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Echinacoside-induced nitric oxide production in endothelial cells: Roles of androgen receptor and the PI3K-Akt pathway

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Abstract. Echinacoside (ECH) is a natural compound with an endothelium-dependent vasodilatory effect. Nitric oxide (NO) is an important vasorelaxant released from endothelial cells. In order to examine the molecular mechanism of ECH-induced NO production in endothelial cells, the present study investigated the involvement of androgen receptor (AR) and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway in the phosphorylation of endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (HUVECs). Using the fluorescent probe DAF-FM, the production of NO was found to be significantly increased, and eNOS was phosphorylated at Ser1177 in a concentration-dependent manner under 0.01-10 μM ECH treatment in HUVECs. In addition, NO production and eNOS phosphorylation induced by ECH were diminished when pretreated with the AR antagonist nilutamide, or when transfected with AR small interfering RNAs. Furthermore, the ECH-induced phosphorylation of the Akt at Ser473 was abrogated by 5 μM wortmannin (a PI3K inhibitor). These data indicated that ECH stimulated NO production via the AR-dependent activation of eNOS in HUVECs, and that the PI3K/Akt pathway may be involved in eNOS phosphorylation induced by ECH.

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Abbreviations: NO, nitric oxide; ECH, echinacoside; AR, androgen receptor; ER, estrogen receptor; PI3K, phosphatidylinositol 3-kinase; Akt, serine/threonine-protein Akt kinase (protein kinase B); eNOS, endothelial isoform of nitric oxide synthase; siRNA, small interfering RNA; cGMP, cyclic guanosine monophosphate; PKG, cGMP-dependent protein kinase; HUVEC, human umbilical vein endothelial cell; ECM, endothelial cell medium; FBS, fetal bovine serum; ECGS, endothelial cell growth supplement; RT-qPCR, reverse transcription-quantitative PCR; DMSO, dimethylsulfoxide

Key words: echinacoside, nitric oxide, endothelial isoform of nitric oxide synthase, phosphatidylinositol 3-kinase/protein kinase B pathway, androgen receptor

Introduction

Cistanche Hoffmg. Et Link is a perennial parasite herb of a genus of the Orobanchaceae family. Herba *Cistanche*, the stem of *Cistanche deserticola* Y. C. MA and *Cistanche tubulosa* (Schenk) R. Weight, have been used as a tonic agent to treat kidney deficiency, impotence, morbid leukorrhea and senile constipation (1), which may be due to its androgen-like or sex hormone regulatory effect (2). Echinacoside (ECH; $C_{35}H_{46}O_{20}$; molecular weight, 786.73; Fig. 1), is one of the phenylethanoid glycosides (PhGs) isolated from the stems of Herba *Cistanche*, and exhibits multiple biological properties, including antioxidant, antiaging, anti-inflammatory, hepatoprotective and neuroprotective effects (3). Modern pharmacology investigations have demonstrated that various constituents of Herba *Cistanche*, including ECH, acteoside, kankanose, kankanoside F and cistanoside F, exhibit vasorelaxant activity (4).

The endothelium is a critical regulator of the vasculature, and nitric oxide (NO) is an important relaxing factor released by endothelial cells. By diffusing into smooth muscle cells, NO activates guanylate cyclase, increases the levels of cyclic guanosine monophosphate (cGMP), and then activates cGMP-dependent protein kinases (PKGs) to promote smooth muscle relaxation (5). It was previously demonstrated that ECH at 350-400 μM directly acts on vascular smooth muscle and inhibits hypoxia-induced proliferation in rat pulmonary artery smooth muscle cells (6), whereas 30-300 μM ECH caused acute vasorelaxation in endothelium-intact rings in a concentration-dependent manner, and enhanced cGMP production in the corpus cavernosum smooth muscle of aortic rings contracted by phenylephrine (7). By opening the NO-cGMP-PKG-BK_{Ca} channels in smooth muscle cells, 100 or 300 μM ECH suppressed noradrenaline-induced contraction in the pulmonary arteries of rats, particularly in endothelium-denuded rings (8). Endothelial cells are key regulators of cardiovascular function and, in several cases, it has been found that the endothelial-dependent relaxation was due to a transferrable substance, such as NO, released from the endothelium (9). However, the upstream molecular mechanism of ECH-induced NO production in vascular endothelial cells requires further investigation.

In the vascular endothelium, NO generation is primarily mediated by endothelial NO synthase (eNOS). Several groups have demonstrated that the phosphatidylinositol 3-kinase (PI3K) pathway activates the serine threonine protein kinase B (Akt), which causes direct eNOS phosphorylation at serine 1177 (Ser1177) (10). The androgen receptor (AR) is a member of the nuclear receptor subfamily s3, which canonically modifies gene expression. AR localization to the caveolae in the cell membrane is involved in the non-genomic regulation of endothelial cell function or gene expression by triggering the c-Src/PI3K/Akt cascade, which ultimately results in eNOS phosphorylation and NO production (11). In human aortic endothelial cells, testosterone has been reported to activate PI3K/Akt signaling and rapidly induce NO production due to the direct interaction of AR and the p85α subunit of PI3K on the cardiovascular system (12). It was reported that ECH aggravates hormone deficiency-related symptoms and exerts its androgen-like effects due to competitive binding to the AR instead of testosterone (2). Therefore, the present study hypothesized that the PI3K/Akt pathway may be involved in NO production through AR-dependent eNOS phosphorylation induced by ECH. The aim of the present study was to evaluate the following effects of ECH: i) Induction of NO production and eNOS phosphorylation; ii) involvement of AR in eNOS phosphorylation; and iii) activation of the PI3K/Akt pathway in human umbilical vein endothelial cells (HUVECs), a well-known experimental model for studying the regulation of endothelial cell functions and angiogenesis (13).

Materials and methods

Chemicals and reagents. ECH (purity, 92.5%) was obtained from the National Drug Reference Standards in National Institute for the Food and Drug Control. Antibodies against p-Akt (Ser473; cat. no. ab8805), Akt (cat. no. ab81283), p-eNOS (Ser1177; cat. no. ab184154) and eNOS (cat. no. ab76198) were purchased from Abcam. The inhibitors of nilutamide and ICI 182780 were obtained from Sigma-Aldrich; Merck KGaA. L-NAME was purchased from Adamas-Beta, Ltd., and wortmannin was purchased from Pribolab.

Cell culture and drug treatment. HUVECs were obtained from ScienCell Research Laboratories Inc. and cultured in endothelial cell medium (ECM; ScienCell Research Laboratories) with 5% (v/v) fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) and 1% endothelial cell growth supplement (ECGS) at 37˚C (5% CO_2 and 95% humidity) (14). Upon reaching confluence, the cells were digested with trypsin and plated in ECM with 1% FBS and 1% ECGS. For all experiments, HUVECs were plated at a concentration of $1x10^4$/ml and grown until reaching confluence. Prior to treatment with ECH or other stimulators, the cells were incubated in phenol red-free ECM without FBS and ECGS for 6 h to induce growth arrest. In the inhibitory experiments, HUVECs were pre-incubated with various antagonists or inhibitors, including 10 μM nilutamide, 10 μM ICI 182780, 0.5 mM L-NAME or 5 μM wortmannin, for 30 min, with or without ECH, at 37˚C. In all groups, including the control, DMSO was used as a solvent at equal concentrations of 0.001%.

Measurement of intracellular NO production. Relative changes in cytosolic NO concentration in HUVECs were monitored using the fluorescent NO probe DAF-FM (Cayman Chemical Company), as previously reported (12). Briefly, the cells were loaded with 5 μM DAF-FM diacetate for 20 min at 37˚C in a black microtiter plate and rinsed several times with PBS (pH 7.4). The fluorescence was determined at excitation and emission wavelengths of 495 and 515 nm, respectively, using a fluorescent microplate reader (Biotek Synergy H4; BioTek Instruments, Inc.) and a compact inverted microscope (Nikon eclipse Ts2R; Nikon Corporation).

Western blot analysis. As previously reported (15), confluent monolayers of cells were washed twice in ice-cold PBS and lysed with RIPA buffer (P0013D; Beyotime Institute of Biotechnology). The protein concentration in the supernatant was measured using the bicinchoninic acid assay method (16). Subsequently, 30 μg protein were loaded per lane, separated using 10% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk for 1 h at 25˚C. After incubation with monoclonal antibodies against Akt (1:1,000 dilution), p-Akt (1:500 dilution), eNOS (1:2,000 dilution), or p-eNOS (1:1,000 dilution) at 4˚C overnight, the membranes were washed with TBST (containing 0.1% Tween-20) 4 times for ~15 min per wash at 25˚C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution; anti-mouse antibody, cat. no. A0216, or anti-rabbit antibody, cat. no. A0208, Beyotime Institute of biotechnology) for 1 h at 25˚C, and detected using an enhanced chemiluminescence kit (cat. no. 32209, Thermo Fisher Scientific, Inc.). Band intensities were quantified using Image J gel analysis software, version 1.8.0_112 (National Institutes of Health).

Small interfering (si) RNA preparation and transfection. The long double-stranded RNAs were synthesized by the target mRNA of the AR and estrogen receptor (ER) with sequences shown in Table I. The conditions for siRNA knockdown involved transfecting HUVECs at 70% confluence maintained in non-antimicrobial culture medium in 60 mm collagen-coated culture dishes. Transfection of 5 nM AR-siRNA or 10 nM ERα-siRNA with Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was performed separately, according to the manufacturer's protocols. Transfection efficiency was evaluated by reverse transcription quantitative-PCR analysis (RT-qPCR), as previously reported (17). The thermocycling conditions were as follows: 10 min at 95˚C; 40 cycles of 95˚C for 5 sec and 60˚C for 1 min, and a melting curve at 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec; three independent biological replicates were performed for each sample. The subsequent experiments were conducted 48 h after transfection. Primer pairs were designed using Primer Premier v5.0 software (PREMIER Biosoft) with the following sequences: AR forward, 5'-GGTTACACCAAAGGGCTAGAA-3' and reverse, 5'-GACTTGTAGAGAGACAGGGTAGA-3'; ERα forward, 5'-CCAGTACCAATGACAAGGGAAG-3' and reverse, 5'-TCACAGGACCAGACTCCATAA-3'; and GAPDH forward, 5'-CAGGGCTGCTTTTAACTCTGGTAA-3' and reverse, 5'-GGGTGGAATCATATTGGAACATGT-3'.

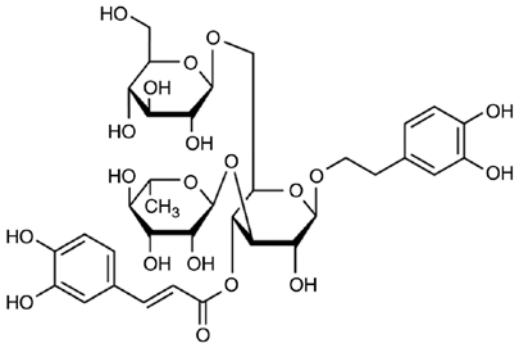

Figure 1. Chemical structure of echinacoside.

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical comparisons between groups were performed using the Kruskal-Wallis test or two-way ANOVA with Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ECH induces NO production and eNOS phosphorylation. As shown in Fig. 2A and B, 1 μM ECH significantly increased intracellular NO production in HUVECs compared with the negative control cells, while the stimulatory effect of ECH was attenuated to the control level by pretreatment with L-NAME. To obtain the optimal concentration, eNOS phosphorylation was tested at 60 min after treatment with ECH at concentrations of 0, 0.01, 0.1, 1 and 10 μM. The results revealed that ECH at concentrations of 0.01-10 μM may significantly induced eNOS phosphorylation at Ser 1177 in a concentration-dependent manner, with the maximal eNOS phosphorylation observed at 10 μM ECH induction (Fig. 2C and D). Furthermore, eNOS phosphorylation at Ser1177 was examined by western blot analysis at 0, 5, 15, 30, 60 and 120 min after incubation with 1 μM ECH. It was observed that eNOS phosphorylation was rapidly triggered by ECH at 5 min, and continued to increase until 30 min of incubation (Fig. 2E and F). Subsequently, despite ECH treatment, the relative magnitude of eNOS phosphorylation remained stable from 30 min onwards; therefore, ECH did not affect total eNOS expression (Fig. 2C and E).

AR mediates ECH-induced NO production and eNOS phosphorylation. RT-qPCR assay demonstrated that the interfering effects of AR-siRNA-1 and ERα-siRNA-2 were the most successful in inhibiting the expression of AR and ER in HUVECs in the present study (Fig. 3). Subsequently, an antagonist for AR inhibition-of-function analysis and siRNA for AR loss-of-function analysis were applied to evaluate the involvement of AR in ECH-induced eNOS activation and NO production. As shown in Fig. 4A, 1 μM ECH significantly increased NO production in HUVECs ($P<0.05$). Pretreatment with the AR antagonist nilutamide (10 μM) abolished ECH-induced NO production, whereas ICI182789 (an ER antagonist) did not exert the same effects. Furthermore, the effects of siRNA-mediated AR knockdown on NO production were examined in cultured cells, indicating that NO production was diminished by transfection with AR siRNAs; however, it was not affected by ER siRNAs or control random siRNAs (Fig. 4B). Representative western blots and semi-quantitative analysis (Fig. 4C and D) revealed that the phosphorylation of eNOS induced by ECH was inhibited by nilutamide and ICI182789, and that the inhibitory effect of nilutamide on ECH-induced eNOS activation was significantly higher compared with that of ICI182789, which indicated that inhibition of AR function had a greater impact than ER on eNOS phosphorylation induced by ECH. Similarly, the phosphorylation of eNOS induced by ECH was reduced significantly in cells transfected with AR-siRNA and ER-siRNA compared with the control random siRNA; the inhibitory effect of AR-siRNA on ECH-induced eNOS activation was significantly stronger compared with that of ER-siRNA (Fig. 4E and F). Therefore, the aforementioned results suggested that ECH may cause AR-dependent activation of eNOS to induce NO production in HUVECs.

ECH activates the PI3K/Akt pathway. In the present study, Akt phosphorylation at Ser473 was tested 60 min after ECH incubation at concentrations of 0, 0.01, 0.1, 1 and 10 μM. The results indicated that ECH (0.01-10 μM) may significantly induce Akt phosphorylation. The relative expression levels of p-Akt peaked at 1 and 10 μM of ECH treatment (Fig. 5A and B). Furthermore, Akt phosphorylation was examined by western blot analysis at 0, 5, 15, 30, 60 and 120 min after the addition of 1 μM ECH to HUVEC cultures. As shown in Fig. 5C and D, ECH rapidly increased Akt phosphorylation after 5 min of incubation, and the maximum protein level of p-Akt was observed at 60 min. By contrast, ECH did not affect the total Akt expression (Fig. 5A and C). Moreover, to investigate the potential effect of PI3K pathway on eNOS phosphorylation, HUVECs were pre-treated with the PI3K inhibitor wortmannin prior to ECH application. Wortmannin was found to reduce ECH-induced NO production to baseline levels (Fig. 5E). A similar phenomenon was observed using fluorescence microscopy when examining ECH-induced DAF-FM fluorescence (Fig. 5F). Furthermore, wortmannin may have the ability to abolish rapid eNOS phosphorylation in HUVECs (Fig. 5G and H). The aforementioned results suggest that ECH may activate eNOS phosphorylation and NO production via the PI3K/Akt pathway.

Discussion

ECH is a natural compound isolated from Herba *Cistanche*, with various pharmacological properties. It was previously demonstrated that ECH exerted an endothelium-dependent vascular relaxation effect by opening NO-cGMP-PKG-BK_{Ca} channels in blood vessel smooth muscle cells (7,8). The current findings provide evidence that ECH exerted AR-dependent activation of eNOS to induce NO production with the involvement of the PI3K/Akt signaling pathway in vascular endothelial cells.

As the innermost layer of the vessel wall, the endothelium can quickly sense and respond to changes in blood flow, in turn resulting in signal transmission to the underlying smooth muscle cells in order to regulate vascular tone (18). It has been widely reported that multiple endothelial-derived vasodilatory compounds exist, with the most prototypical substance

Table I. The sequences of the sense RNA strand targeting AR and ER in siRNA experiments.

Target gene	Name	Sequences (5'-3')
AR	siRNA-1	AAACAGGUACUUCUGUUUCCC
	siRNA-2	AAUGCAAAGGUUCUCUGCUAG
	siRNA-3	AAGGUCUUCUUCAAAAGAGCC
ERα	siRNA-1	GAUCAAACGCUCUAAGAAG
	siRNA-2	GAAUGUGCCUGGCUAGAGA
	siRNA-3	GAUGAAAGGUGGGAUACGA
ERβ	siRNA-1	GGAAAUGCGUAGAAGGAAU
	siRNA-2	UUCAAGGUUUCGAGAGUUA
	siRNA-3	GCACGGCUCCAUAUACAUA
Control	siRNA-random	AAGUGCGAUCUAACUGACCUA

AR, androgen receptor; ER, estrogen receptor.

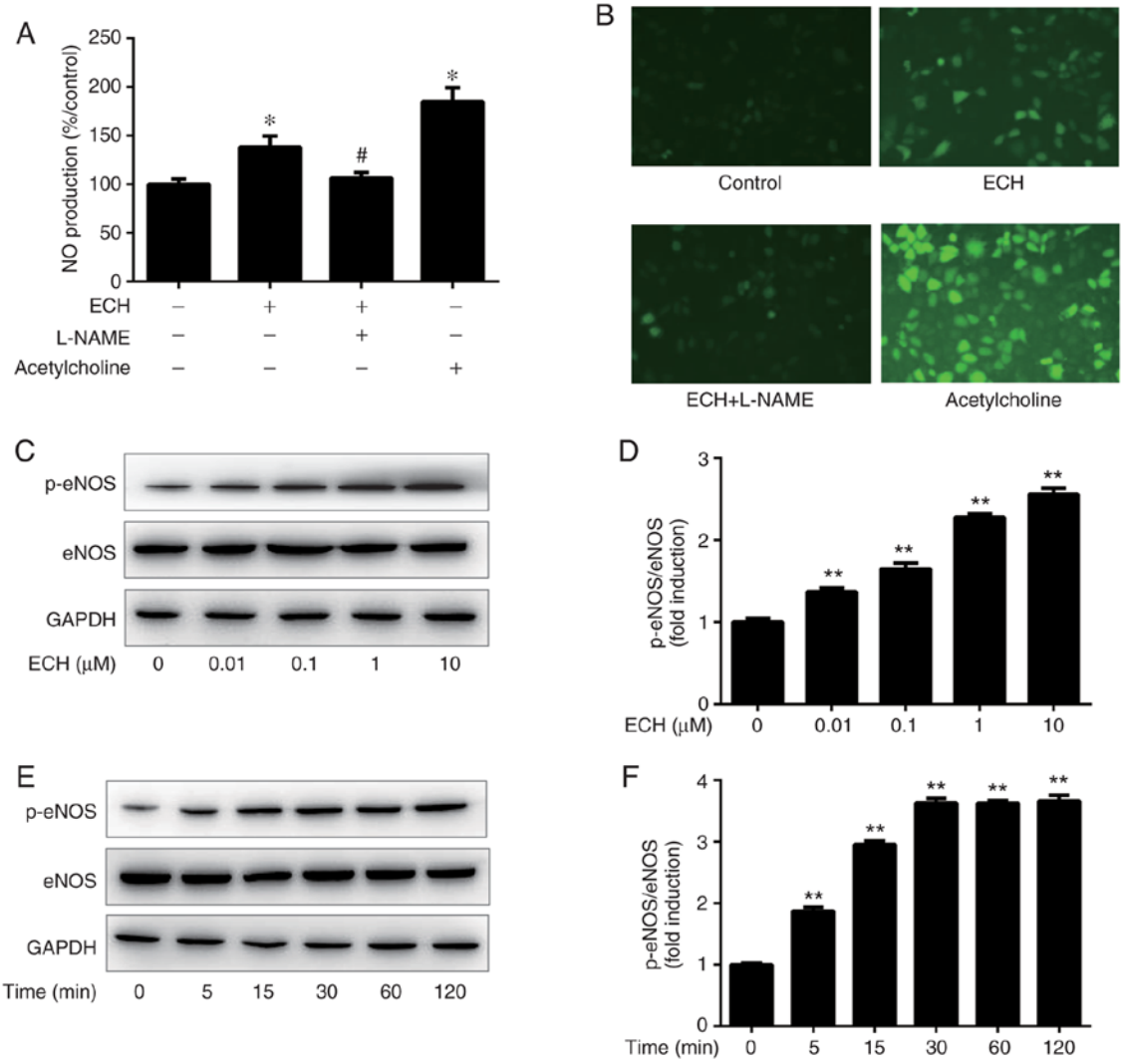

Figure 2. Effects of ECH on NO production and eNOS phosphorylation in HUVECs. (A) Following administration with 0.5 mM eNOS inhibitor L-NAME for 30 min, the cells were treated with 1 μM ECH or 1 μM acetylcholine (positive control). Intracellular NO production was measured using a fluorescence microplate reader; (n=3). *P<0.05 compared with the control group and $^{\#}$P<0.05 compared with the ECH-treated alone group. (B) Optical fluorescence microscopy images of control and ECH-treated cells in the presence or absence of L-NAME at an emission wavelength of 515 nm and an excitation wavelength of 495 nm. Representative (C) western blot and (D) semi-quantitative analysis of the protein levels of total eNOS and p-eNOS in starved cells incubated with different ECH concentrations for 60 min. Representative (E) western blot and (F) semi-quantitative analysis of the protein levels of total eNOS and p-eNOS in starved cells incubated with 1 μM ECH at various time points. **P<0.01 vs. control (n=3). NO, intracellular nitric oxide; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; ECH, echinacoside.

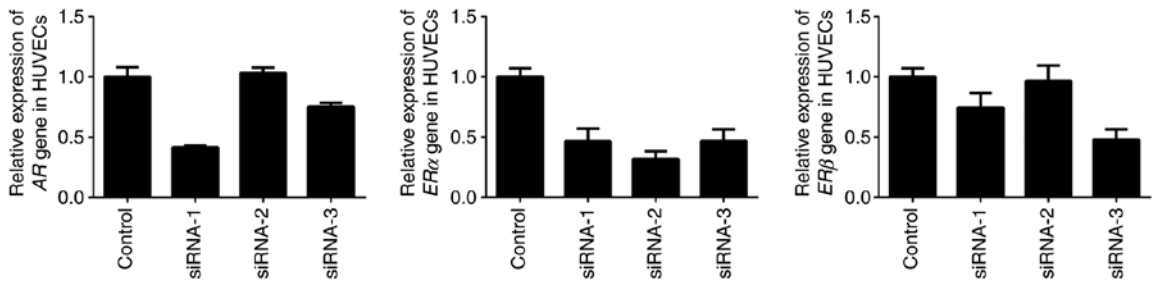

Figure 3. Relative mRNA expressions of androgen receptor (AR) and estrogen receptor (ER) after injecting AR-siRNA-1 and ERα-siRNA-2 in HUVECs. HUVECs, human umbilical vein endothelial cells.

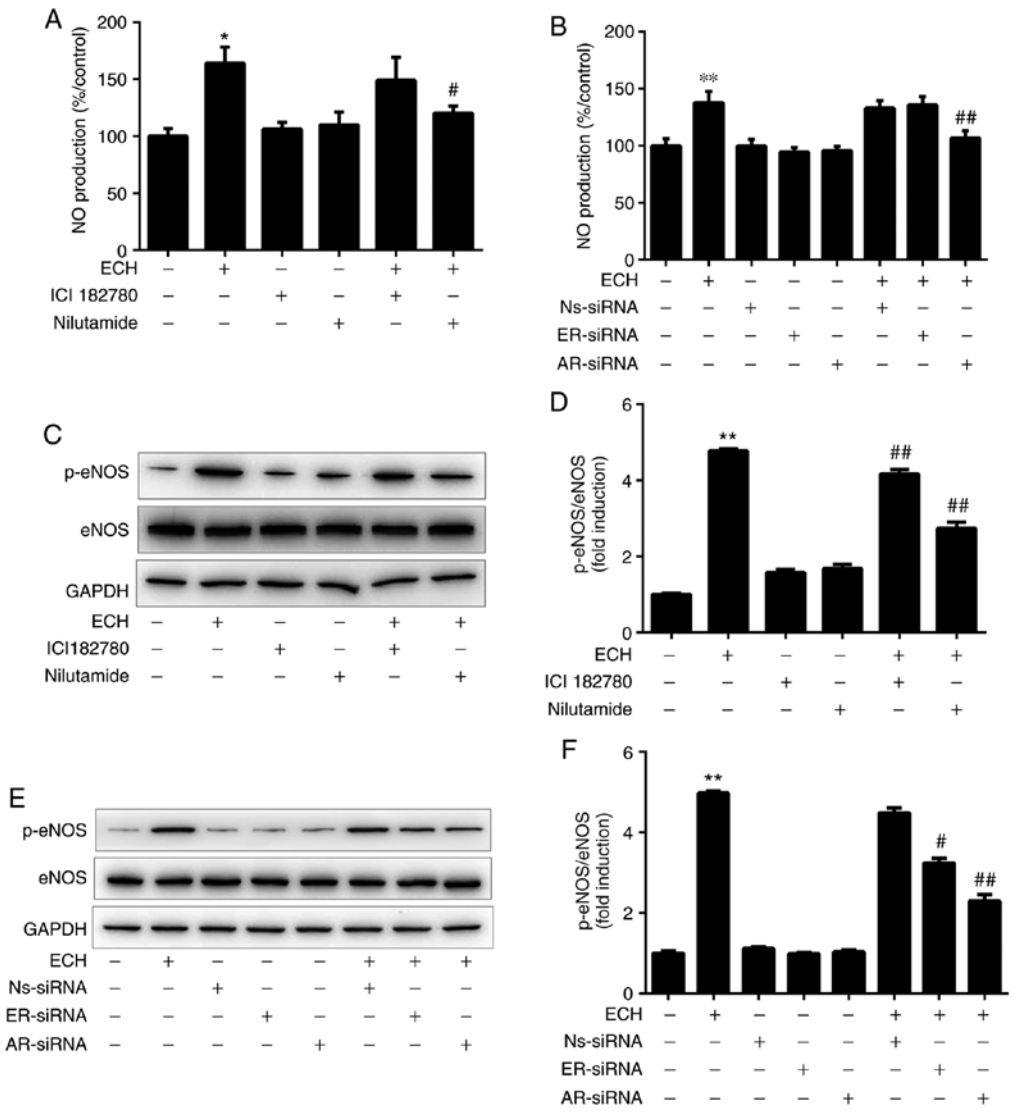

Figure 4. Involvement of AR in ECH-induced NO production and eNOS phosphorylation in HUVECs. (A) Starved cells were pre-treated with nilutamide (AR antagonist) or ICI 182789 (ER antagonist) for 30 min prior to treatment with 1 μM ECH. (B) Intracellular NO production was assessed using the fluorometric method. Representative (C) western blot and (D) semi-quantitative analysis of the protein levels of total eNOS and p-eNOS in starved cells incubated with nilutamide (AR antagonist) or ICI 182789 (ER antagonist) for 30 min prior to treatment with 1 μM ECH. Representative (E) western blot and (F) semi-quantitative analysis of the protein levels of total eNOS and p-eNOS in starved cells treated with or without ECH for 60 min after transfection of the AR siRNA (5 nM), ERα siRNA (10 nM) or non-silencing random siRNA (10 nM). *P<0.05 or **P<0.01 compared with the negative control group and $^{\#}$P<0.05 or $^{\#\#}$P<0.01 compared with the ECH-treated alone group. NO, intracellular nitric oxide; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; ECH, echinacoside; AR, androgen receptor; siRNA, small interfering RNA.

being NO, formed from the endothelial isoform of eNOS, which results in phosphorylation (19). Under normal conditions, eNOS remains inactive when bound to caveolin, and is activated with the following succession of events in endothelial cells: i) eNOS dissociates from caveolin-1 and associates with Ca^{2+}/CaM; ii) heat shock protein (HSP)90 promotes eNOS

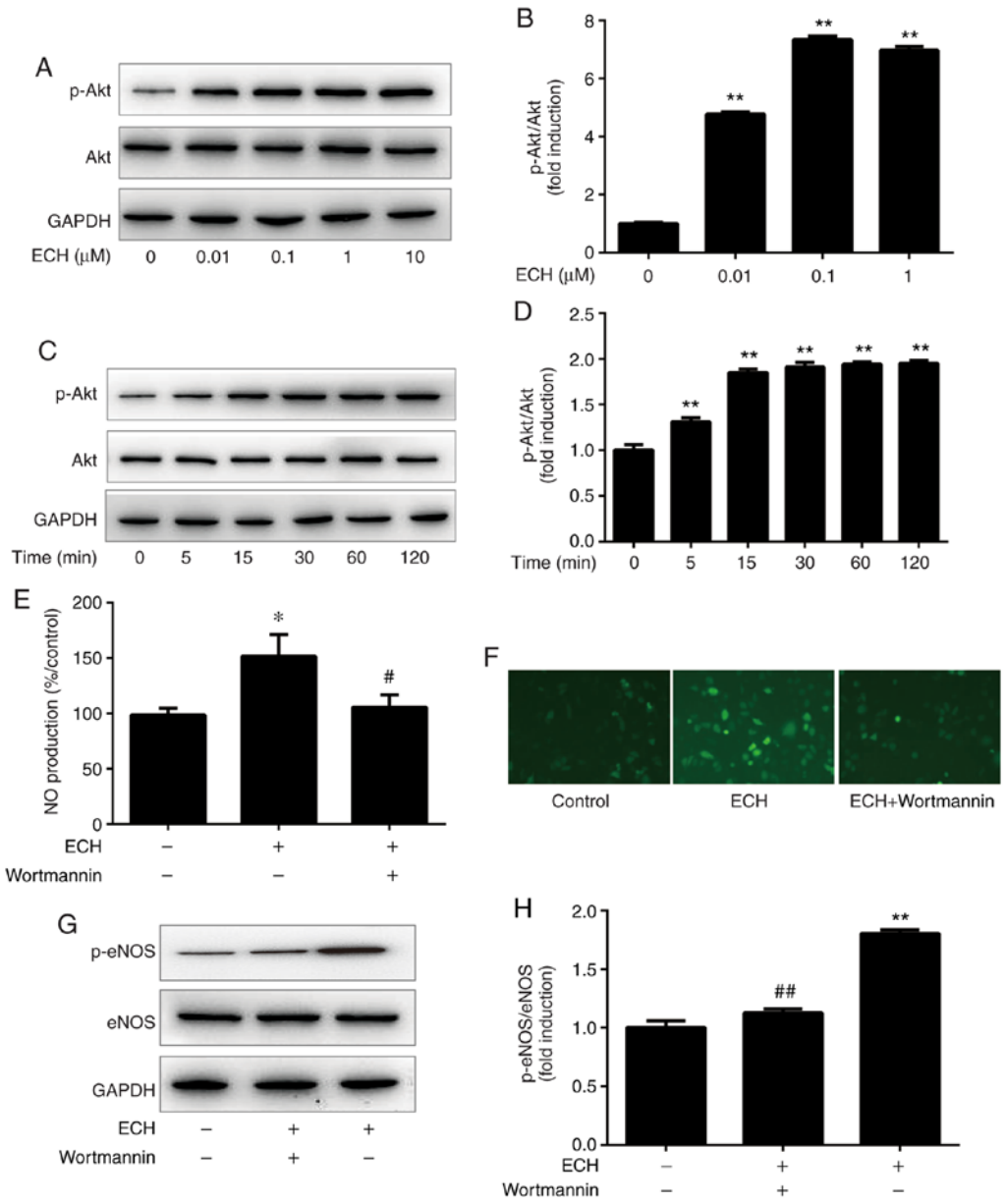

Figure 5. Involvement of the PI3K/Akt pathway in ECH-induced NO production and eNOS phosphorylation in HUVECs. Representative (A) western blot and (B) semi-quantitative analysis of the protein levels of total Akt and p-Akt in starved cells incubated with different ECH concentrations for 60 min. Representative (C) western blot and (D) semi-quantitative analysis of the protein levels of total Akt and p-Akt in starved cells incubated with 1 μM ECH at different time points. **P<0.01 vs. control (n=3). (E) Effects of a PI3K inhibitor on intracellular NO production using a fluorescence microplate reader. (F) Optical fluorescence microscopy images of ECH treated cells in the presence or absence of the PI3K inhibitor wortmannin (5 μM) at an emission wavelength of 515 nm and an excitation wavelength of 495 nm using fluorescent probe DAF-FM. Representative (G) western blot and (H) semi-quantitative analysis of the protein levels of total eNOS and p-eNOS in cells exposed to wortmannin. *P<0.05 or **P<0.01 compared with the control group and $^{\#}$P<0.05 or $^{\#\#}$P<0.01 compared with the ECH treatment alone group. NO, intracellular nitric oxide; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; ECH, echinacoside AR, androgen receptor; siRNA, small interfering RNA; PI3K, phosphatidylinositol 3-kinase.

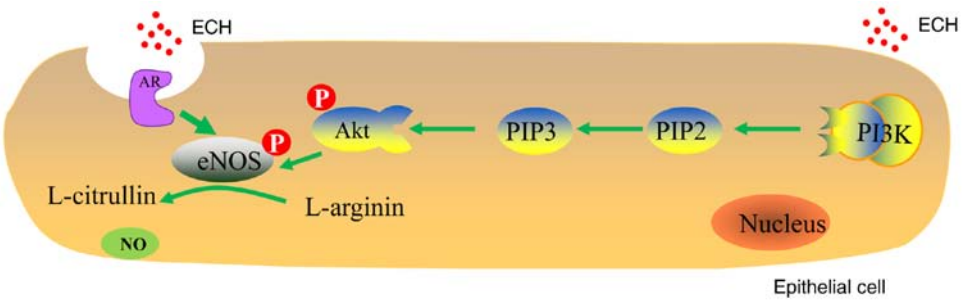

Figure 6. Schematic diagram of the suggested mechanism underlying AR-dependent NO production of ECH. eNOS, endothelial nitric oxide synthase; NO, intracellular nitric oxide; ECH, echinacoside; AR, androgen receptor.

dimerization and favors a steric formation to recruit Akt; and iii) calcineurin dephosphorylates Thr495 to reinforce the activation state (20). In the present study, NO production was significantly increased in HUVECs treated with 1 μM ECH. This phenomenon was similar to previous results, where ECH increased NO release and stimulated the synthesis of cGMP in rat thoracic aortic rings (7). Furthermore, two phosphorylation sites, Ser1177 and Thr495, appear to be particularly important for regulating eNOS activity (20), and the present study revealed that ECH induced acute eNOS phosphorylation at Ser1177 in a concentration-dependent manner. In addition, the stimuli associated with the effects on eNOS protein levels mainly determine eNOS mRNA stability, and if the stimulus is maintained for a longer period of time, eNOS mRNA transcription occurs via mitogen-activated protein kinase and nuclear factor κB (20). Taken together, these observations indicate that the activation of eNOS at Ser1177 is responsible for NO synthesis induced by ECH.

Furthermore, an increasing number of studies have demonstrated that AR is expressed in endothelial cells in a number of human tissues, which suggests a potential role for androgens and their analogues, that act through AR-mediated processes, in the modulation of human endothelial cell homeostasis (21). In the non-classical PI3K/Akt pathway, AR may activate PI3K by directly interacting with PI3K regulatory subunit p85α (22). The present study demonstrated that an AR antagonist or AR siRNA diminished the NO production and eNOS phosphorylation induced by ECH in HUVECs. It has been previously reported that estrogens induce eNOS phosphorylation and stimulate NO production via classic ER activation in endothelial cells (23), and the administration of ECH significantly enhances the expression of ER in the uterus (24). However, in the present study, the effect of AR was more prominent compared with that of ERα on ECH-induced eNOS activation and NO production. In addition, it was observed that ECH caused acute NO production within minutes via AR-involved eNOS activation in HUVECs, which is consistent with the non-genomic nature of the response in endothelial cells. The AR is associated with scaffolding proteins, including HSP90, HSP70 and kinase Src, in the cytoplasm, and it can be transported to the membrane from the AR complex within 5 min of testosterone treatment (23). Based on the 'target fishing' strategy, HSP90 was identified as the PhGs-coupled target, which indicated that ECH may facilitate the dissociation of AR from the scaffolding proteins (25). Another study suggested that, in the hypothalamus, ECH may combine with the AR pocket at amino acids Met-894 and Val-713 and inhibit the transport of cytoplasmic AR to the nucleus (26). However, the underlying mechanism through which ECH mediates cytoplasmic AR translocation to the membrane requires further investigation. Therefore, further research is required to elucidate the mechanism through which ECH achieves AR-dependent eNOS activation and how it may be associated with binding to HSP90 in vascular endothelial cells.

The PI3K/Akt pathway is one of the most important signaling cascades, the activation of which is induced by producing phosphatidylinositol-3,4,5-trisphosphate to bind the N-terminal pleckstrin homology domain of the Ser/Thr kinase Akt. This facilitates Akt recruitment to the plasma membrane (27). The PI3K/Akt pathway may play an important role in the control of NO-dependent relaxation induced by ECH. The present study revealed that ECH-induced NO production was significantly reduced when the cells were incubated with the PI3K inhibitor wortmannin. A previous report demonstrated that 15 mg/kg ECH activated the PI3K/Akt signaling pathway in 5-fluorouracil-suppressed bone marrow cells (28). In the present study, PI3K inhibitors significantly decreased ECH-induced eNOS phosphorylation at Ser1177. Moreover, Akt activity was predominantly regulated by upstream regulatory pathways, particularly PI3K-dependent phosphorylation at Ser473 (20). In the present study, ECH induced Akt phosphorylation at Ser473 in a dose-dependent manner; similarly, a previous study demonstrated that 5, 10 or 20 μM ECH exerted a cardioprotective effect against anoxia/reperfusion treatment in a dose-dependent manner by potentially upregulating p-Akt and SLC8A3 (29). The transcriptional regulation of the Akt gene remains largely unknown (30); therefore, the present study focused on the post-transcriptional regulatory effects of ECH on Akt. Taking the aforementioned findings into consideration, it was inferred that the application of ECH to HUVECs may lead to the activation of the PI3K/Akt pathway, which phosphorylates eNOS and, subsequently, increases NO production.

In conclusion, ECH is a natural product that is mainly isolated from Herba *Cistanche*. The potential mechanism underlying ECH-induced NO production in endothelial cells may include the following (Fig. 6): i) ECH acts as a functional ligand of AR that is localized to the caveolae in the cell membrane; ii) PI3K binds to the hydrophobic domain of Akt at Ser473 and facilitates Akt recruitment to the cell membrane; iii) the recruitment of the PI3K/Akt cascades triggers AR-dependent eNOS phosphorylation; and iv) the generation of NO is mediated by eNOS in endothelial cells. The observation that ECH induces NO production through the AR-dependent phosphorylation of eNOS with the involvement of the PI3K/Akt pathway may contribute to further understanding of the vasorelaxant effects of ECH. Furthermore, the ECH targeting the endothelial-derived NO pathways may be due to non-genomic effects. Therefore, the present study may help elucidate the mechanisms through which ECH exerts its pharmacological effects to prevent cardiovascular disease.

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Availability of data and materials

The data sets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LG and XL conceived and designed the experiments. DL and YZ performed the experiments. WZ and JG analyzed the data. LG drafted the manuscript. XL reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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