Elevation of hypertonicity-induced protein NFAT5 promotes apoptosis of human umbilical vein endothelial cells through the NF-κB pathway

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Abstract. Abdominal aortic aneurysm (AAA) is a great threat to the health of elder (>50 years old) individuals. High salt intake is considered to raise the risk of AAA but the underlying mechanism remains to be elucidated. As endothelial dysfunction in the abdominal aorta is strongly associated with AAA, the present study hypothesized that high salt led to AAA by inducing apoptosis of endothelial cells. The present study verified that hypertonic medium with excess sodium chloride induced apoptosis of human umbilical vein endothelial cells (HUVECs), a commonly used cell model to study aortic endothelial cells. Further mechanism studies suggested that hypertonic conditions elevated the expression of nuclear factor of activated T cells 5 (NFAT5) and a high level of NFAT5 was capable of inducing apoptosis of HUVECs. In the investigation of downstream signals of NFAT5, it was identified that either hypertonic conditions or NFAT5 overexpression promoted the activity of NF-κB signaling pathway and subsequently suppressed the expression of anti-apoptotic protein Bcl-2. Thus, the present study demonstrated a novel mechanism by which high salt induced apoptosis of endothelial cells by enhancing the NFAT5-NF-κB signaling pathway. These findings will extend our knowledge about the pathogenesis of AAA and provide potential drug targets for the treatment of AAA.

Introduction

Abdominal aortic aneurysm (AAA) is a disease characterized by enlargement of the abdominal aorta and commonly occurring in individuals >50 years old, typically in males (1). AAA is usually asymptomatic but can be lethal upon rupture (1,2), which is a considerable threat to the health of the elderly. It has been reported that high-salt diet is positively correlated with the risk of AAA both in patients and in animal models (3,4). However, the underlying mechanism of high salt inducing AAA remains to be elucidated.

Endothelial dysfunction of abdominal aorta is highly associated with AAA (5). Particularly, apoptosis of endothelial cells of blood vessels increases the permeability of vessel walls, enhancing the migration and binding of inflammatory cells to the vascular smooth muscle layer, which is believed to be a major cause of aneurysms and rupture (6,7). Thus, investigation on the apoptosis of endothelial cells should contribute to the understanding of pathogenesis of AAA. Human umbilical vein endothelial cells (HUVECs) are a commonly used cell model in studies related to endothelial cells (8). Blocking NF-κB signaling protects HUVEC cells from high glucose-induced apoptosis (9), indicating the potential involvement of NF-κB pathway in endothelial apoptosis.

Nuclear factor of activated T cells 5 (NFAT5) is a transcriptional factor mainly induced by hypertonic stress (10). Although body fluid usually remains isotonic due to the balance of intra- and extracellular solutes, excess intake of salts causes hypertonicity of blood, leading to various clinical manifestations which may elevate the expression NFAT5 in the cells of vessel walls (11). NFAT5 is closely associated with the NF-κB pathway and enhances NF-κB activity by forming NF-κB-NFAT5 complexes, which promotes the binding of NF-κB to κB elements of NF-κB-responsive genes, including various proinflammatory genes such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and inducible nitric oxide synthase (iNOS) (12). Nevertheless, the role of NFAT5 in endothelial apoptosis remains to be elucidated.

The present study identified that hypertonic culture medium elevated expression of NFAT5 in HUVECs and induced celldeath. Furthermore, overexpression of NFAT5 by plasmid transfection also led to the apoptosis of HUVECs. In addition, knockdown of NFAT5 using specific small interfering (si) RNA relieved cell death induced by hypertonic medium.

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Finally, it was identified that NFAT5 enhanced the activity of NF-κB signaling pathway and inhibited the expression of Bcl-2, an anti-apoptotic protein, in HUVECs. Altogether, the present study demonstrated a novel mechanism underlying hypertonicity-induced apoptosis of HUVECs, mediated by NFAT5 and the NF-κB signaling pathway. These findings extend the current knowledge about the pathogenesis of AAA, especially the role of high-salt diet in the progression of AAA.

Materials and methods

Patient samples. Human AAA (n=9) and adjacent healthy aorta abdominalis (n=9) were collected by aneurysmectomy and prosthetic vascular graft repair at Hangzhou First Affiliated Hospital between January 2015 and December 2019. Six of the patients were male and 3 were female (age range, 57-71 years). Each sample was homogenized for RNA extraction. The study was approved by the Ethics Committee of Hangzhou First Affiliated Hospital and written informed consent was obtained from each patient.

Cell culture. HUVECs were a kind gift from Dr Ye Qiu (College of Biology, Hunan University, China). They were maintained in complete culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich; Merck KGaA), 100,000 U/l penicillin (Sigma-Aldrich; Merck KGaA) and 100 mg/l streptomycin (Sigma-Aldrich; Merck KGaA). The cell culture was incubated in 37˚C with 5% of CO_2.

The hypertonic medium was made by dissolving NaCl (Sigma-Aldrich; Merck KGaA) in the complete culture medium at a final concentration of 100 mM, which was equal to 30 g salt ingestion by a human of 60 kg. This dose was demonstrated to elevate NFAT5 expression in a previous study (13). The hypertonic medium was then filtered using Millex-GP Syringe Filter Unit (0.22 μm; EMD Millipore) to avoid contamination. Normal complete culture medium was subjected to the same process of filtration to serve as control medium for hypertonic treatment.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total cellular RNA was extracted from HUVECs (90% confluence) using PureLink RNA Mini kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was then synthesized by reverse transcription using the SuperScript III First-Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.) and detected by RT-qPCR using the SYBR-Green qPCR kit (Bimake.com) according to the manufacturer's instructions. Briefly, the synthesized cDNA was mixed with SYBR-Green qPCR Master Mix and the primers to a total reaction volume of 20 μl. Then the two-step qPCR program was run as follows: Activation at 95˚C for 30 sec, denaturation at 95˚C for 15 sec, followed by annealing and extension at 60˚C for 60 sec, with a total of 40 cycles. The Cq values were collected and converted to gene expression levels by calculating $2^{-\Delta\Delta Cq}$ (14). GAPDH mRNA levels were used as the endogenous control to normalize the data. All RT-qPCR experiments were performed in triplicate with no template as a negative control. The primers used were: Human NFAT5-forward, 5'-GAAGTGGACATTGAA GGCACT-3' and reverse, 5'-CTGGCTTCGACATCAGCA TT-3'; human VCAM-1-forward, 5'- CAGTAAGGCAGG CTGTAAAAGA-3' and reverse, 5'-TGGAGCTGGTAGACC CTCG-3'; human ICAM-1-forward, 5'-GTATGAACTGAG CAATGTGCAAG-3' and reverse, 5'-GTTCCACCCGTT CTGGAGTC-3'; human GAPDH-forward, 5'-AATCCCATC ACCATCTTCCA-3' and reverse, 5'-TGGACTCCACGACGT ACTCA-3'; human iNOS-forward, 5'-TCATCCGCTATG CTGGCTAC-3' and reverse, 5'-CCCGAAACCACTCGT ATTTGG-3'.

MTS cell viability assay. HUVEC morphology was observed and images captured at room temperature under a TMS-F phase-contrast microscope (Nikon Corporation) connected to Coolpix 8400 a camera (Nikon Corporation). HUVEC viability was further quantified using an MTS assay kit following the manufacturer's instructions (Promega Corporation). Briefly, HUVECs were incubated with MTS solution (2 mg/ml) for 2 h at 37˚C in an atmosphere containing 5% CO_2. Subsequently, the formazan crystals were dissolved in DMSO and diluted to 5X in phospate-buffered saline (PBS). Absorbance of formazan was measured at 492 nm using an ELISA plate reader (Tecan Group Ltd.). The results were normalized to the corresponding controls which were set as a viability of 100%.

Western blot analysis. HUVECs were washed with cold PBS before the addition of an appropriate volume (50 μl) of RIPA lysis buffer (Santa Cruz Biotechnology, Inc.). After incubation for 20 min on ice, the cell lysates were centrifuged at 13,000 x g for 15 min at 4˚C, and protein-containing supernatant was collected. Protein concentration was determined using the Bradford Assay. The isolated proteins (50 μg) were separated by 8-12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich; Merck KGaA). Membranes were blocked with 5% skimmed milk in TBS buffer containing 5% Tween-20 (Sigma-Aldrich; Merck KGaA) at 4˚C for 2h and incubated with one of the following primary antibodies in 4˚C overnight: Monoclonal mouse anti-NFAT5 (cat. no. sc-398171; Santa Cruz Biotechnology, Inc.), polyclonal rabbit anti-Bcl-2 (cat. no. A5010; Bimake.com), polyclonal rabbit anti-caspase-3-p12 (cat. no. A5357; Bimake.com; 1:500 dilution), HRP-conjugated monoclonal mouse anti-β-actin (cat. no. A5092; Bimake.com; 1:3,000 dilution). After several washes with TBST, each blot was further incubated with secondary antibody (goat anti-mouse, cat. no. sc-2005 or donkey anti-rabbit, cat. no. sc-2313; 1:5,000 dilution) conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.) in room temperature for 2 h. Detection was carried out by enhanced chemiluminescence (Amersham; Cytiva) as per the manufacturer's instructions. β-actin was used as a loading control. Signal intensities were quantified using the ImageJ 2 (National Institutes of Health) program and normalized to the control samples. All the western blots were conducted in three biological repeats.

Constructs, siRNAs and transfection. pEGFP-NFAT5 containing the coding region for myc-tagged NFAT5 (EGFP removed during construction; cat. no. 13627; Addgene, Inc.)

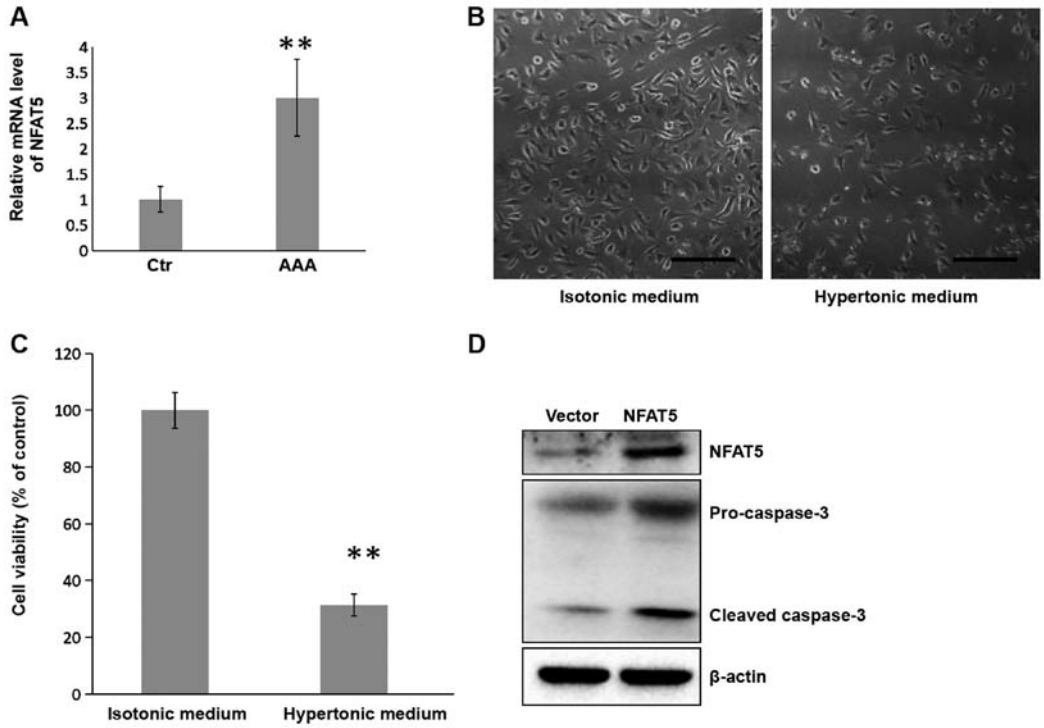

Figure 1. Hypertonic culture medium causes cell death of HUVECs and induces caspase-3 cleavage. (A) AAA tissue samples and adjacent healthy aorta abdominalis Ctr were collected from patients and subjected to RT-qPCR to detect the mRNA levels of NFAT5. Data were normalized to GAPDH mRNA levels. All RT-qPCR experiments were performed in triplicate and the results were presented as the fold change relative to Ctr. **P<0.01, paired t-test. HUVECs were cultured in isotonic or hypertonic medium. After 8 h of incubation, the cells were subjected to (B) phase contrast microscopy (scale bar, 200 μm), (C) MTS cell viability assay and (D) western blotting detection of NFAT5 and cleaved caspase-3. β-actin was used as a loading control. Cell viability is presented as a percentage of the Ctr. n=9 in each group. **P<0.01, Student's t-test. HUVEC, human umbilical vein endothelial cell; AAA, abdominal aortic aneurysm; Ctr, control; RT-qPCR, reverse transcription-quantitative PCR; NFAT5, nuclear factor of activated T cells 5.

was a kind gift from Dr Anjana Rao (Department of Pathology, Harvard Medical School) (15). pEGFP-N1, the empty vector, was a kind gift from Dr Ye Qiu (College of Biology, Hunan University). pSI-Check2-hRluc-NF-κB-firefly, the NF-κB luciferase reporter construct, was a gift from Dr Qing Deng (Department of Biological Sciences, Purdue University) (cat. no. 106979; Addgene, Inc.) (16).

The plasmids were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 1x10^6 HUVEC cells were grown at 37˚C overnight to 80-90% confluence in 6-well plates, washed with PBS and overlaid by the transfection medium (DMEM with 10% FBS and the transfection complex containing plasmids and Lipofectamine® 2000) for 6 h. The transfection medium was then replaced with the complete culture medium for further incubation for 42 h before the following experiments.

The siRNAs targeting human NFAT5 (cat. no. sc-43968; 10 μM) and the scrambled control siRNAs (cat. no. sc-37007; 10 μM) were purchased from Santa Cruz Biotechnology, Inc. All the siRNAs were transfected into cells using Lipofectamine® 2000 according to the manufacturer's instructions. Briefly, HUVEC cells were incubated in 6-well plates at 37˚C overnight to 40% confluence, then washed with PBS and re-cultured in Opti-MEM with transfection complex containing Lipofectamine 2000® and siRNAs. After 6 h of incubation, the transfection medium was replaced with DMEM containing 10% FBS and the incubation was continued for 48 h.

Dual-luciferase assay. pSI-Check2-hRluc-NF-κB-firefly, a luciferase reporter construct for NF-κB activity in which the expression of firefly luciferase (FLuc) is controled by the responsive promoter of NF-κB was used in this assay, and *Renilla* luciferase (RLuc), which is constitutively expressed served as internal control in this assay. HUVECs were transfected with the luciferase reporter constructs together with pEGFP-NFAT5 or the control vector pEGFP using Lipofectamine® 2000. At 48 h post-transfection, HUVECs were lysed by passive lysis buffer (Promega Corporation). The cell lysates were in a Dual-luciferase assay to determine the relative luciferase activity (*Renilla*/firefly) using the Dual-Luciferase Reporter Assay System (Promega Corporation) following the manufacturer's instructions. The luminescence was measured using an ELISA plate reader (Tecan Group, Ltd.).

Statistical analysis. All experiments were repeated three times. Quantitative results are presented in bar graphs as the mean ± SD. Data were analyzed by Microsoft Excel 2010 (Microsoft Corporation) using unpaired Student's t-test unless otherwise stated. P<0.05 was considered to indicate a statistically significant difference.

Results

Hypertonic culture medium induces apoptosis of HUVECs. To explore changes in NFAT5 expression in AAA, AAA samples and adjacent healthy aorta abdominalis samples

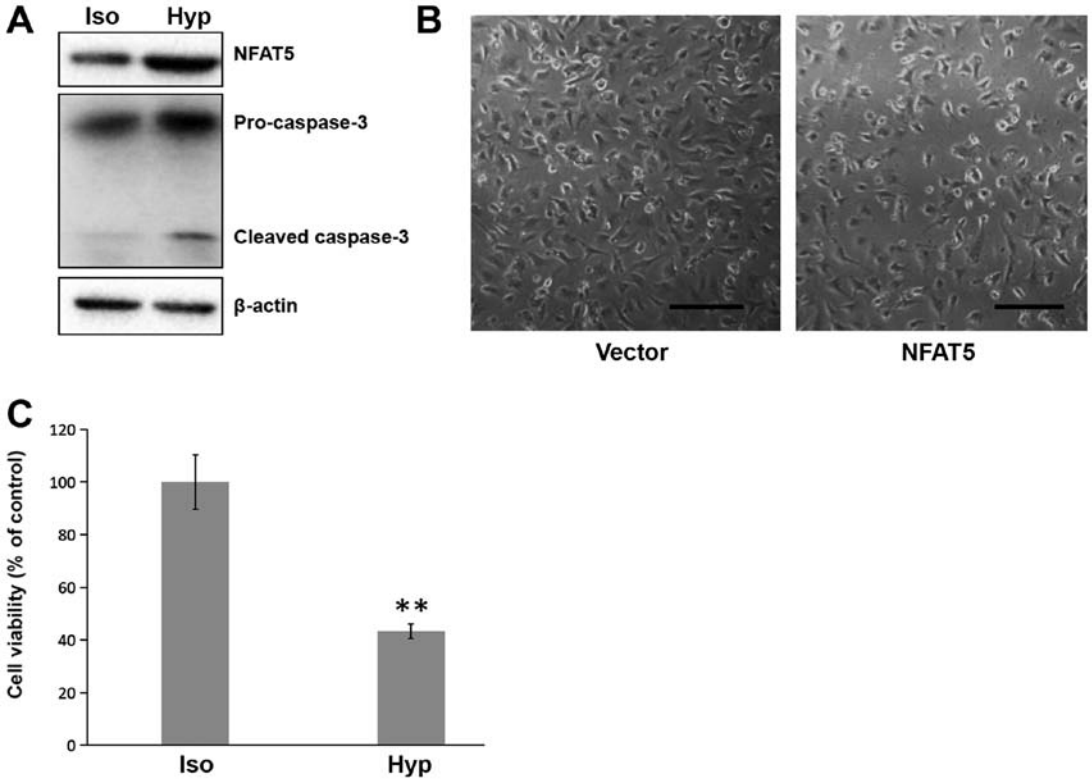

Figure 2. Overexpression of NFAT5 induces caspase-3 cleavage in HUVECs and causes cell death. HUVECs were transfected with pEGFP-NFAT5 (NFAT5) or pEGFP-N1 empty vector (Vector). At 48 h post transfection, the cells were subjected to (A) western blotting detection of NFAT5 and caspase-3 cleavage, (B) phase-contrast microscopy (scale bar, 200 μm) and (C) MTS cell viability assay. β-actin was used as a loading control. Cell viability is presented as a percentage of the Ctr. n=9 in each group. **P<0.01, Student's t-test. NFAT5, nuclear factor of activated T cells 5; HUVEC, human umbilical vein endothelial cell; Iso, isotonic medium; Hyp, hypertonic medium.

were collected from nine patients, and the mRNA levels of NFAT5 detected in the samples by RT-qPCR. As shown in Fig. 1A, compared with the adjacent healthy tissue, NFAT5 was upregulated to ~3 times in the AAA samples, indicating an obvious increase of NFAT5 in AAA.

As NFAT5 is induced by hypertonicity (10) and the dysfunction of the aorta endothelial cells is critical for AAA generation (5), the effect of hypertonicity on endothelial cells was studied. It has previously been suggested that cell culture medium with additional 100 mM of NaCl can elevate the expression of NFAT5 but will not induce apoptosis in HeLa cells within 8 h of treatment (13). In order to elucidate the effect of hypertonic environment on HUVECs, the HUVECs were cultured in the medium with additional 100 mM of NaCl for 8 h. HUVECs in hypertonic medium underwent significant cell death (P=0.00034) compared with those in isotonic medium (Fig. 1B). An MTS assay demonstrated a viability of ~31% for cells cultured in hypertonic medium (Fig. 1C). These results indicate that hypertonicity induces HUVEC death.

To further investigate the underlying mechanism of HUVEC death, the cleavage of caspase-3, an executor of apoptosis, was detected by western blotting. The results demonstrated that the levels of the 17-kDa cleavage band of caspase-3 increased in the cells cultured in hypertonic medium (Fig. 1D), indicating that cell death was due to apoptosis. Notably, an increase of NFAT5 protein in cells subjected to hypertonic culture was also observed (Fig. 1C).

Overexpression of NFAT5 aggravates apoptosis of HUVECs. To explore the role of NFAT5 in the hypertonicity-induced apoptosis of HUVECs, NFAT5 was overexpressed in HUVECs by transfection of pEGFP-NFAT5. The cell viability was evaluated by MTS assay and the protein extracted from the cells was then subjected to western blotting of caspase-3 cleavage. As shown in Fig. 2A, 48 h post transfection, compared with the control cells transfected with pEGFP-N1 (empty vector), the cells transfected with pEGFP-NFAT5 demonstrated an increase of NFAT5 protein and caspase-3 cleavage (Fig. 2A), as well as more severe cell death compared with those in isotonic medium (Fig. 2B). MTS assay further supported this finding by showing a 50% reduction of cell viability upon NFAT5 overexpression (Fig. 2C). These results implied a pro-apoptotic role for NFAT5 in HUVECs.

Knockdown of NFAT5 relieves hypertonicity-induced apoptosis of HUVECs. To verify the role of NFAT5 in hypertonicity-induced apoptosis of HUVECs, the cells were transfected with NFAT5 siRNA, then subjected to the treatment of hypertonic medium as aforementioned. As shown in Fig. 3A, NFAT5 protein was decreased in NFAT5-siRNA-transfected cells, compared with scramble-siRNA-transfected control cells; in addition, the caspase-3 cleavage was reduced in hypertonic medium when NFAT5 was knocked down. MTS assay also demonstrated higher cell viability of ~80% for NFAT5-knocked-down cells, compared with ~43% for control cells (Fig. 3B). These

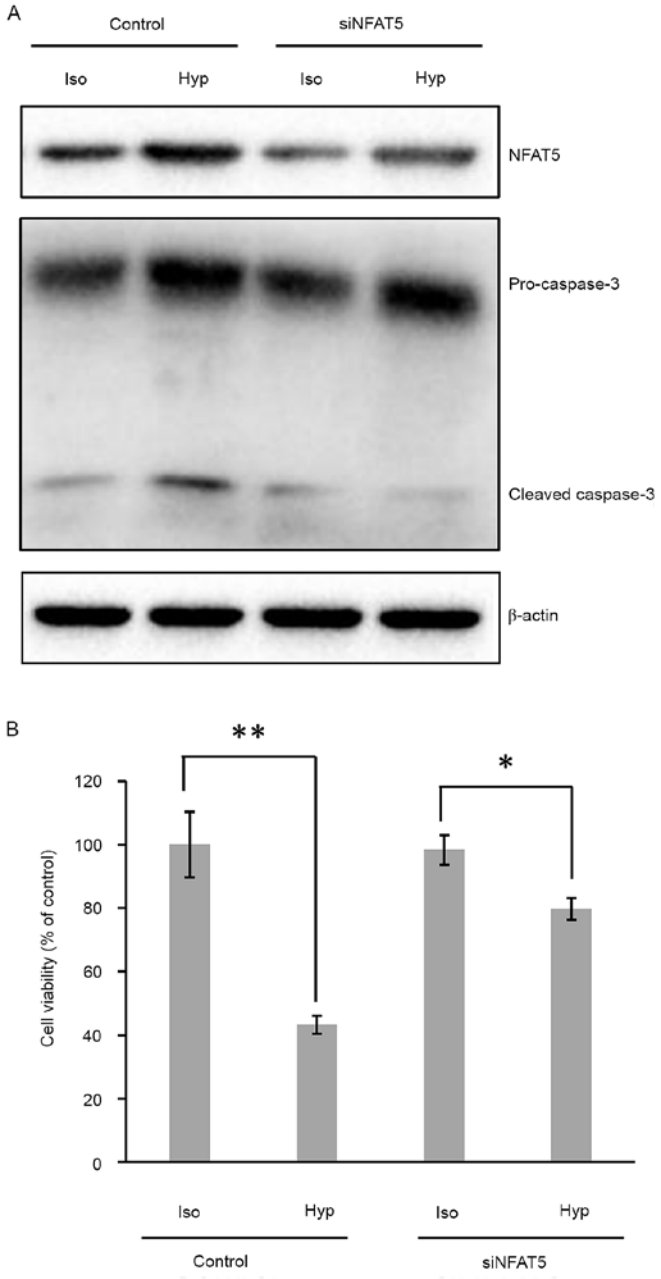

Figure 3. Knockdown of NFAT5 inhibits the apoptosis of HUVECs. HUVECs were transfected with siNFAT5 or scrambled control siRNA (Control). At 40 h post-transfection, the culture medium was replaced with hypertonic medium or isotonic medium as a control. After another 8 h of incubation, the cells were subjected to (A) western blotting detection of NFAT5 and caspase-3 cleavage and (B) MTS cell viability assay. β-actin was used as a loading control. Cell viability is presented as a percentage of the Ctr. n=9 in each group. *P<0.05, **P<0.01, Student's t-test. NFAT5, nuclear factor of activated T cells 5; HUVEC, human umbilical vein endothelial cell; si, small interfering; Iso, isotonic medium; Hyp, hypertonic medium.

results substantiated the involvement of NFAT5 in hypertonicity-induced apoptosis of HUVECs.

NFAT5 activates NF-κB signaling pathway in HUVECs. As aforementioned, the NF-κB signaling pathway is closely associated with the apoptosis of HUVECs, and NFAT5 enhances NF-κB activity. Thus, it was hypothesized that NFAT5 induced apoptosis of HUVECs through the NF-κB pathway. To verify this, the HUVECs were co-transfected with pEGFP-NFAT5 and pSI-Check2-hRluc-NF-κB-firefly. At 48 h post transfection, the cells were used in a Dual-luciferase assay. The results demonstrated that the activity of NF-κB increased 8-fold upon NFAT5 overexpression compared with cells transfected with the empty vector (Fig. 4A). Hypertonic medium treatment demonstrated a similar activation of NF-κB (5-fold). However, this activation was notably reduced when NFAT5 was knocked down using specific siRNA (Fig. 4B), indicating that hypertonicity activated the NF-κB pathway through NFAT5.

NF-κB activation leads to the expression of various pro-inflammatory genes, contributing to the progression of AAA (17). To further clarify the activation of NF-κB signaling cascade induced by NFAT5, the mRNA levels of VCAM-1, ICAM-1 and iNOS, the three pro-inflammatory genes downstream of NF-κB, were detected in HUVECs upon NFAT5 overexpression. As shown in Fig. 4C, when NFAT5 was overexpressed, mRNA levels of all the three genes were significantly elevated (P=0.00058 for VCAM-1, P=0.0012 for ICAM-1 and P=0.0088 for iNOS), indicating an increased expression of these genes induced by NFAT5.

Overexpression of NFAT5 decreases the expression of Bcl-2. Bcl-2 is a typical anti-apoptotic protein. It is reported that NF-κB pathway enhances apoptosis of HUVECs by downregulating the expression of Bcl-2 (9). Thus, the present study verified the effect of NFAT5 on Bcl-2 expression in HUVECs. NFAT5 was overexpressed by transfection as aforementioned. At 48 h post transfection, the cells were subjected to western blotting detection of Bcl-2 protein. These results demonstrated that Bcl-2 was dramatically decreased in NFAT5-overexpressed cells compared with control cells (Fig. 4D), indicating that NFAT5 inhibited the expression of Bcl-2.

Discussion

AAA is the most common form of aortic aneurysm and is characterized by enlargement of the abdominal aorta with a diameter >3 cm or >50% larger than normal (2 cm) (1). The susceptible population of AAA is elderly males and 2-8% of males over the age of 65 are affected by AAA; however, AAA is also identified in women with a rate of one-quarter of that in men (1). AAA usually causes no symptoms but is accompanied by the risk of rupture. Upon AAA rupture, the mortality can be as high as 85-90%, which resulted in 168,200 mortalities around the world in 2013, and the mortality rate continues to increase (18,19). Currently, AAA is a great threat to public health, especially to seniors and surgical treatment is the only option for patients with large AAA (20).

Although the exact pathogenic causes of AAA are still unclear, the risk factors for the progress of AAA usually include tobacco smoking, alcohol, hypertension, genetic background and atherosclerosis (21,22). High salt intake is also reported to be closely associated with increased prevalence of AAA (3). As high salt intake is an important cause of atherosclerosis and hypertension (23,24), it is reasonable to propose that high salt intake contributes to the progression of AAA. However, the pathophysiological link between high salt intake and AAA has rarely been investigated.

Endothelial dysfunction is reported to be critical for the development of AAA (5). Briefly, endothelial dysfunction

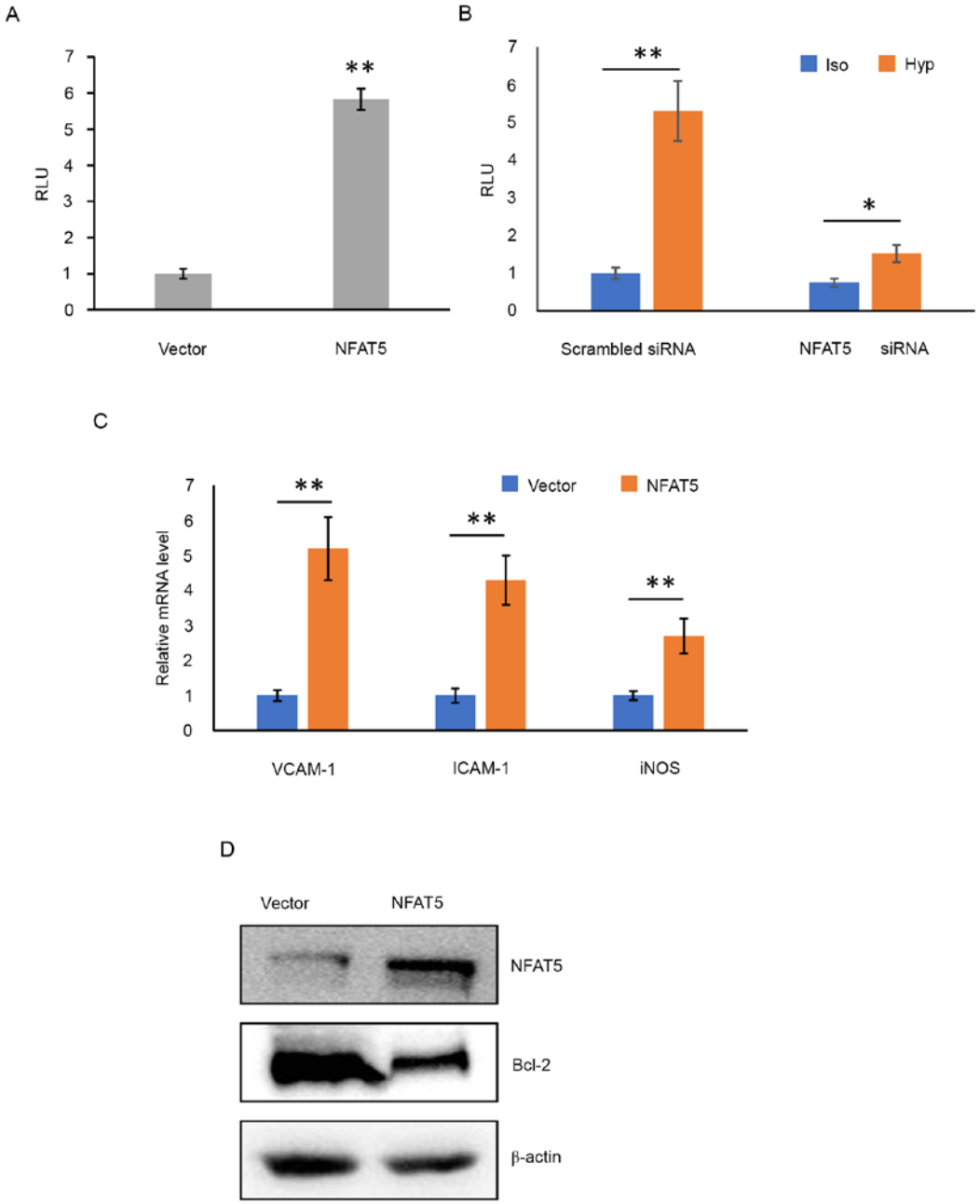

Figure 4. Overexpression of NFAT5 or hyptertonic condition enhances the activity of NF-κB in HUVECs. (A) HUVECs were cotransfected with pSI-Check2-hRluc-NF-κB-firefly and pEGFP-NFAT5 (NFAT5) or pEGFP-N1 empty vector (Vector). At 48 h post transfection, the cells were lysed and subjected to Dual-luciferase assay. (B) HUVECs were co-transfected with pSI-Check2-hRluc-NFκB-firefly and scrambled siRNA or NFAT5 siRNA. At 40 h post transfection, the culture medium was replaced with hypertonic medium or isotonic medium as a control. After another 4 h of incubation, the cells were lysed and subjected to Dual-luciferase assay. The luminescence of firefly luciferase was normalized by the luminescence of *Renilla* luciferase to generate RLU. (C) HUVECs were transfected with pEGFP-NFAT5 (NFAT5) or pEGFP-N1 empty vector. At 48 h post transfection, the cells were lysed and subjected to RT-qPCR detection of VCAM-1, ICAM-1 and iNOS. GAPDH mRNA levels were used as the endogenous control to normalize the data. All RT-qPCR experiments were performed in triplicate and the results were presented as the fold change of each vector group. n=9 in each group. *P<0.05, **P<0.01, Student's t-test. (D) HUVECs were transfected with pEGFP-NFAT5 (NFAT5) or pEGFP-N1 empty vector. At 48 h post transfection, the cells were subjected to western blotting detection of NFAT5 and Bcl-2. β-actin was used as a loading control. NFAT5, nuclear factor of activated T cells 5; HUVEC, human umbilical vein endothelial cell; si, small interfering; RLU, relative luminescence unit; RT-qPCR, reverse transcription-quantitative PCR; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; iNOS, inducible nitric oxide synthase; Vector, empty vector.

impairs the bioavailability of NO, which increases oxidative stress and inflammatory infiltration; the inflammatory cells secretes various proteolytic enzymes and interleukins that degrades the aortic wall, further leading to vascular smooth cells transformation and apoptosis, a typical pathogenic change of AAA. Endothelial dysfunction can be caused by a variety of factors (5). The present study used HUVECs as a model and identified that the extracellular medium with excess NaCl causes the apoptosis of endothelial cells, which may, at least partly, explain how high salt intake raises the risk of AAA.

High sodium in the extracellular fluid leads to a hypertonic environment and brings stress to the cells which will activate NFAT5, the master transcriptional factor regulating hypertonic response (10). NFAT5 transcriptionally regulates the

expression of various downstream genes by directly binding to tonicity-responsive enhancer element in the promoters to relieve hypertonic stress (25). For instance, some genes downstream of NFAT5 are responsible for the biosynthesis of organic osmolytes which balance cellular osmotic pressure, including aldose reductase (26), taurine transporter (27), betaine/GABA transporter (28) and sodium/myo-inositol transporter (29,30). In addition, NFAT5 also induces the expression of molecular chaperones, such as Hsp70-2 (31) and Osp94 (32), in order to prevent the accumulation of misfolding proteins in the stress condition. Therefore, NFAT5 is usually considered as a pro-survival protein that protects the cells in hypertonic stress. However, the present study identified that high level of NFAT5 induced the apoptosis of endothelial cells, indicating diverse functions of NFAT5 on different cells.

The NF-κB pathway is reported to inhibit the proliferation and aggravate the apoptosis of HUVECs (9). The NF-κB pathway is multifunctional and can be either pro-survival or pro-apoptotic for cells in different circumstances (33,34). For HUVECs in stress, the NF-κB pathway is likely to be pro-apoptotic in that it suppresses the expression of Bcl-2, an anti-apoptotic protein, and thus induces apoptosis (9). Notably, NFAT5 positively regulates the NF-κB pathway by forming NF-κB-NFAT5 complexes, which promote the binding of NF-κB to κB elements of NF-κB-responsive genes (12). The results of the present study demonstrated that overexpression of NFAT5 enhanced the activity of NF-κB and reduced the levels of Bcl-2, which may explain the pro-apoptotic role of NFAT5 in HUVECs. In addition, it is also reported that NFAT5 negatively regulates Bcl-2 and promotes cell apoptosis in hepatocellular carcinoma (35), which is consistent with the results of the present study and further supports the mechanism that NFAT5-Bcl2 mediates hypertonicity-induced apoptosis of HUVECs.

Nevertheless, the present study was based on the HUVEC cell model and was not verified in animal models, due to the lack of NFAT5 knockout mice. Conventional NFAT5 knockout mice are reported to be prenatally lethal due to impaired renal and heart development (36,37). Additionally, these mice demonstrate impaired immune responses (38,39), which hinders their application in the study of inflammatory diseases, such as AAA. A tamoxifen-inducible NAFT5 knockout mouse model was established by Kuper *et al* (40), but tamoxifen itself might affect the development of AAA (41). Establishing an adequate NFAT5-deficient animal model for AAA research, perhaps by using a specific NFAT5 inhibitor, is a major direction of our future studies.

In summary, the present study suggested a novel mechanism underlying the apoptosis of endothelial cells in a hypertonic condition. It identified that hypertonicity induced the expression of NFAT5 in HUVECs, subsequently activating the NF-κB pathway and inhibiting Bcl-2 expression, resulting in the apoptosis of the cells. These findings uncovered a distinctive role of NFAT5 in promoting apoptosis of endothelial cells and provided a possible explanation for the risk of high salt intake on AAA. In the prevention and treatment of AAA, more attention should be on hypertonic conditions in the blood, which may also serve as a therapeutic target for drug development.

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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

XX and XF designed the experiments and wrote the manuscript. CH and DX performed the *in vitro* work. YL and MH performed the abdominal aortic bypass surgery and collected patient samples. JL performed the manuscript check and the data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine. Informed consent was signed by all patients who participated in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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