# Regulation of angiotensin II-induced B-cell lymphoma-2-associated athanogene 3 expression in vascular smooth muscle cells

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Abstract. Previous studies have demonstrated that angiotensin II (Ang II) is involved in the process of atherosclerosis and vascular restenosis through its proinflammatory effect. Bcl-2-associated athanogene 3 (BAG3) had been suggested to be associated with proliferation, migration and invasion in many types of tumor. However, the role of BAG3 among the proliferative process of vascular smooth muscle cells (VSMCs) induced by Ang II, to the best of our knowledge, remains to be investigated. The present study demonstrated that in growth-arrested VSMCs, Ang II-induced VSMC proliferation, accompanied by increased BAG3 mRNA and protein expression levels in a dose- and time-dependent manner. BAG3 expression levels were measured in VSMCs treated in the presence or absence of Ang II. The proliferation of VSMCs was assessed using manual cell counting and Cell Counting kit-8 assays. mRNA and protein expression levels of BAG3, Toll-like receptor 4 (TLR4), proliferating cell nuclear antigen, nuclear factor (NF)-KB p65, smooth muscle protein 22a and phosphorylated NF-kB p65 were assessed by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. In non-transfected or scramble short hairpin RNA (shRNA)-transfected VSMCs cells, Ang II significantly induced VSMC proliferation. However, this Ang II-induce proliferation was attenuated when BAG3 was silenced, suggesting that inhibition of BAG3 may somehow reduce proliferation in Ang II-induced VSMCs. Furthermore, the TLR4/NF-KB p65 signaling pathway was involved in BAG3 gene upregulation. In conclusion, to the best of our knowledge, the present study demonstrated for the first time that inhibition of BAG3 attenuates cell proliferation.

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Furthermore, Ang II induced VSMCs proliferation through regulation of BAG3 expression via the TLR4/NF- $\kappa$ B p65 signaling pathway.

## Introduction

Phenotypic switch is a major characteristic of vascular smooth muscle cells (VSMCs), which respond to environmental stimuli via downregulation of contractile marker genes, upregulation of proliferative marker genes, migration ability and synthesis of the cellular matrix (1). Phenotypic transfer of VSMCs from a contractile to a synthetic phenotype is demonstrated to be essential in various vascular diseases, including hypertension, restenosis and atherosclerosis (2,3). Increasing evidence has indicated that as a major peptide hormone of the rennin-angiotensin system, angiotensin II (Ang II) is significant in cardiovascular disease pathogenesis (4). Chronic inflammatory and immunity is significantly associated with atherosclerosis process; according to a previous study, Ang II could generate inflammation in VSMCs via activating Toll-like receptor 4 via the nuclear factor (NF)-kB pathway (5).

Bcl-2-associated athanogene 3 (BAG3), a 576-amino acid anti-apoptotic protein, has been reported to be constitutively expressed in the skeletal muscle, myocardial cells and various tumors, and is associated with multiple pathological process, such as cell survival, cell invasion, and adhesion (6-8). Growing evidence has demonstrated that BAG3 may serve as a key mediator in cardiovascular diseases. Homma *et al* (9) demonstrated that mice with a BAG3 gene defect exhibited myofibrillar degeneration without inflammation, Z-disk architecture disruption, early postnatal apoptotic features and death by 4 weeks of age. Recently, clinical studies have confirmed that BAG3 is associated with cardiomyopathy, such as Takotsubo and dilated cardiomyopathy (10,11).

Growing evidence has confirmed that BAG3 serves a key mediated role in the pathogenesis of cardiovascular damage; however, to the best of our knowledge, there are currently no studies that have investigated whether BAG3 is involved in Ang II-induced VSMC proliferation of VSMCs. Furthermore, the potential underlying mechanism of Ang II inducing BAG3 expression in VSMCs remains unclear. Therefore, the present study aimed to investigate the anti-proliferative effects of BAG3 on Ang II-induced VSMCs, and to elucidate the potential underlying mechanisms.

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*Key words:* Bcl-2 associated athanogene 3, angiotensin II, vascular smooth muscle cells, proliferation

## Materials and methods

Reagents. Fetal bovine serum (FBS) was purchased from Abgent, Inc. (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Hyclone; GE Healthcare Life Sciences (Logan, CT, America). A Cell Counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Ang II, pyrrolidine dithiocarbamate (PDTC), penicillin and streptomycin were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Smooth muscle protein 22a (SM22a; cat. no. PAC781Ra01) antibody was purchased from Cloud-Clone Corp (Katy, TX, USA). GAPDH (cat. no. sc-25778), BAG3 (cat. no. sc-136467), proliferating cell nuclear antigen (PCNA; cat. no. sc-56), NF-κB p65 (cat. no. sc-372), phosphorylated (p)-NF-κB p65 (Ser 536; sc-33020) antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Dallas, TX, USA). Toll-like receptor 4 (TLR4; cat. no. WL00196) antibody was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

*Cell culture*. The A7r5 rat VMSC line was purchased from the China Center for Type Culture Collection (Wuhan, China). Cells were cultured in 25-cm<sup>2</sup> culture flasks (Corning Incorporated, Corning, NY, USA). The cells were developed in DMEM with 100 U/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum, and were stored in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Cells at 80% confluence in culture wells were grown and arrested by serum-starvation for 1 day for subsequent experiments. Cell proliferation and cell number count experiments were conducted as previously described (12). A VMSC suspension ( $0.5x10^5$  cells/ml) was incubated for 1 day without or with Ang II ( $10^{-7}$  M) for 0, 8, 16, 24 or 48 h. Light microscopy was used to count cells in a hemocytometer.

At the density of 5,000 cells per well, CCK-8 assay cells were cultivated in 96-well plates. 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2, 4-disulfophenyl]-2H-tetrazolium, monosodium salt (10  $\mu$ l solution) was added to every well after the treatments. Plates were incubated at 37°C for 4 h. A microplate reader was used to evaluate the absorbance at 450 nm.

*Transfection*. A BAG3 mRNA silencing plasmid, short hairpin (sh)BAG3 (GenBank no. NM\_001011936) was designed and synthesized by Shanghai GeneChem (Shanghai, China). Three different shBAG3 (shRNA-N1, shRNA-N2, and shRNA-N3) targeting the BAG3 gene, or nonspecific shRNA [negative control (NC)-shRNA-N] were transfected into VSMCs for 6 h using Lipofectamine 2000<sup>™</sup> (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. Following this, VMSCs were treated in the presence or absence of 10<sup>-7</sup> M Ang II for 24 h. Plasmid sequences are presented in Table I.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In the present study, TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total cellular RNA. The integrity and purity of the extracted total RNA was evaluated. Reverse transcription was conducted using a Prime Script RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) with 1  $\mu$ g RNA. A

Table I. Primer sequences for Bcl-2-associated athanogene 3.

shBAG3#1 5'-TGCTGAGAAAGTGGAA	
shBAG3#2 5'-GAAGGCAAGAAGACTC shBAG#3 5'-AGGATAAGAAAGGTCC	AGTGAA-3' 'GATAAA-3' CTGAAA-3'

sh, short hairpin.

Table II. Reverse transcription-quantitative polymerase chain reaction primer sequences.

Primer	Sequences
BAG3	
Forward	5'-TAGCTGGACCAGATCTCCCTCCTG-3'
Reverse	5'-CCTTCACTTCCACTTTCTCAGCAG-3'
PCNA	
Forward	5'-TAGAGATGAATGAGCCAGTTCAGC-3'
Reverse	5'-GGGTACATCTGCAGACATACTGAG-3'
SM22α	
Forward	5'-ATCCAAGCCAGTGAAGGTGC-3'
Reverse	5'-CCTCTGTTGCTGCCCATTTG-3'
GAPDH	
Forward	5'-GCCTGGAGAAACCTGCCAAGTATG-3'
Reverse	5'-GAGACAACCTGGTCCTCAGTGTAG-3'

BAG3, Bcl-2-associated athanogene 3; PCNA, proliferating cell nuclear antigen; SM22 $\alpha$ , smooth muscle protein 22 $\alpha$ .

25-µl reaction system was adopted containing 2 µl cDNA products and 12.5 µl SYBR Premix primer mixture (Takara Biotechnology Co., Ltd., Dalian, China). Sangon Biotechnology was assigned to design and synthesize the primers used in the present study, which are presented in Table II. A Thermal Cycler Dice Real Time system (Takara Biotechnology, Co., Ltd.) and a two-step-plus-melting curve program was used to run all the reactions in the following conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Finally, a Thermal Cycle Dice Real Time system was used to analyze gene expression, and the  $\Delta C_q$  method in reference to GAPDH was adopted to analyze the data. The 2<sup>- $\Delta\Delta Cq$ </sup> method was performed to calculate the data based on the measure of the quantitation cycle (13). Three independent experiments were repeated under the same conditions.

Protein extraction and western blot analysis. VSMCs were lysed in cold radioimmunoprecipitation lysis buffer with protease inhibitors. Based on the standard of bovine serum albumin (BSA), a Bicinchoninic Acid assay was used to measure protein concentrations (Beyotime Institute of Biotechnology, Haimen, China). Samples were boiled in 5X sample buffer for 5 min at 100°C. Equal amounts of protein (50  $\mu$ g/sample) were separated by 8-12% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride



Figure 1. Ang II-induced proliferation in VSMCs. Representative microscope images of VMSCs treated with various concentrations of Ang II. Magnification, x50. VMSCs, vascular smooth muscle cells; Ang II, angiotensin II.

membrane with constant current of 100 V. Membranes were blocked with 5% BSA at room temperature for 120 min in TBS with Tween-20 (pH 7.6) and incubated with primary antibodies against TLR4, BAG3, p-NF-KB p65, NF-KB p65, SM22a, PCNA (1:500) and GAPDH (1:1,000). Following incubation with the corresponding horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies or anti-mouse IgG secondary antibodies (SA00001-2 or SA00001-1 respectively; 1:5,000; Wuhan Sanying Biotechnology, Wuhan, China), proteins were detected by the Microchemi 4.2 Bio-imaging system with Enhanced Chemiluminescent-Plus HRP reagents (Beyotime Institute of Biotechnology). Gelpro32 software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to compare the relative grey value of immunoreactive bands. To evaluate protein activation, the densitometry of phosphorylated protein was normalized to total protein. Under the same experimental conditions, the experiments were repeated 3 times.

*NF*-κ*B blocking assay.* To evaluate if TLR4/NF-κB mediated the upregulation of BAG3, a selective inhibitor of NF-κB (PDTC; cat. no. S1808; Beyotime Institute of Biotechnology) was dissolved in dimethyl sulfoxide and was added to the medium at a final concentration of 25  $\mu$ mol/l. A VMSC suspension (0.5x10<sup>5</sup> cells/ml) was incubated for 1 day prior to pre-treatment with PDTC for 1 h and subsequent culture with or without Ang II for 24 h. Following this, western blot analysis was performed as described above.

*Statistical analysis.* The experiments were repeated in triplicate. To analyze data, statistical software package of SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) was

used. Data are expressed as the mean  $\pm$  standard error of three independent experiments. One-way analysis of variance was used to analyze differences between the groups, followed by the Least Significant Difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*VSMC proliferation is induced by Ang II in a dose- and time-dependent manner.* Ang II induced cell proliferation in VSMCs in a dose-dependent manner (Figs. 1 and 2). Furthermore, following treatment with 10<sup>-7</sup> M Ang II, the proliferation of VSMCs increased in a time-dependent manner (Figs. 1 and 2).

Ang II-induced upregulation of BAG3 mRNA and protein expression in VSMCs. As determined by RT-qPCR, BAG3 mRNA expression levels increased in a concentration-dependent manner. PCNA mRNA expression levels peaked following 10<sup>-7</sup> M Ang II treatment. No significant differences were observed in SM22 $\alpha$ mRNA expression levels after 10<sup>-8</sup> and 10<sup>-6</sup> M Ang II treatment; however, 10<sup>-7</sup> resulted in a decrease in expression levels (Fig. 3A). Similar effects were observed at the protein level, except 10<sup>-6</sup> M Ang II treatment resulted in a significant downregulation in SM22 $\alpha$  expression (Fig. 3B and C). Furthermore, Ang II stimulation increased BAG3 and PCNA and decreased SM22 $\alpha$  mRNA and protein expression levels in a time-dependent manner (Fig. 4).

*Knockdown BAG3 expression attenuates Ang II-induced VSMC proliferation*. To assess the potential effect of BAG3 on VSMCs pretreated with Ang II *in vitro*, a BAG3 silencing plasmid was used to knock down BAG3 expression.



Figure 2. Ang II-induced proliferation in vascular smooth muscle cells. Manual cell counts and Cell Counting kit-8 assays were performed. Cell numbers following Ang II treatment (A) at different concentrations and (B) over various time points. Cell viability following Ang II treatment (C) at different concentrations and (D) over various time points. Data are expressed as the mean  $\pm$  standard error of six independent experiments. \*P<0.05, \*\*P<0.01 vs. control (NC, 0 M or 0 h). NC, negative control; Ang II, angiotensin II.





Figure 3. Effects of various concentrations of Ang II on BAG3, SM22 $\alpha$  and PCNA expression. (A) mRNA expression levels, as determined by reverse transcription-quantitative polymerase chain reaction. (B) Representative western blot images and (C) quantification of protein expression levels following Ang-II treatment. Data are expressed as the mean  $\pm$  standard error of three independent experiments. \*P<0.05, \*\*P<0.01 vs. NC. NC, negative control; Ang II, angiotensin II; BAG3, Bcl-2-associated athanogene 3; SM22 $\alpha$ , smooth muscle protein  $\alpha$ ; PCNA, proliferating cell nuclear antigen.

Figure 4. Effects of various incubation times of Ang II on BAG3, SM22 $\alpha$  and PCNA expression. (A) mRNA expression levels, as determined by reverse transcription-quantitative polymerase chain reaction. (B) Representative western blot images and (C) quantification of protein expression levels following Ang II treatment. Data are expressed as the mean  $\pm$  standard error of three independent experiments. \*P<0.05, \*\*P<0.01 vs. 0 h. Ang II, angiotensin II; BAG3, Bcl-2-associated athanogene 3; SM22 $\alpha$ , smooth muscle protein  $\alpha$ ; PCNA, proliferating cell nuclear antigen.



Figure 5. BAG3 gene silencing suppresses the expression of BAG3 protein in VSMCs. VSMCs were transfected with NC, nonspecific shRNA-BAG3 (NC-shRNA-N) or three different shRNA for 6 h and then cultured 24 h. (A) mRNA and (B and C) protein expression levels. Data are expressed as the mean  $\pm$  standard error of three independent experiments. \*P<0.05, \*\*P<0.01 vs. NC. NC, negative control; sh, short hairpin; si, small interfering; BAG3, Bcl-2-associated athanogene 3; VSCMs, vascular smooth muscle cells.

As presented in Fig. 5, shRNA-N2 and shRNA-N3 significantly decreased the expression of BAG3, whereas shRNA-N1 appeared to upregulate BAG3 expression (P>0.05). The shRNA-N3 had the greatest downregulation effect on both mRNA (40.61%; P<0.001; Fig. 5A) and protein (57.47%; P<0.01; Fig. 5B and C) expression levels. Therefore, shRNA-N3 was used in subsequent experiments. Furthermore, no significant differences were observed between BAG3 mRNA and protein expression levels between the NC and NC-shRNA-N groups (Fig. 5); therefore, transfection efficacy was determined.

Compared with the NC group, Ang II significantly increased cell viability (Fig. 6). Furthermore, Ang II upregulated the protein expression levels of BAG3 and PCNA, and decreased SM22 $\alpha$  expression (Fig. 7). However, shBAG3 significantly ameliorated these effects (Figs. 6 and 7), indicating that regulation of Ang II may be partially mediated by BAG3 activity, and may be based on upregulation of proliferative factors.

Ang II induces BAG3 production via the NF-κB p65 signaling pathway. As the NF-κB p65 signaling pathway may be linked to the proliferative, migratory and invasion effects of BAG3, the present study further analyzed the TLR4/NF-κ P65 pathway activity in the process of Ang II-treated VSMCs. When treated with Ang II, the TLR4/NF-κ p65 pathway was activated (Fig. 8). To further estimate whether TLR4/NF-κ p65 mediates the upregulation of BAG3, VSMCs were pretreated with PDTC and were subsequently cultured with or without Ang II for 24 h (14). The results suggested that PDTC pretreatment markedly decreases BAG3 expression in response to the



Figure 6. Ang II induces proliferation in VSMCs. Viability of NC and shRNA-transfected VMSCs, treated in the presence or absence of Ang II, as assessed by Cell Counting kit-8 assay. Data are expressed as the mean ± standard error of three independent experiments. \*P<0.05 vs. Ang II group; \*P<0.05 vs. NC group. NC, negative control; sh, short hairpin; VMSCs, vascular smooth muscle cells; Ang II, angiotensin II.



Figure 7. BAG3 gene silencing suppresses proliferative factors in Ang II-treated VSMCs. VSMCs were transfected with NC or NC-shRNA-N3 in the presence or absence of Ang II. (A) Representative western blot images and (B) quantification of BAG3, PCNA and SM22 $\alpha$  protein expression levels. Data are expressed as the mean ± standard error of three independent experiments. \*P<0.05, \*\*P<0.01 vs. NC; \*P<0.05, \*\*P<0.01 vs. Ang II. NC, negative control; Ang II, angiotensin II; BAG3, Bcl-2-associated athanogene 3; SM22 $\alpha$ , smooth muscle protein  $\alpha$ ; PCNA, proliferating cell nuclear antigen; sh, short hairpin.

treatment of Ang II (P<0.001); however, no such effect was observed on the basic expression under regular conditions (Fig. 9). Therefore, Ang II may regulate BAG3 expression via the TLR 4/NF- $\kappa$  p65 signaling pathway in VSMCs.

## Discussion

The present study demonstrated that Ang II induced cell proliferation and upregulated BAG3 expression in VSMCs. Similarly, a previous study reported that Ang II regulates BAG3 mRNA and protein expression in cultured renal fibroblast (15). Notably, the results of the present study demonstrated that regulation of Ang II on BAG3 expression may be partially mediated via the TLR4/NF- $\kappa$ B p65 signaling pathway in VSMCs. This research firstly presents a potential link between Ang II and BAG3 in VSMCs *in vitro*.





Figure 8. Effects of Ang II on TLR4, p-NF- $\kappa$ B p65 and BAG3 protein expression. VSMCs were incubated with or without Ang II for 24 h. (A) Representative western blot images and (B) quantification of TLR4, p-NF- $\kappa$ B p65 and BAG3 protein expression. Data are expressed as the mean ± standard error of three independent experiments. \*P<0.05, \*\*P<0.01 vs. NC. NC, negative control; TLR4, toll-like receptor-4; NF- $\kappa$ B p65, nuclear factor- $\kappa$ B p65; p, phosphorylated; Ang II, angiotensin II; BAG3, Bcl-2-associated athanogene 3; SM22 $\alpha$ , smooth muscle protein  $\alpha$ ; PCNA, proliferating cell nuclear antigen.



Figure 9. NF- $\kappa$ B p65 inhibitor PDTC suppresses BAG3 protein expression upregulation, stimulated by Ang II in vascular smooth muscle cells. (A) Representative western blot images and (B) quantification of BAG3 and PCNA protein expression levels following PDTC pretreatment. Data are expressed as the mean  $\pm$  standard error of three independent experiments. \*P<0.05 vs. NC group, \*P<0.05 vs. Ang II group. PDTC, pyrrolidine dithiocarbamate; BAG3, Bcl-2-associated athanogene 3; PCNA, proliferating cell nuclear antigen; NC, negative control; Ang II, angiotensin II.

Previous studies have reported that in various tumor diseases, BAG3 may be significant in sustaining cellular survival, proliferation, migration and invasion, and resistance to therapy (16,17). Therefore, BAG3 has been identified as a novel anti-cancerous target in humans (18). However, BAG3 has been reported to only highly expressed in limited cell types, including myocytes and skeletal muscle cells. BAG3 is associated with cell resistance to mechanical stress (6). Du et al (15,19) demonstrated in a rat unilateral ureteral obstruction model that BAG3 expression is associated with the renal fibrosis level. Furthermore, they verified that BAG3 contributes to renal fibrosis via increasing the synthesis and deposition of extracellular matrix protein in vitro (15,19). Similarly, BAG3 mutations have been reported to be associated with familial cardiomyopathy. Furthermore, in end-stage heart failure without a familial history, the level of BAG3 is significantly reduced (20,21). A previous study demonstrated that BAG3 regulates contractility and Ca<sup>2+</sup> homeostasis in adult mouse ventricular myocytes (22). However, to the best of our knowledge, there is no evidence to clarify whether BAG3 is involved in the pathogenic process of VSMCs. Therefore, the present study aimed to estimate the potential effects of Ang II on BAG3 expression in VSMCs in vitro.

A dose- and time-dependent increase in BAG3 mRNA and protein expression levels were observed in the Ang II-induced VSMC proliferative process. Therefore, Ang II may cause vascular remodeling partially by modulation of BAG3 expression. It is well demonstrated that Ang II has a significantly regulative effect on cell proliferation in VSMCs, and serves as a key regulator in the cell proliferative process. A previous study demonstrated that in serum-deprived HK2 cells, the expression of BAG3 mRNA is relatively low. However, transforming growth factor- $\beta$ 1 markedly upregulates this expression (15). Consistent with this, the present study demonstrated relatively low levels of BAG3 under normal conditions. However, treatment with Ang II significantly upregulated BAG3 expression levels, accompanied by increased VMSC proliferation in a dose- and time-dependent manner. This suggested a key regulative role of BAG3 in the proliferative process of Ang II stimulation.

Ang II is significantly associated with the pathophysiological action of atherosclerosis through its pro-inflammatory effect. It has been demonstrated to produce inflammation in VSMCs via the TLR4/NF-KB p65 signaling pathway (5). A previous study demonstrated that suppression of NF-KB p65 activity may prevent proliferation in VSMCs induced by high glucose (23). Furthermore, Rapino et al (24) revealed that NF-kB activation is necessary in BAG3 induction following ST80/Bortezomib treatment in rhabdomyosarcoma cells. Therefore, it was hypothesized that Ang II may induce BAG3 upregulation via activation of the TLR4/NF-KB p65 signaling pathway. The results of the present study suggested that the TLR4/NF-κB p65 pathway was stimulated by Ang II. When VSMCs were pretreated with PDTC, an antagonist of the NF-kB p65 signaling pathway, BAG3 protein expression levels were decreased. Therefore, Ang II may BAG3 expression partially via the NF-kB p65 signaling pathway.

In conclusion, to the best of our knowledge, the present study demonstrated that Ang II induces VSMC proliferation, accompanied with an elevation of BAG3 expression. Furthermore, Ang II elevated BAG3 expression partially via activation of the TLR4/NF- $\kappa$ B p65 pathway in VSMCs. To the best of our knowledge, the present study provides novel evidence to explain the pathological mechanism of Ang II-induced VSMC proliferation, demonstrating that BAG3 may act as a mediator of this mechanism and may be a viable target in the development of future therapies for vascular disease.

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## **Competing interests**

The authors declare that they have no competing interests.

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