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27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen

Michihisa Umetani[1], Hideharu Domoto[1], Andrew K Gormley[2], Ivan S Yuhanna[2], Carolyn L Cummins[1], Norman B Javitt[3], Kenneth S Korach[4], Philip W Shaul[2] & David J Mangelsdorf[1]

The cardioprotective effects of estrogen are mediated by receptors expressed in vascular cells. Here we show that 27-hydroxycholesterol (27HC), an abundant cholesterol metabolite that is elevated with hypercholesterolemia and found in atherosclerotic lesions, is a competitive antagonist of estrogen receptor action in the vasculature. 27HC inhibited both the transcription-mediated and the non-transcription-mediated estrogen-dependent production of nitric oxide by vascular cells, resulting in reduced estrogen-induced vasorelaxation of rat aorta. Furthermore, increasing 27HC levels in mice by diet-induced hypercholesterolemia, pharmacologic administration or genetic manipulation (by knocking out the gene encoding the catabolic enzyme CYP7B1) decreased estrogen-dependent expression of vascular nitric oxide synthase and repressed carotid artery reendothelialization. As well as antiestrogenic effects, there were proestrogenic actions of 27HC that were cell-type specific, indicating that 27HC functions as an endogenous selective estrogen receptor modulator (SERM). Taken together, these studies point to 27HC as a contributing factor in the loss of estrogen protection from vascular disease.

The estrogen receptors, ER-α (NR3A1) and ER-β (NR3A2), are members of the nuclear receptor superfamily that regulate many physiologic processes besides reproduction[1]. One potentially important physiologic, as well as pharmacologic, target of ERs is the cardiovascular system[2], where the action of estrogen in macrophages, smooth muscle cells and endothelial cells is believed to be beneficial[3]. In mice ER-α is required for maintenance and repair of vascular endothelium[4,5], whereas ER-β mediates estrogen-dependent dilation by vascular smooth muscle[6]. Experimental evidence from many studies in animals[7] and in both men and women[8,9] supports the cardioprotective role of estrogen. Nevertheless, estrogen use in postmenopausal hormone replacement therapy (HRT) remains controversial[10,11], mainly owing to the results of two recent clinical trials that did not show a benefit of conjugated estrogens in the prevention of cardiovascular disease[12,13]. Although the interpretation of these trials continues to be debated[14], there are a number of unanswered questions concerning the mechanisms and contributing factors that lead to the loss of cardiovascular protection after menopause and to HRT failure as a cardioprotective measure.

Oxysterols are metabolites of cholesterol that are produced in peripheral tissues as a means to eliminate cholesterol. In macrophages, accumulation of oxysterols and cholesterol is a diagnostic feature of a developing atherosclerotic lesion, and macrophage-derived oxysterols regulate various processes such as cholesterol metabolism and transport, cytokine expression and the induction of apoptosis[15–17]. Certain oxysterols also function as agonists for liver X receptors (LXR-α (NR1H3) and LXR-β (NR1H2)), which govern sterol and lipid homeostasis[18]. The most abundant oxysterol is 27-hydroxycholesterol (27HC), and its concentration correlates well with that of cholesterol[15]. In healthy humans, 27HC circulates at 0.15–0.73 μM (of which ~10% is unesterified[19]), but millimolar concentrations can be reached in developing foam cells and atherosclerotic plaques[15]. Furthermore, 27HC is found in aortic fatty streaks at levels that match the severity of the lesion[20,21]. The enzyme that generates 27HC, sterol 27-hydroxylase (CYP27A1), also is expressed in endothelial cells and macrophages[22]. Despite these correlative findings, it is unknown whether oxysterols such as 27HC have direct actions on vascular cells that might affect vascular health and disease.

In this study we show that 27HC directly antagonizes the transcriptional and nontranscriptional functions of ERs in vascular endothelial and smooth muscle cells, which in mouse and rat models leads to a loss of the cardioprotective effects of estrogen. These findings suggest a mechanism whereby decreased circulating levels of estrogens in the presence of hypercholesterolemia, atherosclerosis, or both may lead to increased risk of heart disease, a condition that may be particularly relevant in postmenopausal women. In addition, the mechanisms we describe may contribute to HRT failure as a cardioprotective measure in older women.

RESULTS

27HC functions as a competitive estrogen receptor antagonist

Using a Gal4-ER cotransfection assay, we examined a series of the most abundant endogenous oxysterols for their abilities to affect ER-α

[1]Department of Pharmacology and Howard Hughes Medical Institute, and [2]Department of Pediatrics, University of Texas Southwestern Medical Center, 6001 Forest Park Road, Dallas, Texas 75390-9050, USA. [3]Department of Pediatrics and Medicine, New York University School of Medicine, New York, New York 10016, USA. [4]Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, PO Box 12233, 111 Alexander Drive, Research Triangle Park, North Carolina 27709, USA. Correspondence should be addressed to D.J.M. (davo.mango@utsouthwestern.edu).

Received 30 April; accepted 8 August; published online 16 September 2007; doi:10.1038/nm1641

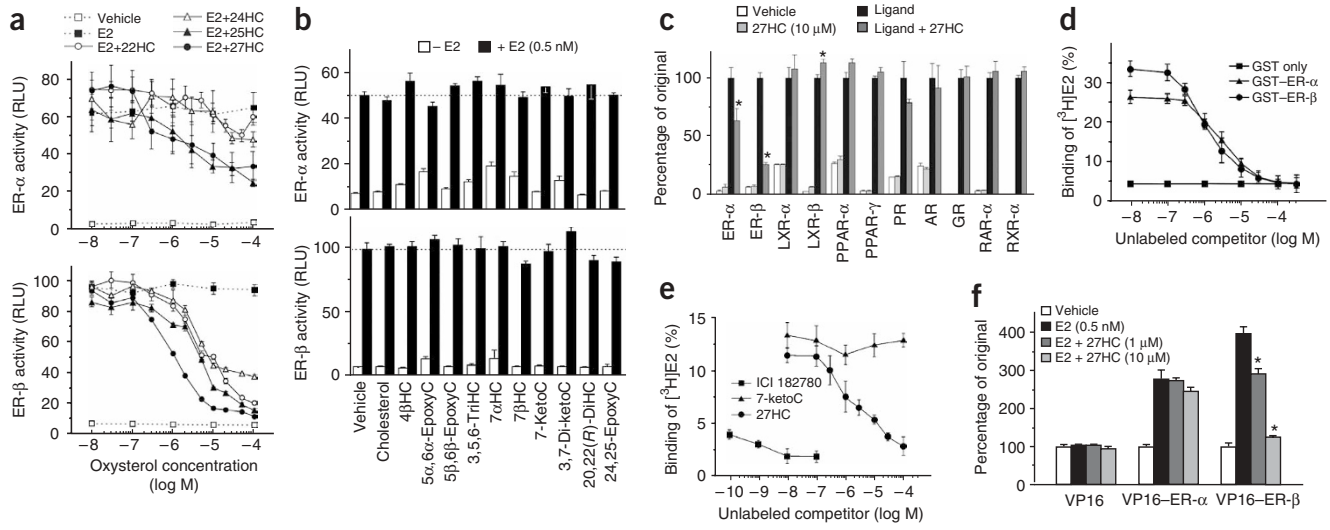

Figure 1 27HC inhibition of estrogen receptors. (**a**) Inhibition of ER-α and ER-β transcriptional activity by side-chain–oxidized sterols in the presence of 0.5 nM E2. HC, -hydroxycholesterol; C, -cholesterol. (**b**) Effect of various other oxysterols (10 μM) on ER-α and ER-β transactivation in the absence or presence of 0.5 nM E2. (**c**) Receptor-specific inhibition by 10 μM 27HC. In **a**–**c**, receptor cotransfection assays were performed with indicated Gal4-receptors in the presence of their cognate ligands ± oxysterol. Ligands used in **c** were 0.5 nM E2 for ER-α and -β, 0.1 μM T0901317 for LXR-α and -β, 1 μM Wy14643 for peroxisome proliferator-activated receptor (PPAR)-α, 0.1 μM troglitazone for PPAR-γ, 10 nM progesterone for progesterone receptor (PR), 50 nM dihydrotestosterone for androgen receptor (AR), 10 nM dexamethasone for glucocorticoid receptor (GR), 100 nM all-*trans* retinoic acid for retinoic acid receptor (RAR)-α, and 100 nM 9-*cis* retinoic acid for retinoid X receptor (RXR)-α. Values in **a**–**c** are expressed as relative light units (RLU) or percentage increases above ligand alone ($n = 4 \pm$ s.e.m.; $^*P < 0.05$ versus ligand control). (**d**) Competitive binding of increasing concentrations 27HC to purified GST-ER proteins in the presence of 1 nM [^{3}H]E2 for GST and GST–ER-α or 0.5 nM [^{3}H]E2 for GST–ER-β ($n = 3$, ± s.e.m.). Values represent percentage of total input [^{3}H]E2 bound. (**e**) Competitive binding of 27HC to endogenous ERs in EA.hy926 cell extracts incubated with ICI 182780, 27HC or 7-ketoC in the presence of 1 nM [^{3}H]E2 ($n = 7$, ± s.e.m.). (**f**) Mammalian two-hybrid assay using Gal4–SRC-1 and VP16–ERs in HEK293 cells. Values expressed as percentage increase above vehicle ($n = 4$, ± s.e.m.; $^*P < 0.05$ versus E2 control).

and ER-β function in HEK293 cells. Of the oxysterols tested, 22*R*-hydroxycholesterol, 24*S*-hydroxycholesterol, 25-hydroxycholesterol and 27HC significantly inhibited estradiol (E2) activation of ER-α and ER-β. The inhibitory effects of these oxysterols were dose dependent and yielded half-maximal inhibitory concentration (IC_{50}) values ranging from 1–5 μM on both ERs (**Fig. 1a**). Other oxysterols had no significant effects on ER activity when tested at concentrations as high as 10 μM (**Fig. 1b**). The most potent oxysterol was 27HC ($IC_{50} = 1$ μM), and its efficacy was greater on ER-β (>90% efficacy) than on ER-α (50% efficacy). Similar results were obtained using full-length ERs and a cognate estrogen responsive element derived from the *TRIM25* gene promoter (data not shown). Although 27HC showed weak agonist activity on LXR-β, its inhibitory effects were ER specific and not observed with any other human nuclear receptor tested (**Fig. 1c**).

To find the mechanism by which 27HC inhibited ER function, we used a [^{3}H]E2 competition ligand-binding assay to demonstrate that 27HC binds directly to purified recombinant ER-α ($K_i = 1.32$ μM) and ER-β ($K_i = 0.42$ μM) proteins (**Fig. 1d**). 27HC also competitively displaced [^{3}H]E2 binding to endogenous ER-α and ER-β proteins in EA.hy926 vascular endothelial cell extracts (**Fig. 1e**). Specificity of binding was demonstrated by competition with the ER-specific antagonist ICI 182780 but not with 7-keto-cholesterol (7-ketoC), an oxysterol found in aorta that does not antagonize ERs (**Fig. 1b**,**e**). Transactivation by ERs is determined in part by their interaction with coactivators such as SRC-1. In a mammalian two-hybrid assay, increasing amounts of 27HC inhibited the E2-dependent interaction of SRC-1 with ER-β but, notably, not with ER-α (**Fig. 1f**), suggesting that 27HC may function similarly to other ER ligands as a selective modulator (see below). These cumulative results implicate 27HC as a naturally occurring ER antagonist.

Vascular 27HC content is elevated with hypercholesterolemia

To begin to examine the conditions associated with greater cardiovascular risk that modify 27HC abundance in vascular cells, we measured 27HC in mouse aorta. Feeding mice a high cholesterol, high fat (HCF) diet for 8 weeks caused a threefold increase in tissue total 27HC (0.313 ± 0.045 ng/mg wet weight on chow versus 0.976 ± 0.096 ng/mg wet weight on HCF, mean ± s.e.m., $n = 3$–4, $P < 0.05$) and a 2.4-fold increase in free 27HC (0.130 ± 0.020 ng/mg wet weight on chow versus 0.313 ± 0.014 ng/mg wet weight on HCF, mean ± s.e.m., $n = 3$–4, $P < 0.05$).

In aorta, the percent of total 27HC that was unesterified was 41.4 ± 0.4% on chow diet and 32.7 ± 1.9% on HCF diet, consistent with previous analyses of the distribution of free and esterified forms among tissues[23]. Estimating aortic tissue density at 0.8–1.0 g/ml, even in the absence of atherosclerotic lesion formation, hypercholesterolemia caused total and unesterified 27HC in vasculature to increase to 1.9 and 0.6 μM, respectively, approaching the IC_{50} for actions on ER. Thus, vascular 27HC content increases with hypercholesterolemia to concentrations that would be expected to modify ER function.

27HC inhibits E2-stimulated NO production and vasodilation

The generation of nitric oxide (NO) by inducible and endothelial nitric oxide synthases (iNOS (NOS2) and eNOS (NOS3)) mediates smooth muscle relaxation in blood vessels, promotes endothelial cell migration and growth, and prevents thrombosis, leukocyte adhesion, and vascular smooth muscle cell proliferation. A number of disorders,

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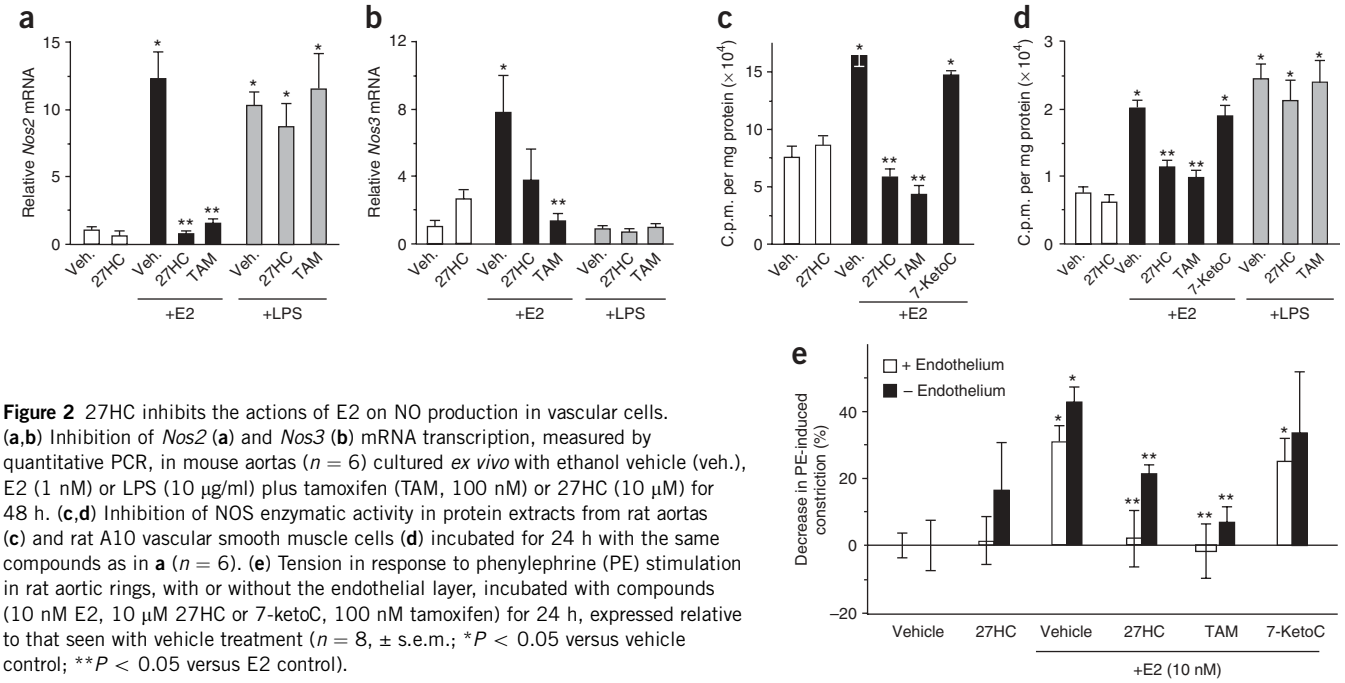

Figure 2 27HC inhibits the actions of E2 on NO production in vascular cells. (**a**,**b**) Inhibition of *Nos2* (**a**) and *Nos3* (**b**) mRNA transcription, measured by quantitative PCR, in mouse aortas ($n = 6$) cultured *ex vivo* with ethanol vehicle (veh.), E2 (1 nM) or LPS (10 μg/ml) plus tamoxifen (TAM, 100 nM) or 27HC (10 μM) for 48 h. (**c**,**d**) Inhibition of NOS enzymatic activity in protein extracts from rat aortas (**c**) and rat A10 vascular smooth muscle cells (**d**) incubated for 24 h with the same compounds as in **a** ($n = 6$). (**e**) Tension in response to phenylephrine (PE) stimulation in rat aortic rings, with or without the endothelial layer, incubated with compounds (10 nM E2, 10 μM 27HC or 7-ketoC, 100 nM tamoxifen) for 24 h, expressed relative to that seen with vehicle treatment ($n = 8$, ± s.e.m.; $^*P < 0.05$ versus vehicle control; $^{**}P < 0.05$ versus E2 control).

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including hypercholesterolemia and diabetes mellitus, are associated with reduced vascular synthesis of NO[24–26]. iNOS and eNOS are also known targets of E2 modulation of vascular functions such as vasodilation and reendothelialization after vascular injury. eNOS is regulated by both ER-α and ER-β, whereas vascular smooth muscle iNOS is regulated primarily by ER-β[6,27]. To determine how 27HC may affect E2 modulation of vascular health, we tested whether 27HC inhibited E2-mediated *Nos2* and *Nos3* mRNA expression in mouse aorta using *ex vivo* organ cultures. 27HC significantly inhibited the E2-dependent upregulation of *Nos2* to an extent comparable to that of the known antiestrogen tamoxifen (**Fig. 2a**). In contrast, 27HC had no effect on *Nos2* expression induced by lipopolysaccharide (LPS), indicating that the 27HC-dependent inhibition was E2-specific. A similar inhibition was seen for expression of *Nos3*, although in this case 27HC was only 50% as efficacious as tamoxifen (**Fig. 2b**). We detected no differences in expression of ERs (**Supplementary Fig. 1a**). The increased efficacy of 27HC on *Nos2* versus *Nos3* transcription may be attributed to the greater ability of 27HC to inhibit ER-β versus ER-α transactivation. We assessed the inhibitory effects of 27HC further by measuring NOS enzymatic activity in homogenates from rat aorta (which express eNOS, iNOS and both ERs) and lysates of the rat vascular smooth muscle cell line A10 (which expresses only iNOS and ER-β)[28]. As expected, 27HC inhibited the increase in NOS activity induced by E2, but not by LPS, providing further evidence that the inhibitory actions of 27HC were E2-specific (**Fig. 2c**,**d**). Furthermore, 7-ketoC did not inhibit E2-dependent NOS activity, supporting the specificity of 27HC as an ER inhibitor.

To assess the functional implications of 27HC inhibition of E2-dependent NOS regulation, we evaluated phenylephrine-induced constriction of rat aorta using an *ex vivo* culture assay[29]. As expected, aortic ring treatment with E2 inhibited phenylephrine-induced contraction, and this effect was reversed by 27HC and tamoxifen but not by 7-ketoC (**Fig. 2e**). Notably, no effects of 27HC were seen in the absence of E2. Although more pronounced in rings with intact endothelium, the effect of 27HC and tamoxifen was also seen in rings denuded of endothelium. As eNOS is expressed exclusively in endothelium and iNOS in vascular smooth muscle[25], 27HC must be altering the function of both eNOS and iNOS.

27HC inhibits ER-dependent NOS expression *in vivo*

We next examined the effects of 27HC on NOS expression *in vivo* under various pharmacologic and dietary conditions that raise 27HC levels in serum and arteries to concentrations that would be expected to inhibit ER signaling. In female wild-type mice and those lacking either ER-α (*Esr1*$^{-/-}$) or ER-β (*Esr2*$^{-/-}$), treatment with 27HC for 7 d significantly increased circulating levels to ~2 μM (**Fig. 3a** and data not shown), but did not alter vascular *Esr* mRNA abundance or plasma estrogen or cholesterol (**Supplementary Fig. 1b** and data not shown). Administration of 27HC, but not 7-ketoC, decreased aortic *Nos2* and *Nos3* mRNA expression in wild-type mice (**Fig. 3b**). Notably, the effects of 27HC were lost in *Esr1*$^{-/-}$ and *Esr2*$^{-/-}$ mice, indicating that both ERs are required for the full inhibitory action of 27HC.

We also examined the effect of hypercholesterolemia-related 27HC elevation on vascular NOS expression. In male mice, chow supplemented with 2% cholesterol for 10 d significantly increased circulating plasma cholesterol (105.9 ± 6.9 mg/dl on chow versus 181.7 ± 14.2 mg/dl on 2% cholesterol diet, mean ± s.e.m., $n = 5$–6, $P < 0.01$) and 27HC (70.5 ± 4.0 ng/ml (0.17 μM) on chow versus 169 ± 29.8 ng/ml (0.42 μM) on 2% cholesterol diet, mean ± s.e.m., $n = 5$–7, $P < 0.01$), but did not alter plasma E2 (24.2 ± 7.0 pg/ml on chow versus 26.3 ± 6.6 pg/ml on 2% cholesterol) or *Esr* mRNA levels (**Supplementary Fig. 1c**). As expected, circulating 27HC reached concentrations expected to inhibit ER function. Hypercholesterolemia decreased iNOS expression at both mRNA and protein levels (**Fig. 3c**,**d**), although under these conditions suppression of eNOS expression did not reach statistical significance (data not shown). Downregulation of iNOS also occurred in mice deficient in both LXR-α- and LXR-β (*Nr1h3*$^{-/-}$*Nr1h2*$^{-/-}$), but not in ER-α-deficient mice, demonstrating that this process was dependent on ER function and not on oxysterol-mediated LXR activation (**Fig. 3c**). To further exclude a role of LXRs, we examined the impact of an LXR agonist,

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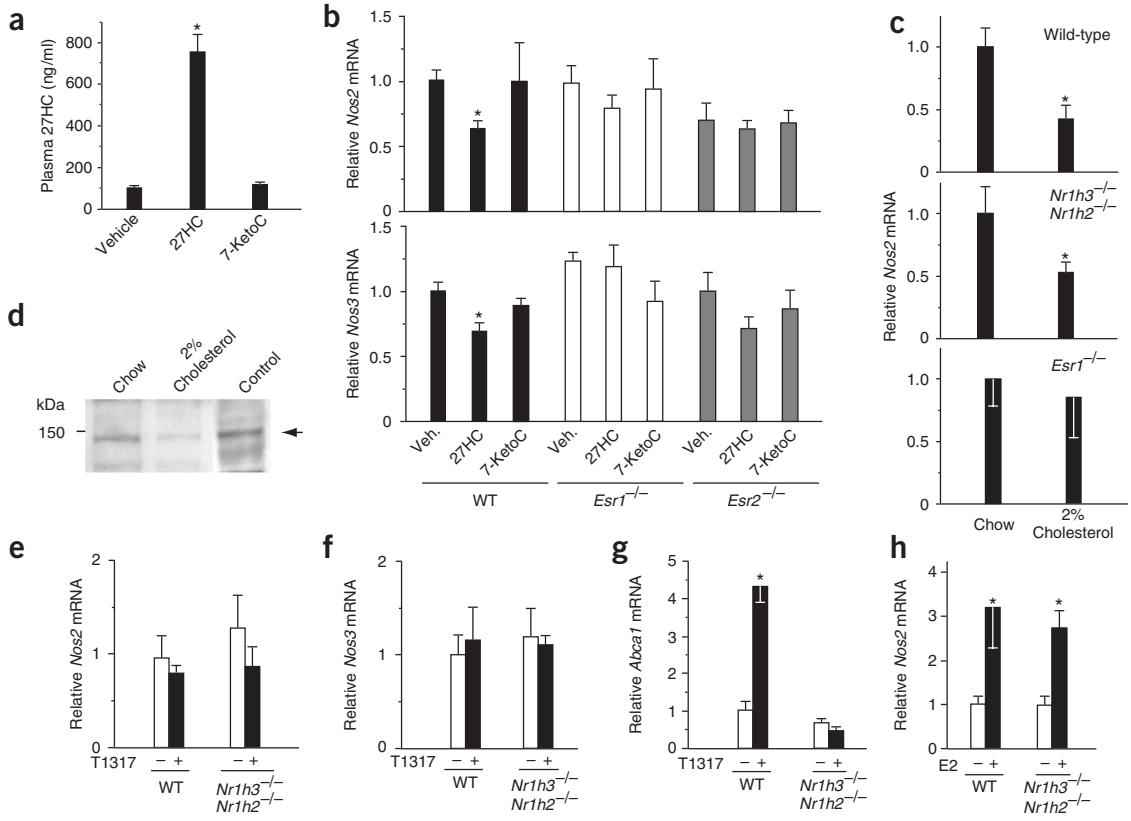

Figure 3 27HC decreases vascular NOS expression in an ER-dependent manner. (**a**,**b**) Results in female wild-type (WT), *Esr1*$^{-/-}$ and *Esr2*$^{-/-}$ mice injected daily with vehicle, 27HC or 7-ketoC subcutaneously for 7d. (**a**) Plasma 27HC measured by LC/MS in WT mice after treatment ($n = 5$, ± s.e.m.; $^*P < 0.05$). Note that 800 ng/ml = 2 μM 27HC. (**b**) Aortic *Nos* mRNA expression after treatment, measured by quantitative PCR ($n = 5$–7, ± s.e.m.; $^*P < 0.05$). (**c**,**d**) *Nos* mRNA and NOS protein levels in male mice fed standard chow or 2% cholesterol diets for 10 d. (**c**) Aortic expression of *Nos2* mRNA assayed by quantitative PCR in WT, LXR-α-, LXR-β-null (*Nr1h3*$^{-/-}$*Nr1h2*$^{-/-}$) or *Esr1*$^{-/-}$ mice ($n = 5$, ± s.e.m.; $^*P < 0.05$). (**d**) Immunoblot of iNOS protein (arrow, iNOS-specific band) in WT mice. Control, macrophage cell extract stimulated by LPS. Equal amounts of total protein were loaded in each lane. A representative of three independent experiments is shown. (**e**–**g**) *Nos2* (**e**), *Nos3* (**f**), or *Abca1* (**g**) mRNA abundance, assayed by quantitative PCR, in aortas taken from WT and LXR-α-, LXR-β-null mice administered vehicle (open bars) or 50 mg/kg of LXR agonist T0901317 (T1317; black bars) for 24 h ($n = 6$, ± s.e.m.; $^*P < 0.05$). (**h**) Induction of *Nos2* mRNA in aortas from WT or LXR-α-, LXR-β-null mice cultured *ex vivo* with 1 nM E2 (black bars) or with vehicle (white bars) for 24 h ($n = 6$, ± s.e.m.; $^*P < 0.05$).

T0901317, on aortic *Nos2* and *Nos3* mRNA expression in wild-type and LXR-deficient mice. Whereas the LXR agonist-receptor pair did not alter *Nos2* or *Nos3* expression (**Fig. 3e**,**f**), it induced expression of *Abca1*, a known LXR target gene (**Fig. 3g**). In *ex vivo* incubation experiments, E2-dependent upregulation of *Nos2* in aorta was LXR-independent (**Fig. 3h**). These cumulative results support the conclusion that under conditions that raise 27HC levels, *Nos2* and *Nos3* expression in the vasculature is suppressed in an ER-dependent and LXR-independent manner.

27HC inhibits nontranscriptional effects of estrogen

In addition to transcriptional effects on *Nos2* and *Nos3*, E2 is known to induce a rapid, non-transcription-mediated stimulation of eNOS enzymatic activity that is ER dependent[27]. 27HC caused a robust, dose-dependent inhibition of the nontranscriptional activation of eNOS by E2 in intact bovine aortic endothelial cells (BAECs), which express high levels of ER-α relative to ER-β (**Fig. 4a**,**b**). Inhibition was also observed with the potent ER antagonist ICI 182780, which decreased activity below basal levels; this may be due to the known ability of ICI 182780 to promote ER degradation[30]. Inhibition of the rapid effects of E2 on eNOS enzyme activity was observed at 27HC concentrations as low as 200 nM (**Fig. 4b**), and was not observed with other oxysterols (**Fig. 4c**). To address the ER subtype selectivity of the nontranscriptional effects of 27HC, we transfected COS-7 cells with expression plasmids for eNOS and either ER-α or ER-β, and subsequently tested for E2-stimulated NOS enzyme activity. In contrast to the stronger effects of 27HC on ER-β-dependent transcription (**Fig. 1a**), 27HC worked equally well at inhibiting the rapid, non-transcription-mediated effects of both ER-α and ER-β on eNOS enzyme activity (**Fig. 4d**). Therefore, 27HC is an effective antagonist of the rapid, nontranscriptional effects as well as the transcriptional effects of E2 on eNOS and iNOS that mediate vascular function.

Oxysterols have also been reported to regulate scavenger receptor (SR)-BI expression (encoded by *Scarb1*), which may in turn alter eNOS activity in endothelial cells[31,32]. However, *Scarb1* mRNA expression in aorta was not changed by elevating sterol levels *in vivo*, and the stimulation of NOS activity in endothelial cells by E2 was inhibited by 27HC equally in the presence or absence of a small interfering RNA targeted against SR-BI (**Supplementary Fig. 2a–c**). Furthermore, increasing plasma 27HC levels did not alter hepatic expression of LXR target genes or SR-BI (**Supplementary Fig. 2d**). These results

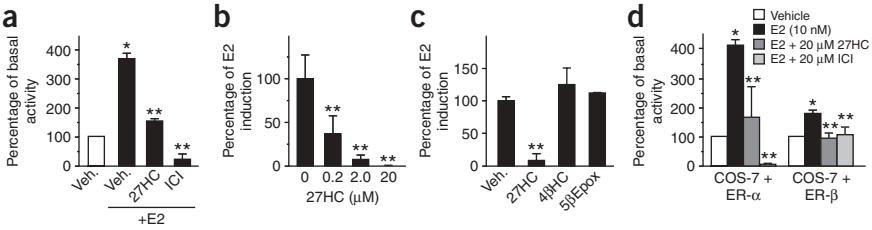

Figure 4 27HC Inhibits nontranscriptional effects of E2 on NOS enzyme activity in vascular cells. (**a**) eNOS enzymatic activity during 15-min incubations of intact BAECs treated with various compounds: 10 nM E2, 20 μM 27HC, 100 nM ICI 182780 (ICI). (**b**) Dose response of 27HC inhibition of 10 nM E2–induced eNOS enzyme activity in BAECs, expressed as percentage of induction in absence of 27HC. (**c**) Inhibitory effect of different oxysterols (20 μM) on 10 nM E2–induced eNOS enzyme activity in BAECs, expressed as percentage of induction in the absence of oxysterols. 4βHC, 4β-hydroxycholesterol; 5βEpox, 5β,6β-epoxycholesterol. (**d**) eNOS activation tested as in **a** after 15-min incubations of various compounds with COS-7 cells expressing ER-α or ER-β and eNOS ($n = 4$, ± s.e.m.; $^*P < 0.05$ versus vehicle control; $^{**}P < 0.05$ versus E2 control).

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further support the conclusion that the inhibitory effects of 27HC are not mediated by SR-BI or LXR.

27HC inhibits estrogen-stimulated reendothelialization

Another mechanism by which E2 protects the vasculature is through enhancement of reendothelialization after injury, a process that is mediated by eNOS[33]. Therefore, we examined the effect of 27HC on E2-induced endothelial cell migration. 27HC and the E2 antagonist ICI 182780 inhibited E2-stimulated migration of endothelial cells (**Fig. 5a,b**). This effect was specific to 27HC and not seen with oxysterols that do not bind ERs. Next, we evaluated the effect of 27HC on vascular function *in vivo* by comparing aortic NOS expression in wild-type versus *Cyp7b1*$^{-/-}$ mice. CYP7B1 catalyzes the metabolic clearance of 27HC (ref. 23). The loss of *Cyp7b1* caused a six- to eightfold elevation in blood 27HC (males 62.2 ± 8.9 ng/ml (0.15 μM) in wild-type versus 509.8 ± 68 ng/ml (1.24 μM) in *Cyp7b1*$^{-/-}$ mice, mean ± s.e.m., $n = 4$–5, $P < 0.01$; females 45.7 ± 8.7 ng/ml (0.11 μM) in wild-type versus 305.0 ± 51.4 ng/ml (0.75 μM) in *Cyp7b1*$^{-/-}$ mice, $n = 3$, mean ± s.e.m., $P < 0.05$) without affecting cholesterol levels. As expected, *Nos2* and *Nos3* mRNA abundances in aorta were lower in *Cyp7b1*$^{-/-}$ female mice compared to wild-type controls (**Fig. 5c**). Notably, when aortas were removed from mice and treated *ex vivo*, *Nos2* and *Nos3* expression were induced similarly by E2 treatment in aortas from both wild-type and *Cyp7b1*$^{-/-}$ mice, indicating that the vasculature of *Cyp7b1*$^{-/-}$ mice is not intrinsically affected and can respond normally to E2 (**Supplementary Fig. 3a,b**). In addition, *Esr1* and *Esr2* expression was similar in aortas of wild-type and *Cyp7b1*$^{-/-}$ female mice, and unaffected by E2 treatment *ex vivo* (**Supplementary Fig. 3c,d**).

We next looked at the effect of 27HC on reendothelialization in female and male mice. Female wild-type and *Cyp7b1*$^{-/-}$ mice were ovariectomized and then implanted with an E2 pellet or vehicle control pellet 3 weeks before perivascular electric injury. Five days after injury the area of endothelial denudation was quantified on the intimal surface. As expected from previous work[4], the area of remaining denudation was significantly less in E2-treated compared to placebo-treated wild-type mice (**Fig. 5d,e**). In contrast, E2 did not promote reendothelialization within the same time period in *Cyp7b1*$^{-/-}$ mice. Further supporting the conclusion that the effects were due to elevation of 27HC and not to some other phenotype of the *Cyp7b1*$^{-/-}$ mice, treatment of wild-type ovariectomized female mice with 27HC, but not 7-ketoC, prevented the reendothelialization response to E2 (**Fig. 5f**). Reendothelialization also was significantly impaired in *Cyp7b1*$^{-/-}$ male mice, which showed a 50% increase in remaining area of endothelial denudation 5 d after injury (**Supplementary Fig. 4a,b**). Likewise, *Nos2* and *Nos3* mRNA abundances were

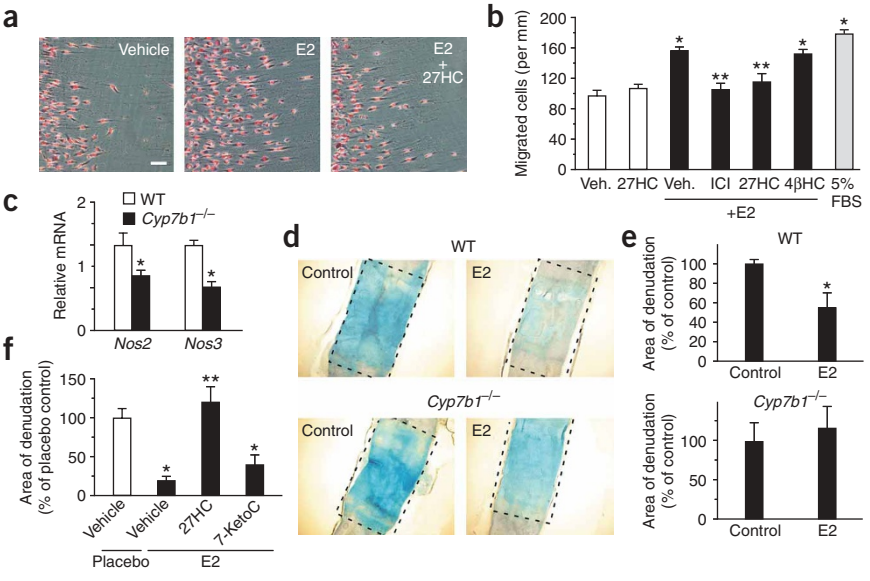

Figure 5 27HC inhibits E2-induced endothelial cell migration and reendothelialization in mice. (**a,b**) Human endothelial cell migration in the presence of various compounds: 10 nM E2, 10 μM 27HC or 4β-hydroxycholesterol (4βHC), 100 nM ICI 182780 (ICI). Cell migration is shown from scraping at left edge of panels in **a** (scale bar, 0.1 mm). Results quantified in **b** by counting cells per mm length of scraped area ($n = 9$, ± s.e.m.; $^*P < 0.05$ versus vehicle control; $^{**}P < 0.05$ versus E2 control). (**c**) *Nos* expression in aortas of wild-type (WT) and *Cyp7b1*$^{-/-}$ female mice ($n = 5$–6; $^*P < 0.05$ versus WT). (**d**) Representative Evans blue staining showing reendothelialization of carotid arteries 5 d after injury (see Methods). Dotted line, area initially denuded. (**e**) Results from experiment in **d** quantified by area remaining denuded (arbitrary pixel units of blue stain) expressed as percentage of vehicle-treated controls ($n = 5$–7 ± s.e.m. per group; $^*P < 0.05$ versus vehicle control). (**f**) Reendothelialization assay in ovariectomized wild-type female mice implanted with vehicle or E2 pellets, followed by oxysterol treatment. Starting three days before artery injury, vehicle, 27HC or 7-ketoC (40 mg/kg mouse body weight) was injected subcutaneously daily for 7 d ($n = 5$–8 ± s.e.m. per group; $^*P < 0.05$ versus vehicle control; $^{**}P < 0.05$ versus E2 control).

diminished in aortas of *Cyp7b1*$^{-/-}$ male mice (**Supplementary Fig. 4c**). Together, these results support the conclusion that elevation of 27HC levels inhibits reendothelialization induced by E2 after vascular injury.

The ability of 27HC to inhibit responses to estrogen in the vasculature raises the possibility that this oxysterol might also affect estrogen action in other tissues. Therefore, we evaluated the effects of 27HC on several more cell lines, including HepG2 (hepatoma), MCF-7 (breast cancer) and Caco-2 (colon cancer). Whereas 27HC antagonized ER transactivation in BAECs and MCF-7 cells, it showed agonist activity in HepG2 and Caco-2 cells (**Supplementary Fig. 5a**). 27HC administration also augmented mRNA expression of the ER target gene complement component 3 (*C3*) in livers of male and female mice, whereas 7-ketoC had no effect (**Supplementary Fig. 5b**). In addition, although a report in a different line of *Cyp7b1*$^{-/-}$ mice showed a hyperestrogenic phenotype in mammary gland and uterus[34], the *Cyp7b1*$^{-/-}$ mice used in our experiments did not show any known reproductive tract abnormalities associated with estrogen action[23]. This tissue-specific, bidirectional response to 27HC is similar to that of other ER pharmacophores such as tamoxifen, and indicates that 27HC functions as an endogenous SERM.

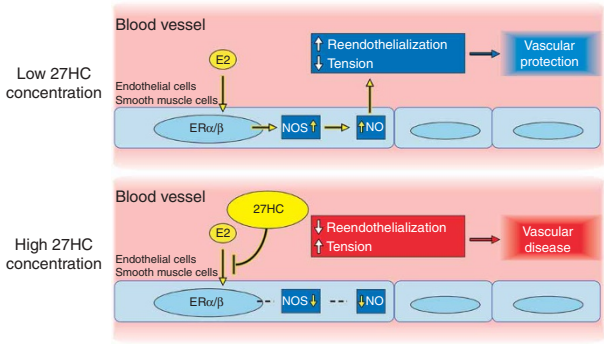

Figure 6 Model of 27HC inhibition of the vasoprotective effects of estrogen. Under conditions of low vascular 27HC and physiologic concentrations of estrogen, vascular production of NO leads to decreased (down-arrow) aortic tension and increased (up-arrow) reendothelialization, which in turn leads to vascular protection (upper panel). When 27HC concentrations are increased relative to estrogen levels, the oxysterol inhibits ER-mediated increases in NO production, leading to conditions that favor the progression of vascular disease (lower panel).

DISCUSSION

In this study, we found that 27HC, a predominant endogenous metabolite of cholesterol, can effectively inhibit estrogen function in vascular tissue by antagonizing both the transcriptional and nontranscriptional effects of ER-α and ER-β. The effects were tissue-specific and due to direct competitive binding of 27HC to ERs. ERs have relatively large ligand binding pockets, permitting several pharmacophores, including SERMs, to bind and modulate their function[30,35,36]; however, no endogenous compounds with this activity have to our knowledge been previously reported. Thus, we believe the discovery that 27HC inhibits vascular ER function represents the first identification of an endogenous selective modulator for a steroid receptor.

An intriguing aspect of the SERM-like activity of 27HC was an ability to discriminate between the rapid non-nuclear effects and the slower transcriptional effects of estrogen signaling through ER-α versus ER-β. Although the binding affinity of 27HC to ER-α and ER-β is similar (K_i = 0.4–1.3 μM), the inhibitory effect of 27HC on E2-induced transcription was stronger for ER-β than ER-α, perhaps owing to the selective activity of the ligand on recruitment of cofactors. In contrast to the preferential impact on ER-β versus ER-α transcriptional function, 27HC caused comparable inhibition of the rapid, non-transcription-mediated effects of ER-α and ER-β on eNOS enzyme activity. The inhibitory effects of 27HC on both transcriptional and nontranscriptional processes in the vasculature consistently required the presence of E2 and one of the ER subtypes, providing further evidence that the effects of 27HC are mediated through antagonism of ER function.

The antagonistic effects of 27HC on both the E2-mediated induction of *Nos* gene expression and non-transcription-mediated induction of *Nos* enzymatic activity provide compelling evidence for the mechanism by which 27HC compromises the vasoprotective effects of estrogen. To evaluate the impact of these effects in vasculature, we used two different physiologic models, aortic tension and reendothelialization after vascular injury. In both models, involvement of NOS regulation by ERs has been reported[33,37]. At the transcriptional level, the inhibition of E2 action by 27HC *in vivo* was comparable between *Nos2* and *Nos3* mice, and our experiments in *Esr*$^{-/-}$ mice indicated that both ER isoforms contribute to iNOS and eNOS expression. These data are consistent with the transcriptional effects of 27HC on both ER-α and ER-β, with the latter isoform known to be a principal regulator of vascular iNOS expression and consequently of arterial tension[6]. Therefore, our results showing that 27HC antagonizes E2-stimulated vasodilation would be expected to have pathophysiologic effects. Furthermore, at the nontranscriptional level, 27HC markedly repressed ER-α-dependent stimulation of eNOS enzymatic function, providing an explanation for the observed inhibition of endothelial cell migration and reendothelialization after vascular injury. Indeed, most estrogenic effects after vascular injury have been attributed to ER-α[4,5,33], although it is worth noting that ER-β, which was similarly antagonized by 27HC, may also have a role. ER-β expression is abundant in vascular smooth muscle cells, is more prevalent in females than in males and is increased after vascular injury[3–5].

The results presented here support a model in which the relative levels of 27HC and estrogen in the vasculature are contributing factors to the risk for cardiovascular disease (**Fig. 6**). Under normal conditions when the amount of 27HC generated from cholesterol is low and the level of estrogen is physiologic (that is, at premenopausal levels), ER function would be preserved, leading to enhanced vasoprotection. In contrast, when the level of 27HC is higher relative to that of estrogen, such as during the postmenopausal period or as a consequence of hypercholesterolemia, the vascular function of ER would be inhibited, resulting in loss of protection. This model may help explain why women are better protected than men from cardiovascular disease until they reach the age of menopause.

As importantly, our work may also explain the repeated observation that estrogen replacement therapy is less likely to be of benefit in the presence of existing vascular disease. In previous studies in hyperlipidemic mice, E2 prevented the initiation of new lesions, but it did not regress established lesions or prevent lesion progression[38]. Likewise, loss of E2 protection in hypercholesterolemic rabbits was proportional to the severity of preexisting atherosclerosis[39]. In nonhuman primates and in humans, E2-related protection from coronary artery disease was evident only when hormone replacement was administered soon after estrogen deficiency, and was absent when therapy was implemented 6 years after menopause[40]. Whereas most US women have only fatty streaks and minimal atherosclerotic plaques in their

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coronary arteries at age 35, there is progression of lesion formation between ages 45 and 55, and more complex lesions are present by age 65 (ref. 41). Although the women in the Women's Health Initiative study were free of known cardiovascular disease, subclinical atherosclerosis was undefined and many of them may have had atherosclerosis[11]. Thus, our findings delineating the actions of a cholesterol metabolite that accumulates in atherosclerotic lesions may provide a partial explanation for why the Women's Health Initiative clinical trial failed to show a cardioprotective benefit, as the age of the women who began taking HRT in this trial was 50–79 years and was an average of 13 years after menopause[13]. Finally, we note that the combination of hormone replacement with a statin resulted in significant improvement of cardiovascular disease compared to estrogen replacement alone[42]. Our results may provide a better understanding of the precise mechanisms by which the beneficial effects of estrogen on vascular function are inhibited.

METHODS

Cell assays. Cells were maintained at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS. Transfections were performed in 96-well plates in phenol red–free DMEM containing 5% dextran-charcoal–stripped (ds) FBS by calcium phosphate precipitation in HEK293 cells[43] or using FuGENE6 (RocheDiagnostics) for all other cells. Six hours after transfection, we treated cells with compounds (Research Plus) for 24 h. We constructed an estrogen response element–luciferase reporter plasmid by inserting three copies of the estrogen response element (5′-TTCAGGGTCATGGTGACCCTGAT-3′) from the human *TRIM25* gene promoter[44] into the *Hin*dIII-*Bam*HI sites of the TK-luc vector. Results represent the mean ± s.e.m. ($n \geq 4$) normalized to a β-galactosidase internal control. For cell migration assays, we grew EA.hy926 human endothelial cells to confluency in 1% dsFBS in six-well plates, then scraped half the well, washed it with PBS, treated it with compounds, and 20 h later stained with hematoxylin and counted migrated cells[45].

Ligand binding assay. We expressed human estrogen receptor (*ESR1* and *ESR2*) cDNAs and affinity purified the resulting glutathione *S*-transferase (GST) fusion proteins in BL21DE3 cells (Promega) using pGEX5X-2 vectors (Pharmacia). We assayed ligand binding with EA.hy926 whole-cell extracts as described[43] using 0.5 or 1.0 nM (18.8 Ci/mmol) [^{3}H]E2 (Sigma) and the various competitors incubated overnight at 4 °C in lysate buffer. Unbound [^{3}H]E2 was removed by adsorption to dextran-coated charcoal, and the supernatant analyzed for tritium. Values are reported as percentage of total input [^{3}H]E2 bound. In preliminary experiments, we determined K_d values for ER-α (0.5 nM) and ER-β (0.2 nM), and then used them to calculate the mean K_i values for 27HC with Prism Software (GraphPad) ± s.e.m., $n \geq 3$.

Mouse studies. *Esr1*$^{-/-}$, *Esr2*$^{-/-}$, *Nr1h3*$^{-/-}$*Nr1h2*$^{-/-}$ and *Cyp7b1*$^{-/-}$ mice have been described[23,46,47]. We administered 27HC to mice daily by subcutaneous injection of 9% (2-hydroxypropyl)-β-cyclodextrin solution with or without 40 mg per kilogram body weight of 27HC or 7-ketoC. We performed studies with chow (4% milkfat, ≤0.04% cholesterol (Teklad 7001)), high cholesterol (Teklad 7001 plus 2% cholesterol) and Western (21% milkfat, 0.2% cholesterol (Teklad 88137)) diets as previously described[47]. Circulating E2 was determined by ELISA (Cayman). We assessed vascular reendothelialization after perivascular electric injury on the distal portion of the common carotid artery in 4–8 month-old male or female mice on identical genetic backgrounds as previously described[4,45]. One or 5 d after injury, we injected 5% Evans blue dye solution into the heart and allowed it to circulate for 10 min. The stain was fixed with a perfusion of 4% phosphate-buffered formalin and the denuded area measured in *en face* images of the carotid intima. Areas of initial denudation (1 d after injury) were similar in all comparison groups. We removed mouse ovaries and implanted vehicle or E2 pellets (Innovative Research of America), and performed perivascular electric injury 3 weeks later. In these experiments, plasma E2 in the control and E2-treated group was <7.8 pg/ml and 637.6 ± 13.9 pg/ml, respectively. There were no differences in plasma E2 between wild-type and *Cyp7b1*$^{-/-}$ mice. Animal experiments were approved by the Institution Animal Care and Research Advisory Committee of University of Texas Southwestern Medical Center at Dallas.

Liquid chromatography/mass spectrometry (LC/MS) analysis of oxysterols. We spiked mouse plasma (100–300 μl) or aortic tissue extracts (50 mg) with 85 ng of 25-hydroxycholesterol-D_3 internal standard (Avanti Polar Lipids) and then extracted lipids[48]. We quantified sterols by LC/MS (Agilent Technologies) with atmospheric pressure chemical ionization in positive ion mode. We loaded samples onto the analytical column at 0.6 ml/min (Eclipse XDB-C18, 4.6 × 150 mm, 5 μm, Agilent). The mobile phase consisted of methanol (A) and water (B) containing 5 mM ammonium acetate. The following gradient was run for a total run time of 30 min: 0–1 min 85% (A), 1–6 min, 85 to 100% (A), 6–24.5 min 100% (A), 25 min 85% (A). Mass spectrometric parameters were as follows: gas temperature 350 °C, vaporizer temperature 450 °C, nebulizer pressure 60 p.s.i. (gauge), drying gas (nitrogen) 5 liters/min, capillary voltage 3,750 V, fragmentor voltage 150 V. We used selective ion monitoring to detect 27HC (*m/z* 385 $[M\text{-}H_2O]^+$, retention time 10.3 min), 7-ketoC (*m/z* 401 $[M\text{-}H]^+$, retention time 13.4 min), 7α/β-hydroxycholesterol (*m/z* 385 $[M\text{-}H_2O]^+$, retention time 12.3 min) and 25-hydroxycholesterol-D_3 (*m/z* 370 $[M\text{-}2H_2O]^+$, retention time 10.1 min). We quantified oxysterols by computing the ratios of the areas of their peaks to that of the internal standard peak and comparing the results against an external calibration curve prepared in methanol.

Real-time PCR analysis. We evaluated mRNA abundance in triplicate assays by real-time quantitative PCR on an Applied Biosystems Prism 7900HT Sequence Detection System using the comparative cycle-time method as previously described[49].

NOS enzymatic activity. We removed thoracic aortas from 3-month-old C57BL/6 male mice and cultured them in DMEM plus 10% dsFBS in the presence of various treatments. RNA was analyzed as described above. Immunoblots were performed using tissue or whole cell extract with iNOS-specific antibodies (Santa Cruz)[6]. Enzymatic activity was assessed by measuring conversion of [^{3}H]L-arginine (Amersham) to [^{3}H]L-citrulline as previously described[32]. eNOS activation was assessed in intact BAECs or transfected COS-7 cells in a similar fashion. We transfected small interfering RNAs for SR-BI into BAECs as described[45].

Aortic tension. We measured aortic tension in rat thoracic aorta as reported elsewhere[29]. Aortic rings were incubated at 37 °C, 5% CO_2 in phenol red–free DMEM with compounds for 24 h, then transferred to organ chambers and phenylephrine (100 nM)-stimulated tension was measured. In selected studies, we removed the endothelial layer using a cotton swab and confirmed removal by loss of reactivity to 10 μM acetylcholine.

Statistical analysis. Results are presented as mean ± s.e.m., and differences between groups were assessed by analysis of variance using the Mann-Whitney *U*-test.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

The authors thank Y. Mizuno, K. Kamm, J. Stull and D. Seetharam for help with NOS activity, aortic tension measurement and cell migration assays; C.J.S. Edgell (University of North Carolina) for providing EA.hy926 cells; A. Liverman (University of Texas Southwestern) for aorta samples; D.W. Russell (University of Texas Southwestern) for *Cyp7b1*$^{-/-}$ mice; and S. Kliewer and members of the Mangelsdorf lab for discussions and suggestions. D.J.M. is an investigator at the Howard Hughes Medical Institute. This work was funded by the Howard Hughes Medical Institute, the Robert A. Welch Foundation (grant I-1275), and US National Institutes of Health grants HL87564 (P.W.S., D.J.M.) and U19DK62434 (D.J.M.).

AUTHOR CONTRIBUTIONS

M.U. designed, executed and interpreted most of the experiments and prepared the manuscript. H.D. performed initial experiments showing that 27HC is an ER ligand. A.K.G. performed reendothelialization experiments. I.S.Y. performed NOS enzyme assays. C.L.C. designed and performed the LC/MS experiments. N.B.J. synthesized and formulated 27HC. K.S.K. supplied the *Esr*$^{-/-}$ mice and contributed to the design of the mouse studies. P.W.S. and D.J.M. conceived, planned and supervised the project and wrote the paper.

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1. Deroo, B.J. & Korach, K.S. Estrogen receptors and human disease. *J. Clin. Invest.* **116**, 561–570 (2006).
2. Mendelsohn, M.E. & Karas, R.H. The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med.* **340**, 1801–1811 (1999).
3. Lindner, V. *et al.* Increased expression of estrogen receptor-beta mRNA in male blood vessels after vascular injury. *Circ. Res.* **83**, 224–229 (1998).
4. Brouchet, L. *et al.* Estradiol accelerates reendothelialization in mouse carotid artery through estrogen receptor-alpha but not estrogen receptor-beta. *Circulation* **103**, 423–428 (2001).
5. Pare, G. *et al.* Estrogen receptor-alpha mediates the protective effects of estrogen against vascular injury. *Circ. Res.* **90**, 1087–1092 (2002).
6. Zhu, Y. *et al.* Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. *Science* **295**, 505–508 (2002).
7. Hodgin, J.B. & Maeda, N. Minireview: estrogen and mouse models of atherosclerosis. *Endocrinology* **143**, 4495–4501 (2002).
8. Mendelsohn, M.E. & Karas, R.H. Molecular and cellular basis of cardiovascular gender differences. *Science* **308**, 1583–1587 (2005).
9. Colditz, G.A. *et al.* Menopause and the risk of coronary heart disease in women. *N. Engl. J. Med.* **316**, 1105–1110 (1987).
10. Turgeon, J.L., McDonnell, D.P., Martin, K.A. & Wise, P.M. Hormone therapy: physiological complexity belies therapeutic simplicity. *Science* **304**, 1269–1273 (2004).
11. Karas, R.H. Current controversies regarding the cardiovascular effects of hormone therapy. *Clin. Obstet. Gynecol.* **47**, 489–499 (2004).
12. Hulley, S. *et al.* Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *J. Am. Med. Assoc.* **280**, 605–613 (1998).
13. Rossouw, J.E. *et al.* Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *J. Am. Med. Assoc.* **288**, 321–333 (2002).
14. Clark, J.H. A critique of Women's Health Initiative studies (2002–2006). *Nucl. Recept. Signal. [online]* **4**, e023 (2006).
15. Brown, A.J. & Jessup, W. Oxysterols and atherosclerosis. *Atherosclerosis* **142**, 1–28 (1999).
16. Schroepfer, G.J., Jr. Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol. Rev.* **80**, 361–554 (2000).
17. Russell, D.W. Oxysterol biosynthetic enzymes. *Biochim. Biophys. Acta* **1529**, 126–135 (2000).
18. Janowski, B.A. *et al.* Structural requirements of ligands for the oxysterol liver X receptors LXRα and LXRβ. *Proc. Natl. Acad. Sci. USA* **96**, 266–271 (1999).
19. Dzeletovic, S., Breuer, O., Lund, E. & Diczfalusy, U. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. *Anal. Biochem.* **225**, 73–80 (1995).
20. Garcia-Cruset, S., Carpenter, K.L., Guardiola, F., Stein, B.K. & Mitchinson, M.J. Oxysterol profiles of normal human arteries, fatty streaks and advanced lesions. *Free Radic. Res.* **35**, 31–41 (2001).
21. Upston, J.M. *et al.* Disease stage-dependent accumulation of lipid and protein oxidation products in human atherosclerosis. *Am. J. Pathol.* **160**, 701–710 (2002).
22. Crisby, M., Nilsson, J., Kostulas, V., Bjorkhem, I. & Diczfalusy, U. Localization of sterol 27-hydroxylase immuno-reactivity in human atherosclerotic plaques. *Biochim. Biophys. Acta* **1344**, 278–285 (1997).
23. Li-Hawkins, J., Lund, E.G., Turley, S.D. & Russell, D.W. Disruption of the oxysterol 7alpha-hydroxylase gene in mice. *J. Biol. Chem.* **275**, 16536–16542 (2000).
24. Christopherson, K.S. & Bredt, D.S. Nitric oxide in excitable tissues: physiological roles and disease. *J. Clin. Invest.* **100**, 2424–2429 (1997).
25. Papapetropoulos, A., Rudic, R.D. & Sessa, W.C. Molecular control of nitric oxide synthases in the cardiovascular system. *Cardiovasc. Res.* **43**, 509–520 (1999).
26. Li, H. & Forstermann, U. Nitric oxide in the pathogenesis of vascular disease. *J. Pathol.* **190**, 244–254 (2000).
27. Chambliss, K.L. & Shaul, P.W. Estrogen modulation of endothelial nitric oxide synthase. *Endocr. Rev.* **23**, 665–686 (2002).
28. Takahashi, K. *et al.* Both estrogen and raloxifene cause G1 arrest of vascular smooth muscle cells. *J. Endocrinol.* **178**, 319–329 (2003).
29. Ravi, J., Mantzoros, C.S., Prabhu, A.S., Ram, J.L. & Sowers, J.R. In vitro relaxation of phenylephrine- and angiotensin II-contracted aortic rings by beta-estradiol. *Am. J. Hypertens.* **7**, 1065–1069 (1994).
30. Parker, M.G., Arbuckle, N., Dauvois, S., Danielian, P. & White, R. Structure and function of the estrogen receptor. *Ann. NY Acad. Sci.* **684**, 119–126 (1993).
31. Yu, L., Cao, G., Repa, J. & Stangl, H. Sterol regulation of scavenger receptor class B type I in macrophages. *J. Lipid Res.* **45**, 889–899 (2004).
32. Yuhanna, I.S. *et al.* High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nat. Med.* **7**, 853–857 (2001).
33. Iwakura, A. *et al.* Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. *Circulation* **108**, 3115–3121 (2003).
34. Omoto, Y., Lathe, R., Warner, M. & Gustafsson, J.A. Early onset of puberty and early ovarian failure in CYP7B1 knockout mice. *Proc. Natl. Acad. Sci. USA* **102**, 2814–2819 (2005).
35. Brzozowski, A.M. *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**, 753–758 (1997).
36. Shiau, A.K. *et al.* Structural characterization of a subtype-selective ligand reveals a novel mode of estrogen receptor antagonism. *Nat. Struct. Biol.* **9**, 359–364 (2002).
37. Rubanyi, G.M. *et al.* Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *J. Clin. Invest.* **99**, 2429–2437 (1997).
38. Rosenfeld, M.E. *et al.* Estrogen inhibits the initiation of fatty streaks throughout the vasculature but does not inhibit intra-plaque hemorrhage and the progression of established lesions in apolipoprotein E deficient mice. *Atherosclerosis* **164**, 251–259 (2002).
39. Hanke, H. *et al.* Effect of 17-beta estradiol on pre-existing atherosclerotic lesions: role of the endothelium. *Atherosclerosis* **147**, 123–132 (1999).
40. Clarkson, T.B. & Appt, S.E. Controversies about HRT–lessons from monkey models. *Maturitas* **51**, 64–74 (2005).
41. Grodstein, F., Clarkson, T.B. & Manson, J.E. Understanding the divergent data on postmenopausal hormone therapy. *N. Engl. J. Med.* **348**, 645–650 (2003).
42. Herrington, D.M. *et al.* Statin therapy, cardiovascular events, and total mortality in the Heart and Estrogen/Progestin Replacement Study (HERS). *Circulation* **105**, 2962–2967 (2002).
43. Makishima, M. *et al.* Vitamin D receptor as an intestinal bile acid sensor. *Science* **296**, 1313–1316 (2002).
44. Klinge, C.M. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* **29**, 2905–2919 (2001).
45. Seetharam, D. *et al.* High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circ. Res.* **98**, 63–72 (2006).
46. Couse, J.F., Yates, M.M., Walker, V.R. & Korach, K.S. Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERα but not ERβ. *Mol. Endocrinol.* **17**, 1039–1053 (2003).
47. Repa, J.J. *et al.* Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* **289**, 1524–1529 (2000).
48. Lund, E.G. & Diczfalusy, U. Quantitation of receptor ligands by mass spectrometry. *Methods Enzymol.* **364**, 24–37 (2003).
49. Bookout, A.L. & Mangelsdorf, D.J. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nucl. Recept. Signal. [online]* **1**, e012 (2003).