The sympathetic transmitter norepinephrine inhibits VSMC proliferation induced by TGFβ by suppressing the expression of the TGFβ receptor ALK5 in aorta remodeling

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Abstract. The sympathetic system is involved in the arterial diseases, but its mechanism remains poorly understood. The present study aimed to explore the impact of the sympathetic neurotransmitter norepinephrine (NE) on transforming growth factor (TGF) β signaling and the role of NE in aortic remodeling. Guanethidine was used to induce a regional chemical sympathetic denervation (CSD) in angiotensin II (AngII) and β-aminopropionitrile (BAPN)-induced aortic aneurysm models. The diameter of the aorta was measured, and elastic fiber staining was performed. TGFβ type I receptor kinase (ALK5) expression in rat aortic NE-treated vascular smooth muscle cells (VSMCs) was detected by reverse transcription-quantitative PCR and western blotting. The effects of NE and ALK5 overexpression on migration, proliferation, apoptosis and TGFβ signaling were also evaluated. Furthermore, adrenergic receptor blockers were used to determine which receptor was involved in the modulation on TGFβ signaling by NE. The results of the present study demonstrated that CSD protected rats from AngII+BAPN-induced aortic remodeling and aneurysm formation. Compared with the control group, NE inhibited VSMC proliferation and migration, but promoted apoptosis by suppressing ALK5 expression, reversing the effects of TGFβ signaling through the suppression of the SMAD-dependent canonical pathway and promotion of the non-canonical pathway. These effects were prevented by ALK5 overexpression. The inhibition of α- or β-adrenergic receptors alleviated the NE-mediated suppression of ALK5 expression. In conclusion, regional CSD protected rats from aortic aneurysm. NE inhibited SMAD2/3-dependent TGFβ signaling by suppressing ALK5 expression, which may serve an important

role in VSMC biological functions. Both α- and β-adrenergic receptors were involved in the regulation of ALK5 expression by NE. Abnormal sympathetic innervation of the aorta may be used as a therapeutic target in aortic diseases.

Introduction

Aortic aneurysm, dissection and dilation share a common pathological feature of cystic medial necrosis and the histological characteristics of cystic medial necrosis include the loss of contractile vascular smooth muscle cells (VSMCs), leading to apoptosis and phenotypic switching, elastic fiber degradation and inflammatory cell infiltration (1). To date, the mechanism contributing to aortic remodeling remains unclear (2).

Previous studies have demonstrated that sympathetic activation and over-innervation promotes aortic dissection (3,4) and that norepinephrine (NE) released by sympathetic nerve endings can upregulate the expression of matrix metallopeptidase-2 (MMP2) and promote aortic remodeling (5). However, the signaling pathway involved in NE regulation of aortic remodeling is still unknown.

Transforming growth factor (TGF) β signaling serves a central role in aortic remodeling. Mutations of TGFβ family members such as TGFR-1/TGFR-2 result in a hereditary aortic aneurysm such as Loeys-Dietz syndrome (6). Marfan syndrome is a result of fibrillin-1 modification due to a defect or a mutation of the gene that encodes it, which is considered to regulate TGFβ bioavailability and activity by controlling access to, or the efficiency of, TGFβ activators (7). The dysregulation of the downstream TGFβ pathway signaling is also associated with aortic aneurysm (7). Since the homeostasis of TGFβ signaling is important in maintaining a normal structure of the aortic wall, the present study aimed to explore whether the sympathetic system may also regulate aortic remodeling via the TGFβ pathway.

Little is known regarding the effect of the sympathetic system on TGFβ signaling. Yang *et al* (8) investigated the interaction between the α1 adrenergic receptor and TGFβ type I receptor kinase (ALK5) pathways; however, the study was insufficient to clarify the relationship between the sympathetic system and TGFβ signaling. Therefore, the present study was designed to test a new hypothesis that the sympathetic system may regulate ALK5-mediated TGFβ signaling, thus

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serving a role in aortic remodeling. Previous studies have provided evidence on the use of ALK5 as a therapeutic target; for example, galunisertib, an ALK5 inhibitor, has antitumor activity in tumor-bearing animal models of breast, colon and lung cancers, and hepatocellular carcinoma (9); a phase II study has revealed that galunisertib treatment exerts hematologic improvements in low- and intermediate-risk myelodysplastic syndromes (10). Thus, the possibility of using ALK5 as a therapeutic target in aortic aneurysm was also explored in the present study.

Materials and methods

Animal experiments. As previously described (5), 50 male Sprague-Dawley rats (8 weeks, weight 267-299 g) were brought from ABLIII experimental animal laboratory of Wuhan university and housed in an animal room under controlled conditions of 20-26°C and 40-70% humidity on a 12/12-h light/dark cycle. Normal chow was supplied to the control group, where as 0.25% β-aminopropionitrile (BAPN) chow was supplied to the angiotensin II (AngII) and BAPN group to loosen the cross-link among elastic fibers (11-13). Chemical sympathetic denervation (CSD) was performed under pentobarbital anesthesia (1%; 30 mg/kg) through a left paraspinal chest incision. The descending aorta between the left subclavian artery and the diaphragm was dissected and covered by a gauze pre-soaked in 20 μg/μl guanethidine for 30 min. An osmotic minipump (Alzet, Durect Corp.) was implanted into the peritoneal cavity to infuse 1,000 ng/kg/min AngII continuously for 4 weeks. The same operation and osmotic minipump was used in the control group where saline was used instead of guanethidine or AngII. At the end of 4 weeks, all surviving mice were sacrificed by CO_2 (100% CO_2, 2.5 liters per min, 5 min) and survival rate was calculated as survived/total. The experiments were approved by The Ethics Committee of Renmin Hospital (Wuhan, China).

Cell culture and treatment. Mouse VSMC cell line (MOVAS) was obtained from ATCC and cultured in DMEM (Procell Life Science & Technology Co., Ltd.) containing 10% FBS (Procell Life Science & Technology Co., Ltd.) at 37°C with 5% CO_2 and 95% air. The cells were sub-cultured to 70% confluence and subsequently cultured in DMEM without serum for 12 h before treatment; 1% FBS was added to the medium during any treatment.

ALK5 overexpression. Mouse ALK5 coding sequence was cloned into a pcw107 (V5) vector (Hanbio Biotechnology Co., Ltd.). A lentivirus was obtained using the PPMD2.G (Hanbio Biotechnology Co., Ltd.) and psPAX2 vectors (Hanbio Biotechnology Co., Ltd.) in 293T cells (China Center for Type Culture Collection). The lentivirus was aliquoted and transfected to the mouse VSMCs at the unified concentration using polybrene (8 μg/ml, Sigma-Aldrich; Merck KGaA) for 72 h.

Histology and immunostaining. Histology and immunostaining were performed as previously described (14). Briefly, sections were cut at 4 μm from the paraffin-embedded aortic specimens of the rat model or control. The sections were stained with hematoxylin and eosin or elastica Van Gieson

staining and immunostained with antibodies against each target protein (TH; 1:100, CST Biological Reagents Co., Ltd.; cat. no. 58844S; ALK5; 1:200, Abcam cat. no. ab31013). For the cell staining, having been seeded on the slides for 24 h, the cells (~10^5 cells/cm^2) were fixed in 4% paraformaldehyde (MACKLIN, China, Cat No. 30525-89-4) at 4°C for 20 min and stained using the same antibodies (incubated overnight at 4°C) as above. An Olympus BX53 fluorescent microscope (Olympus Corporation) was used to investigate and capture images. Sympathetic nerve densities were determined by Image-Pro Plus 6.0 (Media Cybernetics, Inc.,) in tyrosine hydroxylase (TH) staining slides as previously described (3). Nerve density was calculated as the nerve area divided by the total area examined (μm^2/mm^2).

Realtime quantitative PCR (RT-qPCR). Total RNA was extracted from cells (MOVAS) using RNAiso plus (Takara Bio, Inc.) according to the manufacturer's instructions followed by reverse transcription (SMART MMLV cDNA synthesis kit, Takara Biotechnology Co., Ltd.). A total of 20 μl was used (2 μl cDNA, 10 μl SYBR® Green (Thermo Fisher Scientific, Inc.), 2 μl primer (Servicebio), 6 μl water) on an ABI 9700 qPCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The settings were: 93°C for 40 sec, 58°C for 30 sec and 72°C for 60 sec (35 cycles). The primers are listed in Table I. The result was calculated by the $2^{-\Delta\Delta Cq}$ method (15).

Western blotting. RIPA lysis buffer (Beyotime Biotechnology, Inc. cat no. P0013B) was used to extract total proteins from cells and bicinchoninic acid assay was used to measure the protein concentration. Protein (20-30 μg) was loaded onto a 15% SDS-PAGE gel and ran at 100 V. Then the protein was transferred onto PVDF membranes and blocked by 5% milk at room temperature for 1 h. Primary antibodies were incubated with the membrane overnight at 4°C: ALK5, Abcam, cat. no. ab31013, 1:1,000; SMAD2/3, CST Biological Reagents Co., Ltd., cat. no. 5678, 1:2,000; p-SAMD2/3, Santa Cruz Biotechnology, Inc., cat. no. sc-11769,1:200; ERK1/2, Biorbyt Technology, Inc., cat. no. orb216186, 1:500; p-ERK1/2, CST Biological Reagents Co., Ltd., cat. no. 8544, 1:1,000; JNK1/2, Santa Cruz Biotechnology, Inc., cat. no. sc-7345, 1:200; p-JNK, Invitrogen (Thermo Fisher Scientific, Inc.), cat. no. 700031, 1:1,000; GAPDH, Santa Cruz Biotechnology, Inc., cat. no. sc47724, 1:1,000. The membrane was then incubated with the secondary antibodies and chemiluminescence method was used to acquire images. Image-Pro Plus 6.0 (Media Cybernetics, Inc.) was used to analysis the bands.

Cell migration assay. Cell migration assay was performed using mouse VSMCs. Cells were cultured in DMEM supplemented with 1% FBS as VSMCs stopped proliferating in 1% FBS (Fig. S1). The scratch was created with a 200 μl pipette tip and the scratch closure was monitored at 24 and 48 h. Scratch closure was quantified using ImageJ 1.52t (National Institutes of Health).

NE concentration assay. Small segments of the aorta were immediately placed in 0.1 M Perchloric acid (HClO4) solution after harvest and kept overnight and then stored under -80°C. The samples was ground and dissolved in the same volume

Table I. Primers used in the present study.

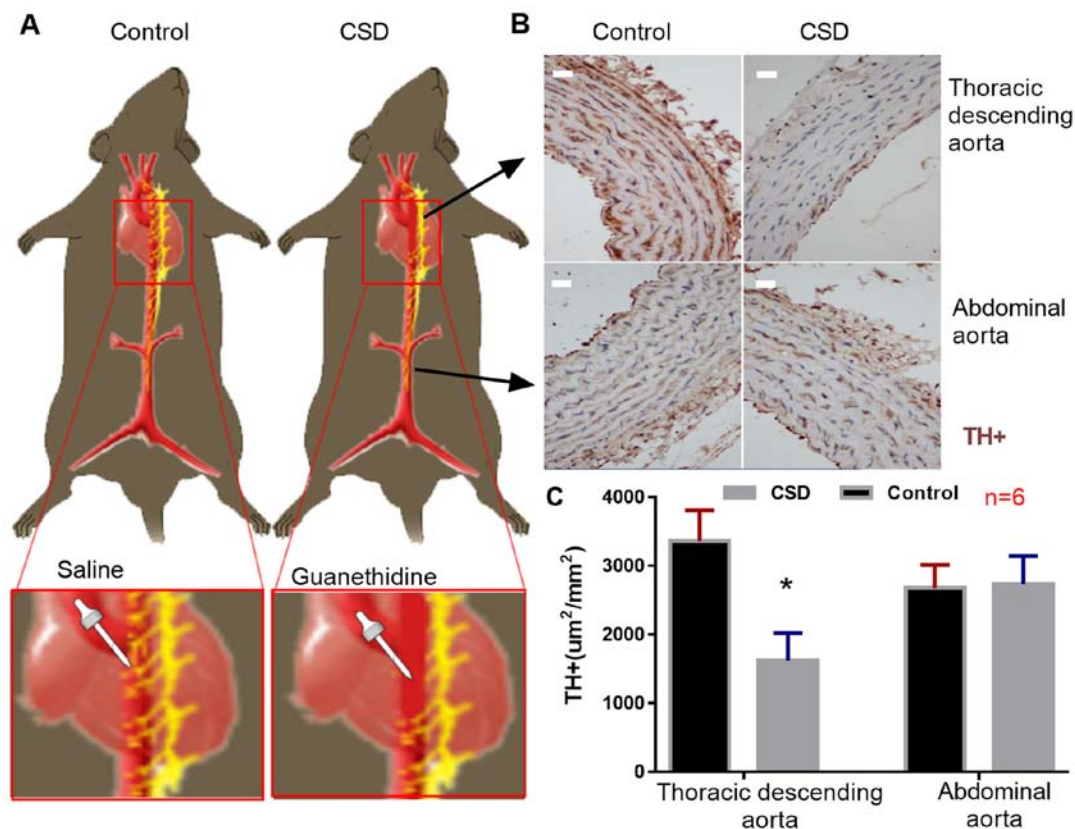| Target genes	Forward 5'-3'	Reverse 5'-3'
Mouse ALK5	GAAAAGCAGTCAGCTGGCCTT	CTTCATTTGGCACACGGTGG
Mouse TGFβ1	CTGCTGACCCCCACTGATAC	AGCCCTGTATTCCGTCTCCT
Mouse INHBA	AAATCAGAACGCCTCCGCTA	TCCCGAGTGTAGAGTTCGGT
Mouse BMP4	TCCGTCCCTGATGGGATTCT	TGGTGTCTCATTGGTTCCTGC

Figure 1. Schematic diagram and the results of CSD. (A) Schematic diagram: CSD was performed on descending aorta with guanethidine but not on abdominal aorta. Saline was used in the control group. (B and C) TH⁺ sympathetic nerve endings were stained. The number of TH⁺ cells was significantly decreased after CSD in the thoracic descending aorta compared with the control group, whereas no change was observed in the abdominal aorta. Scale bars, 400 μm; n=6 in each group. *P<0.01 vs. control. CSD, chemical sympathetic denervation; TH⁺, tyrosine hydroxylase-positive.

of saline (50 mg/ml). The concentration of AngII and NE in the aorta were detected using ELISA kits (AngII, Cloud-Clone Corp. (CEA005Ra); NE, Eagle Biosciences, Inc. (SKU: NOR31-K01) according to the manufacturers' instructions.

Cell proliferation assay. Cell Counting Kit-8 (CCK8) proliferation assay kit was obtained from Biosharp Life Sciences (cat. no: BS350B) and used according to the manufacturer's protocol. Mouse VSMCs were seeded in 96-well plates at a density of 3×10^3 cells/well. After 24 h, DMEM containing 1% FBS and 10 μl CCK8 was added into each well and incubated for 4 h at 37°C. The optical density was read at 450 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc.). The NE and FBS concentration gradient experiment was performed with different dosages of NE or FBS in DMEM by cell counting. Briefly, MOVAS cells were passaged and synchronized in 1% FBS for 12 h and 1, 10, 50, 100 and 200 nm NE and

0, 1, 2, 5 and 10% FBS DMEM was used to culture cells. Cell numbers were counted at 24, 48 and 72 h.

Apoptosis assay. DMEM containing 1% FBS was applied to cells treated with NE, NE+ALK5 or PBS in each group. According to the manufacturer's instructions of the apoptosis assay kit (Nanjing KeyGen Biotech Co., Ltd.), after trypsinization, cells were washed twice, centrifuged (800 x g, 4°C for 5 min) and resuspended in 500 μl Binding Buffer from the kit). Next, the suspension was mixed with 5 μl AnnexinV-FITC, and 5 μl propidium iodide (PI) was added. After incubation in the dark for 5-15 min, flow cytometry assay was performed using a CytoFLEX flow cytometer with Cytexpert software (Beckman Coulter, Inc.; version 2.3). The negative control was without AnnexinV-FITC and PI. Late apoptosis was assessed and the most significant apoptotic group was used as a positive control, as suggested by the kit manufacturer.
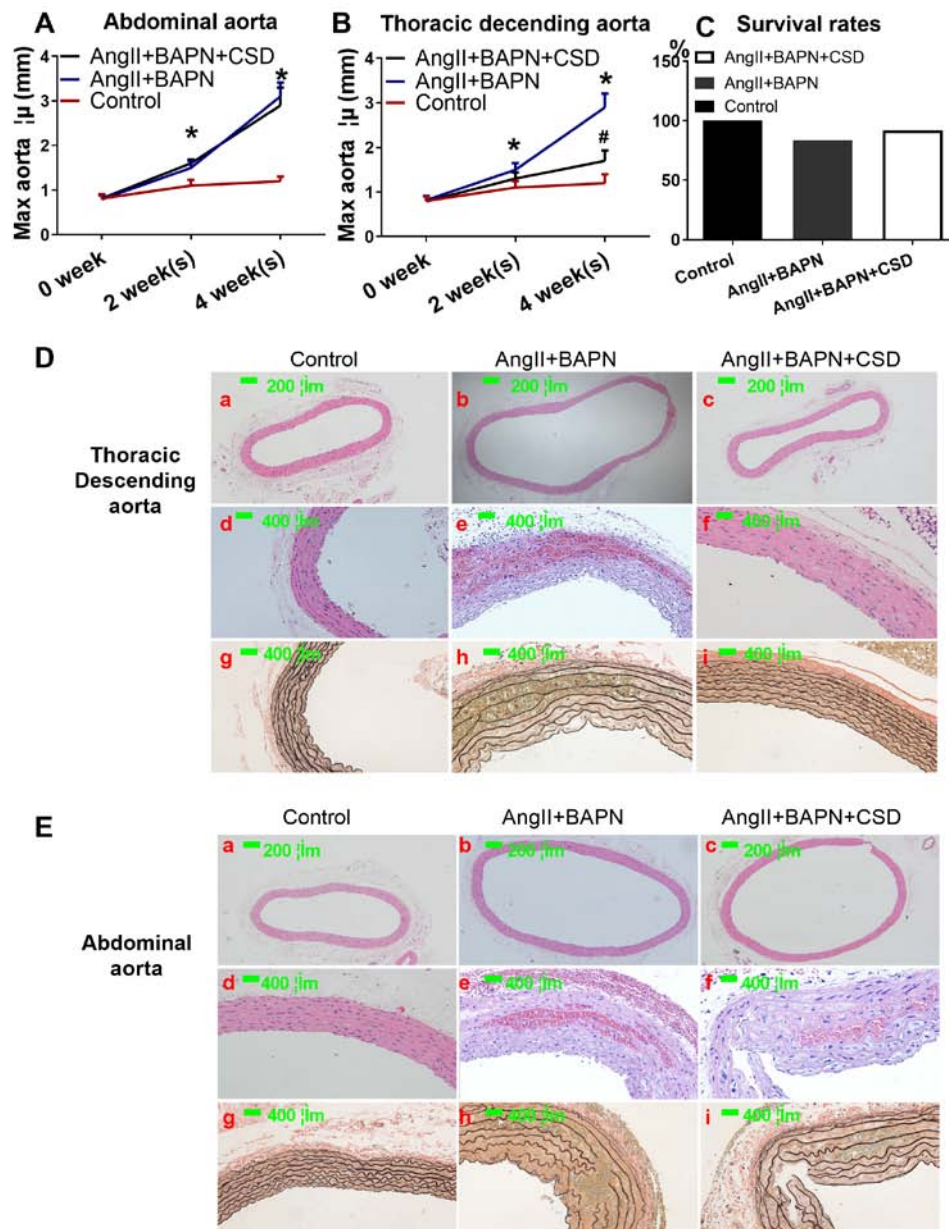
Figure 2. Impact of CSD on aorta dilation and aortic remodeling induced by AngII+BAPN. (A and B) AngII+BAPN induced significant aortic dilation in the thoracic descending and the abdominal aortas. When CSD was performed, the AngII+BAPN-induced aortic dilation was alleviated in (B) the thoracic descending aorta, but not in (A) the abdominal aorta. (C) The survival rate of rats in each group was not significantly different. (D and E) HE and elastica Van Gieson staining demonstrated significant aortic dilation and elastic fiber destruction, which was rescued by CSD in (D) the thoracic descending aorta, but not in (E) the abdominal aorta. n=12 in each group. *P<0.01 vs. control. #P<0.01 vs. AngII+BAPN group. AngII, angiotensin II; BAPN, β-aminopropionitrile; CSD, chemical sympathetic denervation; HE hematoxylin and eosin.

Statistical analysis. All quantitative data are presented as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software (version 6; GraphPad Software Inc.). Unpaired Student's t-test was performed to calculate the differences between two groups, and one-way ANOVA was performed when three groups were compared. Tukey's post hoc test was used for multiple comparisons. P<0.01 was considered to indicate a statistically significant difference.

Results

Aortic CSD protects AngII-induced aortic remodeling. At the end of the animal experiment, TH-positive (TH+)

cells (sympathetic nerve endings) were immunostained. The number of TH+ cells were significantly decreased in the descending aortas, but not in the abdominal aortas of the CSD group compared with those in the control groups (Fig. 1). No aortic rupture was observed in the control group, but two (16.7%) ruptures in the AngII+BAPN group and one in the AngII+BAPN+CSD group (8.3%) were present (Fig. 2C). The diameter of both the descending and abdominal aorta increased with time in the AngII- and BAPN-treated groups (Fig. 2A, B, D-a, D-b, E-a and E-b). CSD rescued the aortic dilation in the descending, but not in the abdominal aorta (Fig. 2A, B, D-c and E-c). AngII and BAPN induced intramural aortic hematoma and elastic fiber destruction,
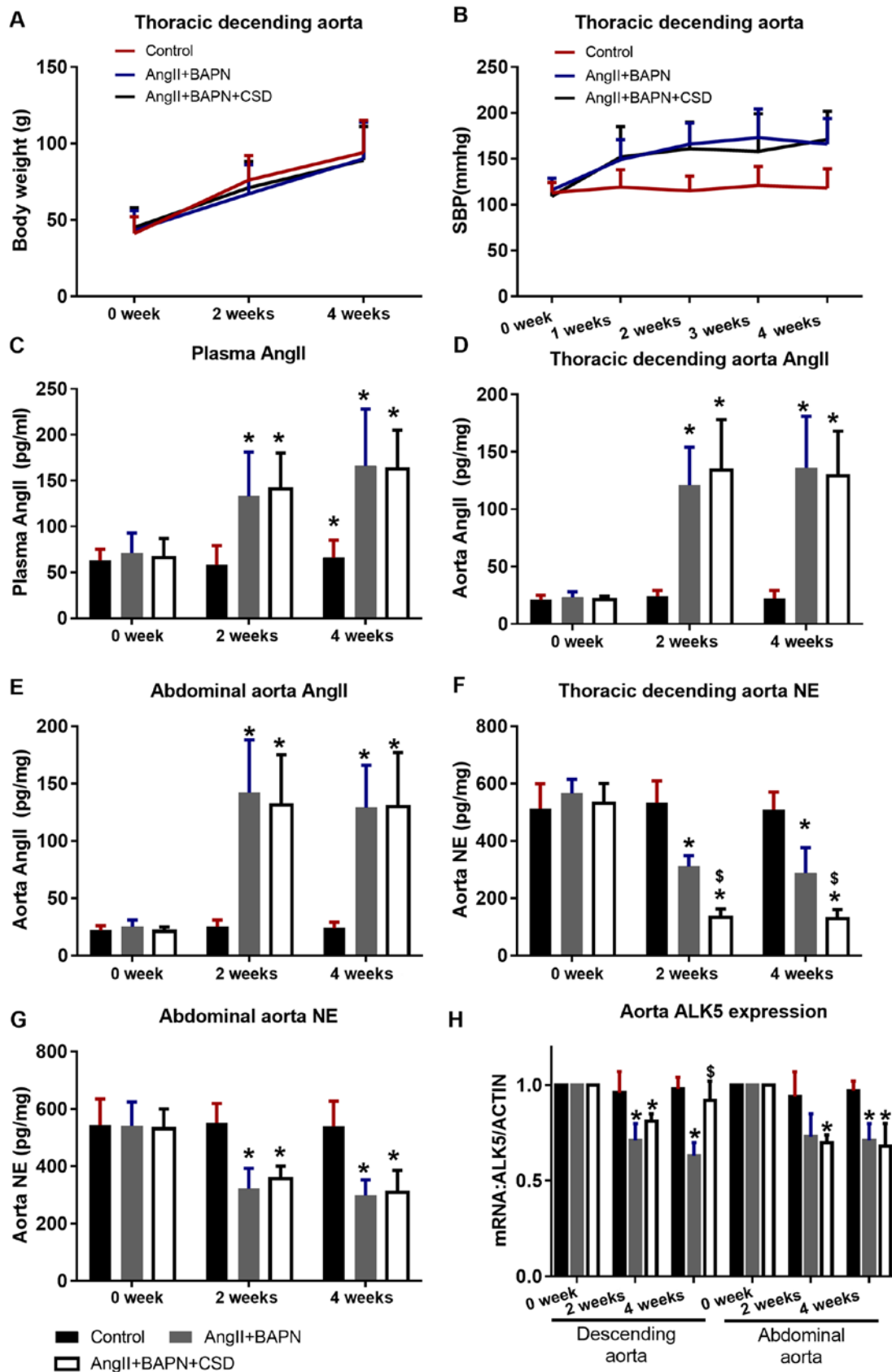
Figure 3. Effects of CSD on AngII-induced NE release and ALK5 expression. (A) Rat body weight was similar among all groups. (B) SBP was higher in the AngII-treated groups. CSD had no significant effect on SBP. (C) Plasma and (D and E) aortic tissue concentration of AngII in (D) the thoracic descending and (E) the abdominal aorta was higher in the AngII-treated groups compared with those in the control group. CSD had no significant effects on AngII concentration. (F and G) Tissue NE concentration in (F) the thoracic descending aorta and (G) the abdominal aorta was lower in the AngII-treated groups compared with the control group. CSD further decreased NE in (F) the thoracic descending aorta, but not in (G) the abdominal aorta. (H) ALK5 expression in the thoracic descending and the abdominal aortas was downregulated in the AngII-treated groups. CSD further downregulated ALK5 in the thoracic descending aorta, but not in the abdominal aorta. $^{*}P<0.01$ vs. control; $^{\$}P<0.01$ vs. AngII+BAPN. SBP, systolic blood pressure; AngII, angiotensin II; BAPN, β-aminopropionitrile; ALK5, transforming growth factor β type I receptor kinase; NE, norepinephrine.
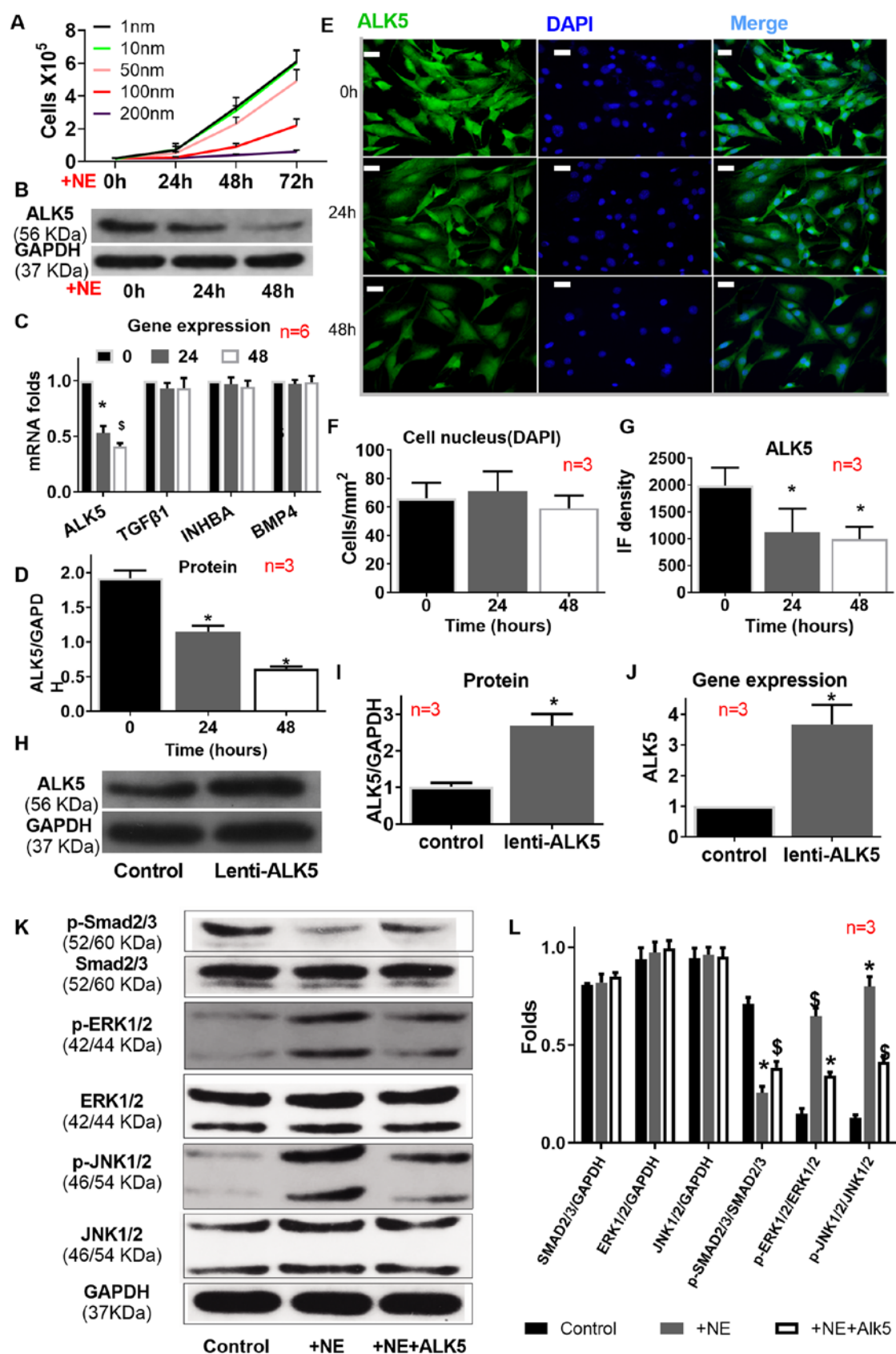
Figure 4. Impact of ALK5 overexpression on ALK5 expression and signaling transduction. (A) NE (100 nm) significantly decreased VSMC proliferation and suppressed ALK5 expression, as indicated by (B-D) western blotting (C) reverse transcription-quantitative PCR and (E-G) immunofluorescence, but (C) did not affect TGFβ1, INHBA or BMP4 expression. (H-J) ALK5 was successfully overexpressed.(K and L) NE suppressed SMAD2/3 signaling, but activated ERK1/2 and JNK1/2 signaling. ALK5 overexpression partially rescued the effect of NE. Scale bar=400 μm. *P<0.01 vs. control; $P<0.01 vs. AngII+BAPN. NE, norepinephrine; ALK5, transforming growth factor β type I receptor kinase; TGF, transforming growth factor; AngII, angiotensin II; BAPN, β-aminopropionitrile; p, phosphorylated; IF, immunofluorescence; INHBA, inhibin subunit β A; BMP4, bone morphogenetic protein 4.
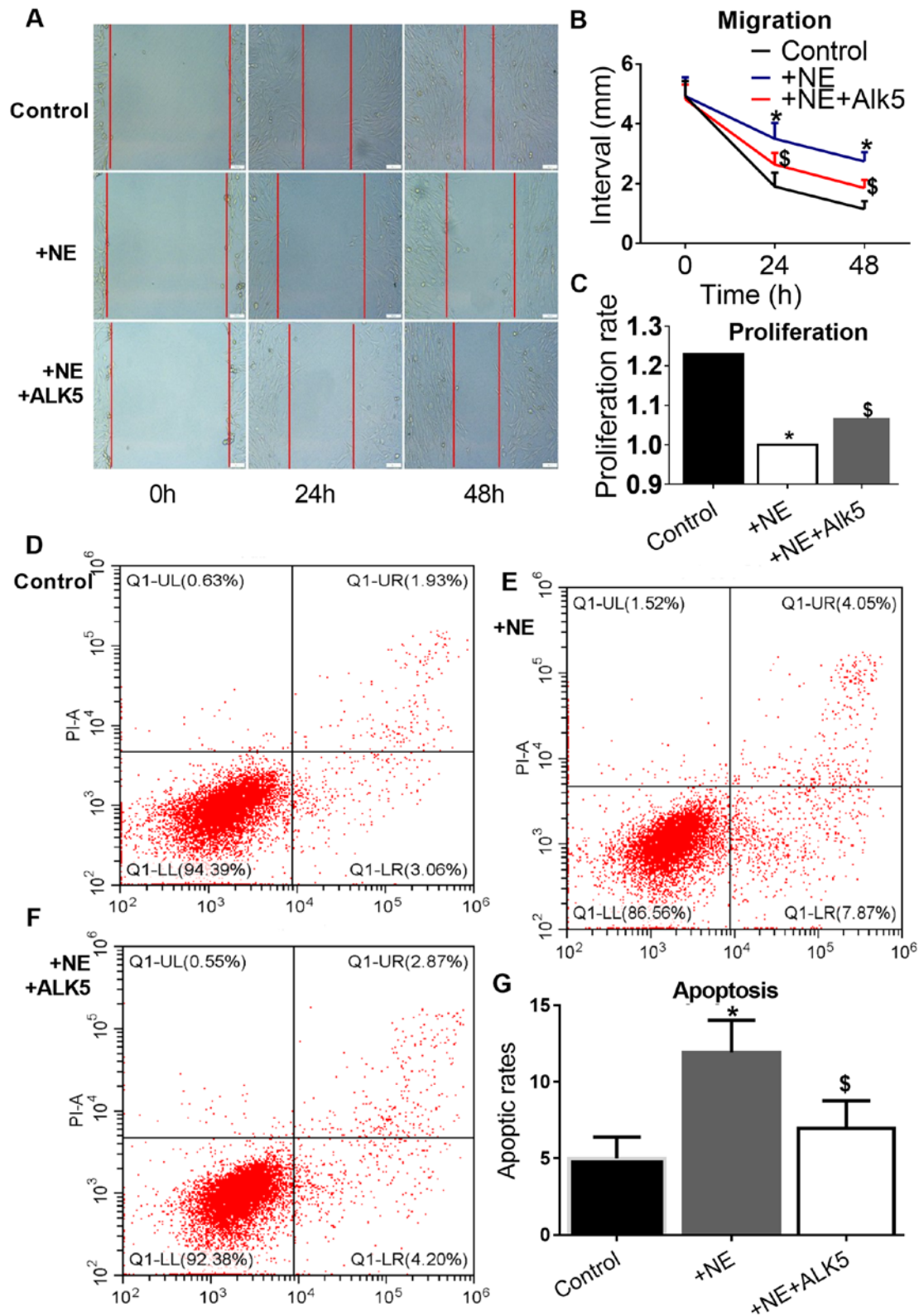
Figure 5. Effect of NE and ALK5 overexpression on VSMCs migration, proliferation, and apoptosis. NE inhibits VSMCs (A and B) migration and (C) proliferation, but (D-G) promotes apoptosis. ALK5 overexpression partially rescued the effects of NE. Scale bars=400 μm. *P<0.01 vs. control; $P<0.01 vs. AngII+BAPN. VSMCs, vascular smooth muscle cells; ALK5, transforming growth factor β type I receptor kinase; NE, norepinephrine; AngII, angiotensin II; BAPN, β-aminopropionitrile; PI, propidium iodide.

which was not observed in the control group. In addition, CSD rescued intramural aortic hematoma and elastic fiber destruction in the descending, but not in the abdominal aorta (Fig. 2D-d-i and E-d-i).

CSD alleviates AngII-induced NE release and ALK5 downregulation in the aorta. No significant difference in body weight was observed among the treatment groups (Fig. 3A). AngII increased the systemic blood pressure compared with

the control group, and regional CSD did not alleviate the AngII-induced hypertension (Fig. 3B). In the AngII-pumped groups, the concentrations of AngII in the plasma, the thoracic descending and the abdominal aorta significantly increased compared with those in the control group, and regional CSD did not affect AngII concentration in the plasma and aortic tissue (Fig. 3C-E). AngII also reduced the NE concentration in the thoracic descending and abdominal aortas of the AngII-pumped groups compared with the control group. In the CSD segment, the NE concentration in the thoracic descending aorta was lower compared with that in the aorta of the AngII+BAPN group, and this difference was not observed in the abdominal aorta (Fig. 3F and G). ALK5 expression was downregulated in the aortas of the rats in the AngII+BAPN group compared with those in the control group, and CSD rescued this effect in the thoracic descending aorta (significant at the end of 4 weeks), but not in the abdominal aorta (Fig. 3H).

NE modulates TGFβ signaling by suppressing ALK5 expression in VSMCs. Based on the concentration gradient experiment, 100 nM NE was used to treat mouse VSMCs. Compared with the control group the expression of ALK5 was significantly downregulated at 24 h after NE treatment was further downregulated at 48 h according to the results obtained by western blotting (Fig. 4B and C), qPCR (Fig. 4D), and immunofluorescence (Fig. 4E-I); however the expression of the TGFβ receptor ligands; TGFβ1, Inhibin Subunit β A (INHBA) and Bone Morphogenetic Protein 4 (BMP4) as determined by qPCR did not change (Fig. 4D). NE treatment also altered the dominance of TGFβ signaling, as it suppressed the phosphorylation of SMAD2/3 and promoted the phosphorylation of ERK1/2 and JNK1/2, which was partially reversed by ALK5 overexpression (Fig. 4K and L).

ALK5 overexpression reverses the effects of NE on VSMC proliferation, migration and apoptosis. CCK8 assay was used to investigate the effects of 100 nM NE on VSMC proliferation. A significant inhibition of mouse VSMC proliferation and migration in the wound healing assay by NE was observed. In addition, a promoting effect of NE on VSMC apoptosis was observed. However, the effect of NE on VSMC proliferation, migration and apoptosis was reversed by ALK5 overexpression (Fig. 5).

α- and β-adrenergic receptors are involved in NE-ALK5 signaling. Among the adrenoceptors, $α_1$ and $β_1$ receptors are very important in maintaining artery structure homeostasis (16,17). To clarify which adrenergic receptors were involved in NE-ALK5 signaling, selective receptor antagonists 10 μM prazosin and metoprolol were used to block $α_1$- or $β_1$-adrenergic receptors, respectively. Both prazosin and metoprolol partially reversed the inhibition of NE on ALK5 expression, with metoprolol exerting a slightly stronger effect (Fig. 6).

Discussion

A number of studies have focused on the regulation of the vessel tone and blood pressure by the sympathetic system, but have neglected its impact on aortic wall structure (18,19). Evidence
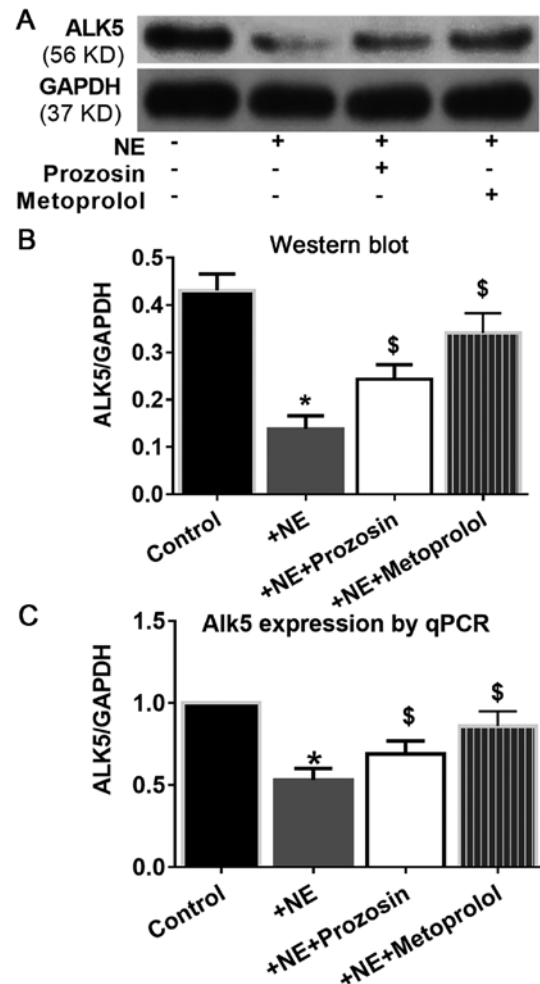

Figure 6. Involvement of $α_1$ and $β_1$ adrenergic receptor in the modulation of NE on ALK5 expression. The inhibitory effects of NE on ALK5 expression are partially blocked by $α_1$ and $β_1$ adrenergic receptor inhibitors, prozosin and metprolol, respectively, indicated by (A and B) western blot and (C) qPCR. *P<0.01 vs. control; $^{\$}$P<0.01 vs. +NE. ALK5, transforming growth factor β type I receptor kinase; NE, norepinephrine.

indicates that the sympathetic system not only regulates the arteries from a functional aspect, but also from a structural aspect. First, there is a clear difference in the sympathetic innervation of arteries and veins; sympathetic innervation is rich in arteries but poor in veins (20). Furthermore, sympathetic innervation increases during development (21). Chronic hypoxia is a risk factor of aortic (22-24) and small artery (25,26) diseases, as well as an inducer of arterial sympathetic innervation (27). Thus, a regional CSD method was used in the present study to investigate the role of aortic sympathetic innervation. Compared with global sympathetic denervation or surgical aortic sympathetic denervation (thoracic sympathectomy), this method is less likely to affect systemic blood pressure (28). Blood pressure is a strong factor promoting aortic remodeling (29). The histological and pathological features of the descending (CSD segment) and abdominal aorta (non-CSD segment) were compared in order to exclude the possibility that CSD may also affect the non-CSD area. In the descending aorta, the number of TH+ cells (sympathetic nerve endings) was significantly decreased, and the intramural aortic hematoma and elastic fiber destruction were rescued compared with the
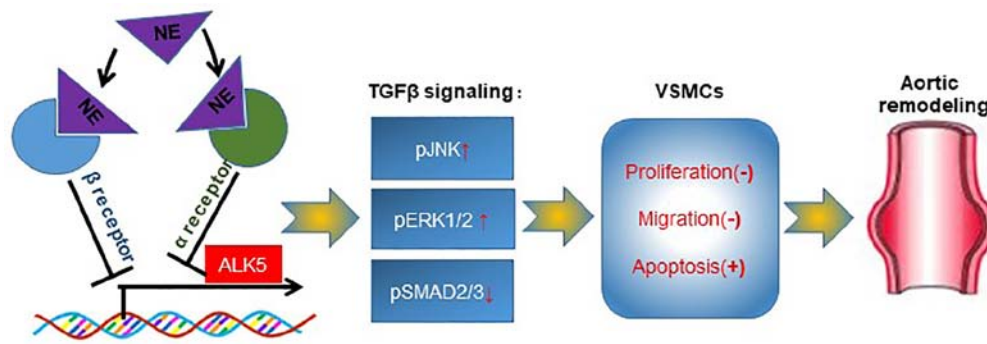
Figure 7. Schematic diagram of the new findings of the present study. NE, norepinephrine; TGF, transforming growth factor; VSMCs, vascular smooth muscle cells; ALK5, transforming growth factor β type I receptor kinase.

control group. By contrast, in the abdominal aorta (non-CSD segment), the number of TH^{+} cells was not decreased, and the intramural aortic hematoma and elastic fiber were not rescued. These results indicated that regional CSD was only effective in the treated region.

The present study identified ALK5 as a target of the sympathetic nervous system. NE suppressed TGFβ receptor1 expression without any impact on the TGFβ receptor ligands (although the expression of TGFβ receptor ligands was only tested by qPCR, not western blotting). To date, only a limited number of studies have explored whether and how the autonomic nervous system regulates ALK5 expression and signaling. In a rat cerebral ischemia/reperfusion model, Ma et al (30) have demonstrated that stimulation of the vagus nerve regulates Growth Differentiation Factor 11 (GDF11) and ALK5 expression, and hypothesized that GDF11/ALK5 may represent a potential target for stroke therapy. Neuropilins1, an axon elongation inducer, inhibits the expression of both ALK1 and ALK5 (31). Yang et al (8) demonstrated that α₁-adrenergic receptor/ALK5 interaction contributes to doxazosin-induced apoptosis, which is further enhanced by TGFβ1 in association with attenuating SMAD3 phosphorylation in H9C2 cells. By contrast, the results of the present study demonstrated that both α- and β-adrenergic receptors were involved in the regulation of NE on ALK5 expression. The present study and the afore-mentioned previous studies further confirmed the interaction between TGFβ signaling and the autonomic nervous system.

ALK5 is a membrane-bound receptor protein of the TGFβ superfamily of signaling ligands (32). When bound to TGFβ, ALK5 transduces the TGFβ signal from the cell surface to the cytoplasm. Abnormal expression and/or activation of ALK5 induce changes in the downstream signaling transduction and diseases (30). Mutations in the ALK5 gene are associated with the Loeys-Dietz aortic aneurysm syndrome (6). The present study revealed that postnatal modulation of ALK5 by a sympathetic transmitter also contributes to aortic remodeling.

TGFβ signaling consists of SMAD2/3-dependent and non-SMAD2/3-dependent cascades; the former is termed canonical TGFβ signaling, and the latter is termed non-canonical TGFβ signaling (33,34). Inhibition of the non-canonical TGFβ signaling molecules such as ERK1/2 and/or JNK1 may rescue aortic aneurysms (33,35,36). The results of the present study suggested that ALK5 repression by NE also induced TGFβ signaling dominance switch and

aortic remodeling. This indicated a strong impact of NE and the sympathetic nervous system on ALK5 expression and downstream signaling. TGFβ signaling serves broad biological functions (34). Its normal status is essential in the development and maintenance of the physiological balance, but when disturbed, may result in a number of diseases (34). Thus, it is possible that NE and the sympathetic nervous system may also exert important roles in other diseases, such as arterial diseases, via modulating ALK5 expression and TGFβ signaling.

A possible limitation of the present study was the NE tissue concentration. Our previous study has demonstrated that AngII promotes NE release from the sympathetic nerve endings (5). Subsequently, cells in the tissue (such as VSMCs) interact with more NE, and the NE in the extracellular fluid is washed away by the blood flow. Thus, more NE release results in lower tissue NE concentration (33). CSD also reduces tissue NE by reducing the number of sympathetic nerve endings and NE release, resulting in less NE acting on cells (VSMCs and others). This is consistent with another study (37). The mortality rate in the present study was lower compared with some studies (12,38) as older rats were used in the present study instead of 3-week-old rats. Tatsuo Kawai et al (11) used a similar protocol in modeling abdominal aortic aneurysm and observed similar mortality.

The biological function of the sympathetic nervous system is broad. NE and ALK5 may not be the only molecules in the sympathetic nervous system to regulate TGFβ signaling, and TGFβ signaling modulation may not be the only mechanism of the sympathetic nervous system to serve a role in aortic disease pathogenesis. However, based on previous and the present research, the sympathetic nervous system, or even other autonomic nervous systems, such as the vagal nerve system, may serve as a therapeutic target to combat aortic diseases. Whether directly inhibiting ALK5 in control cells without NE treatment also produces the same results requires further research. Although this was a limitation of the present study, it did not affect the conclusions as previous studies have demonstrated similar findings on the role of ALK5 in VSMC proliferation and migration (32,35).

The present study confirmed the important role of sympathetic system dysregulation in aortic remodeling. In addition, the present study also revealed a new signaling pathway regulating the sympathetic-adrenergic system. This pathway and its in vivo function have not been previously investigated.

In conclusion, the results of the present study demonstrated that regional CSD protected the rats from aortic aneurysm. The sympathetic transmitter NE modulated TGFβ signaling by suppressing ALK5 expression, serving an important role in the biological functions of VSMCs. Both α- and β-adrenergic receptors may be involved in the regulation of NE on ALK5 expression. Thus, abnormal sympathetic innervation of the aorta may serve as a therapeutic target in aortic diseases (Fig. 7).

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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

ZW and ZH conceived the hypothesis and designed the study protocol. ZH wrote the manuscript. BL, RC, QW and ZH performed most of the experiments. XH, MZ, and FJ participated in some of the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the ethics committee of Renmin hospital, Wuhan University (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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