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Contribution of acid sphingomyelinase to angiotensin II-induced vascular adventitial remodeling via membrane rafts/Nox2 signal pathway

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ABSTRACT

Aims: Vascular adventitial fibroblasts (AFs) in the vascular remodeling during atherosclerosis are increasing arousing attention. Acid sphingomyelinase (ASM) is a soluble glycoprotein which is involved in the development and progression of atherosclerosis. However, it remains unknown if ASM is expressed in vascular AFs and regulates vascular adventitial remodeling and underlying mechanisms.

Main methods and key findings: ASM downregulation with gene silencing was used in the rat AFs treated with angiotensin (Ang) II, which is universally demonstrated to induce vascular adventitia remodeling. It was showed that ASM was indeed expressed in vascular AFs and ASM downregulation resulted in a significant decrease in the protein level of PCNA and collagen I and cell migration under Ang II stimulation. Such improvement of adventitial remodeling was not further augmented by Ang-(1–7), which is deemed as an endogenous Ang II blocker. We further found that ASM downregulation blocked the Nox2-dependent superoxide ($O_2^{-}\cdot$) generation, which regulated vascular remodeling in AFs under Ang II. ASM siRNA decreased the aggregation of membrane rafts (MRs) and the consequent recruiting of ceramide and Nox2 in MRs.

Significance: In conclusion, these results suggested that ASM downregulation could improve vascular adventitial remodeling which was attributed to inhibiting MRs/Nox2 redox signaling pathway in AFs. Thus, these data supported the idea that ASM is a potential therapeutic target for diabetic vascular complication.

1. Introduction

Vascular remodeling contributes to diverse cardiovascular diseases due to its association with atherosclerosis and hypertension. Recently, the vascular adventitia has been recognized as playing an important role in vascular remodeling [1]. Some studies suggest vascular AFs are activated prior to atherosclerotic lesion formation during the early stage of atherosclerosis [2]. Even though abundant researches have been reported, the underlying mechanisms of vascular adventitial remodeling are not fully understood.

Acid sphingomyelinase(ASM) is a soluble glycoprotein of a calculated molecular mass of 64KDa, which is best known for Nieman-Pick disease caused by its deficiency [3]. It was reported that ASM induced vascular smooth muscle cells to a more differentiated contractile phenotype, thereby decreasing cell proliferation and preventing fibrosis [4]. In macrophages, ASM-mediated sphingomyelin hydrolysis prevented the retention of cholesterol in lysosomes and foam cell formation [5]. Conversely, ASM promotes aggregation and uptake of lipoproteins by arterial-wall macrophages leading foam cell formation [6]. Our previous studies demonstrated that ASM contributed to the formation of MRs/redox signalosomes and mediates redox signaling in coronary arterial endothelial cells [7]. Ceramide, the production of ASM catalyzing hydrolysis of sphingomyelin, leaded to the vascular remodeling due to oxidative stress and the unbalance of apoptosis and proliferation [8]. ASM inhibitor amitriptyline prevented AngII-induced the proliferation of pulmonary artery smooth muscle cells [9]. Thus, ASM is involved in the development and progression of atherosclerosis, however, it seems that ASM has either anti- or pro-atherogenic roles, which is dependent on various stages or cell type. The expression and function of ASM in vascular AFs are poorly unknown. It is intriguing to explore the expression and function of ASM in vascular AFs. We hypothesize that ASM expressed in vascular AFs and is activated by Ang II, which induced vascular adventitia remodeling.

In this study, we firstly explored whether ASM existed in vascular

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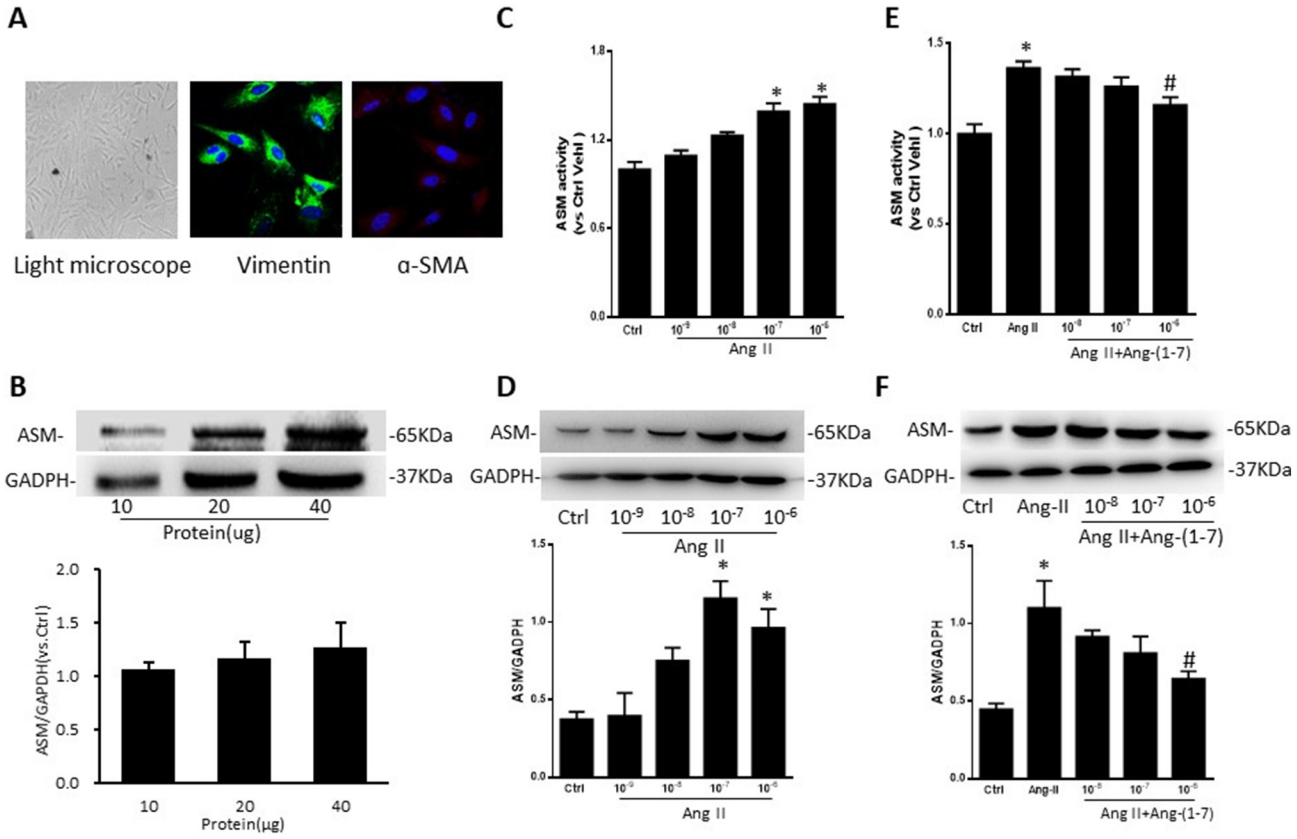

Fig. 1. Ang-(1–7) inhibited Ang II-induced ASM activation.
(A) Cell morphology under light microscope and typical fluorescent images of AFs which were incubated with anti-vimentin antibody and anti-α-SMA antibody followed by Alexa-488-conjugated second antibody. (B) Representative Western blot gel documents and summarized data showed the expression of ASM in AFs. (C, D) The activity and protein expression of ASM in AFs treated with Ang-II from 10^{-9} to 10^{-6} M. (E, F) Ang-(1–7) (10^{-8}–10^{-6} M) inhibited the activity and protein expression of ASM in AFs treated with Ang-II. $^{*}P < 0.05$ vs. Control (Ctrl); $^{\#}P < 0.05$ vs. Ang-II treated group (n = 3).

AFs, and whether participated in adventitial remodeling process. ASM downregulation with gene manipulation was used in our research. We further detected the mechanisms of ASM downregulation alleviating vascular remodeling, which was associated with the formation of MRs/redox signaling platforms. Ang-(1–7) as a newly fragment of renin-angiotensin system (RAS) is generated by angiotensin-converting enzyme 2(ACE2) hydrolysis of Ang II, which is deemed as an endogenous Ang II blocker [10]. Ang-(1–7) was reported to be a critical therapy for vascular dysfunction related to vascular remodeling [11], thus Ang-(1–7) was used in current study to test our hypothesis.

2. Material and methods

2.1. Culture of the primary vascular AFs

The vascular AFs were isolated from 8-week-old male Sprague-Dawley rats. Rats were obtained from Qinglongshan Lab Animal Ltd., Nanjing, China. The vascular AFs were isolated from rat thoracic aorta. Briefly, after anesthesia, the aortas were removed from the rats and then the media and intima layer were scraped off and the adventitia was cut into small pieces in DMEM. The adventitia was digested with 0.2% collagen I (Beyotime Biotechnology, China) for 5 h and was centrifuged for 1000 rpm at 5 min.The sediment was cultured with high glucose DMEM containing 10% FBS, 100 U/ml penicillin and streptomycin at 37 °C with 5% CO_2. The vascular AFs grew out and reached 80%–90% after 8–10 days. AFs in passages 3 to 7 were used in the current experiments. AFs were identified according to their morphology and immunohistological staining of marker proteins. In these experiments, α-smooth muscle actin (α-SMA, 1:1000, Abways) and vimentin (1:1000, Abways) were used as positive and negative markers of AFs, respectively [12].

2.2. Western blotting

The protein expression was analyzed by Western blot which was performed as we described previously[13]. The vascular AFs were washed twice by PBS and lysed in RIPA lysis buffer (BU Technology CO, LTD). The cells were centrifuged at 10000rmp for 10 min at 4 °C. The protein concentrations were determined by the BCA protein quantitative kit (BU). 20ul proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (PVDF membrane, and blocked as we described previously [14]). The membranes were probed with the primary antibodies of ASM (1:500; BIOword, China), PCNA (1:1000; Abways), Collagen I (1:1000; Wanleibio, Shenyang, China), Nox2 (1:1000; Abways) overnight at 4 °C followed by incubation with the secondary antibody at room temperature for 1.5 h. GADPH (1:5000; Wanleibio, Shenyang, China) were used as control protein. The blot was detected by chemiluminescent detection systems with LumiGlo and Peroxide (1:1, BU). Densitometric analysis of the images was performed with ImageJ software (NIH, Littleton, CO, USA).

2.3. RNA interference of ASM and Nox2

siRNA of ASM and Nox2 were purchased from Santa Cruz (sc-41,650) and Ribobio (SIG140804140821). Their targeting sequences were as follows: 5′-GCAGGUCACACUUGCUGUTT-3′ and 5′-GCACCAT GATGAGGAGAAA-3′. The scrambled small RNA was used as control in

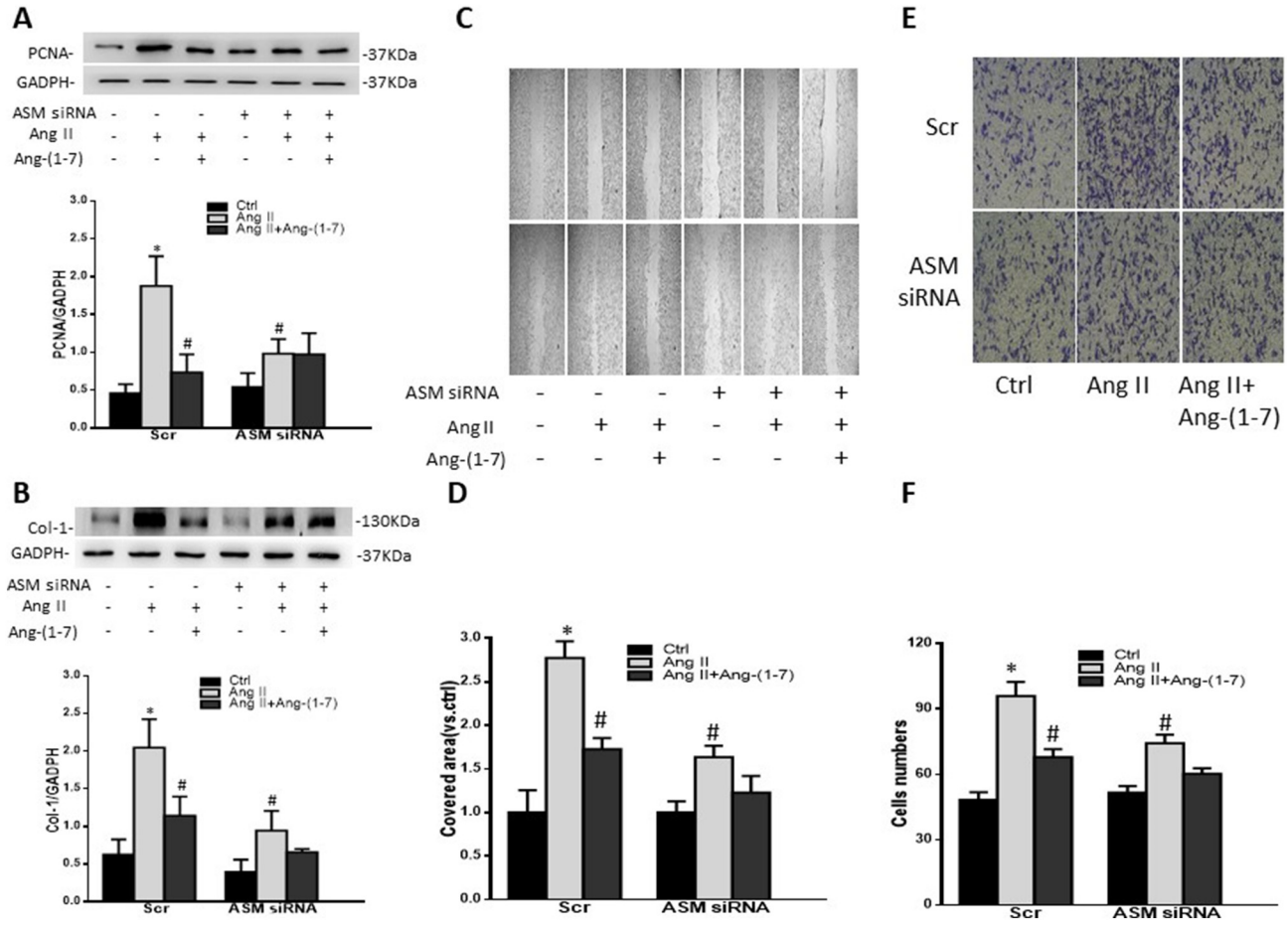

Fig. 2. ASM siRNA inhibited Ang II-induced vascular remodeling in AFs.
ASM siRNA were transfected into AFs, which were incubated with Ang II (10^{-7} M) for 24 h with or without Ang-(1–7) (10^{-8} M) for 12 h. (A, B) Immunoblotting and summarized data showed the expression of PCNA and collagen I in AFs. (C, D) The wound healing assays was used to test cell migration and summarized data showed the scraped areas. (E, F) The transwell migration assay and summarized data showed the number of migrated cells. $^{*}P < 0.05$ vs. Scramble Control (Scr Ctrl); $^{\#}P < 0.05$ vs. Scr Ang-II treated group (n = 3).

this study. Transfection of Nox2 siRNA and scramble RNA was performed using the Gene TranTM III (BIOMIGA) according to the manufacturer's instructions. The siRNA of ASM and Nox2 was confirmed to be effective with Western blot analysis [14].

2.4. Cell migration assay

Two separate experiments were used to assess cell migration in vitro. In wound healing assays, AFs were seeded into 6-well plates. After 12 h, the wound of cells was made using a sterile toothpick and washed three times with PBS. The cells were cultured with serum-free DMEM and the wound area was photographed with a light microscope (magnification, 10×). After 24 h, the wood area was photographed again. The scraped areas were measured using ImageJ software (NIH, Littleton, CO, USA).

In the transwell migration assay, AFs were seeded into the transwell system (8-mm pore size, Corning Costar, USA) and DMEM with 10% FBS (400 μl) was added in the bottom chamber. After incubating for 6 h, the cells in the upper layer of the membrane (not migrated) were gently wiped off with a cotton swab, and the migrated cells were fixed on the lower membrane with methanol for 20 min. The migrated cells were stained with 0.2% crystal violet for 1 h and the number of migrated cells was calculated under a light microscope (magnification, 100×).

2.5. Co-immunoprecipitation assay

300 μg protein extracted from vascular AFs was incubated with 2 μl primary antibody against ASM (1:500; BIOword,China), CtxB (1:1000; BIOword,China) overnight at 4 °C. Then 20 μl protein A/G agarose beads (Beyotime Biotechnology, China) were added to each sample and the immunoprecipitation was rocked gently for 3 h at 4 °C. The cell lysate-beads mixture was centrifuged for 2500 rpm at 5 min and washed five times with PBS. The cell lysate-beads mixture boiled for 5 min 95 °C in a 5 × loading buffer (BU Technology CO, LTD). The protein was analyzed by Western blotting.

2.6. Measurement of $O_2^-\cdot$ production

Total $O_2^-\cdot$ production by AFs was measured using the fluorescent probe dihydroethidium (DHE; Sigma, CAS 104821-25-2). AFs were cultured in six-well plates in 5% CO_2–95% O_2 at 37 °C. After treatment, each well was washed twice with 2 ml PBS, while DHE was diluted with serum free-medium to a final concentration of 10 μM. 2 ml dilution was evenly added to each well and incubated for 30 min in 5% CO_2 at 37 °C without light. After the incubation, AFs were washed with 2 ml PBS three times, and red fluorescence images were observed by fluorescent microscopy (Olympus IX53, Olympus, Tokyo, Japan). The fluorescent intensity was analyzed and processed by ImageJ software and the ratio of fluorescent intensity to that at basal level was quantified to represent cellular $O_2^-\cdot$ level.

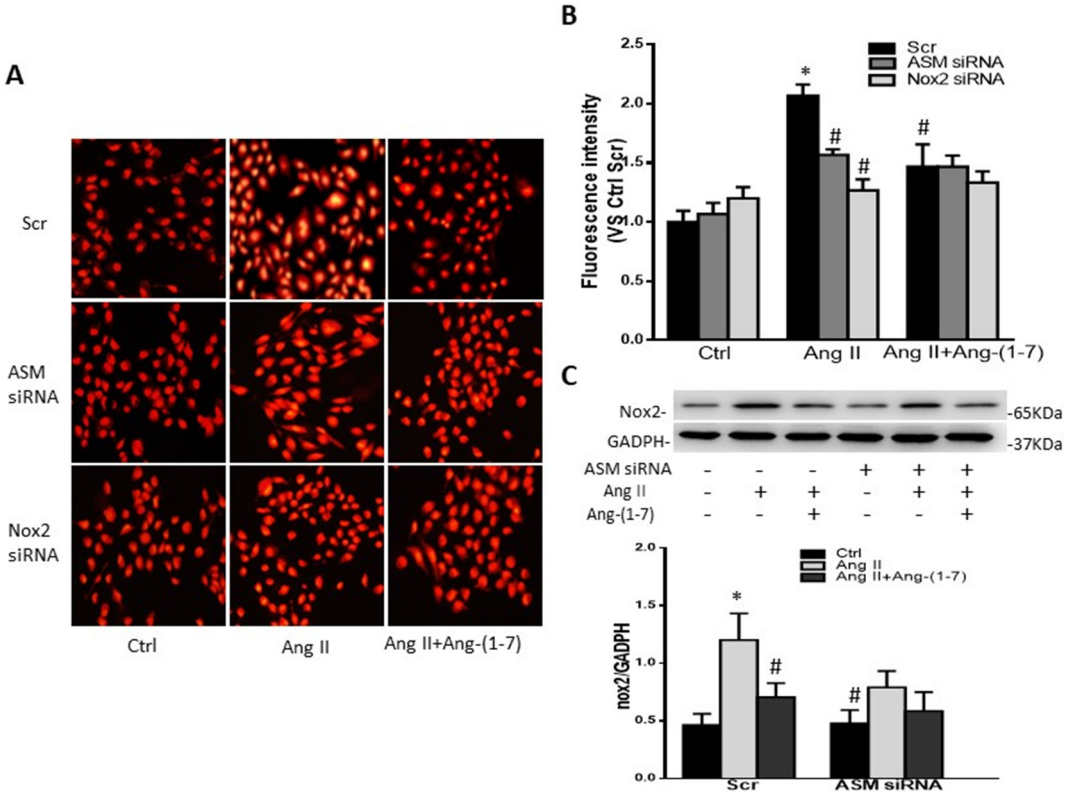

Fig. 3. ASM siRNA decreased Nox2-dependent $O_2^-\cdot$ production inside the AFs.
ASM siRNA and Nox2 siRNA were respectively transfected into AFs, which were incubated with Ang II (10^{-7} M) for 24 h with or without Ang-(1–7) (10^{-8} M) for 12 h. (A, B) Typical representative fluorescent images and summarized data showed $O_2^-\cdot$ production in AFs stained by DHE. (C) Immunoblotting and summarized data showed the expression of Nox2 in AFs. $^{*}P < 0.05$ vs. Scramble Control (Scr Ctrl); $^{\#}P < 0.05$ vs. Scr Ang-II treated group (n = 3).

2.7. Confocal microscopy of the colocalization of MRs cluster and ceramide or Nox2

Immunofluorescent assay was used to determine the MRs as we described previously [7]. Briefly, cells fixed with 4% paraformaldehyde solution for 15 min at room temperature and washed with PBS three times. Then, the cells were incubated with 5% bovine serum albumin for 1 h and washed with PBS three times again, and incubated with the primary antibodies of CtxB overnight at 4 °C. After washing three times with PBS, the cells were stained with secondary antibodies conjugated to Alexa 594 (1:100; Abways). For dual-staining detection of the colocalization of MRs marker with ceramide (1:200; ENZO, Alexis, Carlsbad, CA, USA) or Nox2, the AFs were first incubated with the primary antibodies of CtxB and ceramide or Nox2, and then stained with secondary antibodies conjugated to Alexa 488 (1:100; Abways) or Alexa 594(1:100; Abways), respectively. Cells were washed with PBS and visualized using a confocal microscopy. The colocalizations were visualized by confocal microscopic analysis.

2.8. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 20 software. Data are presented as means ± SE. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test. The Independent-Samples *t*-test was used to detect significant differences between two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ang-(1–7) inhibited Ang II-induced ASM activation

As shown in Fig. 1A, AFs elongated or spindle shaped under the light microscopy. Immunocytochemistry showed that AFs were positively stained with anti-vimentin antibody and negatively anti-α-SMA antibody followed by Alexa-488-conjugated second antibody, which identified the extracted AFs. The intensity of individual ASM bands was proportional to the amounts of AFs homogenates loaded by Western blot analysis (Fig. 1B), which demonstrated the presence of ASM in the AFs.

To investigate the role of Ang-II and Ang-(1–7) on ASM, we assessed the protein expression and activity of ASM. The protein expression and activity of ASM were dose-dependently enhanced in AFs treated with Ang-II from 10^{-9} to 10^{-6} M, which was most significant in the dose of 10^{-7} M (Fig. 1C and D). Moreover, we found that 10^{-6} M Ang-(1–7) significantly inhibited the expression and activity of ASM (Fig. 1E and F). Thus, Ang-(1–7) was regarded as an endogenous Ang II blocker to suppress the activation of ASM.

3.2. ASM siRNA inhibited Ang II-induced vascular remodeling in AFs

Vascular adventitial remodeling is characterized by AFs proliferation, migration, phenotypic transformation and extracellular matrix collagen deposition [16]. Type I and Type III collagen are the major vascular collagen, while collagen I plays a crucial role during vascular remodeling. The levels of collagen I and PCNA, an excellent marker for cell proliferation, increased in AFs under Ang II stimulation compared with control (Fig. 2A and B). To elucidate the role of ASM on vascular remodeling in AFs, we examined whether AFs lacking ASM gene exhibited a less proliferative status and collagen synthesis. ASM siRNA

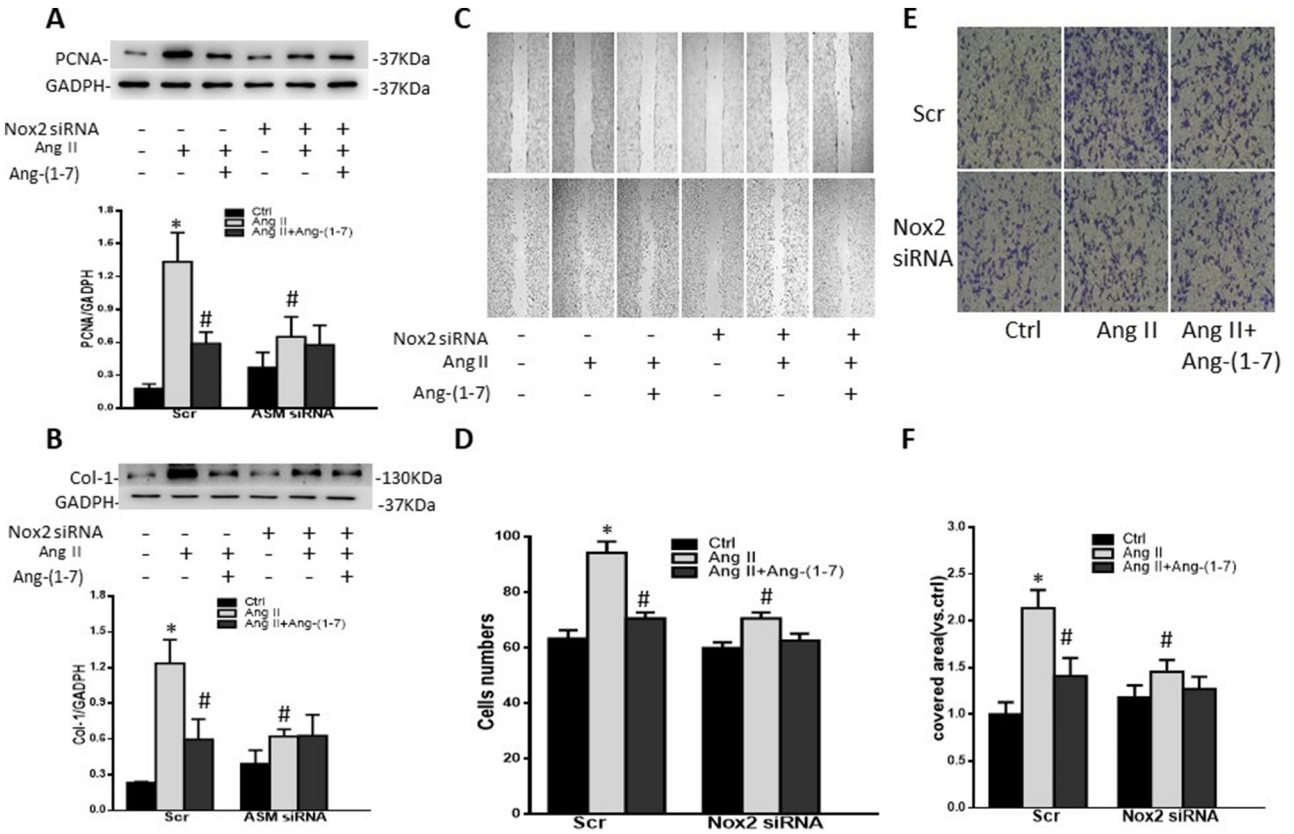

Fig. 4. Nox2 siRNA inhibited Ang II-induced vascular remodeling in AFs.
Nox2 siRNA were transfected into AFs, which were incubated with Ang II (10^{-7} M) for 24 h with or without Ang-(1–7) (10^{-8} M) for 12 h. (A, B) Immunoblotting and summarized data showed the expression of PCNA and collagen I in AFs. (C, D) The wound healing assays was used to test cell migration and summarized data showed the scraped areas. (E, F) The transwell migration assay and summarized data showed the number of migrated cells. $^{*}P < 0.05$ vs. Scramble Control (Scr Ctrl); $^{\#}P < 0.05$ vs. Scr Ang-II treated group (n = 3).

was transfected into AFs to inhibit the protein expression of ASM by 67.4% (data not shown). As showed in Fig. 2A and B, ASM deficiency resulted in a significant decrease in the protein level of PCNA and collagen I under Ang II stimulation, which was similar with the role of Ang-(1–7). In addition, the wound healing assays and the transwell migration assay were used to assess cell migration in vitro. Similarly, Ang II-induced cell migration was dramatically prevented in AFs with silencing of ASM gene and the pretreatment with Ang-(1–7) (Fig. 2C–F). However, Ang II-induced cell proliferation, collagen I synthesis and cell migration were not further augmented by Ang-(1–7) in AFs with ASM gene silencing, which indicated that ASM inhibition was involved in the amelioration of vascular remodeling by Ang-(1–7).

3.3. ASM siRNA decreased Nox2-dependent $O_2^-\cdot$ production in the AFs

To further explore the mechanisms how ASM deficiency improved vascular adventitial remodeling, the anti-oxidant capacity of ASM deficiency was considered in our study. Dihydroethidium enters the cells through the cell membranes, and it is oxidized by intracellular $O_2^-\cdot$and then it is incorporated into the chromosomal DNA to produce red fluorescence. Thus, DHE is widely used for intracellular $O_2^-\cdot$ production. As showed in Fig. 3A–B, ASM siRNA significantly decreased Ang II-induced $O_2^-\cdot$ production inside the AFs. Among NADPH oxidase, Nox2 was highly expressed in vascular AFs. The transfection of Nox2 siRNA also showed the inhibitory effects on $O_2^-\cdot$ production inside the AFs treated with Ang II. Furthermore, the protein expression of Nox2 was decreased after the transfection of ASM siRNA (Fig. 3C). Ang-(1–7) decreased the Nox2 protein expression and $O_2^-\cdot$ generation during Ang II stimulation. Such results suggested that ASM deficiency blocked the Nox2- dependent $O_2^-\cdot$ generation during Ang II stimulation.

3.4. Nox2 siRNA inhibited Ang II-induced vascular remodeling in AFs

To further verify Nox2 as the downstream molecular mediated the vascular adventitial remodeling in the AFs, Nox2 siRNA was transfected in AFs. As expected, Ang II-induced the expression of PCNA, collagen I synthesis and cell migration were also blocked by gene silencing targeting Nox2 in AFs. However, the cell proliferation, collagen 1 and cell migration were not further augmented by Ang-(1–7) in AFs with silencing of Nox2 gene, which indicated that Ang-(1–7) ameliorated vascular remodeling through inhibiting the expression of Nox2 (Fig. 4).

3.5. ASM siRNA decreased the formation of MRs on the AFs

Our previous studies have demonstrated that ASM and its product of ceramide contributed to the formation of MR/redox signalosomes to mediate redox signaling in coronary arterial endothelial cells [7]. Here, we studied if MRs were linked with ASM and Nox2 in AFs. As shown in Fig. 5A, methyl-β-cyclodextrin (MCD), a MRs inhibitor significantly decreased the $O_2^-\cdot$ generation during Ang II stimulation. The primary antibody of CtxB followed secondary antibodies conjugated to Alexa 488 was used to label MRs observed by fluorescence microscopy in current study [7]. Under the resting condition, there was only a little diffuse fluorescent staining on the cell membrane, showing an even distribution of single MRs. Large fluorescent dots were tested on the cell membrane after Ang II stimulation, indicating the formation of MRs cluster. After the transfection of ASM siRNA, the formation of MRs was markedly inhibited, which had no further reduction after the

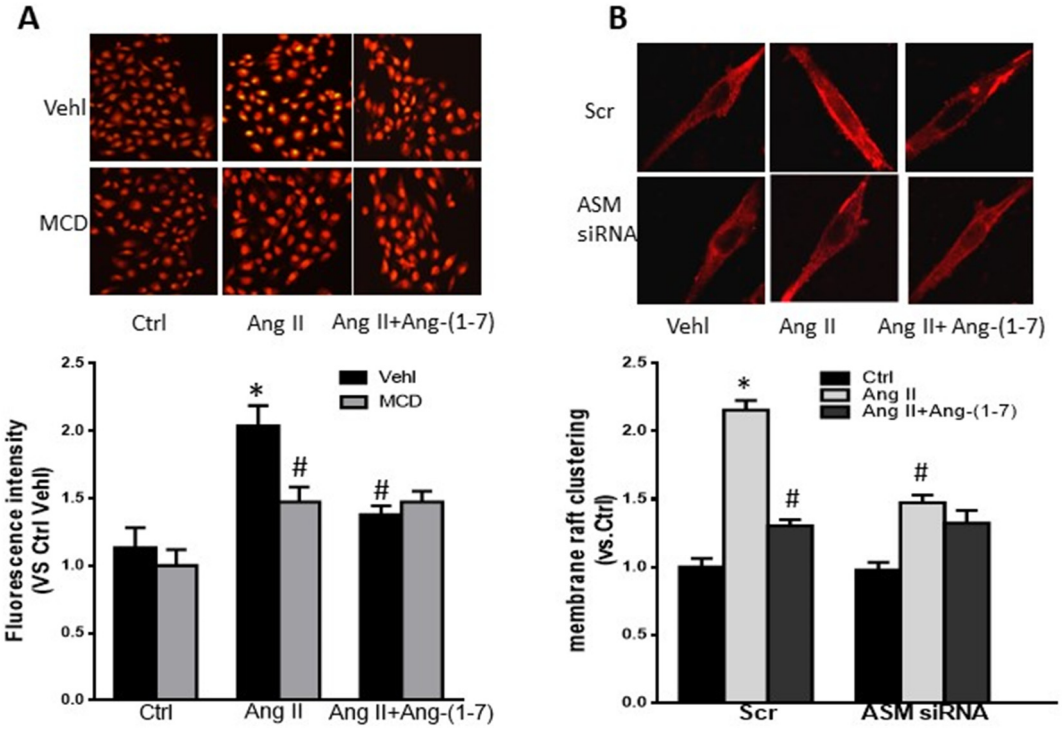

Fig. 5. ASM siRNA decreased the formation of MRs on the AFs.
Methyl-β-cyclodextrin (MCD,100 μM) were pretreated into AFs, which were incubated with Ang II (10^{-7} M) for 24 h with or without Ang-(1–7) (10^{-8} M) for 12 h. Typical representative fluorescent images and summarized data showed $O_2^-\cdot$ production in AFs stained by DHE (A) or MRs formation in AFs incubated with stained by CtxB followed with secondary antibodies conjugated to Alexa 594 (B). $^*P < 0.05$ vs.Vehicle Control (Vehl Ctrl); $^\#P < 0.05$ vs. Vehl Ang-II treated group (n = 3).

pretreatment with Ang-(1–7).

3.6. MRs clustered with ASM, ceramide and Nox2 in Ang II-treated AFs

It was reported that lysosomal trafficking and translocation of ASM into MRs to result in ceramide production [7]. As showed in Fig. 6A, Ang II caused a strong colocalization of ceramide and CtxB, which showed yellowed dots or patches around the AFs. Our previous studies showed that ASM mediated the aggregation of membrane raft, forming NADPH oxidase-mediated $O_2^-\cdot$ generation [7]. Here, we found that Ang II contributed an aggregation of Nox2 in MRs clustering domain around the AFs in Fig. 6B. When AFs were treated with Ang-(1–7), Ang II-induced ceramide and Nox2 aggregation in MRs was markedly blocked. To further demonstrate the aggregation of MRs with ASM and Nox2 during Ang II treatment, the co-immunoprecipitation assay was performed. As showed in Fig. 6C–E, co-immunoprecipitation using CtxB antibody produced significantly stronger bands of ASM or Nox2 in Ang II-treated AFs. Also, co-immunoprecipitation using ASM antibody produced significantly stronger bands of Nox2 in Ang II-treated AFs. All the co-immunoprecipitation results suggested an increased binding of CTxB with ASM and Nox2 in Ang II-treated AFs. The increased colocalization of CtxB/ASM or CtxB/Nox2 was equally blocked by Ang-(1–7). Thus, ASM activation and ceramide production contribute MRs aggregation to recruit Nox2 in MRs cluster.

4. Discussion

In the present study, we provided novel evidences that ASM activation was critical for vascular remodeling of AFs triggered by Ang II. Further, we elucidated that ASM-ceramide signaling could aggregate MRs and further recruited Nox2, which contributed to vascular remodeling of AFs. Ang-(1–7) could prevent vascular adventitial remodeling via inhibiting the ASM and downstream MRs/Nox2 signaling pathway in AFs.

Vascular remodeling is defined as structural change in the vascular wall because of vascular cell proliferation and extracellular matrix deposit. The previous studies on vascular remodeling focused on the intimal endothelial cells and medial smooth muscle cells. Some studies found that the vascular adventitia played a key role in regulating cardiovascular functions and vascular homeostasis. AFs were activated during the early stages of vascular remodeling through cell migration, proliferation, phenotypic differentiation and the accumulation of collagen [17].Thus, the role of vascular AFs in the vascular remodeling during atherosclerosis is increasing arousing attention. Ang II, as an important part of RAS system, plays a crucial role in adventitial remodeling via activating type 1(AT1R) and type2 (AT2R) angiotensin receptors [16]. In the present study, we confirmed that the stimulation of Ang II significantly activated AFs characterizing by cell proliferation, and collagen I deposition and cell migration.

Through binding to specific Mas receptor, ACE-Ang-(1–7)-Mas contends with ACE-Ang II-AT1R and maintains the stability of the environmental homeostasis. ACE-Ang-(1–7)-Mas axis is highly expressed in vascular endothelial cells and smooth muscle cells. As a protective role of RAS, Ang-(1–7) exerts the opposite effect of Ang-II such as vasodilatation and inhibiting smooth muscle cell proliferation, migration and inflammation [11]. Recently, Ang-(1–7) was reported to have a protective role in the development of cardiovascular disease [19]. Here, we found that Ang-(1–7) inhibited the activation of AFs induced by Ang II. These results provided initial evidence for Ang-(1–7) to reduce vascular remodeling in AFs, which expanded the pharmacological effects of Ang-(1–7) and confirmed Ang-(1–7) as an endogenous Ang II blocker.

ASM encoded by Smpd1 gene, is a lysosome hydrolase that metabolizes sphingomyelin to ceramide and phosphorylcholine [8]. Clinical studies reported that the Niemann-Pick disease patients with deficient ASM activity had high incidences of coronary atherosclerosis, suggesting that ASM activity is crucial for preventing atherogenesis in humans [20]. Consistently, adenovirus-mediated ASM expression

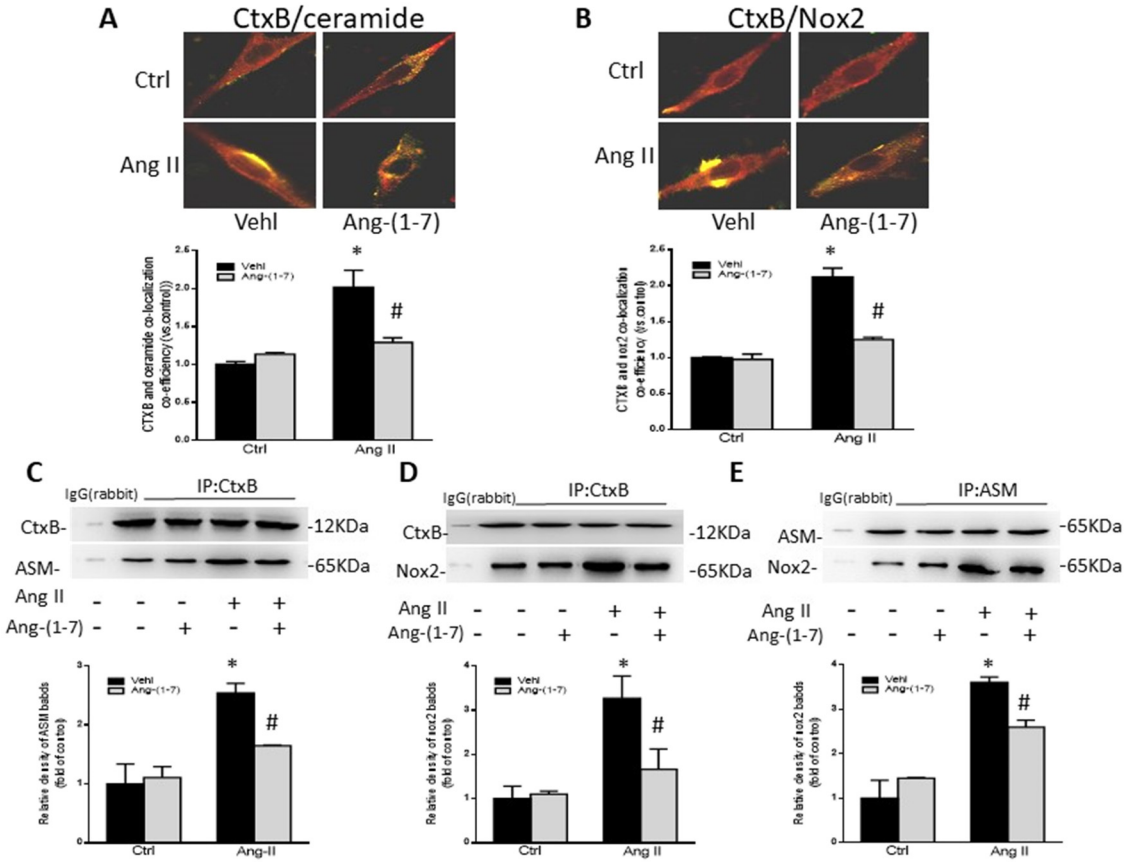

Fig. 6. MRs clustered with ASM, ceramide and Nox2 induced by Ang II.
(A, B) Representative confocal microscopic images and summarized coefficient of the colocalization of CtxB-staining patches and ceramide or Nox2. Yellow spots in the overlaid images were defined as patches of the colocalization in CtxB and ceramide or Nox2. (C, D, E) Immunoprecipitation with anti-CtxB or anti-ASM antibodies and immunoblotting with anti-ASM or anti-Nox2 antibodies. $^{*}P < 0.05$ vs. Vehicle Control (Vehl Ctrl); $^{\#}P < 0.05$ vs. Vehl Ang-II treated group (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreased the lesion formation in atherosclerotic ApoE−/− mice [21]. However, some studies found that ASM promoted foam cell formation and dysfunction of coronary arterial endothelial cells [7,22]. Thus, it is intriguing to explore the precise role of ASM in vascular AFs. We found that ASM indeed expressed in AFs and Ang II dose-dependently increased the expression and the activity of ASM, which was dose-dependently blocked by Ang-(1–7). It suggests that ASM activation was possibly involved in vascular adventitial remodeling induced by Ang II and the improvement of Ang-(1–7) was associated with ASM inhibition. Indeed, cell proliferation and collagen I deposition and cell migration in AFs treated Ang II could be improved by ASM gene silencing. It is consistent with the reports that Ang II induced ASM activity and the ASM inhibitor amitriptyline improved Ang II-induced impairment of vasodilatation [9,23]. Thus, it seems that ASM plays an anti- or pro-atherogenic role depending on the cell type and the pathological stages. The increase in intracellular Ca^{2+} responding to Ang II induced lysosome fusion, which is a crucial mediator of MRs clustering in the endothelial cells [23]. During lysosome fusion, lysosome-targeted ASM is able to traffic and expose to cell-membrane, which may lead to MRs clustering and Nox activation [24]. Thus, it was suggested that Ang II induced ASM via Ca^{2+}-mediated lysosome fusion in vascular AFs. Ang-(1–7) as an endogenous ligand of MAS, counteracted with Ang II through competitively inhibiting AT1R. Thus, ACE-Ang-(1–7)-Mas axis was associated with ASM-ceramide signaling pathway via Ang II-AT1R. However, no evidences showed the ACE-Ang-(1–7)-Mas was directly associated with ASM-ceramide signaling pathway. The current results also provided initial evidence for Ang-(1–7) to improve vascular remodeling through ASM inhibition in AFs, which expanded the pharmacological effects of Ang-(1–7).

The key mechanisms of ASM in vascular remodeling have not been determined. It has been shown that vascular adventitial remodeling is closely link to the presence of oxidative stress [25]. Here, we demonstrated that ASM down-regulation inhibited the production of $O_2^{-}\cdot$, which was mainly derived from NADPH oxidase in Ang II-induced AFs. Our present study also demonstrated that ASM indeed increased $O_2^{-}\cdot$ production through activating NADPH oxidase [14]. Ang-(1–7) improved Ang II-induced proliferation, migration and inflammation through inactivation of ROS-mediated signaling pathways [11,26]. In the present study, Ang-(1–7) abrogated Ang II-induced $O_2^{-}\cdot$ production. We further explored the isoforms of NADPH oxidases to mediate the oxidative stress originated from ASM activation in AFs. It was reported that Nox1, 2 and 4 are expressed in AFs [27]. Nox2 contributes to generation of adventitial [28], which was also confirmed in current study. We verified that ASM down-regulation or Ang-(1–7) pretreatment alleviated the protein expression of Nox2 stimulated by Ang II. It has been reported that ASM could activate the Nox2 isoform in non-muscle and endothelial cells [14,16]. Thus, Nox2 was the main source of $O_2^{-}\cdot$ responsible for the regulation of Ang-(1–7) on vascular remodeling in AFs. The gene deletion of Nox2 alleviated the vascular remodeling of AFs, which mimicked the effects of Ang-(1–7) under Ang II stimulation. It is consistent with the researches that Nox2 activation induced vascular remodeling [26,29]. It was reported that Nox4 in pulmonary artery adventitia contributed to hypertensive vascular remodeling[30]. Thus, Nox4 seemed to play a similar role with Nox2 in vascular adventitia remodeling. Due to the low expression of Nox1 in vascular AFs, few studies detected the relationship between Nox1 and

vascular adventitial remodeling. In smooth muscle cell, medial-Nox1 upregulation has been implicated in cardiovascular pathologies such as hypertension and restenosis [31].

At last, we analyzed the possible mechanisms of ASM regulating Nox2 in AFs. MRs, which consist of dynamic assemblies of cholesterol and lipids with saturated acyl chains in the membrane of cells, play an essential role in signal transduction [32]. The clustered MRs could recruit or aggregate various signaling molecules to form platforms that initiate receptor mediated transmembrane signal transduction in a variety of mammalian cells. Our previous studies showed that lysosome-targeted ASM is able to traffic to and become exposed on the cell membrane surface, which may lead to MRs clustering and NADPH oxidase activation to form MRs/redox signalosomes in vascular cells [33].In the present study, Ang-(1–7) prevented Ang II-induced MRs clustering via restraining the activation of ASM. Moreover, Ang II induced ceramide and Nox2 aggregation in MRs domain, which could be blocked by Ang-(1–7). Thus, MRs clustering is a vital mechanism determining Nox2 activity associated with ASM in AFs.

In summary, the present study provided evidences that ASM gene silencing remarkably attenuated Ang II-induced adventitial remodeling in vitro. The beneficial effects were associated with the attenuation of Nox2-dependent $O_2^-\cdot$ generation via clustering MRs. Ang-(1–7) could improve Ang II-induced adventitial remodeling via restraining ASM and downstream MRs/Nox2 signal pathway in AFs. These results provide evidences that ASM serves as a novel therapeutic strategy for adventitial remodeling related diseases.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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