17β-estradiol promotes cholesterol efflux from vascular smooth muscle cells through a liver X receptor α-dependent pathway

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Abstract. Estrogen has pleiotropic effects on the cardiovascular diseases, yet the underlying mechanisms remain incompletely understood. Cholesterol efflux is a key mechanism through which to prevent foam cell formation and the development of atherosclerosis. Recent studies highlight the role of vascular smooth muscle cell (VSMC)-derived foam cells in atherogenesis. However, it remains unclear whether estrogen promotes cholesterol efflux from VSMCs and inhibits VSMC-derived foam cell formation. In the present study, we demonstrated that 17β-estradiol (E2) markedly enhanced cholesterol efflux to apolipoprotein (apo)A-1 and high-density lipoprotein (HDL) and attenuated oxidized low-density lipoprotein (ox-LDL) induced cholesteryl ester accumulation in VSMCs, which was associated with an increase in the expression of ATP-binding cassette transporters ABCA1 and ABCG1. The upregulation of ABCA1 and ABCG1 expression by E2 resulted from liver X receptor (LXR)α activation, which was confirmed by the prevention of the expression of ABCA1 and ABCG1 after inhibition of LXRα with a pharmacological inhibitor or small interfering RNA (siRNA). Furthermore, E2 increased LXRα, ABCA1 and ABCG1 expression in VSMCs via the estrogen receptor (ER), and the involvement of ERβ was confirmed by the use of selective ERα or ERβ antagonists (MPP and PHTPP) and agonists (PPT and DPN). These findings suggest that E2 promotes cholesterol efflux from VSMCs and reduces VSMC-derived foam cell formation via ERβ- and LXRα-dependent upregulation of ABCA1 and ABCG1 and provide novel insights into the anti-atherogenic properties of estrogen.

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Introduction

Cardiovascular disease (CVD), a leading cause of morbidity and mortality in Western society, is caused mainly by atherosclerosis (1). Epidemiological studies suggest that estrogen protects women against CVD before menopause (2), and numerous animal studies have shown that estrogen significantly protects against the development of atherosclerosis (3-5). However, randomized controlled trials of postmenopausal estrogen therapy have demonstrated mixed CVD effects (6,7). These findings underscore the complexity of the cardiovascular effects of estrogen, and the underlying mechanisms by which estrogen regulates cardiovascular biology are of great value and warrant further investigation.

Reverse cholesterol transport (RCT) is proposed to be a primary atheroprotective property of high-density lipoprotein (HDL) and its major protein, apolipoprotein (apo)A-1, which promote efflux of excess cholesterol from macrophages in atherosclerotic lesions and then transport it to the liver for degradation and excretion (8). Cholesterol efflux is the initial and most likely rate-limiting step in RCT and plays a pivotal role in maintaining intracellular cholesterol levels and preventing foam cell formation (9-11). Recent studies have indicated that vascular smooth muscle cells (VSMCs) are also capable of accumulating lipid and form foam-like cells *in vitro* and in atherosclerotic plaques (12-14). Moreover, VSMC-derived foam cells have been demonstrated to acquire phagocytotic activity similar to macrophages (15) and express cytokines or chemokines to promote intimal foam cell accumulation (16). These studies suggest that lipid accumulation in VSMCs contributes to atherosclerosis development. Thus, promotion of cholesterol efflux from VSMCs may potentially inhibit VSMC-derived foam cell formation and the development of atherosclerosis. However, unlike macrophages, little is known about the regulation of cholesterol efflux and cholesterol transporters such as ATP-binding cassette transporters ABCA1 and ABCG1 and scavenger receptor B1 (SR-B1) (8) in VSMCs.

Previous studies have focused on the anti-proliferative and anti-migratory effects of estrogen on VSMCs (17-19). Nevertheless, the contributions of estrogen to cholesterol efflux and VSMC-derived foam cell formation are relatively unexplored. In the present study, we aimed to ascertain whether 17β-estradiol (E2) promotes cholesterol efflux from VSMCs and inhibits VSMC-derived foam cell formation, as well as the underlying mechanisms.

Materials and methods

Chemicals. E2, monoclonal anti-α-smooth muscle actin (F-3777), geranylgeranyl pyrophosphate (GGPP), ICI 182,780, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP) and 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a] pyrimidin-3-yl] phenol (PHTPP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenol red-free Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone (Logan, UT, USA). Charcoal stripped fetal bovine serum (FBS) was obtained from Gibco-BRL (Grand Island, NY, USA). 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol) and 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY® 493/503) were obtained from Invitrogen (Grand Island, NY, USA). ApoA-1 was purchased from Calbiochem (San Diego, CA, USA). HDL and oxidized low-density lipoprotein (ox-LDL) were obtained from XieSheng Biotechnology (Beijing, China). Propyl-pyrazole triol (PPT), diarylpropionitrile (DPN) and the cholesterol assay kit (catalog no. 10007640) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Rabbit polyclonal antibodies against ABCA1 and ABCG1 were obtained from Novus Biologicals (Littleton, CO, USA). Rabbit polyclonal antibody against liver X receptor (LXR)α was obtained from Abnova (Taipei, Taiwan). Rabbit polyclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Epitomics (Burlingame, CA, USA). Horseradish peroxidase (HRP) goat-anti-rabbit IgG was obtained from Abcam (Cambridge, MA, USA).

Cell culture. Eight-week-old female C57BL/6 mice were purchased from the Experimental Animal Center of Medical School of Xi'an Jiaotong University (Shaanxi, China). Primary mouse VSMCs were obtained using the tissue explant method, as previously published (20). Briefly, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. The protocol for the present study was approved by the Institutional Ethics Committee for Animal Experiments of Xi'an Jiaotong University, China. The aortic segments were placed into an ice-cold 60-mm dish containing DMEM. The media of aorta was isolated surgically and minced into small pieces. The pieces were then placed into 60-mm dishes and cultured in phenol red-free DMEM, supplemented with 10% charcoal-stripped FBS containing 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in 5% CO_2. The VSMCs used between passages 2 and 5 had a VSMC purity >95% (determined by immunofluorescence staining for α-smooth muscle actin). Quiescent VSMCs were obtained by incubation with serum-free medium for 24 h prior to performing all of the experimental procedures.

Cholesterol efflux assay. The cholesterol efflux assay was performed as previously described with minor modifications (21). The VSMCs ($4x10^4$ cells/well) cultured in 12-well plates were treated with various concentrations of E2 (1-100 nM) or vehicle (ethanol at a final concentration <0.01%) for 18 h, followed by the equilibration of NBD-cholesterol (1 μg/ml) for an additional 6 h in the presence of E2. NBD-cholesterol-labeled cells were washed with phosphate-buffered saline (PBS) and incubated in DMEM, DMEM containing apoA-1 (15 μg/ml) or HDL (50 μg/ml) for 6 h. The fluorescence-labeled cholesterol released from the cells into the medium was measured with a multilabel counter (PerkinElmer, Waltham, MA, USA). Cholesterol efflux was expressed as a percentage of fluorescence in the medium relative to the total amount of fluorescence (cells and medium). Specific efflux to apoA-1 or HDL was calculated by subtracting the non-specific efflux to DMEM alone.

Detection of cellular lipid droplets in VSMCs and VSMC-derived foam cells. The VSMCs were seeded in chamber slides at a density of $1x10^5$ cells/chamber and pretreated with either E2 (1-100 nM) or vehicle for 2 h and then incubated with ox-LDL (50 μg/ml) for 72 h in either the presence or absence of E2. For detecting lipid accumulation in VSMCs, BODIPY staining was performed as previously described with minor modifications (22). The cells were washed twice with ice-cold PBS, followed by 4% paraformaldehyde fixation for 1 h at room temperature. Then the cells were stained with BODIPY® 493/503 working solution (10 μg/ml in PBS) for 30 min at room temperature and rinsed twice with PBS. The images were captured and analyzed with NIS-Elements imaging software (Nikon, Tokyo, Japan).

Quantitative measurement of intracellular cholesteryl ester content. The VSMCs were plated into 6-well plates ($1x10^5$ cells/well) and treated as described above. The cellular lipids were extracted with hexane/isopropanol (3/2, v/v). The levels of total and free cholesterol were determined by an enzymatic, fluorometric method, using a cholesterol assay kit according to the kit instructions (Cayman Chemical). Fluorescence intensity was measured using excitation at 540 nm and emission at 590 nm. Cholesteryl ester content was calculated as total cholesterol minus free cholesterol and normalized to the cellular protein content, which was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Real-time RT-PCR analysis. The VSMCs ($5x10^5$ cells) plated on 6-cm culture dishes were incubated with E2 at various concentrations (1-100 nM) or vehicle (ethanol) for 24 h and then rinsed twice with PBS. Total cellular RNA was extracted by TRIzol reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription was carried out with 2 μg total RNA using RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, CA, USA), and the iQ SYBR-Green Supermix kit (Takara, Tokyo, Japan) was used on an iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). Primers for the genes tested are shown in Table I. Expression data were normalized to GAPDH levels.

Western blot analysis. Western blot analyses were carried out as previously described (23). VSMC lysates were prepared in

Table I. Primers sequences for real-time RT-PCR.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
ABCA1	CTCAGTTAAGGCTGCTGCTG	TCAGGCGTACAGAGATCAGG
ABCG1	TGTGCTGTTCG CTGCTCTGG	GGTAGGCTGGGATGGTGTCAAAG
SR-B1	GTTTGGTGCGCCTCTGTTTC	CGATGCCCTTGACAGATTAG
LXRα	ACGTGCAGGACCAGCTCCAA	GCAGGCGA AGGGC AAACACT
GAPDH	TCAACGGCACAGTCAAGG	ACTCCACGACATACTCAGC

ABCA1, ABC transporter A1; ABCG1, ABC transporter G1; SR-B1, scavenger receptor B1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM dithiothreitol, complete protease inhibitor cocktail). The protein concentration was assayed using a BCA protein assay kit. An equal amount of total proteins (30 μg) was loaded onto either 8 or 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Bio-Rad). After blocked with 5% nonfat milk in TBST (20 mM Tris-HCl, 150 mM NaCl, pH 7.5 and 0.1% Tween-20), blots were incubated with anti-ABCA1 (1:500), anti-ABCG1 (1:500), anti-LXRα (1:500) or anti-GAPDH (1:1,000) antibodies overnight at 4˚C. After washing with TBST, the blots were incubated with the HRP-conjugated secondary antibody (1:5,000) for 1 h at room temperature. Immunoreactive bands were quantified using an enhanced chemiluminescent system of detection (Pierce).

Transfection of siRNA. For downregulation of LXRα expression, LXRα siRNA and negative control siRNA were synthesized by GenePharm (Shanghai, China). The sequences for LXRα siRNA were: sense, 5'-GGCUGCAACACACAU AUGUTT-3' and antisense, 5'-ACAUAUGUGUGUUGCAGC CTT-3'. The sequences for negative control siRNA were: sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCG GAGAATT-3'. One day prior to transfection, the VSMCs ($1x10^5$/well) were seeded in 6-well plates in 2 ml DMEM containing 10% FBS. LXRα siRNA and control siRNA were transfected into VSMCs using Lipofectamine™ 2000 reagent (Invitrogen) at a final concentration of 100 nM according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were treated with E2 (10 nM) for an additional 24 h and lysed for western blot analysis.

Statistical analysis. All experiments were repeated at least three times. Data are expressed as the mean ± standard error of the mean (SEM). Intergroup differences were analyzed using one-way analysis of variance (ANOVA) for the comparison of 3 or more groups. The Student's t-test was used for comparison between 2 groups. A value of $P<0.05$ was considered to indicate a statistically significant result.

Results

E2 promotes cholesterol efflux from VSMCs and attenuates cholesteryl ester accumulation in VSMC-derived foam cells. To investigate the effects of E2 on cholesterol efflux from VSMCs, cells were pretreated with different concentrations of E2 for 18 h, followed by incubation with NBD-cholesterol for 6 h in the presence of E2. Cholesterol efflux was initiated by the addition of apoA-1 (15 μg/ml) or HDL (50 μg/ml). The results (Fig. 1A and B) revealed that cholesterol efflux to apoA-1 was significantly increased in response to E2, and E2 dose-dependently promoted HDL-mediated cholesterol efflux from the VSMCs. At 10 nM of E2, the cholesterol efflux from the VSMCs to apoA-1 and HDL was increased by 176 and 95%, respectively, compared with the controls (apoA-1 or HDL only; $P<0.01$).

In addition, BODIPY staining and an enzymatic colorimetric method were employed to assess the effect of E2 on cholesteryl ester accumulation in VSMC-derived foam cells. Administration of 50 μg/ml ox-LDL for 72 h significantly increased cellular lipid droplets in the VSMCs (Fig. 1C). Treatment with E2 markedly attenuated the ox-LDL-induced accumulation of lipid droplets in a dose-dependent manner (Fig. 1C). By directly measuring the intracellular cholesteryl ester content, it was evident (Fig. 1D) that E2 dose-dependently decreased cholesteryl ester content in the VSMCs, compared with the oxLDL-treated group. At 10 nM of E2, the cholesteryl ester content in the VSMCs was decreased by 70%, compared with the cells treated with ox-LDL only ($P<0.01$).

E2 increases the expression of ABCA1 and ABCG1 in VSMCs. To study the possible mechanisms responsible for E2 action, VSMCs were treated with different concentrations of E2 for 24 h. Changes in ABCA1, ABCG1 and SR-B1 mRNA in response to E2 were assessed by real-time RT-PCR. E2 significantly increased ABCA1 and ABCG1 mRNA levels, whereas E2 had little effect on SR-B1 mRNA expression in the VSMCs (Fig. 2A-C).

To study if the increased mRNA levels of ABCA1 and ABCG1 by E2 can lead to an increase in ABCA1 and ABCG1 protein expression, after treatment VSMCs were determined for ABCA1 and ABCG1 protein levels by western blot analysis. Protein levels of ABCA1 and ABCG1 had a trend of increase similar to mRNA levels when treated by E2 (Fig. 2D). At 10 nM of E2, the ABCA1 and ABCG protein levels had an increase of 266 and 164%, respectively, when compared with the controls ($P<0.01$). Moreover, the upregulation of ABCA1 and ABCG1 by E2 (10 nM) peaked at 24 h, when compared with the control group (Fig. 2E).

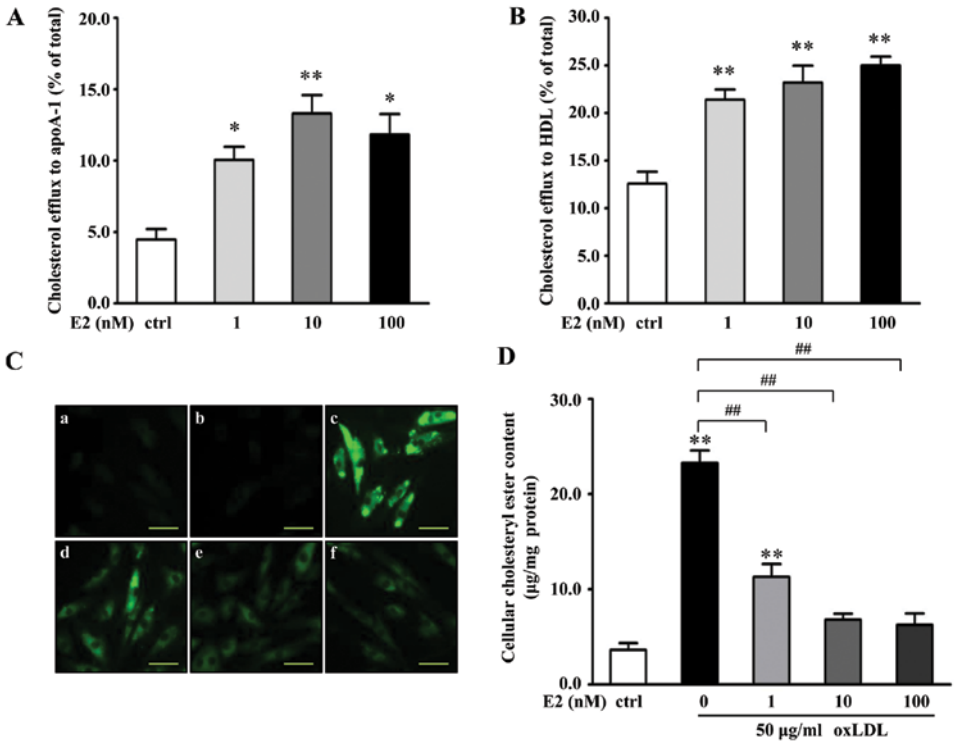

Figure 1. Effects of E2 on cholesterol efflux and cellular cholesteryl ester content in vascular smooth muscle cells (VSMCs). (A and B) Cholesterol efflux to apoA-1 and high-density lipoprotein (HDL) from VSMCs, respectively, in response to treatment with different concentrations of E2. (C) Lipid droplet accumulation in response to different concentrations of E2 was observed by BODIPY staining: (a) control; (b) E2 10 nM; (c) oxidized low-density lipoprotein (ox-LDL); (d) E2 1 nM + ox-LDL; (e) E2 10 nM + ox-LDL; (f) E2 100 nM + ox-LDL. Scale bar, 50 μm. (D) The effects of E2 on cellular cholesteryl ester content in VSMCs were assayed by an enzymatic fluorometric method. The data represent the mean ± standard error of the mean (SEM). *P<0.05 and **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the ox-LDL-treated alone group. Each experiment was performed three or four times. E2, 17β-estradiol.

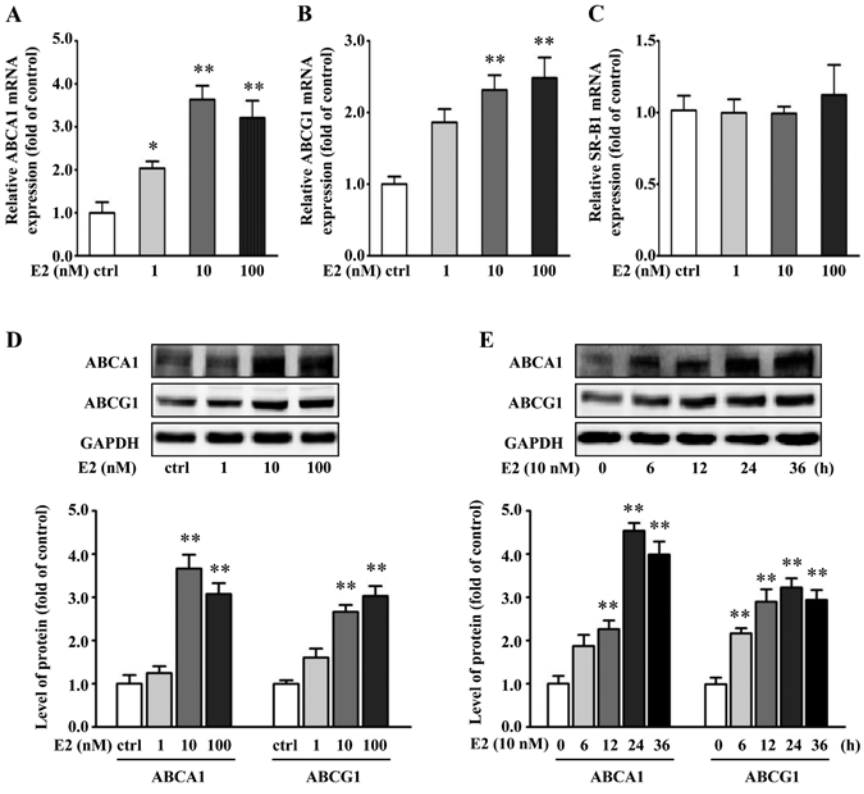

Figure 2. Effects of E2 on the expression of ATP-binding cassette transporters ABCA1 and ABCG1 and scavenger receptor B1 (SR-B1) in vascular smooth muscle cells (VSMCs). VSMCs were treated with different concentrations of E2 for 24 h. (A-C) The mRNA expression of ABCA1, ABCG1 and SR-B1 was determined by real-time RT-PCR. (D) The protein expression of ABCA1 and ABCG1 was determined by western blot analysis. (E) VSMCs were treated with E2 (10 nM) for different exposure times, and the protein expression of ABCA1 and ABCG1 was determined by western blot analysis. The data represent the mean ± standard error of the mean (SEM). *P<0.05 and **P<0.01 vs. the control group. Each experiment was performed three times. E2, 17β-estradiol.

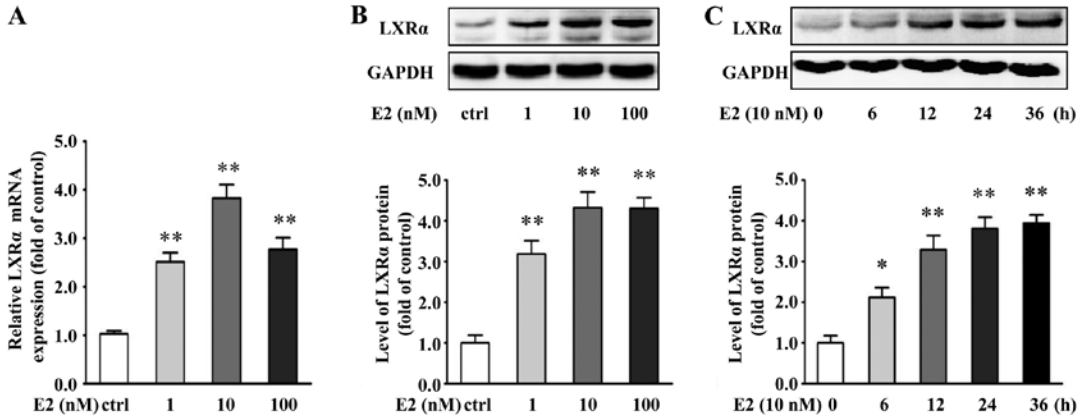

Figure 3. Effects of E2 on LXRα expression in vascular smooth muscle cells (VSMCs). (A and B) VSMCs were incubated with different concentrations of E2 for 24 h. The mRNA and protein expression of LXRα was determined by real-time polymerase chain reaction (PCR) and western blot analysis, respectively. (C) VSMCs were treated with E2 (10 nM) for different time periods. The protein levels of LXRα were determined by western blot analysis. The data represent the mean ± standard error of the mean (SEM). *P<0.05 and **P<0.01 vs. control group. Each experiment was performed three times. E2, 17β-estradiol.

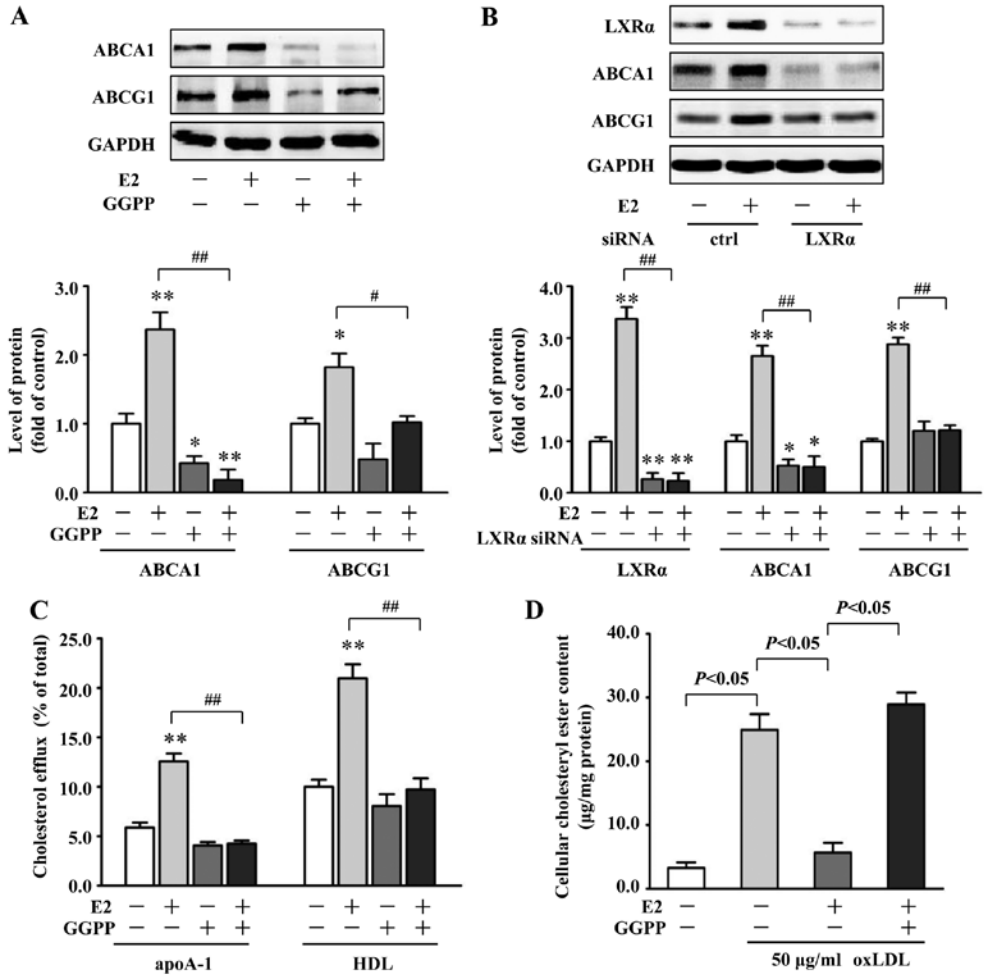

Figure 4. Role of LXRα in E2 activity in vascular smooth muscle cells (VSMCs). (A) VSMCs were pretreated with geranylgeranyl pyrophosphate (GGPP) (20 μM) for 2 h, and then treated with E2 (10 nM) for 24 h. The protein expression of ATP-binding cassette transporters ABCA1 and ABCG1 was determined by western blot analysis. (B) VSMCs were transfected with either control or LXRα siRNA (100 nM) for 24 h, followed by E2 (10 nM) for an additional 24 h. LXRα, ABCA1 and ABCG1 protein expression was determined by western blot analysis. (C and D) Prior to treatment with E2 (10 nM), VSMCs were treated with GGPP for 2 h. Cholesterol efflux and intracellular cholesteryl ester content were assessed. The data represent the mean ± standard error of the mean (SEM). *P<0.05 and **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the E2-treated alone group. Each experiment was performed three or four times. E2, 17β-estradiol.

E2 induces ABCA1 and ABCG1 expression and cholesterol efflux from VSMCs through an LXRα-dependent pathway. To address whether LXRα is involved in the E2-induced expression of ABCA1 and ABCG1, we determined the mRNA and

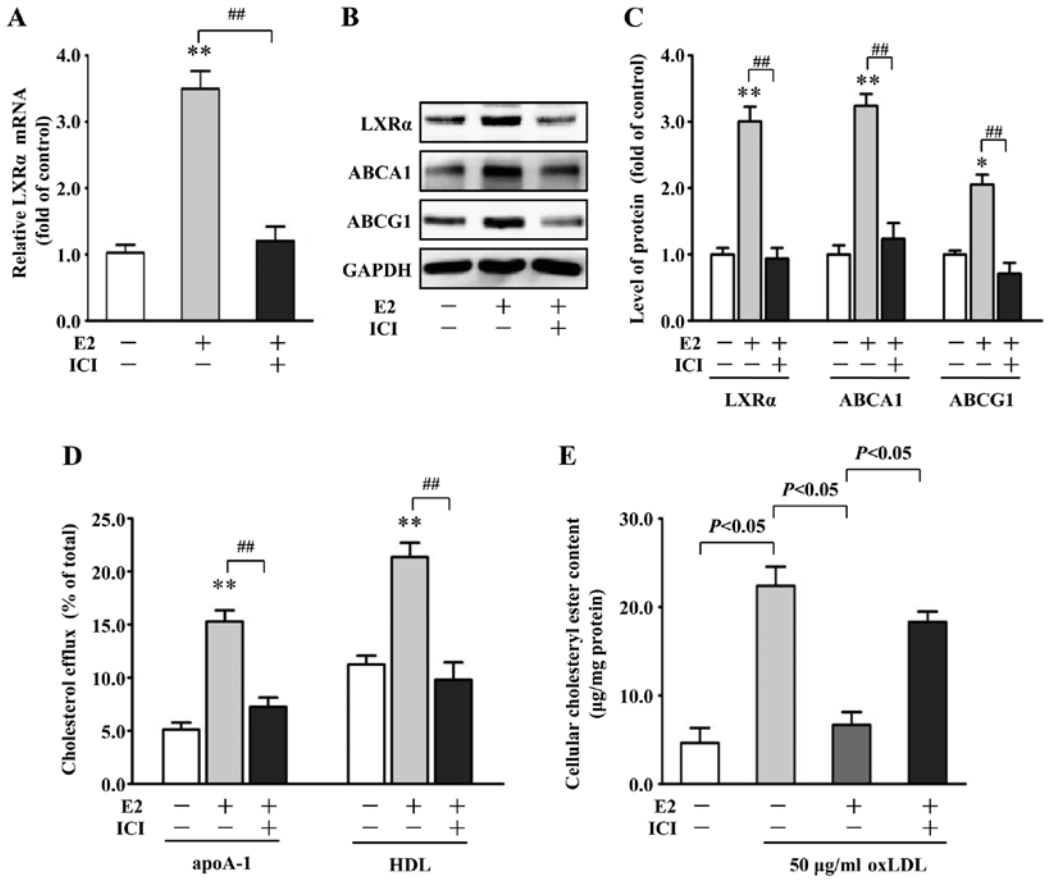

Figure 5. Role of the estrogen receptor (ER) in E2 activity in vascular smooth muscle cells (VSMCs). VSMCs were pre-incubated with the non-selective receptor antagonist ICI 182,780 (1 μM) 30 min prior to stimulation with E2 (10 nM). (A) LXRα mRNA expression was determined by real-time polymerase chain reaction (PCR). (B and C) The expression of LXRα, ATP-binding cassette transporters ABCA1 and ABCG1 was determined by western blot analysis. (D and E) Prior to treatment by E2 (10 nM), the VSMCs were treated with ICI 182,780 for 30 min. Cholesterol efflux and intracellular cholesteryl ester content were assessed. The data represent the mean ± standard error of the mean (SEM). *P<0.05 and **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the E2-treated alone group. Each experiment was performed three or four times. E2, 17β-estradiol.

protein levels of LXRα in the E2-treated VSMCs. E2 significantly increased the mRNA and protein expression of LXRα (Fig. 3A and B). At 10 nM of E2, LXRα mRNA and protein levels had an increase of 282 and 333%, respectively, compared with the controls (P<0.01). Furthermore, E2 (10 nM) upregulated the protein levels of LXRα in a time-dependent manner (Fig. 3C).

To study whether the induction of ABCA1 and ABCG1 expression by E2 is through an LXRα-dependent pathway, VSMCs were pretreated with GGPP (20 μM), a pharmacological inhibitor of LXRα, followed by treatment with E2 (10 nM). Compared with the VSMCs treated with 10 nM E2 alone, coincubation with GGPP significantly decreased ABCA1 and ABCG1 protein expression by 90% (P<0.01) and 44% (P<0.05), respectively (Fig. 4A). Moreover, we investigated the effects of LXRα siRNA on ABCA1 and ABCG1 expression induced by E2. LXRα siRNA reduced the amount of LXRα protein in VSMCs by 73%, compared with the control siRNA (P<0.01) (Fig. 4B). Concomitantly, LXRα siRNA treatment abolished E2-induced upregulation of ABCA1 by 81% and ABCG1 by 58% in VSMCs, compared with the control siRNA treatment (P<0.01) (Fig. 4B). Additionally, inhibition of LXRα activation by GGPP (20 μM) blocked the promotive effects of E2 (10 nM) on cholesterol efflux to apoA-1 and HDL (Fig. 4C) and further abrogated the inhibitory effect of E2 on cholesteryl ester accumulation in VSMCs (Fig. 4D). Together, these results imply the critical role of LXRα in E2-regulated expression of ABCA1 and ABCG1 and subsequent changes in cholesterol efflux and cholesteryl ester accumulation in VSMCs.

Estrogen receptor β (ERβ) mediates the stimulatory effects of E2 on LXRα, ABCA1 and ABCG1 expression in VSMCs. To investigate whether upregulation of LXRα by E2 is ER-dependent, we determined LXRα mRNA expression after pre-incubation with the nonselective ER antagonist ICI 182,780 (1 μM). ICI 182,780 effectively abolished the E2-induced increase in LXRα mRNA expression by 71% in VSMCs, compared with the E2 alone group (P<0.01) (Fig. 5A). Moreover, in the presence of ICI 182,780, the increased protein expression of LXRα, ABCA1 and ABCG1 by E2 (10 nM) was abrogated (Fig. 5B and C). E2-induced promotion of apoA-1- and HDL-mediated cholesterol efflux and reduction of cholesteryl ester content in the VSMCs were also effectively blocked by ICI 182,780 (Fig. 5D and E).

To more explicitly decipher the ER-subtype involved in the E2-mediated upregulation of LXRα, ABCA1 and ABCG1, we evaluated the blocking effects of a selective ERα antagonist MPP and a selective ERβ antagonist PHTPP on the E2-induced upregulation of LXRα, ABCA1 and ABCG1 expression. Pretreatment with PHTPP (1 μM), but not MPP

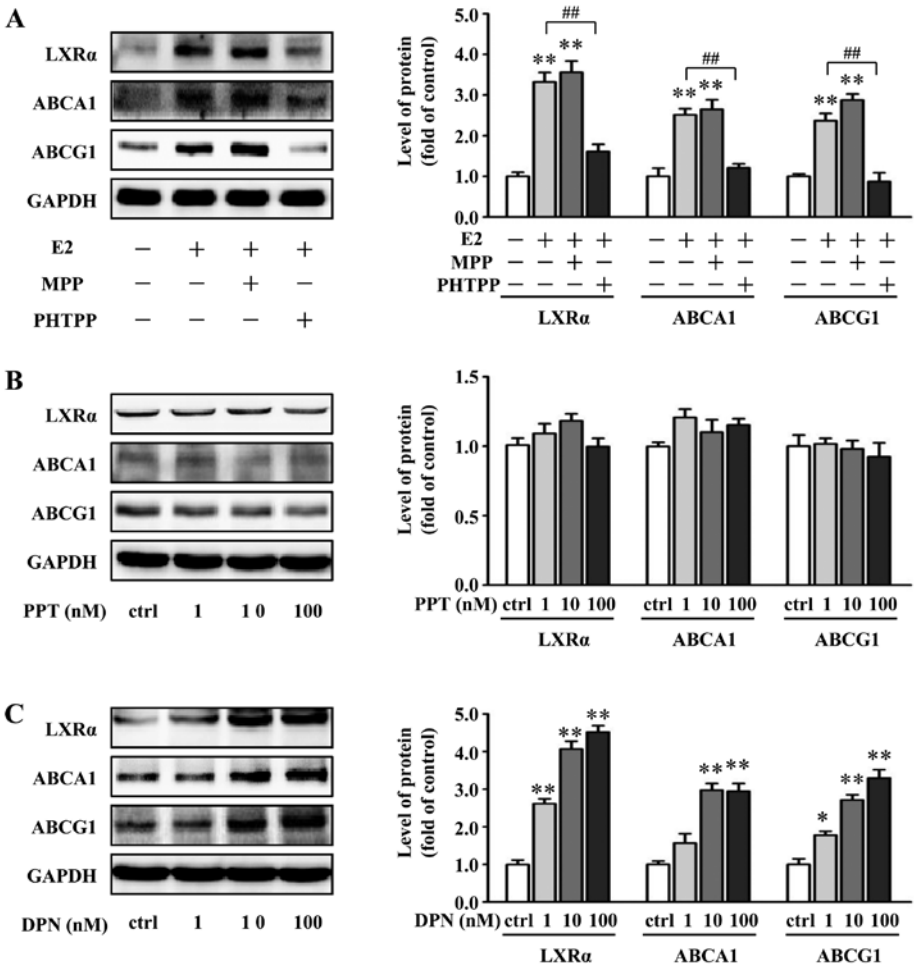

Figure 6. Role of the estrogen receptor (ER)β on E2 induced upregulation of the protein expression of LXRα and ATP-binding cassette transporters ABCA1 and ABCG1. (A) VSMCs were treated with MPP (1 μM) or PHTPP (1 μM) in the presence of E2 (10 nM) for 24 h. The protein expression of LXRα, ABCA1 and ABCG1 was determined by western blot analysis. (B and C) VSMCs were treated with different concentrations of PPT or DPN for 24 h. The protein expression of LXRα, ABCA1 and ABCG1 was determined by western blot analysis. The data represent the mean ± standard error of the mean (SEM). *P<0.05 and **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the E2-treated alone group. Each experiment was performed three times. E2, 17β-estradiol.

(1 μM), completely inhibited the E2-induced responses in the VSMCs (Fig. 6A). Moreover, ERα-specific agonist PPT and ERβ-specific agonist DPN were used to further delineate the role of specific ERs. Importantly, DPN dose-dependently increased LXRα, ABCA1 and ABCG1 protein expression in the VSMCs, while stimulation with PPT did not alter the protein expression of LXRα, ABCA1 and ABCG1 (Fig. 6B and C). These results indicate that upregulation of LXRα, ABCA1 and ABCG1 by E2 is likely mediated by ERβ.

Discussion

The formation of foam cells within the arterial wall is thought to play a pivotal role in the development of atherosclerotic lesions (24). In the last decade, several studies have shown that in addition to macrophages, VSMCs accumulate excess intracellular cholesteryl (25,26) and give rise to a significant number of foam cells in atherosclerotic lesions (14,27). Moreover, VSMC-derived foam cells *in vitro* lose the expression of VSMC contractile markers, transform into macrophage-like cells (15) and express monocyte chemoattractant protein-1 (MCP-1) (16,28). These data suggest that lipid accumulation in VSMCs may contribute to the development of atherosclerosis. Previous research has focused on the anti-proliferative and anti-migratory properties of estrogen on VSMCs (29). Here, we elucidated a novel atheroprotective effect of estrogen that E2 promotes cholesterol efflux from VSMCs and suppresses VSMC-derived foam cell formation.

Accumulation of cholesteryl ester stored as cytoplasmic lipid droplets is the main characteristic of foam cells derived from both macrophages and VSMCs (15,30). The removal of excess cholesterol from macrophages by apoA-1 and HDL is thought to play an important role in preventing foam cell formation (31). Unlike macrophages, little is known regarding the regulation of cholesterol efflux and lipid accumulation in VSMCs. Our data showed that treatment with E2 at physiological concentrations promoted cholesterol efflux to both apoA-1 and HDL from VSMCs and attenuated intracellular cholesteryl ester accumulation in VSMCs. This suggests that reduced lipid accumulation in VSMCs by E2 is, at least in part, due to an increase in cholesterol efflux from VSMCs.

Cholesterol removal from macrophages is mediated by ABC transporters and SR-B1. ABCA1 and ABCG1 have been demonstrated to primarily promote cellular cholesterol efflux to apoA-1 and HDL, respectively (8). In the present study, we showed that E2 treatment significantly augmented both

mRNA and protein expression of ABCA1 and ABCG1 without an alteration in SR-B1 mRNA expression in VSMCs. These data indicate that E2 increases cholesterol efflux through the upregulation of ABCA1 and ABCG1 in VSMCs.

LXRα, which plays a pivotal role in maintaining macrophage cholesterol homeostasis, is the main regulator of ABCA1 and ABCG1 gene transcription (32,33). We, therefore, examined whether LXRα is involved in the upregulation of ABCA1 and ABCG1 by E2 in VSMCs. Notably, E2 increased LXRα expression in VSMCs. Moreover, inhibition of LXRα activation by either GGPP or LXRα siRNA diminished the E2-mediated ABCA1 and ABCG1 induction. GGPP also blocked E2-induced cholesterol efflux and abrogated the inhibitory effect of E2 on cholesteryl ester accumulation in VSMCs. These results demonstrated the essential role of LXRα in E2-regulated gene expression of ABCA1 and ABCG1 and promotion of cholesterol efflux from VSMCs.

Although a number of findings regarding the gene expression and cholesterol efflux of E2 action on human macrophages have been previously reported, these data are controversial. Corcoran *et al* (34) reported that E2 induced a modest reduction in ABCG1 and did not affect cholesterol efflux and ABCA1 levels in human monocyte-derived macrophages (HMDMs). Cerda *et al* (35) reported that hormone therapy reduced the mRNA levels of ABCA1, without alteration in ABCG1 and LXRα in peripheral blood mononuclear cells (PBMCs). Kramer and Wray (36) observed that LXRα expression was significantly increased after estrogen withdrawal in human macrophages. However, in a mouse study, Ribas *et al* (5) reported that ABCA1 protein expression was reduced in both bone marrow-macrophages from ERα$^{-/-}$ mice and peritoneal macrophages from mice with a myeloid-specific ERα deletion. In addition, Srivastava (37) reported that estrogen treatment significantly increased hepatic and intestinal ABCA1 mRNA levels in mice. Moreover, a previous study by our group also demonstrated that E2 increased ABCA1 levels both in atherosclerotic lesions in ApoE$^{-/-}$ mice and in murine Raw 264.7 macrophages (38). Together with our present results in VSMCs, these findings suggest species differences in the regulation of cholesterol efflux and related gene expression between the human and the mouse and may explain why an atheroprotective benefit of estrogen is strongly supported by research using animals, particularly mouse models of atherosclerosis, but cannot be established in humans participating in randomized controlled trials of estrogen replacement treatment.

Estrogen exerts most of its biological action via estrogen receptors (ERs), which are prominent members of the nuclear receptor superfamily (39). Pre-incubation with ICI 182,780 to antagonize ERs blocked the E2-mediated upregulation of LXRα, ABCA1 and ABCG1 expression and attenuation of cholesteryl ester accumulation in VSMCs, indicating ER dependence. There are two different isforms of ERs, ERα and ERβ, which are expressed in endothelial cells, VSMCs and macrophages (29). Previous research found that ERα may be the major mediator of the atheroprotective effects of estrogen (40). However, recent evidence suggests that ERα may not be involved in estrogen-mediated protection from early lesion development (41). Moreover, Rayner *et al* (42) reported that E2 via ERβ induced an extracellular release of HSP 27 in macrophages and prevented the development of atherosclerosis in apoE$^{-/-}$ mice. In addition, Xing *et al* (43) showed that estrogen modulated TNF-α-induced inflammatory responses in rat VSMCs through ERβ activation. In the present study, treatment with specific antagonists and agonists for ERα and ERβ clearly showed that upregulation of LXRα, ABCA1 and ABCG1 by E2 was ERβ-dependent, suggesting a novel mechanism for the regulation of atherosclerosis specifically through ERβ.

In conclusion, our results indicate that in mouse VSMCs, E2 promotes apoA-1- and HDL-mediated cholesterol efflux from VSMCs and prevents VSMC-derived foam cell formation via upregulation of ABCA1 and ABCG1 expression, which is mediated by ERβ and LXRα activation. These findings provide novel insight into the anti-atherogenic properties of estrogen and to some extent, may explain the complexity of the cardiovascular effects of estrogen in the human and mouse.

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