REVIEW

Estrogens and atherosclerosis: insights from animal models and cell systems

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Abstract

Estrogens not only play a pivotal role in sexual development but are also involved in several physiological processes in various tissues including vasculature. While several epidemiological studies documented an inverse relationship between plasma estrogen levels and the incidence of cardiovascular disease and related it to the inhibition of atherosclerosis, an interventional trial showed an increase in cardiovascular events among postmenopausal women on estrogen treatment. The development of atherosclerotic lesions involves complex interplay between various pro- or anti-atherogenic processes that can be effectively studied only *in vivo* in appropriate animal models. With the advent of genetic engineering, transgenic mouse models of atherosclerosis have supplemented classical dietary cholesterol-induced disease models such as the cholesterol-fed rabbit. In the last two decades, these models were widely applied along with *in vitro* cell systems to specifically investigate the influence of estrogens on the development of early and advanced atherosclerotic lesions. The present review summarizes the results of these studies and assesses their contribution toward better understanding of molecular mechanisms underlying anti- and/or pro-atherogenic effects of estrogens in humans.

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Introduction

Sexual hormones are intimately involved in the pathogenesis of atherosclerosis. The strikingly lower incidence of myocardial infarction (MI) in premenopausal women than in men in the same age group, together with the dramatic increase in coronary risk after menopause is strongly suggestive of an important role of estrogen levels in the etiology of MI in women (Lerner & Kannel 1986, Pérez-López *et al.* 2009). Studies on males with defective estrogen action add further support to the notion that these hormones exert potent atheroprotective effects. Actually, accelerated development of atherosclerosis has been observed in male subjects deficient in P450 aromatase, an enzyme that converts androgens to estrogens, and characterized by estradiol (E₂) levels below the detection limit (Maffei *et al.* 2004). More importantly, estrogen treatment in aromatase-deficient subjects reversed atherosclerotic changes in the carotid artery. While the above results provide a strong argument for the atheroprotective effects of estrogens, they are in a remarkable contradiction to other data suggesting that, under certain

circumstances, these hormones promote the development of atherosclerosis. First, epidemiological studies examining the efficacy of menopausal hormone therapy demonstrated that E₂ applied together with progestins increases coronary risk in postmenopausal women both with and without clinically established coronary heart disease (Hulley *et al.* 1998, Rossouw *et al.* 2002). Secondly, no or even positive correlation between plasma estrogen levels and atherosclerosis has been found in male subjects with intact E₂ synthesis (The Coronary Drug Project Research Group 1970, Muller *et al.* 2004). In addition, administration of E₂ in such males has been reported to increase the risk of MI and stroke. While reasons for these profound discrepancies remain obscure, they may be related to the following:

- divergent estrogen levels – various treatment regimens have been used in both clinical and animal studies for estrogen substitution. It cannot, therefore, be excluded that estrogens exert both anti- and pro-atherogenic effects depending on actual plasma hormone concentrations;

- distinct estrogen receptors – estrogens act via distinct receptors including nuclear receptors estrogen receptor α (ER α) and β (ER β) as well as the recently identified plasma membrane-bound and G protein-coupled GPR30 (GPER). The exact involvement of these receptors in processes related to the pathogenesis of atherosclerosis has not been fully explored;
- distinct cell targets of estrogen action – atherosclerotic lesion arises as a consequence of complex interplay between lipoproteins, inflammatory mediators, and various cell types (such as macrophages, endothelial cells, smooth muscle cells, B-cells, dendritic cells, platelets, myofibroblasts, mast cells, etc.). It is conceivable that estrogens simultaneously exert both pro- and anti-atherogenic effects depending on their cellular target.

Examination of vascular lesions in animals represents an integral part of experimental approaches helping to study arteriosclerotic processes. With the advent of genetic engineering, transgenic mouse models have supplemented classical dietary cholesterol-induced disease models such as cholesterol-fed hamster, rabbit, pig, or monkey. In the last two decades, these transgenic models were widely used to specifically investigate the influence of estrogens on the development of atherosclerotic lesions. The present review summarizes the results of these studies and assesses their contribution toward better understanding of molecular mechanisms underlying anti- and/or pro-atherogenic effects of estrogens in humans.

Effects of estrogens on atherosclerosis in animal models

Atherosclerosis is often defined as a multifactorial disease. While selected pro- or anti-atherogenic processes can be effectively studied under *in vitro* conditions, the complex interplay between them can be explored only in appropriate animal models. Even though there is no one perfect animal model that completely replicates the stages of human atherosclerosis, cholesterol feeding is a common feature shared by most of them. Increased dietary cholesterol feeding produces a permanent hyperlipidemic state, which favors penetration of pro-atherogenic lipoproteins into the arterial wall and produces chronic inflammation accompanied by monocytes promoting the recruitment of monocytes into the vessel wall. While a cholesterol-rich diet induces the development of atherosclerotic lesions in the disease-prone C57Bl6 mouse strain, these are usually small and do not progress to resemble human lesions. However, mice deficient in apolipoprotein E (APOE) or LDL receptor (LDLR), which were generated

on a C57Bl6 background, are characterized by highly pro-atherogenic dyslipidemia with high LDL and low HDL levels in plasma and develop human-like lesions.

The effects of estrogens on diet-induced atherosclerosis have been studied in atherosclerosis-prone B6 mice as well as in *Apoe* KO and *Ldlr* KO mice under hypercholesterolemic conditions (Bourassa *et al.* 1996, Elhage *et al.* 1997a,b, 2001, Hodgkin *et al.* 2001, 2002, Mayer *et al.* 2005, Tsuda *et al.* 2005, Seli *et al.* 2007, Villablanca *et al.* 2009). In female animals, the study design usually encompassed surgical or pharmacological castration followed by E₂ substitution to assure E₂ concentrations in plasma comparable to those seen in premenopausal women before ovulation (ca. 100 and 200 pg/ml). Under such experimental conditions, ovariectomy exerted no effect or aggravated, while E₂ substitution almost invariably ameliorated atherosclerosis. Additional studies documented that varying dose and timing of estrogen replacement critically affect the disease development. Elhage *et al.* (1997a) have shown that low-dose E₂ fails to prevent fatty streak formation in *Apoe* knockout (KO) mice, while Freudenberg *et al.* (2010) reported even increased atherosclerosis in the same animal model treated with low E₂ concentrations. Cann *et al.* (2008) found that E₂ is ineffective in reducing atherosclerotic burden in *Apoe* KO mice, when administered 45 days after ovariectomy. The latter observation corroborates the results of interventional studies showing that women undergoing a period of low estrogen levels followed by reintroduction of estrogen treatment are more likely to experience a cardiovascular event than those on continuous treatment.

In addition to E₂, natural E₂ metabolites or phytoestrogens also exert anti-atherogenic effects in animal models. For instance, 2-methoxyestradiol (2-ME), which is the major metabolite of E₂ formed via sequential conversion of E₂ to 2-hydroxyestradiol and 2-ME by cytochrome P450 and catechol-*O*-methyltransferase (COMT) and is produced in various tissues in addition to ovary (Zacharia *et al.* 2004), was demonstrated to attenuate atherosclerosis in female *Apoe* KO mice (Bourghardt *et al.* 2007). Similar favorable effects were also seen after administration of isoflavones exerting weak estrogenic effects (Adams *et al.* 2002a,b).

Anti-atherogenic effects of estrogens in males were studied less intensely than those of females. Available results indicate, however, that estrogens reduce atherosclerotic burden in orchidectomized *Apoe* KO mice on cholesterol-rich diet or infused with angiotensin II (Ang-II) as well as in diabetic *Apoe* KO mice (Elhage *et al.* 1997a, Tse *et al.* 1999, Martin-McNulty *et al.* 2003). It ought to be emphasized that plasma E₂ concentrations in treated male animals were comparable to those encountered in normal female animals. There is, however, compelling evidence suggesting that estrogens exert atheroprotective effects in males also under

conditions in which E₂ in plasma is not elevated to supraphysiological levels. For instance, anti-atherogenic effects exerted by dehydroepiandrosterone in oophorectomized rabbits or by testosterone in orchidectomized wild-type B6 mice or in testicular feminized mice exhibiting a nonfunctional androgen receptor were reduced by pharmacological inhibitors of aromatase – an enzyme that converts testosterone to E₂ (Hayashi *et al.* 2000, Nathan *et al.* 2001, Nettleship *et al.* 2007). More recently, reduction of atherosclerosis under testosterone treatment could be observed in orchidectomized *Apoe* KO mice lacking androgen receptor (Bourghardt *et al.* 2010). Collectively, these results indicate that estrogens exert atheroprotective effects in male animals acting locally within vasculature.

The biological effects of estrogens are mediated on a genomic level by ER α and ER β . The full-length ER α (66 kDa) is composed of six domains termed A to F that harbor two transactivation functions AF1 and AF2 located in the domains B and E respectively. A truncated ER α isoform (46 kDa) lacking the N-terminal portion (domain A/B) has been identified in the uterus and endothelial cells. In addition, a G protein-coupled ER (GPR30) has been identified, which is localized in the endoplasmic reticulum and mediates nongenomic estrogen signaling (Maggiolini & Picard 2010). The identity of ER involved in the protective effects against atherosclerosis is not entirely clear. Early studies suggested that the prevention of fatty streaks require the full-length ER α , as administration of E₂ or isoflavones to ovariectomized *ER α /Apoe* double KO mice only insignificantly reduced atherosclerotic lesion size (Hodgin *et al.* 2001, Adams *et al.* 2002a). Later investigations, however, documented that anti-atherogenic effects of E₂ are preserved in female mice lacking full-length ER α (Villablanca *et al.* 2009), which might be attributed to the presence of the 46 kDa ER α isoform as well as the expression of the 55 kDa ER α isoform arising as a consequence of a non-natural alternative splicing in these mice. Actually, *Ldlr* KO mice engineered to specifically lack the AF1 transactivation function remained responsive to E₂ treatment, while E₂ protective effects were absent in animals lacking both the 66 and 46 kDa isoforms (Billon-Galés *et al.* 2009a). Interestingly, these effects were also missing in mice lacking AF2 transactivation function, pointing to its crucial role in the estrogen-mediated atheroprotection at least in animal models of disease (Billon-Galés *et al.* 2011). In major contrast to female animals, deletion of ER α in male B6 mice fed a high-cholesterol diet dramatically reduced atherosclerotic burden (Villablanca *et al.* 2004). As increased ER α -mediated susceptibility to atherosclerosis was found in male mice lacking the 66 kDa but maintaining the 46 kDa ER α isoform, it cannot be excluded that the full-length

ER α isoform may exert pro-atherogenic effects under certain experimental conditions.

While the preponderant majority of studies suggest that ER α exerts protective effects in animal models of atherosclerosis, less information is available regarding the involvement of ER β and GPR30. Unfortunately, the direct effect of E₂ on the formation of early atherosclerotic lesions has not been studied in *ER β* KO mice on an *Apoe*-deficient or *Ldlr*-deficient background. However, atheroprotective effects of isoflavones are persevered in *ER β /Apoe* double KO mice (Adams *et al.* 2002a,b). On the other hand, the isoflavone estrogen genistein, which shows a 20-fold higher binding affinity to ER β than to ER α , inhibited atherosclerosis development in *Ldlr* KO mice (Wang *et al.* 2008). In addition, 8 β -VE2 – a selective ER β agonist – reduced atherosclerotic lesions in *Apoe* KO mice (Sun *et al.* 2011). The contribution of GPR30 to the pathogenesis of atherosclerosis has not as yet been evaluated. As already mentioned, the E₂ metabolite, 2-ME, exerts atheroprotective effects, although it shows little affinity to classical ERs (Bourghardt *et al.* 2010).

Mechanisms underlying anti-atherogenic effects of estrogens

Involvement of endothelial cells

Endothelial monolayer separates blood, where lipoproteins and cellular participants (monocytes and lymphocytes) of atherosclerotic processes normally occur from the arterial wall. Consequently, the intact function of endothelial cells is critical for maintaining arterial integrity. Alterations of endothelial homeostatic mechanisms, which are known as endothelial dysfunction and precede the loss of endothelial integrity and denudation of the arterial wall, are initiated by several pro-atherogenic factors such as hypercholesterolemia or hyperhomocysteinemia and are encountered in pathological states favoring atherosclerosis such as hypertension or diabetes mellitus. Conversely, plasma-borne anti-atherogenic factors (HDLs and laminar blood flow) help to preserve integrity of the endothelial monolayer.

In vitro studies repeatedly demonstrated that E₂, E₂ metabolites, and synthetic estrogens exert favorable effects on endothelial function. One of the best-described endothelial actions of E₂ is stimulation of nitric oxide (NO) production, which depends on both genomic (expression of endothelial NO synthase (eNOS)) and nongenomic effects (activation of phosphatidylinositol 3-kinase and protein kinase AKT, phosphorylation of eNOS) (Arnal *et al.* 2010, Chow *et al.* 2010). Because the ability of E₂ to promote an increase in eNOS activity and NO-dependent

vasorelaxation are abolished in ER α KO mice and are emulated by highly selective ER α agonists, modulation of NO production by E₂ is likely mediated by ER α (Arnal *et al.* 2010, Bolego *et al.* 2010). However, treatment of endothelial cells with E₂ also stimulates covalent adduction of a nitrosyl group to cysteins (S-nitrosylation), which represents a key route for NO to directly modulate protein functions and which depends on ER β (Zhang *et al.* 2010, 2012). In addition, 2-ME, which shows affinity neither to ER α nor to ER β , abrogates vascular constriction by increasing the expression and the redistribution of eNOS and by promoting local NO production (Dubey & Jackson 2009, Fenoy *et al.* 2010). Another vasorelaxing substance liberated from endothelial cells under the influence of E₂ is prostacyclin (PGI₂; Mikkola *et al.* 1995, 1996, Seeger *et al.* 1999, Sherman *et al.* 2002). The latter effect depends on the combined increased synthesis of cyclooxygenase 1 and/or 2 (COX1 (PTGS1) and COX2 (PTGS2)) and prostaglandin synthase (PGH₂), which ultimately leads to a shift from COX-dependent vasoconstriction to vasodilation (Jun *et al.* 1998, Akarasereenont *et al.* 2000, Kawagoe *et al.* 2007, Sobrino *et al.* 2009, 2010, Su *et al.* 2009). Experiments with ER agonists and endothelial cells transfected with COX promoter suggest the involvement of both ER α and ER β in E₂-dependent PGI₂ production (Gibson *et al.* 2005, Su *et al.* 2009, Sobrino *et al.* 2010), albeit 2-ME was also demonstrated to enhance PGI₂ liberation (Barchiesi *et al.* 2006). In addition to the release of vasorelaxants, estrogens also prevent the activation of endothelium, which constitutes an essential component of endothelial dysfunction. Both E₂ and its metabolites such as estron and 17-epiestriol, as well as 5 α -androstane-3 α ,17 β -diol-(3 α -diol) and 5 α -androstane-3 β ,17 β -diol-(3 β -diol), two dihydrotestosterone metabolites that are unable to bind the androgen receptor but exhibit affinity for ERs, blunt the cell-surface expression of vascular cell adhesion molecule-1 (VCAM1), intercellular cell adhesion molecule (ICAM-1), E-selectin, P-selectin, as well as CD40 and CD40L in endothelial cell exposed to pro-atherogenic factors such as TNF α , IL1B, lipopolysaccharide (LPS), IFN γ or lysophosphatidylcholine (LPC; Nathan *et al.* 1999, Simoncini *et al.* 1999, 2000, Mukherjee *et al.* 2002, 2003, Mori *et al.* 2004, Gerald *et al.* 2006, Ling *et al.* 2006, Wang *et al.* 2008, Norata *et al.* 2010, Thor *et al.* 2010, Rauschemberger *et al.* 2011). At the same time, E₂ reduces the monocyte expression of $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins, binding partners for VCAM1 and ICAM-1, respectively, in a process involving downregulation of RAC1 activity (Friedrich *et al.* 2006). As a consequence, estrogens reduce both the monocyte and neutrophil adhesion to the endothelial monolayer and the transendothelial migration of monocytes under *in vitro* conditions (Alvarez *et al.* 2002,

Geraldes *et al.* 2006). In addition, exposure of endothelial cells to estrogens suppresses the NADPH oxidase activity and thereby the intracellular production of reactive oxygen species (ROS), which is instrumental for the expression of adhesion molecules in response to pro-atherogenic factors (Wagner *et al.* 2001). Experiments using ER agonists and antagonists point to both ER α and ER β as mediators of suppressing effects of E₂ on endothelial activation (Mukherjee *et al.* 2003, Mori *et al.* 2004, Gerald *et al.* 2006), though again these effects could also be observed in the presence of 2-ME (Kurokawa *et al.* 2007, Dubey & Jackson 2009).

In addition to beneficial effects on endothelial function, estrogens promote endothelial integrity. E₂, 2-ME, and isoflavones enhance endothelial barrier function and reduce its permeability to pro-atherogenic factors such as native and oxidized LDLs (Carbajal & Schaeffer 1998, Delarue *et al.* 1998, Fujimoto *et al.* 1998, Gardner *et al.* 1999, Chi *et al.* 2004, Liu *et al.* 2005, Sumanasekera *et al.* 2007, Dubey & Jackson 2009). The latter effect depends on the modulation of tight junction proteins such as occludin and/or claudin and is mediated via both ER α and ER β (Ye *et al.* 2003, Burek *et al.* 2010, Sandoval & Witt 2011). Furthermore, estrogens promote endothelial cell proliferation and survival. Several studies documented mitogenic effects of E₂ either acting alone or in combination with serum-derived growth factors in endothelial cell cultures (Gerald *et al.* 2002, Sengupta *et al.* 2004, Williams *et al.* 2004, Kawagoe *et al.* 2007, Oviedo *et al.* 2011). Moreover, endothelial apoptosis initiated by TNF α , H₂O₂, or oxidized LDL was consistently inhibited in the presence of estrogens in a process involving activation of protein kinases MAPK and AKT, increased expression of anti-apoptotic proteins BCL2 and BCL-XL (BCL2L1), and disabling of the pro-apoptotic protein BAD (Alvarez *et al.* 1997, Spyridopoulos *et al.* 1997, Liu *et al.* 2002, Lu *et al.* 2006, Florian & Magder 2008, Yu *et al.* 2009). In addition, acting through ER α , estrogens were demonstrated to prevent mitochondrial ROS production in endothelial cells as well as ROS-induced apoptosis by interfering with cytochrome *c* release from mitochondria (Sudoh *et al.* 2001, Razmara *et al.* 2008). Finally, E₂ was found to promote formation of the eNOS-ER α complex that interacts with telomerase catalytic subunit (hTERT) gene promoter, enhances hTERT transcription, and thereby increases activity of telomerase, which plays pivotal role in the determination of the cell lifespan and counteracts cellular senescence (Hayashi *et al.* 2006, Grasselli *et al.* 2008).

Several putatively protective effects of estrogens observed in endothelial cells under *in vitro* conditions could be recapitulated in animal models in the context of atherosclerotic disease. For instance, E₂ was found to counteract detrimental effects of hypercholesterolemia on arterial vasorelaxation by augmenting COX2

expression and induction, and production of PGI₂ by estrogens was postulated to play an important role in the prevention of fatty streak formation (Ghanam *et al.* 2000, Egan *et al.* 2004). Somewhat unexpectedly, increasing NO bioavailability in ovariectomized *Apoe* KO mice failed to affect development of fatty streaks, suggesting that prevention of early atherosclerosis by E₂ is independent of NO production (Elhage *et al.* 1997b, Hodgins *et al.* 2002). However, chronic induction of endothelial dysfunction by prolonged administration of NOS inhibitor aggravated atherosclerosis and specifically abolished atheroprotective effects of E₂ in intact but not in de-endothelialized aorta in female rabbits on a hypercholesterolemic diet (Holm *et al.* 1997). In addition, a close inverse correlation between NO production and the arterial lesion extent was observed in rabbits under the condition of severe hypercholesterolemia combined with or without endothelial dysfunction (Nascimento *et al.* 1999, Hayashi *et al.* 2000). In a similar rabbit model of atherosclerosis, hypercholesterolemia-induced monocyte adhesion and penetration into the subendothelial area were more pronounced in male than in female rabbits (Nathan *et al.* 1999). Both monocyte adhesion and VCAM1 expression were aggravated by oophorectomy and these were reversed after treatment of female animals with E₂. Later studies performed on hypercholesterolemic *Apoe*-deficient animals confirmed the pivotal role of endothelial VCAM1 expression for the atheroprotective effects of estrogens (Gourdy *et al.* 2003, Martin-McNulty *et al.* 2003).

The central role of endothelium for the atheroprotective effects of estrogens was initially suggested by Holm *et al.* (1997), who demonstrated complete loss of anti-atherogenic effects of estrogens in hypercholesterolemic rabbits after destructing the endothelial layer in the aorta using a balloon catheter. However, the ultimate proof for the obligatory involvement of endothelial cells in the estrogen-exerted atheroprotection in animal models was provided by Billon-Galès *et al.* (2009a). Using an approach utilizing CreLox strategy (with Cre recombinase under control of the endothelial-specific Tie promoter), these authors have clearly shown that elimination of ER α in endothelial cells fully abolishes the atheroprotective action of E₂ in ovariectomized *Ldlr*-deficient mice and this was accompanied by a complete abrogation of the E₂-induced NO release. These results not only define the endothelial monolayer as a key target of estrogens in the vasculature but also pinpoint ER α as a principal mediator of estrogen action. In this regard, they fully corroborate with previous findings that ER α is necessary and sufficient for E₂-induced NO and PGI₂ production and that it likely mediates the suppressive effects of E₂ on VCAM1 expression in endothelial cells (Javeshghani *et al.* 2006, 2007).

Involvement of macrophages, dendritic cells, and lymphocytes

Macrophages and lymphocytes are intimately involved in the pathogenesis of atherosclerosis. Monocytes are recruited to the arterial wall, where they (after conversion to macrophages) initiate a processes culminating in fully fledged intra-arterial inflammation. The contribution of macrophages to this process is manifold (see Ley *et al.* (2011) and Moore & Tabas (2011) for the most recent reviews) and encompasses

- uncontrolled ingestion of oxidatively modified LDLs and formation of cholesterol-loaded foam cells. HDL, a potent anti-atherogenic factor in humans, acts as a cholesterol acceptor and counterbalances cholesterol accumulation in macrophages in a process dubbed cholesterol efflux;
- production of pro-inflammatory cytokines and chemokines, such as IL1B and TNF α , that activate endothelial and smooth muscle cells and promote further recruitment of mononuclear cells. In addition, IL1B, IL12 (IL12A), and IL18 activate T-cells and facilitate the adaptive immune response;
- antigen presentation via MHC-II, which further contributes to the activation of T-cells. This mechanism is also thought to account for the pro-atherogenic effects of dendritic cells, which are present in atherosclerotic lesions.

The number of macrophages recruited into the arterial wall is controlled by mutually opposed processes of proliferation and apoptosis. Macrophage colony-stimulating factor (M-CSF) – a cytokine promoting monocyte–macrophage transition and macrophage growth – increases macrophage numbers in plaques, while its partial absence protects against the development of atherosclerotic lesions. Conversely, suppression of macrophage apoptosis was found to increase macrophage number in plaques and to enhance plaque formation in atherosclerosis-prone mice. In advanced atherosclerotic lesions, however, apoptotic macrophages become secondarily necrotic and coalesce over time into a key feature of vulnerable plaques – the necrotic core. This event is critically important, as necrotic core formation is thought to promote plaque disruption and ultimately acute atherothrombotic vascular disease.

Despite the key relevance of cholesterol accumulation in macrophages for the formation of atherosclerotic lesions, little effort has been devoted toward better understanding of the influence of estrogens on macrophage cholesterol homeostasis. Currently available information suggests that estrogens favor discharging of cellular cholesterol and reduce the cholesterol burden of atherosclerotic plaques. Actually, exposure of human or murine macrophages to E₂ decreased

their cholesterol ester content by stimulation of neutral cholesterol ester hydrolase and inhibition of acylCoA-cholesterol transferase (Cheng *et al.* 1999, Napolitano *et al.* 2001, 2002, Wilson *et al.* 2008, Corcoran *et al.* 2011). In addition, E₂ was reported to reduce macrophage uptake of modified LDLs such as acetylated, oxidized, or aggregated LDL, and this latter effect was found to specifically depend on the ER α -mediated suppression of the expression of scavenger receptor CD36 (Sulistyani & St Clair 1997, McCrohon *et al.* 1999, Allred *et al.* 2006, Wilson *et al.* 2008). More recently, ER α -deficient macrophages were shown to express reduced amounts of proteins involved in cholesterol efflux such as ABCA1 and APOE and to export less cholesterol when incubated with HDL, whereas E₂ associated with HDL particles was found to potentiate HDL-induced cholesterol efflux mediated by scavenger receptor class B type I (SR-BI (SCARB1)) (Badeau *et al.* 2009, Ribas *et al.* 2011). Several studies documented that HDL carries E₂ in the form of fatty acyl esters produced in a reaction catalyzed by lecithin-cholesterol acyltransferase (LCAT; Helisten *et al.* 2001, Höckerstedt *et al.* 2002, 2004). HDL-derived E₂ esters are internalized via LDLR- and/or SR-BI-mediated mechanisms and subsequently hydrolyzed intracellularly to unfold their effects (Badeau *et al.* 2007a, 2009). In addition, E₂ esters are transferred by cholesterol ester transfer protein to LDL particles, where they reduce oxidation by free radicals produced in copious amounts by activated macrophages (Helisten *et al.* 2001, Höckerstedt *et al.* 2002). Overall, these results suggest that estrogens not only favorably affect cholesterol homeostasis and reduce macrophage cholesterol burden but also protect LDL particles against oxidation, thereby further limiting LDL retention and cholesterol accumulation in the arterial wall.

Additional atheroprotective effects exerted by estrogens in macrophages may be related to modulation of growth and apoptotic processes. While evidence for the direct regulation of macrophage proliferation by estrogens is lacking, these hormones were shown to modulate bone marrow M-CSF production and reduced aortic expression of M-CSF was found in *ApoE*-deficient mice treated with E₂ (Sarma *et al.* 1998, Lea *et al.* 1999, Martin-McNulty *et al.* 2003). E₂ was also demonstrated to promote apoptosis of monocytes, monocyte-derived macrophages, and macrophage-derived osteoclasts, and these effects were largely attributed to the upregulation of Fas and Fas ligand (FasL (FASLG)) as well as the activity of caspases 8 and 3 (Carruba *et al.* 2003, Mor *et al.* 2003, Thongngarm *et al.* 2003, Saintier *et al.* 2006, Nakamura *et al.* 2007, Montagna *et al.* 2009). In this context, it is worth noting that Fas/FasL effectively regulate apoptosis of cholesterol-loaded macrophages and that Fas and FasL-mediated macrophage apoptosis coincides with decreased lesion cellularity and regression of atherosclerosis in mice

(Esaki *et al.* 2000, Yao & Tabas 2000). Hence, it would be tempting to speculate that estrogens countervail the early lesion development by reducing the number of intimal macrophages. However, in one instance, E₂ has also been demonstrated to inhibit macrophage apoptosis in a process dependent on the cytoplasmic ER α receptor and mediated by the increased expression of anti-apoptotic molecule BCL2 (Subramanian & Shaha 2009). This latter process might be interpreted as favorable in the late lesion scenario, where the prevention of macrophage apoptosis attenuates necrotic core formation.

With respect to macrophage and dendritic cell functions related to initiation, propagation, and/or resolution of inflammation, investigations on the impact of estrogens led to equivocal results. Both E₂, phytoestrogens, and ER agonists were reported to attenuate pro-inflammatory activation induced by toll-like receptor (TLR) agonists such as LPS or by receptor activator of nuclear factor- κ B ligand in primary human and murine macrophages, in monocyte-derived macrophage lines (RAW247.6, J774, and THP-1), as well as in primary microglia and in microglial cell lines (Frazier-Jessen & Kovacs 1995, Deshpande *et al.* 1997, Bruce-Keller *et al.* 2000, Vegeto *et al.* 2004, Bellosta *et al.* 2007, Hämäläinen *et al.* 2007, Yoshitake *et al.* 2008, Yuan *et al.* 2008, Lee *et al.* 2008, Rettew *et al.* 2010). This has been inferred from reduced expression of iNOS, decreased production of pro-inflammatory cytokines and chemokines (TNF α , IL6, IL12, MCP1 (CCL2), MIP-1 α (CCL3)), and the decreased production and release of the matrix metalloproteinase 9. In addition, E₂ was reported to inhibit MHC-II expression in macrophages exposed to INF γ (Adamski *et al.* 2004). While no single mechanism underlying these effects could be identified, estrogens were found to interfere with several intracellular pro-inflammatory signaling pathways including phosphorylation, nuclear translocation, and DNA binding of the transcription factor NF- κ B as well as phosphorylation and activation of signal transducers and activators of transcription (STAT) 1 and 3 (Deshpande *et al.* 1997, García Palacios *et al.* 2005, Ghisletti *et al.* 2005, Chen *et al.* 2006, Hämäläinen *et al.* 2007, Lee *et al.* 2008, Yoshitake *et al.* 2008, Xiu-li *et al.* 2009). In particular, inhibitory effects of E₂ on NF- κ B activation were uniformly found in several macrophage cell systems and were attributed to both genomic (regulation of let-7a and miR-125b micro-RNAs) and nongenomic (regulation of protein kinases MAPK and AKT) action (Ghisletti *et al.* 2005, Murphy *et al.* 2010). Moreover, the reduced iNOS expression, TNF α production, and NF- κ B activation seen in the presence of E₂ were partially abolished in macrophages lacking peroxisome proliferation-activating receptor α (PPAR α ; Crisafulli *et al.* 2009). The latter observation, which suggests that PPAR α contributes to the

anti-inflammatory activity of E_2 , is of particular relevance to the pathogenesis of atherosclerosis, as PPAR α is a molecular target of fibrates – drugs with established anti-atherogenic and anti-inflammatory properties.

The anti-inflammatory effects of estrogens on macrophages have been confirmed in several animal models of inflammation. For instance, E_2 treatment blocked NF- κ B translocation and prevented glial reactivity in rats undergoing chronic spinal cord injury (Sribnick *et al.* 2010). Similarly, both E_2 and synthetic ER agonists normalized NF- κ B activation and pro-inflammatory cytokine production as well as reduced TLR4 expression in Kupfer cells following trauma–hemorrhage in rats and mice, and these effects were shown to be specifically mediated by ER α (Hsieh *et al.* 2007, Suzuki *et al.* 2007, 2008). In addition, transplant arteriosclerosis in rats or rabbits as well as macrophage infiltration into the allograft intima were potently reduced in E_2 -treated recipients, and this was associated with almost complete suppression of MHC-II antigen expression in transplanted arteries (Lou *et al.* 1996, Saito *et al.* 1998). Most recently, ER α -deficient macrophages were found refractory to IL4-induced activation of anti-inflammatory phenotype *in vitro* and presented with elevated expression of chemokines and markers of immune cell activity, when isolated from animals with myeloid-specific ER α deficiency (Ribas *et al.* 2011). Importantly, *Ldlr* KO animals lacking ER α in myeloid cells showed twofold increase in atherosclerotic lesion area. In the most striking contrast to these findings, Rettew *et al.* (2009) observed pro-inflammatory effects of estrogens in a murine model of sepsis. In this model, removal of endogenous estrogens by means of ovariectomy decreased production of pro-inflammatory cytokines and concomitantly reduced cell surface expression of TLR4 on macrophages. Conversely, E_2 replacement significantly elevated macrophage TLR4 expression, and this effect was accompanied by significantly higher inflammatory cytokine levels after *in vivo* LPS challenge. Potentiating effects of E_2 on macrophage activation were also observed by Calippe *et al.* (2008), who found that administration of E_2 to ovariectomized mice markedly increased the expression of IL1B, IL6, IL12p40, and iNOS by peritoneal macrophages in response to LPS *ex vivo*. Moreover, E_2 treatment of animals resulted in the inhibition of phosphoinositide kinase-3 (PI3K) activity and AKT phosphorylation in LPS-activated macrophages, whereas NF- κ B p65 transcriptional activity was concomitantly increased. Further studies showed that targeted disruption of ER α gene in macrophages totally abolished the effect of E_2 on the expression of inflammatory mediators by peritoneal macrophages, thus indicating that E_2 directly targeted these cells to exert pro-inflammatory effects (Calippe *et al.* 2010). The

same group noticed that ER α -mediated signaling is required for optimal dendritic cell function as assessed by MHC-II and CD86 expression and pro-inflammatory cytokine production (IL6 and IL12) and that estrogens enhance susceptibility to experimental myasthenia gravis by augmenting dendritic cell activation and pro-inflammatory Th1 response (Delpy *et al.* 2005, Douin-Echinard *et al.* 2008). The pivotal role of estrogens in regulating dendritic cell differentiation and activation was also highlighted in other studies, in which E_2 was shown to support GM-CSF-initiated transition of bone marrow progenitor cells into dendritic cells, to prevent apoptosis, and to augment the production of pro-inflammatory and pro-atherogenic cytokines IL12 and IFN γ by dendritic cells in response to TLR4 or TLR7 agonists (Carreras *et al.* 2008, 2010, Kawasaki *et al.* 2008, Siracusa *et al.* 2008, Li *et al.* 2009). The reasons why estrogens differentially affect macrophage and dendritic cell activation *in vitro* and *in vivo* and thereby exert dual effects on inflammation are currently unclear (see Straub (2007) for exhaustive discussion). It may be speculated that the duration of treatment plays a role in this respect. Actually, short-term cell or animal treatment was used in most *in vitro* and *in vivo* experiments, in which suppressing effects of estrogens on macrophage activation were studied, while prolonged administration (over 35–60 days) was applied in experiments performed by Calippe *et al.* or Rettew *et al.*

In yet another twist in the estrogens and atherosclerosis story, Rayner *et al.* (2008) showed that estrogens dose-dependently promote the release of heat-shock protein 27 (HSP27 (HSPB1)) from macrophages. The latter protein binds to scavenger receptor A (SR-A) and thereby inhibits ingestion of cholesterol-rich lipoproteins and formation of foam cells. In addition, HSP27 promotes anti-inflammatory macrophage response characterized by decreased and elevated productions of IL1B and IL10 respectively. Studies *in vivo* documented reduced atherosclerosis in HSP27-overexpressing female but not in male mice and additionally revealed the inverse relationship between plasma HSP27 and E_2 concentrations. Further investigations demonstrated that E_2 treatment is more effective in preventing atherosclerotic lesion formation in ovariectomized HSP27-overexpressing mice than wild-type mice (Rayner *et al.* 2009). Most strikingly, the atheroprotective effects of E_2 in HSP27-overexpressing mice were mimicked by selective ER β agonist but not by ER α -specific agonist. Taken together, these results reveal an entirely new mechanism, by which estrogens may protect against the development of atherosclerosis and emphasize a previously overseen role of ER β as potential therapeutic target in vascular atherosclerotic diseases (Fig. 1).

Involvement of smooth muscle cells

Vascular smooth muscle cells (VSMC) represent a third cell type in addition to endothelial cells and macrophages that determine atherosclerotic plaque development (Doran *et al.* 2008, Orr *et al.* 2010). After intimal injury, endothelial cells, platelets, and macrophages release mediators, such as growth factors and cytokines, that promote VSMC phenotype change from the quiescent ‘contractile’ to the active ‘synthetic’ and induce their proliferation and migration from the media to the intima. Like macrophages, VSMC can express a variety of receptors for lipid uptake and can form foam-like cells, thereby participating in the early accumulation of plaque lipid. In addition, activated VSMC secrete pro-inflammatory cytokines and chemokines and release free radicals that modify lipoproteins. Like endothelial cells, VSMC express adhesion molecules to which monocytes and lymphocytes can

adhere and migrate into the vessel wall. Hence, VSMC essentially contribute to development of early atherosclerotic plaques. However, VSMC are also the major source of the extracellular matrix and at later stages of atherosclerosis provide a substantial input to plaque stability (Clarke & Bennett 2006).

Studies on interactions between estrogens and VSMC clearly point to the profound influence of E₂ and synthetic estrogens on VSMC function. Inhibitory effects of E₂ on VSMC proliferation induced by serum or serum-derived pro-atherogenic factors such as fibroblast growth factor, PDGF, Ang-II, or LPS were shown in the overwhelming majority of *in vitro* studies and were also accompanied by the G₁ arrest, reduced expression of cell cycle regulatory proteins including cyclin D, attenuated phosphorylation of retinoblastoma (Rb) protein, as well as reduced activation of mitogen-activated kinases such as ERK1/2 (MAPK3/6) and

Figure 1 Molecular modes of estrogen action in atherosclerotic disease. Green and red boxes indicate pro- and anti-atherogenic effects respectively. CINC, cytokine-induced neutrophil chemoattractant; CycD, cyclin D; ER, estrogen receptor; ET, endothelin; HSP, heat-shock protein; ICAM, intercellular cell adhesion molecule; IL, interleukin; MCP, monocyte chemoattractant protein; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; NO, nitric oxide; PGI₂, prostacyclin; PPAR, peroxisome proliferator-activated receptor; Rb, retinoblastoma protein; ROS, reactive oxygen species; SOD, superoxide dismutase; SR-A, scavenger receptor type A; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; TLR, toll-like receptor; VCAM, vascular cell adhesion molecule.

MEK1/2 (MAP2K1/2) (Dai-Do *et al.* 1996, Espinosa *et al.* 1996, Suzuki *et al.* 1996, Kappert *et al.* 2006, Wang *et al.* 2007). In parallel, E₂ tended to promote VSMC apoptosis by activating p42/44 and/or p38 MAPK and upregulating the pro-apoptotic protein BAX (Mori-Abe *et al.* 2003, Yang *et al.* 2007, Ding *et al.* 2009). E₂ was also shown to inhibit VSMC migration toward pro-atherogenic chemoattractants such as PDGF or Ang-II, and this was likely dependent on the inhibition of the small G protein RAC1 (Dai-Do *et al.* 1996, Kolodgie *et al.* 1996, Yoon *et al.* 2001, Hwang *et al.* 2002, Ling *et al.* 2002, Takahashi *et al.* 2003, Kappert *et al.* 2006). In addition, estrogens were demonstrated to reduce inflammatory activation of VSMC. For instance, the expression of *COX2*, *MCPI*, and endothelin 1 (*ET1* (*EDN1*)) in response to serum, LPS, or Ang-II, respectively, was markedly attenuated in VSMC exposed to E₂ (Seli *et al.* 2001, Hong *et al.* 2004, Kawagoe *et al.* 2007, Jiang *et al.* 2010). Xing *et al.* (2007) found that E₂ inhibits TNF α -stimulated expression of the neutrophil-specific chemokine cytokine-induced neutrophil chemoattractant (CINC)-2 β and suggested that this effect might be responsible for the reduced infiltration of neutrophils, which is observed in animal models after vessel injury. Finally, estrogens are also known to reduce oxidative stress in VSMC by increasing expression of antioxidative enzymes such as superoxide dismutase and reducing expression and/or activity of NADPH oxidase (Strehlow *et al.* 2003, Wassmann *et al.* 2005, Wing *et al.* 2009, Broughton *et al.* 2010, Sivritas *et al.* 2011). The anti-proliferatory, pro-apoptotic, anti-migratory, anti-oxidative, and anti-inflammatory *in vitro* effects of estrogens collectively translate into reduced neointima formation under *in vivo* conditions. The latter effect has been repeatedly observed in ligation-injured carotid arteries of E₂-treated ovariectomized mice as well as in balloon-injured rabbit aortas or rat carotid arteries.

ER β is a predominant mRNA transcript in VSMC (Register & Adams 1998). However, the identity of ER mediating the anti-atherogenic effects of estrogens in VSMC remains enigmatic. For instance, the pro-apoptotic effect of E₂ could be observed in the A10 VSMC line only after overexpressing ER α in these cells (Mori-Abe *et al.* 2003). Furthermore, stimulatory effects exerted by E₂ on the superoxide dismutase expression seem to depend on ER α (Sivritas *et al.* 2011). By contrast, treatment of porcine VSMC with antisense oligonucleotides complementary to ER β mRNA abrogated the inhibitory effects of E₂ on ERK1/2 activity and proliferation (Geraldine *et al.* 2003). Likewise, the inhibitory effects exerted by estrogens on Ang-II-induced VSMC proliferation and TNF α -stimulated CINC-2 β expression were prevented by ER β but not by ER α receptor inhibitors (Xing *et al.* 2007). ER β mRNA was found to be markedly expressed after balloon injury in rats, and local delivery of selective ER β agonists was

shown to more effectively reduce neointima formation than ER α agonists (Lindner *et al.* 1998, Aavik *et al.* 2001, Krom *et al.* 2007). Collectively, these results point to ER β as a major mediator of estrogen action in VSMC. More recently, GPR30 was found to be expressed on VSMC and to modulate vascular tone in rats and in isolated human arteries (Haas *et al.* 2009). GPR30 activation was shown to inhibit VSMC proliferation and NADPH oxidase activation, and *GPR30* expression was required for the estrogen-mediated VSMC apoptosis (Ding *et al.* 2009, Haas *et al.* 2009, Broughton *et al.* 2010). Hence, GPR30 activation in VSMC may be tentatively added to the list of cellular effects exerted by estrogens, which are considered atheroprotective. Finally, 2-ME, an estrogen metabolite with no affinity to classical ERs, was shown to prevent injury-induced neointima formation in rats (Barchiesi *et al.* 2006). Several studies *in vitro* documented that 2-ME inhibits VSMC proliferation at both G₀/G₁ and G₂/M cell cycle phase and downregulates cyclones D and B, while upregulating p27 – a negative regulator of VSMC growth, and that these effects are missing in VSMC lacking COMT that do not effectively convert E₂ to 2-ME (Zacharia *et al.* 2003, Barchiesi *et al.* 2006, Dubey & Jackson 2009). Collectively, these results suggest that at least part of the atheroprotective effects related to the effects of estrogens on VSMC are mediated in a fashion independent of classical ERs.

Conclusions and directions for future research

Estrogen-dependent regulation of vascular gene expression and vascular physiology encompasses complex processes involving both nuclear and membrane-associated ER signaling pathways. Recent years have witnessed major progress in understanding how these regulatory processes contribute to the atheroprotective effects exerted by naturally occurring estrogens and synthetic ER agonists. Studies on animal models of atherosclerosis provided compelling evidence that physiological estrogen levels potentially attenuate both early and advanced stages of atherosclerosis lesion development in females and suggested similar protective effects in males. Using tissue-specific KO and/or highly selective ER agonists, these studies clearly defined endothelial cells as a primary target of the anti-atherogenic action of estrogens, demonstrated that non-nuclear ER α signaling plays a crucial role in this respect, and allowed dissecting the effects on cardiovascular endpoints from uterotrophic response (Arnal *et al.* 2010, Bolego *et al.* 2010). It could also be established that ER β signaling contributes to atheroprotective effects exerted by estrogens and this has been attributed primarily to the modulatory effects on smooth muscle and probably also on macrophage

function. New insights into molecular mechanisms underlying vascular effects of estrogens improved our capacity to correctly interpret surprising and sometimes contradictory data from large interventional studies, in which hormone-replacement therapy failed to reduce cardiovascular risk despite favorable effects on plasma lipid profile and several other coronary risk factors. For instance, results of animal studies demonstrating that estrogens may exert both pro- and anti-atherogenic effects depending on the disease stage provide experimental support to the increasingly accepted 'timing hypothesis' according to which continuous treatment with estrogens may result in different outcomes than reinstatement of therapy several years after menopause. By the same token, dual pro- or anti-inflammatory effects of estrogens, which seem to depend on the duration of treatment, corroborate well with the results of clinical studies demonstrating protective effects of short-term (<10 years) menopausal hormone therapy and gradual disappearance of protection in succeeding years (Rossouw *et al.* 2007). Finally, it seems possible that some mechanisms instrumental to presumed atheroprotection exerted by estrogens may be less effective in women after menopause. For instance, BH4 – an obligatory cofactor for eNOS – is reduced with aging and its absence may at least partially prevent estrogens to exert atheroprotective effects via the endothelium. Similar effects antagonizing the beneficial action of estrogens on endothelial cells may be exerted by 27-hydroxycholesterol, which binds to and blocks ER α and increased concentrations of which are observed in postmenopausal women (Umetani & Shaul 2011). It also cannot be excluded that estrogens are less effective in postmenopausal women due to increased esterification by LCAT and estrogen ester sequestration in adipose tissue (Badeau *et al.* 2007b).

Despite the immense progress in understanding the role of estrogens in atherosclerosis, a number of questions regarding mechanisms underlying their protective effects remain unanswered. We still need to know whether various estrogen concentrations, which physiologically vary over two orders of magnitude, exert distinct signaling effects (either pro- or anti-atherogenic) in diverse cells involved in the pathogenesis of atherosclerosis. We also need to know to what extent and how the cessation of endogenous estrogen production affects ER expression and function in the vasculature and thereby enhances or attenuates their pro- or anti-atherogenic effects. Finally, the potential pro- or anti-atherosclerotic effects of newly identified ERs such as GPR30 have not been addressed in animal models of disease. Clearly, further experimental work including development of new animal models better reflecting estrogen secretion during menopause, animal models specifically expressing ER (ER β and GPR30) only in selected cells and tissues relevant to the

pathogenesis of atherosclerosis (e.g. smooth muscle cell, macrophage, and dendritic cell), as well as systematic assessment of the pro- or anti-atherogenic effects of various concentrations of natural estrogens and synthetic ER agonists will be necessary to address these questions.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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