Estrogen prevent atherosclerosis by attenuating endothelial cell pyroptosis via activation of estrogen receptor α-mediated autophagy

Qinghai Menga,1, Yu Lic,1, Tingting Ji a, Ying Chao a, Jun Li a, Yu Fu a, Suyun Wang a, Qi Chen a, Wen Chen e, Fuhua Huang e, Youran Wang e, Qichun Zhang a,b, Xiaoliang Wanged, Wen Chen e, Huimin Bian a,b,*

a School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China
b Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China

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Excessive inflammation and the pyroptosis of vascular endothelial cells caused by estrogen deficiency is one cause of atherosclerosis in post-menopausal women. Because autophagy is highly regulated by estrogen, we hypothesized that estrogen can reduce vascular endothelial cell pyroptosis through estrogen receptor alpha (ERα)-mediated activation of autophagy to improve atherosclerosis in post-menopausal stage. Aortic samples from pro-menopausal and post-menopausal women with ascending aortic atherosclerosis were analyzed, and bilateral ovariectomized (OVX) female ApoE-/- mice and homocysteine (Hcy)-treated HUVECs were used to analyze the effect of estrogen supplementation therapy. The aortic endothelium showed a decrease in ERα expression and autophagy, but presented an increase in inflammation and pyroptosis in female post-menopausal patients. Estrogen treatment accelerated autophagy and ameliorated cell pyroptosis in the cardiac aortas of OVX ApoE-/- mice and Hcy-treated HUVECs.

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Introduction

Compared to young women, the role of estrogen in protecting the cardiovascular system in women during menopause is significantly diminished, leading to a sharp increase in the risk of atherosclerosis [1,2]. The abnormal depressed expression of estrogen receptors (ERs) in women along with the reduced estrogen level is one factor of the pathological basis of atherosclerosis in post-menopausal stage [3].

Studies have shown that estrogen can inhibit cardiomyocyte apoptosis and reduce cell damage via ERs to regulating autophagy [4–6]. A review article summarized the effect of estrogen in autophagy and extensively summarized the function of ERs and autophagy in breast, ovary, kidney, and lung, tissue as well as the nervous system [7]. There is an increasing evidence showing that autophagy occurs in atherosclerotic plaques [8]. Some typical features of autophagy can be observed in atherosclerosis-associated cells, including vascular smooth muscle cells, macrophages or endothelial cells [9–11]. However, whether estrogen supplement can prevent atherosclerosis during the post-menopausal period by regulating autophagy through ERs in vascular endothelial cells has not been studied.

Atherosclerosis is an inflammatory disease accompanied by endothelial dysfunction [12,13]. Interestingly, pyroptosis has been reported to take part in the accretion of atherosclerotic plaque [14]. The important functional molecules involved in pyroptosis, including NLRP3, ASC and caspase-1, are associated with the emergence and accretion of atherosclerotic plaque [15,16]. Unlike apoptosis, pyroptosis is a response to pathogen-associated molecular patterns (PAMPs) that result in programmed cell death following cytokine release [17]. NLRP3 inflammasome induced endothelial cell pyroptosis accompanied by aortic atherosclerosis was shown to be aggravated in mice with hyperlipidemia [15,18,19]. In another study, ovariectomy was observed to promote the expression of Il-1β, IL-18, and NLRP3, and an increased active of caspase-1 in the hippocampus of female mice [20]. E2 and estrogen receptor agonists could reverse the expression of NLRP3, IL-1β, and IL-18. However, the dysfunction accused by pyroptosis in atherosclerosis during post-menopausal period remains to be developed.

Studies have certified that autophagy regulates the activation of inflammatory [21,22]. After knockdown of autophagy regulates genes, such as ATG16L1 or ATG7, increased NLRP3-mediated inflammation induced by lipopolysaccharide (LPS), suggesting that autophagy is likely involved in inhibiting NLRP3 activation [23]. During inflammation, microglia or macrophages can activate caspase-1 mediated release of inflammatory factors such as IL-1β and IL-18, while the administration of autophagy agonists could inhibit the release of inflammatory factors and the expression of caspase-1 [24]. The results of the above studies prompted us to consider whether estrogen can regulate autophagy through ERs and exert an inhibitory effect on the pyroptosis of vascular endothelial cells to against atherosclerosis during post-menopausal period. In the present study, we analyzed aortic samples from pro-menopausal and post-menopausal women with ascending aortic arteriosclerosis, used estrogen to treat high-fat diet fed bilateral ovariectomized (OVX) female ApoE-/- mice, and used estrogen to treat homocysteine (Hcy)-treated HUVECs to investigate whether estrogen can reduce vascular endothelial cell pyroptosis through estrogen receptor mediated autophagy activation to improve atherosclerosis during menopause.

Materials and methods

Reagents

Estradiol (E2758, purity ≥ 98%), MPP dihydrochloride hydrate (M7068, purity ≥ 97%), HPTPP (SML1355, purity ≥ 98%) and 3-Methyladenine (3-MA, M9281, purity ≥ 99%) were purchased from Sigma (Saint Louis, USA). Antibody for NLRP3 (19771-1-AP) and IL-18 (10663-1-AP) were purchased from Proteintech (Wuhan, China). Antibody for caspase-1 (48847) was purchased from SAB signalway antibody (Maryland, USA). Antibody for GSDMD (A18281) and IL-1β (A16288) were purchased from ABclonal (Wuhan, China). Antibody for Beclin 1 (ab210498), LC3B (ab192890), SQSTM1 (ab56416), Hcy (ab15154), estrogen receptor alpha (ERα) (ab32063), estrogen receptor beta (ERβ) (ab3576), GSDMD (ab219800), and β-actin (ab8226) were purchased from Abcam (Cambridge, UK). FITC - goat Anti-IgG (ab6785), TRITC - goat anti-rabbit IgG (BS10250), FITC - goat anti-rabbit IgG (BS10590), HRP - goat anti-rabbit IgG (BS13278), and HRP - goat anti-mouse IgG (BS12478) were purchased from BioWorld (Bloomington, USA). The prestained protein MW marker (26617) were purchased from Thermo scientific. Control siRNA and ERα siRNA (sc-29305) were purchased from Santa Cruz (Santa Cruz, USA).

Human subjects

From May 2019 to November 2019, female ascending aorta samples were collected from 24 subjects who underwent ascending artery replacement surgery. All surgeries and sample collection were conducted at Nanjing First Hospital. The screening criteria were as follows: female, ≥ 30 years old, ascending aortic aneurysm accompanied by aortic atherosclerosis, and the need for ascending aortic replacement surgery. Ascending aorta samples were histologically assessed by an experienced pathologist for diagnosis. Informed consent was obtained from all patients. The patients were divided into groups based on the age and whether in post-menopausal stage, 12 pro-menopausal female patients aged 40–48 years old in one group, and 12 post-menopausal female patients aged 58–65 years old in another group.

Animal experimental design

Female ApoE-/- mice weighing 20 ± 2 g (8 weeks old) were purchased from Beijing HFK Bioscience Co, Ltd. The mice were raised...
in the animal experimental center (SPF level) of Nanjing University of Traditional Chinese Medicine.

All female ApoE-/- mice were anesthetized with 3% pentobarbital sodium (40 mg/kg, SCRC 69020180, Sinopharm chemical reagent, Shanghai, China). Then, a cut was made in the skin along the sides of the leg on the backs of the mice to expose the ovaries. Then, a small amount of fat around the ovaries was removed, and the wounds were sutured. These female ApoE-/- mice were used as the sham group. After a week of being fed a normal diet (1022, Beijing HK Bioscience Co., Ltd., Beijing, China), they were switched to a high-fat diet (Beijing HK Bioscience Co., Ltd., Beijing, China) containing 0.3% cholesterol and 20% pork fat.

The post-menopausal models used in this study were female ApoE-/- mice that underwent a bilateral ovariectomy and were fed a high-fat diet. The female ApoE-/- mice were anesthetized with 3% pentobarbital sodium (40 mg/kg). Then, a cut was made in the skin along the sides of the leg on the back of the mice to expose the ovaries. A nylon thread was used to ligature the uterus near the ovaries. The bilateral ovaries were cut off, after which the wounds of the mice were sutured. Vaginal smears of the mice were observed to determine whether the ovaries were completely removed. The lack of an estrus cycle (many white blood cells continuously observed), demonstrated that the ovaries were completely removed. After a week of being fed a normal diet, the bilateral ovarietomized ApoE-/- mice were switched to being fed a high-fat diet [25–27].

Bilaterally ovarietomized ApoE-/- mice were randomly placed into two groups: the model group (n = 6) and the bilaterally ovariectomized ApoE-/- mouse group (n = 6) and the bilaterally ovariectomized ApoE-/- mouse group were provided an equal volume of water. Mice were cally treated once a day for 90 days. The ApoE-/- mouse sham group were provided an equal volume of water. Mice were cally treated once a day for 90 days. The ApoE-/- mouse sham group were provided an equal volume of water. Mice were cally treated once a day for 90 days. The ApoE-/- mouse sham group were provided an equal volume of water. Mice were cally treated once a day for 90 days. The ApoE-/- mouse sham group were provided an equal volume of water. Mice were

Transfection of siRNA oligonucleotides

As described in our previous study [27], and following the manufacturer's instructions, siRNA oligonucleotide transfection was performed as follows. To prepare the transfection solution, 6 μL of control siRNA or ERα siRNA was added into 100 μL transfection medium. Then, the transfection working solution was obtained by mixing the transfection reagent and transfection solution. After the HUVECs were washed with transfection medium, they were covered with transfection working solution and incubated for 5 h. Subsequently, growth medium was added, and the HUVECs were incubated for 24 h. Finally, the supernatant was discarded, serum-free DMEM was added, and the HUVECs were incubated for 12 h to allow for gene silencing.

Transfection of adenovirus expressing GFP-LC3B

HUVECs (5 × 10⁵ cells/well) were added to a 6-well plate containing cell slides. After 12 h, the 6-well plate was removed to confirm that the cells had grown well. Then, the medium was discarded, 1.2 mL of fresh fetal bovine serum free medium was added to each well, and a viral solution at a MOI value of 10 was added into the plate, and wells with no virus were used as a control group. Approximately 24 h after infection, the virus-containing medium was removed, and 2 mL of 10% fetal bovine serum medium was added to each well. After 24 h of cultivation, the HUVECs were incubated in the appropriate reagents for a specific amount of time. Next, the HUVECs were fixed with 4% paraformaldehyde, and then cells were stained with a 50 μL of DAPI staining solution (62248, Thermofisher, Waltham, USA) in the dark for 5 min. The HUVECs were then washed with PBS three times, and the slides with HUVECs were prepared and covered inward onto the microslides. The changes in LC3B fluorescence were observed under a fluorescence microscope (Observer.Z1, Zeiss, Germany) according to previous reports [28,29].

Serum lipid detection

The mice were fasted and free for water for 12 h before collecting from the eyelids of the mice to tubes containing 4% sodium citrate (R23210, Shanghai Yuanye Biological Technology, Shanghai, China). The tubes were centrifuged at 3000 rpm/min for 15 min to collect the plasma. The TC, TG, HDL-c and LDL-c levels in the plasma were determined using a total cholesterol determination kit (A111-1-1, Nanjing Jiaocheng Bioengineering Institute, Nanjing, China), a triglyceride assay kit (A110-1-1, Nanjing Jiaocheng Bio- engineering Institute, Nanjing, China), a high-density lipoprotein assay kit (A112-1-1, Nanjing Jiaocheng Bioengineering Institute, Nanjing, China), and a low-density lipoprotein assay kit (A113-1- 1, Nanjing Jiaocheng Bioengineering Institute, Nanjing, China), respectively, following the manufacturer's instructions. The absorbance (OD) value was measured using a microplate reader (XSS-02, BioTek, America), and lipid levels were calculated following the manufacturer's instructions.
Enzyme-linked immunosorbent assay

Blood samples were centrifuged at 3000 rpm/min for 15 min to collect the serum. Then, serum levels of IL-1β, IL-18 and Hcy were determined using an interleukin 1β (IL-1β) assay kit (H002, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), an interleukin 18 (IL-18) assay kit (H015, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), a homocysteine (Hcy) assay kit (E031-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer’s instructions. In these assays, the standard substance was dissolved in deionized water as a standard stock solution, which was subsequently diluted to 7 different gradient concentrations. The different concentrations of standards and samples were then added to the antibody-covered microplates, and after incubating for 2 h, the microplates were washed 5 times. The chromogenic substrate was then added to the wells, and after incubating for 30 min in the dark, stop solution was added to terminate the reaction. The absorbance values were then detected using a microplate reader (XSZ-02, BioTek, America). IL-1β, IL-18, and Hcy levels were calculated based on their corresponding standard curves.

Hematoxylin and eosin (HE) staining

The arterial tissues of human females and mice, and the liver tissues of mice were fixed with 4% paraformaldehyde. Then, the samples were dehydrated in a gradient of ethyl alcohol solutions, after which the samples were infiltrated with xylene (1330-20-7, Shanghai Macklin Biochemical Co., Ltd, Shanghai, China) before they were embedded in paraffin (8002-74-2, Shanghai Macklin Biochemical Co., Ltd, Shanghai, China). After slicing (5 μm) the samples using a microtome (RM2245, Leica, Germany), the microslides with tissues were dewaxed and rehydrated. Then, hematoxylin and eosin (15086-94-9, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) staining solutions were used to stain the tissues. The tissues were imaged under an optical microscope (N-MSI-MANTRA, Perkin Elmer, America). Additional details for the procedures mentioned above are described in our previous study [27].

Oil red O staining

The method for aorta oil red O staining was described in our previous study [27]. Briefly, after anesthetizing mice, the aorta connected to the heart was taken and fixed in 4% paraformaldehyde. Subsequently, it was dyed with an oil red O staining solution for 3 h and then soaked in 70% ethanol. After cleaning, 4% paraformaldehyde was used to fix aorta, before being imaged with a digital camera (DMC-GX85, Panasonic, Japan).

For oil red O staining of the liver and aortic root sections from mice, fresh tissues were dehydrated using a sucrose solution (57-50-1, Shanghai Macklin Biochemical Co., Ltd, Shanghai, China). Then, the tissues were embedded with optimal cutting temperature compound (4583, Sakura Finetek Japan Co., Ltd, Tokyo, Japan). A frozen slicer (CM1950, Leica, Germany) was used to slice the samples, and after being washed with PBS, the tissues were stained with oil red O staining solution. Then, 70% isopropyl alcohol (112012, Aladdin, Shanghai, China) was used to wash the slices, which were then sealed with gelatin gelatin (C018, Beyotime Biotechnology, Shanghai, China), observed and imaged under an optical microscope (N-MSI-MANTRA, Perkin Elmer, America).

Flow cytometry

A FAM FLICA Caspase 1 Assay kit (ICT-097, BIO-RAD, California, America) was used to detect the caspase-1 levels in HUVECs following the manufacturer’s instructions, the detection procedure for which was briefly described as follow. DMSO was added to each FLICA vial to generate the 30 × FLICA stock solution. Wash buffer (an isotonic solution for washing cells after exposure to FLICA) was prepared by adding 135 mL of ddH2O to 15 mL 10 × wash buffer. Then, 290 μL of cells was transferred to new tubes, and 10 μL of a 30 × FLICA stock solution was added to the tubes. The cells were then incubated for 2 h in the dark, and were gently resuspended every 20 min. Subsequently, 2 mL of wash buffer was added to each tube and mixed gently, after which the tubes were centrifuged at 200g for 5 min and the supernatant was discarded. The cells were then washed with 1 mL of wash buffer, resuspended in 400 μL of wash buffer, and stained with 2 μL of PI. The samples were analyzed using a flow cytometer (Accuri C6, BD Accuri, America), where the FL-1 channel was used to detect FAM (caspase-1) and the FL-2 channel was used to detect red fluorescence (PI).

Western blot

For each sample, 30 mg of tissue (humans or mouse artery) and 300 μL of lysis buffer (P0013B, Beyotime, Shanghai, China) was added to a tissue grinder to extract the proteins. For protein extraction from HUVECs, after collecting the cells, 50 μL of lysis buffer per 10^6 cells was added to the EP tube. After mixing and incubating on ice for 2 h, the samples were centrifuged, and the protein concentration was measured using a BCA kit (P0012S, Beyotime, Shanghai, China). The 5 × protein loading buffer (P0015L, Beyotime, Shanghai, China) was added to the samples, which were then boiled for 10 min. Different concentrations of SDS-PAGE gels were prepared according to the molecular weight of the target proteins, and the volume of the sample was calculated according to the measured protein concentration. The samples were added to the sample wells of SDS-PAGE gels for electrophoresis, after which the proteins were transferred to PVDF membranes (IPV/H00010, Fcmacs, USA). Subsequently, the membranes were blocked in 5% BSA (9048-46-8, Biosharp, Heifei, China) for 1 h. After washing the membranes three times with PBS, the membranes were incubated with different antibodies at 4 °C overnight, including antibodies against NLRP3, caspase-1, GSDMD, IL-1β, IL-18 and Hcy were calculated based on their corresponding standard curves.

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Immunofluorescence staining

The serial sections of human arteries were subjected to antigen retrieval after hydration using sodium citrate solution. After being washed with PBS, the sections were incubated with 5% BSA for 30 min, and then incubated with antibodies against Beclin 1, LC3B, SQSTM1, NLRP3, caspase-1, GSDMD, IL-1β, IL-18, Hcy, ERα, or ERβ overnight. After washing, the sections were incubated with TRITC - goat anti-rabbit IgG or FITC - goat anti-rabbit IgG for 2 h. After washing the sections 3 times with PBS, 50 μL of DAPI solution was added to dye nucleus for 5 min. After washing the sections 3 times with PBS, they were covered using a glycerin- tablet (C018, Beyotime Biotechnology, Shanghai, China). Then, the tissues were imaged under a fluorescence microscope (Observer.Z1, Zeiss, Germany).

HUVECs (5 × 10^5 cells/well) were added to a 6-well plate containing cell slides, incubated for 12 h, and then treated with control siRNA or ERα siRNA and different reagents. Subsequently, the cells
were fixed with 4% paraformaldehyde, washed 3 times with PBS, and then incubated with antibodies against caspase-1, ERα or/and GSDMD overnight. After washing and incubating the cells with goat Anti-mouse IgG (H + L) (FITC), goat anti-rabbit IgG (H + L) - FITC, or/and goat anti-rabbit IgG (H + L)-TRITC for 2 h, 50 μL of DPAI solution was added to dye nucleus for 5 min. Subsequently, after washing the cells 3 times with PBS, a glycercin- tablet was used to cover the samples. The HUVECs were imaged under a fluorescence microscope (Observer.Z1, Zeiss, Germany).

**Transmission electron microscopy (TEM)**

TEM (H-7650 TEM system, Hitachi Limited, Japan) was used to detect autophagosomes and autophagic lysosomes in the endarterial samples of female humans and mice, and in HUVECs as described in a previous study [30]. Briefly, fresh samples of human artery, mouse artery, and HUVECs were fixed overnight at 4 °C with 2.5% glutaraldehyde (g105905, Aladdin, Shanghai, China)-phosphate buffer (pH 7.2). After being fixed with 1% osmium tetroxide for 4 h, and then washed with PBS, the samples were dehydrated with gradient series of ethanol solutions. Then, the samples were embedded in epoxy resin and sliced with an ultramicrotome. Then, the samples were sprayed with gold to make them electrically conductive, placed on the sample platform, and imaged using a transmission electron microscope.

**Statistical analyses**

The measurement data are expressed as the means ± standard deviation (SD). The comparisons between 2 groups were analyzed by two-tailed Student's t-test, and data from 2 groups were analyzed using one-way analysis of variance (ANOVA) Bonferroni post hoc. Statistical analyses were performed using GraphPad PRISM 7.0 software (GraphPad Software, La Jolla, USA). Differences with a P value < 0.05 were considered significant.

**Results**

**Female post-menopausal patients present reduced estrogen receptor levels and autophagy and increased NLRP3 inflammasome and pyroptosis in the ascending aorta**

Histological evaluation of the pro-menopausal and post-menopausal female ascending aortas was performed to determine the damage of the blood vessels. The HE staining results showed a significantly thinner endothelium and disorganized vascular smooth muscle cells present in the ascending aorta of post-menopausal females than that of pro-menopausal females (Fig. 1A). Compared with pro-menopausal women, the TEM results revealed that the ascending aortas of post-menopausal women harbored fewer autophagosomes (Fig. 1B). The expression of the autophagy-related factors Beclin 1 and LC3B were consistently decreased, while that of SQSTM1 was increased (Fig. S1A and S1D) in both the endothelium and vascular smooth muscle cells of ascending aortas of post-menopausal female compared to those from pro-menopausal female. The steroid hormone E2 is the primary estrogen produced by female ovaries [31] and works to regulate gene transcription via estrogen receptors, including ERα and ERβ [32]. Our results showed that ERα and ERβ expression was decreased in the vascular smooth muscle cells of the ascending aortas of post-menopausal females, whereas in the endothelium of the ascending aortas, only ERα expression was displayed significantly decreased (Fig. S1B and S1D). In addition, aortic samples from post-menopausal women exhibited increased expression of NLRP3, cleaved caspase 1, and GSDMD (Fig. S1C and S1D). Increased expression of NLRP3 is a marker of enhanced inflammation [33], and promotes more IL-1β and IL-18 secretion (Fig. 1C and 1D). We also observed that the level of homocysteine (Hcy), an independent risk factor for early stage atherosclerosis [34,35], was increased in the ascending aorta of post-menopausal female patients (Fig. 1C and 1D).

**Estrogen supplementation ameliorates pyroptosis and inflammation in cardiac aorta of OVX ApoE-/- mice and Hcy-treated HUVECs**

As female post-menopausal patients presented reduced estrogen receptor levels and autophagy accompanied by increased NLRP3 inflamasome and pyroptosis, we speculated that estrogen supplementation may ameliorate these changes. Therefore, we studied the therapeutic effects of estrogen on OVX ApoE-/- mice fed a high-fat diet and on Hcy-treated HUVECs. First, we assessed the effect of estrogen on the pyroptosis of endothelialocyte in the cardiac aortas of OVX ApoE-/- mice and in Hcy-treated HUVECs. The bilateral ovariectomy in ApoE-/- mice resulted in increased NLRP3, cleaved caspase 1, and GSDMD expressions. Estradiol supplementation (0.13 mg/kg) of OVX ApoE-/- mice resulted in a significant decreasing in NLRP3, cleaved caspase 1, and GSDMD protein expressions (Fig. 2A). The changes in the levels of IL-1, IL-18 and Hcy in plasma showed the same tendency in the OVX ApoE-/- mice and in those with estrogen supplementation (Fig. 2B-D). We used Hcy to induce injury of HUVECs in vitro. HUVECs treated with different concentrations (40, 60, 80, and 100 nM) of Hcy or 60 nM Hcy for different amounts of time (0, 3, 6, 12, 24, and 48 h) resulted in different degrees of cell damage (Fig. S2A-C). Estradiol (10 nM) treatment had a protective effect on cell viability (Fig. S2D) and suppressed pyroptosis (Fig. S2E) in HUVECs treated with 60 nM Hcy for 12 h. HUVECs incubated with 60 nM Hcy for 12 h showed upregulated NLRP3, cleaved caspase 1, and GSDMD protein expression and increased IL-1 and IL-18 secretion, whereas the addition of estradiol (10 nM) suppressed the expression and secretion of these proteins (Fig. 2E-F).

**Estrogen supplementation accelerates autophagy in the cardiac aortas of OVX ApoE-/- mice and Hcy-treated HUVECs**

Autophagy is involved in endothelial cell damage and the progression of atherosclerosis [36], and is significantly regulated by estrogen [7]. To verify whether the prevention of pyroptosis induced by estrogen in endothelial cells is associated with autophagy, we used OVX ApoE-/- mice and Hcy-treated HUVECs to assess the effect of estrogen intervention on autophagy signals. Compared with ApoE-/- mice, autophagy signals in the aortas of OVX ApoE-/- mice were significantly inhibited, presenting decreased LC3 and Beclin1 expression and an accumulation of SQSTM1. After 90 days of estradiol supplementation (0.13 mg/kg), the OVX ApoE-/- mice showed increased expression of LC3 and Beclin1 and decreased expression of SQSTM1 in the cardiac aorta (Fig. 3A). Hcy not only caused pyroptosis of endothelial cells [37], but also the inhibited autophagy signalling in HUVECs (Fig. 3B). When HUVECs were treated with 10 nM estradiol, the inhibitory effects of Hcy on LC3 and Beclin1 expression and on the accumulation of SQSTM1 were suppressed (Fig. 3B). Furthermore, we used a GFP-LC3B adenovirus to transfect HUVECs to confirm that LC3B was inhibited by Hcy and improved by estrogen supplementation (Fig. 3C).
Fig. 1. Female post-menopausal patients present reduced estrogen receptor levels and autophagy and increased NLRP3 inflammasome and pyroptosis in the ascending aorta. (A) Representative histological images of HE staining in ascending aorta in pre-menopausal women and post-menopausal women. (B) Representative transmission electron microscopy images of ascending aorta in pre-menopausal women and post-menopausal women. The boxed area is showed by a higher magnification. Arrow in red, autophagosome. (C) Representative immunohistochemical staining images of IL-1, IL-18 and Hcy in ascending aorta in pre-menopausal women and post-menopausal women. Up boxed area showed a higher magnification of the intima and the down boxed area showed a higher magnification of the media. (D) Related to C, the intensity analysis of IL-1, IL-18 and Hcy in the endothelium (left panel) and vascular smooth muscle cells (right panel) of ascending aorta in pre-menopausal women and post-menopausal women. In all experiments, n = 12. *P < 0.05, **P < 0.01.
Estrogen supplementation ameliorates atherosclerosis in post-menopausal mice via autophagy induction

We next assessed the protective effects of estrogen on atherosclerosis in post-menopausal mice. The results showed that estradiol (10 nM) significantly attenuated the plaque area (Fig. 4B-C) in the aorta, decreased the levels of TC, TG and LDL-c and increased that of HDL-c in the plasma of OVX ApoE-/- mice (Fig. 4D). TEM results revealed that OVX ApoE-/- mice had fewer autophagosomes in cells of the cardiac aorta than ApoE-/- mice. After estrogen supplementation with 10 nM estradiol, the number of autophagosome in the cardiac aorta in OVX ApoE-/- mice decreased.

Fig. 2. Estrogen supplementation ameliorates pyroptosis and inflammation in cardiac aorta of OVX ApoE-/- mice and Hcy-treated HUVECs. (A) Representative western blots and relative quantitative analysis of NLRP3, cleaved caspase 1, and GSDMD in cardiac aorta of three groups of mice. (B) Levels of IL-1β in plasma of three groups of mice. (C) Levels of IL-18 in the plasma of three groups of mice. (D) Levels of Hcy in plasma of three groups of mice. (E) Representative western blots and relative quantitative analysis of NLRP3, cleaved caspase 1, and GSDMD in HUVECs cells. (F) Representative western blots and relative quantitative analysis of IL-1 and IL-18 in HUVECs cells. In all experiments, n = 6. **P < 0.01.
increased (Fig. 4A). In addition, we observed changes in body weight and lipid accumulation in the livers of mice after 90 days of estradiol treatment. The body weights of the mice in the three groups showed no significant differences (Fig. S3A). The HE and oil red O staining of the livers showed that after bilateral ovaries were removed from ApoE-/- mice, the fatty liver-induced damage was further increased. Interestingly, the 10 nM estradiol treatment relieved fatty liver damage caused by lipid accumulation in OVX ApoE-/- mice (Fig. S3B and 3C). TEM analysis of the livers of the mice revealed that the number of lipid droplets in the livers was consistent with the observed fatty liver-induced damage revealed by the HE staining and oil red O staining in the different groups (Fig. S3D).

**Estrogen supplementation induces autophagy via estrogen receptor α**

We next studied the changes in ERα and ERβ levels in OVX ApoE-/- mice and Hcy-treated HUVECs. The expression of ERα and ERβ was significantly reduced in both the in vivo and in vitro models, suggesting that the function of ERα and ERβ in the vascular endothelium may decrease during post-menopausal stage. The treatment of both OVX ApoE-/- mice and Hcy-treated HUVECs with estradiol resulted in a significant increase in ERα and ERβ expressions (Fig. 5A-B). These changes suggest that ERs may be involved in the upregulation of autophagy caused by estrogen supplementation. Furthermore, we assessed the effects of ERα and ERβ specific
inhibitors on Hcy-treated HUVECs. When Hcy-treated HUVECs were treated with the ERα-specific inhibitor MPP (10 μM) and estradiol (10 nM), the upregulation of autophagy induced by estrogen supplementation was abolished (Fig. 5E), as observed by decreased LC3B and Beclin1 expression and increased SQSTM1 expression (Fig. 5C). Interestingly, when Hcy-treated HUVECs were treated with ERβ-specific inhibitor PHMTPP (10 μM) and estradiol (10 nM), no changes were observed in LC3, Beclin1 or SQSTM1 expression compared to the group treated with estradiol (10 nM) alone (Fig. 5D). Furthermore, PHMTPP has no effect on the estrogen-induced autophagy increase in Hcy-treated HUVECs cells (Fig. 5F). These results suggested that the increased autophagy in the vascular endothelium caused by estrogen supplementation may be related to ERα.

**Estrogen supplementation ameliorates cell pyroptosis by upregulating autophagy via estrogen receptor α**

To further confirm that estrogen could induce autophagy via ERα in the vascular endothelium, we silenced ERα expression in HUVECs using ERα siRNA. Similar to the effect of the ERα inhibitor...
MPP on Hcy-treated HUVECs, the silencing of ERα abolished the upregulation of LC3B and Beclin1 protein expressions and the down-regulation of SQSTM1 protein expression caused by estrogen supplementation in Hcy-treated HUVECs (Fig. 6A). In addition, the protein expression of pyroptosis-associated factors in HUVECs were also affected by the silencing of ERα, including NLRP3, cleaved caspase 1, and GSDMD. Compared to the control siRNA group, the inhibitory effect of estrogen supplementation on the protein expression of NLRP3, cleaved caspase 1, and GSDMD was abolished in the ERα siRNA groups (Fig. 6B), and the same changes in IL-1 and IL-18 protein expression were observed (Fig. 6C).

Immunofluorescence assays were performed to assess the localization of GSDMD and ERα expression in HUVECs. The results showed that ERα was barely expressed in HUVECs after ERα siRNA transfection. **P < 0.01.
Inhibition of autophagy abrogates the protective effect of estrogen supplementation on pyroptosis by negatively affecting ERα expression

To assess the role of autophagy in the estrogen supplementation-mediated inhibition of pyroptosis of the vascular endothelium, Hcy-treated HUVECs were treated with the autophagy inhibitor 3-MA (10 μM) together with estradiol (10 nM). The results showed that 3-MA decreased LC3B and Beclin1 expression and increased that of SQSTM1 in estradiol (10 nM)- and Hcy-treated HUVECs (Fig. 7A). The inhibitory activity of estradiol (10 nM) on NLRP3, cleaved caspase 1, and GSDMD protein expression was disrupted in the presence of 3-MA, similar to that of IL-1β and IL-18 expression (Fig. 7C). Furthermore, the upregulation of autophagy induced by estrogen supplementation was abolished by 3-MA, as indicated by the absence of GFP-LC3B in HUVECs (Fig. 7D). These results suggest that autophagy plays an important role in the protection of vascular endothelium caused by estrogen supplementation. Fluorescence staining of caspase-1 was performed to assess caspase-1-dependent pyroptosis in different groups of cells. The results showed that 3-MA treatment disrupted the inhibitory effect of caspase-1 expression induced by estradiol (10 nM) supplementation (Fig. 7E). In addition, the localization of GSDMD and ERα in HUVECs revealed that GSDMD was significantly and highly expressed in estrogen-treated HUVECs after autophagy inhibitor 3-MA (10 μM) was added, while the level of ERα was comparable to that observed in cells treated with estradiol (10 nM) alone (Fig. 7F). Taken together, these results suggest that autophagy may play a key role in the estrogen-mediated protection against vascular endothelial pyroptosis.

Discussion

In this study, we showed that estrogen attenuates pyroptosis of vascular endothelial cells through the ERα-mediated activation of autophagy to improve atherosclerosis in post-menopausal women and mice. In human, we observed a significant reduction in estrogen receptor levels and autophagy and increased NLRP3 inflammation and pyroptosis in the vascular endothelium in post-menopausal female ascending aorta with arteriosclerosis. We demonstrated that estrogen ameliorated pyroptosis in cardiac aorta of OVX ApoE−/− mice and in Hcy-treated HUVECs. We further showed that estrogen accelerates autophagy in the aortas of OVX ApoE−/− mice and Hcy-treated HUVECs. In mice, we observed that estrogen ameliorated atherosclerosis. In HUVECs, the use of ERα and ERβ inhibitors led to the determination that ERα, not ERβ, is involved in the up-regulation of autophagy caused by estrogen. The inhibition of autophagy disrupted the protective effect of estrogen supplementation on pyroptosis, confirming that autophagy has a protective effect on vascular endothelium.

Dyslipidemia is associated with the occurrence of atherosclerosis [38]. In post-menopausal women, the function of ovarian begins to decline, the secretion of estrogen is gradually reduced and the metabolism of blood lipids is becomes disrupted [39]. Furthermore, ERs expression is also reduced in women during menopause [40]. Consistent with our findings, bilaterally ovariectomized ApoE−/− mice that could no longer produce estrogen had elevated plasma levels of TC, TG, LDL-c, and HDL-c, aggradated lipid deposition in the aorta and liver, and increased area of lipid plaques in the aortic root that promoted the development of atherosclerosis. In contrast, estrogen supplementation of these mice resulted in reduced plasma lipid levels, and lipid deposition in the aorta and liver to prevent the development atherosclerosis (Fig. 4 and Fig. 52). Until midlife, the cardiovascular disease incidence in men is consistently higher than that observed in women, which may be due to the protective effect of estrogen on the cardiovascular system [41]. Interestingly, the blood vessels of women are more likely to be damaged when menopause occurs, which may be caused by a reduction of estrogen secretion and the low expression of estrogen receptors [42]. We obtained some evidence that post-menopausal women with ascending aortic arteriosclerosis have significantly elevated arterial intimal injury and low levels of estrogen receptors expression in arteries (Fig. 1 and Fig. 51).

During menopause, in addition to decreased endogenous estrogen and ERs expression and disrupted lipid metabolism, inflammation is remarkable increasing [43]. The inflammatory response accelerates the development of atherosclerosis. Local stimulus in ascending aortic atherosclerotic plaques activate caspase-1 via NLRP3 inflammatory, resulting in the cleavage of pro-IL-1β, pro-IL-18 to produce active IL-1β and IL-18 [44], which causes the pyroptosis of vascular endothelial cells and further aggravates atherosclerosis [45]. IL-18 localizes to human atherosclerotic lesions and is a pro-inflammatory and atherogenic cytokine that induces the expression of other pro-inflammatory cytokines and adhesion molecules to promote pyroptosis of endothelial cells [46–48]. In the early stages of atherosclerosis, some independent risk factors, such as homocysteine, promote the secretion of IL-1β and IL-18, leading to the destruction of the endothelial barrier [35,49]. The results of the present study showed that the levels of NLRP3, cleaved caspase 1, GSDMD, IL-1β, IL-18 and Hcy in the vascular endothelium of the ascending aorta in post-menopausal female were significant increased compared to that observed in pre-menopausal women (Fig. 1 and Fig. 51), while estrogen supplementation of OVX ApoE−/− mice and Hcy-treated HUVECs had an inhibitory effect on NLRP3, cleaved caspase 1, GSDMD, IL-1β, and IL-18 levels (Fig. 2 and Fig. 52).

Caspase-1 induces pyroptotic cell death [44], and caspase-1 dependent pyroptosis requires the activation of the typical inflammatory response [44,50]. As a representative inflammasome, the NLRP3 polyprotein complex induces pyroptosis by releasing cytokines. Activated caspase-1 cleaves inflammatory factors and...

Fig. 6. Estrogen supplementation ameliorates cell pyroptosis by upregulating autophagy via estrogen receptor α. (A) Representative western blots and relative quantitative analysis of Beclin1, LC3B, and SQSTM1 in HUVECs cells that transfected with Control siRNA or ERα siRNA. (B) Representative western blots and relative quantitative analysis of NLRP3, cleaved caspase 1, and GSDMD in HUVECs cells that transfected with Control siRNA or ERα siRNA. (C) Representative western blots and relative quantitative analysis of IL-1β and IL-18 in HUVECs cells that transfected with Control siRNA or ERα siRNA. (D) Immunofluorescence images of cells that transfected with Control siRNA or ERα siRNA were taken with or without 60 nM Hcy for 12 h and in the presence or absence of 10 nM E2. Green: GSDMD, red: ERα. DAPI was used to stain the nucleus. Mean optical density analysis of GSDMD and ERα was performed. Scale bar: 50 μm. (E) Immunofluorescence images of cells that transfected with Control siRNA or ERα siRNA were taken with or without 60 nM Hcy for 12 h and in the presence or absence of 10 nM E2. Red: Caspase-1. DAPI was used to stain the nucleus. Mean optical density analysis of Caspase-1 was performed. Scale bar: 50 μm. In all experiments, n = 6. *P < 0.01.
Fig. 7. Inhibition of autophagy abrogates the protective effect of estrogen supplementation on pyroptosis without negatively affecting ERα expression. (A) Representative western blots and relative quantitative analysis of Beclin1, LC3B, and SQSTM1 with or without 12 h of application of 60 nM Hcy to HUVECs in the presence or absence of 10 nM E2 or/and 10 μM 3-MA. (B) Representative western blots and relative quantitative analysis of NLRP3, cleaved caspase 1, and GSDMD with or without 12 h of application of 60 nM Hcy to HUVECs in the presence or absence of 10 nM E2 or/and 10 μM 3-MA. (C) Representative western blots and relative quantitative analysis of IL-1β and IL-18 with or without 12 h of application of 60 nM Hcy to HUVECs in the presence or absence of 10 nM E2 or/and 10 μM 3-MA. (D) After transfection of HUVECs with GFP-LC3 adenovirus, immunofluorescence images of cells were taken with or without 60 nM Hcy for 12 h and in the presence or absence of 10 nM E2 or/and 10 μM 3-MA. DAPI was used to stain the nucleus. Mean optical density analysis of GFP was performed. Scale bar: 50 μm. (E) Immunofluorescence images of cells were taken with or without 60 nM Hcy for 12 h and in the presence or absence of 10 nM E2 or/and 10 μM 3-MA. Green: GSDMD, red: ERα. DAPI was used to stain the nucleus. Mean optical density analysis of GSDMD and ERα was performed. Scale bar: 50 μm. (F) Immunofluorescence images of cells were taken with or without 60 nM Hcy for 12 h and in the presence or absence of 10 nM E2 or/and 10 μM 3-MA. Red: Caspase-1. DAPI was used to stain the nucleus. Mean optical density analysis of Caspase-1 was performed. Scale bar: 50 μm. In all experiments, n = 6. **P < 0.01.
GSDMD, allowing the domain of GSDMD to penetrate the cell membrane and promote the release of inflammatory factors [50]. GSDMD is a marker of pyroptotic cell death [50], and a previous study observed that the inhibition of autophagy by genetic deletion of Atg16L1 enhanced the inflammatory responses induced by TLR4 signaling [23]. Autophagy can indirectly inhibit the activation of IL-1β by reducing the levels of endogenously activated NLRP3 inflammasome, which inhibits the expression of caspase-1 [51]. The block of autophagy enhances the activity of the inflammasome, while increased autophagy inhibits the activity of inflammasome [52]. The results of these studies suggest that autophagy may be an inhibitor of the NLRP3 inflammasome. Our present data showed that the protective effect of estrogen on the vascular endothelium may be associated with up-regulation of autophagy.

Autophagy in atherosclerosis has been extensively studied, primarily in vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and macrophages [9,10,53]. There are a variety of autophagy triggers in atherosclerotic plaques, such as inflammatory mediators, reactive oxygen species, low-density lipoprotein, tumor necrosis factor-α (TNF-α), and advanced glycation end products (AGEs) [10]. Autophagy is activated when HUVECs are treated with oxidized low-density lipoprotein (ox-LDL), and the activation of autophagy helps to degrade the damage from low-density lipoprotein to allow HUVECs to survive in harsh environments [54]. In addition, the exposure of vascular smooth muscle cells to relatively moderate concentrations of ox-LDL also resulted in notable autophagy, while high levels of ox-LDL inhibited autophagy and resulted in the increased apoptosis of VSMCs [55]. Ox-LDL increases the activity of active oxygen, which promotes the activation of inflammasome and caspase-1 [55] and may lead to pyroptosis. The autophagy inducer 7-ketocholesterol promotes the protective effect of autophagy in VSMCs [56]. We used Hcy to induce HUVECs injury, and OVX ApoE−/− mice combined with a high-fat diet to establish a post-menopausal model with atherosclerosis. We observed that estrogen up-regulated beclin/LC3 levels and decreased SQSTM1 expression in both in vivo and in vitro experiments (Fig. 3). These data suggest that estrogen supplementation reduces vascular endothelial pyroptosis and prevents atherosclerosis during the post-menopausal stage by up-regulating autophagy.

The physiological effects of estrogen are mediated by ERs. A growing number of studies have shown that estrogen participates in various diseases by regulating autophagy [7]. Some studies have shown that estrogen plays a key role in the protection of the cardiovascular system through autophagy [5,57]. However, at present, the effects of different types of estrogen receptors on the activation or inhibition of autophagy to exert cardiovascular protective effects is debatable [7]. The results of the present study showed that ERα and ERβ expression in aortas of OVX ApoE−/− mice and in Hcy-treated HUVECs was significantly reduced, while estrogen significantly increased the expression of ERα and ERβ. Interestingly, the results of our study also showed that the effect of estrogen in-promoting autophagy was reversed after the inhibition of ERα. Despite the observed change in ERβ expression after estrogen supplementation, the inhibition of ERβ had no effect on the estrogen-mediated promotion of autophagy in HUVECs (Fig. 5). The observed co-localization of GSDMD and ERα in HUVECs in the presence or absence of an ERα inhibitor (MPP) or ERα siRNA demonstrated that estrogen up-regulates autophagy via ERα, not, ERβ in Hcy-treated HUVECs (Figs. 5 and 6).

The autophagy inhibitor 3-MA has been used to aggravate the AGE-induced injury of endothelial cell, indicating that autophagy plays a role in protecting endothelial cells [58]. Wei et al [59] used ox-LDL to induce vascular endothelial cell injury, and showed that appropriate autophagy restrains inflammation and slows the progression of atherosclerosis. In addition, Razani et al [60] observed that the interruption of autophagy leads to an accumulation of cholesterol crystals in atherosclerotic plaques, promotes the activation of the NLRP3 inflammasome, increases the death of vascular endothelial cells, and aggravates the development of atherosclerosis. In the present study, we demonstrated that the ability of estrogen supplementation to prevent the pyroptosis of vascular endothelial cells was disrupted by the autophagy inhibitor 3-MA. In addition, the NLRP3 inflammasome, caspase1, IL-1β, and IL-18 levels were elevated by 3-MA treatment in estrogen-treated HUVECs (Fig. 7). These results suggest that autophagy may play a protective role in the development of atherosclerosis during post-menopausal stage, and the interruption of autophagy eliminates the beneficial effects of estrogen supplementation on the vascular endothelium.

Conclusion

The results of the present study demonstrated that estrogen improves atherosclerosis during the post-menopausal period by upregulating ERα and the subsequent induction of autophagy. Estrogen-induced autophagy activation down-regulated the NLRP3 inflammasome-IL1β-mediated inflammation and pyroptosis in vascular endothelial cells. Based on these data, our results indicate that estrogen supplementation may be a potential approach for preventing the development of atherosclerosis in post-menopausal women.

Compliance with ethics requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. Informed consent was obtained from all patients for being included in the study. The human research protocol was approved by the medical ethics committee of Nanjing First Hospital affiliated to Nanjing Medical University (NO. KY20190404-03-KS-01).

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed. The animal research was approved by Nanjing University of Chinese Medicine Animal Care and Use Committee (NO. ACU-40-20141226).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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