Understanding the controversy about hormonal replacement therapy: insights from estrogen effects on experimental and clinical atherosclerosis.

Summary

Whereas hormonal replacement/menopause therapy (HRT) in post-menopausal women increases the coronary artery risk, epidemiological studies (protection in pre-menopausal women) suggest and experimental studies (prevention of the development of fatty streaks in animals) demonstrate a major atheroprotective action of estradiol (E2). The understanding of the deleterious and beneficial effects of estrogens is thus required.

The immuno-inflammatory system plays a key role in the development of fatty streak deposit as well as in the atherosclerotic plaque rupture. Whereas E2 favors an anti-inflammatory effect in vitro (cultured cells), it rather elicits pro-inflammatory in vivo, at the level of several subpopulations of the immuno-inflammatory system, which could contribute to plaque destabilization.

Endothelium is another important target for E2, since it stimulates endothelial NO and prostacyclin production, thus promoting beneficial effects of vasorelaxation and platelet aggregation inhibition. Prostacyclin, but not NO, appears to be involved in the atheroprotective effect of E2. Estradiol accelerates also endothelial regrowth, thus favoring vascular healing. Finally, most of these effects of E2 are mediated by estrogen receptor α, and are independent of estrogen receptor β.

In summary, a better understanding of the mechanisms of estrogen action is required not only on the normal and atheromatous arteries, but also on innate and adaptive immune responses. This should help cardiovascular disease prevention optimization after menopause. These mouse models should help to screen existing and future Selective Estrogen Receptor Modulators (SERMs).

Résumé

Mieux comprendre la controverse concernant le traitement hormonal substitutif, à partir de la revue des effets de l’estrogène sur l’athérosclérose expérimentale et clinique.

Alors que le traitement hormonal substitutif (THS) augmente le risque coronaire chez la femme ménopausée, les études épidémiologiques (protection des femmes en pré-ménopause) sont en faveur d’une action athéromatrice, démontrée en utilisant l’estradiol (E2) dans des études expérimentales. La compréhension de ces effets délétères ou bénéfiques des œstrogènes est donc nécessaire.

Le système immunitaire et inflammatoire a un rôle clef dans le développement des stries lipidiques ainsi que dans la rupture de plaque. Alors que E2 a un effet anti-inflammatoire in vitro (sur des cultures cellulaires), il présente un effet pro-inflammatoire in vivo, sur plusieurs sous-populations de cellules du système immuno-inflammatoire, ceci pouvant contribuer à la déstabilisation de la plaque.

Une autre cible importante de l’E2 est l’endothélium. L’E2 stimule la production endothéliale dépendante du NO ainsi que celle de la prostacycline, tous deux ayant des effets vasodilatateurs et inhibant l’agrégation plaquettaire. La prostacycline (mais pas le NO) apparaît aussi être impliqué dans l’effet athéroprotecteur de l’E2. L’E2 accélère aussi la régénération endothéliale, favorisant la cicatrisation vasculaire. Enfin, la plupart des effets de l’E2 sont médiés par le récepteur œstrogénique α et sont indépendants du récepteur œstrogénique β.
Estrogens play a pivotal role in the sexual development and reproduction and are also implicated in a number of physiological processes in various tissues including the cardiovascular system. Numerous epidemiological studies suggest that estrogens protect women against cardiovascular diseases prior to the age of menopause. After menopause, the cardiovascular risk of women becomes progressively closer to that of men, reinforcing the hypothesis of an atheroprotective effect of estrogens. However, the two controlled prospective and randomized studies published so far did not demonstrate any beneficial effect of hormone replacement therapy (HRT), neither in secondary prevention (Heart and Estrogen/Progestin Replacement Study, HERS) [1] nor in primary prevention (Women’s Health Initiative study, WHI) [2]. This is in contrast to the large amount of data from experimental models of atherosclerosis, where estradiol (E2) treatment prevents the development of fatty streaks in comparison with castrated animals given a placebo [3].

The American Heart Association [4] recently defined 5 main priorities in the area of menopause treatment and cardiovascular (CV) risk including 1) the determination of the mechanisms of the CV events during the first year of the HRT, 2) the understanding of the beneficial effects of endogenous estrogens.

After a brief summary of our current knowledge of atherosclerosis, we will detail the atheroprotective action of E2 in experimental models, and the cell populations that are the target of E2 and that could mediate the protective effect. Unexpectedly, we found that E2 elicited a proinflammatory action at the level of several cell populations of the inflammatory-immune system. Whereas this effect cannot account for the prevention of the fatty streak deposit in experimental models, it could contribute to plaque destabilization in postmenopausal women. Endothelium, another major target for E2, and endothelial messengers, such as NO and prostacyclin, are both increased by E2. Although the atheroprotective effect of E2 appears independent of NO production, induction of COX-2 and prostacyclin production play an important role in the prevention of fatty streak. We finally detail the respective roles of estrogen receptor α and β in the vascular effects of E2. As for conclusion, we will try to provide a comprehensive view of experimental and clinical studies.

THE ATEROMATOUS PROCESS: NUMEROUS CELLULAR ACTORS FOR SEVERAL SCENARIOS

The first step of the atheromatous process is the penetration of atherogenic lipoproteins, in particular low density lipoproteins (LDL) through the endothelial monolayer [5]. LDL oxidation occurs in the subendothelial space and probably represents a necessary modification to the subsequent steps, because oxidized LDL induces in turn an activation of the endothelium, consisting in particular of an increased expression of adhesion molecules, such as intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1). These molecules are required to slow down circulating monocytes, to stop them and to allow their subsequent migration into the intima. In the subendothelial space, the activation of the monocytes induces their differentiation into macrophages, and this probably contributes to increase the level of LDL oxidation. These modified LDLs can be recognized by scavenger receptors expressed by macrophages. Thus, macrophages tempt to clean the intima, thereby preventing the accumulation of oxidized LDL. As oxidized LDL accumulates intracellularly, macrophages progressively turn into foam cells which constitute the major component of fatty streaks.

Blood flow shear stress represents a crucial protective factor, where abnormal shear stress promotes endothelial activation and dysfunction which represent so far the best and well recognized explanation for a focal location of atheroma [6-7]. Classical risk factors (high blood pressure, hypercholesterolemia, smoking, diabetes) appear to favor and/or even aggravate endothelial dysfunction. They can also favor the production of the chemokines and cytokines by different cellular actors (endothelium, monocytes-macrophages, smooth muscle cells as well as lymphocytes). Moreover, abnormal in vivo local shear stress appears to favor endothelial cell apoptosis and may be a major determinant of plaque erosion and thrombosis [8]. Protective factors are less recognized, although High Density Lipoproteins (HDL) appear of major importance.

Expansion of the fatty streak tends to be limited and circumvented by a scaring reaction of the smooth muscle cells migrating into the intima, secreting collagen and other extracellular matrix proteins. The balance between the inflammation level and the strength of fibro-muscular cap determines the stability of the atheromatous plaque [9]. Plaque rupture exposes thrombogenic materials leading to thrombus formation, which threatens the viability of the tissue down-stream the occluded artery. Unfortunately, the mouse model is not a satisfactory one for plaque rupture, and this is probably the most important limitation of the current experimental approach.

Many groups have been working to describe the cellular and molecular mechanisms leading to the aggregation or to the protection from atheroma [9-12]. This was allowed by the generation of the two major mod-
els of hypercholesterolemic mice: mice deficient in apolipoprotein E (apoE-KO) and mice deficient in LDL-receptor (LDLR-KO). ApoE-KO mice are hypercholesterolemic (3-4 g cholesterol/liter) under a chow diet and have very low levels of HDL cholesterol [13]. Accordingly, they spontaneously and rapidly (within a few weeks) develop fatty streaks at the root of the aorta. Mice deficient in LDL-receptor must be given a Western diet (fat plus cholesterol) to develop fatty streaks, because their lipoprotein profile under chow diet is less severe than the apoE-KO mice.

So far, the cellular and molecular dissection of the pathophysiology of atheroma was explored by breeding hypercholesterolemic mice and mice deficient in another specific gene. For instance, hypercholesterolemic mice also deficient either in monocyte-macrophage (through a deficit of Macrophage Colony Stimulating Factor) [14], or in mature B and T lymphocytes (RAG-1 gene deficient) [15] develop respectively 10- and 2-fold less fatty streaks than control hypercholesterolemic mice. It has been proposed that T cell-mediated and antibody-mediated responses directed against plaque associated antigens could influence the progression of arterial disease in a variety of animal models [16]. CD4 + T cells can be divided into functional subsets according to the type of cytokines they produce. T helper (Th) 1 cells mainly produce IFN-γ, activate macrophages, and are implicated in the elimination of intracellular pathogens and tumours. This Th subset is also implicated in the development of autoimmune diseases. On the other hand, Th2 cells produce IL-4, IL-5, IL-10 and IL-13, are associated with allergic diseases, and down-regulate Th1-mediated responses. Both subsets promote humoral immune responses with Th1 cells inducing the IFN-γ-dependent IgG2a isotype production, whereas Th2 cells preferentially induce IgG1 and IgE production through IL-4 synthesis. Experiments from several laboratories have shown that Th1-driven immune responses are deleterious in the context of atherogenesis [16]. The production of the Th1 cytokine IFN-γ appears to play a central role in the atherogenic process through its capacity to activate macrophages, to inhibit smooth muscle cell proliferation and collagen synthesis, promoting thereby plaque destabilization.

Mice deficient in various cytokines in general demonstrated an aggravating role of pro-inflammatory cytokines (such as Interferon γ (IFN-γ), interleukins (IL)-1α and β, IL-12, IL-18 and a protective role of anti-inflammatory cytokines (mainly IL-10) in the development of the atherosclerotic process [10].

Platelets, as key actors of arterial thrombosis, have been recognized to play a major role in the complication of atheroma, after endothelial erosion or plaque rupture. More recently, they have also been shown to participate in the constitution of fatty streak lesions at a very early stage, in particular at the level of the carotid bifurcation, a lesion prone site, by interacting with the activated endothelium before any macrophage infiltration [17]. This process involves the platelet GPIbα (Glycoprotein 1b, alpha polypeptide), and the adhesive proteins P-selectin and/or von Willebrand factor which mediate the attachment of platelets to activated endothelial cells. Blocking these interactions completely (-100%) prevented fatty streak formation at the level of the carotid bifurcation in apoE-KO mice, but only partially (-30%) at the level of the aortic sinus [17]. While this points out the platelets as a target for anti-atherosclerotic therapies, it also suggests that the modulation of the adhesive properties of the endothelium may be of pathophysiological relevance, especially at the level of the carotid bifurcation.

**ESTRADIOL IS AHEROPROTECTIVE IN EXPERIMENTAL MODELS DESPITE PROINFLAMMATORY ACTIONS**

Estradiol prevents the development of fatty streaks in all animal species

Studies in primates, mainly conducted by Clarkson, et al., have provided convincing evidence for the primary prevention of coronary artery atherosclerosis when estrogens are administered soon after the development of estrogen deficiency [18]. Equally convincing are the data from studies in cynomolgus monkeys indicating the total loss of the beneficial effects of estrogens if the treatment is delayed for a period equal to six postmenopausal years for women [18]. Moreover, in the monkey model, an attempt has been made to identify the most effective hormone treatment regimen in preventing the progression of coronary artery atherosclerosis. By far, the most successful approach is that of using estrogen containing oral contraceptive during the perimenopausal transition, followed directly by hormone replacement therapy in postmenopause. However, monkey models do not allow the understanding of the cellular or molecular mechanisms of E2 action.

We and several other groups have been working on the vascular effects of E2 and their cellular and molecular mechanisms [3, 19-20]. Ovariectomy of ApoE-KO or LDLR-KO mice is followed by an increase in fatty streak lesion area and E2 prevents in both models the fatty streak deposit. However, serum E2 concentrations of the order of those encountered during gestation are necessary for maximal protection [21, 22]. Although E2 treatment induces a decrease in serum cholesterol concentrations, the decrease involves both LDL and HDL fractions and is too minor to explain the hormone atheroprotective effect [3, 22]. This effect seems rather to be the consequence of a direct effect of E2 on the arterial wall cells [19]. A similar conclusion was previously obtained by other groups [23, 24]. Using hypercholesterolemic rabbits. In addition, they nicely showed the crucial role of intact endothelium, as the antiatherogenic effect of E2 was abolished, or even reversed, after balloon catheter injury [24].
Involvement of the inflammatory-immune system

As mentioned above, cell populations of the inflammatory-immune system (monocytes-macrophages, lymphocytes, etc.) play crucial roles in the pathophysiology of atherosclerosis [10-12]. Indeed, we demonstrated that E2 prevents the deposit of fatty streaks in immunocompetent apoE-KO mice, whereas it has no effect in mice deficient in both apoE and RAG-2 gene expression, lacking mature B and T lymphocytes [25]. One hypothesis resulting from these observations was that lymphocytes, or at least a subpopulation of them, were the mediators of the atheroprotective effect. After crossing ApoE-KO mice with mice deficient in either TCRαβ, CD4, CD8 or TCRβγ T lymphocytes, we reported that TCRαβ T-lymphocytes play a major role in fatty streak development [26]. However, the protective effect of E2 persisted in all these strains, showing that none of these lymphocyte subpopulations specifically mediated the atheroprotective effect of E2 [27]. This led us to investigate the effect of E2 on the cytokine production of different cell populations of the inflammatory-immune system, as it was shown to play a crucial role in the pathophysiology of atheroma [10].

Pro- or anti-inflammatory effect of E2?

Cell populations of the inflammatory-immune system are heterogenous and difficult to be isolated, especially in mouse models. At variance with macrophages in fatty streaks, peritoneal macrophages represent a cell population that can be obtained in considerable amounts, and thus the chronic effect of E2 on the inflammatory-immune system can be easily studied in these cells. It should first to be mentioned that a chronic in vivo treatment of mice by E2 led to a proinflammatory response in peritoneal macrophages (our unpublished data), whereas an acute (only few hours of E2) in vitro treatment of the macrophage cell line RAW 264.7 by E2 led to an anti-inflammatory effect [28]. In vitro experiments have also suggested a selective inhibitory effect of E2 on the production of some inflammatory cytokines, such as TNF-α, IL-6, but not IL-12 or IL-10 by LPS-activated splenic macrophages [29]. Similar differences were also reported by comparing the effect of E2 on microglial cells, the resident macrophages of the brain [30-32]. Whereas E2 was found to prevent LPS-induced microglial reactivity both in vitro and in vivo, based on the changes in inducible NO synthase, PGE2, and metalloproteinase-9 levels [30-31]; another study has clearly shown that E2, through ERα signalling, was required for optimal transcriptional activation of TNF-α and IL-12 genes in the brain-resident microglia upon endotoxin challenge [32]. Although all these parameters were not simultaneously assessed in these two studies, the anti-inflammatory effect of E2 could depend on the target genes. Altogether, these striking discrepancies illustrate the importance of the in vivo approach to understand the physiopathological effects of E2.

We have thus evaluated the influence of a chronic stimulation of E2 on the production of pro- and anti-inflammatory cytokines in lymphocytes and macrophages. We have demonstrated that the profile of cytokine secretion in CD4+ [33] as well as in NK-T lymphocytes [34] is altered by E2. In these studies, an increase in IFNg production and a decrease in anti-inflammatory cytokine production were observed, resulting in a strong bias towards a Th1 profile. This effect of E2 was observed in vivo upon induction of T cell responses but not in T cells stimulated in vitro, suggesting that E2 may not directly up-regulate cytokine production in T cells as previously suggested. Likewise, we recently found that starting hormonal treatment at the time of immunization had almost no effect on the establishment of T-cell responses [35]. By contrast, E2 therapy, limited to the 3 weeks period before immunization, was necessary and sufficient to promote antigen (Ag)-specific Th1 cell expansion and the production of type-I-dependent IgG2a and IgG2b isotypes [39]. Furthermore, we found that the E2-mediated increase in Ag-specific Th1 cell development was abolished in IL-12-R-deficient mice. These observations suggest that E2 acts on the antigen presenting cell (APC) compartment rather than T cells, although the latter cell type cannot be excluded as a potential target. In favor of a role for E2 on APC, it has been shown that estrogens were needed for the optimal development of mouse dendritic cells (DC) from bone marrow precursors in vitro [36]. The requirement for estrogens during DC differentiation suggests a mechanism by which E2 levels in peripheral tissues may modulate both number and functional properties of DC in vivo, thereby influencing immune responses. Indeed, splenic DC numbers were increased in E2-treated mice, and this increase seems to affect preferentially CD8α+ DCs that have been shown to secrete higher amounts of IL-12 [35]. Similarly, we observed an increased production of IL-1 (α and β), IL-12 and IL-18 by macrophages obtained from chronically E2-treated mice compared with those from ovarietomized mice (Calippe B, et al. in preparation).

According to our current knowledge, the proinflammatory effect of E2 cannot account for its preventive effect of fatty streak accumulation [10-12]. In contrast, it could contribute to the atheromatous plaques destabilization, and thus represent a good candidate to explain the cardiovascular events increase during the year following the onset of HRT [1, 2].

Understanding the protective and deleterious effects of E2 in atheroma (fig.)

In summary, E2 exerts an atheroprotective effect in all experimental models and most likely in women before menopause. Although E2 decreases serum cholesterol in all animal species including humans, this influence on lipid metabolism is negligible in hypercholesterolemic mouse models [3, 21, 22]. Endothelium is involved in the regulation of coagulation, leukocyte adhesion in inflammation, transvascular flux of cells, vascular smooth muscle growth, etc. and also represents a major target for E2. Endothelial messengers, such as NO [37] and prostacyclin are increased by E2. Indeed, E2 can increase NO bioactivity acutely.
E2 influences coagulation factors through complex pathways, i.e. it promotes venous thrombosis and tends to prevent arterial thrombosis. In addition, it seems to influence platelet physiology: E2 can elicit various alterations of platelet adhesion and increase the number of circulating platelets, these effects being mediated by ERα [42,43]. To which extent chronic E2 influences platelet adhesion and aggregation in vivo is currently under investigation (MC. Valera, M.P. Gratapa, P. Sié, B. Payrastre). Since blocking the interactions between endothelium and platelets prevents fatty streak formation in apoE-KO mice [17], we hypothesized that E2 could interfere with this interaction. However, using an intravital microscopy approach, we could not detect any significant effect of E2 on the endothelium-platelet interaction at the level of the carotid (AD Terrisse, P. Sié, unpublished data).

Blocking these interactions completely (-100%) prevented fatty streak formation at the level of the carotid bifurcation in apoE-KO mice, but only partially (-30%) at the level of the aortic sinus [17]. While this points out the platelets as a target for anti-atherosclerotic therapies, this also suggests that the modulation of the adhesive properties of the endothelium may be of pathophysiological relevance, especially at the level of the carotid bifurcation.

E2 also promotes the production of interferon γ, a prototypic pro-inflammatory cytokine, by enhancing antigen-specific Th1 cell development. This proinflammatory effect could have been prominent in advanced atheromatous plaques in post-menopausal women, favoring a destabilization of the most unstable plaques through IFN-γ-dependent activation of macrophages (fig.: Red arrow 1). This previously unrecognized effect could significantly contribute for the increase in the frequency of cardiovascular events in post-menopausal women during the first year of the HRT, as observed in the HERS and WHI studies. Red arrow (2) : the progestin (medroxyprogesterone acetate) used in HERS and WHI [46] clearly contributes to the early deleterious effect, as hysterectomized patients of any age treated with ‘estrogens alone’ do not present any increase in coronary risk [45].

Le double effet des œstrogènes sur l’athérosclérose initiale (effet bénéfique, Green arrow) et sur le stade évolué (red arrow 1) atherosclérose.

Schematic representation of a normal artery, fatty streak, stable plaque, unstable plaque and plaque rupture and thrombosis (adapted from P. Libby). Green arrow : the endothelial effect of E2 could represent one key mechanism of the atheroprotective effect of E2, which is prominent in intact arteries, as experimental models and premenopausal women. Red arrows : in advanced atheromatous plaques in post-menopausal women. Red arrow (1) : the proinflammatory effect of E2 could favour a destabilization of unstable plaques through IFN-γ-dependent activation of macrophages. This previously unrecognized effect could significantly contribute for the increase in the frequency of cardiovascular events in post-menopausal women during the first year of the HRT, as observed in the HERS and WHI studies. Red arrow (2) : the progestin (medroxyprogesterone acetate) used in HERS and WHI [46] clearly contributes to the early deleterious effect, as hysterectomized patients of any age treated with ‘estrogens alone’ do not present any increase in coronary risk [45].

Thus, it is likely that exogenous estrogens protect against the coronary risk, while this benefit is completely lost when using exogenous estrogens after 10 years of untreated menopause. In addition, hysterectomized patients treated at any age with ‘estrogens alone’ do not present any coronary risk increase [45], underlining a potential deleterious role of the progestin (medroxyprogesterone acetate) used in HERS and WHI [46] (fig.: red arrow 2). Indeed, this non-natural progestin used in these clinical trials possesses undesirable, deleterious effects [47].
Estrogen receptor α mediates most of the vascular effects of E2

We previously mentioned that E2 can increase acutely the NO bioactivity through a stimulation of endothelial NO synthase activity [37] and chronically through a decreased breakdown of NO, as a consequence of a reduced production of reactive oxygen species [38, 39]. Estradiol effects can be mediated by estrogen receptor alpha (ERα) and beta (ERβ), two members of the nuclear receptor superfamily, that are encoded by 2 distinct genes [48]. A collaborative effort with the Krust/Chambon group led to the clearcut demonstration of a prominent role of ERα in vascular physiology in vivo. Full length ERα (66 kD) is composed of 6 ‘domains’ (named from A to F). The two transcription function domains, AF1 and AF2, are found within domain B and E, respectively [49, 50]. Both the ERα and ERβ genes have been subjected to targeted mutagenesis [48]. The first gene disruption mice model of ERα was generated by the group of K. Korach in 1993, through the insertion of the Neomycin resistance gene in exon 1 (thus named ER-αNeoKO) [51]. These mice were subsequently shown to present a transcriptional leakage due to a non-natural alternative splicing of the ERα mRNA resulting in the expression of a truncated 55kD isoform [52-54]. However, such an ERα isoform, lacking a major part of the B domain and thus the AF-1 transactivating function, was sufficient to mediate the E2 effect on the endothelial NO production [53]. Interestingly, an ERα 46kD-isoform, lacking the N-terminal portion (domains A/B), can be physiologically expressed through an alternative splicing [55-56] in the uterus [53, 57] and in cultured endothelial cells [58]. This isoform is also expressed in the ER-αNeoKO mice [53].

In contrast, the generation and studies of mice that fully and unambiguously lack ERα [59] showed that ERα is necessary in the response of E2 on NO production [53]. ERβ deficient mice had, however, a normal NO production [60]. Altogether, these data allow us to conclude that an ERα lacking the AF-1 function is sufficient to mediate some of the vascular effects of estrogen.

However, the prevention of fatty streaks appears to require the full length ERα (66 kD) [61]. Indeed, in contrast to the E2 protection elicited in apoE-KO mice, E2 treatment of ovariectomized ERα-Neo/apoE double KO female mice caused a non-significant (p = 0.12) reduction in lesion size and no reduction in total plasma cholesterol [61]. It should be however mentioned that E2 treatment significantly reduced the complexity of plaques in ERα-Neo/apoE double KO female mice, although not to the same degree as in apoE-KO female mice. Although this could have been due to the existence of ERβ-dependent atheroprotective effects of E2, the expression of the truncated 55kD ER-α isoform could also have been responsible of this E2 effect.

As mentioned previously, the loss of the integrity of the endothelial monolayer represent another important aspect at early steps of atherosclerosis, but also after the destruction provoked by endoluminal angioplasty (often followed by stent implantation) [62]. In this context, the acceleration of vascular healing, with re-endothelialization playing a key role, is considered as a major protective event against short- and long-term complications of endovascular therapy.

Endovascular desendothelialization in mice is a complex and delicate manipulation as a consequence of the very small size of carotid artery. P Carmeliet et al. [63] proposed to destroy the endothelium using perivascular electric injury approach, and an adaptation of this model was proposed by our group [64]. Endovascular and electric perivascular injury are identical for their efficiency to destroy the endothelium, which will be temporarily replaced by a monolayer of platelets, but they differ in at least two major points. First, whereas endovascular injury preserves most of the medial smooth muscle cells as well as cells in the adventitia [65], electric injury destroys the cells of the three layers of the injured area, and in particular the smooth muscle cells which do not recolonize the media even after several weeks. Second, the main cell population in the perivascular electric injury model susceptible to interact with regenerating endothelium is immuno-inflammatory cells.

The acceleration effect of E2 on reendothelialization is mediated by ERα [64] and endothelial NO synthase appears absolutely required for this effect [66]. Interestingly, the ERα isoforms (55 kD and 46kD) expressed in the ER-αNeoKO mice [51] is sufficient to mediate the E2 effect on the post-injury medial hyperplasia [67,68]. Thus, in analogy with vascular NO production [53], the AF-1 trans-activating function may be dispensable to mediate the beneficial effect of E2 on artery healing. Finally, E2 increases the number of circulating EPCs [66, 69], and this effect could be a key actor of the re-endothelialization acceleration by E2.

Towards a comprehensive view of experimental and clinical studies

Overall, experimental studies demonstrate and epidemiologic studies suggest that estrogens prevent atherosclerosis, whereas randomized clinical trials did not confirm a cardioprotective effect. Although observational studies may have overestimated the coronary benefit conferred by HRT, there are other plausible explanations for the apparent discrepancy between previous results and the less favorable findings from the WHI study. It is likely that age or time after onset of the menopause may importantly influence the cardiovascular benefit-risk ratio associated with HRT, and that the method of administration, dose, and formulation of exogenous hormones may also be relevant [70, 20].

Compared to oral route, transdermal E2 administration allows to avoid the hepatic first-pass and consequently to limit certain deleterious effects. The ESTHER case-control study [71] confirmed that oral estrogens favor a significant increase in the thrombo-
embolic risk, and suggests that the transdermal route is not associated to an increase in the incidence of thrombo-embolic events. However, no large controlled study provided definite evidence for this difference. Whatever the informative value of C-reactive protein (CRP) levels in terms of cardiovascular risk, it is of interest to mention that, whereas the oral estradiol increases these levels, the transdermal route does not [72].

Although increasing amount of evidence indicates that older women and those with subclinical or overt coronary heart disease should not take HRT, estrogen remains the most effective treatment currently available for vasomotor symptoms (vasomotor flushes). The HRT effects on the development of coronary disease in newly postmenopausal women remain unclear. An apparently surprising absence of effect of HRT on the quality of life was reported in the WHI study [73]. However, it is worth to mention that women with hot flushes were discouraged to participate to this WHI trial, investigators considering as opposed to ethics to be without the most active treatment for years, as mentioned in the accompanying editorial [74].

Clearly, the effects of HRT on quality of life and cognitive function in recently postmenopausal women deserve further studies. These unresolved clinical issues provide the rationale for the design of the Kro nos Early Estrogen Prevention Study, a 5-year randomized trial that will evaluate the effectiveness of low-dose oral estrogen and transdermal estradiol in preventing progression of atherosclerosis in recently postmenopausal women [75].

Finally, HRT decreases the risk of bone fracture in menopausal women, as clearly established in WHI study with a reduction of about 40% of all fractures. This effect is noteworthy since the population included was at low risk of osteoporosis (in particular as a high percentage of overweight women were included). Thus, less than 15% of the women had a densitometric osteoporosis, at variance with other large trials conducted to evaluate osteoporosis or effects of bisphosphonates, that included women with a higher risk of fracture.

Unresolved questions, present and future treatments.

Breast cancer represents the most obvious risk feared by women taking HRT. Indeed, cohort or intervention trials (HERS and WHI 2002) consistently showed a 1.3-fold increase in breast cancer by HRT. Unexpectedly, WHI 2004 ‘estrogens alone’ in hysterectomized women showed an almost significant trend (p = 0.05) toward protection against breast cancer (RR: 0.77 [45]). Even if this study raised the question of a potential role of the consequences of associated ovariectomy or the nature of the estrogens used (potential protective role of conjugated equine estrogens), most of the recent studies underline again the deleterious role of progesterone. Indeed, a cohort study [76] and more recently the E3N INSERM study [77] showed no increased risk when HRT included natural progesterone, contrasting strikingly with an increased risk of breast cancer when estrogen are associated with synthetic progestin having androgenic (19-nortestosterone) or anti-inflammatory (medroxyprogesterone acetate) properties.

Although ERs are classically defined as ligand-activated transcription factors [50], it has become clear that ‘extragenomic membrane short-term’ responses play an important role in cultured endothelial cells [37], but also in osteoblasts (as for instance the activation of PI3kinases-AKT pathways as well as MAP kinase pathways) [78]. An important challenge for the next years will be to characterize the respective roles of these ‘membrane’ effects and the ‘classical’ effects [37, 79-80, 20].

These new acquisitions constitute a basis for new pharmacological developments allowing the prevention of deleterious effects and preserving the beneficial ones [70, 81]. The effects of Selective Estrogen Receptor Modulators (SERMs) on the different actors of the atheroma plaque formation have now to be analyzed on the basis of their specific regulation of the ERα but also of the ERβ, which specific activation elicit some anti-inflammatory actions in vivo. Various classes of estrogens and SERMs have been described according their molecular actions through ERα [82-84]. Due to the complexity of the mechanisms of action of estrogens and SERMs, their effect on various cell types and tissues cannot be predicted from their structure. Hence, integrated models that allow the screening of present and future SERMs in terms of the beneficial and deleterious effects will be valuable and important tools. Theoretically, it is conceivable to design a SERM (or a combination of molecules) which would retain most (if not all) of the desired effects of E2 (on the central nervous system to prevent vasomotor flushes, on bone, on endothelium, etc.), but which should be devoid of the undesirable effects of E2 (mainly breast cancer, thrombo-embolism and probably pro-inflammatory effect). SERMs currently available (tamoxifen, raloxifen) prevent breast cancer, but are devoid of effect on menopause symptoms and on cardiovascular risk. Prevention of both breast cancer and cardiovascular diseases by novel SERMs thus represents the major challenges of the future treatment of menopause.

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