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Protective effects of astragaloside IV against hypertension-induced vascular remodeling involves the DNMT1 and TET2 signaling pathway

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Abstract: Proliferation, migration, and the phenotypic switch of vascular smooth muscle cells (VSMCs) play an important role in vascular remodeling induced by hypertension. Astragaloside IV (AS-IV), the active ingredient of *Astragalus membranaceus*, has been shown to exert a beneficial role in cardiovascular disease. The present study aimed to investigate the mechanism responsible for the protective effects of AS-IV on hypertension-induced vascular remodeling. AS-IV significantly reduced blood pressure and aortic media thickness in two-kidney, one-clip (2K1C) hypertensive rats. AS-IV administration downregulated the expression levels of DNA methyltransferase1 (DNMT1), matrix metalloproteinase (MMP2) and proliferating cell nuclear antigen (PCNA) and upregulated the expression of smooth muscle 22α protein (SM22α), α-smooth muscle actin (ACTA2) and ten-eleven translocation 2 (TET2) in the aorta of hypertensive rats. AS-IV inhibited the proliferation and migration in VSMCs treated with angiotensin II (Ang II). AS-IV increased the expression of SM22α, ACTA2 and TET2, and decreased the expression of collagen Ia (COL-1a), collagen IIIa (COL-3a), DNMT1, MMP2 and PCNA *in vitro*. Reduction in 5-methylcytosine (5-mC) was observed in VSMCs treated with AS-IV. Knockdown of DNMT1 induced the expression of TET2, while the level of DNMT1 did not change after knockdown of TET2. These results suggest that AS-IV reversed hypertension-induced vascular remodeling by inhibiting DNMT1 and upregulating TET2.

Keywords: hypertension; vascular remodeling; vascular smooth muscle cells (VSMC); astragaloside IV; DNA methyltransferase1 (DNMT1); ten-eleven translocation 2 (TET2)

Abbreviations: vascular smooth muscle cells (VSMCs); astragaloside IV (AS-IV); extracellular matrix (ECM); angiotensin II (Ang II); DNA methyltransferase1 (DNMT1); ten-eleven translocation 2 (TET2) enzyme; systolic blood pressure (SBP); diastolic blood pressure (DBP); smooth muscle 22 protein α (SM22α); α-smooth muscle actin (ACTA2); proliferating cell nuclear antigen (PCNA); matrix metalloproteinase 2 (MMP2); collagen Ia (COL-1a); collagen IIIa (COL-3a); two-kidney and one-clip (2K1C); 5-hydroxymethylcytosine (5-hmC); 5-methylcytosine (5-mC); negative control (NC); sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE)

INTRODUCTION

Hypertension is a chronic disease worldwide, which is considered to be the strongest risk factor for various cardiovascular diseases, such as stroke, coronary atherosclerosis, myocardial infarction and heart failure [1-3]. The persistent elevation in blood pressure causes pathological vascular remodeling and cell dysfunction characterized by excessive migration, proliferation, phenotypic switching of VSMCs, and deposition of the ECM. An increasing number of studies have indicated that VSMCs play a critical role in vascular remodeling; therefore, improving VSMCs function represents a strategy for the treatment of hypertension [4,5].

DNA methylation is an important epigenetic modification that is closely related to biological functions and various diseases. DNA methyltransferases (DNMTs – DNMT1, DNMT3a and DNMT3b) catalyze the methylation of the 5th carbon atom of cytosine to form 5-mC, which ultimately leads to gene silencing and the inhibition of transcription [6,7]. It has been demonstrated that DNMT1 has *de novo* methyltransferase activity and carries out *de novo* DNA methylation along with maintaining methylation [8]. Dysregulation of DNMT1 is associated with vascular diseases [9]. For example, a high-fat diet was shown to induce the expression of DNMT1 in rat aorta [10]; DNMT1 was upregulated in pulmonary smooth muscle cells in cigarette smoke-induced pulmonary hypertensive rats [11]. An increased DNMT1 expression level was found in VSMCs treated with homocysteine [12]. Knockdown of DNMT1 inhibited the proliferation, migration and differentiation of VMSCs and improved pathological vascular remodeling [13]. Therefore, DNMT1 plays an important role in regulating VSMC function.

While DNMTs contribute to the formation of 5-mC, demethylation of DNA is mainly performed by the ten-eleven translocation (TET) family of enzymes [14]. The TET enzymes (TET1, TET2 and TET3) belong to a large family of Fe2+/α-ketoglutarate-dependent oxygenases that catalyze consecutive oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC) and further to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), which can be replaced by unmethylated cytosine via base excision repair leading to demethylation and gene reactivation [15]. TET2, which is abundantly expressed in VSMCs, is an important regulator of VSMC function [16]. TET2 was found to be downregulated in vessels of the murine model of pulmonary hypertension and vascular injury [17,18]. Deletion of TET2 inhibited 5-hmC enrichment and affected the proliferation, migration and phenotypic switch of VSMCs. In contrast, overexpression of TET2 inhibited proliferation and migration and induced differentiation of VSMCs [19,20].

Astragaloside IV (AS-IV) is a triterpene saponin extracted from the *Astragalus* root and the main active ingredient of *Astragalus* [21]. AS-IV has been shown to exert a cardiovascular protective role [22]. Previous studies reported that AS-IV inhibited the proliferation and migration of VSMCs stimulated with high glucose or angiotensin II (Ang II), a vasoconstrictor that induces growth of VSMCs by acting on the AT1 receptor [23,24]. Recent studies have reported that AS-IV protects against vascular remodeling induced by hypoxia and monocrotaline [25,26]. An early study showed that AS-IV relaxed the aorta of spontaneous hypertensive rats through endothelium-dependent and endothelium-independent ways [27]. Although the protective effects of AS-IV on VSMCs have been investigated, the underlying mechanisms have not been fully elucidated.

In this study, we explored the potential protective effects of AS-IV on 2K1C hypertension-induced vascular remodeling and investigated whether they involve DNMT1 and TET2 activation. The present study may help to improve the understanding of the mechanism of AS-IV effects on VSMCs function.

MATERIALS AND METHODS

Renovascular hypertensive rat model and histological analysis

Male Sprague-Dawley (SD) rats weighing 200±15 g were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China). All experimental procedures were carried out in accordance with the Animal Management Rules of China (55, 2001, Ministry of Health, China) and were approved by the Ethics Committee of Shanghai Chinese Traditional University. The hypertensive rat model was established by the 2K1C method as previously described [28]. Briefly, rats were anesthetized with isoflurane, the left renal artery was exposed, a silver clip with 0.2-mm cleft diameter was placed in the left renal artery. Control rats underwent the same operation procedure except for clip placement. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by the tail-cuff method with a BP-2010 Series Blood Pressure Meter (Softron Biotechnology, Beijing, China). SBP above 160 mmHg was considered as high blood pressure [29]. Eighteen rats were randomly divided into three groups (n=6) as follows: control group, hypertensive group, and the hypertension+AS-IV group. AS-IV (Weikeqi Biotechnology Co, Sichuan, China) was injected intraperitoneally at 50 mg/kg daily. Aortas were harvested 7 weeks after surgery for

further experiments. A segment of the thoracic aorta was fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 3-μm thickness were stained with hematoxylin and eosin (H&E). Images of the sections were photographed by an inverted microscope (BX71; Olympus Corporation, Tokyo, Japan). The wall thickness of the aorta was measured using ImageJ software.

Cell culture and treatments

Primary VSMCs of rats were isolated from the aorta by the explant method, as previously described [30]. VSMCs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Massachusetts, USA) at 37°C and 5% CO_2 in a humidified incubator (Thermo Fisher Scientific, Massachusetts, USA). The purity of the VSMCs was confirmed by staining of smooth muscle α-actin. VSMCs in passages 3-8 were used. After serum starvation for 24 h, the cells were divided into five groups as follows: vehicle, 1 μM Ang II (MedChemExpress, New Jersey, USA) treatment, 1 μM Ang II treatment with 10 μM AS-IV (Selleck, Shanghai, China), 1 μM Ang II treatment with 25 μM AS-IV and 1 μM Ang II treatment with 1 μM losartan (MedChemExpress, New Jersey, USA), which served as the positive control.

Transfection of a stable DNMT1 and TET2 cell line

pLent-U6-GFP-Puro lentiviral vectors containing shRNA targeting TET2 and a pLent-U6-GFP-Puro non-silencing shRNAmir lentiviral control vector were purchased from Vigene Biosciences (Jinan, Shandong, China). In addition, psi-LVRU6GP lentiviral vectors containing shRNA targeting DNMT1 and a psi-LVRU6GP non-silencing shRNAmir lentiviral control vector were purchased from GeneCopoeia Inc. (Guangzhou, Guangdong, China). The lentiviral vectors were packaged in HEK293T cells using the GM easy™ Lentiviral packaging kit (Genomeditech, Shanghai, China). The supernatant was collected after transfection. The stable cell lines were established by transducing VSMCs cells with lentiviral supernatants. Stable pools of cells were selected with 3 μg/mL puromycin. Expression of DNMT1 and TET2 was confirmed by Western blotting.

Cell proliferation analysis

VSMC proliferation was examined using the BeyoClick™ EdU Cell Proliferation Assay Kit (Beyotime Biotechnology, Shanghai, China). Briefly, VSMCs were seeded into 96-well culture plates at an initial concentration of 3×10^3 cells/well. After 2 h, EdU reagents were added to the medium, VSMCs were fixed in 4% paraformaldehyde and labeled with Click staining solution for 30 min, and finally counterstained with Hoechst 33342. VSMCs were imaged using a fluorescence microscope (IX71 Olympus Corporation, Tokyo, Japan) and analyzed with Image J software.

Wound healing assay

VSMCs (1×10^6 cells/well) were seeded in six-well plates. A linear wound was made with a 200-μL pipette tip. The debris of cells was removed by washing with phosphate buffered saline (PBS). The cells were then treated with vehicle, Ang II or Ang II with AS-IV at different concentrations for the indicated time. The scratch wound was photographed with an inverted microscope (Olympus IX 73, Tokyo, Japan) at 0 h, 12 h, 24 h and 48 h. The wound healing area was calculated using ImageJ software.

Western blotting

Aortas and VSMCs were lysed using RIPA buffer. The proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and incubated overnight with the appropriate primary antibody. The following primary antibodies were used: anti-α-tubulin (1:1000), anti-DNMT1 (1:1000), anti-TET2 (1:1000), anti-MMP2 (1:1000), anti-SM22α (1:1000), anti-ACTA2 (1:1000), anti-PCNA (1:1000), anti-COL-1a (1:1000), and anti-COL-3a (1:1000). The membranes were then incubated with corresponding HRP-conjugated goat anti-mouse (1:10000) or goat anti-rabbit secondary antibodies (1:10000) at room temperature for 1 h. Finally, the proteins were visualized by enhanced chemiluminescence (ECL) (Absin Bioscience, Shanghai, China), and the signals were quantified with a Tanon image analyzer (Tanon Science, Shanghai, China). All of the antibodies were purchased from Proteintech Group, Inc. (Chicago, IL, USA).

Dot blot analysis

DNA was extracted from VSMCs using an Axy Prep Multisource Genomic DNA Miniprep Kit (Axygen Scientific, NY, USA). DNA was denatured in 0.1 M NaOH at 95°C for 10 min and neutralized with 1 M NH_4OAc on ice. Next, 5 μL of denatured DNA was spotted on a nylon membrane. After heating at 80°C for 30 min, the membrane was blocked with 5% nonfat milk in tris-buffered saline (TBS) and Tween 20 (TBST) for 1 h. The membrane was incubated with mouse 5-mC monoclonal antibody (1:1000, EpiGentek, Farmingdale, NY, USA) in TBST containing 5% nonfat milk at 4°C overnight. The membranes were then incubated with peroxidase conjugated goat anti-mouse antibody in TBST for 1 h at room temperature. The blot was developed by ECL (Millipore, Billerica, MA, USA). The signals were quantified using a Tanon image analyzer (Tanon Science, Shanghai, China). To ensure equal loading of extracted DNA, another blot was stained in 0.02% methylene blue (Sangon Biotech, Shanghai, China).

Statistical analysis

Statistical analysis was conducted using GraphPad 7 software. The results were expressed as the mean±standard deviation (SD). Student's *t* test was performed to analyze the difference between the experimental and control groups. Data from multiple groups were analyzed using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison tests. Blood pressure and wound healing assay were analyzed using two-way ANOVA followed by the Bonferroni post-test, with $P<0.05$ regarded as statistically significant.

RESULTS

AS-IV reduces blood pressure and improves vascular remodeling in the aorta of hypertensive rats

Blood pressure increased progressively in 2K1C rats and reached a peak on the 3rd week after the surgery. There was a significant difference in blood pressure compared with the sham group. AS-IV administration (50 mg/kg) decreased the systolic and diastolic blood pressure in hypertensive rats. Although blood pressure of the AS-IV group began to rise slowly at the 6th and 7th weeks, blood pressure remained lower than that of 2K1C rats (Fig. 1A and B).

To assess the effects of AS-IV on vascular remodeling, morphometric analyses were performed in the aorta. Arterial wall thickness was significantly increased in 2K1C hypertensive rats compared with the sham group. However, AS-IV significantly decreased arterial wall thickness in hypertensive animals (Fig. 1C and D).

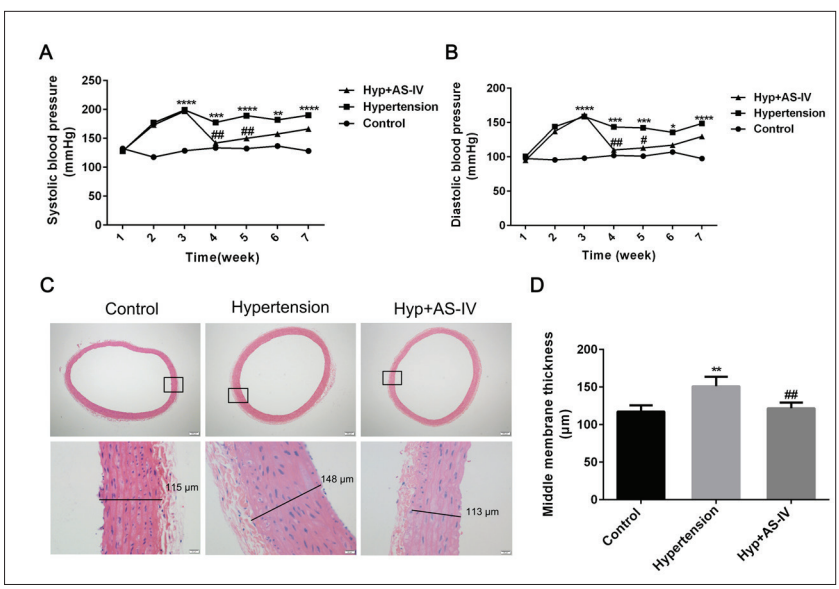

Fig. 1. Effect of AS-IV on blood pressure and vascular remodeling. AS-IV decreased SBP (**A**) and DBP (**B**) in hypertensive rats. Values represent the mean ± SD, n=6. * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001 compared with the control group, # P<0.05 and ## P<0.01 compared with the hypertension group. **C**, **D** – AS-IV alleviated vascular remodeling; the upper panel shows representative images of thoracic aortas; bar=200 μm; the lower panel shows an enlarged view of the red box part of the upper panel; bar=20 μm. n=6. ** P<0.01 compared with the control group, ## P<0.01 compared with the hypertension group.

AS-IV modulates the expression of DNMT1, TET2 and phenotypic molecules in the aorta of hypertensive rats

As DNMT1 and TET2 are the critical regulators of DNA methylation and demethylation in mammalian cells, their protein level in the aorta was measured. Compared with the vehicle, the expression level of DNMT1 in the aorta of hypertensive rats increased while the expression of TET2 decreased. After AS-IV administration, the expression levels of DNMT1 and TET2 in the aorta of hypertensive rats were reversed (Fig. 2A and B). Meanwhile, we also tested the effect of AS-IV on the expression of SM22α, ACTA2, PCNA, MMP2. SM22α and ACTA2 are the contractile phenotypic marker proteins of VSMCs; PCNA is a nuclear protein recognized as a marker protein for cell proliferation; MMP2 is related to the degradation of collagen and cell migration. As shown in Fig. 2C and D, AS-IV reduced PCNA and MMP2 expression in hypertensive rats, whereas it increased the protein levels of SM22α and ACTA2.

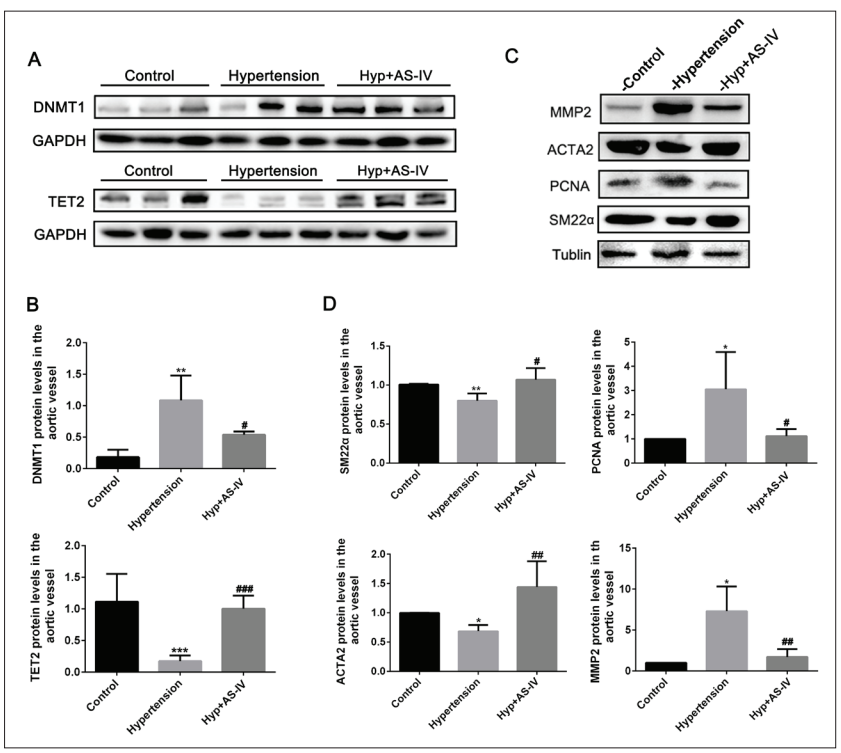

Fig. 2. Effects of AS-IV on the protein expression levels of DNMT1 and TET2 as well as SM22α, ACTA2, PCNA and MMP2 in the aorta of hypertensive rats. **A** – Western blot, **B** – densitometric quantification of DNMT1 and TET2 expression in the aorta; **C** – Western blot; **D** – densitometric quantification of SM22α, ACTA2, PCNA and MMP2 protein expression in each group. Values represent the mean±SD, n=6. Significant differences among different groups are indicated as * P<0.05, ** P<0.01, *** P<0.001 compared with the control group, $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001 compared with the hypertension group.

AS-IV inhibits the proliferation and migration of VSMCs induced by Ang II

The effect of AS-IV on the proliferation and migration of VSMCs was detected in a different group: vehicle, Ang II, Ang II+AS-IV 10μM, Ang II+AS-IV 25μM, Ang II+losartan 1μM and AS-IV 25μM. The results of the 5-ethynyl-2'-deoxyuridine (EdU) assay showed that Ang II induced proliferation of VSMCs. However, growth of VSMCs was suppressed by AS-IV (Fig. 3A). Ang II-stimulated VSMCs were treated with losartan, an Ang II receptor 1 antagonist. Losartan significantly inhibited proliferation of VSMCs. In addition, there was no significant difference between the AS-IV alone treatment and the vehicle group, showing that AS-IV did not inhibit the proliferation ability of normal cells. Western blot analysis showed that AS-IV significantly inhibited the expression of PCNA (Fig. 3B).

The results of wound healing showed a significant difference in cell migration between the Ang II+AS-IV group and the Ang II group. Similarly, losartan inhibited the migration of VSMCs (Fig. 3D). As MMP2 is related to cell migration, protein expression of MMP2 was also examined. The results showed that Ang II increased the MMP2 protein level, while the expression of MMP2 decreased after AS-IV treatment. (Fig. 3C).

AS-IV modulates the expression of SM22α, ACTA2, COL-1a, and COL-3a in VSMCs treated with Ang II

Previously, we demonstrated that AS-IV inhibited the proliferation and migration of VSMCs. Next, we verified whether AS-IV affects the phenotypic transition of VSMCs and reduces the synthesis

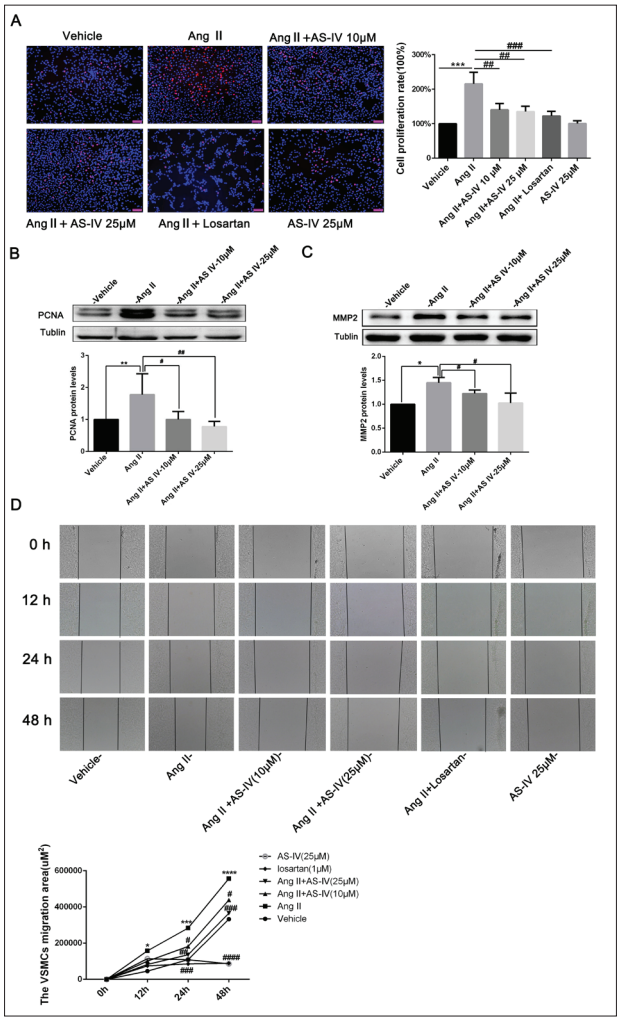

Fig. 3. AS-IV suppresses Ang II-induced VSMCs proliferation and migration. **A** – EdU assay performed to evaluate the effects of AS-IV on VSMC proliferation (100×); the values represent the mean±SD, n=4. Significant differences among different groups are indicated by *** P<0.001 compared with the vehicle group, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001 compared with the Ang II treatment group. **B** – Protein expression of PCNA evaluated by Western blot analysis. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by ** P<0.01 compared with the vehicle group, $^{\#}$ P<0.05 and $^{\#\#}$ P<0.01 compared with the Ang II treatment group. **C** – Protein expression of MMP2 evaluated by Western blot analysis. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by * P<0.05 compared with the vehicle group, $^{\#}$ P<0.05 compared with the Ang II treatment group. **D** – Wound healing assay performed to evaluate the effects of AS-IV on VSMC migration (20×); the migration areas on the images were quantified; values represent the mean±SD, n=4. Significant differences among different groups are indicated by * P<0.05, *** P<0.001, **** P<0.0001 compared with the vehicle group, $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001, $^{\#\#\#\#}$ P<0.0001 compared with the Ang II treatment group.

of COL-1a and COL-3a, the major structural component of the ECM in vessels. Our results showed that Ang II significantly reduced the expression of SM22α and ACTA2, prompting the conversion of VSMCs from contraction to the synthetic phenotype, while AS-IV treatment inhibited this abnormal phenotypic conversion (Fig. 4A). The expression levels of COL-1a and COL-3a, which were overproduced by Ang II stimulation, were significantly reduced after treatment with AS-IV (Fig. 4B).

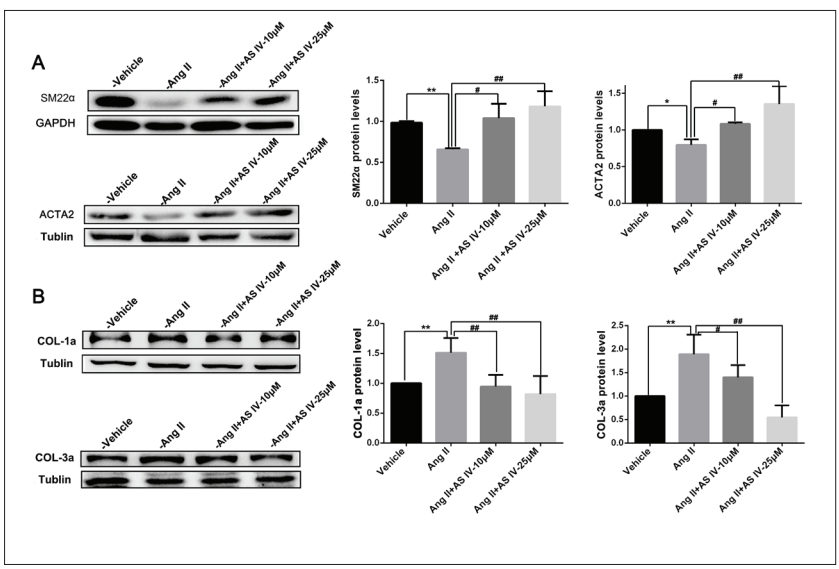

Fig. 4. AS-IV inhibits Ang II-induced VSMCs phenotypic transition and ECM deposition. **A** – Protein expression of SM22α and ACTA2 evaluated by Western blotting. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by * P<0.05 and ** P<0.01 compared with the vehicle group, $^{\#}$ P<0.05 and $^{\#\#}$ P<0.01 compared with the Ang II treatment group. **B** – Protein expression of COL-1a and COL-3a evaluated by Western blotting. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by ** P<0.01 compared with the vehicle group; $^{\#}$ P<0.05 and $^{\#\#}$ P<0.01 compared with the Ang II treatment group.

AS-IV modulates DNA methylation and expression of DNMT1 and TET2 in Ang II-treated VSMCs

Because we observed that the expression levels of DNMT1 and TET2 were different between 2K1C hypertensive rats and normal rats, we next measured DNMT1 and TET2 in VSMCs stimulated by Ang II with or without AS-IV treatment by Western blotting. Consistent with the results of the previous *in vivo* experiments, the expression of DNMT1 protein in VSMCs increased significantly after Ang II stimulation, while the expression of TET2 significantly decreased (Fig. 5A). Consistent with the changes in DNMT1 protein expression in VSMCs, the content of 5-mC increased significantly after Ang II stimulation by dot blotting. However, the content of 5-mC decreased after the AS-IV treatment and reverted to the normal level. In contrast, the content of 5-hmC did not change significantly (Fig. 5B).

Silencing DNMT1 or TET2 modulates expression of SM22α, ACTA2, PCNA, and MMP2

Encouraged by the observed role of AS-IV in the inhibition of 5-mC content and TET2 expression, we further explored the interplay between DNMT1 and TET2. The results revealed that knockdown of DNMT1 increased the level of TET2, but the level of DNMT1 did not change significantly after knockdown of TET2. Furthermore, we also explored the protein levels related to the proliferation and migration of VSMCs after knockdown of DNMT1 and TET2. Compared with the negative control (NC), shDNMT1 significantly reduced the protein expression of MMP2 and PCNA and significantly increased the expression of ACTA2 and SM22α. In contrast, compared with the NC, shTET2 significantly increased the protein expression of MMP2 and PCNA and significantly reduced the expression of ACTA2 and SM22α proteins (Fig. 6A and B). These results suggest that DNMT1 and TET2 are involved in modulating VSMC proliferation, migration and the phenotypic switch.

DISCUSSION

In the present study, AS-IV was found to reverse hypertension-induced vascular remodeling. AS-IV administration improved proliferation, the phenotypic switch of VSMCs and the deposition of ECM *in vivo* and *in vitro*. Our study indicated that the beneficial effects of AS-IV on vascular remodeling were associated with the DNMT1/TET2 signaling pathway.

2K1C hypertension has been widely used as a model for the study of hypertension. The renin-angiotensin-aldosterone system is activated by renal artery stenosis, resulting in an increased level of Ang II [31]. It is well established that Ang II promotes the proliferation, migration, phenotypic switch and secretion of the ECM in VSMCs, thereby inducing hypertrophic vascular remodeling and enhancing arterial contraction and stiffness [32]. Our results showed that renovascular hypertension increased the thickness of the media, augmented the expression level of PCNA and downregulated the expression of

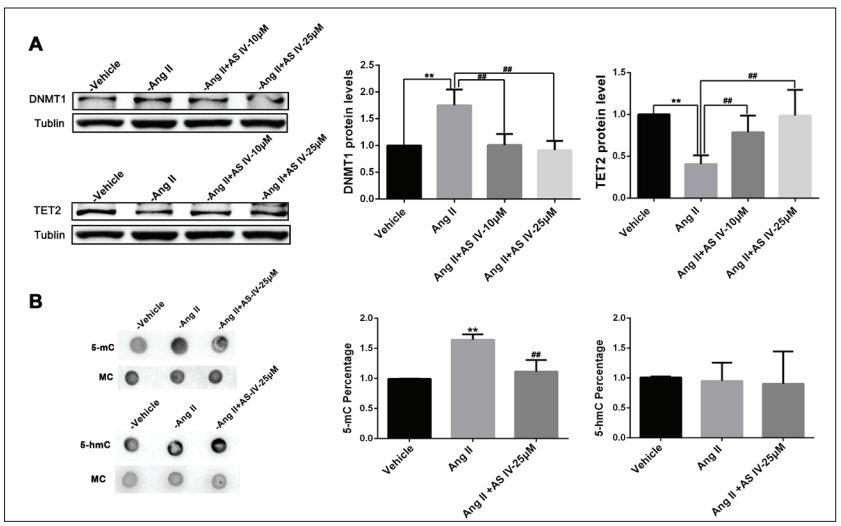

Fig. 5. Effects of AS-IV on the expression levels of DNMT1 and TET2 as well as DNA hypermethylation in VSMCs induced by Ang II. **A** – Protein expression of DNMT1 and TET2 evaluated by Western blot analysis. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by ** P<0.01 compared with the vehicle group, ## P<0.01 compared with the Ang II treatment group. **B** – Expression of 5-mC evaluated by dot blot analysis. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by ** P<0.01 compared with the vehicle group, ## P<0.01 compared with the Ang II treatment group.

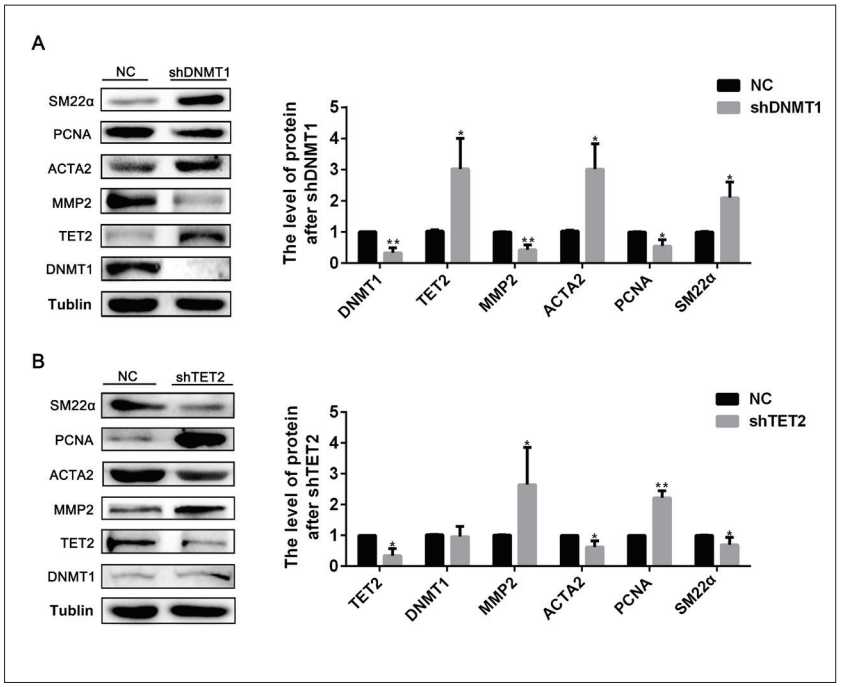

Fig. 6. The effect of knockdown of DNMT1 or TET2 on the function-related proteins of VSMCs. **A** – Protein expression of TET2, MMP2, ACTA2, PCNA and SM22α after DNMT1 knockdown evaluated by Western blot analysis. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by * P<0.05 and ** P<0.01 compared with the NC group. **B** – Protein expression of DNMT1, MMP2, ACTA2, PCNA and SM22α after TET2 knockdown evaluated by Western blot analysis. Values represent the mean±SD, n=4. Significant differences among different groups are indicated by * P<0.05 and ** P<0.01 compared with the NC group.

contractile proteins, such as SM22α and ACTA2 *in vivo*. We also observed that Ang II induced proliferation and migration of VSMCs, increased the expression of PCNA, COL-1a and COL-3a, and decreased the expression of SM22α and ACTA2 *in vitro*. AS-IV administration lowered SBP and DBP, attenuated media thickness and the expression of PCNA triggered by hypertension and upregulated the expression of SM22α and ACTA2. Similarly, treatment of VSMCs with AS-IV reduced proliferation, migration, expression levels of PCNA, COL-1a and COL-3a but elevated the expression of SM22α and ACTA2. Consistent with our findings, a recent study demonstrated that AS-IV reduced blood pressure in rats fed with a high-fat diet [33]. AS-IV has been also found to attenuate pulmonary artery pressure induced by hypoxia or monocrotaline as well as pulmonary artery structural remodeling [25,26,34,35]. The decrease in blood pressure can be associated with vascular relaxation mediated by AS-IV. Several studies showed that AS-IV significantly inhibited vascular contraction and induced the dilation of the aortic rings [27,36,37]. In addition, AS-IV was reported to impede VSMCs proliferation and migration and regulate phenotypic modulation caused by Ang II, PDGF-BB, or high glucose [23,24,38]. Collectively, these findings suggest that AS-IV impedes the progress of pathological vascular remodeling.

As a demethylase, TET2 has been implicated in vascular diseases. The expression of TET2 is reduced in atherosclerosis plaques [13]. Dysfunction of endothelial cells was associated with TET2. Downregulation of TET2 in endothelial cells was induced by homocysteine, oxidized low-density lipoprotein and low shear stress [39-41]. Growing evidence indicates that TET2 is involved in modulating the function of VSMCs. According to Liu et al. [18], TET2 controls differentiation of VSMC; loss of TET2 exacerbates neointimal formation after vascular injury, while ectopic expression of TET2 attenuates intimal hyperplasia. An *in vitro* study showed that knockdown of TET2 diminished the expression of procontractile genes [18]. Zhong et al. [42] showed that tumor necrosis factor α downregulated TET2 in VSMCs, enhanced miR-145 and exacerbated plaque formation and inflammation in mice. Recently, germline mutations in TET2 were found in patients who develop pulmonary hypertension. Likewise, TET2-knockout mice spontaneously developed pulmonary arterial hypertension [43]. Given the importance of TET2 in modulating VSMC plasticity, we detected the effect of AS-IV on TET2 expression. *In vivo* and *in vitro* studies confirmed that AS-IV upregulates TET2 in the aorta of hypertensive rats and in VSMCs treated with Ang II. Silencing of TET2 reduced the expression of SM22α and ACTA2, while it induced PCNA and MMP2 expression. These data suggested that the effect of AS-IV on VSMC function were mediated by TET2.

It is well known that DNMT1 is the main methylase that regulates DNA methylation, and the abnormal expression of DNMT1 is associated with disease progression. DNMT1 was upregulated in atherosclerotic aorta, which was responsible for increased DNA methylation. In addition, 5-aza-2'-deoxycytidine, a DNMT1 inhibitor, enhanced TET2 expression and influenced the vascular smooth muscle phenotype and behaviors resulting from platelet-derived growth factor treatment [13]. DNMT1 has also been implicated in the pulmonary arterial remodeling in pulmonary hypertension. SD rats exposed to cigarette smoke developed pulmonary hypertension and exhibited upregulation of DNMT1 [11]. The current results demonstrated that an increased DNMT1 protein level was observed in rat aorta and VSMCs treated with Ang II. AS-IV administration inhibited protein expression of DNMT1 in rat aorta and VSMCs, and downregulated DNA methylation in VSMCs. Furthermore, DNMT1 knockdown decreased the expression of PCNA and MMP2 but increased the expression of SM22α and ACTA2. These results suggest that DNMT1 is involved in AS-IV alleviated hypertension-induced vascular remodeling.

While arteries undergo hypertrophic and eutrophic remodeling in hypertension, VSMCs switch their phenotype from contractile to synthetic. VSMCs with synthetic phenotype are hyperproliferative and hypermigratory, which is accompanied by ECM resynthesis and downregulation of contractile proteins such as SM22α and ACTA2. It is well established that MMP2 contributes to maladaptive vascular remodeling [44]. Mechanical stress and Ang II augment the expression and activity of MMP2 in VSMCs as well as in arteries [45-47]. MMP2 induces collagen deposition, which results in an increase in vascular stiffness [48]. MMP2 also facilitates VSMCs migration and contributes to VSMC phenotypic switch [49]. It was also reported that AS-IV inhibits the PDGF-BB-induced expression of MMP2, but not matrix metallopeptidase 9 (MMP-9) in human VSMCs [38]. Consistent with these findings, our results showed that AS-IV diminished the expression of MMP2 in aorta of hypertensive rats and Ang II-stimulated VSMCs. In addition, silencing of DNMT1 reduced the expression of MMP2, while TET2 knockdown increased the expression of MMP2. AS-IV also reduced the expression levels of COL-1a and COL-3a. Taken together, our study suggests that MMP2 is involved in AS-IV protective role in maladaptive vascular remodeling.

CONCLUSIONS

Herein we demonstrated that treatment with AS-IV notably decreased blood pressure and ameliorated hypertension-induced vascular remodeling. AS-IV modulated VSMC proliferation and migration by inhibiting DNMT1 expression and subsequently promoting TET2 expression. Therefore, AS-IV may have a potential therapeutic role in preventing vascular alterations caused by hypertension.

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