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Original Paper

NLRP3 Gene Deletion Attenuates Angiotensin II-Induced Phenotypic Transformation of Vascular Smooth Muscle Cells and Vascular Remodeling

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Key Words
Nlrp3 inflammasome • Angiotensin • Vascular remodeling • Vascular smooth muscle cells • Phenotypic transformation • Hypertension

Abstract
Background/Aims: Angiotensin (Ang) II plays vital roles in vascular inflammation and remodeling in hypertension. Phenotypic transformation of vascular smooth muscle cells (VSMCs) is a major initiating factor for vascular remodeling. The present study was designed to determine the roles of NLRP3 inflammasome activation in Ang II-induced VSMC phenotypic transformation and vascular remodeling in hypertension. ***Methods:*** Primary VSMCs from the aorta of NLRP3 knockout ($NLRP3^{-/-}$) mice and wild-type (WT) mice were treated with Ang II for 24 h. Subcutaneous infusion of Ang II via osmotic minipump for 2 weeks was used to induce vascular remodeling and hypertension in WT and $NLRP3^{-/-}$ mice. ***Results:*** NLRP3 gene deletion attenuates Ang II-induced NLRP3 inflammasome activation, phenotypic transformation from a contractile phenotype to a synthetic phenotype and proliferation in primary mice VSMCs. Ang II-induced hypertension and vascular remodeling in WT mice were attenuated in $NLRP3^{-/-}$ mice. Furthermore, Ang II-induced NLRP3 inflammasome activation, phenotypic transformation and proliferating cell nuclear antigen (PCNA) upregulation were inhibited in the media of aorta of $NLRP3^{-/-}$ mice. ***Conclusions:*** NLRP3 inflammasome activation contributes to Ang II-induced VSMC phenotypic transformation and proliferation as well as vascular remodeling and hypertension.

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Introduction

Vascular smooth muscle cells (VSMCs) are highly specialized cells whose main function is contraction, and thus regulation of blood flow and blood pressure. VSMCs are not terminally differentiated, and are able to switch from a differentiated phenotype to a dedifferentiated phenotype in response to various stimuli [1, 2]. The phenotypic transformation of VSMCs is involved in reduced contractile proteins, and increased extracellular matrix and inflammatory cytokines [3]. This phenotypic transformation plays a major pathophysiologic role in the vascular remodeling in hypertension, atherosclerosis, vascular restenosis and diabetic vascular complications [1, 3, 4].

Renin-angiotensin system (RAS) plays a critical role in the development of hypertension and the end-organ damage in hypertension [5]. Ang II is a powerful vasoconstrictor and contributes to hypertension [6]. VSMC phenotypic transformation is widely accepted as the pivotal process in the vascular remodeling in hypertension [7]. The remodeling of large and small arteries contributes to the development and complications of hypertension [8]. Ang II-induced direct vasoconstriction and VSMC phenotypic transformation are involved in hypertension. Pharmacological blockade of Ang II with angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists attenuates hypertension and produces beneficial organ protective effects in hypertension [9]. Chronic low-grade vascular inflammation has been proposed to play a vital role in the initiation, development and progression of hypertension in humans and experimental models [10-12]. Ang II is known to act as a powerful pro-inflammatory mediator [13-16]. It stimulates the production of inflammatory cytokines and causes oxidative stress via AT_1 receptors, affecting the vasculature, kidney and brain to promote hypertension [17].

Inflammasomes are high-molecular-weight cytosolic complexes that mediate the activation of caspases and induce inflammation [18]. Nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome can be activated by many and diverse stimuli making NLRP3 the most importantly and clinically implicated inflammasome [19]. The NLRP3 inflammasome is activated by forming a complex of NLRP3 and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which processes pro-caspase-1 to active caspase-1. The activated caspase-1 facilitates the conversion of pro-interleukin (IL)-1β into its mature form IL-1β and thus triggers an inflammatory response [20]. ASC serves as a signal amplification mechanism for inflammasome-mediated cytokine production [21]. Inflammasome activation during infection can be protective, but an unregulated activation in response to non-pathogenic endogenous or exogenous stimuli can lead to unintended pathology [22]. It has been found that inflammatory cytokines, IL-1β and IL-18, in plasma and vessels are increased in hypertension [23]. Inflammasome is essential for one kidney/deoxycorticosterone acetate/salt-induced hypertension in mice [24]. Hydrogen sulfide suppresses high glucose-induced cardiomyocyte inflammation by inhibiting the TLR4/NF-κB pathway and its downstream NLRP3 inflammasome activation [25]. Ang II-induced hypertension is prevented in NLRP3 gene deletion mice but not in ASC deletion mice, indicating that NLRP3 contributes to gestational hypertension independently of ASC-mediated inflammasomes [26]. However, it is not known whether NLRP3 is involved in Ang II-induced VSMC phenotypic transformation and proliferation as well as vascular remodeling. The present study was designed to investigate the roles of NLRP3 in Ang II-induced inflammation, phenotypic transformation and proliferation in VSMCs, and vascular remodeling in NLRP3 knockout ($NLRP3^{-/-}$) mice and wild-type (WT) mice.

Materials and Methods

Animals

$NLRP3^{-/-}$ mice on the C57BL/6 background and WT mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) [27]. Experimental protocols were conducted in male WT mice and $NLRP3^{-/-}$ mice

aged 12 weeks. Experiments were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University. The procedures were conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication, 8th edition, 2011). Animals were housed in a temperature-controlled room with 12-h light/dark cycle and a free access to tap water and standard chow.

Ang II-induced hypertensive mice model

Angiotensin II (Ang II)-induced hypertension model was used in WT and NLRP3$^{-/-}$ mice to determine the roles of NLRP3 in hypertension and vascular remodeling. This model is believed to recapitulate several features of human essential hypertension [28]. Subcutaneous infusion of Ang II (400 ng/kg/min) or saline via osmotic minipump (Alzet 1002, Durect Corporation, Mountain View, CA, USA) for 2 weeks. The infusion rate was selected on the basis of previous study that this dose induces a slowly progressive hypertension in which the Ang II effects of appetite suppression and depressed body weight gain are avoided [28]. Blood pressure of tail artery was measured weekly in a conscious state with a noninvasive computerized tail-cuff system (NIBP, AD Instruments, Sydney, Australia), and blood pressure of carotid artery was measured with an direct intubation method under anesthesia as we previously reported [29]. Mice were sacrificed using 4% isoflurane in air and subsequent exsanguination or decapitation.

VSMC culture and Ang II treatment

Primary VSMCs were isolated from thoracic aorta of WT and NLRP3$^{-/-}$ mice aged 12 weeks using a modified enzyme dispersal method [30, 31]. Ang II was used to induce inflammation and proliferation of VSMCs *in vitro* [32]. VSMCs were used at passages 3-5 for the experiments to minimize phenotypic switching which can occur with prolonged culture period and at later passages. The VSMCs were maintained in vascular cell basal medium, vascular smooth muscle cell growth supplement (SMGS), and antibiotic-antimycotic solution at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After the attainment of confluence, the VSMCs were trypsinized, and seeded onto 60 mm Petri dishes. Subconfluent VSMCs were maintained in medium devoid of serum (SMGS) for 24 h to achieve quiescence, and then stimulated by Ang II (1 μmol/L) incubation for 24 h according to previous studies [33, 34].

Western blot analysis

Samples were homogenized in lysis buffer, and the supernatant was extracted for the measurement of total protein with a protein assay kit (BCA; Pierce, Santa Cruz, CA, USA). Equal amounts of total protein were separated by SDS-PAGE, and transferred to PVDF membranes in Trisglycine methanol buffer. The bands were visualized with Enhanced Chemiluminescence Detection Kit (Thermo Scientific, Rockford, IL, USA). The antibodies against NLRP3, ASC and procaspase-1 were purchased from Abcam (Cambridge, MA, USA). Antibodies against α-SMA, SM22α, OPN and GAPDH were obtained from Cell Signaling Technology (Beverly, MA, USA). IL-1β and PCNA were purchased from Protein Tech Group Inc. (Chicago, IL, USA).

Immunohistochemistry

The aorta was fixed in 4% formaldehyde, embedded in paraffin and transversely cut into 5-μm sections using a cryostat (Leica, Solms, Germany). The sections were washed 3 times with 0.1 M PBS after de-paraffinization, and blocked with blocking buffer (Dual Endogenous Enzyme Block; Dako, Carpinteria, CA, USA) for 5 min. The sections were incubated with rabbit primary anti-NLRP3 antibody (1:100; Abcam, Cambridge, UK), anti-IL-1β antibody (1:200; Proteintech Group Inc., Chicago, IL, USA) or anti-PCNA antibody (1:500; Proteintech Group Inc.) for 24 h at 4°C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody for 30 min in room temperature. 3, 3-diaminobenzidine was used to develop the positive cells in arteries. Sections were counterstained with hematoxylin, and then covered with glass coverslips with xylene-based mounting medium.

VSMC proliferation assay

VSMC proliferation was evaluated by Cell Counting Kit-8 (CCK-8) and EdU incorporation assay [35, 36]. Cell counting kit-8 kits (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China) was used according to the manufacturer's instructions. The absorbance was conducted at 450 nm using a microplate reader (ELX800, BioTek, Vermont, USA). Furthermore, VSMC proliferation was evaluated with 5-ethynyl-

2'-deoxyuridine (EdU) incorporation assay with *In vitro* Imaging Kit (Guangzhou RiboBio, Guangzhou, China). The DNA synthesis of VSMCs was measured using a Cell-Light™ EdU Apollo®567. The EdU positive cells were counted and normalized by the total number of Hoechst 33342 stained cells [29]. In addition, expression of proliferating cell nuclear antigen (PCNA) was measured with Western blotting and used as an marker of proliferation in aortic media of mice [37, 38].

Masson's staining

Paraffin-embedded sections were stained with Masson's trichrome staining under standard protocols [39]. The images were collected with a light microscope (BX-51, Olympus, Tokyo, Japan). The media thickness and media area were used as indexes of vascular remodeling.

Statistical analysis

Two-way ANOVA followed by post hoc Bonferroni test was used when multiple comparisons were made. All data are expressed as mean±SE. A value of $P<0.05$ was considered statistically significant.

Results

NLRP3 inflammasome and inflammation in VSMCs

There were almost no NLRP3 protein expression in VSMCs from NLRP3$^{-/-}$ mice. Ang II increased NLRP3 expression in VSMCs from WT mice, but had no significant effect on NLRP3 expression in those from NLRP3$^{-/-}$ mice. There were no significant difference in ASC, procaspase-1 and IL-1β protein expressions between the VSMCs from WT mice and the VSMCs from NLRP3$^{-/-}$ mice. However, Ang II-induced ASC, procaspase-1 and IL-1β protein expressions were significantly inhibited in VSMCs from NLRP3$^{-/-}$ mice compared with those from WT mice (Fig. 1A & 1B).

VSMC phenotypic transformation

There were no significant difference in the expressions of osteopontin (OPN), α-smooth muscle actin (α-SMA), smooth muscle 22α (SM22α) between the VSMCs from WT mice and the VSMCs from NLRP3$^{-/-}$ mice. Ang II upregulated synthetic protein OPN, and downregulated contractile proteins α-SMA and SM22α in VSMCs from WT mice. NLRP3 gene deletion almost prevented the Ang II-induced VSMC phenotypic transformation (Fig. 2A & 2B).

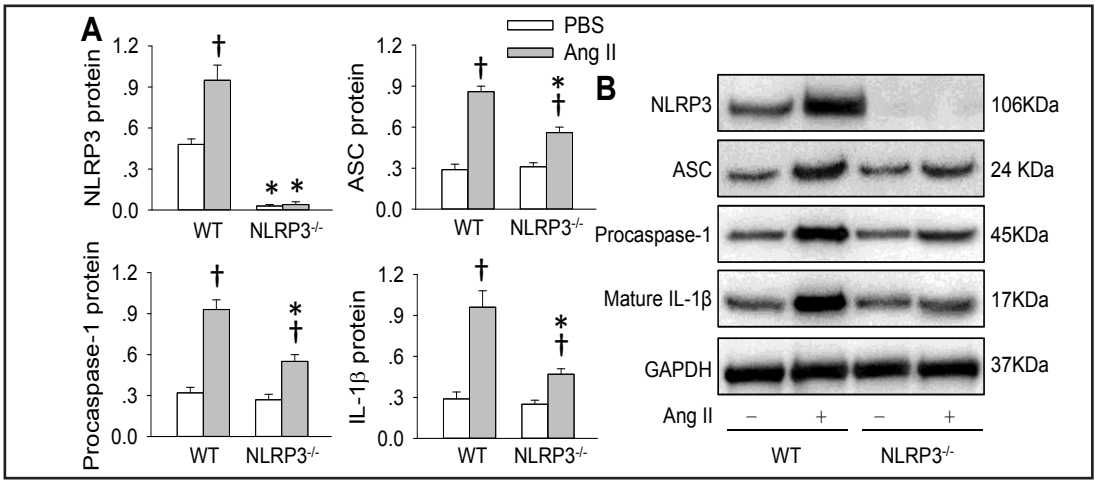

Fig. 1. NLRP3 gene deletion attenuates Ang II-induced inflammasome activation in VSMCs. A, expressions of NLRP3, ASC, procaspase-1 and IL-1β proteins; B, representative images of Western blotting. Values are mean±SE. *P<0.05 vs. WT; †P<0.05 vs. PBS. n=6.

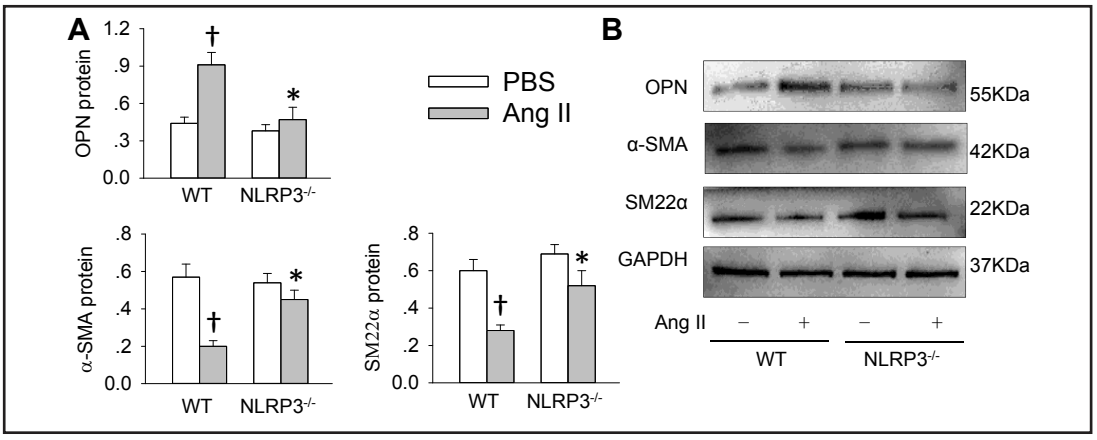

Fig. 2. NLRP3 gene deletion inhibits Ang II-induced phenotypic transformation in VSMCs. A, expressions of OPN, α-SMA and SM22α proteins; B, representative images of Western blotting. Values are mean±SE. *P<0.05 vs. WT; †P<0.05 vs. PBS. n=6.

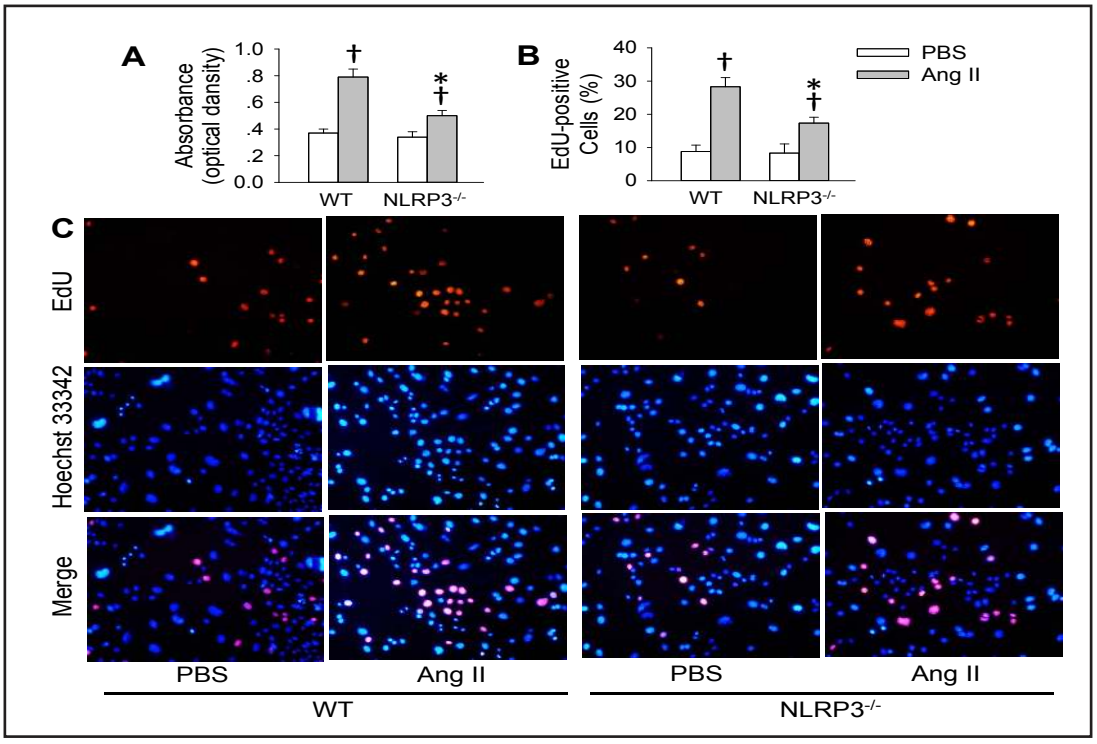

Fig. 3. NLRP3 gene deletion inhibits Ang II-induced proliferation in VSMCs. A, VSMC proliferation was evaluated with changes of absorbance measured with CCK-8 kits; B, percentage of EdU-positive cells in VSMCs. C, representative images showing EdU-positive cells measured with Edu incorporation assay. Blue fluorescence shows cell nuclei and red fluorescence stands for cells with DNA synthesis. Values are mean±SE. *P<0.05 vs. WT; †P<0.05 vs. PBS. n=6.

VSMC proliferation

VSMC proliferation was evaluated with CCK-8 kit and EdU assay. CCK-8 kit assay showed that Ang II-induced VSMC proliferation was attenuated in VSMCs from NLRP3$^{-/-}$ mice compared with those from WT mice (Fig. 3A). The role of Ang II in increasing the number of EdU-positive cells was inhibited in VSMCs from NLRP3$^{-/-}$ mice (Fig. 3B & 3C).

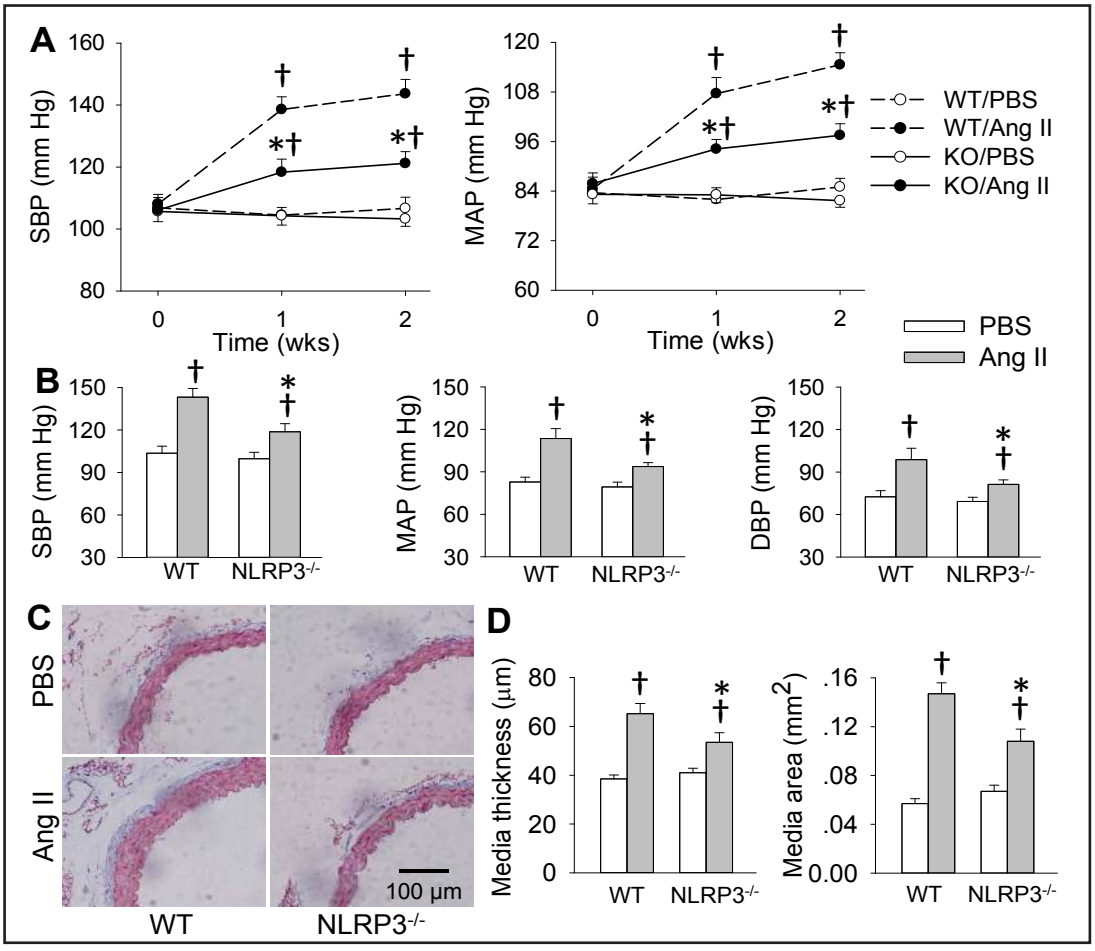

Fig. 4. Deletion of NLRP3 gene attenuates Ang II-induced hypertension and vascular remodeling of aorta in mice. Hypertension was induced by subcutaneous infusion of Ang II (400 ng/kg/min) for 2 weeks. A, systolic blood pressure (SBP) and mean arterial pressure (MAP) measured in conscious state with a noninvasive computerized tail-cuff system; B, SBP, MAP and diastolic blood pressure (DBP) measured with carotid artery intubation method under anesthesia; C, representative sections of Masson's staining for the aorta; D, media thickness and media area of aorta. Values are mean±SE. *P<0.05 vs. WT; †P<0.05 vs. PBS. n=6.

Blood pressure and vascular remodeling in mice

The roles of NLRP3 in Ang II perfusion-induced hypertension and vascular remodeling were determined in age-matched WT and NLRP3$^{-/-}$ mice. Deletion of NLRP3 gene attenuated Ang II-induced hypertension determined in conscious state with a noninvasive computerized tail-cuff system (Fig. 4A) and under anesthesia with a direct carotid artery intubation method (Fig. 4B). Ang II increased media thickness and area of aorta, which was reduced in NLRP3$^{-/-}$ mice, suggesting that NLRP3 gene deletion attenuates vascular remodeling caused by Ang II (Fig. 4C & 4D).

NLRP3 inflammasome and inflammation in aortic media of mice

In the media of aorta, there were no significant difference in ASC, procaspase-1 and IL-1β protein expression between WT mice and NLRP3$^{-/-}$ mice, but almost no NLRP3 expression in NLRP3$^{-/-}$ mice. The role of Ang II in upregulating NLRP3, ASC, procaspase-1 and IL-1β expression was almost completely prevented by NLRP3 gene deletion (Fig. 5A & 5B). The changes of NLRP3 and IL-1β expression was further confirmed by immunohistochemical analysis. It is noted that the changes induced by NLRP3 gene deletion or Ang II were not only localized in the media, but occurred in all three layers of aorta including vascular intima,

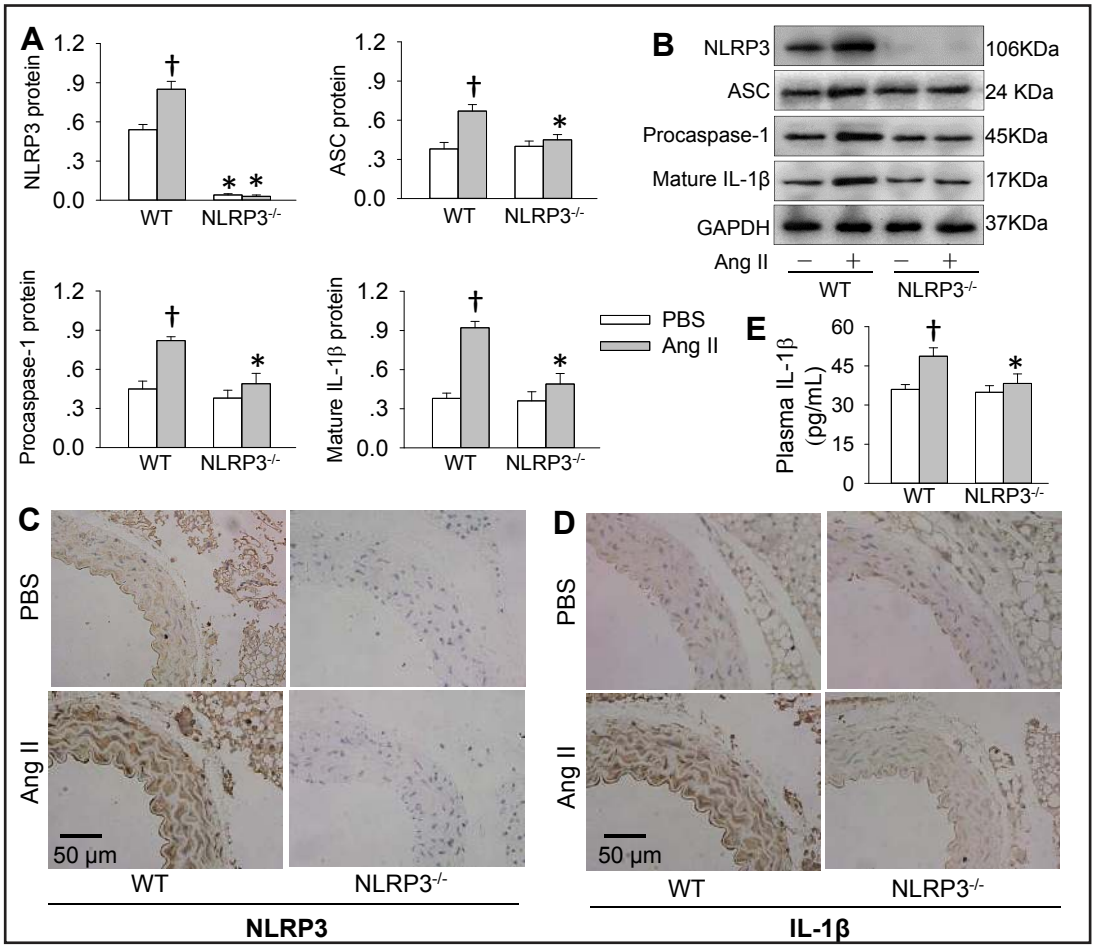

Fig. 5. Deletion of NLRP3 gene attenuated Ang II-induced NLRP3 inflammasome activation in aortic media of mice. Measurements were carried out after 2 weeks of subcutaneous infusion of Ang II (400 ng/kg/min) via osmotic minipump. A, expressions of NLRP3, ASC, procaspase-1 and IL-1β proteins; B, representative images of Western blotting; C, immunohistochemical analysis for NLRP3 in aorta of mice; D, immunohistochemical analysis for IL-1β in aorta of mice. E, plasma IL-1β levels in mice. Values are mean±SE. *P<0.05 vs. WT; †P<0.05 vs. PBS. n=6.

media and adventitia (Fig. 5C & 5D). Furthermore, Ang II increased plasma IL-1β levels, which was prevented by NLRP3 gene deletion (Fig. 5E).

Phenotypic transformation and proliferation of vascular smooth muscle in mice

NLRP3 gene deletion did not affect the phenotype of vascular smooth muscle, but attenuated the Ang II-induced phenotypic transformation (Fig. 6A & 6C). On the other hand, NLRP3 gene deletion reduced the Ang II-induced upregulation of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation (Fig. 6B & 6C). Immunohistochemical analysis showed that Ang II upregulated PCNA, which was inhibited by NLRP3 gene deletion in all three layers of aorta (Fig. 6D).

Discussion

Ang II plays an important role in the pathogeneses of hypertension [5]. Ang II exerts proinflammatory effects on vasculature, which deteriorates hypertension and related tissue damage [6, 13]. Blockage of Ang II actions produces beneficial protective role via its depressor

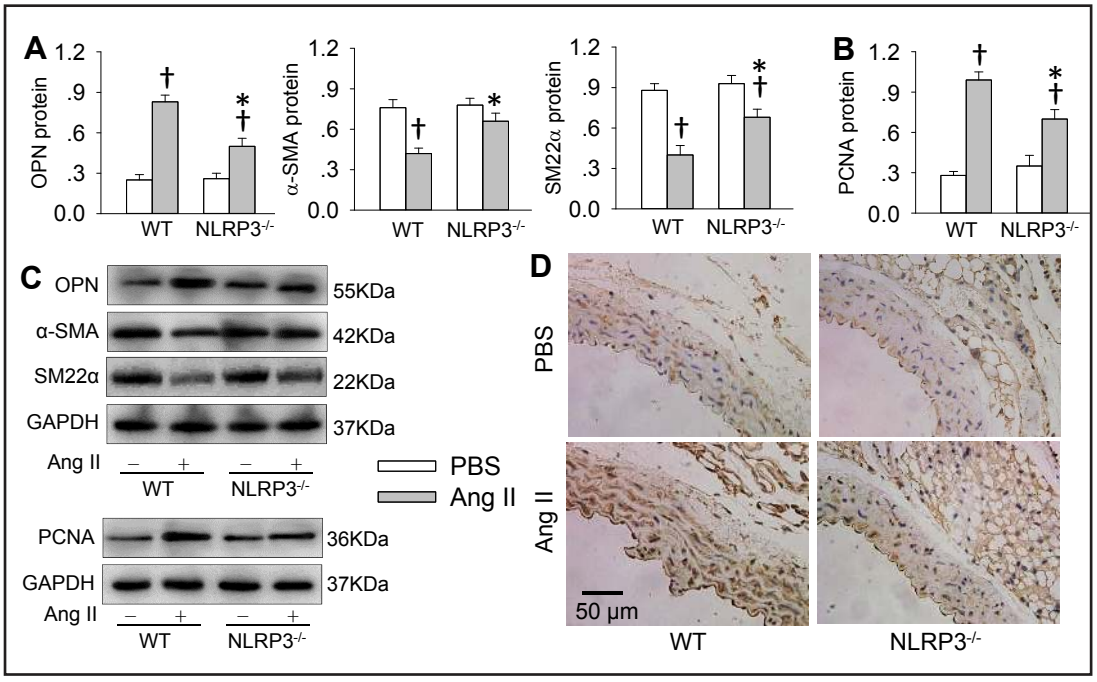

Fig. 6. Deletion of NLRP3 gene attenuated Ang II-induced phenotypic transformation and proliferation in aortic media of mice. Measurements were carried out after 2 weeks of subcutaneous infusion of Ang II (400 ng/kg/min) via osmotic minipump. A, expressions of synthetic protein (OPN) and contractile proteins (α-SMA, SM22α); B, expression of PCNA protein, a marker of proliferation; C, representative images of Western blotting; D, Immunohistochemical analysis for PCNA in aorta of mice. Values are mean±SE. *P<0.05 vs. WT; †P<0.05 vs. PBS. n=6.

effect and blockade of the Ang II-induced inflammatory response [40]. The primary novel findings are that NLRP3 deficiency attenuates Ang II-induced inflammation, phenotypic transformation, proliferation in VSMCs and vascular remodeling in mice, indicating the importance of NLRP3 in mediating Ang II-induced inflammation and vascular remodeling. NLRP3 may be a novel target for the intervention of hypertension and vascular remodeling.

Vascular inflammation contributes to vascular remodeling in hypertension and several other vascular diseases [41-43]. It has been found that plasma IL-1β levels was increased in renovascular hypertensive rats [44] and stroke-prone SHR [45]. IL-1β accelerated the onset of stroke concomitant with severe hypertension in spontaneously hypertensive rats (SHR) [45]. VSMCs retain a capacity of phenotype transformation. Differentiated phenotype (contractile phenotype) VSMCs do not proliferate or produce inflammatory mediators [1, 3]. In response to injury, VSMCs change their gene expression profile to a differentiated phenotype (synthetic phenotype or inflammatory phenotype), and begin to produce inflammatory mediators which stimulates VSMC proliferation and migration [46]. On the other hand, some inflammatory mediators ultimately activate transcription factors to initiate gene expression pattern changes associated with VSMC phenotypes to a differentiated phenotype [47]. It seems to be a vicious circle between VSMC phenotype transformation and inflammation in pathological status. VSMC phenotypic transformation is responsible for the pathogeneses and progression of hypertension and related vascular pathologies [4]. The present study provides evidence that NLRP3 gene deletion inhibited Ang II-induced inflammation, VSMC phenotypic transformation and proliferation *in vitro*, which was further confirmed by the *in vivo* findings that persistent subcutaneous infusion of Ang II in mice induced vascular inflammation, phenotypic transformation and proliferation of vascular smooth muscle, which were attenuated by NLRP3 gene deletion. It is noted that NLRP3 gene deletion almost prevented the Ang II-induced VSMC phenotypic transformation, but only

partially inhibited the Ang II-induced VSMC proliferation. The results suggest that NLRP3 and inflammation play primary roles in Ang II-induced VSMC phenotypic transformation, but partially contributes to Ang II-induced VSMC proliferation. On the other hand, VSMC phenotypic transformation participates in the increase in arterial stiffening, arterial pulse pressure, and dilatation of conduit arteries in ageing [48]. However, it is unknown whether the reduced VSMC contractile proteins including α-SMA and SM22α in hypertension would be involved in the alteration of vasoconstriction vascular reactivity, which need further investigation.

Remodeling of arteries contributes to the development and complications of hypertension [4, 6, 49]. Phenotypic transformation of VSMCs is a major initiating factor for vascular remodeling in hypertension [1]. VSMC proliferation, migration and hypertrophy contributes to vascular remodeling and hypertension [50, 51]. In the present study, NLRP3 gene deletion attenuated Ang II-induced vascular remodeling and hypertension in mice, indicating that NLRP3 inflammasome plays an important role in hypertension and vascular remodeling. The attenuation of vascular remodeling may be partially attributed to the inhibition of inflammation and VSMC phenotypic transformation and proliferation. The antihypertensive effect of NLRP3 gene silencing may be secondary to the improvement of vascular remodeling.

It has been reported that Ang II-induced hypertension is prevented in NLRP3 gene deletion mice but not in ASC deletion mice [26]. In their study, there was a tendency of reduction in systolic blood pressure in Ang II-treated ASC deletion mice compared with that in Ang II-treated WT mice, but the values did not reach statistic difference between the two groups (about -15 mmHg, n=3-6 per group). There is a possibility that the statistic difference might reach its significant level with more number of mice per group. In the present study, NLRP3 gene deletion attenuates Ang II-induced ASC upregulation, suggesting that ASC may be partially involved in the effects of NLRP3 gene deletion. However, we cannot exclude the possibility that the ASC downregulation caused by NLRP3 gene deletion might be secondary to its role in attenuating inflammation. A limitation in the present study is that Ang II was administered subcutaneously in the universal NLRP3 gene knockout mice and WT mice. Thus, the Ang II-induced NLRP3 inflammasome activation in other tissues and organs might be also involved in the roles of NLRP3 gene deletion in vascular remodeling and hypertension. Furthermore, the study was carried out in aorta but not in resistance blood vessels. There is a possibility that the findings might not entirely applicable to resistance blood vessels.

Conclusion

NLRP3 gene deletion attenuates the Ang II-induced inflammation, VSMC phenotypic transformation and proliferation, as well as the Ang II-induced hypertension and vascular remodeling. Intervention of NLRP3 may be an effective strategy to attenuate hypertension and related end-organ damage.

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Disclosure Statement

The authors declare that they have no competing interests.

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