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17 β -Estradiol Prevents Early-Stage Atherosclerosis in Estrogen Receptor-Alpha Deficient Female Mice

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Abstract Estrogen is atheroprotective and a high-affinity ligand for both known estrogen receptors, ER α and ER β . However, the role of the ER α in early-stage atherosclerosis has not been directly investigated and is incompletely understood. ER α -deficient (ER $\alpha^{-/-}$) and wild-type (ER $\alpha^{+/+}$) female mice consuming an atherogenic diet were studied concurrent with estrogen replacement to distinguish the actions of 17 β -estradiol (E₂) from those of ER α on the development of early atherosclerotic lesions. Mice were ovariectomized and implanted with subcutaneous slow-release pellets designed to deliver 6 or 8 μ g/day of exogenous 17 β -estradiol (E₂) for a period of up to 4 months. Ovariectomized mice (OVX) with placebo pellets (E₂-deficient controls) were compared to mice with endogenous E₂ (intact ovaries) and exogenous E₂. Aortas were analyzed for lesion area, number, and distribution. Lipid and hormone levels were also determined. Compared to OVX, early lesion development was significantly ($p < 0.001$) attenuated by E₂ with 55–64% reduction in lesion area by endogenous E₂ and >90% reduction by exogenous E₂. Compared to OVX, a decline in lesion number (2- to

4-fold) and lesser predilection (~4-fold) of lesion formation in the proximal aorta also occurred with E₂. Lesion size, development, number, and distribution inversely correlated with circulating plasma E₂ levels. However, atheroprotection was independent of ER α status, and E₂ atheroprotection in both genotypes was not explained by changes in plasma lipid levels (total cholesterol, triglyceride, and high-density lipoprotein cholesterol). The ER α is not essential for endogenous/exogenous E₂-mediated protection against early-stage atherosclerosis. These observations have potentially significant implications for understanding the molecular and cellular mechanisms and timing of estrogen action in different estrogen receptor (ER) deletion murine models of atherosclerosis, as well as implications to human studies of ER polymorphisms and lipid metabolism. Our findings may contribute to future improved clinical decision-making concerning the use of hormone therapy.

Keywords Estrogen · Vascular · Hormone · Lipid · Atherosclerosis

Introduction

Coronary heart disease is relatively rare in premenopausal women compared to postmenopausal women or men. After menopause, the sex protection in women decreases, and cardiovascular risk parameters are adversely impacted [1]. Pathogenic consequences of menopause and sex differences in disease prevalence are attributed to differences in estrogen levels supplied to cardiovascular tissues. Estradiol (17 β -estradiol, E₂) atheroprotection has been documented in many animal models of atherosclerosis [2–5], including murine models with targeted mutations (reviewed in [6]).

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In humans, observational epidemiological studies strongly support an atheroprotective role for endogenous E_2 [7], largely attributed to reductions in low-density lipoprotein (LDL) and increases in high-density lipoprotein (HDL) cholesterol [8, 9], antioxidant actions [10, 11], and a direct biological effect on cardiovascular tissue [12–14]. In contrast, clinical trials have failed to confirm the benefits of combined or unopposed estrogen reported by earlier observational studies [15–17]. The hypothesis that hormone therapy is an effective preventive measure for coronary heart disease has been challenged by these trials, perhaps in part due to the inclusion of postmenopausal women with subclinical coronary artery plaque. The weight of the evidence indicates that older women and those with subclinical or overt coronary heart disease should not take hormone therapy. However, timing of initiation of hormone therapy in relation to menopause onset and age might reduce coronary risk over time, presumably by affording atheroprotection prior to the onset of established vascular disease [18]. In addition, factors including the method of administration, dose, and duration of use of exogenous hormone may also be relevant. Nonetheless, the overall clinical utility of hormone therapy for atheroprotection remains controversial and complex.

Estrogen's long-term effects are generally ascribed to transcriptional modulation of target genes through estrogen receptors (ERs) [19]. Two ERs, $ER\alpha$ (ESR1) and $ER\beta$ (ESR2), have been characterized [20, 21]. They are encoded on two separate genes (ESR1 and ESR2), are distinct functionally and structurally, and have overlapping though not identical tissue expression patterns [21–23]. Functional estrogen receptors are expressed in vascular endothelial cells [24], smooth muscle cells [25, 26], and macrophages [27]. Normal coronary arteries of premenopausal women demonstrate normal ER expression, whereas in atherosclerotic vessels of postmenopausal women, ERs are downregulated [28]. Thus, changes in expression of ERs accompany atherogenesis, though the direction and magnitude of the association needs clarification.

In humans and rodents, both ERs may mediate protection against vascular injury [25, 29]. However, the individual contribution of sex steroid hormones and their cognate receptors to the atherosclerosis and its progression remains poorly understood. In LDL-receptor (LDL-R) and apolipoprotein (Apo) E-deficient mice, $ER\alpha$ is a major mediator of E_2 's atheroprotective effects on advanced atherosclerotic lesions [30–33]. However, studies to distinguish the atheroprotective effects of E_2 from those of $ER\alpha$ in the development of early atherosclerosis and examine the mechanism of E_2 protection in fatty-streak formation are limited. In comparing the effects of E_2 treatment on spontaneous development of atherosclerotic lesions in $ApoE^{-/-}$ mice (with or without the $ER\alpha$), the major

atheroprotective effect of E_2 was substantially abrogated in mice lacking both ApoE and $ER\alpha$ [33]. However, some of the protective effects of E_2 appeared to be $ER\alpha$ independent.

Thus, there is continued need to define the individual contribution(s) of estrogen and estrogen receptors to atherosclerosis, the stage(s) of atherosclerosis when estrogen is atheroprotective, and the stage-specific mechanism(s) involved in vasculoprotection. The goal of this study was to directly examine the action of the $ER\alpha$ in E_2 -mediated atheroprotection, using a murine model of early-stage atherosclerosis (fatty streaks) deficient for the $ER\alpha$, in order to directly examine the action of the $ER\alpha$ in early E_2 -mediated atheroprotection, identify the individual contribution(s) of estrogen and/or the $ER\alpha$ to early atherogenesis, and address mechanisms of E_2 protection in early vascular disease. The study design permitted us to evaluate three potential individual and/or interrelated mechanisms for atheroprotection in early-onset disease: [1] 17 β -estradiol (E_2), [2] the estrogen receptor-alpha ($ER\alpha$), and [3] serum lipids.

Materials and Methods

All experiments were performed in compliance with the National Institutes of Health (NIH) Guidelines and in accordance with protocols approved by the Animal Care and Use Committee of the University of California, Davis.

Generation and Use of Mice Mice heterozygous for the $ER\alpha$ gene ($ER\alpha^{+/-}$), obtained from Dr. Dennis Lubahn (University of Missouri) and mated to yield a breeding colony and progeny lacking the $ER\alpha$ gene ($ER\alpha^{-/-}$), and wild-type littermate controls having intact $ER\alpha$ ($ER\alpha^{+/+}$) were used. The correct genotype was confirmed by polymerase chain reaction (PCR) amplification of genomic DNA. Tail DNA from progeny of heterozygous matings was genotyped as previously described [34] using DNeasy spin columns (Qiagen kit) per the manufacturer's instructions. PCR amplification was used to distinguish homozygous mutants from heterozygotes and wild-type animals. Primers used to amplify products specific for the wild-type gene and disrupted $ER\alpha$ gene were synthesized as custom primers (Life Technologies). Primer sequences for presence of the targeted $ER\alpha$ gene in homozygous mutants (a 649-bp fragment only) and animals with the normal wild-type gene (a 239-bp fragment only) were as follows:

$ER\alpha^{-/-}$ (KO) forward 5' TGAATGAACTGCAG GACGAG 3' and reverse 5' AATATCACGGGTAGC CAACG 3'

ER α ^{+/+} (WT) forward 5' CTACGGCCAGTCGGG
CAT 3' and reverse 5' AGACCTGTAGAAGGCGG
GAG 3'

Mice were housed in a temperature-, 12 h light-, and humidity-controlled environment in a dedicated pathogen-free barrier facility at the University of California, Davis. ER α gene deletion mice have a mixed genetic background from strains 129/J and C57BL/6 J, an atherosclerosis-susceptible strain. They are infertile but otherwise phenotypically normal [34]. They do not produce a fully functional ER α and have no demonstrable full-length wild-type ER α protein [34]. However, the disrupting neo-sequence used to generate the knockout results in a variant ER α protein (not the full-length ER α wild-type protein) contained an internal deletion of a portion of the N-terminal region of the receptor. This alternative ER α protein has residual low-level, high-affinity, estradiol-binding activity [35, 36] but, importantly, lacks the specificity to be from either ER α or ER β (Liu and Lubahn, unpublished data).

Atherogenesis Model and Experimental Design Experimental animals were weaned at 4 weeks. To manipulate estrogen status, age-matched ER α ^{-/-} and ER α ^{+/+} female mice were castrated at 6–7 weeks of age by bilateral ovariectomy (OVX) under anesthesia (2.5% Avertin). OVX mice were then implanted with subcutaneous hormone pellets (Innovative Research, Toledo, OH, USA) designed for slow release of 6 μ g/day of exogenous E₂ as 17 β -estradiol (exogenous E₂-6; $n=19$ and $n=18$ for ER α ^{-/-} and ER α ^{+/+} mice, respectively), or 8 μ g/day of exogenous E₂ as 17 β -estradiol (exogenous E₂-8; $n=21$ and $n=20$ for ER α ^{-/-} and ER α ^{+/+} mice, respectively) for a total of up to 4 months. The 6- μ g/day E₂ dose was chosen based on analogous models indicating that this dosage attains blood levels at peak estrus necessary to lower plasma lipids and plaque size [30–32]. The 8- μ g/day E₂ dose was chosen to assess the dose-response of exogenous E₂ and the impact of higher E₂ dose and levels on atheroprotection. OVX ER α ^{-/-} ($n=22$) and ER α ^{+/+} ($n=20$) mice implanted with placebo vehicle pellets served as E₂-deficient controls. In addition, ER α ^{-/-} ($n=18$) and ER α ^{+/+} ($n=19$) female mice with ovaries left intact served as an additional study group for evaluating the comparative vascular effect of endogenous E₂. Intact ER α -deficient females have generally been excluded from prior studies due to plasma E₂ levels that are higher than wild-type mice as a consequence of their estrogen insensitivity [37]. However, we chose to include these mice to evaluate the effect of higher endogenous estrogen in our model.

To induce atherosclerotic lesions, mice were fed a high-fat, high-cholesterol, atherogenic diet [38, 39], herein referred to as the atherogenic diet (Purina; 15% wt/wt butter

fat, 1.25% wt/wt cholesterol, and 0.5% cholic acid) for 4 months starting 1 week after surgical recovery from OVX. The diet was formulated to be phytoestrogen-free. Aortas (five to eight from each genotype) were sampled for lesion formation at baseline (age 6–7 weeks, immediately before cholesterol feeding), and after 2 and 4 months on the atherogenic diet.

Aortic Atherosclerotic Lesion Analysis To determine extent of characteristics and extent of atherosclerotic lesions in aortas and determine any regional differences in atherosclerosis susceptibility, we adapted and optimized previously published and reproducible methods for atheroma quantification [40], as previously described [41]. Oil Red O-stained (Sigma) cryosections (10 μ m thick) defined and characterized neutral lipid in the vessel wall, permitting histological assessment and quantitative evaluation of lesions in three artery segment locations (proximal segment=proximal aortic arch; mid segment=suprarenal aorta; distal segment=infraarenal aorta proximal to the iliac bifurcation). Fatty streaks (herein, early lesions) were analyzed for area, location, and quantity by direct imaging (Olympus BX-40 Microscope, Olympus DP11 Camera with 64 MB smart media chip). Quantitative total lesion area and mean lesion size (μ m²±SEM) for each early lesion was determined morphometrically (Image Pro Plus, version 4.1). Qualitative morphological assessment of lesion complexity used, as parameters, extent of subintimal lipid deposition and ORO staining and absence or presence of foam cells. Wall thickness, disruption of vessel wall architecture, and distortion of nuclear proliferation were not assessed as they were not part of the early-lesion pathology in the study's early-lesion model. For each mouse, a total of 36 cryosections (discarding every other section) were obtained from the proximal, mid, and distal aortic segments for extensive sampling of a total of >2,100 μ m per aorta.

Measurement of Uterine Weight The entire uterus was collected at baseline and 2 and 4 months to assess in vivo exposure to estradiol. Serosal fat was removed, the uterus divided from the utero-tubal junction bilaterally, transected at the junction of the uterine body and cervix, and uterine wet weight recorded relative to body weight.

Quantification of Plasma Estradiol Plasma levels of free 17 β -estradiol were determined in study mice at baseline and 2 and 4 months on the atherogenic diet. Assays were performed in duplicate by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) on nonpooled plasma samples.

Quantification of Plasma Lipids Plasma lipid levels were measured in study mice at baseline and 2 and 4 months on

the atherogenic diet and analyzed as follows: triglyceride (TG) with Triglyceride (INT) Reagent (Sigma); high-density lipoprotein cholesterol (HDL-C) with a colorimetric reagent kit (Wako) after precipitating the ApoB-containing particles; and total cholesterol (TC) using a GM7 Analox Analyzer (Analox). All assays were performed in duplicate on nonpooled plasma samples.

Statistical Analysis Lesion area and lesion number were quantified by calculating the total lesion area and total number of lesions, respectively, in each of the 36 aortic sections for each mouse aorta. Comparisons between study groups were made using Student's *t* test for independent samples (two-tailed), and analysis of variance, using MS-Office 2000 Excel software for PC and Sigma Stat version 2.03 (SPSS). Genotype ($ER\alpha^{-/-}$ versus $ER\alpha^{+/+}$) and E_2 status (OVX, Intact, E_2-6 , and E_2-8) were the grouping variables. Correlations between parameters were analyzed using simple linear regression. Probability values at $\alpha < 0.05$ identified all statistically significant comparisons.

Results

Plasma Estradiol We determined plasma estradiol levels in study mice. We successfully achieved the desired hormonal status in all of the mice (Fig. 1a). OVX reduced circulating plasma E_2 to very low levels in $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ mice. E_2 levels in intact $ER\alpha^{-/-}$ mice were more than twice those of intact $ER\alpha^{+/+}$ mice ($p < 0.05$), consistent with the previously reported ER-dependent functional estrogen insensitivity of $ER\alpha^{-/-}$ mice [42]. Plasma E_2 in exogenous E_2 -replaced $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ mice were at or above peak estrus levels, previously reported necessary for physiologic atheromatous plaque reduction [30–32]. With E_2 replacement, female mice have been previously reported to develop carcinoma of the cervix and urinary tract and reproductive tract pathology [43]. None of our study mice developed cervical carcinoma with E_2 . However, three mice developed endometritis, hydronephrosis, and bladder enlargement unassociated with infection. These mice were excluded from subsequent analysis.

Uterine weights were measured to functionally assess in vivo E_2 action in our system (Fig. 1b). In $ER\alpha^{+/+}$ mice, the very small, atrophic uteri of E_2 -deficient OVX female mice confirmed the absence of estrogenic stimulation. $ER\alpha^{+/+}$ mice with endogenous E_2 had significantly ($p < 0.01$) larger uteri than OVX females. Uterine weights of OVX $ER\alpha^{+/+}$ female mice replaced with exogenous E_2-6 and E_2-8 were over 8-fold larger than OVX $ER\alpha^{+/+}$ females ($p < 0.05$) and 4-fold larger than intact $ER\alpha^{+/+}$ mice ($p < 0.05$). As expected, in $ER\alpha^{-/-}$ mice, uteri were consistently small

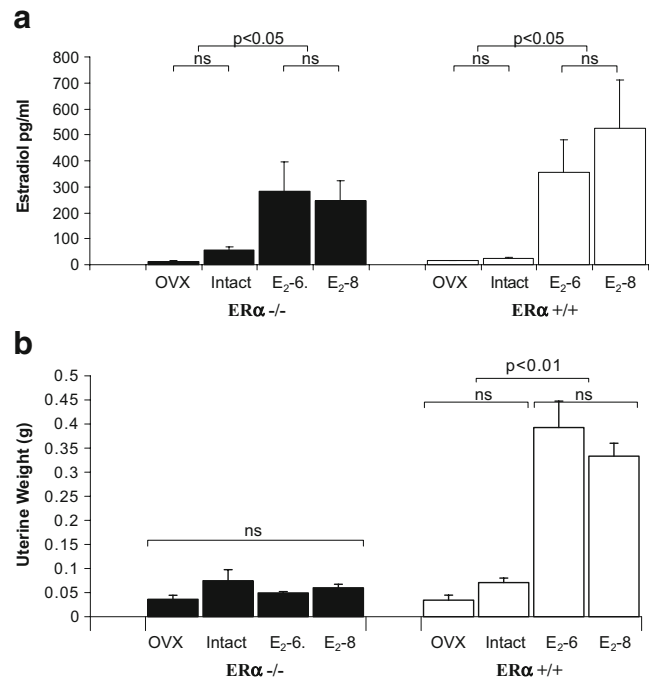


Fig. 1 **a** Plasma estradiol levels in $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ female mice. Levels of plasma 17β -estradiol (pg/ml; mean \pm SEM) in $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ female study mice are shown for ovariectomized (OVX, $n = 14-15$), intact ($n = 6-10$), and ovariectomized replaced with estradiol pellets designed to deliver $6\mu\text{g/day}$ (E_2-6 ; $n = 10-12$) and $8\mu\text{g/day}$ (E_2-8 ; $n = 8-13$). Statistically significant ($p < 0.05$) and non-significant (ns) comparisons are shown for each genotype. There were no significant differences for treatment group comparisons between genotypes. **b** Uterine weights in $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ female mice. Uterine weights (g; mean \pm SEM) in $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ female study mice are shown for ovariectomized (OVX, $n = 4-6$), intact ($n = 5-7$), and ovariectomized replaced with estradiol pellets designed to deliver $6\mu\text{g/day}$ (E_2-6 ; $n = 13-14$) and $8\mu\text{g/day}$ (E_2-8 ; $n = 6-14$). Statistically significant ($p < 0.01$) and non-significant (ns) comparisons are shown for each genotype. In addition, there were significant differences ($p < 0.01$) for uterine weight comparisons between $ER\alpha^{+/+}$ E_2-6 and E_2-8 and all $ER\alpha^{-/-}$ treatment groups

and did not significantly enlarge with E_2 treatment. Although E_2 has reported effects on body weight in humans and several animal species [44, 45], there were no significant differences in body weight between $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ mice in our different treatment groups and experimental time points (data not shown).

Plasma Lipids Figure 2 summarizes fasting plasma lipids for total cholesterol, triglycerides, and high-density lipoprotein cholesterol for OVX, intact, and E_2 -replaced $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ female mice while on the atherogenic diet. TC was lower at baseline in $ER\alpha^{+/+}$ mice than in $ER\alpha^{-/-}$ mice (73 ± 11 mg/dl versus 104 ± 5 mg/dl, respectively, $p = \text{ns}$). Compared to baseline, after 4 months on the atherogenic diet, fasting TC increased significantly ($p < 0.05$) in OVX, endogenous E_2 , and exogenous E_2-6 $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ mice (32%, 28%, and 30% versus 55%, 58%, and 55% for

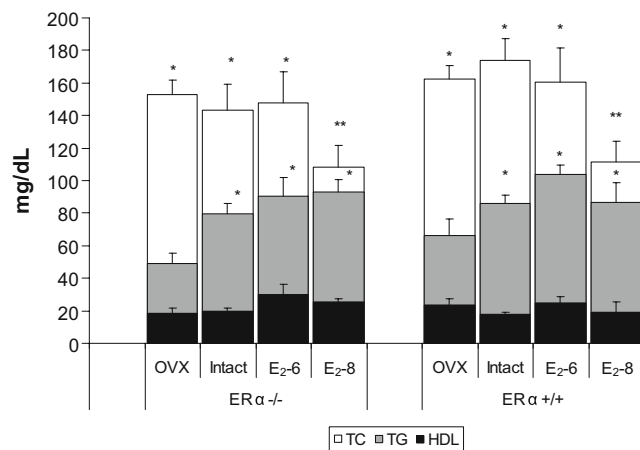


Fig. 2 Plasma lipid levels in ERα^{-/-} and ERα^{+/+} female mice. Fasting lipid levels (mg/dl; mean±SEM) for total cholesterol (TC), triglyceride (TG), and high-density lipoprotein (HDL) were determined in non-pooled plasma of ERα^{-/-} (n=50 samples for each lipid) and ERα^{+/+} (n=49 samples for each lipid) female mice on the atherogenic diet for animals ovariectomized (OVX), intact, and ovariectomized replaced with estradiol pellets designed to deliver 6 μg/day (E₂-6) and 8 μg/day (E₂-8). *p<0.05 versus lipid baseline values (not shown, see text) prior to cholesterol feeding for each genotype; **p<0.05 versus TC in other treatment groups for a given genotype

each genotype, respectively; p=ns for treatment group comparisons between genotypes). However, the atherogenic diet-associated increase in TC in ERα^{-/-} and ERα^{+/+} mice was not affected by estrogen treatments in any of the study groups, except for a significant (p<0.05) reduction in TC with exogenous E₂-8 only observed in both ERα^{-/-} and ERα^{+/+} mice.

In addition, TC levels on the atherogenic diet in OVX and intact ERα^{+/+} mice did not correlate with lesion area and correlated poorly with lesion area in exogenous E₂-6 ERα^{+/+} mice (r=0.56; p=ns). However, in ERα^{+/+} mice, the reduction in TC with exogenous E₂-8 correlated strongly with reduction in fatty streak size (r=0.83; p<0.05). In ERα^{-/-} mice, aortic lesion area did not correlate with TC in any of the treatment groups. Thus, with the sole exception of E₂-8 in ERα^{+/+} mice, the dramatic reduction in lesion size with E₂ (endogenous or exogenous) in ERα^{-/-} and ERα^{+/+} mice was not a consequence of an estrogen-mediated reduction in TC levels.

There were no significant differences in baseline fasting plasma TG levels between ERα^{-/-} and ERα^{+/+} female mice (75±6 and 81±13 mg/dl, respectively). While on the atherogenic diet, fasting levels of plasma TG increased significantly (p<0.05) in all E₂ mouse groups, compared to baseline or OVX (Fig. 2). However, increases in TG did not correlate with lesion formation in any of the treatment groups or genotypes.

Fasting levels of HDL-C were similar at baseline in ERα^{-/-} and ERα^{+/+} mice (13±2 and 17±4 mg/dl,

respectively). Compared to baseline, HDL levels tended to be significantly greater in hormone replaced ERα^{-/-} mice than ERα^{+/+} mice. However, there were no significant changes in HDL-C levels between treatment groups in ERα^{+/+} mice on the atherogenic diet (Fig. 2). Furthermore, there was no correlation between lesion area and HDL-C levels in either genotype.

Lesion Area, Number, and Distribution ERα^{-/-} and ERα^{+/+} mice developed aortic lesions typical of fatty streaks [38]. The fatty-streak lesion area (averaged throughout the period on the atherogenic diet) for all treatment groups is summarized in Fig. 3. Endogenous and exogenous E₂ were both very effective in inhibiting early lesion formation. In both ERα^{-/-} and ERα^{+/+} female mice, endogenous E₂ in mice with intact ovarian function and exogenous E₂ in estrogen-replaced OVX mice significantly reduced lesion area compared with E₂-deficient OVX mice. Compared to OVX, lesions in mice with endogenous E₂ were 64% and 55% smaller in ERα^{-/-} and ERα^{+/+} mice, respectively (p<0.001). In addition, following exogenous E₂ replacement, lesions were more than 90% smaller (p<0.001) in both ERα^{-/-} and ERα^{+/+} mice compared to OVX mice. No significant difference in lesions was observed between the two doses of exogenous estradiol (E₂-6 and E₂-8) in ERα^{-/-} or ERα^{+/+} mice. In addition, the rate at which lesions developed over time appeared to

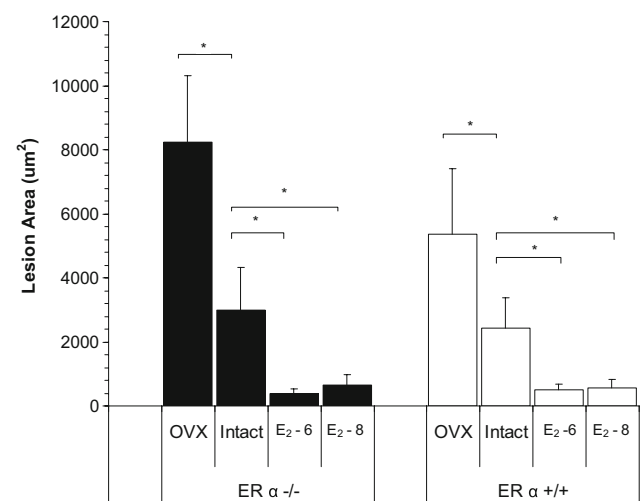


Fig. 3 Early lesion formation in ERα^{+/+} and ERα^{-/-} female mice. Lesion area (μm²±SEM) of early atherosclerotic fatty streaks in the aortas of ERα^{-/-} (n=80) and ERα^{+/+} female mice (n=77) was measured at baseline and 2 and 4 months of cholesterol feeding (n=5–7 mice each per genotype, treatment group, and time point). Lesion area is shown for mice ovariectomized (OVX), intact, and ovariectomized replaced with estradiol pellets designed to deliver 6 μg/day (E₂-6) and 8 μg/day (E₂-8). Data are mean±SEM lesion area (in μm²). *p<0.001 compared to the indicated treatment groups for a given genotype

decline with increasing estrogenic action in both ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ mice but did not differ between the two genotypes (data not shown).

For each of the E₂ treatment groups, representative histological lesions of female ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ mice after 4 months on the atherogenic diet are shown (Fig. 4). Early atherosclerotic lesions were present in the intimal layer of aortas of both ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ mice as early as 2 months on the atherogenic diet, with more extensive lesions developing over the 4 months of the study. Lesions were distinguished by lipid infiltration into the vessel wall and the presence of fatty streaks and did not have characteristics of advanced lesions, confirming our model of early atherosclerosis. Compared to endogenous and exogenous E₂, OVX ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ mice had early lesions that were more extensive and lipid rich and with larger deposits of intra- and extra-cellular lipid. Lesions in ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ endogenous E₂ mice had less lipid deposits than OVX mice but more fatty streaking of the sub-endothelium than exogenous E₂ mice. Lesion formation was essentially absent in mice replaced with exogenous E₂.

We also characterized early lesions by quantity and segment location in the aorta. The segmental distribution of early lesions in aortas of ER $\alpha^{-/-}$ ($n=51, 48, 54,$ and 54 total segments for OVX, intact, E₂-6, and E₂-8, respectively) and ER $\alpha^{+/+}$ mice ($n=51, 51, 51,$ and 54 total segments for OVX, intact, E₂-6, and E₂-8, respectively) is shown in Fig. 5. All mice showed predilection for early lesion formation in the proximal aortic segment, with 83–100% of all early lesions localized to this region of the aorta. The mid aorta contained 2–18% of early lesions, whereas <3% of early lesions were in the distal aorta. Differences in distribution of lesions were significant ($p<0.01$) between segments (proximal versus mid versus distal aorta), and compared to endogenous or exogenous E₂, there was significantly greater (~4-fold) proximal distribution of lesions in OVX mice. However, lesion distribution was not significantly affected by ER α status.

The number of lesions in aortas of exogenous (E₂-6 and E₂-8) ER $\alpha^{-/-}$ mice was significantly lower ($p<0.05$) than

in aortas of OVX ER $\alpha^{-/-}$ mice. In ER $\alpha^{-/-}$ mice, the mean number of lesions in endogenous E₂, E₂-6, and E₂-8 mice was $12\pm 5, 8\pm 2,$ and $10\pm 2,$ respectively, compared to a mean of 41 ± 7 lesions in the aortas of OVX mice ($p<0.05$). In ER $\alpha^{+/+}$ mice, a similar pattern was observed with

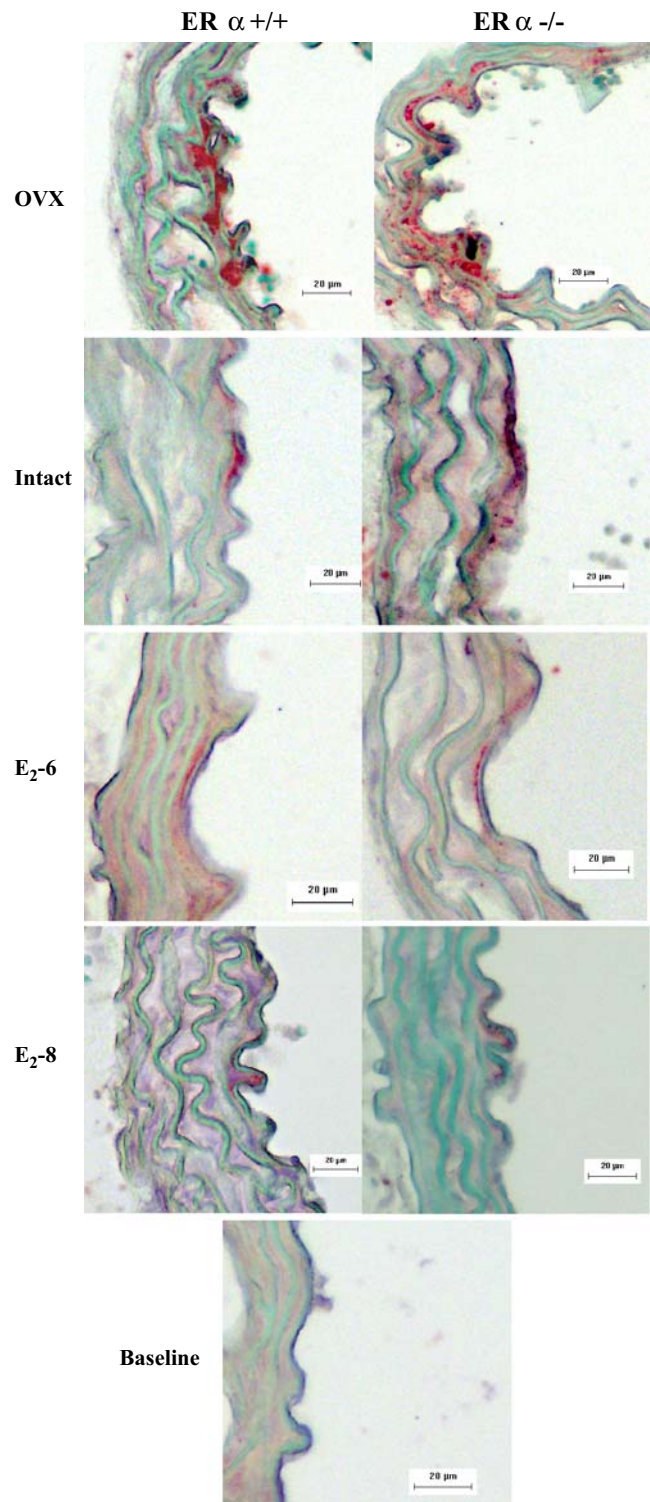


Fig. 4 Representative cross sections of lesions from the aortas of ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ mice. Histochemistry of examples of representative cross sections (10 μ m) of lesions from the proximal aorta of cholesterol fed (4 months) ER $\alpha^{-/-}$ (right panel) and ER $\alpha^{+/+}$ (left panel) female mice for the following experimental groups: ovariectomized (OVX), ovaries intact, and ovariectomized replaced with estradiol pellets designed to deliver 6 μ g/day (E₂-6) and 8 μ g/day (E₂-8). The vessels have been stained for lipid with Oil-red-O and counterstained with Gill's Hematoxylin. A normal vessel prior to the initiation of cholesterol feeding, *baseline*, is shown for comparison. The representative images ($\times 20$ magnification) demonstrate red-staining subintimal lipid deposits and fatty streaks. Cross sections are oriented with the lumen to the right and the adventitial surface to the left of each image. Size markers=20 μ m

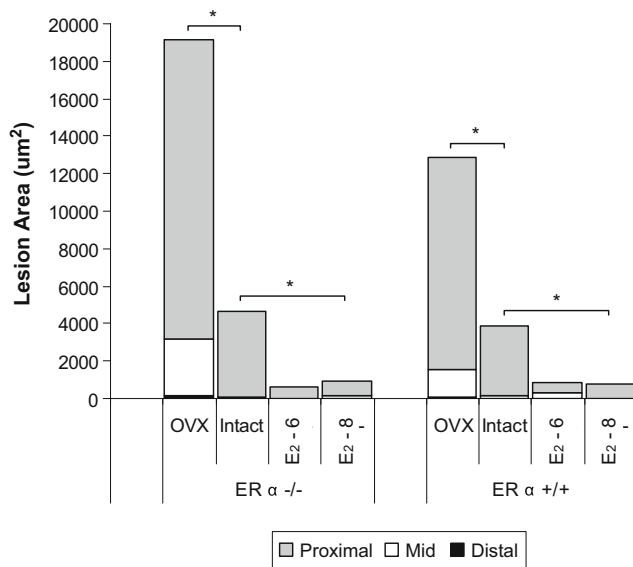


Fig. 5 Lesion distribution in aortic segments of ERα^{-/-} and ERα^{+/+} female mice. Lesion area (µm²; mean±SEM) was determined in the proximal, mid, and distal aortic segments of female ERα^{-/-} and ERα^{+/+} mice ovariectomized (OVX, n=29), intact (n=23), and ovariectomized replaced with estradiol pellets designed to deliver 6 µg/day (E₂-6; n=25) and 8 µg/day (E₂-8; n=24). See text for details. *p<0.01 versus other treatment groups of the same genotype

exogenous E₂ mice having fewer lesion than OVX or intact mice.

Relationship of Plasma Estradiol and Lesions In our system, early lesion formation was inversely correlated with plasma estradiol levels in both ERα^{-/-} and ERα^{+/+} mice. This relationship is demonstrated in Fig. 6. A step-wise reduction in lesion size was observed with a rise in plasma estradiol, and a converse step-wise increase in lesion size was seen with a drop in plasma estradiol. OVX animals had a mean plasma estradiol of only 13 pg/ml (we assume that the residual measurable estradiol in OVX mice is due to adrenal sex steroid production and conversion to estradiol) and the greatest extent of lesions. Intact mice with endogenous E₂, in which mean plasma estradiol levels were 58 pg/ml, demonstrated nearly 60% reduction in lesion size (p<0.05). As expected, intact ERα^{-/-} females had relatively greater endogenous E₂ levels than intact ERα^{+/+} mice due to their relative insensitivity to estrogen. However, the differences in endogenous E₂ levels between these mice were relatively small and not sufficient to result in significant differences in aortic lesions in our studies. However, with higher plasma estradiol levels (mean >247 pg/ml attained in exogenous E₂ mice), lesion development was essentially eliminated compared to endogenous E₂ and OVX mice (<0.05). The inverse relationship between mean serum estradiol and mean lesion area was unaffected by ERα

status as it did not significantly differ between the two ERα genotypes.

Discussion

The important new finding of our studies is that protection from development of early atherosclerotic lesions (fatty streaks) is dependent on estrogen but independent of the ERα. In addition, estrogen atheroprotection was estrogen-level dependent, with greater atheroprotection offered by exogenous estrogen compared to endogenous estrogen and loss of atheroprotection with ovariectomy.

Atheroprotection by E₂ in atherogenic transgenic mouse models with targeted inactivation of the low-density lipoprotein receptor [32] and apolipoprotein E [30, 31] has been demonstrated. In these models, mice spontaneously develop advanced atherosclerosis. In a combined ApoE/ERα deletion model [33], ERα was reported to be a major mediator of E₂ protection in advanced atherosclerotic lesions. However, whether the same occurs in early-stage lesions is unknown. In this paper, we demonstrate several novel findings: (1) E₂ inhibits and delays the development of early lesions by ERα-independent mechanisms, as there was no significant interaction between ERα genotype and hormone treatment or hormone status. (2) There was an estrogen concentration-dependent response to atheroprotection. And (3) Endogenous and exogenous E₂ are both highly, though not equally, effective in attenuating aortic lesion development and progression. In our model, inhibition of early lesions using exogenous E₂ in ovariectomized

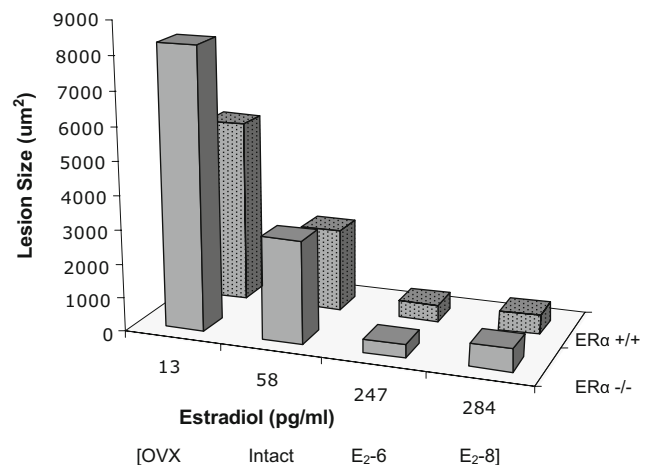


Fig. 6 Reduction in lesion area as a function of plasma estradiol. Lesion area (µm²±SEM) as a function of plasma 17β-estradiol (E₂; pg/ml; mean) in ERα^{-/-} and ERα^{+/+} female study mice. Estradiol values (mean, pg/ml) are indicated for mice ovariectomized, OVX (E₂=13 pg/ml), intact (E₂=58 pg/ml), and ovariectomized and replaced with E₂ pellets designed to deliver 6 µg/day (E₂-6=247 pg/ml) and 8 µg/day (E₂-8=284 pg/ml)

animals was more atheroprotective than leaving the ovaries (and thus endogenous E_2 levels) untouched. This was somewhat surprising since the dosage used was chosen to obtain blood E_2 levels similar to those at peak estrous, though actual serum E_2 levels attained were at times higher. However, we do not think that this is the reason that inhibition of early lesions using exogenous E_2 therapy in ovariectomized animals was more effective at inhibiting early lesion formation than endogenous E_2 . A more likely possibility to consider is that cycling estrogen levels may attenuate the growth inhibitory effect of endogenous E_2 , in contrast to continuous therapy in our model.

Our findings also extend recent observations reported in ApoE KO mice in which E_2 treatment failed to reduce aortic lesions in ApoE-deficient mice also lacking ER α [33]. We postulate that the differences between our findings and previous are due to two principal factors: (a) the difference in the animal model used and (b) the differences in estradiol effect in early- versus late-stage atherosclerosis. In the previous report, the experimental strain used was the heterozygous ER α deletion on the atherogenic ApoE null transgenic mouse background. In contrast, the current study used ER α -Neo null mice on a mixed 129/J and C57Bl/6J background. Such mixed background mice are relatively protected from atherosclerosis and thus, a cholesterol-containing diet was used to induce aortic fatty streaks that did not progress to more mature atheroma. We took advantage of this model to specifically study E_2 and ER α effects on early-stage disease. Secondly, the current study examined the early stages of atherosclerosis, whereas the previous report examined more advanced lesions of atherosclerosis. The differential effects of E_2 treatment on early-stage atherosclerosis as opposed to the more advanced stages and how ER α is associated with one but not the other are very important observations and distinctions with keen relevance to better understanding atherosclerotic cardiovascular disease. Sex steroid hormones are critical determinants of cardiovascular sex differences. Moreover, the effects of estrogens on the blood vessel wall depend upon the extent and complexity of atherosclerotic disease present at the time hormone therapy is initiated. As atherosclerosis evolves, the early vascular protective mechanisms of estrogen (e.g., increased NO and cyclooxygenase 2 and decreased TNF- α , cell adhesion molecules, LDL oxidation/binding, platelet activation, and vascular smooth muscle cell proliferation) recede and are replaced by estrogen responses that may be deleterious (decrease in ER function/expression and vasodilation and increase in inflammatory activation and plaque instability). These events have recently been comprehensively reviewed by Turgeon et al. [46], Clarkson [47], and Manson [48]. Thus, it is becoming increasingly clear that the pleiotropic effects of hormone therapy on the vascular system and cells differ,

depending not only on hormone status, but also importantly on the stage of atherosclerosis in the underlying blood vessel.

We also examined whether the atheroprotective effects of E_2 on early atherosclerosis were lipid mediated. However, the extent of atheroprotection attributable to E_2 in our study and previous studies [30–33] is clearly greater than can be accounted for by changes in lipid parameters alone. In LDL-R $^{-/-}$ mice [32], exogenous E_2 , but not endogenous E_2 , lowers cholesterol. In the ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ mice, lipid levels at baseline and following an atherogenic diet were similar to those previously published for atherosclerosis-susceptible mouse strains [38] and concur with those reported in ApoE-deficient mice, where only high doses of exogenous E_2 reduced plasma cholesterol [30]. However, the protective effects of endogenous and exogenous E_2 against fatty-streak formation in our studies were not correlated with plasma levels of TC, TG, or HDL-C. Thus, in our model, the traditional factors that promote lesion formation, such as a high-fat diet and high plasma cholesterol, did not interact with ER α genotype as the changes in serum lipoproteins were independent of ER α expression and occurred in both ER $\alpha^{-/-}$ and $+/+$ female mice.

Our studies had some limitations. The ER α -deficient mouse used (ER α -Neo KO) is not fully null [35, 49, 50]. In this mouse, the ER α gene was disrupted by the insertion of the neomycin gene into the first coding exon, resulting in deficiency of the full-length wild-type ER α protein [34]. This approach also results in the expression of two alternatively spliced transcripts of the disrupted ER α gene, E1 and E2 [35], and the expression of two variant ER α proteins (only one of them found in the aorta) [35, 49]. This alternative ER α protein has residual low-level, high-affinity, estradiol-binding activity [35, 36] but lacks the specificity to be from either ER α or ER β (Liu and Lubahn, unpublished data). This variant also has some estrogen-dependent transcriptional activity [35, 49, 50]—ER α transcriptional activity is mediated through two activation functions, AF1 and AF2, whose activity is tightly regulated in a cell-specific manner through yet unknown processes. More recently, mice completely deficient of the ER α gene by excision of exon 2 (ER α - Δ 2KO) [51] resulted in the complete abrogation of an estradiol effect [49]. Although our studies used the ER α Neo KO model, the results presented clearly indicate that any residual activity of the variant of ER α present in the aorta of this mouse model is not sufficient to protect against atherosclerosis in female mice, indicating that the expression of the full-length ER α is not required and that ER α transcriptional activity mediated through the AF transactivation function is dispensable in protection against fatty-streak formation in the vessel wall.

Although our findings indicate that E_2 protects against the development of atherosclerosis by an $ER\alpha$ - and lipid-independent mechanism, we cannot conclude that the early atheroprotective effects of E_2 occurred entirely via non-receptor-mediated pathways. In C57BL/6J mice, E_2 prevents the vascular injury response to carotid artery denudation independent of $ER\alpha$ [52], perhaps proceeding via the $ER\beta$ [53]. Although the response to vascular injury differs from that of the atherosclerotic response to high fat feeding, a mechanistic role for $ER\beta$ seems unlikely in our system as the $ER\beta$ does not appear to be involved in early stages of atherosclerosis development [6]. Estrogen receptor $ER\beta$ localized to the vascular intima is correlated with coronary calcification and thus more advanced atherosclerosis in pre- and postmenopausal women [54], suggesting a role for this ER subtype in more advanced vascular disease. Recently, however, heat shock protein (HSP) 27, an $ER\beta$ -associated protein, was demonstrated to have attenuated expression in coronary atherosclerosis and to modulate estrogen signaling in minimally diseased arteries [55]. Thus, in our system, vascular protective effects mediated by $ER\beta$ receptor, its isoforms [56], an as yet uncharacterized ER, HSPs, or other factors cannot be excluded and remain the work for future studies. Lastly, $ER\alpha$ - or $ER\beta$ -independent effects of E_2 have been demonstrated for catechol estrogens (estrogens converted to catechol estrogens via oxidation) where inhibition of cell growth by E_2 is independent from ERs [57].

Furthermore, in clinical studies, the common estrogen receptor-alpha ($ER\alpha$) IVS1-401 C/C genotype polymorphism identifies a group of women (approximately 20%) who have augmented response to hormone therapy. This has been demonstrated on levels of HDL cholesterol and intermediate cardiovascular risk markers, such as E-selectin [58, 59]. Although the impact of this polymorphism on $ER\alpha$ transcription and other estrogen-sensitive intermediate and clinical end points has not yet been established, the augmented effects of ER polymorphisms on hormone therapy regulation of lipids and other vascular mediators may be additional possible mechanisms by which ER polymorphisms might influence estrogen action in early atheroprotection. Thus, the implications of differing experimental results in various genetic models as extrapolations to human disease suggests that a polygenomic approach will be needed to fully understand whether exogenous estrogen treatments will be appropriate for all women and the timing of this treatment in relationship to atherosclerosis development.

In summary, this study addresses the atheroprotective role of estradiol that is uncoupled to that of the $ER\alpha$ and whether the mechanism of action of estradiol in atheroprotection is $ER\alpha$ dependent, or not, in early-stage lesions. Our findings demonstrate that, in early lesions of atherosclerosis,

the mechanism of atheroprotection is estradiol-mediated and not dependent on the extent of hyperlipidemia, a reduction in serum lipids by estrogen, or on the presence or absence of the $ER\alpha$. Our findings are important and provide new clues to the stage of vascular atherosclerosis at which estradiol and $ER\alpha$ protection occurs. Specifically, our observations establish that both endogenous and exogenous E_2 retard the development of fatty-streak formation characteristic of early atherosclerosis in a manner that is closely tied to estrogen level, yet independent of a functional $ER\alpha$. In contrast, and based on prior studies, the $ER\alpha$ is required for a major part, if not all, of the E_2 -mediated reduction in lesion size in plaques with more advanced lesion characteristics and is a major mediator in plaque maturation. Furthermore, the inhibitory effects of E_2 on atherogenesis appear to be lost once atherosclerotic lesions are established [60].

In conclusion, our studies contribute to a better understanding of $ER\alpha$ -dependent and -independent mechanisms of E_2 atheroprotection, linked to an emerging concept coupling estrogen action to the timing of its initiation in relationship to critical periods in the stages of atherogenesis. This knowledge is potentially very important, as one of the most critical areas of new focus in this field is understanding the molecular and phenotypic interface of estrogens with their receptors in the vascular wall vis-à-vis the stage(s) of atherosclerosis. A better understanding of this system may lead to a better understanding of how to optimize and when to target hormone therapies for cardiovascular disease protection in women.

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