Estrogen receptor α is a major mediator of 17β-estradiol's atheroprotective effects on lesion size in *Apoe*$^{-/-}$ mice

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The inhibitory effects of estrogen (17β-estradiol) on atherosclerosis have been well documented in numerous animal models, and epidemiological evidence supports this protective effect in humans. The detailed mechanisms for this protection are not understood, but most are thought to be mediated through estrogen receptors (ERs), of which two are known (ERα and ERβ). To investigate the role of ERα in the atheroprotective effect of 17β-estradiol (E2), we ovariectomized female mice that lack apoE (*AAee*) or lack both apoE and ERα (*ααee*), and treated half of them with E2 for three months. E2 treatment of ovariectomized *AAee* females dramatically reduced the size of the lesions as well as their histological complexity. Plasma cholesterol was significantly reduced in this group, although the observed extent of protection by E2 was greater than could be explained solely by the change in lipid levels. In contrast, E2 treatment of ovariectomized *ααee* females caused minimal reduction in lesion size and no reduction in total plasma cholesterol compared with *ααee* mice without E2, demonstrating that ERα is a major mediator of the atheroprotective effect of E2. Nevertheless, E2 treatment significantly reduced the complexity of plaques in the *ααee* females, although not to the same degree as in *AAee* females, suggesting the existence of ERα-independent atheroprotective effects of E2.

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Introduction

Coronary heart disease (CHD) is the leading cause of death in the Western world (1). Epidemiological studies indicate that premenopausal women are protected, but that the risk of CHD rises sharply after menopause (2). This protection is thought to be in part through endogenous estrogen (17β-estradiol, or E2) as the risk is significantly reduced by estrogen replacement therapy (2), although a recently completed clinical trial using hormone replacement therapy did not show protection in women with preexisting CHD (3). Exogenous E2 clearly inhibits lesion progression in numerous animal models of atherosclerosis including the apoE-deficient mouse (*Apoe*$^{-/-}$) (4–8). While E2 causes favorable alterations in lipoprotein metabolism, these changes appear to account for not more than 50% of the protection in humans (9). In addition, several studies in animals show full atheroprotection at E2 doses that do not alter the lipid profile (2). Thus attention has focused on the direct effects of E2 on the vessel wall and on the processes involved in the inflammatory and fibroproliferative components of atherosclerosis including: endothelial permeability to LDL (10), LDL oxidation (11, 12), cytokine and cell adhesion molecule expression (13–16), macrophage cholesterol homeostasis (17), vascular smooth muscle cell and neointimal proliferation and migration (18, 19), calcification (20, 21) and platelet adhesion and aggregation (22).

The long-term effects of E2 are generally ascribed to transcriptional modulation of target genes through estrogen receptors (ERs). These receptors are members of the steroid hormone receptor superfamily of ligand-activated transcription factors (23) that modulate gene expression directly through estrogen response elements in the promoter regions of target genes, or indirectly through interaction with transcription factors, coactivators, and transcription complexes, such as activating protein-1 (AP-1) (23–26). In addition, rapid, nongenomic, vasodilatory effects of E2 have been reported that may also involve ERs (27).

Two estrogen receptors, ERα and ERβ, have been characterized (28, 29). They are encoded in two separate genes (*Esr1* and *Esr2*), are distinct structurally and functionally, and have tissue expression patterns that overlap but are not identical (29–31). Both receptors are expressed in cell types important in atherogenesis, including endothelial cells (32) and vascular smooth muscle cells (33, 34). Estrogen receptors have also been

demonstrated in macrophages and T lymphocytes (35, 36). Determining the relative contribution of the two receptors to atheroprotection is of considerable interest in its own right, as well as in relation to the potential use of pharmaceutical agents specific to each receptor type.

Considerable success has been achieved in demonstrating the atheroprotective effect of E2 in *Apoe*$^{-/-}$ mice (7, 8) and in LDL-receptor deficient mice (37). For example, dose-response studies with E2 continuously administered to ovariectomized apoE-deficient mice have demonstrated that high physiological levels result in near complete inhibition of atherosclerotic lesion progression. We have therefore employed a similar study design to compare the effects of E2 treatment on the spontaneous development of atherosclerotic lesions in ovariectomized *Apoe*$^{-/-}$ mice (*ee*) that have or do not have ERα (*AAee* versus *ααee*). We find that the atheroprotection provided by E2 administration is substantially abrogated in the *ααee* mice, which lack ERα, but atheroprotection by non-ERα mechanisms is still clearly demonstrable.

Methods

Mouse experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Generation and use of AAee and ααee mice. Mice heterozygous for an insertional mutation in ERα (*Aα*) and *Apoe*$^{-/-}$ mice (*ee*), each backcrossed over 99% to C57BL6/J, were intercrossed to yield males and females heterozygous for both mutations (*AαEe*). These double heterozygotes were further mated with *ee* mice to generate mice heterozygous for ERα and homozygous for the insertional mutation in ApoE (*Aαee*). Intercrossing the *Aαee* mice produced mice lacking ERα (*ααee*) and littermate controls having intact ERα (*AAee*). At day 30, females were weighed, ovariectomized, and randomly implanted subcutaneously with either pellets designed to release E2 at 6 μg/day for 60 days or a placebo control (C) (Innovative Research of America, Sarasota, Florida, USA). The four study groups were *AAee* with E2 (*AAee*/E2; n = 17), *ααee* with E2 (*ααee*/E2; n = 14), and their respective control groups (*AAee*/C; n = 17 and *ααee*/C; n = 11). Pellets were replaced 60 days after surgery and the mice were sacrificed 30 days later (day 120, or 4 months of age) after a 2- to 4-hour fast. Total body weight and uterine weight were recorded. Four of 17 *AAee*/E2 mice died during the third month of treatment and the data from another four mice were excluded for other reasons (see Results), leaving 9 *AAee*/E2 mice. Mice were maintained in specific-pathogen-free conditions on standard mouse chow (Prolab Isopro RMH 3000; PMI Nutrition International, Brentwood, Missouri, USA).

Serum E2 concentration. E2 levels were assayed on plasma from individual mice obtained at sacrifice using a radioimmunoassay kit for E2 and following the manufacturer's instructions (Diagnostic Systems Laboratories Inc., Webster, Texas, USA).

Atherosclerotic lesion analysis. Hearts were perfusion fixed with 4% paraformaldehyde under physiological pressure (pH 7.4). The proximal aorta and the part of the heart containing the aortic root were removed, embedded, sectioned, and stained with hematoxylin and eosin (H&E) and Sudan IV (Fisher Scientific, Fair Lawn, New Jersey, USA) as described (38). Lesion size was measured using NIH 1.59 Imaging Software from four sections chosen by strict anatomical criteria. The average of the four sections was taken as the lesion size of each animal and logarithmically transformed for statistical analyses. The plaques in the aortic sinus were morphologically graded in a blinded fashion by R.L. Reddick, an experienced cardiovascular pathologist, for lesion complexity using as parameters absence or presence of foam cells, fully formed or developing fibrous cap, calcifications, cholesterol clefts, acellular cores, inflammatory cells, and medial extensions.

Plasma lipid analysis. Total cholesterol, HDL, and triglycerides were measured colorimetrically (Sigma-Aldrich, Milwaukee, Wisconsin, USA) on plasma collected at sacrifice. HDL cholesterol levels were determined after precipitating the apoB-containing particles. Plasma samples were stored at –20°C until assayed.

Statistics. Statistical analyses were performed with the PC-based software package SYSTAT 5.0 (SPSS, Chicago, Illinois, USA), JMP (SAS Institute Inc., Cary, North Carolina, USA), and LogXact 2.0 (Cytel Corp., Cambridge, Massachusetts, USA). Data were analyzed with a factorial ANOVA, using genotype (*AAee* versus *ααee*) and E2 status (E2 versus C) as the two grouping variables. Further analyses to interpret significant main effects and/or interactions were conducted using the Tukey-Kramer honestly significant difference multiple comparison procedure. Nonparametric analyses of lesion histopathological characteristics were conducted with multiple logistic regression. Comparisons of females with E2 versus C across each genotype were with Fisher's exact test using the Bonferoni procedure to correct for multiple comparisons. Correlations between different parameters were analyzed using simple linear regression. P values less than 0.05 were considered statistically significant for all comparisons.

Results

Experimental design. The experimental animals used for investigating the role of ERα in atheroprotection by E2 were female apoE-deficient mice with or without ERα (*AAee* or *ααee*) on a C57BL6/J genetic background. However, intact ERα-deficient females have plasma E2 levels three times higher than wild-type mice as a consequence of their lack of an ERα-dependent functional negative feedback mechanism (39) and also have increased plasma levels of testosterone, which inhibits lesion progression in *Apoe*$^{-/-}$ mice (8). We therefore chose, as a means of removing these confounding variables, to ovariectomize all experimental animals and replace their endogenous E2 with subcutaneously implanted hormone-releasing pellets at 30 days of age.

Table 1
Plasma estradiol levels, uterine weight, and body weight in *AAee* and *ααee* mice

Study Group	*n*	Plasma E2 (pg/ml)	Uterine wt (mg/g body wt)	Body wt (at ovx) (g)	Body wt (at sacrifice) (g)
AAee/C (ovx)	17	<20	1.01 ± 0.26	15.3 ± 0.3	24.3 ± 0.7
AAee/E2 (ovx)	9	72 ± 20[A]	5.45 ± 0.58[B]	16.0 ± 0.5	22.2 ± 0.6
ααee/C (ovx)	11	<20	0.56 ± 0.12	15.9 ± 0.7	22.0 ± 0.8
ααee/E2 (ovx)	14	104 ± 17[A]	1.62 ± 0.17	17.0 ± 0.5	24.3 ± 0.7
AAee (intact)	13	ND	3.4 ± 0.54	ND	21.2 ± 0.7
ααee (intact)	11	ND	1.7 ± 0.18	ND	24.2 ± 3.9

Study group includes ovariectomized (ovx) females treated with control pellets (C) or E2 (E2), or females with ovaries intact (intact). [A]$P < 0.01$ versus control. [B]$P < 0.01$ versus all other groups. Data represent mean ± standard error. ND, not determined.

Pellets which nominally release 6 μg of E2 a day and attain plasma levels within the range of animals at peak estrus (7) were chosen, based on previously published work demonstrating that this dosage was the lowest able to reproducibly lower plasma lipids and plaque size in *Apoe*$^{-/-}$ mice (7, 8).

The data presented in Table 1 demonstrate that we were successful in achieving the desired hormonal status of the experimental animals. Ovariectomy reduced plasma E2 to below detection limits, and the E2 replacement attained plasma levels in the high physiological range. On gross inspection, the uteri were smaller and atrophic in *AAee*/C, *ααee*/C, and *ααee*/E2 females compared with *AAee* mice with intact ovaries, while uteri in *AAee*/E2 mice were enlarged. Uterine weight in the ovariectomized females was increased about fivefold by E2 treatment in *AAee* mice ($P < 0.0005$) and was 60% increased over intact *AAee* females ($P < 0.005$). The uteri in *ααee*/E2 females tended to be larger than in *ααee*/C ($P = 0.075$) but were similar to the uterine size of *ααee* females with intact ovaries. There were no differences in total body weight between groups at ovariectomy or at the end of the study. ERα-deficient mice have been reported to gain more body weight than wild-type controls, but the difference is not significant until after 4 months of age (39).

All of the *ααee*/E2 and control females completed the study. E2 treatment in some of the *AAee* females, however, was associated with decreased survival (four animals) and urinary and reproductive tract pathology (enlarged bladder, cystitis, hydronephrosis, and uterine hyperplasia and endometritis; four animals), most likely secondary to an increased susceptibility to infection. Data collected from these mice were excluded from the final analysis (final $n = 9$), though inclusion of the data did not alter the mean values for lesion size or lipid profiles.

ERα is required for the major atheroprotective effect of E2. To determine the role of ERα in the inhibition of atherosclerotic progression by E2, lesion size in the proximal aorta was measured at 4 months of age in *AAee* and *ααee* females ovariectomized at 1 month of age and treated with or without E2 from the time of ovariectomy. As expected, exogenous E2 was highly effective in inhibiting lesion progression in *AAee* females. Lesions in *AAee*/E2 females were more than 80% smaller than those of *AAee*/C females ($P < 0.001$) (Figure 1). In contrast, E2 only minimally reduced lesion size in *ααee* mice. Mean lesion size in *ααee*/E2 females was 35% less than the mean lesion size in *ααee*/C females, but the effect did not reach significance ($P = 0.12$). Interaction between genotype and hormone treatment was highly significant by two-way ANOVA ($P < 0.0001$), demonstrating that ERα is important for the E2 effect. Furthermore, while all E2-deficient females and *ααee*/E2 females had at least one plaque in the descending aorta that was readily visible under a dissecting microscope, the *AAee*/E2 mice had none, correlating with the markedly reduced lesion sizes in the aortic sinus. Thus, ERα is required for a major part, if not all, of the E2-mediated reduction in lesion size.

E2 reduces advanced-lesion characteristics in the absence of ERα. Figure 2 shows examples of atherosclerotic lesions chosen from females whose mean lesion size was near the mean for its respective group (actual lesion size for each example is shown in panels). The lesions from *AAee*/C (Figure 2a) and *ααee*/C females (Figure 2b), which are not distinguishable, are substantially more complex than lesions from *AAee*/E2 females (Figure 2c), which are essentially fatty streaks containing lipid-laden foam cells and have few advanced characteristics. Lesions in *ααee*/E2 females (Figure 2, d and e) are also reduced by the E2 treatment not only in size but also in complexity compared with the untreated *ααee*/C females (Figure 2b).

To analyze the effects of E2 and ERα on the development of lesion complexity, tabulations of lesion characteristics from the aortic sinus were conducted in a blinded fashion. Figure 3 presents the percentage of mice in

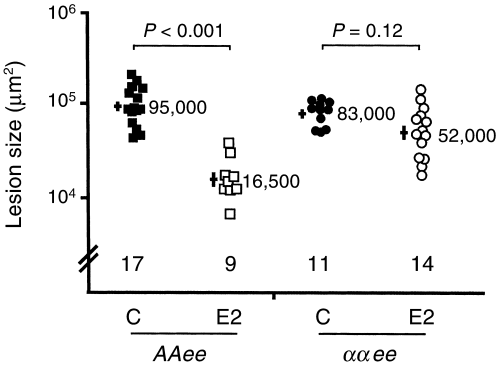

Figure 1
Atherosclerotic lesion size expressed on a log scale in ovariectomized 4-month-old *AAee* and *ααee* females after 3 months of treatment with control pellets (C) (filled squares and circles, respectively) or with E2 pellets (E2) (open squares and circles, respectively). Each point represents one mouse. Bars on the left represent the logarithmic mean ± standard error with mean lesion size (in μm²) shown on the right.

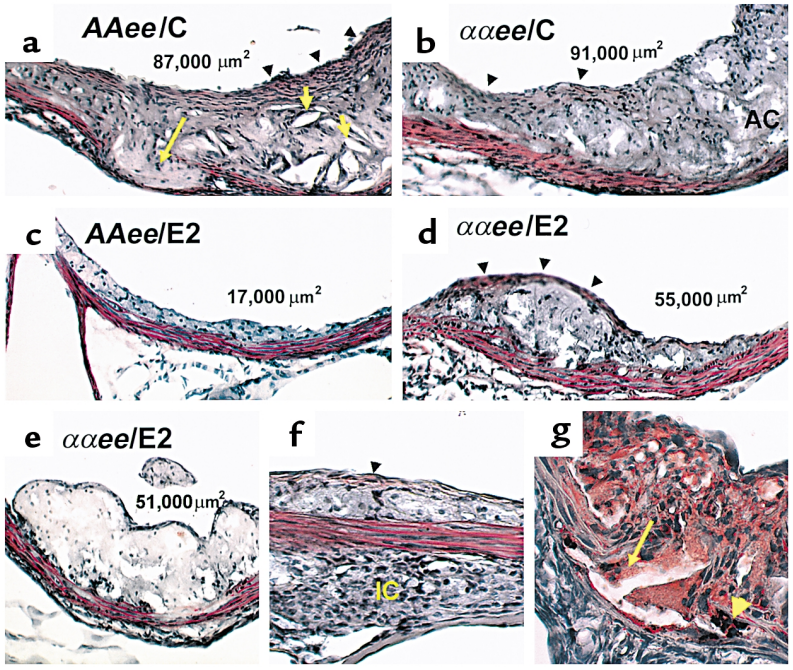

Figure 2
H&E (**a**–**f**) and Sudan IV (**g**) staining of representative lesions from individual mice, 10× (**a**–**e**) and 20× (**f** and **g**) magnification. Numbers in lumen (top) indicate the lesion size for each example. Lesions from E2-deficient *AAee*/C (**a**) and *αα*ee/C (**b**) females have multiple features of complex lesions including fibrous caps (black arrowheads), acellular cores (AC) with cholesterol clefts (short yellow arrows), and medial extension into the adventitia (long yellow arrow). Note that the lesion in the *AAee*/E2 female (**c**) is essentially a fatty streak composed of lipid-laden macrophages (foam cells). A lesion from an *αα*ee/E2 female (**d**) has a fibrous cap (black arrowheads) in addition to many foam cells. A second example of a lesion from an *αα*ee/E2 female (**e**) is composed primarily of foam cells. In (**f**), a focal collection of inflammatory cells (IC) is present adjacent to a small lesion that contains foam cells and a thin fibrous cap (black arrowhead) in an *αα*ee/C female. Sudan IV staining of the base of a lesion found in an *αα*ee/C female (**g**) illustrate examples of fragmentation of the media, calcifications (yellow arrowhead), and extension of the lesion into the adventitia (yellow arrow). Lipid-laden foam-cell macrophages are stained red.

each group that had evidence of advanced lesions (absence or presence), including developing or well-formed fibrous caps (vascular smooth muscle cells and extracellular matrix deposition), calcifications, cholesterol clefts (extracellular crystallized cholesterol), acellular cores, inflammatory cells in the adventitia and plaque shoulders, and medial extension (vessel wall involvement by lesion) (see Figure 2 for specific examples). While the lesions in all four groups of mice contained lipid-laden foam cells (by Sudan IV and H&E staining, see Figure 2f for example), the frequencies of advanced lesion characteristics varied across the different groups. Thus when *AAee*/E2 females are compared with *AAee*/C females, the frequencies of all advanced characteristics were reduced, although only reductions in fibrous cap formation, inflammatory cells, and calcifications reached significance ($P < 0.05$). Similarly E2 treatment reduced the frequencies of all the advanced lesion characteristics in *αα*ee/E2 females compared with their *αα*ee/C controls ($P < 0.05$ for fibrous cap and calcifications only). The effect of E2 treatment was statistically significant by multiple logistic regression for all characteristics (fibrous caps, $P < 0.00005$; calcifications, $P < 0.00005$; cholesterol clefts, $P < 0.02$; acellular cores, $P < 0.02$; inflammatory cells, $P < 0.02$) except medial extension ($P = 0.095$). However, interaction between genotype and E2 treatment was not statistically significant, nor was there a statistically demonstrable effect of genotype. These results show that E2 reduces lesion complexity, but that the effect is probably independent of ERα. However, we note that lesion size and complexity are not entirely independent measures of atherosclerotic progression, and factors that affect the growth of the lesion at any stage are likely to have an impact on the development of complex features.

*E2 alters plasma lipids in AAee but not in αα*ee *females.* To determine the role of ERα in lipid metabolism, fasting plasma was obtained at sacrifice from E2-treated and control ovariectomized *AAee* and *αα*ee animals on normal chow and assayed for total cholesterol and HDL (Figure 4). Fasting plasma total cholesterol was significantly reduced by exogenous E2 in the *AAee* females ($P < 0.005$), but not in the *αα*ee females ($P = 0.17$). Interaction between genotype and E2 treatment was highly significant by two-way ANOVA ($P < 0.001$), indicating that significant cholesterol reduction by E2 requires ERα. Fast performance liquid chromatography demonstrated that the reduction of total cholesterol by E2 was a result of decreases in VLDL, IDL, and LDL range fractions (not shown). Plasma HDL–cholesterol (the cross-hatched bases of the bars in Figure 4) was unchanged across all groups.

In the *AAee* group (/C and /E2 combined), but not the *αα*ee group, lesion area was positively correlated with plasma total cholesterol ($r^2 = 0.51$, $P < 0.0001$ and $r^2 = 0.06$, $P = 0.25$, respectively). This was expected since E2 was able to reduce both lesion size and total cholesterol only in *AAee*/E2 females. Subgroup analysis revealed a positive correlation between lesion size and plasma total cholesterol only in *AAee*/E2 females ($r^2 = 0.47$, $P = 0.041$). These results should be interpreted cautiously because E2 release in individual mice may vary, although it could suggest a small reduction in size consequent to small reductions in total cholesterol. Comparison of plasma total cholesterol between individual females of the *AAee*/C and *AAee*/E2 groups demonstrated an overlap in about half of each group (*AAee*/C = 9 and *AAee*/E2 = 5) within the 300–400 mg/dl range. Total cholesterols above

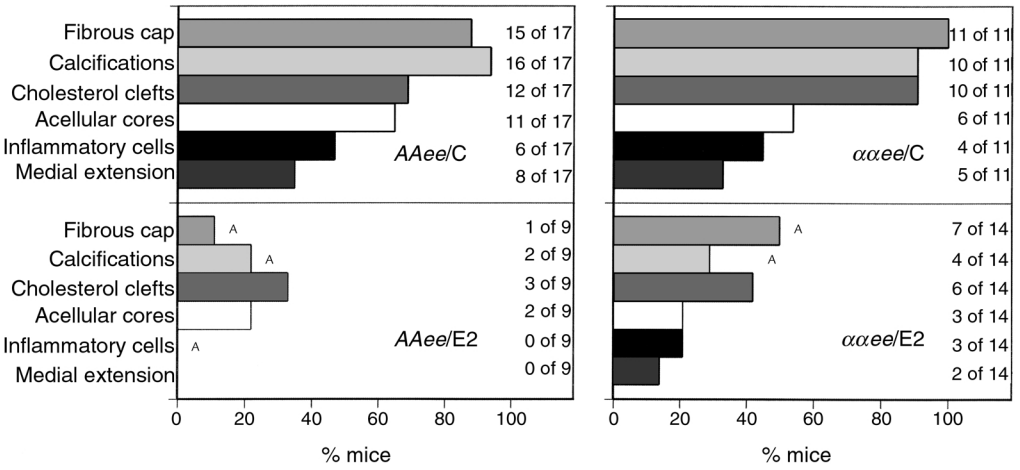

Figure 3
Histopathological lesion characterization of *AAee* (left) and *αααee* (right) females treated with control pellet (C) or E2 pellets (E2) shown as percentage of mice with the presence of the characteristic. [A]Significant differences ($P < 0.05$). The number of animals scoring positively for each characteristic are indicated.

400 mg/dl included only *AAee*/C females and cholesterols below 300 mg/dl included only *AAee*/E2 females. In this midrange group of total cholesterol values in control and E2-treated *AAee* females, there was no overlap in lesion size. This demonstrates that a substantial reduction in lesion size occurs with E2 treatment in ERα-intact females, and that this effect cannot entirely be explained by the reduction in plasma levels of total cholesterol.

ERα and E2 are required for xanthoma formation in Apoe$^{-/-}$ mice. At sacrifice, we observed that the dorsal skin of all the E2-treated *Apoe*$^{-/-}$ females with wild-type ERα (*AAee*/E2) was difficult to remove and frequently required sharp dissection. Histological sections of the skin from the *AAee* females given E2 (Figure 5, c and e), when compared with *AAee*/C (Figure 5a) and *αααee*/C (Figure 5b) females not treated with E2, demonstrated subcutaneous infiltration of lipid-laden macrophages. The foam cells stained intensely with Sudan IV (Figure 5f) and had obliterated most of the subcutaneous fat. In general, the *αααee*/E2 females (Figure 5d) did not show this phenotype, although two of the 14 showed minor collections of foam cells in the flank areas (not shown). These observations demonstrate that the absence of ERα prevents the development of xanthomas in ovariectomized *Apoe*$^{-/-}$ females treated with E2.

Discussion

The genetic and treatment studies described here demonstrate that continuous administration of E2 to ovariectomized *αααee* mice, which lack ERα, largely fails to reduce the size of their atherosclerotic lesions compared with the effects of E2 in ovariectomized *AAee* mice, which have the receptor. This establishes ERα as a major mediator of the atheroprotective effects of exogenous E2 in atherosclerosis-prone mice. Our histological analysis reveals that the complexity of the lesions in the *αααee* mice is nevertheless reduced by E2 treatment, demonstrating that some protective effects of E2 are still present in the absence of ERα. Finally, we demonstrate that the cholesterol-lowering effects of E2 in ovariectomized *Apoe*$^{-/-}$ mice is entirely dependent on the presence of ERα.

Our experiments provide some clues to the stage at which the ERα protection occurs. Thus our observation that the lesions in the *AAee*/E2 mice at 4 months of age have rarely progressed beyond small and uncomplicated fatty streaks indicates that E2 must be affecting an early stage of atherogenesis. A possible factor in the protection is nitric oxide, since multiple studies have shown that estrogen increases the synthesis of nitric oxide through increased expression and activity of endothelial nitric oxide synthase (27, 40, 41). Previous work by Rubanyi et al. (42) has also shown that the basal release of nitric oxide is compromised in ERα-deficient male mice, implicating the importance of ERα for this effect. It is likely that nitric oxide can scavenge harmful oxidants in the subendothelial space and thereby protect vascular cells from injuries that activate the cascade of atherosclerotic processes (43, 44). Protection from injurious oxidants would be expected to delay the formation of fatty streaks and so reduce their sizes at a defined time.

The role of ERα on the effects of E2 in fatty-streak formation has not been directly investigated, but stud-

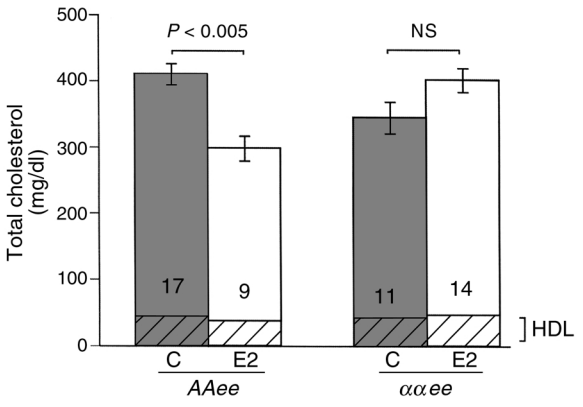

Figure 4
Total cholesterol in plasma of *AAee* and *αααee* mice after 3 months of treatment with control pellets (C) or E2 pellets (E2). Vertical bars represent ± SEM. Filled bars, C; open bars, E2; cross-hatched bases of bars, HDL.

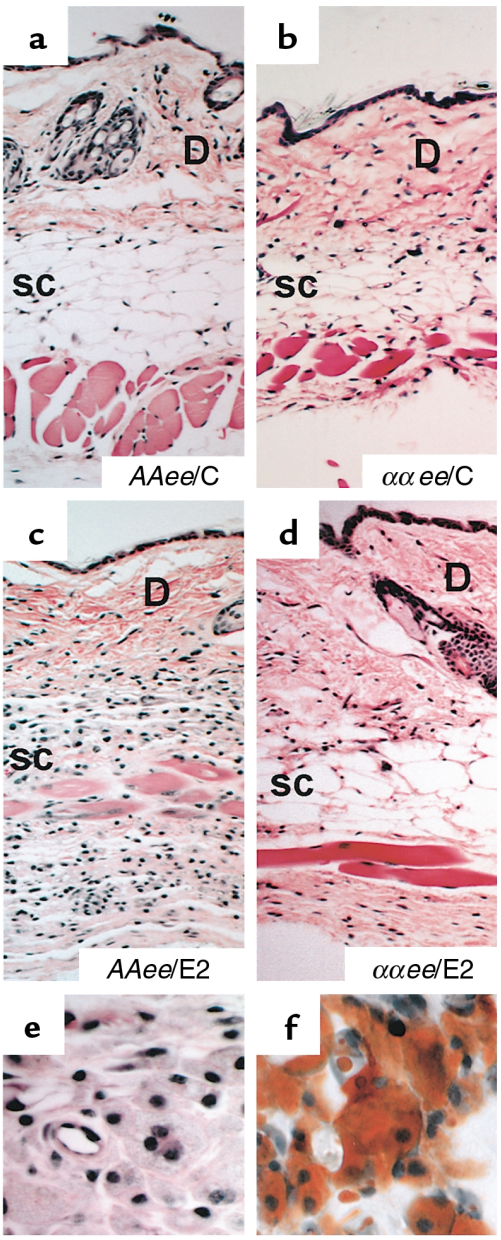

Figure 5
Histological sections of dorsal skin at sacrifice. H&E stain; 10×. (**a**) *AAee*/C; (**b**) *αααee*/C; (**c**) *AAee*/E2; (**d**) *αααee*/E2; (**e**) 40× magnification of *AAee*/E2 skin; (**f**) Sudan IV stain of *AAee*/E2 skin, 40× magnification. Note the infiltration of lipid-laden macrophages or foam cells (xanthoma formation) in the subcutaneous fat (SC) of *AAee*/E2 skin. The dermis (D) is positioned at the top.

ies using estrogen receptor antagonists have suggested receptor-dependent effects of E2 on mechanisms that contribute to macrophage foam-cell accumulation. Thus E2 decreases mRNA levels of vascular adhesion molecules (13) and monocyte chemoattractant protein 1 (15), both of which effects would likely reduce the recruitment of macrophages to the intimal area and their activation. Furthermore, Tomita et al. (45) have demonstrated that E2 stimulates neutral cholesterol esterase activity, thereby inhibiting the accumulation of cholesterol esters in macrophages, a key process of foam-cell formation. Finally, the ERα-dependent reduction of plasma cholesterol may still be contributing to a delay in atherosclerosis development in *AAee*/E2 mice, although the extent of protection is clearly greater than can be accounted for by the lipid changes, as suggested by our data and previously shown by others (2, 7, 8, 37).

The effect of E2 on the complexity of lesions requires comment. Previous investigators have shown that E2 inhibits smooth muscle cell proliferation, a component of fibrous cap formation, both in vitro (46, 47) and in various animal models of vascular injury in vivo (18, 48–50). E2 also reduces connective tissue deposition (51, 52), and possibly inhibits calcium phosphate deposition in the atherosclerotic plaque (21). Furthermore, Tse et al. (53) have demonstrated that E2 prevents calcified cartilaginous metaplasia in the aorta of streptozotocin-treated *Apoe*$^{-/-}$ mice. How much of each of these effects of E2 is mediated by the estrogen receptors remains to be determined. Though ERα is clearly a major mediator of atheroprotection by E2, there remains a demonstrable ERα-independent protective effect of E2 seen mainly as a decrease in plaque complexity in *αααee* females. This protection may be mediated by ERβ or by a yet-unknown ER, or it may be through a receptor-independent action of E2 (27, 40, 41). One possibility is that E2 may be inhibiting early steps of lesion formation even in the absence of ERα. Since lesion complexity is generally correlated with lesion size, a delay in lesion formation would also delay the development of advanced lesion characteristics. Receptor-independent antioxidant properties of E2 are possible factors (40), but recent evidence suggests that direct antioxidant actions of physiological concentrations of E2 are unlikely (54). Changes in plasma cholesterol levels can be excluded as a non-ERα mechanism, since we find that E2 does not lower total cholesterol in the *αααee* females.

A second possibility is that, by an ERα-independent mechanism, E2 may play a role in a later stage of atherosclerosis lesion progression. Our finding of a significant reduction of fibrous caps and calcification in the plaques developing in *αααee* mice treated with E2 compared with the *αααee* control mice could be secondary to a general delay in lesion formation, or it could be due to a contribution of ERα-independent mechanisms to these later processes. In this context, it is notable that E2 reduces the proliferative response of smooth muscle cells to carotid injury in mice even in the absence of either ERα or ERβ (19, 55). Further study is needed to determine whether this effect of E2 is receptor-independent or is due to overlapping contributions of ERα and ERβ.

E2 alters the lipid profile in both animals and humans in part by regulating lipoprotein binding and clearance in the liver (40) where ERα is the predominant receptor (30, 39). In C57BL/6J mice, estrogen increases apoB, decreases apoAI (56), and increases

apoE expression through an ERα-dependent mechanism (57). Our data demonstrate that E2 does not lower total plasma cholesterol in *Apoe*$^{-/-}$ mice when ERα is absent.

Although E2 treatment was highly effective in reducing atherosclerosis in *AAee* mice, it had some marked adverse effects at the dose we employed. Thus, in addition to an overt enlargement of uteri, we found evidence of urinary obstruction in 24%, and death in another 24% of *AAee* mice treated with E2. Marsh et al. (37) have reported renal tract obstruction in LDL receptor–deficient mice treated with a similar dose of E2. Our present experiments show that ERα mediates the adverse effects of E2 in *Apoe*$^{-/-}$ mice, because none of the *αaee* mice treated with E2 exhibited urinary or reproductive tract abnormalities. We also observed a striking infiltration of lipid-laden macrophages into the dorsal subcutaneous fat layer in all *AAee* mice treated with E2, even though E2 inhibits foam-cell accumulation in the aorta of *AAee* mice. Only two of 14 *αaee*/E2 mice demonstrated any infiltration of subcutaneous macrophages, and the extent was considerably less than in the *AAee* mice. We conclude that the development of xanthomas in our animals requires E2 and that ERα is the primary mediator of this E2-induced collection of macrophages in the fat of the skin. It is therefore conceivable that E2 induces alterations in the expression of cytokines from subcutaneous tissues that induce extravasation of monocytes. Hyperlipidemia is associated with cutaneous foam-cell formation (xanthoma) in humans, and Maeda and colleagues have previously shown that cholesterol feeding exacerbates planary xanthoma in the skin of *Apoe*$^{-/-}$ mice (58). Whether E2-induced xanthomatosis is related to the cholesterol-induced xanthoma remains to be determined.

In conclusion, our data show a dramatic loss of the atheroprotection induced by E2 in *Apoe*$^{-/-}$ mice lacking functional ERα, and demonstrate that ERα is the major mediator in this protection. The role played by the second receptor, ERβ, in the remaining protective effects of E2 remains to be established; mice lacking ERβ (59) should facilitate this investigation. Understanding the relative roles of the two known estrogen receptors and of other factors should further the development of improved therapies for preventing CHD in postmenopausal women.

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