Estrogens and atherosclerosis: insights from animal models and cell systems

Jerzy-Roch Nofer1,2

1Center for Laboratory Medicine, University Hospital Münster, Albert Schweizer Campus 1, Gebäude A1, 48129 Münster, Germany
2Department of Medicine, Endocrinology, Metabolism and Geriatrics, University of Modena and Reggio Emilia, Modena, Italy

(Correspondence should be addressed to J-R Nofer at Center for Laboratory Medicine, University Hospital Münster; Email: nofer@uni-muenster.de)

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.

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 (ERα) and β (ERβ) 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, 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 monocytosis 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, Hodgin 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 Freudenberger 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 ERα and ERβ. The full-length ERα (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 ERα 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 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 ERα, as administration of E2 or isoflavones to ovariectomized ERα/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 ERα (Villablanca et al. 2009), which might be attributed to the presence of the 46 kDa ERα isoform as well as the expression of the 55 kDa ERα 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 ERα in male B6 mice fed a high-cholesterol diet dramatically reduced atherosclerotic burden (Villablanca et al. 2004). As increased ERα-mediated susceptibility to atherosclerosis was found in male mice lacking the 66 kDa but maintaining the 46 kDa ERα isoform, it cannot be excluded that the full-length ERα isoform may exert pro-atherogenic effects under certain experimental conditions.

While the preponderant majority of studies suggest that ERα exerts protective effects in animal models of atherosclerosis, less information is available regarding the involvement of ERβ and GPR30. Unfortunately, the direct effect of E2 on the formation of early atherosclerotic lesions has not been studied in ERβ KO mice on an Apoe-deficient or Ldlr-deficient background. However, atheroprotective effects of isoflavones are persevered in ERβ/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 ERβ than to ERα, inhibited atherosclerosis development in Ldlr KO mice (Wang et al. 2008). In addition, 8β-VE2 – a selective ERβ 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 ERα KO mice and are emulated by highly selective ERα agonists, modulation of NO production by E2 is likely mediated by ERα (Arnal et al. 2010, Bolego et al. 2010). However, treatment of endothelial cells with E2 also stimulates covalent addition of a nitrosyl group to cysteins (S-nitrosylation), which represents a key route for NO to directly modulate protein functions and which depends on ERβ (Zhang et al. 2010, 2012). In addition, 2-ME, which shows affinity neither to ERα nor to ERβ, 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 and COX2 (PTGS2)) and prostaglandin synthase (PGH2), which ultimately leads to a shift from COX-dependent vasoconstriction to vasodilation (Jun et al. 1998, Akaraseenont 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 ERα and ERβ 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 5α-androstane-3α,17β-diol-(3α-diol) and 5α-androstane-3β,17β-diol-(3β-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 adhesion molecule (ICAM-1), E-selectin, P-selectin, as well as CD40 and CD40L, in endothelial cell exposed to proatherogenic factors such as TNFz, IL1B, lipopolysaccharide (LPS), IFNγ or lysophosphatidylcholine (LPC; Nathan et al. 1999, Simoncini et al. 1999, 2000, Mukherjee et al. 2002, 2003, Mori et al. 2004, Geraldes 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 αβ1 and αβ2 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 ERα and ERβ as mediators of suppressing effects of E2 on endothelial activation (Mukherjee et al. 2003, Mori et al. 2004, Geraldes 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 ERα and ERβ (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 (Geraldes et al. 2002, Sengupta et al. 2004, Williams et al. 2004, Kawagoe et al. 2007, Oviedo et al. 2011). Moreover, endothelial apoptosis initiated by TNFz, 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 ERα, 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–ERα 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 PGI\textsubscript{2} 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 E\textsubscript{2} is independent of NO production (Elhage et al. 1997b, Hodgin et al. 2002). However, chronic induction of endothelial dysfunction by prolonged administration of NOS inhibitor aggravated atherosclerosis and specifically abolished atheroprotective effects of E\textsubscript{2} 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 VCAM\textsubscript{1} expression were aggravated by oophorectomy and these were reversed after treatment of female animals with E\textsubscript{2}. Later studies performed on hypercholesterolemic Apoe-deficient animals confirmed the pivotal role of endothelial VCAM\textsubscript{1} 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 destroying 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-Galés 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\textsubscript{z} in endothelial cells fully abolishes the atheroprotective action of E\textsubscript{2} in ovariectomized Ldb-deficient mice and this was accompanied by a complete abrogation of the E\textsubscript{2}-induced NO release. These results not only define the endothelial monolayer as a key target of estrogens in the vasculature but also pinpoint ER\textsubscript{z} as a principal mediator of estrogen action. In this regard, they fully corroborate with previous findings that ER\textsubscript{z} is necessary and sufficient for E\textsubscript{2}-induced NO and PGI\textsubscript{2} production and that it likely mediates the suppressive effects of E\textsubscript{2} on VCAM\textsubscript{1} 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\textsubscript{z}, 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; and 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 E\textsubscript{2} 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 ERα-mediated suppression of the expression of scavenger receptor CD36 (Sulis-tiyani & St Clair 1997, Mcgrohon et al. 1999, Allred et al. 2006, Wilson et al. 2008). More recently, ERα-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, Lex 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 caspasas 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 counteract 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 ERα 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-kB 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, Deshpane 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 (TNFα, IL6, IL12, MCP1 (CCL2), MIP-1z (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 INFγ (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-kB 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-kB 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, TNFα production, and NF-kB activation seen in the presence of E2 were partially abolished in macrophages lacking peroxisome proliferation-activating receptor α (PPARα; Crisafulli et al. 2009). The latter observation, which suggests that PPARα contributes to the
anti-inflammatory activity of E$_2$, is of particular relevance to the pathogenesis of atherosclerosis, as PPAR$_{A}$ 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-κB 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-κB activation and pro-inflammatory cytokine production as well as reduced TLR4 expression in Kupffer cells following trauma-hemorrhage in rats and mice, and these effects were shown to be specifically mediated by ERa (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, ERa-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 ERa deficiency (Ribas et al. 2011). Importantly, Ldr KO animals lacking ERa 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 ovarioectomy 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 ovarioctomized 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-κB p65 transcriptional activity was concomitantly increased. Further studies showed that targeted disruption of ERa 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 ERa-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 IFNγ 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 ovarioctomized 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 ERβ agonist but not by ERa-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 ERβ 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...
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, Koloğlu 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, MCP1, 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 TNFα-stimulated expression of the neutrophil-specific chemokine cytokine-induced neutrophil chemoattractant (CINC)-2β 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.

ERβ 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 ERα in these cells (Mori-Abe et al. 2003). Furthermore, stimulatory effects exerted by E₂ on the superoxide dismutase expression seem to depend on ERα (Sivritas et al. 2011). By contrast, treatment of porcine VSMC with antisense oligonucleotides complementary to ERβ mRNA abrogated the inhibitory effects of E₂ on ERK1/2/activity and proliferation (Geraldes et al. 2003). Likewise, the inhibitory effects exerted by estrogens on Ang-II-induced VSMC proliferation and TNFα-stimulated CINC-2β expression were prevented by ERβ but not by ERα receptor inhibitors (Xing et al. 2007). ERβ mRNA was found to be markedly expressed after balloon injury in rats, and local delivery of selective ERβ agonists was shown to more effectively reduce neointima formation than ERα agonists (Lindner et al. 1998, Aavik et al. 2001, Krom et al. 2007). Collectively, these results point to ERβ 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 GO/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 potently 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 ERα 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 ERβ 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 ERα 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. 2007).

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 (ERα 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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