MINI REVIEW

Estrogens and atherosclerosis


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Abstract

Numerous epidemiological as well as experimental studies have suggested that estradiol (E2) prevents atherosclerosis development. However two controlled prospective and randomized studies in women using hormone replacement therapy (HRT) did not confirm this beneficial effect. We then decided to use mouse models of atherosclerosis to define the possible mechanisms involved and the reasons for the discrepancy. We have shown that, although serum cholesterol decreases, this influence on lipid metabolism is negligible. Surprisingly, E2 induces an inflammatory–immune response towards a T helper cell (Th1) profile with increasing interferon-γ production that could destabilize atheromatous plaques, and could account for the increase in the frequency of cardiovascular events in women undergoing HRT. At the level of the endothelium, E2 induces an increase in nitric oxide (NO) bioavailability, but this phenomenon does not concern the development of fatty streaks. Nevertheless, the atheroprotective effect is apparently mediated at the level of the endothelium by a mechanism that has still to be characterized in molecular terms. These new acquisitions constitute a basis for new pharmacological developments allowing the prevention of deleterious effects and preserving the beneficial ones.

European Journal of Endocrinology 150 113–117

Introduction

Numerous epidemiological studies suggest that estrogens protect women against cardiovascular diseases before the age of menopause. After menopause, the cardiovascular risk of women becomes progressively closer to that of men, suggesting an atheroprotective effect of estrogens. However, the two controlled prospective and randomized studies published so far did not demonstrate a beneficial effect of hormone replacement therapy (HRT) whether in secondary prevention, i.e. in women who had already presented a cardiovascular event (Heart and Estrogen/Progestin Replacement Study (HERS) study (1)) or in primary prevention (Women’s Health Initiative study (2)). Thus, a better understanding of these non-reproductive effects of estrogens is urgently needed to decide whether or not to treat postmenopausal women who are anxious to prevent bone and vascular pathologies associated with ageing but who also complain of functional manifestations like hot flushes or dyspareunia. Moreover, such studies could provide new information concerning the pathophysiology, diagnosis and treatment of atherosclerosis, and also shed some light on autoimmune diseases, whose appearance and evolution are known to be influenced by estrogen hormones.

Indeed, whereas uncertainties persist concerning the nature and the implications of the effect of estradiol (E2) in clinical use, experimental works are much clearer. In experimental models of atherosclerosis, generally studied at the initial stages of the atherosclerotic process, i.e. at the stage of fatty streak constitution in various animal species, E2 treatment (possibly combined with progestin administration) prevents the development of fatty streaks when results are compared with castrated animals given a placebo (3, 4).

Our group has been working for several years to describe the vascular effects of E2 and to elucidate some of these mechanisms. We have used mice deficient in apolipoprotein E (apoE-KO) which are hypercholes- terolemic (3–4 g cholesterol/l) and spontaneously develop fatty streaks at the root of the aorta within a few weeks. Castration is followed by an increase in lesion area and, in castrated mice, E2 prevents the fatty streak deposit but serum E2 concentrations of the order of those encountered during gestation are necessary for maximal protection (5, 6). In addition, the atheroprotective effect seems to be mainly the consequence of a direct effect of E2 on the cells of the arterial wall. Although E2 treatment induces a decrease in serum cholesterol concentrations, the decrease is too low to explain the hormone protective effect (4–7);
it is also associated with decreases in both low- and high-density (LDL and HDL) fractions of the circulating lipoproteins.

**Estradiol and the inflammatory–immune system**

In recent years, atherosclerosis has come to be recognized as active and inflammatory, rather than simply a passive process of lipid infiltration. The inflammatory–immune system is strongly involved in the development of fatty streaks (8). Despite the obvious role played by inflammation at the different stages of atherosclerosis, classical anti-inflammatory drugs (steroid and non-steroid) have never proved their efficacy in this pathology (aspirin being as efficient at anti-aggregant doses as at anti-inflammatory doses). One of our goals has been to establish, using E2, the specific characteristics of the inflammatory mechanisms involved in atherosclerosis. Indeed, hypercholesterolemic mice also deficient in either monocyte-macrophage (through a deficit of macrophage colony stimulating factor) (9) or in mature B and T lymphocytes (RAG-2 gene deficient) (10) respectively develop ten- and two-fold less fatty streaks than control hypercholesterolemic mice. Mice deficient in various cytokines in general demonstrated the aggravating role of pro-inflammatory cytokines (such as interferon γ (IFN-γ), interleukins (IL)-1α and β, IL-12, IL-18) (11–13) and the protective role of anti-inflammatory cytokines (mainly IL-10) (14) in the development of the atherosclerotic process.

The adhesion of leukocytes to the endothelium is a critical step in the initiation and the development of fatty streaks. Leukocytes first undergo selectin and selectin ligand interaction which allows cell rolling along the endothelial surface. Leukocyte chemokine receptors thereby come into contact with chemokines displayed by the endothelium, leading to activation of integrins. This is necessary for subsequent firm adhesion through adhesion molecules of the immunoglobulins superfamily such as intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1). This process is followed by the leukocyte emigration from the vasculature to the subendothelial space. This initial step in fatty streak constitution could represent a relevant target for estrogen hormones, but experimental and epidemiological studies have until now reported conflicting results. We have investigated the effect of E2 and have demonstrated that P-selectin and ICAM-1 are not involved in the protective effect of estrogens; however it is suggested that VCAM-1 could play a role in this process (15).

We then looked at the influence of E2 on the inflammatory–immune cells constituting the atherosclerotic lesions, especially unstable lesions that are prone to lead to acute incidents such as myocardial infarction (16). In this context, we have demonstrated that, whereas E2 prevents the deposit of fatty streaks in Apo-E KO mice, it has no effect in mice deficient both in apoE and RAG-2 gene expression, lacking mature B and T lymphocytes (17). One hypothesis resulting from these observations is that lymphocytes, or at least a subpopulation of them, were the mediators of the atheroprotective effect. After crossing apoE-KO mice with mice deficient either in B, T cell receptor (TCR)αβ+ T or TCRγδ+ T lymphocytes we looked at the development of fatty streaks. We observed that TCRαβ+ T lymphocytes play a major role in fatty streak development. However, the protective effect of E2 persisted in all the constructed strains showing that none of the lymphocyte subpopulations specifically mediated the atheroprotective effect of E2 (R Elhage et al., unpublished observations). We then evaluated the possibility of an anti-inflammatory cytokine production. Further experiments did not confirm this hypothesis. In collaboration with different groups of immunologists, we have demonstrated that the profile of cytokine secretion of cluster of differentiation 4 (CD4+)(18) as well as natural killer (NK) T lymphocytes (P Gourdy et al., unpublished observations) is altered by E2 resulting in an increase in IFN-γ production and a decrease in anti-inflammatory cytokine production (IL-4, IL-10 and IL-13). Thus, E2 directs the inflammatory response towards a Th1 profile (the increasing interferon γ production). Similarly, we recently observed an increased production of IL-1, α and β, and IL-18 by macrophages obtained from E2-treated mice compared with macrophages obtained from castrated mice. From these studies, it appears that the inflammatory–immune system is not the mediator of the atheroprotective effect of E2. According to our current understanding, an increase in pro-inflammatory cytokine production in response to E2 cannot explain its atheroprotective effect. In contrast, it could destabilize atheromatous plaques, and account for the increase in the frequency of cardiovascular events during the year following the start of HRT (1, 2).

**Estrogens and endothelium**

The endothelium is uniquely positioned at the interface between the blood and the vessel wall and plays a crucial role in the physiology of circulation (19, 20) by performing multiple functions. It is involved in the regulation of coagulation, leukocyte adhesion in inflammation, transvascular flux of cells, liquids and solutes, vessel tone, and vascular smooth muscle growth. One of the main factors in all these activities is endothelium-derived reducing factor (EDRF), which is nitric oxide (NO) or a related nitrosocomound. Besides regulation of vascular tone and smooth muscle cell proliferation, NO might also play a protective role by inhibiting leukocyte adhesion to the endothelium, monocyte chemotaxis and inflammatory
reactions induced by cytokines. Indeed, inhibition of NO production during the initial weeks of cholesterol feeding in rabbits accelerates atherosclerosis and NO production is decreased in atherosclerotic vessels from both human and animal models. Finally, NO probably protects against the late events of atherosclerosis, such as thrombosis, by inhibiting platelet adhesion and aggregation. Different experimental and clinical studies have suggested that the mechanism by which E₂ is active is an increased bioavailability of NO. Such an increase could result from enhanced NO production, because endothelial NO synthase gene expression was reported to be increased by E₂ in different animal species (21, 22). It could also result from a decreased breakdown of NO, because we found that the production of reactive oxygen species (ROS) by cultured bovine aortic endothelial cells was decreased under estrogen treatment. In a poorly understood balance, ROS interacts with NO to form peroxynitrite, thereby destroying NO and diverting ROS away from its dismutation product, hydrogen peroxide (23, 24). Two estrogen receptor (ER) subtypes, encoded by two distinct genes, have been characterized: ERα and ERβ; of these, ERα has been shown to be the major mediator of the atheroprotective effects of E₂ on lesion size in ApoE-KO mice (25). In agreement with these observations, and in collaboration with Chambon’s group (26), we demonstrated that ERα mediates the effect of E₂ on the production of NO (27). A different mouse model, targeted for ERα through the insertion of Neo gene in exon 1, had been generated by Korach’s group in 1993; to date, this mouse model had been considered as ‘deficient’ in ERα (28, 29). In this model, we have observed the persistence of a truncated form (55 versus 66 kD for the wild-type ERα) through a peculiar splicing of ERα mRNA. Such an ERα isoform, lacking the B domain and thus probably the transactivating function of this aminoterminal domain (AF-1), was sufficient to mediate the E₂ effect on the endothelial NO production (30). Despite the clear increase in NO biodisponibility under E₂ treatment, we have been able to demonstrate that this mechanism was not involved in the protective effect of this treatment against atherosclerosis (31).

Using a series of experimental models, E₂ has also been reported to promote endothelial regrowth after endothelial denudation (32), angiogenic activity and endothelial cell migration and proliferation (33). This parameter could also be involved in the phenomenon of atheroprotection. Indeed, the most commonly used therapy of atherosclerotic complications consists of endoluminal angioplasty followed by endovascular stent implantation (34). This procedure consists of impacting the atheromatous plaque in the arterial wall by inflating a balloon. The immediate results are good, but there is deterioration due to a phenomenon of re-stenosis within a few months (in 30–50% of cases) as a consequence of constrictive remodeling and neo-intimal proliferation of smooth muscle cells. The constrictive remodeling can be prevented by an endovascular stent and this is now implanted almost routinely. In contrast the process leading to the re-stenosis, i.e. neo-intimal proliferation of smooth muscle cells, is not prevented by implantation of a stent and is still responsible for re-stenosis in 20–30% of cases. To prevent this intrastent re-stenosis, two approaches have been used in the past few years (34). The first approach consists of irradiating the artery endoluminally. However, irradiation induces radic fibrosis and prevents re-endothelialization, leading to delayed thrombosis. This severe complication led to this therapeutic approach being abandoned. The second approach consists of coating stents with an antimitotic drug (Rapamycin or Taxol) which seems very efficient in preventing neo-intimal hyperplasia. However, this approach inhibits the re-endothelialization, as was demonstrated in large animal models using the balloon technique (35); this could lead, as with radiotherapy, to delayed thrombosis. The best approach, which would consist of favoring re-endothelialization by a functional endothelium with the help of growth factors (such as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF)-1 or -2), has failed until now due to the pleiotropic and deleterious effects of these factors.

To evaluate the possible role of E₂ in re-endothelialization, we developed a novel model of electric arterial injury in mice: the model consists of destroying the endothelial and smooth muscle cells in the distal part (4 mm) of the common carotid artery. This model demonstrated that E₂ accelerates the re-endothelialization and that this effect is also mediated by ERα (36). Moreover, the rate of spontaneous re-endothelialization was normal when the production of NO or the activity of VEGF were inhibited but was abolished in FGF-2-deficient mice. Indeed, FGF2 was a good candidate to be a partner of E₂ because E₂ induces, for example, high levels of Fgf2 mRNA in the rat ovary (37). However, FGF2 expression is complex since five FGF2 isoforms of 18, 22, 22.5, 24, and 34 kDa in man and three isoforms of 18, 21 and 22 kDa in mouse are synthesized through an alternative use of translation initiation codons (38). These isoforms differ only in their NH₂ extremities, which confer a nuclear localization to the high-molecular weight (HMW) CUG-initiated forms, the function of which is largely unknown. In contrast, the smaller AUG-initiated FGF2 (18 kDa) is predominantly cytoplasmic, excreted and stored in the extracellular matrix (39). The AUG-initiated 18 kDa protein is thought to stimulate proliferation and migration and induce the downregulation of the FGF receptors. These are high-affinity transmembrane tyrosine kinase receptors, working in association with low-affinity receptors (heparan-sulfate-containing proteoglycans), activate the mitogen-activated protein (MAP) kinases and/or phospholipase C-dependent pathways. In line with these observations, E₂ has
been reported to stimulate MAP kinase activity via an autocrine loop involving FGF2 (40). Interestingly, the stimulating effect of E2 on the rate of spontaneous re-endothelialization in mice deficient only in low-molecular weight FGF-2, 18 kDa, isoform was normal showing that the HMW forms of FGF-2 are involved in the E2 effect. Cultures of endothelial cells obtained from subcutaneous Matrigel plugs allowed demonstration of a direct effect of E2 on FGF-2 gene expression and cell migration, and confirmed an intracrine effect of HMW forms of FGF-2 on this activity (B Garmy Susini et al., unpublished observations). The molecular mechanisms of these interactions are being investigated.

Finally, acceleration of re-endothelialization was followed by a spectacular decrease in fatty streak formation at the site of the carotid wound (L Brouchet et al., unpublished observations). Altogether, this series of data suggests that the site of atheroprotection could be located at the level of the endothelium. Further work is being pursued to confirm this suggestion and determine its molecular mechanisms.

In conclusion, E2 exerts an atheroprotective effect. As summarized in Fig. 1, although serum cholesterol decreases, the influence on lipid metabolism is negligible. Similarly, although E2 induces an increase in endothelial NO biodisponibility, this phenomenon does not concern the development of fatty streaks. Nevertheless, the atheroprotective effect is apparently mediated at the level of the endothelium by a mechanism that has still to be characterized in molecular terms. In contrast, E2 also induces an inflammatory–immune response towards a Th1 profile with increasing interferon γ production that could destabilize atheromatous plaques. This could account for the increase in the frequency of cardiovascular events in women undergoing HRT. These new acquisitions constitute a basis for new pharmacological developments allowing the prevention of deleterious effects and preserving the beneficial ones.

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