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Elevated luteinizing hormone contributes to atherosclerosis formation by inhibiting nitric oxide synthesis via PI3K/Akt pathway

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ABSTRACT

Background: The contentious effects of estrogen therapy on the risk of postmenopausal cardiovascular disease (CVD) indicate that this type of atherosclerosis is not solely induced by estrogen deficiency. Other sex hormones such as elevated luteinizing hormone (LH) may also affect CVD risk in this population. We therefore explored the relationship between LH and atherosclerosis in ovariectomized (OVX) female mice.

Methods: Aortic atherosclerotic lesions were assessed in OVX ApoE knock out ($ApoE^{-/-}$) female mice administered with LH. Human umbilical vascular endothelial cells (HUVECs) were cultured as cell model. The influence of LH on NO release, phosphorylated endothelial nitric oxide synthase (eNOS) and Akt levels were evaluated. Immunoprecipitation and lentiviral particle transfection were applied to assess the role of Gαq on PI3K activity.

Results: LH increased the atherosclerotic lesion area and carotid artery intima-media thickness (IMT) in OVX $ApoE^{-/-}$ female mice. High levels of LH attenuated vasodilation induced by Ach and inhibited NO release from HUVECs. These effects were related to the findings that LH enhanced interaction between Gαq and p110α, which subsequently inhibited PI3K activity and suppressed the phosphorylation of Akt and eNOS.

Conclusions: Elevated LH promotes atherosclerosis formation in OVX $ApoE^{-/-}$ female mice. This effect may be mediated by inhibiting endothelial NO synthesis via PI3K/Akt signaling pathway.

1. Introduction

Ischaemic heart disease and stroke, mainly resulting from atherosclerosis, are the leading causes of death globally [1]. Women have a lower morbidity of cardiovascular disease (CVD) before menopause compared to men; while the CVD rates of women become similar to those of men after menopause with the gradual decrease of estrogen level [2–4]. Hence, sex hormones, especially estrogen, have long been proposed as treatment of menopausal women to reduce the risk of CVD. Nonetheless, the contentious effects of estrogen therapy on CVD risk [5–9] indicate that postmenopausal atherosclerosis is not solely induced by estrogen deficiency. Other sex hormones may also have an effect on the incidence of CVD in menopausal women.

Two pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), regulate estrogen secretion from ovaries; free estrogen, in a negative feedback loop, inhibits FSH and LH secretion from the pituitary [10]. In addition to the decrease of estrogen, more attention needs to be paid to the fact that the level of FSH and LH in postmenopausal women drastically increase due to reduced negative feedback of estrogen on them. Considering the contradictory effects of hormone therapy on CVD in postmenopausal women, it is reasonable to speculate that elevated FSH or LH might be associated with CVD in this population.

Recently, there were reports showing that FSH levels correlated with vascular inflammation [11], low FSH level was associated with low intima media thickness (IMT) [12]. Our previous study revealed that higher levels of FSH and vascular cell adhesion molecule 1 (VCAM1) were found in blood samples from postmenopausal women; FSH elevated VCAM1 in human umbilical vascular endothelial cells (HUVECs) and increased adhesion of monocytes to HUVECs [13]. These data implicate that FSH also contribute to postmenopausal atherogenesis. Although peripheral levels of

Abbreviations: LH, luteinizing hormone; LHR, luteinizing hormone receptor; CVD, cardiovascular disease; FSH, follicle stimulating hormone; IMT, intima media thickness; OVX, ovariectomized; HUVECs, human umbilical vascular endothelial cells; eNOS, endothelial nitric oxide synthase; PI3K, phosphatidylinositol 3-kinase; GPCR, G protein coupled receptor; Bcl-2, B-cell lymphoma-2

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LH increase three-fold in postmenopausal women [14], it remains unclear whether a relationship between LH and atherosclerosis in postmenopausal women exists until now.

Endothelial dysfunction is an initial step in the development and progression of atherosclerosis [15]. The formation of nitric oxide (NO), a vasodilating molecule synthesized in the endothelia, is considered as one of the key parameters of endothelial dysfunction [16]. In addition to vasodilation, endothelium-derived NO plays crucial roles in vasoprotection, cardioprotection, and anti-atherogenesis [17,18]. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the endothelial nitric oxide synthases (eNOS)/NO pathway are essential for NO synthesis [19–21]. Therefore, we attempted in this study to explore the effects of LH on atherosclerotic lesion formation in ovariectomized (OVX) female mice and NO release from human umbilical vein endothelial cells (HUVECs). We also analyzed the roles of PI3K/Akt pathway and the interaction between LH receptor (LHR) and PI3K in this process, with the aim to clarify the possible underlying mechanisms.

2. Materials and methods

2.1. Animals

Six-week-old homozygous ApoE knock out ($ApoE^{-/-}$) female mice in C57BL/6 background were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). Mice were kept under specific pathogen-free and temperature-controlled conditions on a 12 h light/dark cycle and fed ad libitum on normal diet. At 9 weeks of age, mice were anesthetized and sham-operated (SHAM) or bilaterally ovariectomized (OVX) through a 1 cm abdominal incision. After surgery, mice were deprived of food and water for one day, and then fed ad libitum on an atherogenic diet containing 24% (*w*/w) fat (Guangdong Medical Animal Experimental Center, Guangzhou, China). Mice were randomly divided into 4 groups (8 mice per group) as follows: Sham, OVX, Sham + LH, OVX + LH. Recombinant LH (Luveris, Merck Serono, Sweden) was dissolved in physiologic saline and administered into mice by subcutaneous injection daily. The dose of LH (0.15 IU/day) was established. Mice without LH treatment were injected daily with physiologic saline. After 16 weeks of treatment, the intima-media thickness (IMT) of carotid artery in mice was measured by carotid ultrasound (Vevo 2100 Imaging System), and then those mice were sacrificed by exsanguination under anesthesia and blood samples were collected. LH concentrations were measured by Guangdong Medical Laboratory Animal Center. All procedures were performed in accordance with the guidelines for animal welfare of Guangzhou Medical University.

2.2. Assessment of aortic atherosclerotic lesions

After 16 weeks of treatments, mice were sacrificed by exsanguination under anesthesia. The heart and arterial tree were dissected carefully and were incubated with physiologic saline and fixed in 4% formalin, then dehydrated with sucrose gradient. Tissues were embedded in Tissue Freezing Medium and serially sectioned at 8 μm using a Thermo cryostat (Thermo scientific, Waltham, USA). To quantify the dimensions of atherosclerotic lesions, 10–12 sections with tricuspid valves were stained with oil-red O and counterstained with hematoxylin. Images were captured with Leica CS2 and lesion areas were quantified with Aperio ImageScope software.

2.3. Reagents

LH (#L6420) were purchased from Sigma Aldrich (St. Louis, MO, USA). Endothelial Cell Medium-Phenol Red Free (ECM-prf) (#23907) was purchased from Sciencell (San Diego, California, USA). DMEM-High Glucose (#11995065) was purchased from Gibco (Life technologies, California, USA). All other chemicals were of analytical grade and purchased from Guangzhou Chemical Reagents (Guangzhou, China).

2.4. Cell cultures and treatments

Human umbilical vein endothelial cells (HUVECs, Guangzhou First People's Hospital) and human ovarian cancer cell line OVCAR-3 (KeyGEN BioTECH, #KG040) were cultured as previously described [22]. In brief, cells were seeded in ECM-prf medium supplemented with 10% FBS and 1% streptomycin/penicillin in a humidified atmosphere of 5% CO_2 at 37 °C. They were sub-cultured for experiment when grew to 80–90% confluency. Cells on passage 6 to 10 were used in this study. Before various treatments, HUVECs were cultured for 8 h in ECM-prf containing 1% FBS. The control cells received the same volume of ammonium bicarbonate (solvent for LH). In the dose-effect treatment, cells were exposed to 0 (control), 5, 25, 50, 75, and 100 mIU/ml of LH for 24 h. In the time-effect treatment, cells were exposed to 100 mIU/ml of LH for 2–48 h.

2.5. Western blot

After various treatments, HUVECs were washed twice with ice-cold PBS before addition of the lysis buffer (100 mM Tris-HCl pH = 6.8, 4% SDS, 20% glycerol, 1 mM sodium orthovanadate, 1 mM NaF, 1× protease inhibitors cocktail (sigma, #P8340), 1× phosphatase inhibitors cocktail (sigma, #P0044) and 1 mM phenylmethylsulfonylfluoride (PMSF) to cell-culture dish on ice. Subsequently, cell lysates were scraped, boiled, centrifuged for 2 min at 13000 rpm, and then separated by SDS-PAGE. Anti-LHR (NOVUS, #NBP2-53258), anti-Phosphorylated Akt (CST, #4060s), anti-Akt (CST, #4691s), anti-Phosphorylated eNOS (CST, #9570s), anti-eNOS (CST, #32027s), anti-PI3K p110 (santa cruz, #sc-8010), anti-PI3K p110α (santa cruz, #sc-293172), anti-PI3K p110β (sc-376641), anti-Bcl-2 (CST, #4223T), and anti-β-actin (Santa Cruz, #sc-81178) were used to reveal the protein levels, respectively. Primary and secondary antibodies were incubated with the membranes using the standard technique. Immunodetection was accomplished using enhanced chemiluminescence.

2.6. Immunoprecipitation assay

HUVECs were harvested in NP40 (Invitrogen, #FNN0021), 1× Protease inhibitors cocktail, and 1 mM PMSF. Equal amounts (50 μl) of Dynabead (Invitrogen, #10007D) were incubated with 1 μg of precipitating anti-Gαq antibody for 30 min at 4 °C under gentle agitation. Then the equal amounts (200 μg) cell lysate added to the Dynabead, and the samples were rotated and incubated at 4 °C for another 30 min. The samples were then pelleted, washed, and re-suspended in 20 μl of Elution buffer for Western blot. Antibodies used were mouse anti-Gαq (Santa Cruz, #sc-136181).

2.7. Immunofluorescence staining of cells

HUVECs and OVCAR-3 cells were seeded into a 6-well plate. At the next day they were incubated with 100 mIU/ml of LH for 24 h. Then these cells underwent fluorescent staining according to published methods [13]. In brief, cells were fixed in 4% paraformaldehyde (PFA) for 30 min, washed three times in PBS, permeabilized in 0.3% Trition X-100 on ice for 5 min, blocked in 5% BSA for 30 min, incubated with primary antibody against LHR (NOVUS, #NBP2-53258, 1:200) at 4 °C overnight, and then incubated with FITC-conjugated secondary antibody (Invitrogen, #1858182, 1:1000) for 1 h. Nuclei were stained with DAPI (CST, #4083S, 1:2000). Immunofluorescence images were visualized and photographed by using a laser scanning confocal microscopy (ZEISS LSM 710, ZEISS, Germany).

2.8. Gαq shRNA and Gαq (Q209L) lentiviral particle transduction

The Gαq shRNA (santa cruz, sc-35,429-V) or Gαq (Q209L) (ViGene Biosciences) lentiviral particle transduction was performed according to

the protocol provided by the manufacturer. Briefly, HUVECs were seeded into a 6-well plate at 2.5 × 10^5/well 24 h prior to viral infection and cultured in ECM-prf medium supplemented with 5% FBS and 1% antibiotics. Transduction was performed when cells grew to approximate 80% confluency. At day 2, the culture medium was replaced with 2 ml of fresh ECM-prf medium (without Polybrene). Lentiviral particles were thawed at room temperature. Then 16 μl of these particles were added to the culture medium and incubated overnight to infect cells. At day 3, the culture medium was replaced with 2 ml of fresh ECM-prf medium, and incubated overnight. At day 4, cells were split at 1:3 and incubated for 48 h. At day 6 and forward, stable clones expressing the Gαq shRNA/Gαq (Q209L) were selected via puromycin dihydrochloride (2 μg/ml) selection. Medium was replaced with fresh puromycin-containing medium every 3 days, until resistant colonies could be identified. Colonies with stable Gαq shRNA/Gαq (Q209L) expression were expanded for further experiments.

2.9. NO concentration measurement

HUVECs were seeded into a 6-well plate at 1.5 × 10^5/well. Then they received dose- and time-effect treatments of LH at the next day. Then the culture medium was collected for NO measurement. The data acquisition system is composed of single channel gas molecular biodetector (World Precision Instruments, TBR1025), Four-channel data acquisition system (World Precision Instruments, Lab-Trax-4/16), and NO sensor (World Precision Instruments, ISO-NOP). The NO concentration was measured according to the manufacturer's instructions. Briefly, two solutions, solution 1 (containing 0.1 M KI and 0.1 M H_2SO_4) and solution 2 (containing 50 μM KNO_2), were prepared for calibrating electrode. The electrode was placed in a thermostatically jacketed stirred cuvette and the temperature was set at 37 °C. The NO electrode was calibrated by filling the stirred cuvette with 20 ml of solution 1, and titrating by adding 2, 4, 8, 16, and 32 μl of solution 2 to the stirred cuvette (the corresponding concentrations of NO were 5, 10, 20, 40, and 80 nM). Then, replaced the solution with culture medium collected from 6-well plate cultured with HUVECs for NO measurement.

2.10. Cell apoptosis analysis by flow cytometry assay

Cell apoptosis analysis was performed by flow cytometry assay with Apoptosis Detection Kit (BD Pharmingen, #556547). HUVECs were collected after being incubated with trypsin (without EDTA) for 3 min at 37 °C. The mixture containing cells and trypsin was centrifuged at 1200 rpm for 5 min at RT (25 °C). The supernatant was discarded and cells were washed twice with cold PBS. Then cells were re-suspended in 1× Binding Buffer at a concentration of 1 × 10^6 cells/ml. 100 μl of the re-suspended cell solution (1 × 10^5 cells) was transferred to a 5 ml culture tube, and 5 μl of FITC Annexin V and 5 μl PI was added. The cells were gently vortexed and incubated for 15 min at RT in the dark. Finally, 400 μl of 1× Binding Buffer was added to each tube for flow cytometry assay.

2.11. Statistical analyses

Data are presented as mean ± SD. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test. Student's *t*-test was used to evaluate the significance in differences between two groups of observations. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LH promoted atherosclerosis formation in ovariectomized mice

To investigate the proatherogenic effects of LH in menopausal mice, $ApoE^{-/-}$ mice were ovariectomized (OVX) and administered with LH to imitate postmenopausal state, then fed on atherogenic diet for 16 weeks. Oil red O staining showed that atherosclerotic lesions formed in the aorta roots of all $ApoE^{-/-}$ mice. Obviously, aorta roots from OVX $ApoE^{-/-}$ mice treated with LH exhibited a higher lesion area compared to those without LH treatment or SHAM mice; the area of atherosclerotic plaques in OVX-control mice was higher than that in SHAM-control (Fig. 1A–B). LH treated OVX mice also had an increased carotid artery IMT, while there was no significant difference in carotid

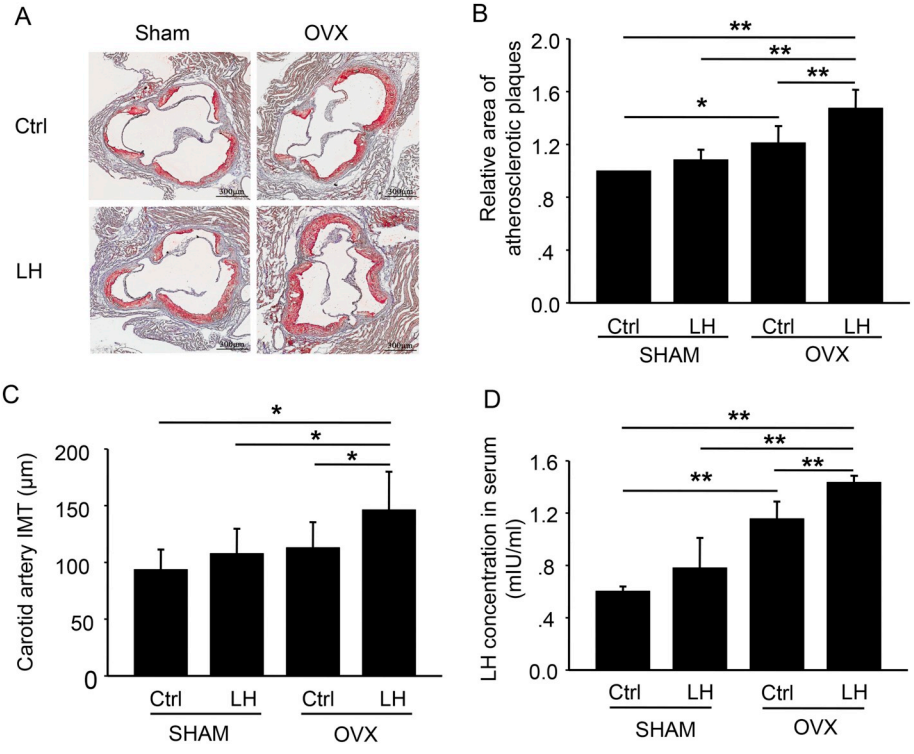

Fig. 1. Elevated LH promoted atherosclerosis formation in OVX $ApoE^{-/-}$ mice fed on atherogenic diet. (A) Oil-red O staining of aorta root (Scale bar: 300 μm). (B) The relative area of atherosclerotic plaques ($n = 8$). (C) Carotid artery intima-media thickness (IMT). (D) LH levels in serum (n = 8). (*, $P < 0.05$; **$P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

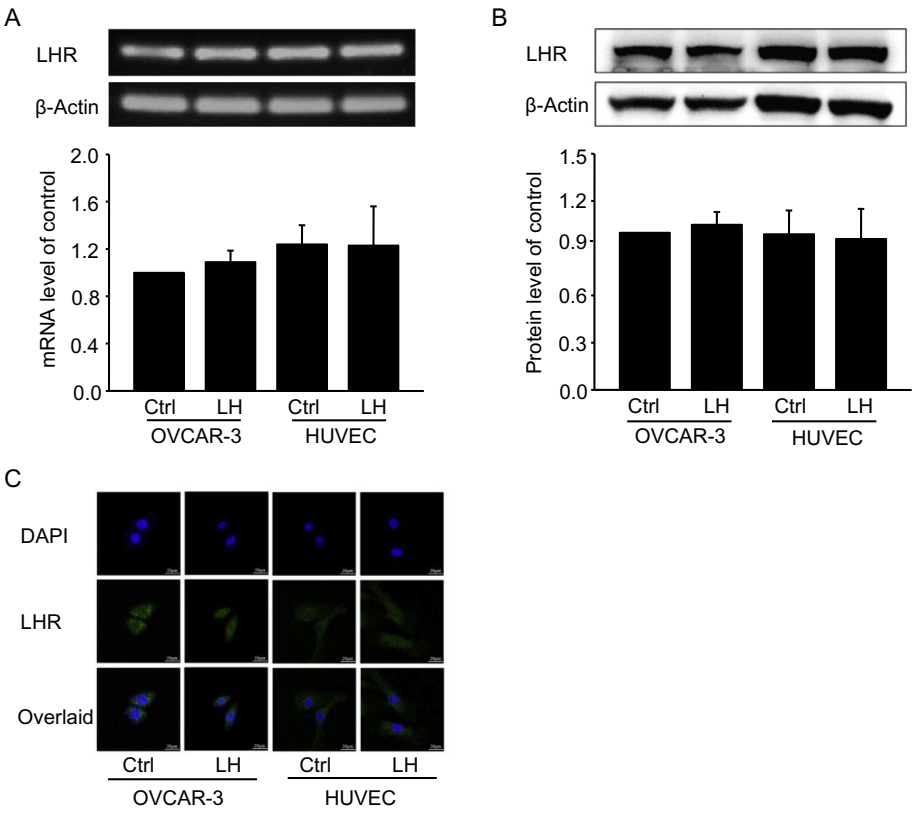

Fig. 2. LH receptor (LHR) was expressed in endothelial cells. Both LHR mRNA (A) and LHR protein (B) were expressed in HUVECs and OVCAR-3 cells. The upper of (A) or (B) is a representative image of Northern or Western blot. Real-time PCR results and quantified results of Western blot were presented at the lower of (A) and (B), respectively. ($P > 0.05$, n = 6). (C) Confocal microscopy images from LHR (green) and DAPI (blue) staining also showed that LHR was expressed in HUVECs and OVCAR-3 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

artery IMT between OVX-control and SHAM-control (Fig. 1C). As expected, LH level in OVX mice increased remarkably as compared with SHAM group; administration of exogenous LH elevated serum LH concentrations in OVX mice (Fig. 1D). These findings implicated that elevated LH promoted aortic atherosclerotic lesion formation.

3.2. LH receptor was expressed in vessel endothelial cells

To elucidate the effects of elevated LH on cardiovascular system and the corresponding mechanism underlying it, it is necessary to determine whether LH receptor (LHR) is located in vascular endothelial cells. Northern blot (the upper of Fig. 2A), real-time PCR (the lower of Fig. 2A) and western blot showed that LHR mRNA and LHR protein (Fig. 2B) expressed in both HUVECs and OVCAR-3 cells; LH had no remarkable effect on the LHR expression as compared to the corresponding control group in both cell lines ($P > 0.05$, $n = 6$). Confocal microscopy images also revealed that LHR existed in HUVECs and OVCAR-3 cells (Fig. 2C).

3.3. LH attenuated vasodilation induced by Ach

Sex hormones exert influences on blood vessels as they alter the functions of endothelium and vascular smooth muscle. To determine the impact of LH on vasomotor effects, thoracic aortas from wild type mice were evaluated for responses to vasoconstrictors and vasodilators (Fig. 3). NO donor, sodium nitroprusside dihydrate (SNP), was regarded as positive control, and Ach was used for control (Fig. 3A). After pre-incubated with high concentration of LH (100 mIU/ml) for 2 h, the relative value of vasodilatory rate induced by Ach was significantly attenuated compared with SNP or Ach group; while LH had no effect on SNP induced vasodilation (Fig. 3B).

3.4. High concentrations of LH inhibited NO release from HUVECs

Given vasodilation induced by SNP and Ach is namely attributed to their stimulation on NO release from endothelia. It is obligated to examine the impact of LH on NO release from endothelial cells. The release of NO from HUVECs did not change remarkably with the

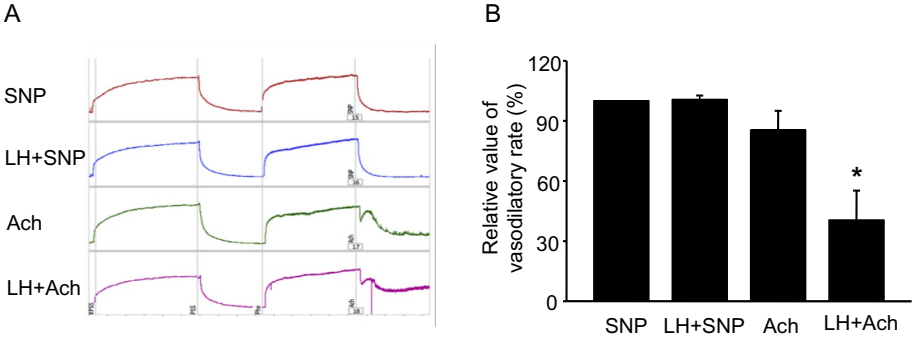

Fig. 3. LH attenuated vasodilation induced by Ach. (A) A representative lab chart recording vasodilatory responses in thoracic aorta segments pre-incubated with LH (100 mIU/ml) or corresponding control reagents. Thoracic aortas were challenged by K-PSS twice followed by PSS wash to check the endothelial function, then were pre-contracted with phenylephrine (PE) (100 nM). After a stable pre-contraction plateau level was reached, vasodilators, SNP (10 μM) or Ach (10 nM), were added to the baths. (B) Summarized relative value of vasodilatory rate. The response to vasodilators was given as the tension remaining after the drug is given, expressed in % of the pre-contraction level; then the data were normalized to SNP group. (*. $P < 0.05$ vs. SNP, LH + SNP and Ach group, $n = 4$).

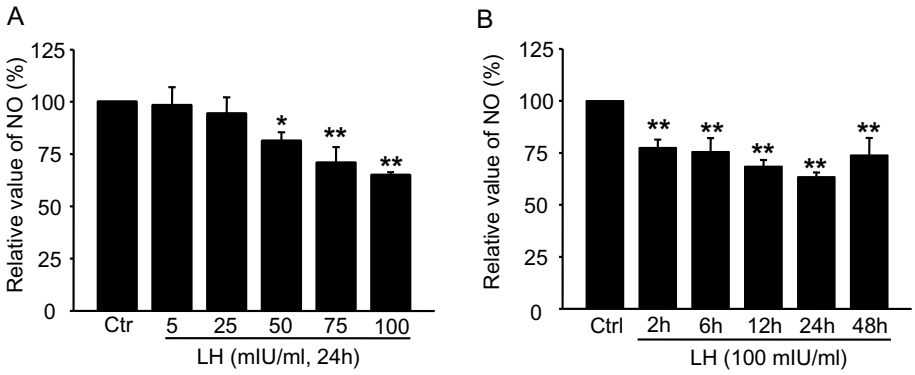

Fig. 4. LH dose- and time-dependently reduced NO release from HUVECs. (A) LH inhibited the production of NO from HUVECs in a dose-dependent manner, with highest effect at 100 mIU/ml. (B) Time-dependent effect of LH on NO release from HUVECs. The release of NO from HUVECs was inhibited by the application of LH for 2–48 h, reached lowest level at 24 h. (*, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control. $n = 6$).

treatment of LH at low concentrations (5–25 mIU/ml), while it significantly decreased when treated with high concentrations (50–100 mIU/ml) of LH for 24 h (Fig. 4A). High concentration (100 mIU/ml) of LH time-dependently inhibited NO release from HUVECs, with highest effect at 24 h (Fig. 4B).

3.5. High concentrations of LH decreased phosphorylated eNOS and Akt levels in HUVECs

To clarify the mechanism by which LH inhibited NO release from HUVECs, the influences of LH on eNOS level, its active phosphorylated form of eNOS (P-eNOS), and the up-stream regulatory factor phosphorylated Akt (P-Akt), were evaluated.

The results showed that phosphorylated eNOS level progressively decreased when treated with LH (100 mIU/ml) for 2 to 48 h, but total eNOS did not show significant changes. Coincidently, phosphorylated Akt, the up-stream regulator of phosphorylated eNOS, also decreased when exposed to LH with the prolonging of treatment time from 2 to 48 h, with highest effect at 24 h (Fig. 5A-B). Dose-effect of LH on total eNOS, phosphorylated eNOS and Akt revealed that low concentrations (5–25 mIU/ml) of treatment had no remarkable influence on the levels of phosphorylated eNOS and Akt, while high concentrations of LH (50–100 mIU/ml) decreased phosphorylated eNOS and Akt levels significantly (Fig. 5C–D). Similarly, no obvious alteration was found in total eNOS levels or total Akt levels (not shown).

3.6. LH enhanced the interaction between Gαq and the p110α subunit of PI3K, silencing Gαq abolished the inhibitory effect of LH on eNOS and Akt phosphorylation

To unveil the relationship between LHR and PI3K/Akt pathway, immunoprecipitation was performed to assess the interaction between Gαq and the catalytic subunits of PI3K, p110α and p110β; Gαq was also silenced by transducing shRNA into HUVECs to confirm the role of Gαq on regulating Akt phosphorylation.

Immunoprecipitation assay revealed that the interaction between Gαq and p110α was markedly elevated by LH, whereas LH decreased the interaction between Gαq and total p110 or p110 β (Fig. 6A), implying that the negative influence of LH on the phosphorylation of Akt was mediated by the interaction between Gαq and p110α. Silencing Gαq remarkably abolished the inhibition of P-Akt and P-eNOS level induced by LH despite the fact that LH still had a negative effect on P-eNOS level (Fig. 6B–C). Gαq shRNA transfection also abolished the inhibitory effect of LH on NO release from HUVECs (Fig. 6D).

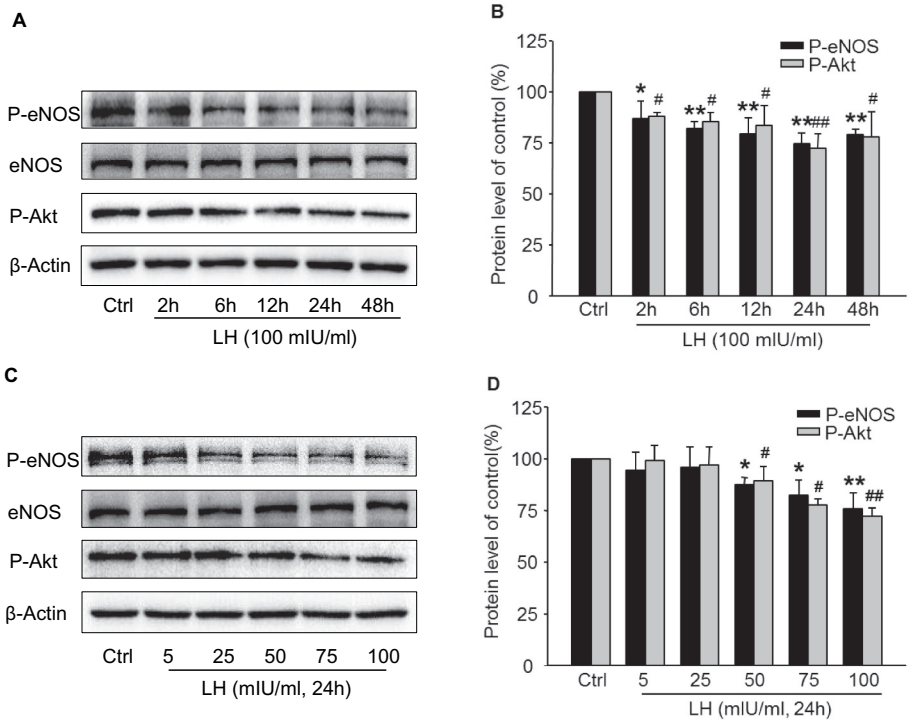

Fig. 5. LH dose- and time-dependently reduced phosphorylated eNOS and Akt level in HUVECs. (A) Representative western blots of the protein levels of phosphorylated eNOS, total eNOS, and phosphorylated Akt in HUVECs exposed to LH at 100 mIU/ml for 2–48 h. (B) Relative eNOS and Akt phosphorylation levels of control in HUVECs cells treated with LH at 100 mIU/ml for different time. (C) Representative western blot images of phosphorylated eNOS, total eNOS, and phosphorylated Akt in HUVECs treated with LH at concentrations of 5–100 mIU/ml for 24 h; the corresponding band intensities of phosphorylated eNOS and Akt in western blot were also summarized (D). (*or #, $P < 0.05$ vs. control; ** or ##, $P < 0.01$ vs. control. $n = 5$).

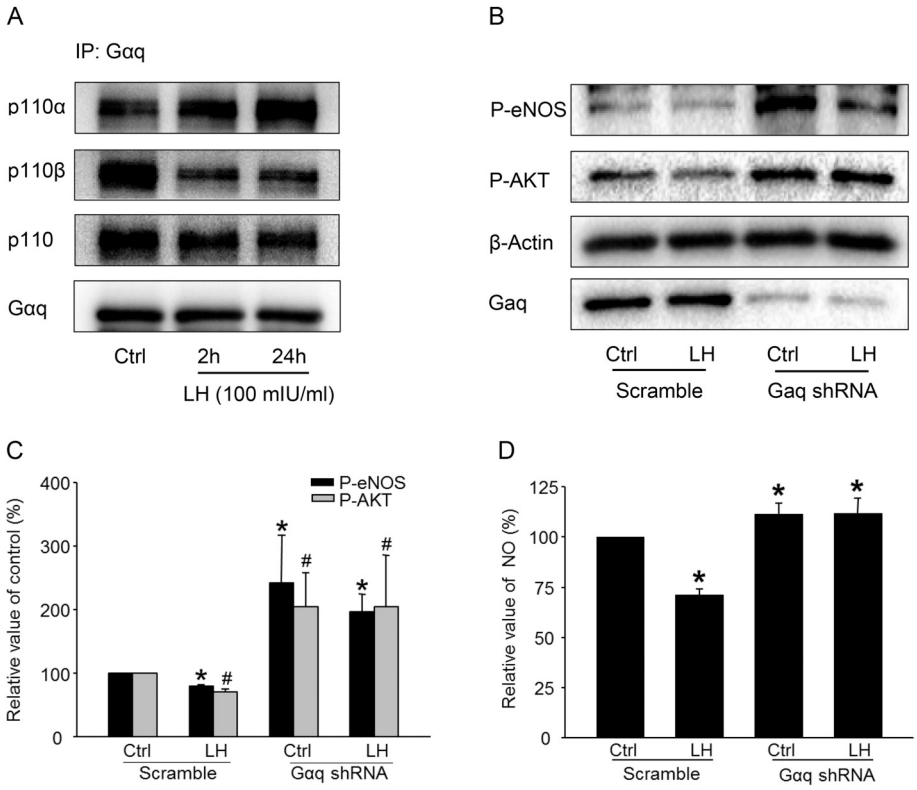

Fig. 6. LH enhanced the interaction between Gαq and the p110α subunit of PI3K and silencing Gαq leaded to elevated levels of phosphorylated eNOS and Akt. (A) Representative co-immunoprecipitation (IP) images of PI3K subunits p110α, p110β and total p110 immunoprecipitated with Gαq antibody. (B) Representative western blot images of phosphorylated eNOS and Akt in HUVECs transfected with Gαq shRNA and then exposed to 100 mIU/ml LH for 24 h; the corresponding band intensities of phosphorylated eNOS and Akt in western blot were also summarized (C). (D) Relative NO concentration in the culture medium of HUVECs transfected with Gαq shRNA and then exposed to 100 mIU/ml LH for 24 h. (* or #, $P < 0.05$ vs. scramble control; $n = 3$).

3.7. Sustained expression of Gαq inhibited eNOS and Akt phosphorylation

To further confirm the role of Gαq on PI3K/Akt pathway, Gαq (Q209L) lentiviral particles were transfected into HUVECs, and the levels of phosphorylated Akt and eNOS were assessed.

Not surprisingly, sustained expression of Gαq (Q209L) markedly reduced the phosphorylation of Akt and eNOS, while it had no remarkable effect on the expression of total Akt or eNOS (Fig. 7A–B). This inhibitory effect was so strong that it concealed the influence of LH. The NO release from HUVECs was also inhibited by the persistent activation of Gαq (Fig. 7C). These results further demonstrated that the activation of Gαq blocked the phosphorylation of Akt and eNOS.

4. Discussion

The increasing evidences that estrogen therapy has not successfully reduced stroke risk in postmenopausal women in all studies, suggest estrogen may not be solely responsible for elevated atherosclerotic diseases in this population [7]. Other sex hormones may therefore also exert effects on CVD risk. In recent years, FSH was found to be associated with atherogenesis in postmenopausal women [11–13]. As for LH, another important hormone acts synergistically with FSH on regulating estrogen and other sex hormones release, its potential effects on postmenopausal atherosclerosis remain to be explored. Interestingly, OVX mice administered with LH had a higher lesion area in aorta root compared to those without LH treatment or SHAM mice. OVX mice without LH administration also exhibited a higher lesion area compare to SHAM-control mice. These results were correlated to the LH circulation level, indicating that high level of LH promotes atherogenesis. Although the LH concentration in serum of SHAM mice showed a trend to be increased compared to SHAM-control, this change was not significant. The reason for it may be that the administration of exogenous LH stimulates ovaries to release sex hormones, which in turn has a negative feedback on the release of endogenous LH from pituitary.

To our knowledge, this is the first study revealing that elevated LH promoted atherosclerotic lesion formation in OVX ApoE$^{-/-}$ female mice, possibly by inhibiting endothelial NO synthesis via PI3K/Akt pathway.

LH plays a critical role in regulating reproductive functions such as ovarian hormone synthesis, ovulation in the female, and testosterone release by the Leydig cells of testis [10]. LHR, which can bind either LH or human chorionic gonadotropin (hCG), is a G protein coupled receptor (GPCR) that belongs to rhodopsin/β2 adrenergic receptor-like family A [23]. In addition to reproductive tissues, LHR is also expressed in a number of extra gonadal sites including brain, pineal gland, spinal cord, neural retina, pituitary gland, breast, skin, adrenal gland, blood vessels in target tissues, gastrointestinal tract, cells of immune system and bone [24,25]. In this study, it was found that LHR also expressed in both HUVECs and OVCAR-3 cells. This finding implicates that LH has a potential effect on regulating vascular endothelial functions.

Keeping vascular endothelia with normal structure and function is essential for maintaining vascular homeostasis, secreting vasodilatory and vasoprotective NO [26]. Endothelial dysfunction induced by atherogenic factors reduces NO secretion and increases superoxide production, resulting in elevated endothelial permeability, lipid accumulation and oxidation, monocyte chemotactic recruitment, foam cell formation, and finally leading to atherosclerotic lesion formation [27]. Clinically, endothelial dysfunction is measured by abnormalities in endothelial-dependent vasodilatation. In this study, high concentration of LH (100 mIU/ml), which is approximately close to the serum LH level in postmenopausal women [28], attenuated vasodilation induced by Ach. It is well known that vasodilation induced by Ach is attributed to its stimulating NO release from endothelia [29]. The subsequent findings that LH dose- and time-dependently decreased NO release from HUVECs, implicated that LH reduced vasodilation by inhibiting NO release from HUVECs. However, a *vivo* study in rat showed an inconsistent result that intraventricular administration of LH caused cutaneous vasodilation, hypertension and tachycardia [30]. This cutaneous vasodilation effect may be due to baroreceptor reflex induced by elevated blood pressure caused by LH. Converse result was also found in bovine granulosa cells that LH stimulated eNOS mRNA expression and increased NO release in a prostaglandin-dependent manner [31]. The

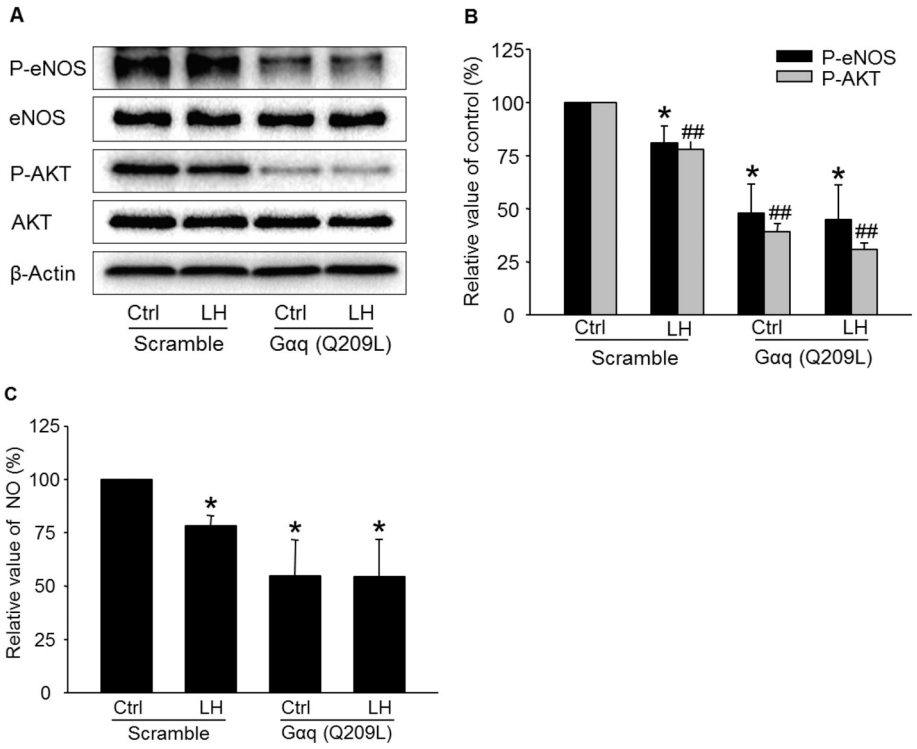

Fig. 7. Persistent expression of Gαq blocked eNOS and Akt phosphorylation. (A) Representative western blots of protein levels in HUVECs transfected with Gαq (Q209L) lentiviral particles with or without LH (100 mIU/ml) treatment for 24 h. (B) Summarized band intensities of phosphorylated eNOS (P-eNOS) and Akt (P-Akt) in western blot. (C) Relative NO concentration in the culture medium of HUVECs transfected with Gαq (Q209L) lentiviral particles and then exposed to 100 mIU/ml LH for 24 h. (*, $P < 0.05$ vs. scramble control; ##, $P < 0.01$ vs. scramble control. n = 3).

underlying mechanism of this effect may be that LH causes the release of the membrane-bound proteins epiregulin (EREG) and amphiregulin (AREG) from granulosa cells that then activate the EGF receptor, resulting in ERK1/2 and Akt activation, and finally leading to the activation of eNOS [31]. These data indicate that actions of LH might be diverse in different cells. Further work is still required to identify the precise mechanisms of actions of LH in different cells.

The PI3K/Akt pathway and the endothelial specific eNOS/NO pathway are pivotal for NO synthesis, and subsequently associated closely with blood pressure regulation, vascular remodeling, angiogenesis, endothelial progenitor cell proliferation and migration [19–21]. The activation of eNOS (eNOS phosphorylation) is mediated by phosphorylated Akt (P-Akt); and P-Akt itself is activated by PI3K in HUVECs [32]. Since elevated LH reduced the NO release from HUVECs, we therefore determined the role of PI3K/Akt pathway in the phosphorylation of eNOS in LH treated HUVECs. The corresponding results showed that LH decreased phosphorylated eNOS (P-eNOS) and P-Akt level, implying that elevated LH reduces NO release from HUVECs by inhibiting PI3K/Akt/eNOS pathway.

Our findings are in contrast to several studies showing that binding of LH to LHR activates the signaling pathway of PI3K/Akt in ovarian epithelial tumor cell [33]; LHR silencing down-regulated expression of PI3K/Akt in prostate cancer cells [34]; LH regulated apoptosis and steroidogenesis in goat theca cells by activating the PI3K/AKT pathway [35]. Given that LHR belongs to GPCR and the versatile functions of GPCR are dependent on the various α-subunits of the coupling G protein [36], the diverse functions of LH in different cells may be related to the α-subunits of G protein.

Like all GPCRs, LHR has seven transmembrane domains and couples to a heterotrimeric G protein which consists of three subunits, α-, β- and γ-subunit. According to the α-subunits: Gαs, Gαi/Gαo, Gαq/Gα11 and Gα12/Gα13, the G proteins can be divided into Gs, Gi, Gq/11, and G12/13 subfamilies [36,37]. Each of the four major subfamilies of G proteins is associated with different signaling pathways: Gs stimulates the adenylyl cyclase (AC); Gi/o inhibits AC; Gq/11 activates the phospholipase C (PLC); and G12/13 activates small GTPases [38]. LHR undergoes a conformational change when binding to LH or hCG, leading to the activation of the stimulatory Gs and activates AC in turn [39]. Whereas, LHR stimulation induced by high level of LH activates PLC [40]. Furthermore, extracellular signal-regulated protein kinases (ERK) and Akt have also been identified as downstream molecules in LHR-mediated signaling pathway [41]. Based on the studies listed above, it is plausible to speculate that the inhibitions of P-Akt and P-eNOS by high levels of LH in our study may attribute to a possible mechanism: the binding of LH to LHR activates a different sub-family of G proteins, which subsequently suppresses PI3K/Akt pathway.

Multiple studies revealed that Gαq-linked receptors negatively influence the growth factor-directed activation of PI3K and Akt isoforms [42–45]. Activated Gαq directly inhibits the PI3K p110α catalytic subunit and Akt in vitro [45,46]. However, it is noteworthy that the effect of Gαq on PI3K/Akt pathway can vary depending on the cell type. Agonists such as bradykinin, thrombin, interleukin-8, and carbachol promote the activity of PI3K/Akt in different cells ([47] and references therein). While, overexpression of Gαq in cardiomyocytes leads to cardiac hypertrophy, cardiomyocyte apoptosis, heart failure and decreased Akt phosphorylation [48]. These biphasic effects of Gαq on PI3K/Akt pathway implies the suggestion that low levels of Gαq activity may promote Akt phosphorylation and cell survival, whereas high or sustained Gαq activity induces an alternative cellular responses including apoptosis [43].

Since postmenopausal women have a relatively high level of LH, which may exert a long and persistent activation on the G protein of LHR, it is possible that the negative effect of elevated LH on Akt phosphorylation in this study is mediate by Gαq. This speculation was supported by our finding that LH significantly increased the interaction between Gαq and p110α. This was further demonstrated by the results that silencing Gαq remarkably abolished the inhibition of phosphorylation of Akt and eNOS induced by LH, while sustained expression of Gαq (Q209L) significantly blocked the phosphorylation of Akt and eNOS.

This finding is consistent with one report documented that activated Gαq inhibits p110α PI3K and Akt [45]. Based on these evidences, we therefore speculate that the LH activated Gαq might bind to PI3K and directly inhibit its catalytic activity, resulting in the inhibition of Akt/

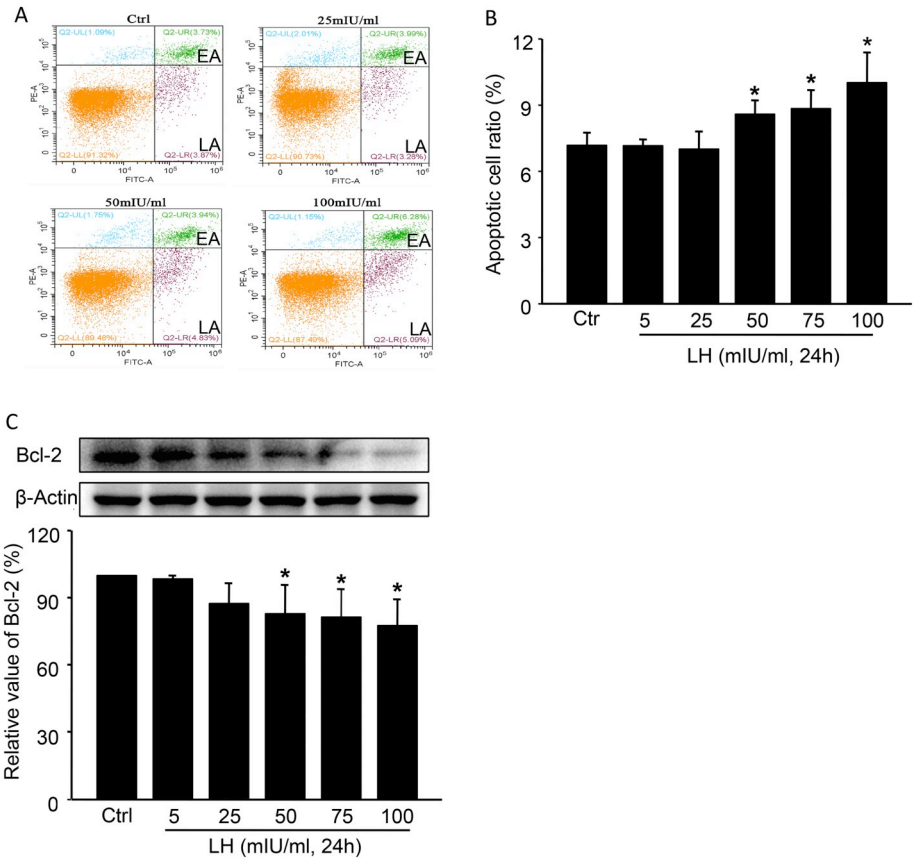

Fig. 8. LH promoted HUVECs apoptosis and decreased Bcl-2 protein expression. (A) Flow cytometry diagram of HUVECs treated with different concentrations of LH for 24 h. The subgroups of early apoptotic (EA) and late apoptotic (LA) cell were marked in the diagram. (B) Summarized flow cytometry results showed that LH (50–100 mIU/ml) increased apoptotic cell ratio markedly. (C) LH dose-dependently decreased anti-apoptotic marker Bcl-2 protein expression. (*, $P < 0.05$ vs. control; $n = 3$).

eNOS phosphorylation, and finally leading to the decrease of NO release which in turn contributes to the atherosclerosis formation.

Interestingly, high level of LH also promoted HUVECs apoptosis (Fig. 8A–B) and decreased Bcl-2 protein expression (Fig. 8C). Bcl-2 protein, an important anti-apoptotic marker, is modulated by PI3K/Akt signaling pathway [49]. Our findings implicate that the inhibition of PI3K/Akt by LH also inhibits Bcl-2 expression and then promotes endothelial cell apoptosis, which in turn benefits atherogenesis. Further works are required to be performed to clarify the precise mechanism underlying it.

In conclusion, our results reveal that elevated LH promotes atherosclerosis formation in OVX ApoE$^{-/-}$ mice. This effect may be mediated by inhibiting endothelial NO synthesis via suppressing PI3K/Akt signaling pathway. LH induced interaction between Gαq and PI3K plays a key role in this pathway. Our findings provide the first experimental evidence that LH directly contributes to atherogenesis. Intervening LHR or the corresponding signaling pathway may be an alternative approach to reduce CVD risk in postmenopausal women. Further investigation into how Gq-coupled receptors inhibit this signaling pathway might reveal important physiological insights into the postmenopausal diseases.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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