Bazedoxifene activates the angiotensin II-induced HUVEC hypertension model by targeting SIRT1

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Abstract. The shift in vascular function to vasoconstriction, pro-inflammatory state, oxidative stress and carbon monoxide deficiency may to endothelial dysfunction and injury, which is the key event in hypertension. Estrogen receptor modulators play a protective role in blood vessels. The present study aimed to investigate the effect of bazedoxifene, a selective estrogen receptor modulator, on human umbilical vein endothelial cells (HUVECs) and its potential underlying mechanism of action. The present study treated endothelial cells with different concentrations of bazedoxifene and determined cell viability using Cell Counting Kit-8 to screen for the optimal working concentration of bazedoxifene. Subsequently, an angiotensin II (AngII)-induced vascular endothelial cell model was established to observe the effect of bazedoxifene on AngII-induced endothelial cells. The concentrations of nitric oxide (NO) and reactive oxygen species (ROS) were detected using NO and ROS kits, respectively. The protein expression of sirtuin 1 (SIRT1), oxidative stress-related proteins and apoptosis-related proteins was detected using western blotting, and apoptosis was detected using a TUNEL assay. The results demonstrated that bazedoxifene promoted AngII-induced HUVEC viability, reduced the expression of stress-related proteins and inhibited apoptosis. Furthermore, bazedoxifene activated SIRT1 to promote the proliferation and inhibit the oxidative stress and apoptosis of AngII-induced HUVECs. These findings suggested that bazedoxifene could effectively promote AngII-induced HUVEC proliferation and inhibit cell apoptosis and oxidative stress. In addition, bazedoxifene protected HUVEC dysfunction induced by AngII by targeting the activation of SIRT1. In summary, bazedoxifene could improve the protective role against hypertension induced by AngII.

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Introduction

The complete mechanism of hypertension is complex and remains unclear. Clinical treatments [such as diet modification, increased exercise and a selection of appropriate blood pressure lowering medications (diuretics combined with angiotensin receptor antagonists or angiotensinase I inhibitors)] can effectively control the increase in high blood pressure in patients with hypertension; however, complications of hypertension can still occur, including stroke, heart failure and kidney damage (1-3). Emerging evidence has indicated that increased oxidative stress is involved in the pathogenesis of hypertension, which results in an abundance of reactive oxygen species (ROS) (4). During this process, nitric oxide (NO), synthesized by one of the NO synthase isoforms, inducible NO synthase, is a marker of oxidative stress (5). As a key enzyme in NO release, endothelial NO synthase (eNOS) also exerts a notable role in mediating oxidative stress (6). The development of hypertension is influenced by the interaction of several components, the most important being the abnormal renin-angiotensin system (RAS) (7). Angiotensin II (AngII), an important component of the RAS, can promote vasoconstriction, increase peripheral resistance and cause vascular dysfunction (8). Therefore, effective inhibition of AngII-induced vascular smooth muscle dysfunction could prevent and help the treatment of cardiovascular diseases such as hypertension.

Estrogen receptor modulators can play a vasoprotective role similar to that of estrogen (9). It has been reported that both tamoxifen and raloxifene can decrease the concentration of total serum cholesterol and low-density lipoprotein (10). Based on the reduction of lipids and inflammatory markers, tamoxifen is effective at reducing the burden of coronary heart disease (11). Following tamoxifen treatment, the mortality rate of myocardial infarction and ischemic heart disease is decreased (12). In a small group of women with high cardiovascular risk during menopause, raloxifene is associated with a reduced incidence of cardiovascular disease (13). In addition, tamoxifen can reduce the risk of heart disease and stroke; however, the risk of deep venous thrombosis development is similar to that with other selective estrogen

receptor modulators, mainly increasing the risk of femoral vein thrombosis, pulmonary embolism and retinal venous thrombosis, although the relative incidence is very low (14).

Bazedoxifene, an estrogen receptor modulator, can alleviate cardiac hypertrophy induced by blood pressure overload *in vivo* by inhibiting interleukin (IL)-6 cytokine family signal transducer signaling transduction and can reduce myocardial fibrosis induced by AngII in mice (15,16). Furthermore, bazedoxifene can inhibit arterial aging and the development of atherosclerosis by increasing the expression level of sirtuin 1 (SIRT1) (17). Furthermore, SIRT1 activation can be used to treat hypertension by enhancing AMP-activated protein kinase activity (18). Therefore, the present study explored whether bazedoxifene may have the ability to attenuate AngII-induced vascular endothelial cell dysfunction by targeting the activation of SIRT1.

Materials and methods

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (cat. no. PCS-100-010). The cells were cultured in DMEM (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and placed at 37˚C in a humidified incubator with 5% CO_2.

The experiments were performed 24 h after induction of cells with 1 μM AngII (19). Cells in the treatment group were treated with bazedoxifene (2, 4, 6, 8 and 10 μM) for 1 h prior to AngII induction for 2 h (20). To block SIRT1, cells were incubated with 1 μM of the inhibitor EX527 for 48 h prior to bazedoxifene treatment (21). All drugs were purchased from Sigma-Aldrich, Merck KGaA.

Cell transfection. SIRT1 small interfering (si)RNA (si-SIRT1) and siRNA negative control (si-NC) were synthesized by Shanghai GenePharma Co., Ltd. Cells were seeded into 6-well plates at a density of $3x10^5$ cells/well and cultured for 24 h at 37˚C. In strict accordance with the manufacturer's instructions, the siRNAs (100 nM) were transfected into the cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The transfection efficiency was detected using reverse transcription quantitative (RT-q)PCR 48 h following transfection. The sequences of the siRNAs were as follows: si-SIRT1-1, 5'-GCTAAGAATTTCAGGATTA-3'; si-SIRT1-2, 5'-ACTTTGCTGTAACCCTGTA-3'; si-NC, 5'-ACUUUC AUAAGUCUUCGUGGG-3'.

Cell Counting Kit (CCK)-8 assay. Trypsin (Beyotime Institute of Biotechnology) was used to digest HUVECs and prepare a single cell suspension. Subsequently, cells were seeded into 96-well plates at the density of $2x10^3$ cells/well for 24 h. A total of 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc., Japan) was then added to the each well of the 96-well plates. Cells were further incubated for 1 h and the absorbance at 450 nm was detected using a spectrophotometer (Thermo Fisher Scientific, Inc.).

NO and ROS determination. NO and ROS production were evaluated using NO assay kit (cat. no. BC1475) and ROS (CA1410-100T) assay kit (Beijing Solarbio Science & Technology). Briefly, for NO determination, cells were treated with the extract from the kit, sonicated in an ice bath for 3 min and centrifuged at 4˚C for 15 min at 12,000 x g. The absorbance of the centrifuged supernatant at 550 nm was measured using a spectrophotometer to determine NO production. For ROS determination, DCFH-DA was diluted 1:1,000 with serum-free medium (HyClone; Cytiva) to a final concentration of 10 μmol/l. After removing the cell culture medium, enough diluted DCFH-DA was added to cover the volume of cells. They were then incubated for 20 min at 37˚C in a cell incubator. DCFH-DA was removed, and the cells were washed with serum-free medium. Finally, the fluorescence intensity at 525 nm emission wavelength (the absorbance) was measured.

TUNEL assay. Apoptosis was detected using a TUNEL Apoptosis Assay kit (Beyotime Institute of Biotechnology). Briefly, HUVECs were washed with PBS, fixed with 4% paraformaldehyde for 20 min and treated with 0.1% Triton X-100 for 10 min, all at room temperature. Cells were incubated with TUNEL assay solution (TdT enzyme: Fluorescent labeling solution=1:9) at 37˚C for 1 h. Cell nucleus were stained with 10 μg/ml DAPI for 5 min at 37˚C. Fluorescence expression of the apoptotic cells was observed under an EVO fluorescence microscope (Advanced Microscopy Group), and five fields of view were randomly observed in each group. The green fluorescence was considered to stain apoptotic cells and the blue stained nuclei, and the cells were counted using ImageJ (V1.8.0.112; National Institutes of Health). The apoptotic rate was calculated as follows: Apoptosis rate = (average number of apoptotic cells/average number of total cells) x100%.

RT-qPCR. Total RNA was extracted from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). A reverse transcription kit (Takara Bio, Inc.) was subsequently used to reverse transcribe the RNA into cDNA according to the manufacturer's instructions. RT-qPCR reactions were performed using the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The relative expression levels were normalized to endogenous control and were expressed as $2^{-\Delta\Delta Cq}$ (22). The sequences of the primers were as follows: SIRT1 forward, 5'-TATGGCTGACTTCGCTTTGG-3', reverse, 5'-TCGGGG CACTGATTTCTGTA-3'; and GAPDH forward, 5'-GGAGCG AGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGT CATACTTCTCATGG-3'.

Western blotting. Cells were lysed with RIPA lysate (Beyotime Institute of Biotechnology) containing 1% protease inhibitor and 1% phosphatase inhibitor (both from Solarbio), centrifuged at 4˚C for 5 min with 3,220 x g and BCA (Beyotime Institute of Biotechnology) was used to quantify the protein concentration in the supernatant. Proteins (40 μg/lane) were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto PVDF membranes (MilliporeSigma). Subsequently, membranes were blocked with 5% skimmed milk for 2 h at room temperature and incubated with primary antibodies against phosphorylated-eNOS (p-eNOS; 1:300; cat. no. ab215717; Abcam), eNOS (1:400; cat. no. ab252439;

Abcam), p47 phox (1:300; cat. no. ab181090; Abcam), Bcl-2 (1:400; cat. no. ab182858; Abcam), Bax (1:400; cat. no. ab32503; Abcam), cleaved caspase-3 (1:500; cat. no. ab32042; Abcam), caspase 3, (1:500; cat. no. ab32351; Abcam), SIRT1 (1:400; cat. no. ab189494; Abcam), β-actin (1:1,000; cat. no. ab8227; Abcam) and GAPDH (1:1,000; cat. no. ab181602; Abcam) overnight at 4˚C. Subsequently, the membranes were washed with PBS-10% Tween-20 three times and were incubated with the goat anti-rabbit IgG H&L (HRP; 1:1,000; cat. no. ab7090; Abcam) for 2 h at room temperature. Pierce Western Blotting Substrate (Thermo Fisher Scientific, Inc.) was used to detect the signal on the membrane. The data were analyzed via densitometry using ImageJ software and normalized to expression of the internal controls β-actin or GAPDH.

Statistical analysis. Each experiment was repeated independently at least three times. The data are presented as the means ± standard deviation. Statistical analyses were performed using SPSS v19.0 software (IBM Corp.). One-way ANOVA followed by Tukey's post hoc test was used for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Bazedoxifene promotes the proliferation of HUVECs induced by AngII. The effect of bazedoxifene on normal endothelial cells was determined, as shown in Fig. 1A. The results from CCK-8 assay demonstrated that low concentrations of bazedoxifene had little effect on cell viability. However, when the concentration of bazedoxifene was >6 μM, cell viability was significantly decreased, demonstrating a cytotoxicity of bazedoxifene. Subsequently, the concentrations of 2-6 μM bazedoxifene were selected in subsequent experiments. The cells were then pretreated with bazedoxifene for 1 h, followed by treatment with 1 μM AngII for 24 h. The results from CCK-8 assay showed that bazedoxifene promoted the viability of HUVECs induced by AngII (Fig. 1B).

Bazedoxifene reduces AngII-induced oxidative stress in endothelial cells. The effect of bazedoxifene on AngII-induced endothelial cells was further investigated, and NO content was detected using a NO kit. As presented in Fig. 1C, bazedoxifene upregulated the decreased NO content in Ang-II-induced HUVECs in a concentration-dependent manner. Furthermore, bazedoxifene treatment decreased the ROS content in AngII-induced endothelial cells (Fig. 1D). Subsequently, the expression levels of oxidative stress-related proteins were detected using western blotting. The protein expression levels of p-eNOS and eNOS were decreased, while the protein expression levels of p47 phox were increased in AngII-induced endothelial cells. Furthermore, the protein expression levels of p-eNOS and eNOS were increased, while the protein expression level of p47 phox was decreased following the addition of bazedoxifene (Fig. 1E). These findings indicated that bazedoxifene may reduce AngII-induced oxidative stress in endothelial cells.

Bazedoxifene inhibits AngII-induced apoptosis of endothelial cells. To determine the effect of bazedoxifene on the apoptosis of AngII-induced HUVECs, TUNEL staining was performed. The results demonstrated that the apoptotic rate in the AngII-treated alone group was significantly higher compared with that in the control group; however, bazedoxifene treatment could decrease cell apoptosis (Fig. 2A and B). These results were further confirmed by the detection of apoptosis-related protein expression levels. As presented in Fig. 2C and D, bazedoxifene could decrease Bax and cleaved caspase-3 protein expression levels in the AngII-induced HUVECs compared with the AngII-induced HUVECs alone group; however, bazedoxifene increased the expression level of the anti-apoptosis protein Bcl-2. These findings demonstrated that bazedoxifene may inhibited the AngII-induced apoptosis of endothelial cells.

Bazedoxifene promotes AngII-induced endothelial cell proliferation and inhibits oxidative stress by activating SIRT1. It has been reported that SIRT1 activation can be used to limit hypertension by enhancing AMP-activated protein kinase activity (23). In the present study, si-SIRT1 was transfected into HUVECs and the results from western blotting (Fig. 3A) and RT-qPCR (Fig. 3B) demonstrated that the transfection efficiency of si-SIRT1-1 was higher than of si-SIRT1-2. si-SIRT1-1 was therefore selected for use in subsequent experiments. The expression level of SIRT1 was decreased in AngII-induced endothelial cells, while the expression level of SIRT1 was increased with bazedoxifene in a concentration-dependent manner. The results showed that when the concentration of bazedoxifene was 4 μM, the inhibitory effect of AngII on SIRT1 was counteracted (Fig. 3C and D). Therefore, this concentration was selected for use in subsequent experiments.

The SIRT1 inhibitor, EX527, was added to the AngII-induced endothelial cells. The results from CCK-8 assay demonstrated that si-SIRT1 could partially counteract the promoting effect of bazedoxifene on the proliferation of AngII-induced endothelial cells (Fig. 3E). These results indicated that bazedoxifene may activate SIRT1 and promote AngII-induced endothelial cell proliferation.

Furthermore, as presented in Fig. 3F, bazedoxifene could increase the concentration of NO, while si-siRT1 and EX527 could partially reduce the concentration of NO. Furthermore, bazedoxifene could decrease ROS content, whereas both si-SIRT1 and EX527 partially increased the concentration of ROS (Fig. 3G). The results from western blotting further demonstrated that bazedoxifene could activate SIRT1 to inhibit AngII-induced oxidative stress in endothelial cells (Fig. 3H).

Bazedoxifene activates SIRT1 to inhibit AngII-induced apoptosis of endothelial cells. As presented in Fig. 4A and B, the cell apoptosis rate in the bazedoxifene treatment group was significantly decreased compared with that in the AngII group, and both si-SIRT1 and EX527 could partially increase the apoptosis rate. These results were further confirmed by detecting the expression levels of apoptosis-related proteins (Fig. 4C and D). Both si-SIRT1 and EX527 partially increased the protein expression of the pro-apoptotic protein Bax, cleaved caspase 3/caspase 3, while promoting the degradation of the anti-apoptotic protein Bcl-2 in endothelial cells in comparison with the AngII + 4 μg/ml + si-NC goup and the AngII + 4 μg/ml group, respectively. These findings

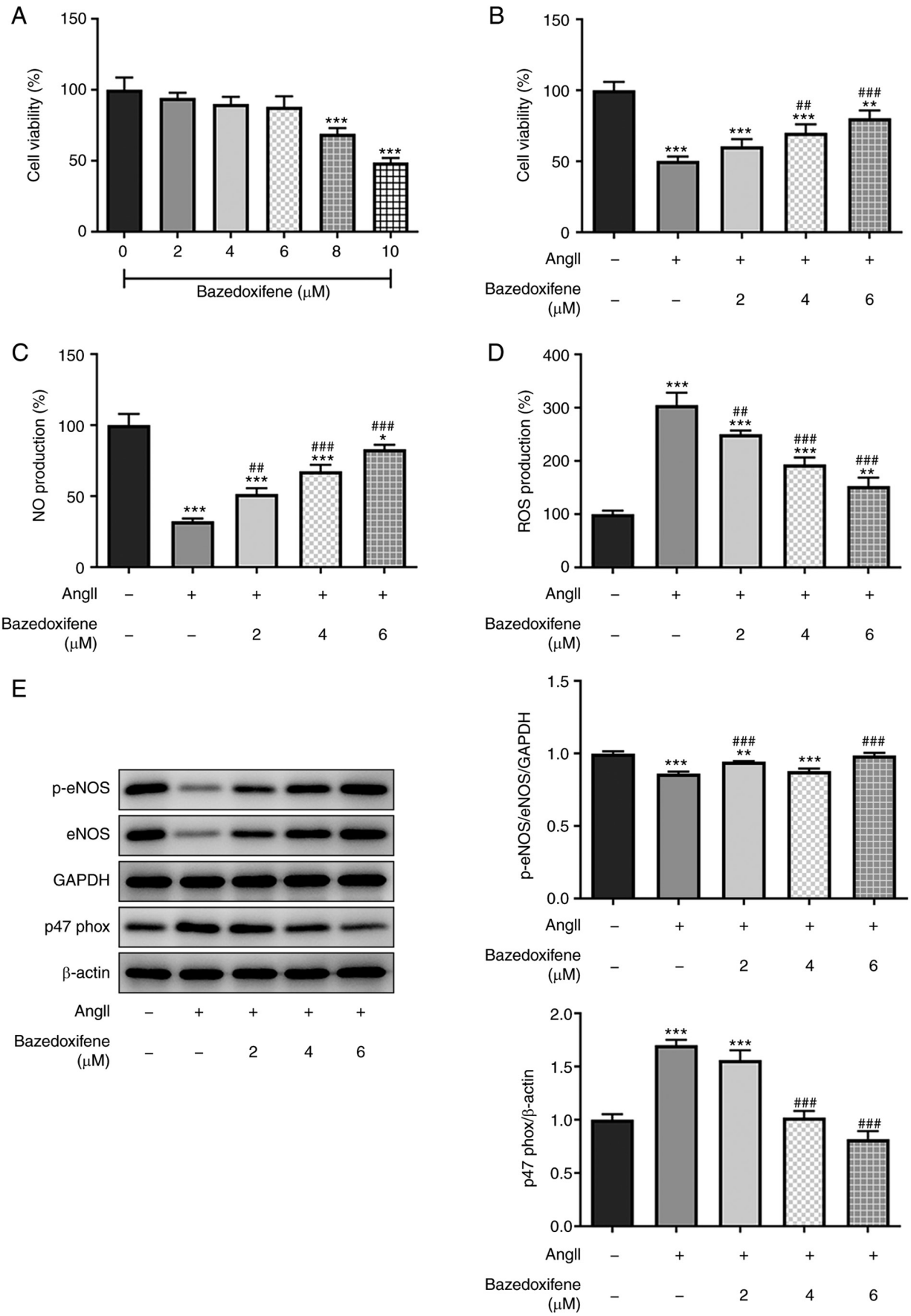

Figure 1. Bazedoxifene promotes the proliferation and decreases oxidative stress in HUVEC cells induced by AngII. (A) CCK-8 assay was used to detect cell viability of HUVEC cells treated with bazedoxifene. (B) CCK-8 assay was used to detect the cell viability of HUVEC cells induced by AngII. (C) NO content was detected using a NO kit. (D) ROS content was detected using a ROS kit. (E) Expression of oxidative stress related proteins was detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. Control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. AngII + 0 μg/ml. AngII, angiotensin II; CCK-8, Cell Counting Kit-8; NO, nitric oxide; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; p, phosphorylated.

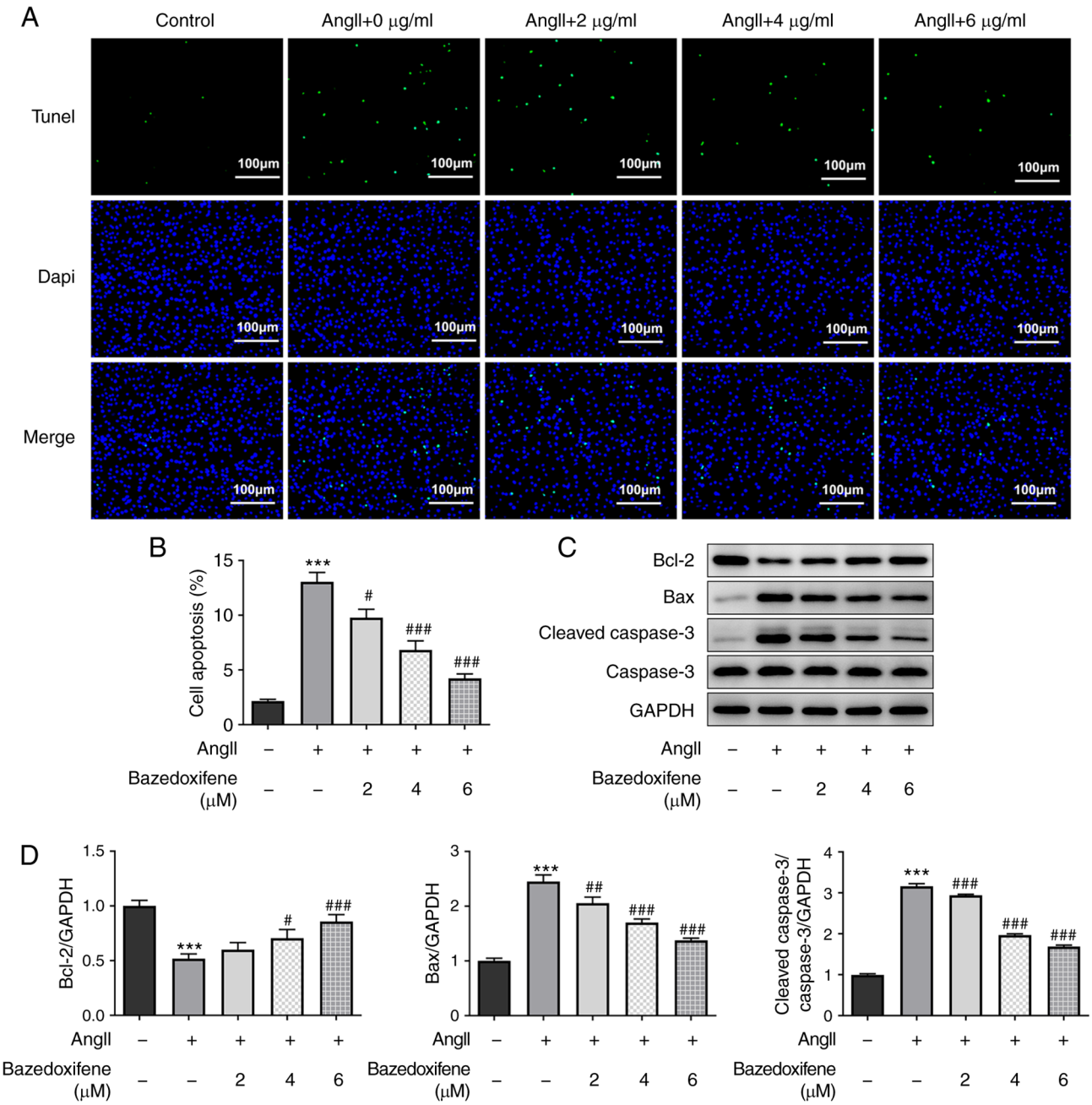

Figure 2. Bazedoxifene inhibits AngII-induced HUVEC apoptosis. (A and B) TUNEL staining was performed to detect the apoptosis of AngII-induced HUVEC cells. Magnification, scale bars, 100 μm. (C and D) Expression of apoptosis-related proteins was detected by western blotting. ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. AngII + 0 μg/ml. AngI, angiotensin II.

suggested that bazedoxifene may activate SIRT1 to inhibit the AngII-induced apoptosis of endothelial cells.

Discussion

AngII is the main active peptide of RAS, and AngII produced by both classical and non-classical pathways can reduce endothelial cell function (24). Endothelial cells are the main components of the vascular system, which serve a crucial role in vascular homeostasis by secreting and releasing vasodilators (such as NO and prostaglandins) and vasoconstrictors (such as AngII and endothelin) (25-27). The shift of vascular function to vasoconstriction, proinflammatory state, oxidative stress and NO deficiency may lead to endothelial dysfunction and injury. This shift is a key event in the pathophysiological process of cardiovascular diseases, including hypertension, diabetes, atherosclerosis, arterial hypertension and pulmonary hypertension (28,29). In the present study, HUVECs were treated with 1 μM AngII for 24 h to establish an AngII-induced endothelial cell model. The results demonstrated that endothelial cell viability was decreased, while oxidative stress levels and apoptosis were increased in AngII-induced cells, which was consistent with a previous report (30).

Statins are mainly used to reduce blood lipid and cholesterol levels and treat patients with cardiovascular diseases (31). At present, the commonly used statins in

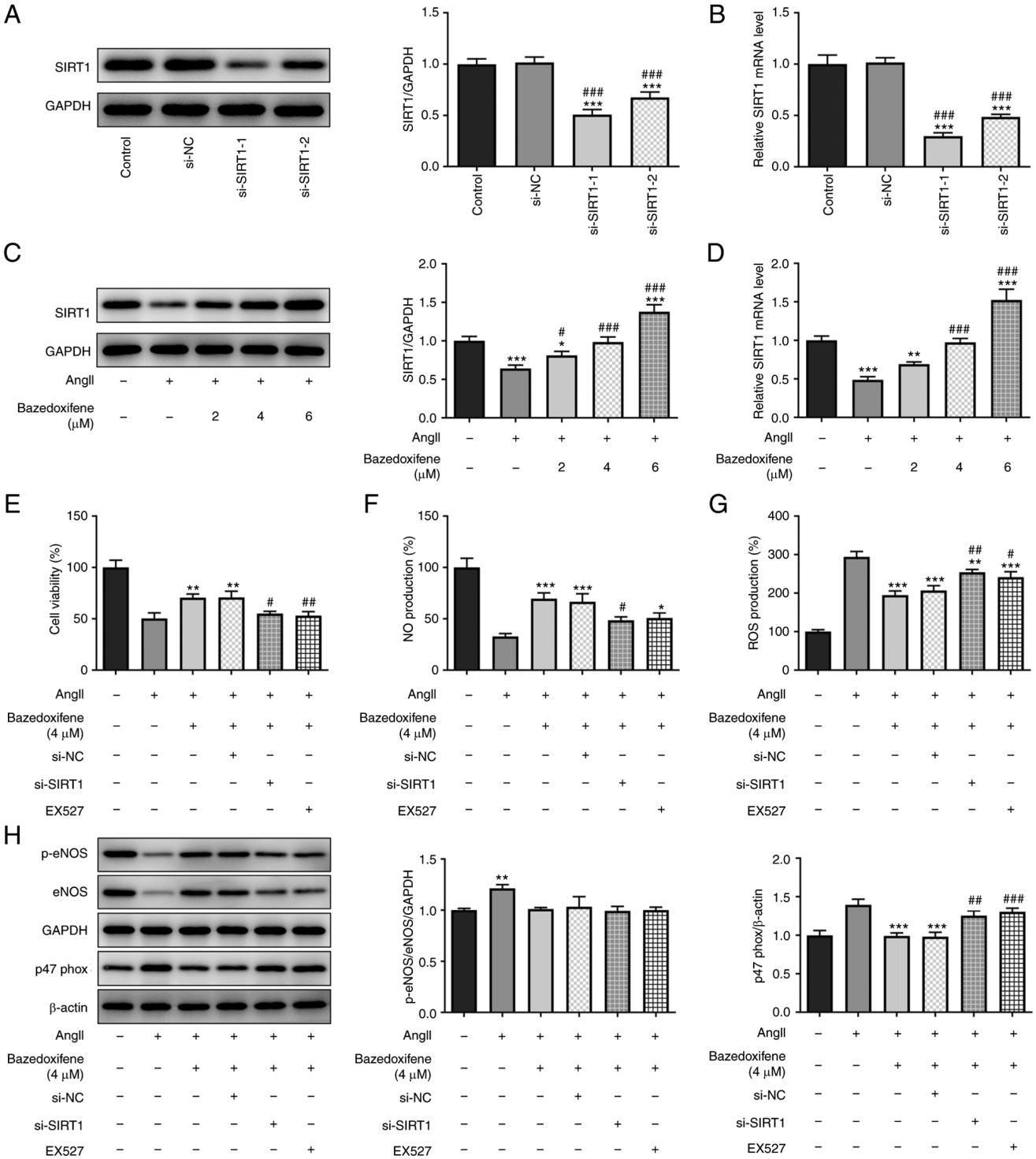

Figure 3. Bazedoxifene stimulates AngII-induced HUVEC proliferation and inhibits oxidative stress by activating SIRT1. (A and B) Cell transfection efficiency with si-SIRT1 was confirmed by (A) western blotting (A) and (B) RT-qPCR. (C and D) Expression of SIRT1 in AngII-induced endothelial cells was detected by (C) western blotting and (D) RT-qPCR. (E) Cell Counting Kit-8 assay was used to determine the cell viability. (F) NO content was detected using a NO kit. (G) ROS content was detected using a ROS kit. (H) Expression of oxidative stress related proteins was detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. Control or AngII + 0 μg/ml; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. si-NC or AngII + 4 μg/ml + si-NC. RT-qPCR, reverse transcription quantitative PCR; ANGII, angiotensin II; SIRT1, sirtuin 1; NO, nitric oxide; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; p, phosphorylated; si, small interfering; NC, negative control.

clinical practice include atorvastatin, fluvastatin, lovastatin, simvastatin and pravastatin. Recent studies have reported that statins exert anti-inflammatory and anti-angiogenesis effects and can inhibit endothelial cell migration (32,33). The results from the present study demonstrated that bazedoxifene could promote AngII-induced HUVEC proliferation and inhibit cell apoptosis, indicating that bazedoxifene could enhance the viability of AngII-induced HUVEC.

When the endothelium suffers damage and dysfunction (such as ischemia and oxygen deprivation reperfusion), the

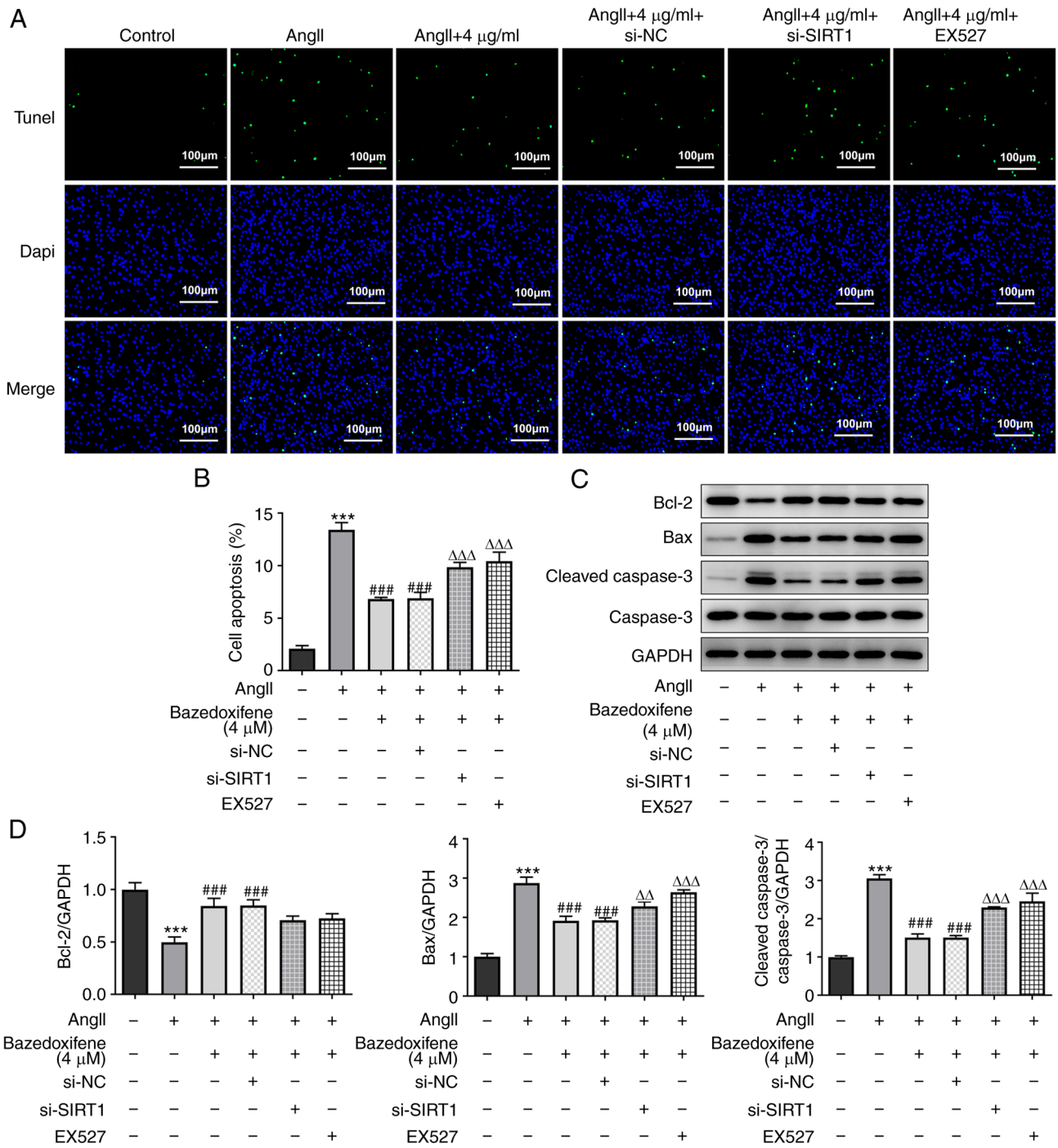

Figure 4. Bazedoxifene activates SIRT1 to inhibit AngII-induced apoptosis of HUVECs. (A and B) TUNEL staining was performed to detect the apoptosis of AngII-induced HUVEC cells. Scale bars, 100 μm. (C and D) Expression of apoptosis-related proteins was detected by western blotting. ***P<0.001 vs. Control or AngII + 0 μg/ml; $^{###}$P<0.001 vs. AngII + 0 μg/ml or AngII + 4 μg/ml + si-NC; $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 vs. AngII + 4 μg/ml + si-SIRT1. AngI, angiotensin II; SIRT1, sirtuin 1; si, small interfering; NC, negative control; eNOS, endothelial nitric oxide synthase; p, phosphorylated.

excessive production of oxidants exceeds the antioxidant capacity of the cell and the balance between oxidants and antioxidants is disrupted, resulting in increased oxidative stress (34). The endothelium releases NO, which relaxes the surrounding smooth muscle to increase blood flow and relax the vessels. NO is synthesized by e-NOS, with L-arginine, oxygen and NADPH as endogenous sources, and is capable of vasodilating, which can be balanced with vasoconstriction produced by the sympathetic nervous system and RAS (35). AngII stimulates NADPH oxidase, which then increases the formation of superoxide anions in the blood vessels, resulting in increased oxidative stress. Excessive ROS can easily inactivate NO via the formation of peroxynitrite, thus further damaging vascular function (36). The present study demonstrated that bazedoxifene could decrease the oxidative stress of endothelial cells induced by AngII, suggesting that it could protect AngII-induced endothelial cells and enhance the function of damaged endothelial cells. It has been reported that bazedoxifene stimulates the activation of eNOS and upregulates SIRT1 in bilateral ovariectomy mice and delays the development

of atherosclerosis and vascular senescence (37). In addition, SIRT1 can reduce systolic blood pressure elevation and inhibit AngII-induced vascular remodeling in mice (38). These results might indicate whether bazedoxifene mechanism of action could be mediated by targeted agonism of SIRT1. Therefore, to further investigate bazedoxifene underlying mechanism, the present study determined the effect of bazedoxifene on AngII-induced cells using STIR1 inhibitor and si-SIRT1. The results demonstrated that both EX527 and si-SIRT1 could counteract the apoptosis-inhibiting effect and anti-oxidative stress effect observed with bazedoxifene. Subsequently, our study suggested that bazedoxifene may be able to counteract AngII-induced endothelial cell apoptosis, increased oxidative stress and NO release dysfunction by agonizing SIRT1. However, the present study only focused on the protective effect of bazedoxifene on AngII-induced cells by inhibiting or downregulating STIR1. Future investigation will involve animal experiments to confirm these results, refine our understanding and provide an experimental basis for the application of bazedoxifene in the treatment of AngII-induced diseases.

In summary, the present study demonstrated that bazedoxifene may promote AngII-induced HUVEC proliferation and inhibit cell apoptosis and oxidative stress by activating SIRT1, thereby playing a protective role. These findings could provide an important basis for the clinical treatment of AngII-induced hypertension with estrogen.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QY and BL designed the study and wrote the manuscript. QY and BL confirm the authenticity of all the raw data. QY, JZ and BL performed experiments and participated in data collection and analysis. All authors have 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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