IKKε knockout alleviates angiotensin II-induced apoptosis and excessive autophagy in vascular smooth muscle cells by regulating the ERK1/2 pathway

GANYI CHEN*, YUEYUE XU*, YIWEI YAO, YIDE CAO, YAFENG LIU, HAO CHAI, WEN CHEN and XIN CHEN

Department of Thoracic and Cardiovascular Surgery, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu 210006, P.R. China

Received October 18, 2020; Accepted June 18, 2021

DOI: 10.3892/etm.2021.10485

Abstract. Inhibitor of nuclear factor-κB kinase subunit ε (IKKε) is an important signal regulator in the formation of abdominal aortic aneurysm (AAA). However, the underlying mechanism remains to be elucidated. Therefore, the present study aimed to investigate the mechanism underlying IKKε function in AAA formation by studying apoptosis and autophagy in angiotensin II (Ang II)-induced vascular smooth muscle cells (VSMCs). AngII was used to stimulate VSMCs for 24 h to simulate the process of AAA formation. VSMCs were transfected with IKKε small interfering RNA to investigate the effect of IKKε on AAA formation, cell apoptosis and autophagy. IKKε deficiency led to reduced mitochondrial damage and apoptosis in VSMCs in the early stage of apoptosis *in vitro*, as demonstrated using a JC-1 probe. IKKε deficiency also reduced autophagy and decreased the formation of autophagic vacuoles in VSMCs, demonstrated using transmission electron microscopy. The decrease in apoptosis caused by IKKε knockdown was reversed when the autophagic flow was blocked using bafilomycin A1. Western blot analysis further revealed that IKKε deficiency negatively regulated the ERK1/2 signaling pathway to reduce autophagy. Collectively, the results of the present study revealed that IKKε played a key role in apoptosis by inducing excessive autophagy, thereby potentially contributing to AAA formation. These findings further revealed the mechanism underlying IKKε function in the formation of AAA.

Correspondence to: Dr Wen Chen or Professor Xin Chen, Department of Thoracic and Cardiovascular Surgery, Nanjing First Hospital, Nanjing Medical University, 68 Changle Road, Nanjing, Jiangsu 210006, P.R. China
E-mail: cxacf@163.com
E-mail: stevecx@njmu.edu.cn

*Contributed equally

Key words: abdominal aortic aneurysm, inhibitor of nuclear factor-κB kinase subunit ε, autophagy, apoptosis, vascular smooth muscle cells

Introduction

Abdominal aortic aneurysm (AAA) is a complicated and dangerous cardiovascular disease characterized by high morbidity and mortality globally (1,2). In developing countries, the incidence of AAA rises to 3-4% in people aged over 65, and rupture of AAA causes ~4,500 deaths in the United States (1,2). Most AAA-related deaths are attributed to AAA rupture (3). Currently, surgery is the only treatment option for AAA; however, the benefits of surgery are limited by the aortic diameter (4). Moreover, AAA pathogenesis during formation and development is yet to be fully elucidated. Apoptosis of vascular smooth muscle cells (VSMCs) aggravates AAA progression at the histopathological level (5). Thus, it is necessary to explore new therapeutic approaches, such as inhibition of apoptosis in VSMCs, in order to minimize AAA progression.

Findings of previous studies report that AAA and atherosclerosis share similar histopathological characteristics (6,7). Inhibitor of nuclear factor-κB kinase subunit ε (IKKε) regulates a variety of pathophysiological processes associated with the occurrence of atherosclerosis, such as morphological changes and lipid accumulation in the aorta (8). It is also an important signal regulator that plays a crucial role in tumorigenesis, inflammation, metabolic disorders and the reduction-oxidation process (9). AAA is characterized by inflammatory cell infiltration and apoptosis of VSMCs (5). Therefore, it was inferred that IKKε plays an important role in AAA development. A previous study revealed that IKKε expression is significantly increased in patients with AAA (10). The results of a another study indicated that the lack of IKKε reduced AAA formation by reducing apoptosis and inflammation in the abdominal aorta of mice (11). However, the mechanism underlying IKKε function remains unclear.

Autophagy and apoptosis are two key forms of cell death (12). In the cardiovascular system, autophagy protects blood vessels from dysfunction (13). However, excessive autophagy leads to cell death during the pathological state under ER stress (14). Myocardin regulates the apoptosis of VSMCs by regulating autophagy to promote the occurrence of aortic aneurysms (15). A previous study has reported that

the targeted knockdown of autophagy-related protein (ATG)7 or the autophagy inhibitor 3-methyladenine inhibited caspase activation and reduced apoptosis (16). Furthermore, a recent study postulated that IKKε is an autophagy-activating gene in breast cancer (17). Multiple autophagy-related genes, such as ATG5 and ATG7 are markedly upregulated in human aortic aneurysm disease (18). The present study investigated the effects of IKKε on autophagy and apoptosis in a VSMC model of AAA.

In the present study, loss of IKKε ameliorated angiotensin II (AngII)-induced cellular AAA model by inhibiting autophagy and apoptosis in VSMCs. This phenomenon was partially dependent on activated ERK1/2 signaling.

Materials and methods

Cell line and culture. Mouse thoracic aortic vascular smooth muscle cell (VSMCs; cat. no. CRL-2797) were obtained from the American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotic solution (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were incubated in a humidified incubator at 37˚C with 5% CO_2 (Thermo Fisher Scientific, Inc.). VSMCs were passaged 3-5 times and seeded in six-well plates at a density of $1.0x10^4$ cells/well. The following day, cells were treated with AngII (1 μmol/l; Sigma Aldrich; Merck KGaA) for 24 h.

Transfection. VSMCs at 60% confluence were cultured in six-well plates and transfected with IKKε small interfering (si) RNA (20 μmol/l; Shanghai GenePharma Co., Ltd.) for 6 h using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The siRNA was used to infect cells for 6 h at 37˚C and cell medium was replaced with complete culture medium. The following day, cells were exposed to AngII for 24 h. The sequences were as follows: A sense, 5'-GCAUACUGAUGACCUGCUATT-3' and antisense, 5'-UAGCAGGUCAUCAGUAUGCTT-3'; b sense, 5'-CCCACAACACGAUUGCCAUTT-3' and antisense 5'-AUGGCAAUCGUGUUGUGGGTT-3'; c sense, 5'-GCAACCUAUGGCUCCUCAUTT-3' and antisense, 5'-AUGAGGAGCCAUAGGUUGCTT-3' and the control sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'.

Western blot analysis. Total proteins of VSMCs were extracted using RIPA buffer (Beyotime Institute of Biotechnology) and their concentrations were determined using the BCA protein assay kit (cat. no. KGP902; Nanjing KeyGen Biotech Co., Ltd.). The proteins (30 μg per lane) were then separated via 10 and 12% SDS-PAGE, and the resultant bands were transferred onto PVDF membranes (Sigma-Aldrich; Merck KGaA). The membranes were blocked with 5% skimmed milk powder for 1 h at room temperature and subsequently incubated with primary antibodies at 4˚C overnight. The antibodies included anti-IKKε (1:1,000; cat. no. 3416S; Cell Signaling Technology, Inc.), anti-LC3B (1:2,000; cat. no. ab51520; Abcam), anti-P62 (1:1,000; cat. no. 23214S; Cell Signaling Technology, Inc.), anti-ATG7 (1:1,000; cat. no. 8558S; Cell Signaling Technology, Inc.), anti-Bax (1:1,000; cat. no. 2772S; Cell Signaling Technology, Inc.), anti-cleaved (c)-caspase-3 (1:1,000; cat. no. 9661S; Cell Signaling Technology, Inc.), anti-caspase-9 (1:1,000; cat. no. 9508T; Cell Signaling Technology, Inc.), anti-p38 (1:1,000; cat. no. 9212S; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-p38 (1:1,000; cat. no. 4511S; Cell Signaling Technology, Inc.), anti-MEK1/2 (1:1,000; cat. no. 9122S; Cell Signaling Technology, Inc.), anti-p-MEK1/2 (1:1,000; cat. no. 9154S; Cell Signaling Technology, Inc.), anti-ERK1/2 (1:1,000; cat. no. 4695S; Cell Signaling Technology, Inc.), anti-p-ERK1/2 (1:1,000; cat. no. 4370S; Cell Signaling Technology, Inc.) and anti-GAPDH antibody (1:5,000; cat. no. 8884S; Cell Signaling Technology, Inc.). The PVDF membranes were then incubated with secondary antibodies, including HRP-anti-mouse IgG (1:5,000; cat. no. HRP-60004; ProteinTech Group, Inc.) and HRP-anti-Rabbit IgG (1:5,000; cat. no. 7074P2; Cell Signaling Technology, Inc.) for 1 h at room temperature. The protein bands were subsequently visualized using chemiluminescent HRP Substrate (cat. no. P90720; MilliporeSigma) and captured on a Hyperfilm (Amersham; Cytiva), and the results were analyzed using ImageJ software (version 1.8.0; National Institutes of Health) for semi-quantitation of the mean gray value of each blot.

Flow cytometry analysis. Apoptosis was measured using the Annexin-V/PI double staining method. VSMCs were seeded in six-well plates at a density of $1.0x10^4$ cells/well and treated with AngII for 24 h, washed with PBS, and subsequently trypsinized to obtain a single-cell suspension. The Annexin V-FITC Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Ltd.) was used to stain the cells according to the manufacturer's protocol. Apoptotic cells were subsequently evaluated using a flow cytometer (BD FACSCalibur™; BD Biosciences). The results were analyzed using ModFit LT version 5.0 (Verity Software House, Inc.).

Transmission electron microscopy. VSMCs were centrifuged to form clusters at 67 x g and room temperature for 5 min, and subsequently fixed with 2.5% glutaraldehyde at 4˚C overnight. Cells were further fixed in 1% buffered osmium tetroxide and dehydrated in graded ethanol. Cluster sections (60-70 nm) were double stained with 2% uranyl acetate for 2 h at room temperature and lead citrate for 5 min at room temperature. The samples were examined under a JEOL JEM-1010 transmission electron microscope (JEOL, Ltd.). The autophagosomes were manually counted.

Mitochondrial membrane potential analysis. The JC-1 probe (Beyotime Institute of Biotechnology) was employed to measure mitochondrial depolarization in VSMCs. The cells were first cultured with AngII in six-well plates at a density of $1.0x10^4$ cells/well for 24 h and subsequently incubated with an equal volume of JC-1 staining solution (5 pg/ml) at 37˚C for 20 min. Cells were then rinsed twice with PBS, followed by monitoring of the mitochondrial membrane potentials by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers and aggregates. Mitochondrial membrane potentials were monitored using an Olympus fluorescent microscope (Olympus Corporation) under Argon-ion 488 nm laser excitation. An increase in the ratio

of green/red (488/594 nm) fluorescence intensity indicated mitochondrial depolarization. The results were analyzed using ImageJ software (version 1.8.0; National Institutes of Health).

Autophagic flux analysis. VSMCs were first treated with bafilomycin A1 (Baf-A1; 400 nM; Selleck Chemicals) for 4 h prior to AngII treatment as previously described, to block autophagosome-lysosome fusion. Autophagic flux was measured via western blot analysis, as previously described.

Statistical analysis. Data are presented as the mean ± standard error. Differences between groups were determined using ANOVA followed by Tukey's post hoc test. Comparisons between two groups were performed using a paired Student's t-test. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

IKKε knockdown attenuates AngII-induced apoptosis in VSMCs. IKKε deficiency has been indicated to attenuate AAA formation in mice by inhibiting apoptosis (11). In the present study, VSMCs were transfected with siRNA to knock down IKKε, and were subsequently exposed to AngII for 24 h to elucidate the role of IKKε in apoptosis *in vitro*. The sequences of IKKε siRNA with the highest knockdown efficiency were selected (Fig. S1A and B). IKKε knockdown efficiency in VSMCs was 40-60% compared with the control group. (Fig. 1A and B). Western blot analysis revealed that IKKε knockdown reduced the expression of caspase-9, c-caspase-3 and Bax in VSMCs after induction with AngII (Fig. 1A and B). The analysis of the apoptosis of VSMCs detected by flow cytometry revealed that IKKε knockdown reduced the apoptosis of VSMCs following AngII treatment (Fig. 1C and D). Furthermore, JC-1 staining revealed that IKKε deficiency reduced mitochondrial damage after AngII treatment, which occurs at an early stage of apoptosis (19) (Fig. 1E and F). Collectively, the results of the present study demonstrated that IKKε knockdown reduced apoptosis of VSMCs at an early stage with AngII treatment.

IKKε knockdown attenuates AngII-induced excessive autophagy in VSMCs. Autophagy in VSMCs was further investigated to verify the role of IKKε on apoptosis in VSMCs. Western blot analysis of LC3B, **ATG7** and P62 revealed that IKKε knockdown reduced autophagy in AngII-induced VSMCs (Fig. 2A and B). Transmission electron microscopy further revealed that autophagic vacuoles were significantly reduced in AngII-induced VSMCs in the SiIKKε group compared with the control group (Fig. 2C and D).

IKKε plays an important role in the association of autophagy and apoptosis. To detect the autophagy flow *in vitro*, Baf-A1 was used to block the autophagy flow in AngII-induced VSMCs. The expression levels of LC3B and P62 in the VSMCs were significantly increased following autophagy blocking with Baf-A1 and AngII compared with the AngII group, indicating that the autophagic process was active in the AngII-induced VSMC autophagosomes (20) (Fig. 3A and B). A smaller decrease in the expression of LC3B and P62 was demonstrated in the IKKε deficiency with Baf-A1 + AngII group compared with the control + AngII + Baf-A1 group, indicating that IKKε potentially played a role in autophagy (Fig. 3A and B). Notably, there was no significant difference in the expression levels of caspase-9 and Bax in the SiIKKε + AngII + Baf-A1 group compared with the control + AngII + Baf-A1 group (Fig. 3C and D). Thus, it was hypothesized that IKKε increased apoptosis by promoting autophagy in VSMCs.

IKKε regulates autophagy and apoptosis via the ERK1/2 signaling pathway. The activation of the ERK1/2 pathway in VSMCs was assessed to further investigate the mechanism underlying IKKε in autophagy regulation *in vitro*. The level of p-ERK1/2 was decreased in SiIKKε cells exposed to AngII compared with control cells exposed to AngII (Fig. 4A and B). The phosphorylation levels of MEK1/2 and p38 were investigated, and no significant difference was demonstrated in the phosphorylation of MEK1/2 and p38 in VSMCs with AngII treatment (Fig. S2A and B). These results indicated that IKKε knockdown reduced autophagy and apoptosis by inhibiting the ERK1/2 signaling pathway.

Discussion

AAA is a disease associated with serious complications such as the rupture of aneurysm and death, and is more severe than common heart disease (21). However, the specific pathogenesis of AAA is yet to be elucidated. The results of the present study demonstrated the important role of IKKε in autophagy, apoptosis and AAA development. Knockdown of IKKε attenuated AngII-induced apoptosis and excessive autophagy in VSMCs. IKKε also increased apoptosis by promoting the autophagy of VSMCs. Moreover, IKKε knockdown reduced autophagy and apoptosis by inhibiting the ERK1/2 signaling pathway.

Apoptosis of VSMCs is a key biomarker of AAA formation (5). The results of a previous study demonstrated that IKKε played an important role in regulating apoptosis of VSMCs in mice (11). In the present study, the *in vitro* results were consistent with previous findings (8). It has been previously revealed that when the membrane potential of mitochondria decreases, the permeability of the mitochondrial membrane increases and the proapoptotic factors are released into the cytoplasm (22). In the present study, IKKε deficiency reduced mitochondrial damage and apoptosis *in vitro*, indicating that IKKε regulated the apoptosis of VSMCs via the mitochondrial apoptosis pathway.

Activated autophagy is generally considered a cell protection mechanism due to the promotion of cell survival (23). However, excessive activation of autophagy has been indicated to result in autophagic cell death (24). Functional autophagy is essential for cardiac homeostasis (25); however uncontrolled autophagy induction in ischemia-reperfusion injury response resulted in excessive cardiomyocyte apoptosis, thereby aggravating the injury (26). VSMCs may exhibit excessive autophagy leading to cell death when exposed to severe stimuli (27). This mechanism serves a role in the occurrence of a number of vascular diseases, such as atherosclerosis. A previous study postulated that suppressing autophagy inhibited AAA development (28).

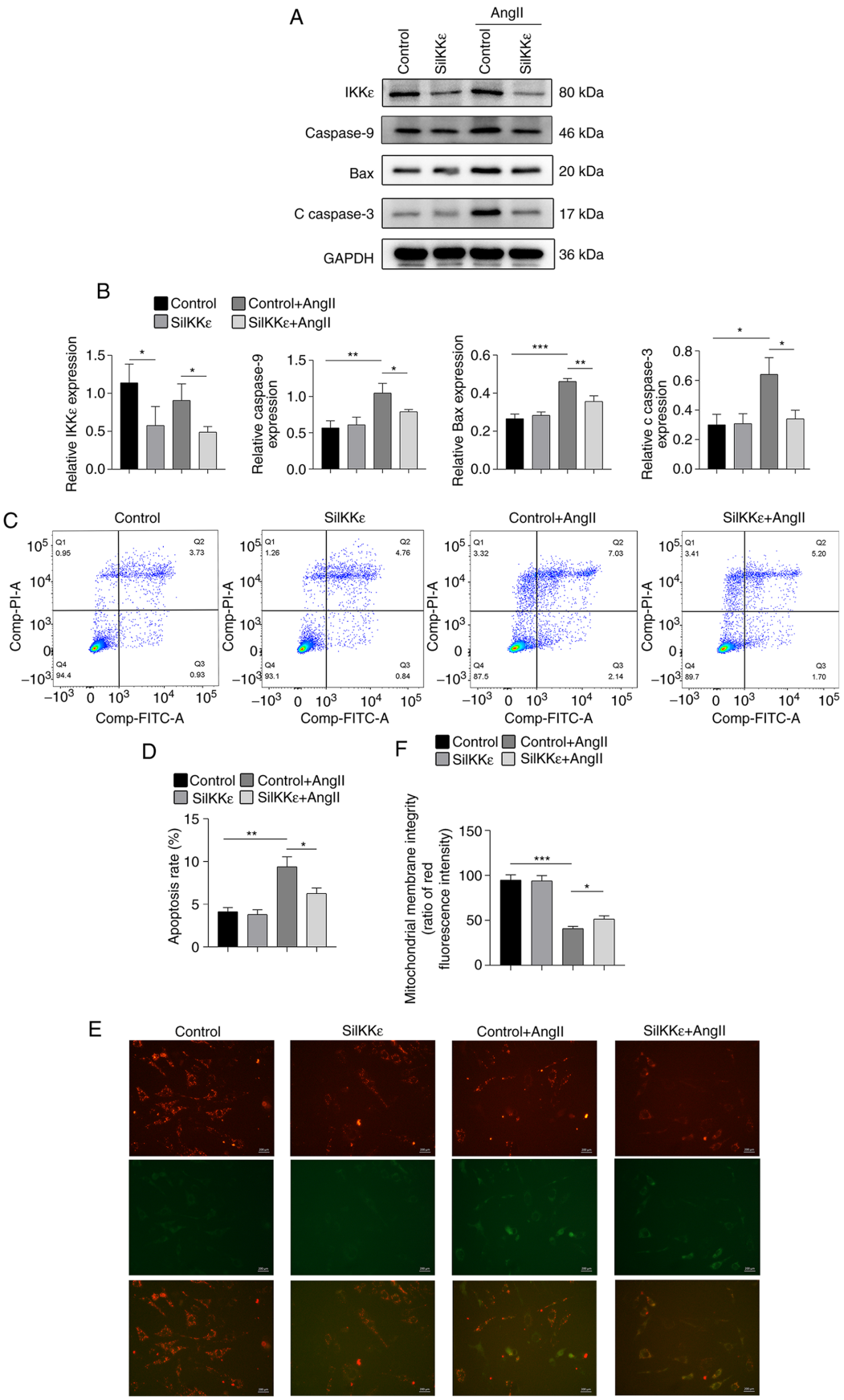

Figure 1. Knockdown of IKKε attenuates AngII-induced apoptosis in VSMCs. (A) Bax, caspase-9, c-caspase-3 and IKKε expression levels were detected via western blot analysis in AngII-induced VSMCs following siRNA transfection for 24 h (n=4-5 per experimental group). (B) Quantitative results of expression levels of Bax, caspase-9 and c-caspase-3 (n=4-5 per experimental group). (C and D) Quantitative flow cytometry results of the apoptosis rate of VCMCs exposed to AngII for 24 h (n=4-5 per experimental group). (E) Representative images and (F) quantitative results of JC-1 staining in VSMCs exposed to AngII for 24 h (n=6 per experimental group; magnification, x400). Scale bar, 200 μm. Each experiment was repeated three times. *P<0.05; **P<0.01; ***P<0.001. IKKε, inhibitor of nuclear factor-κB kinase subunit ε; AngII, angiotensin II; VSMCs, vascular smooth muscle cells; si, small interfering; c, cleaved.

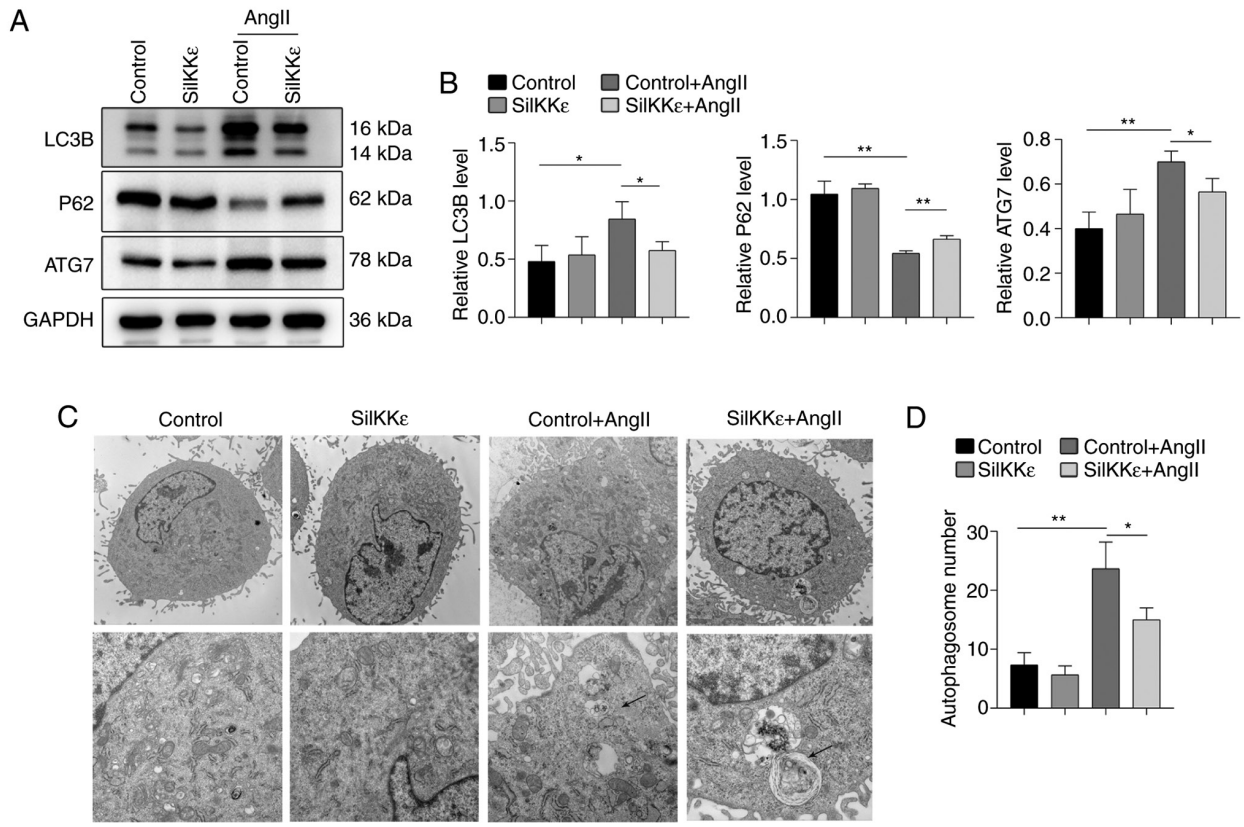

Figure 2. Knockdown of IKKε attenuates AngII-induced excessive autophagy in VSMCs. (A) LC3B, P62 and ATG7 expression levels detected via western blot analysis in AngII-induced VSMCs following siRNA transfection for 24 h (n=4-5 per experimental group). (B) Quantitative results of the expressions levels of LC3B, P62 and ATG7. (C) Representative transmission electron microscopy images and (D) autophagosome quantification in VSMCs exposed to AngII for 24 h (n=4-5 per experimental group; magnification, x10,000). Each experiment was repeated three times. *P<0.05; **P<0.01. IKKε, inhibitor of nuclear factor-κB kinase subunit ε; AngII, angiotensin II; VSMCs, vascular smooth muscle cells; ATG7, autophagy-related protein 7; si, small interfering.

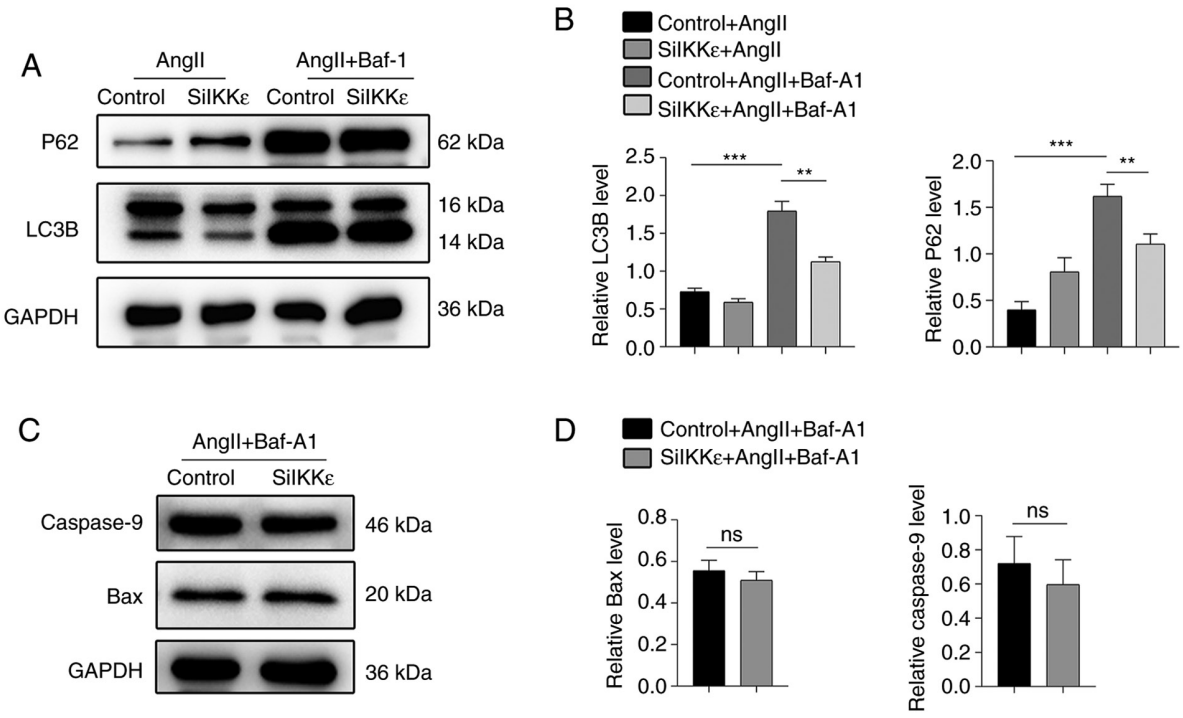

Figure 3. IKKε plays an important role in the association of autophagy and apoptosis. (A) Representative western blot images and (B) quantitative results of LC3B and P62 expression in VSMCs pretreated with Baf-A1 for 4 h, followed by siRNA transfection and AngII-induction for 24 h (n=4-5 per experimental group). (C) Representative western blot images and (D) quantitative results of caspase-9 and Bax in VSMCs pretreated with Baf-A1 for 4 h, followed by siRNA transfection and AngII-induction for 24 h (n=4-5 per experimental group). Each experiment was repeated three times. **P<0.01; ***P<0.001. IKKε, inhibitor of nuclear factor-κB kinase subunit ε; AngII, angiotensin II; VSMCs, vascular smooth muscle cells; si, small interfering; Baf-A1, bafilomycin A1; ns, not significant.

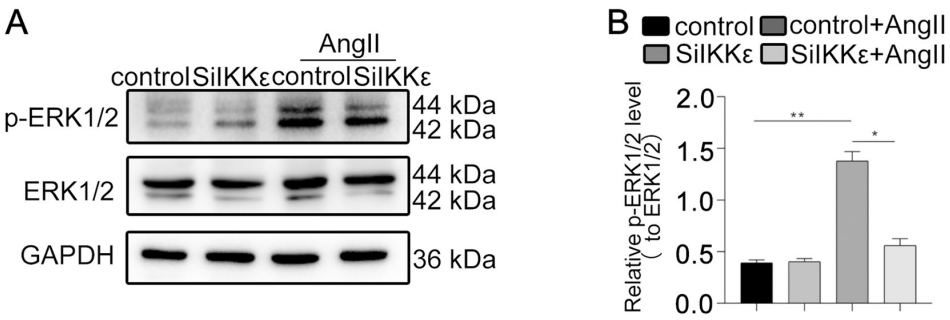

Figure 4. IKKε-regulated autophagy involves the ERK1/2 signaling pathway. (A) Representative western blot images and (B) quantitative results of phosphorylated and total protein levels of ERK1/2 following siRNA transfection in AngII-induced vascular smooth muscle cells for 24 h (n=4-5 per experimental group). Each experiment was repeated three times. *P<0.05; **P<0.01. IKKε, inhibitor of nuclear factor-κB kinase subunit ε; si, small interfering; AngII, angiotensin II; p, phosphorylated.

Although previous findings have suggested that the IKK family plays an important role in autophagy (29), few studies have reported the role of IKKε in autophagy. A previous study demonstrated that IKKε played a protective role against cardiovascular diseases (30). Thus, inhibition of the autophagy of VSMCs may provide a novel treatment against AAA.

The autophagic flow plays an important role in autophagy (31). In the present study, autophagy was blocked with Baf-A1 to prevent the downstream events of autophagy by inhibiting lysosomal degradation (32). IKKε knockdown suppressed the synthesis of autophagy initiation-related autophagy vesicles and the accumulation of autophagosomes; therefore, it was hypothesized that IKKε played an important role in the early stages of autophagic flow in VSMCs.

Apoptosis and autophagy are important biological activities that contribute to the stability of the internal environment, thus promoting the organism's survival (33). Maintaining a balance between autophagy and apoptosis is critical for cell development (33). Abnormal induction of the autophagic flux has been indicated to promote apoptotic neuronal cell death (34). Autophagy has been demonstrated to play a protective role in attenuating AngII-induced oxidative stress and inflammation (35). However, autophagy has been indicated to contribute to apoptosis in cardiac microvascular endothelial cells (36). In the present study, treatment with Baf-A1 inhibited the expression levels of AngII-induced Bax and Caspase-9 in VSMCs. Therefore, it was hypothesized that IKKε deficiency reduced the apoptosis of VSMCs by decreasing autophagy to alleviate AAA formation.

The results of a previous study indicated that activation of ERK signaling is detected in a number of tumor cells, such as melanomas and gastric carcinoma (37). Furthermore, it has been reported that the phosphorylation of ERK induced during tumor development is dependent on IKKε (38). Continuous activation of the MEK/ERK signaling pathway directly induced autophagy (39) and induced apoptosis in tumor cells (40,41). The findings of the present study revealed that the ERK1/2 pathway was activated in parallel with AngII-induced apoptosis in VSMCs, consistent with the findings generated from a previous study (11). The ERK1/2 pathway was possibly associated with AngII-induced autophagy. Furthermore, there was no significant difference in the phosphorylation of MEK1/2 and p38 in VSMCs after AngII treatment. Thus, the present study revealed that IKKε knockdown inhibited the phosphorylation of ERK1/2 in VSMCs.

In conclusion, the present study demonstrated that IKKε induced excessive autophagy in VSMCs, leading to increased apoptosis and potentially AAA formation. Therefore, inhibition of IKKε has the potential to act as a therapeutic target, to inhibit autophagy and apoptosis to reduce AAA occurrence.

Nonetheless, the present study was limited by several factors. The VSMC cell line used differs from primary cells, and the levels of IKKε were not increased by AngII treatment in the present study. The findings of the present study should be extended by analyzing both upstream and downstream mechanisms of IKKε and the ERK1/2 signaling pathway. Future studies should therefore focus on these shortcomings to provide more comprehensive results.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant. No. 81870193), the Project of Invigorating Health Care through Science, Technology and Education, Jiangsu Provincial Key Medical Discipline (grant. no. ZDXKA2016021) and the Young Program of National Natural Science Foundation of China (grant. no. 81700415).

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

GC, YC, YX and YY performed the experiments. GC, YL and HC conducted data analysis. YX, WC and XC designed the experiments and aided in data analyses. WC and XC confirm the authenticity of all the raw data. 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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