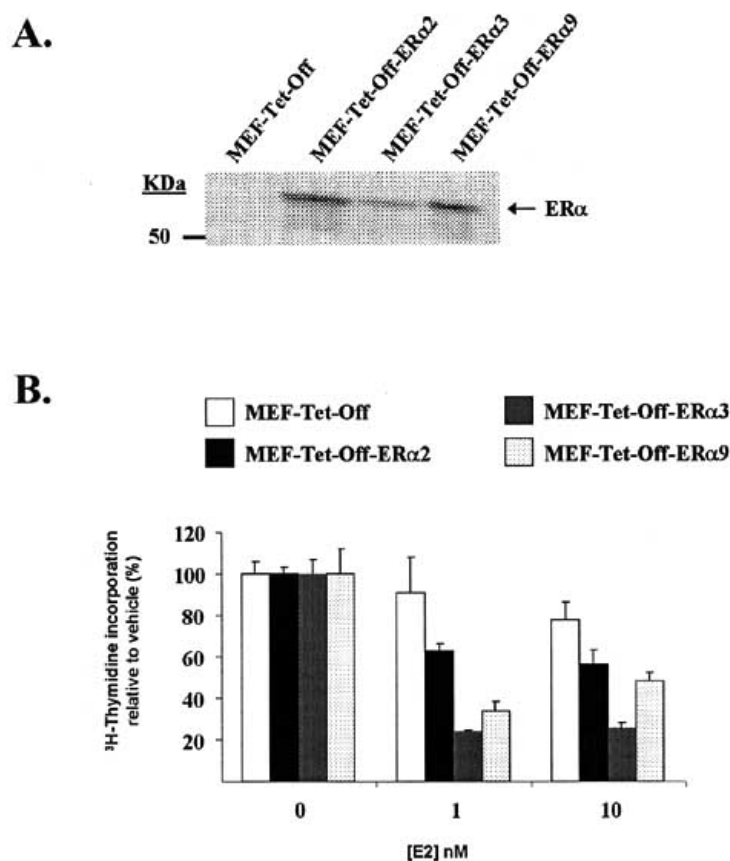
Fig. (2). Mouse embrionary fibroblasts-Tet-Off (MEF-Tet-Off, Clontech) were infected with a recombinant retrovirus encoding human ERα. Cells were selected with hygromycin and G418 and 3 resistant clones were isolated (MEF-Tet-Off-ERα 2, 3, and 9). A. Western blot analysis demonstrated ERα expression in the 3 isolated clones, but not in the parental MEF-Tet-Off cells. B. ^{3}H-Thymidine incorporation assay in asynchronously growing cultures. Cells were maintained in phenol red-free DMEM supplemented with 10% charcoal/dextran-treated FBS (HYCLONE). Cultures were exposed to the indicated concentrations of E2 during 24 hours and were labelled with ^{3}H-Thymidine during the last 4 hours. For each cell, bars represent ^{3}H-Thymidine incorporation relative to control without E2 (n = 3). Basal incorporation (in cpm, 100%) was 36964 ± 2017 (MEF-Tet-Off), 27239 ± 843 (MEF-Tet-Off-ERα 2), 31492 ± 2086 (MEF-Tet-Off-ERα 3) and 34167 ± 4028 (MEF-Tet-Off-ERα 9).

reduced proliferative capacity of cultured VSMCs by estrogen [43,86-90]. This inhibitory effect occurs in a dose-dependent manner [91] and through activation of ERs [92]. 2-methoxyestradiol, an endogenous metabolite of E2, inhibits EC proliferation and migration *in vitro* as well as angiogenesis *in vitro* [93]. Regarding potential gender differences, Dai-Do *et al.* reported similar E2-dependent growth arrest and migration blockade of human VSMCs obtained from postmenopausal women and age-matched men [94]. Likewise, E2 and progesterone, but not 17α-estradiol, estrone, or estriol, inhibited serum-induced proliferation of cardiac fibroblasts in a concentration-dependent manner and to a similar extent in male and female [95].

In contrast, other studies have shown estrogen-dependent induction of VSMC proliferation [96,97]. It is noteworthy in this regard that the growth-regulating effect of estrogen on VSMCs may be dependent upon cellular phenotype. Indeed, addition of E2 to the culture medium delays the cell cycle reentry of contractile (differentiated) VSMCs by retarding their phenotypic modulation, however it promotes the replication of VSMCs displaying a synthetic phenotype [98].

6. EFFECT OF ESTROGENS ON THE CARDIO-VASCULAR SYSTEM: LESSONS FROM ANIMAL STUDIES

The role of estrogens in the cardiovascular system has been investigated in animal models of angiogenesis, mechanical arterial injury, diet-induced atherosclerosis, and graft atherosclerosis. More recently, the analyses of ERα- and ERβ-deficient mice has added significant insight into the role of each ER isoform on the pathophysiology of the cardiovascular system (Table **1**). The majority of these studies have been carried out with gonadectomized animals that received exogenous estrogens or SERMs.

6.1. Angiogenesis

The formation of new blood vessels is abundant during embryonic development and during several pathophysiological processes in adult life, including those involving the reproductive organs (ovary, testes, endometrium and placenta), wound healing, tumor growth, inflammatory vasculitides, and atheroma development [99,100].

E2 enhances *in vitro* activities of ECs that are important during neovascularization (e.g., proliferation, migration and organization into tubular networks) [41,42]. Importantly, ovariectomiced mice display reduced basic fibroblast growth factor (bFGF)-dependent vascularization, and estrogen replacement therapy restored angiogenesis to the levels observed in nonovariectomiced animals [42]. Expression of the proangiogenic protein vascular endothelial growth factor (VEGF) and its receptor (fetal liver kinase-1) were diminished in coronary vessels of ovariectomiced rats, and estrogen replacement therapy restored it to intact levels. Analysis of ERα$_{CH}$-null mice have suggested that functional ERs are essential for the augmentation of bFGF-induced angiogenesis by exogenous E2 in female mice [101].

Blackwell *et al.* have demonstrated the antiangiogenic effects of the ER ligand drug tamoxifen [102]. Likewise,

Table 1. **Murine Knockout Models to Investigate the Role of ERα and ERβ on E2-dependent Protective Functions in the Cardiovascular System. All Studies were Performed on Ovariectomized Females Treated with E2.**

Model	ER-null mouse	Effect	References
Angiogenesis	ERα KO$_{CH}$	Abolished E2-dependent augmentation of bFGF-induced angiogenesis seen in wild-type mice	[101]
Mechanical injury carotid artery	ERα KO$_{CH}$	E2-dependent inhibition of the increase in medial area similar to wild-type mice	[11]
Mechanical injury carotid artery	ERα KO$_{St}$	Abolished E2-dependent inhibition of the increase in medial area seen in wild-type mice	[126]
Mechanical injury carotid artery	ERβ KO$_{CH}$	Estradiol-dependent inhibition of medial area and VSMC proliferation similar to wild-type mice	[123]
Mechanical injury carotid artery	ERα/ERβ KO$_{CH}$ double KO	Abolished E2-dependent inhibition of the increase in medial area seen in wild-type mice (although did not prevent E2-dependent VSMC growth arrest)	[127]
Electric injury carotid artery	ERα KO$_{St}$	Abolished E2-dependent increase in reendothelization as compared to wild-type mice	[121]
Electric injury carotid artery	ERβ KO$_{St}$	Increase in estradiol-dependent reendothelization similar to wild-type	[121]
Atherosclerosis	ERα$_{CH}$apoE double KO	Abolished E2-dependent atheroprotective effects on lesion size and plasma cholesterol level	[154]

ERα KO$_{CH}$: ERα-null mice generated in Chapel Hill [209]. These mice appear to have an incomplete disruption of ERα [124,125].
ERβ KO$_{CH}$: ERβ-null mice generated in Chapel Hill [210].
ERα/ERβ KO$_{CH}$: Double ERα/ERβ-null mice generated by extensive crossbreeding of parental ERα KO$_{CH}$ and ERβ KO$_{CH}$ mice.
ERα KO$_{St}$: ERα-null mice generated in Strasbourg [211].
ERβ KO$_{St}$: ERβ-null mice generated in Strasbourg [211].

tamoxifen reduced VEGF-dependent EC proliferation *in vitro* and attenuated VEGF-mediated angiogenesis in the rat [103]. In addition, 2-methoxyestradiol enhanced apoptosis and reduced proliferation and migration of ECs, and these effects correlated with reduced angiogenesis [104].

6.2. Restenosis and Reendothelization

Restenosis remains the major long-term limitation of percutaneous transluminal coronary angioplasty. According to the response-to-injury hypothesis [85], endothelial denudation at the site of angioplasty plays a critical role in triggering this rapid form of neointimal lesion growth. Recruitment of leukocytes into the denuded arterial wall elicits an inflammatory response, which in turn promotes abundant arterial cell proliferation and migration.

The efficacy of estrogens in inhibiting neointimal thickening has been documented in several experimental models of mechanical vascular injury, including vessel transection and anastomosis [105], cuff placement [91], and vessel denudation after balloon angioplasty [106-109] or wire injury [110]. However, Finking *et al.* did not found inhibition of neointimal formation by estrogen in normo-cholesterolemic rabbits subjected to balloon angioplasty [111]. Moreover, when examined in hypercholesterolemic animals with preexisting atherosclerosis, estrogen suppressed neointimal formation after balloon angioplasty in rabbits [111], but not in primates [112].

Using a rabbit model of balloon angioplasty in cholesterol-clamped rabbits, Holm *et al.* have provided evidence suggesting that the state of the arterial endothelium is a major determinant of the vascular response to estrogen [38]. Gender differences also appear to influence the effect of estrogens on neointimal formation after balloon angioplasty [113]. Neither E2 nor medroxyprogesterone acetate (MPA) altered the neointimal hyperplastic response after balloon angioplasty in intact males. In contrast, estradiol reduced and MPA enhanced the response in intact females, whereas addition of MPA to estradiol blocked the vasoprotective effects of estrogen.

Recently, New *et al.* investigated the effect of E2-eluting, phosphorycholine-coated stent implanted in porcine coronary arteries [114]. As compared with control stents, E2-eluting stents reduced by 40% neointimal formation without affecting endothelial regeneration. The efficacy of E2-eluting Biodiv Ysio stents is under evaluation in 30 patients with de novo coronary lesions [115]. A second phase of the EASTER trial is ongoing to assess the potential benefit of estrogen-coated stents in the prevention and treatment of in-stent restenosis.

The mechanisms that have been proposed to contribute to estrogen-dependent inhibition of neointimal thickening after mechanical injury include inhibition of VSMC proliferation [116], increase in NO production and reduced mononuclear-EC binding [38], and increased rate of reendothelialization of the damaged vessel by means of a mechanism that may depend on endogenous VEGF [117-119]. Consistent with this notion, idoxifene-dependent inhibition of neointimal formation after vascular injury correlated with reduced

VSMC proliferation and enhanced reendothelialization [120]. Treatment with MPA alone did not alter vascular remodelling in the rat carotid injury model, but its addition abolished the antiproliferative effects of estrogen in this model [107]. Using a model of electric arterial injury, it has been shown that ER but not ER mediates the beneficial effect of E2 on reendothelialization [121].

Studies using the nonselective ER antagonist ICI 182,780 have suggested that the vasoprotective effect of E2 in the balloon-injured rat carotid artery model is mediated by ER [122]. Regarding the expression of both ER isoforms in the aorta of male rats, little or no change in ER expression was observed after vascular injury in either ECs or VSMCs at any time point [12]. In contrast, ER mRNA expression was markedly induced after vascular injury, supporting a role for ER in the direct vascular effects of estrogen [12]. Disruption of either ER [11] or ER [123] did not abolish E2-dependent inhibition of neointimal thickening in the mouse, suggesting that either of the two known ERs is sufficient for the vasoprotective function of E2, or that an ER /ER -independent pathway is involved in these effects. It is important to note in this regard that the ER -null mouse model used in these studies (ER $_{CH}$) [11] appears to have an incomplete disruption of ER [124,125]. Indeed, Pare *et al.* have demonstrated the requirement of ER for the protective effects of estrogen against vascular injury using a new ER –null mouse model (ER $_{St}$) [126].

The phenotype of ovariectomized female ER /ER KO$_{CH}$ (double) ER knockout mice is complex [127]. Although E2 failed to inhibit the increase in medial carotid area when these mice underwent vascular injury, it elicited both a reduction in VSMC proliferation and a significant increase in uterine weight.

6.3. Atherosclerosis

The protective effects of estrogen on atherosclerosis have been well documented in numerous animal models, and epidemiological studies support their atheroprotective effects in humans [23-26]. In a study using postmortem coronary artery specimens that were obtained from pre and postmenopausal women, Losordo *et al.* detected ER expression in the majority of normal arteries (15 positive of 21 total) [14]. In contrast, a minority (6 of 19) of atherosclerotic arteries demonstrated evidence of ER . Importantly, the relation between absence of atherosclerosis and ER expression was most evident in premenopausal women. It is also noteworthy that both endothelial dysfunction and premature coronary artery disease were observed in a man bearing a disruptive mutation in ER [128,129]. In this section we will discuss animal models of graft atherosclerosis and diet-induced atherosclerosis that have shed significant insight into the molecular mechanisms underlying the vasoprotective effects of estrogen.

6.3.1. Graft Atherosclerosis

Accelerated atherosclerosis is a major complication after heart transplantation and coronary bypass surgery. E2 treatment inhibits neointimal hyperplasia and protects the endothelium from the degenerative changes normally seen in

aorta allografts of control rabbits [130]. This study also demonstrated reduced number of vacuolized macrophages and VSMCs (foam cells) in animals treated with estrogen. Using a rat model of aorta allograft transplantation, Saito *et al*. demonstrated E2-dependent elevation of eNOS expression in the intima and suppression of inducible NOS (iNOS) in all three vessel layers in the early phase following trans-plantation [131]. Local administration of estrogen in rat aorta inhibits transplant arteriosclerosis accelerated by topical exposure to IGF-I [132].

E2 protects against experimental cardiac transplant atherosclerosis [109,133]. Chronic E2 treatment of cardiac allograft recipient rabbits abolishes major histocompatibility complex class II antigen expression in the coronary arteries and decreases macrophage and T cell infiltration, suggesting that immune mechanisms also contribute to the beneficial effect of E2 on graft arteriosclerosis [134].

6.3.2. Diet-induced Atherosclerosis

The protective effects of estrogens against the development of diet-induced atherosclerosis have been demonstrated in several animal models, including rabbits [33,35,135-139] and monkeys [28,31].

Regarding the mechanisms underlying the atheroprotective effects of estrogen, it has been shown that E2 preserves endothelial vasodilator function and limits LDL oxidation in hypercholesterolemic swine, thus suggesting a favourable effect of E2 on vascular function and coronary artery disease by virtue of its antioxidant properties [140]. Wagner *et al*. reported that estrogen and progesterone replacement therapy reduces LDL accumulation in the coronary arteries of surgically postmenopausal cynomolgus monkeys, suggesting that one mechanism by which sex hormone treatment inhibits the initiation of atherosclerosis is a direct effect at the level of the arterial wall by suppressing the uptake and/or degradation of LDL [31]. However, several studies have suggested that estrogen-mediated cardiovascular protection is incompletely explained by its beneficial lipid-modifying effects [33,35,137,141-143]. Selzman *et al*. have suggested that estrogens may provide atheroprotection independently of its effects on serum lipids both by modulating local production of bFGF and by attenuating the influence of this mitogen on VSMC growth [144].

Hanke *et al*. have compared the effect of estrogen and progesterone on the development of atherosclerosis in female versus male rabbits to assess possible sex-specific differences [145]. An inhibitory effect of estrogen on atheroma size was found in female rabbits, which correlated with reduced arterial cell proliferation. In contrast, E2 administration to castrated male rabbits was not associated with an inhibitory effect on cellular proliferation or intimal thickening compared with controls.

Conjugated equine estrogen (CEE) reduced the extent of coronary artery atherosclerosis in ovariectomized cynomolgus monkeys fed atherogenic diets [142,146,147]. However, addition of CEE or CEE plus MPA to a lipid-lowering diet did not further augment the improvements of the dietary modification alone, except for the dilator capacity of the coronary microcirculation [148]. Raloxifene, 17 alpha-dihydroequilin sulfate (DHES), a water-soluble estrogen of conjugated estrogens (Premarin), and ethynylestradiol, a commonly used estrogen found in many oral contraceptives, reduced atheroma development in cholesterol-fed rabbits [135,136,143,149].

Several animal studies have investigated the efficacy of combined hormone replacement therapy (HRT). Haarbo *et al*. found that norethisterone acetate or levonorgestrel, two commonly prescribed progestogens, do not attenuate the beneficial effect of E2 on coronary atherosclerosis in cholesterol-fed rabbits [35]. In contrast, Adams *et al*. reported that levonorgestrel, at doses used in modern oral contraceptive formulations, antagonizes the atheroprotective effect of E2 on coronary atherosclerosis of cholesterol-fed cynomolgus monkeys [150]. Doses of progestin (hydroxyprogesterone caproate) that are able to successfully reduce the proliferative effect of estrogen on endometrium do not diminish the desirable antiatherosclerotic properties of estrogen in cholesterol-fed rabbits [139]. In contrast, progesterone was dose-dependently able to completely inhibit the beneficial effect of estrogen in cholesterol-fed rabbits, suggesting that progesterone exerts a direct inhibitory effect on the atheroprotective action of estrogen [141,145]. Combined, continuously administered CEE plus MPA is a prescribing pattern that has gained favour. Adams *et al*. reported that continuously administered oral MPA antagonizes the beneficial effect of CEE monotherapy on coronary atherosclerosis extent in cholesterol-fed ovariectomized cynomolgus monkeys [142]. In contrast, Clarkson *et al*. found that both CEE and CEE+MPA reduced coronary atherosclerosis in this animal model [147].

Several studies have investigated the role of estrogens on atherosclerosis using mice with targeted inactivation of the apolipoprotein E (apoE) [151,152] and LDL receptor (LDLR) [153] genes, which respond to moderate amounts of dietary cholesterol with severe hypercholesterolemia and develop lipid-rich vascular lesions resembling human atherosclerotic plaques.

Bourassa *et al*. provided evidence suggesting that E2 protects against atherosclerotic lesion formation in apoE-null mice, and this could only be partially explained through effects on plasma lipoprotein levels [29]. Both in animals fed control or atherogenic diet, ovariectomy resulted in augmented aortic atherosclerosis compared with female mice with intact ovarian function without changes in plasma cholesterol. E2 replacement therapy significantly decreased atherosclerosis both in male and ovariectomized female mice. In contrast, neither E2 nor tamoxifen affected lesion progression in ovariectomized female mice. In the atherogenic diet-fed group, exogenous estradiol markedly reduced plasma cholesterol and triglycerides, whereas, in animals fed the chow diet, exogenous estrogen and tamoxifen treatment only decreased plasma cholesterol and VLDL triglycerides. However, lesion area was only weakly correlated with plasma cholesterol and triglycerides. Likewise, Hodgin *et al*. reported that the extent of atheroprotection by E2 in ovariectomized apoE-null female mice was greater than could be explained solely by the change in lipid levels [154].

Tangirala *et al*. found significantly more atherosclerotic lesions in male LDLR- and apoE-null mice than females [155]. Elhage *et al*. have investigated the effect of exogenous E2 on atherosclerosis in castrated male and female apoE-null mice fed with a low-fat chow diet [156]. E2 treatment resulted in decreased concentrations of total serum cholesterol, LDL-cholesterol, and HDL-cholesterol in each sex, however these parameters were weakly correlated with lesion area. A highly significant correlation between serum E2 levels and atheroma size suggested a direct action of E2 on cells of the vascular wall. These authors provided evidence suggesting that these activities are sex-dependent, with females being nearly twice as sensitive to E2 as males. E2 also protected LDLR-deficient female mice from atherosclerosis, and this protection appears to be independent of changes in plasma cholesterol levels [157].

Oral tamoxifen attenuated atheroma development in apoE-null mice, whether the mice were fed with a control diet or a diet with high-fat content [158]. This inhibitory effect of tamoxifen correlated with changes in lipoprotein profile (a shift from LDL to HDL cholesterol and a decrease in total triglycerides) and with elevated levels of transforming growth factor- , both of which are thought to be protective against atherosclerosis in humans and animal models.

Hodgin *et al*. investigated the effect of E2 on aortic atherosclerosis in ovariectomized mice lacking apoE or both apoE and ER α (ER α KO$_{CH}$apoEKO mice) [154]. As compared to apoE-null mice, E2 treatment of ER α KO$_{CH}$apoEKO mice caused minimal reduction in lesion size and did not reduce total plasma cholesterol, demonstrating a critical role of ER α in the atheroprotective effect of E2. Nevertheless, E2 treatment significantly reduced atheroma complexity in the ER α KO$_{CH}$apoEKO females, although not to the same degree as in apoE-null females, suggesting the existence of ER α-independent atheroprotective effects of E2.

Dietary soy isoflavones reduced atherosclerosis both in apoE-null mice and doubly deficient ER β $_{CH}$apoEKO mice [159]. In contrast, the atheroprotective effects of this dietary regime was unaffected in ER β KO$_{CH}$apoEKO mice, demonstrating a necessary role for ER β-dependent pathways in mediating the atheroprotective effects of dietary soy isoflavones.

Hodgin *et al*. investigated the interactions between eNOS and E2 in vascular protection in mice [160]. They found that exogenous E2 has strong blood pressure-lowering and atheroprotective effects both in apoE-null mice and doubly deficient eNOSapoEKO mice, indicating that eNOS is not essential for either effect. However, endogenous sex hormones cause significant damage to the vasculature in the absence of eNOS, but these effects are overridden by interactions between eNOS and sex hormones.

Fatty streak development in immunodeficient mice with homozygous disruption of both the apoE and the recombinase activating gene 2 loci is insensitive to E2, thus suggesting that lymphocytes are required for development of the atheroprotective effect of estradiol [161].

Elhage *et al*. investigated the role of interleukin-6 (IL-6) in the atherosclerotic process and in the atheroprotective effect of E2 by examining double deficient mice at the apoE

and IL-6 loci [162]. At 1 year of age, IL-6apoEKO mice showed similar hypercholesterolemia compared to apoE-null mice but disclosed larger and more calcified lesions. IL-6 disruption nor did affect fatty streak formation in younger mice, neither prevented E2-dependent decrease of fatty streak formation. Thus, IL-6 appears to be involved at the fibrous plaque stage of the atherosclerotic process but does not participate in early fatty streak formation in this animal model. Moreover, IL-6 is not required for E2-dependent inhibition of fatty streak formation.

7. HRT TO PREVENT ISCHEMIC CORONARY DISEASE IN POSTMENOPAUSAL WOMEN

Much of the interest in the action of estrogens on the vascular tree derives from the relevance of CVD, a broad concept including coronary heart disease (CHD), stroke and venous thromboembolic disease as principal entities. As confirmed for men, CHD is the leading cause of death in women. The observation that, as compared with men, women maintain some level of protection, especially before menopause, has nourished a debate about a possible favorable effect associated with exposition to estrogens. Furthermore, estrogens have been associated with many benefits both in experimental and clinical models at the level of intermediate indicators. The hypothesis that a certain estrogenic threshold might be protective, therefore, has been consistently proposed in recent years. However, only the availability of unequivocal clinical trials may definitively confirm whether the final balance of estrogens is beneficial or not. Consequently, much interest has been concentrated on the data obtained in several recently published clinical studies.

There is a variety of research strategies addressing the association of estrogens with CHD. Some epidemiological studies have investigated the modifications of risk as a function of the changes in endogenous estrogens. The exposure to high estrogenic intervals, as in pregnancy, or the length of ovarian life has been studied as eventual modulators of risk [163-165]. Attention has also been given to the changes in risk occurring during menopausal transition [164,166]. Most effort, however, has concentrated on studying the effect that HRT may have on CHD risk. This has been for two main reasons: first, because this therapy is used in women of an age where CHD risk starts to be manifest, and second, because the number of hormone users is potentially enormous, thus converting the issue into one of particular significance for public health.

Given the beneficial profile observed for estrogens on a range of coronary risk factors, HRT has been proposed both for primary and secondary prevention. As a result, a series of studies has been accumulating in recent years. Most of them, however, have been observational in design, and only recently have randomized, placebo-controlled studies been available. The objective of this review is to briefly revise the main evidence on the effects of HRT on CHD risk as observed in primary and secondary prevention trials.

7.1. Primary vs. Secondary Prevention

The large body of knowledge rapidly gathered on many mechanisms and effects set in motion by estrogens on

coronary risk factors, together with the global beneficial profile obtained, moved investigators to initiate trials in both primary and secondary prevention settings. Against the seeming results obtained in most observational trials, the publication of the Heart Estrogen/Progestin Replacement Study (HERS) in 1998 [167], the first randomized, placebo-controlled trial on secondary prevention, defined a critical point. Against the almost unanimous protection reported in the previous secondary prevention observational studies, the HERS trial found an overall neutral effect (see later). Interestingly, the total null effect found in HERS resulted from a pattern of consistent risk reduction that during the first year, involved a 50% increase in risk. This observation gave way to the "early harm" hypothesis, which has been used in the analysis of subsequent experimental data. One main contribution of HERS was that in an attempt to conciliate its results with the large body of experimental and clinical data favoring benefit, effort was made to distinguish between primary and secondary prevention.

Investigators soon paid attention to the concept that against the healthy status of the vessel wall expected in the women of primary prevention trials, a high prevalence of atherosclerosis should be encountered in the women participating in secondary prevention studies. This point is crucial, since sufficiently advanced atherosclerosis and more specifically plaque composition, is the key factor conditioning coronary events. Atheromatous plaques favor acute focal thrombosis, thus leading to partial or complete occlusion of the vessel lumen. Either erosion of the endothelial surface or, particularly, the disruption of the cap of a lipid-rich plaque, are the triggering factors of thrombus initiation (for a review see [168]). Consequently, the presence or absence of atherosclerotic lesions at a stage capable of promoting thrombus initiation, i.e., advanced lesions, is pivotal for determining coronary risk. Since the prevalence of advanced lesions should be higher in women with previous coronary disease, it can be advanced if the local environment in the coronary vessel changes in women depending on whether studies are of primary or secondary prevention. Further, following this logic, one can hypothesize that the effect of hormones may vary in one or the other setting. We will analyze, therefore, primary and secondary prevention studies separately. When describing the available literature, the information will be presented as a function of the quality of the evidence, separating the observational from the randomized studies. For obvious reasons, more attention will be paid to the latter studies.

7.2. Secondary Prevention

7.2.1. Observational Studies

Until relatively recent time, this has been the only source of information available. In agreement with the global beneficial profile of intermediate risk factors associated with the use of estrogens, either alone or in combination with progestogens, the observational studies confirmed a protective effect [169,170]. These clinical and laboratory data were in agreement with classical studies on primates, which showed a consistent reduction of atherosclerosis in animals with surgical menopause that were supplemented

with hormones [28,171,172]. Investigators critical of these observational data have claimed the well-proved selection bias of women having hormones [173,174]. As an example, high socioeconomic status is associated with lower rates of cardiovascular disease and higher rates of HRT [175]. Thus, rather than HRT keeping women healthy, healthy women were taking HRT. Despite these criticisms, the overall agreement between trials, plus the concordant experimental and biological studies, determined an almost unanimous support of the protective hypothesis.

7.2.2. The HERS Study

Although not the only evidence available on secondary prevention at present, the HERS Study [167] set a milestone in the understanding of the relationship of HRT to CHD. A multi-centre, double-blind, randomized trial, the HERS included 2763 postmenopausal women with previous CHD. Women were randomized to either HRT or placebo and were followed for an average of 4.1 years. Contrary to what might be anticipated from the previous evidence, the use of HRT turned out to be neutral (relative hazard (RH): 0.99, 95% confidence interval (CI): 0.81-1.22). Interestingly, a time-trend sub-analysis of these data demonstrated an increased risk for CHD events in the first year of therapy followed by a decreased risk for CHD events in years 4 and 5.

The surprising results of HERS set in motion a critical movement against the previous evidence from observational trials and, at the same time, a very critical judgement of the HERS development. It was claimed that the trial was significantly underpowered as a result of a defective completion of the design conditions [176]. For example, the crossover rate between groups was too high, and the event rate was lower than expected in both the placebo and the HRT groups. It was argued that the use of other preventive therapies by the women, such as statins, aspirin, or others, might have been a difficulty to permit HRT the development of its potential protective action. The possibility that the early increase in CHD events might have been influenced by pro-thrombotic susceptibility in some women was also claimed. Nonetheless, the most solid criticisms were perhaps those underlining the advanced age of the women participating in the study (average age 67 years) or the particular HRT combination selected. The treated women received 0.625 mg/d CEE and 2.5 mg/d of MPA, a combination that has raised concern because of the progestogen component: MPA has been shown to decrease the beneficial effects of estrogen on coronary vasoreactivity and inhibition of atherosclerosis in primates [142,177].

In consonance with these data, on July 24, 2001, the American Heart Association released a consensus statement addressing the use of HRT in women with heart disease [178]. The summary recommendations stated, "HRT should not be initiated for the secondary prevention of cardiovascular disease".

More recently, data from two other studies have become available. First, the HERS follow-up Study (HERS II) [179], which consisted of an unblinded follow-up of patients participating in HERS. The purpose of this study was to clarify whether the risk reduction observed in the later years

of HERS persisted and resulted in a reduced risk of CHD events with additional years of follow-up. A total of 2321 women (93% of those surviving) accepted to follow-up for 2.7 years. Contrary to the declining trend observed in HERS, there were no significant decreases in rates of primary CHD events or secondary cardiovascular events among women assigned to the hormone group compared with the placebo group in HERS II or overall. A second study was developed in England on 1027 women who had survived a first myocardial infarction (MI) [180]. The participants were randomized to estradiol (2 mg/d, orally) or placebo. There was no progestin included in the protocol. After 24 months follow-up the frequency of reinfarction or cardiac death did not differ between treatment groups (RH: 0.99, 95% CI: 0.70-1.41).

Other Trials of HRT for Secondary Prevention

7.2.3.1. Studies Measuring Artery Wall Thickness

a. The Estrogen Replacement and Atherosclerosis (ERA) Trial

This was a randomized, placebo-controlled study, in which women who had at least one coronary stenosis of 30%, as confirmed by quantitative coronary angiography, were subjected to estrogen (CEE 0.625 mg/d), an estrogen plus progestogen combination (CEE plus MPA 2.5 mg/d), or placebo [181]. Again, women were not in their early postmenopausal period (mean age 65.8 years). The follow-up attained an average of 3.2 years. The primary endpoint was mean minimum coronary artery diameter at baseline and follow-up. There were clear improvements in the lipid profile in women ascribed to any of the two hormone arms of the study. Nonetheless, the three groups evolved similarly, since no difference in mean minimum artery diameter was found. This annotation is of interest because it might be taken to suggest that the prejudicial effect ascribed to progestogens is not well supported.

b. The Postmenopausal Hormone Replacement Against Atherosclerosis (PHOREA) Trial

This German study included 321 postmenopausal women aged between 40 and 70 years [182]. The objective of the study was to discern whether HRT slow could down the progression of atherosclerosis, measured as intima-media thickness in carotid arteries by ultrasound. In addition to the placebo group, there were two other groups receiving 1 mg/d oral estradiol with gestodene that was administered for 12 days of either each 4-week cycle or each third month. After 48 weeks of treatment, no differences were found between placebo and any of the two treatment arms of the study.

7.2.3.2. Not Concluded Studies: Papworth HRT Atherosclerosis Study Enquiry (PHASE)

PHASE [183] was stopped before completing recruitment due to the publication of the HERS trial. The interesting feature of this study was that the estrogen (estradiol) was administered transdermally. Angiographically proven coronary artery disease was the inclusion requisite, and hospitalization for unstable angina, MI, and death were the primary endpoints. The mean duration of follow up was 30.8 months. Although there was a more than expected drop out rate in the HRT group (40%), no statistical significance could be detected between both arms of the study when an intention-to-treat analysis or a per-protocol analysis were performed.

7.2.3.3. HRT and Stroke: WEST

Stroke defines another form of CVD which, together with CHD, exhibits a high prevalence in women [184]. The Women's Estrogen for Stroke Trial (WEST) included 652 postmenopausal women (mean age 71 years) with cerebro-vascular disease [185]. The trial had two arms, HRT (1 mg/d estradiol) or placebo. During a mean follow-up period of 2.8 years, there were 99 strokes or deaths among the women in the estradiol group, and 93 among those in the placebo group (RH: 1.1; 95% CI: 0.8-1.4). Estrogen therapy did not reduce the risk of death alone (RH 1.2; 95% CI: 0.8-1.8) or the risk of nonfatal stroke (RH: 1.0; 95% CI: 0.7-1.4). The main conclusion of this study was that estradiol does not reduce mortality or the recurrence of stroke in postmenopausal women with cerebrovascular disease. The authors then suggested that this therapy should not be prescribed for the secondary prevention of cerebrovascular disease.

7.2.3.4. Observational Evidence Revisited: The Nurses' Study

The contrast between data from placebo-controlled trials and the previous observational studies has been attributed to the susceptibility of the latter to distinct potential biases. Nonetheless, the remarkable unanimity found in obser-vational studies has raised questions among investigators. In an attempt to enlighten this apparent contradiction, the data from the Nurses' Health Study, a well-reputed observational study including a high number of postmenopausal women, were re-examined [186]. The purpose was to ascertain which had been the effect of hormones on the subset of women with medical conditions similar to those of participants in secondary prevention trials. Among 2489 women with previous MI or documented atherosclerosis, 213 cases of recurrent nonfatal MI or coronary death were identified. A significant increase for recurrent CHD (multivariate-adjusted RH: 1.25, 95% CI: 0.78 to 2.00) was detected for hormone users when compared with never users. In sharp agreement with HERS, the rate of second events was lower in current users than in never users in the long term.

One notable message from this study was that an adequate examination of data could fit the results from observational and randomized studies. As an additional conclusion, the observational studies seemed reinforced as an analytical tool. The reflection was of interest since the unanimous beneficial effect detected in observational studies on primary prevention gained credibility. The hypothesis that hormones may be protective in primary prevention but prejudicial in secondary prevention was then released.

7.2.4. Lessons from the Secondary Prevention Trials

The results of HERS, together with subsequent evidence reviewed above, altered the view of hormone action on CVD

to a high degree. Despite the many questions raised by the unsatisfactory rendition of HERS, the rather concordant results of the subsequent studies has strengthened the notion that the dogma of the protective effect of HRT against CHD should be subjected to a new interpretation. The large amount of evidence in favour of a direct action of hormones on the vascular wall proved to be particularly useful to understanding something that, being obvious, had perhaps been overlooked, that is, the realization that the environment defined by an atherosclerotic wall differs from that conditioned by a healthy vessel. A diseased endothelium, in close partnership with one or several atheroma plaques, constitutes both a source of inflammatory mediators as well as a pro-aggregant surface for platelets. Both conditions are considered to be crucial for thrombosis and subsequent reduction/occlusion of the lumen. Disruption of the cap of a lipid-rich plaque is a more frequent determinant of thrombus formation than endothelial erosion [187]. A rapid growth of the knowledge of plaque biology has made clear that the action of estrogens may be less than protective, even clearly deleterious for the stability of the lipid-rich plaques. It has been shown that oral, but not transdermal estrogens, increase the circulating level of C-reactive protein [188,189], an inflammatory marker that has been shown to stimulate cytokine production, activate the complement pathway in arteries, and induce expression of adhesion molecules [190,191]. Factors leading to the vulnerability of plaque have been investigated, too. Matrix metalloproteinases, derived from macrophages within the lesion, are very active in causing the fibrous cap to thin and become prone to rupture. Recent work has shown that HRT increases the level of matrix metalloproteinase-9 [192]. When this group of evidence is analyzed in light of the previous data favoring a beneficial effect of the hormones, it is rather clear that most of that evidence may be categorized as protective against atherosclerosis initiation or progression.

In conclusion, the concept that hormones may be protective in primary prevention, but neutral or clearly prejudicial in secondary prevention was gaining momentum in the understanding of the role of HRT in CHD. This consensus was in agreement with the clinical evidence available at that moment which, for primary prevention, consisted of observational studies suggesting HRT would be beneficial.

7.3. Primary Prevention

7.3.1. Observational Studies

Studies including several hundred thousand woman-years of follow-up were unanimously confirming that the use of HRT was associated with a 40% to 60% reduction in CHD events. A paradigm of that evidence has been the Nurse's Health Study, which has been re-updated regularly, and has been discussed above. Elegant meta-analyses have systematized this evidence and confirmed the benefit [193,194].

7.3.2. The WHI Study

The absence of high-level evidence studies on the effects of HRT on CHD in healthy postmenopausal women prompted the National Institutes of Health to set up the Women's Health Initiative study (WHI), a huge trial with two main arms, depending on the hysterectomy status of the participating women. A total of 27,347 women aged 50 to 79 years accepted to enter into this randomized, placebo-controlled study. Non-hysterectomized women (n=16,608) received the same oral HRT combination as in HERS (0.625 mg/d CEE plus 2.5 mg/d MPA) or placebo, while hysterectomized women received only 0.625 mg/d CEE in the treatment arm. While this second arm of the study is still open, the combined treatment arm was stopped early based on health risks that exceeded health benefits over an average follow-up of 5.2 years [195].

Concerning CHD, the estimated RH was 1.29 (95% CI: 1.02-1.63, p<0.05), an increase that had already been detected during the first year of treatment. The estimates of cumulative hazard indicated that there was no evidence for convergence through 6 years of follow-up. Stroke rates were also higher in women receiving hormones with an estimated RH of 1.41 (95% CI: 1.07-1.85) but, in contrast to CHD, the cumulative hazard curves began to diverge between 1 and 2 years after randomization.

One main conclusion of WHI was that, contrary to the interpretations raised after HERS, HRT seemed not only neutral, but also slightly prejudicial in primary prevention.

7.3.3. Other Trials of HRT for Primary Prevention

7.3.3.1. WISDOM

The Women's International Study for long Duration Oestrogen after the Menopause (WISDOM) was designed and initiated in the United Kingdom [196]. The objective was to include 22,000 postmenopausal women (16000 women from the United Kingdom plus 6000 from Australia and New Zealand) who had to be randomized to either HRT (same protocol as in WHI), or placebo. The planned follow-up period was 10 years that, in 1999, was reduced to 5 years as emerging evidence suggested that the optimum follow-up time to determine the effect on breast cancer would be 5 years after treatment was discontinued. Soon after publication of the WHI data, however, WISDOM was stopped by the British Medical Research Council for "scientific and practical reasons" [197]. It was added, "the Independent International Committee was concerned by the slow progress of WISDOM and considered that the results of the trial, which would not be available for another decade, would be unlikely to influence clinical practice". WISDOM had the potential to recruit younger and healthier women than those recruited in WHI and might have better investigated the still untested hypothesis that HRT may have a primary cardioprotective role in the early postmenopausal woman, by helping to prevent atherosclerosis [198,199].

7.3.3.2. Studies Measuring Artery Wall Thickness: EPAT

The Estrogen in the Prevention of Atherosclerosis Trial (EPAT) has the great interest of having recruited healthy women [200]. It tested, consequently, whether HRT reduces the risk of atherosclerosis under randomized and placebo-controlled conditions. Two hundred and twenty-two post-menopausal women 45 years of age or older were subjected

to either estradiol (1 mg/d) or placebo. The primary endpoint was the rate of change in intima-media thickness of the right distal common carotid artery far wall for a period of 2 years. The progression of subclinical atherosclerosis was lower in women taking estradiol who did not associate lipid-lowering medications, a combination imposed only in women whose LDL levels exceeded 160 mg/dL.

7.3.4. Lessons from the Primary Prevention Trials

The WHI Study provides the most frequently used information presently used to take a position about the role of HRT in CHD prevention. Comments and opinions have arisen in the literature to underscore the prejudicial effect of HRT on the distinct forms of CVD, CHD being the most important, as well as stroke and venous thrombosis [201-204]. To further support the conclusions of the WHI Study, additional elaboration of the available data has subsequently confirmed the lack of evidence in favor of a protective effect of HRT against either CVD or CHD [205,206]. Despite this apparent unanimity, however, some debate has emerged to stress that, despite the value of the WHI data, uncertainty still exists [207].

It has been commented that as WHI evolved a number of weaknesses seriously affecting the validity of data arose. A substantially high drop out rate was detected in the HRT group, where 42% of women stopped treatment due to bleeding or other unwanted effects. As a consequence, unmasking occurred in 40.5% of the treated group and in 6.8% of the placebo group. In order to respect randomization, intention-to-treat analysis was applied. All participants were assigned to their original group, regardless of the number of drop outs and drop ins. Therefore, it can be concluded that assessing the long-term benefits of a treatment based upon results from subjects who received treatment for only a short period is a risky exercise.

Finally, some other points need to be addressed. Together with the strong argument that the estrogen-only arm of the WHI trial is still open, the consistency across studies of the several beneficial effects of HRT on crucial risk markers cannot be dismissed. There is also the possible influence of variables related with the particular combination of HRT that was used, as commented for HERS. The age of patients is another important concern. As mentioned above, women in both the WHI Study, as well as those in HERS, exceeded the age of most of the women starting HRT by a decade. The higher prevalence of atherosclerosis in women at that age, as confirmed by the identification of 400 women with established coronary artery disease upon entry, raises the question of whether or not this was a primary prevention study. Every argumentation on the altered behavior of a diseased artery wall subjected to hormones has to be reexamined. In fact, one cannot state that the data from WHI have shown that HRT is or is not cardioprotective in women treated from menopause.

8. CONCLUDING REMARKS

Many doubts persist regarding the benefits of HRT on CVD, despite the availability of such apparently unequivocal data, as those derived from the WHI Study. Nonetheless, it is also clear that the expectations generated by the combination of biological data and observational studies have not been fulfilled. Consequently, the indication of CVD prevention cannot be sustained for HRT. The accumulation of the biological benefits of hormones on the side of atherosclerosis prevention seems to indicate that perhaps the utility of HRT may be understood from that point of view. The epidemiological evidence commented above is supported by recent experimental data on monkeys, where the atherosclerotic development associated with social subordination may be interfered with concomitant use of oral contraceptives [208]. Adequately designed studies recruiting women in the early postmenopausal period may answer this crucial question, something that cannot be clarified with the information available at present. Whether other hormonal preparations or alternative routes of administration may improve the risk/benefit profile remains unanswered too. If trials as WISDOM had continued, some additional light might have been shed on this important matter.

ABBREVIATIONS

apoE	=	Apolipoprotein E
bFGF	=	Basic fibroblast growth factor
CVD	=	Cardiovascular disease
CHD	=	Coronary heart disease
CI	=	Confidence interval
CEE	=	Conjugated equine estrogen
DHES	=	17 -dihydroequilin sulfate
E2	=	17 -estradiol
EC	=	Endothelial cell
eNOS	=	Endothelial nitric oxide synthase
ER	=	Estrogen receptor
ERE	=	Estrogen-responsive element
ERK	=	Extracellular signal-regulated kinase
HDL	=	High-density lipoprotein
HRT	=	Hormone replacement therapy
HSP	=	Heat shock protein
IL-6	=	Interleukin-6
iNOS	=	Inducible nitric oxide synthase

LDL	=	Low density lipoprotein
LDLR	=	Low density lipoprotein receptor
mER	=	Membrane estrogen receptor
MI	=	Myocardial infarction
MPA	=	Medroxyprogesterone acetate
NO	=	Nitric oxide
PI3K	=	Phosphatidylinositol 3-kinase
RH	=	Relative hazard
SERM	=	Selective estrogen receptor modulator
VEGF	=	Vascular endothelial growth factor
VSMC	=	Vascular smooth muscle cell

CLINICAL TRIALS

EASTER	=	Estrogen and Stent to Eliminate Restenosis
EPAT	=	Estrogen in the Prevention of Atherosclerosis Trial
ERA	=	Estrogen Replacement and Atherosclerosis
HERS	=	Heart Estrogen/Progestin Replacement Study
PHASE	=	Papworth HRT Atherosclerosis Study Enquiry
PHOREA	=	Postmenopausal Hormone Replacement against Atherosclerosis
WEST	=	Women's Estrogen for Stroke Trial
WHI	=	Women's Health Initiative
WISDOM	=	Women's International Study for long Duration Oestrogen after the Menopause.

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