

Inhibition of SRF/myocardin reduces aortic stiffness by targeting vascular smooth muscle cell stiffening in hypertension

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Received 15 May 2016; revised 14 September 2016; editorial decision 10 October 2016; accepted 14 October 2016; online publish-ahead-of-print 23 October 2016

Time of primary review: 31 days

Aims

Increased aortic stiffness is a fundamental manifestation of hypertension. However, the molecular mechanisms involved remain largely unknown. We tested the hypothesis that abnormal intrinsic vascular smooth muscle cell (VSMC) mechanical properties in large arteries, but not in distal arteries, contribute to the pathogenesis of aortic stiffening in hypertension, mediated by the serum response factor (SRF)/myocardin signalling pathway.

Methods and results

Four month old male spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats were studied. Using atomic force microscopy, significant VSMC stiffening was observed in the large conducting aorta compared with the distal arteries in SHR ($P < 0.001$), however, this regional variation was not observed in WKY rats ($P > 0.4$). The increase of VSMC stiffness was accompanied by a parallel increase in the expression of SRF by 9.8-fold and of myocardin by 10.5-fold in thoracic aortic VSMCs from SHR vs. WKY rats, resulting in a significant increase of downstream stiffness-associated genes (all, $P < 0.01$ vs. WKY). Inhibition of SRF/myocardin expression selectively attenuated aortic VSMC stiffening, and normalized downstream targets in VSMCs isolated from SHR but not from WKY rats. *In vivo*, 2 weeks of treatment with SRF/myocardin inhibitor delivered by subcutaneous osmotic minipump significantly reduced aortic stiffness and *then* blood pressure in SHR but not in WKY rats, although concomitant changes in aortic wall remodelling were not detected during this time frame.

Conclusions

SRF/myocardin pathway acts as a pivotal mediator of aortic VSMC mechanical properties and plays a central role in the pathological aortic stiffening in hypertension. Attenuation of aortic VSMC stiffening by pharmacological inhibition of SRF/myocardin signalling presents a novel therapeutic strategy for the treatment of hypertension by targeting the cellular contributors to aortic stiffness.

Keywords

Atomic force microscopy • Hypertension • Myocardin • Serum response factor • Vascular smooth muscle cell stiffness

1. Introduction

Aortic stiffening is strongly associated with hypertension, as well as aging, and has been proved to be of critical importance as an early predictor of subsequent cardiovascular diseases.^{1,2} Despite a widely held belief that

increased aortic stiffness in hypertensive patients is largely a manifestation of long-standing hypertension-related damage, a recent statement from the American Heart Association asserts that aortic stiffness is a cause rather than a consequence of hypertension in middle-aged and older individuals.³ This new concept further highlights the importance of better

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defining the mechanism and pathogenesis of aortic stiffening in hypertension. Although the aetiology of aortic stiffness has been thought to involve changes in extracellular matrix proteins and endothelial dysfunction,^{4–6} increasing attention has been attracted to the crucial role of vascular smooth muscle cells (VSMCs) in aortic wall stiffness.^{7–9} In particular, recent studies showed that intrinsic mechanical properties of thoracic aortic (TA) VSMCs, independent of VSMC proliferation and migration, are important contributors to increased aortic stiffness during aging and hypertension.^{7–9} However, key molecular mechanisms regulating VSMC mechanical properties remain largely unknown.

Our previous studies showed that increased aortic stiffness during aging was accompanied by an activation of genes encoding smooth muscle cytoskeletal proteins.^{7,10} A search for the governing transcription regulatory mechanisms found that serum response factor (SRF), a MADS-box transcription factor, was strongly associated with these up-regulated genes in VSMCs from stiffening aorta. Recent studies from other groups also showed that SRF and its transcriptional cofactors, the myocardin-related transcription factors (MRTFs), act as key mediators of gene transcription and function of VSMCs.^{11,12} Moreover, it has been shown that inactivation of SRF in a mouse model resulted in a decreased arterial stiffness.¹³ These studies together suggested that SRF may act as a master regulator of VSMC mechanical properties in hypertension. Building on these findings, we tested our hypothesis that SRF, accompanied by its transcriptional cofactor myocardin, is a crucial mediator of aortic VSMC stiffening in hypertension, and that inhibition of this pathway provides a new avenue for the therapy of aortic wall stiffening and high blood pressure.

In the present study, by integrating atomic force microscopy (AFM) and molecular biological examination, we investigated the regional variation of VSMC stiffness between the large conduction vessels and the distal medium-sized arteries in spontaneously hypertensive rats (SHR) compared to normotensive Wistar-Kyoto (WKY) control rats, and further identified the molecular basis underlying the regional heterogeneity of VSMC stiffening. By using both *in vitro* isolated VSMCs and *in vivo* animal models, we also provided evidence that pharmaceutical targets aimed at VSMC stiffness can effectively rectify aortic vascular stiffening and high blood pressure in hypertensive animals.

2. Methods

A comprehensive description of the Methods can be found in the online [Supplementary Data](#).

2.1 Animal model

Adult (16- to 18-week-old) male SHR and normotensive control WKY rats (Charles River Laboratories, San Diego, CA) were studied. All animal experiments conformed to NIH guidelines (Guide for the care and use of laboratory animals) (NIH Publication No. 85-23, revised 2011) and the local ethics review board. For *in vivo* drug treatments, CCG-100602 (1-[3,5-bis(trifluoromethyl)benzoyl]-N-(4-chlorophenyl)-3-piperidinecarboxamide) (1.5 mg/kg/day, Cayman Chemical, MI) or vehicle control (DMSO, Sigma-Aldrich) were continuously administered for 2 weeks, by Alzet osmotic minipump (Model 2ML2, DURECT, CA) implanted subcutaneously under anaesthesia with an inspired concentration of 2% isoflurane (JD Medical, AZ).¹⁴

2.2 Measurement of blood pressure

Systemic blood pressure was measured in the conscious status by restraint tail cuff every 2 days for 2–3 weeks using the CODA system (Kent Scientific, CT). Direct aortic pressure was measured in ascending

thoracic aorta via inserting a Millar catheter (SPR 320, Millar Instruments, TX) through right common carotid artery under anaesthesia with an inspired concentration of 2% isoflurane (JD Medical, AZ). The transducer was connected to a Powerlab system (AD Instruments, Castle Hill, Australia) to record systolic aortic pressure (SAP) and diastolic aortic pressure (DAP). Mean aortic pressure (MAP) and pulse pressure (PP) were then calculated accordingly ($MAP = DAP + (SAP - DAP)/3$, $PP = SAP - DAP$).

2.3 Measurements of aortic stiffness *in vivo*

Under 2% isoflurane anaesthesia, aortic stiffness was measured non-invasively at time points before and after initiating the treatments at Days 7 and 14. Performed by two independent researchers, the diameter and wall thickness of the ascending thoracic aorta were measured by echography while blood pressure was measured by tail cuff simultaneously.^{15,16} Aortic stiffness was confirmed using aortic pressure measured directly via pressure catheter on treatment Day 14. Regional aortic stiffness was evaluated by multiple indexes: pressure-strain modulus ($E_p = 1333 \times PP / (\Delta D/D)$), arterial stiffness index ($ASI = \ln[(SBP/DBP)/(\Delta D/D)]$), and Young's modulus ($E_Y = D/h/DC$).^{17,18} PP: pulse pressure; D: diastolic diameter of the thoracic aorta; ΔD : systolic minus diastolic diameter change; SBP: systolic blood pressure; DBP: diastolic blood pressure; h: thickness of aortic wall; DC: distensibility = $\Delta A/A \times PP$,¹⁹ while A: the minimal cross-sectional area of the aorta, ΔA : the maximal minus minimal cross-sectional area of the aorta.

2.4 VSMC isolation, culture and treatments

Rats were euthanized with carbon dioxide inhalation and tissues collected. Primary VSMCs were isolated from arteries of rats including thoracic aorta (TA), abdominal aorta (AA), renal artery (RA) and iliac artery (IA), then serially cultured for two to three passages as described previously.^{7,8} Selected VSMC cultures were treated with CCG-100602 (25 $\mu\text{mol/L}$) for 24 h and then collected for RNA and protein extraction or immunostaining. DMSO served as vehicle control.

2.5 VSMC stiffness measured by AFM

Single-cell micromechanical measurements were performed using a biological AFM system (Asylum Research, MFP-3D-BIO, CA) with a silicon nitride AFM probe (nominal spring constant, $k = 0.1 \text{ N/m}$) with a pyramidal tip (radius 40 nm). The targeted VSMCs were indented to a threshold force of 2–3 nN, at a rate of 2 $\mu\text{m/s}$, in an intermediate region between the nucleus and the cell edge. Two nanoindentation protocols were used to determine the cellular micromechanics: (i) spatial variation, which indented multiple locations per cell between the nucleus and periphery, and (ii) temporal variation, which repeatedly indented one site every 10 s for 30 min to assess spontaneous oscillations in VSMC mechanical properties. The local apparent elastic modulus (E_{ap}) was determined using Hertz contact analysis for a cone to model the indentation force curve (advancing curve); the maximum adhesion force (F_{ad}), due to non-specific steric interaction between the uncoated AFM tip and the cell surface, was obtained from the retraction force curve. Spatial variations were averaged to obtain a single representative E_{ap} or F_{ad} value per cell.

Time-dependent variations in E_{ap} were decomposed using a sum of three sinusoidal functions as detailed in the online Detailed Methods. The primary component (maximum amplitude) of the sinusoidal decomposition function dominates the overall temporal oscillation behaviour. All comparisons in this study were focused on this primary sinusoidal component, including the primary amplitude and period of the oscillation, as well as the mean elastic modulus (E_{ap}).

To assess the effects of drug intervention on VSMC stiffness, cultured cells were treated for 24 h with CCG-100602 (1.12 $\mu\text{mol/L}$) or vehicle control (DMSO) prior to AFM indentation testing as described above.

2.6 RNA extraction and real-time PCR

RNA was extracted from isolated VSMCs and artery tissues using Quick-RNA MiniPrep kit (Genesee Scientific, Cat No. 11-327) according to the manufacturer's instructions. Quantitative real-time PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Cat No. 1725121) according to the manufacturer's instructions. All real-time PCRs were performed in triplicate, with expression normalized to GAPDH.

2.7 Protein extraction and Western blot

Total protein was extracted from VSMCs and arteries as described previously.⁷ Subcellular fractions were extracted using the Nuclear Extraction Kit (Millipore Inc., USA). Proteins were measured by Western blotting using the LI-COR Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). HDAC1 and GAPDH were used as loading control of nuclear and cytoplasmic fraction or total protein, respectively.

2.8 Cyto-immunostaining

Immunostaining was used to detect the expression and distribution of SRF, myocardin and α -SMA in cultured VSMCs using respective primary antibodies as described previously.⁷

2.9 Histology

Samples were taken from the thoracic aorta and preserved in 4% phosphate buffered formaldehyde in the unloaded state for histology as described previously.^{5,10} Sections were stained with haematoxylin and eosin (H&E) and medial layer thickness and lumen diameter were measured and collagen staining was performed with Picric acid sirius red as previously described.⁵ Images were analysed using Image-Pro Plus software (Media Cybernetics).²⁰ Total area of each aortic medial layer was measured in pixels. Collagen content was estimated as a ratio of integrated optical density (IOD) to a total area of each aortic medial layer. Counting criteria and software settings were identical for all slides.

3.0 Statistical analysis

Results are presented as the mean \pm SEM for the number of samples indicated in the figure legends. One- or Two-way and/or repeated measure ANOVA were used to test effects of group, region, and drug intervention, and Student-Newman-Keuls post hoc correction was applied for multiple pairwise comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Increase of VSMC stiffness in SHR is more prominent in the large conducting aorta

Our previous study in an aging model showed that VSMC stiffness increased in the stiffening thoracic aorta.^{7,8} To test whether this characteristic also exists in other arteries in hypertension, VSMCs were isolated from different regions along the artery tree of both SHR and WKY rats. The elastic modulus of isolated VSMCs was measured using

nanindentation with AFM. While no significant difference in VSMC stiffness was found between the large elastic arteries (TA and AA), nor between the muscular arteries (RA and IA), a significant difference between large and small arteries was observed in SHR ($P < 0.001$ for each pairwise comparison), e.g. the elastic modulus of VSMCs from TA and AA were significantly higher than that in RA and IA VSMCs (Supplementary material online, Figure S1). In contrast, the effect of vessel location on VSMC modulus was not significant for WKY. Of particular interest, VSMC stiffness was significantly higher in SHR vs. WKY in the TA ($P < 0.001$) but not in the RA or IA (Supplementary material online, Figure S1) nor in arterioles. To examine the intriguing regional variations of VSMC intrinsic mechanical properties, TA and RA were selected for detailed comparison in this study. As shown in Figure 1A, a steeper advancing curve and a larger snap-off feature in the retraction curve were observed in TA VSMCs from SHR compared with normotensive WKY rats, indicating increased cell stiffness and stronger surface adhesion force in SHR TA VSMCs. However, such differences were not observed between VSMCs from SHR RA versus WKY RA (Figure 1B).

The average local E_{ap} of individual VSMC from the TA was significantly higher in SHR (7.0 ± 0.4 kPa) vs. WKY rats (5.3 ± 0.5 kPa); ($P < 0.01$, Figure 1C). In addition, the F_{ad} was significantly higher in TA VSMCs from SHR (39.9 ± 2.2 pN) vs. WKY rats (30.6 ± 3.4 pN; $P < 0.01$, Figure 1D), which is consistent with our previous studies.^{7,8} However, no difference was observed in VSMCs between SHR RA and WKY RA in terms of E_{ap} (4.5 ± 0.3 kPa vs. 4.8 ± 0.3 kPa, $P = 0.6$, Figure 1C), nor F_{ad} (23.1 ± 1.6 pN vs. 28.0 ± 2.3 pN, $P = 0.2$, Figure 1C). E_{ap} and F_{ad} were significantly higher in TA VSMCs compared to RA in SHR (all, $P < 0.01$), but not in WKY rats ($P > 0.4$) (Figure 1C and D). This finding indicated that the altered mechanics of VSMCs in SHR are more pronounced in the large conducting vessels (TA) compared to the distal arteries (RA).

Our previous study showed that VSMCs from stiffer aortas associated with aging also had a different pattern of spontaneous temporal oscillation of stiffness.⁸ To determine, if such temporal variations are exhibited in the VSMCs from different regions of the aortic tree, time-dependent changes of elastic modulus in VSMC were tested by AFM with repeated indentation measurements. As shown in Figure 1E, dynamic temporal variations in VSMC elastic modulus were observed in both TA and RA from either SHR or WKY rats. However, VSMCs from SHR TA were distinct from WKY TA, and also from SHR RA and WKY RA by showing a significantly higher value of elastic modulus. This observation is consistent with the results of spatial measurements (Figure 1C). Furthermore, the reconstructed oscillatory curves based on the primary magnitude and period showed a distinct pattern of SHR TA elasticity (Figure 1F). On average, the amplitude of the primary sinusoidal decomposition function (A_1) was significantly higher in SHR TA VSMCs versus WKY TA VSMCs ($P < 0.01$) (Figure 1G) and the corresponding period was significantly longer in SHR TA VSMCs vs. WKY TA VSMCs ($P < 0.01$) (Figure 1H), indicating larger and slower spontaneous stiffness oscillations in SHR TA VSMCs. Compared to RA, the oscillation amplitude was significantly higher in TA of SHR ($P < 0.01$) (Figure 1G), but the corresponding difference in oscillation period was not statistically significant (Figure 1H). There was no significant difference in amplitude or period in RA VSMCs between SHR and WKY rats.

3.2 SRF/myocardin signalling is up-regulated in aortic VSMCs from SHR

We next examined the molecular basis underlying the heterogeneity of mechanical properties in VSMCs between TA and RA described above.

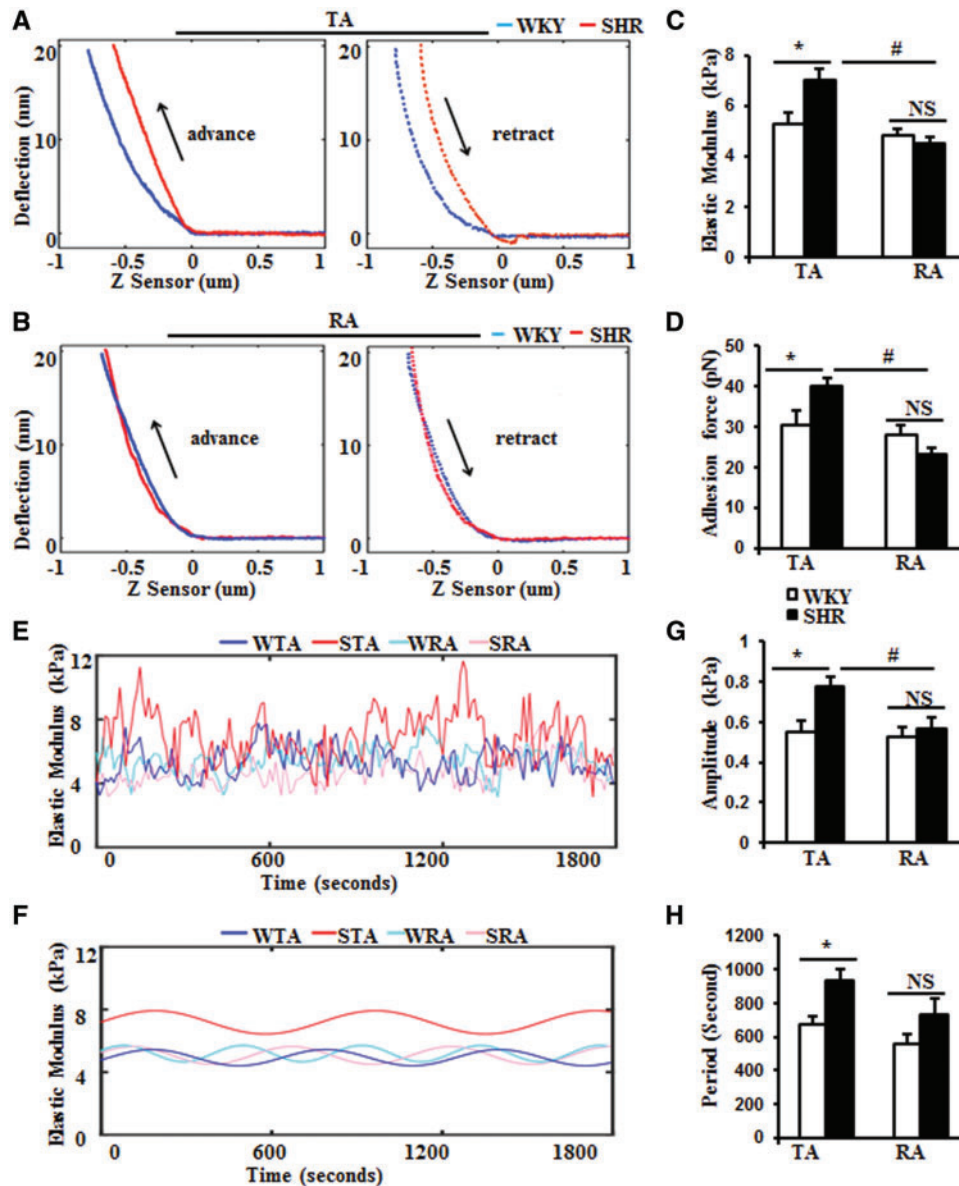


Figure 1 Variation in VSMC mechanical properties between TA and RA by AFM. (A and B): Representative force curves during advance and retraction from AFM nanoindentation of VSMCs from (A) the thoracic aorta (TA) and (B) renal artery (RA) of WKY (blue) and SHR (red). (C) Average apparent elastic modulus and (D) non-specific adhesion force ($n = 10$ cells/group from four different animals/group). (E) Representative raw time-dependent stiffness of VSMCs from WKY TA (WTA), SHR TA (STA), WKY RA (WRA), and SHR RA (SRA). (F) Primary component of sinusoidal decomposition of raw signals in panel E. (G) Amplitude and (H) period of the primary sinusoidal component of cell stiffness oscillations ($n = 15\sim 23$ cells/group from four different animals/group). Data are shown as mean \pm SEM, * $P < 0.01$ vs. WKY; # $P < 0.01$ vs. corresponding TA. Two-way ANOVA was used for panels C, D, G, and H.

As shown in Figure 2, compared to WKY TA VSMCs, SHR TA VSMCs showed a significant increase in expression of SRF and myocardin at both mRNA and protein levels (all, $P < 0.01$ vs. WTA). However, such differences were not detected in RA VSMCs between SHR and WKY. Differences between TA and RA VSMCs were only observed in SHR (Figure 2A–D). In addition, the alteration of SRF/myocardin gene expression resulted in a parallel alteration in their downstream target genes, e.g. alpha-smooth muscle actin (α -SMA), myosin heavy chain 11 (MYH11), smooth muscle 22 (SM22) and smoothelin (SMTN) (Figure 2E). The increase of the corresponding proteins of these downstream targets was further confirmed by western blotting (Figure 2F and G). Similar alterations were also observed in other SRF/myocardin

downstream targets, such as caldesmon, calponin, and RhoA (Supplementary material online, Figure S2A–F).

To ensure the differences observed in VSMCs are not artefacts of cell isolation and culture, these proteins were also measured in freshly harvested artery tissue samples. Patterns of expression of SRF and myocardin as well as their downstream targets detected in vessel tissues were similar to observations in cultured VSMCs (Supplementary material online, Figure S3). Thus, the regional variations of SRF/Myocardin signalling in VSMCs are highly consistent with the regional heterogeneity of VSMC mechanical properties (Figure 1). These data support that SRF/myocardin signalling may present a mediator of the increased aortic VSMC stiffness in SHR TA.

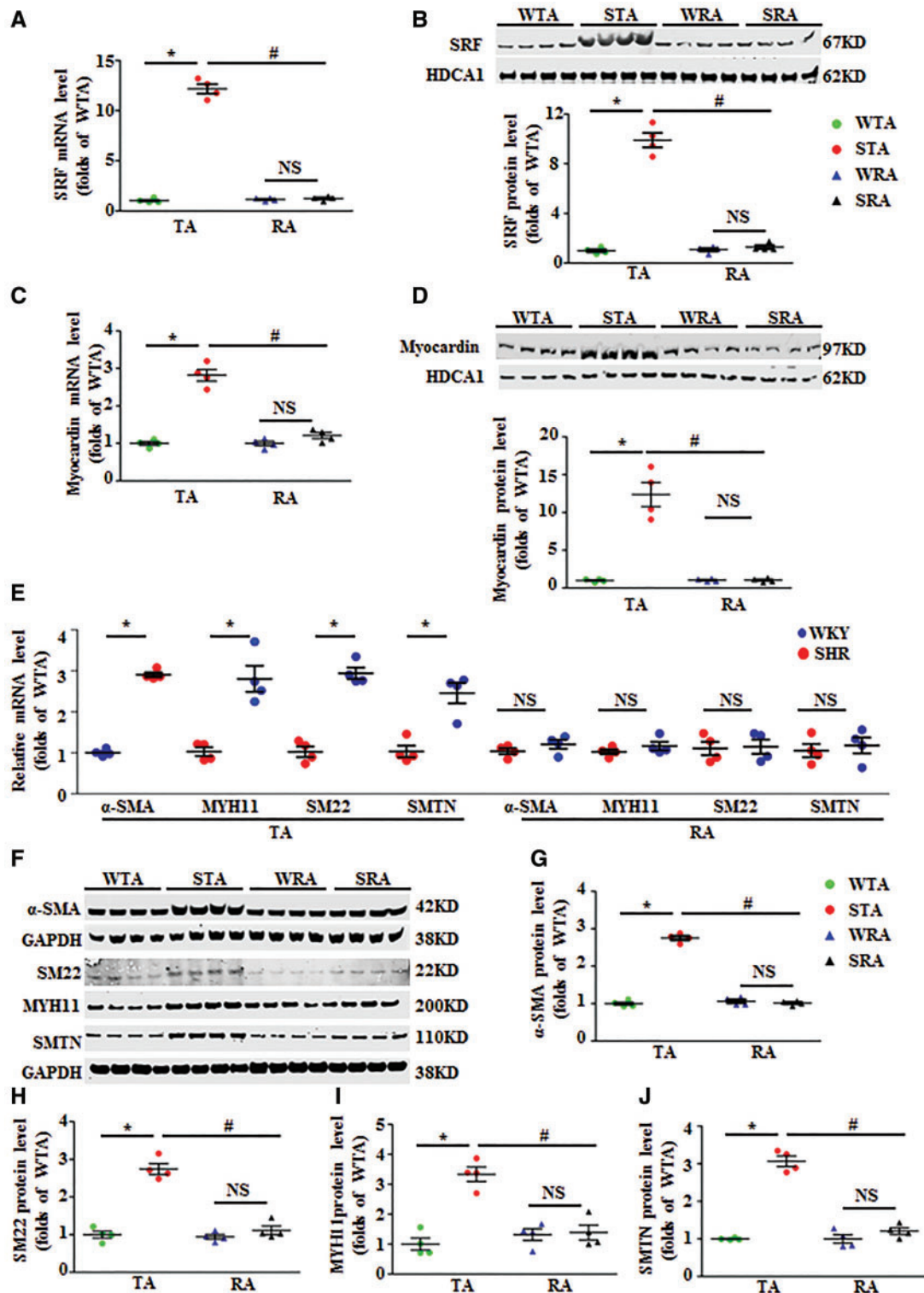


Figure 2 Regional variation of SRF/myocardin signaling in VSMCs from TA and RA. (A–D) The relative mRNA of SRF (A) and of myocardin (C) and their protein levels in the nuclear fraction of VSMCs (B and D). HDAC1 was used as a loading control of nuclear fraction. (E). The relative mRNA levels of SRF/myocardin target genes in VSMCs. α -SMA: alpha-smooth muscle actin; MYH11: myosin heavy chain 11; SM22: smooth muscle 22; SMTN: smoothelin. (F) The western blots of corresponding proteins. GAPDH was used as a loading control. G to J: The level of corresponding proteins in total cell lysis. Data are shown as mean \pm SEM, * P < 0.01 vs. WKY; # P < 0.01 vs. corresponding TA. All, n = 4 rats/group. NS: no significant difference. Two-way ANOVA was used for panels A–J.

3.3 CCG-100602 attenuates the activation of SRF/myocardin in aortic VSMCs specifically from SHR

To test the specific role of SRF/myocardin in VSMCs stiffness, cells isolated from TA were treated with CCG-100602, a specific inhibitor of SRF/myocardin signalling. Under CCG-100602 treatment, the elevated protein levels of SRF and myocardin in SHR TA VSMCs were significantly reduced to levels similar to WKY TA VSMCs (Figure 3A and B), which was further confirmed by immunostaining in individual VSMC isolated from TA (Figure 3C). Notably, similar CCG-100602-induced reduction of SRF and myocardin was not observed in TA VSMCs from WKY rats (Figure 3A–C), indicating a specificity of CCG-100602 for normalizing the increased SRF/myocardin in VSMCs from the thoracic aorta of hypertensive SHR without changing normotensive VSMCs from healthy WKY rats. Moreover, CCG-100602 did not alter SRF and myocardin expression in RA VSMCs from either SHR or WKY rats (Figure 3D and E).

To test whether the suppression of SRF/myocardin in SHR TA VSMCs by CCG-100602 results in a parallel reduction of SRF/myocardin activity, the transcriptions of their downstream target genes were detected by qPCR. Consistent with the alteration of SRF/myocardin expression, the hypertension-related increase of mRNA levels in SRF/myocardin target genes in SHR TA VSMCs, including α -SMA, MYH11, SM22, SMTN, *caldesmon*, and *calponin*, was also abolished by treatment with CCG-100602 compared to untreated cells (Figure 4A and B). Reduced expression of the corresponding proteins by CCG-100602 was also confirmed by western blotting (Figure 4C–I) and by immunostaining (Figure 4J) in cultured SHR TA VSMC. Again, these effects on SRF/myocardin downstream genes were not observed in WKY TA VSMCs under the treatment of CCG-100602 (Figure 4A–J), nor in RA VSMCs from either group of animals (Supplementary material online, Figure S4A–H), supporting the specificity of this intervention.

3.4 Inhibition of SRF/myocardin signalling selectively ameliorates the stiffening not only in VSMCs but also in aortic wall in SHR

To test whether molecular alterations caused by inhibition of SRF/myocardin signalling result in corresponding changes of VSMC mechanical properties, the intrinsic stiffness of individual VSMCs was measured by AFM after treatment with CCG-100602. AFM indentation revealed that CCG-100602 significantly reduced the elevated VSMC E_{ap} in SHR TA without affecting that from WKY TA, which effectively eliminated the disease-associated difference of microelastic properties between SHR and WKY TA VSMCs (Figure 5A).

Based on our observations *in vitro*, we next tested whether a correction of VSMC stiffening by the inhibition of SRF/myocardin results in a decrease of aortic wall stiffness in SHR *in vivo*. CCG-100602 was subcutaneously delivered by osmotic minipumps in both SHR and WKY rats for 2 weeks, and compared to the respective vehicle controls. Aortic wall stiffness was evaluated non-invasively by echocardiography. As shown in Figure 5B–D, aortic wall stiffness quantified by E_p , ASI and E_T was significantly increased in SHR compared to WKY rats at baseline. Treatment with CCG-100602 significantly reduced aortic stiffness in SHR as early as the 7th day after initiating treatment and more so at day 14, in terms of E_p (Figure 5B), ASI (Figure 5C), and E_T (Figure 5D). However, there were no significant effects of CCG-100602 on aortic stiffness of WKY rats, indicating a selective anti-stiffening effect of CCG-100602 in

hypertension. The aortic stiffness was also confirmed using aortic pressure measured by invasive catheter on day 14 post-treatment (Figure 5E–G). These data are consistent with the *in vitro* observations of TA VSMC stiffness (Figure 5A), suggesting an important role of VSMC mechanical properties in aortic wall stiffening in hypertension.

To test whether the reduction of aortic stiffness by CCG-100602 also involves aortic wall remodelling, collagen content and aortic wall thickness were measured by histology. As shown in Figure 5H–J, the medial layer thickness of aortic wall and the ratio of medial thickness to lumen diameter were significantly greater in SHR compared to WKY; however, 2-week treatment with CCG-100602 did not change the wall thickness in SHR nor in WKY rats. The deposition of total collagen was significantly higher in SHR aorta compared to WKY, but there was no difference detected between the CCG-100602 treated group versus vehicle rats in SHR or WKY (Figure 5K and L). These findings indicate no measureable effects of CCG-100602 on aortic wall dimensions or on collagen content during the 2-week term of the treatment.

3.5 Inhibitor of SRF/myocardin signalling selectively reduces blood pressure in SHR

Finally, we tested whether the reduction of aortic stiffness by CCG-100602 resulted in a decrease of blood pressure in SHR. Systemic blood pressure was tracked every 2 days for 2 weeks by tail cuff in conscious animals. As shown in Figure 6A and B, both systolic blood pressure (SBP) and diastolic blood pressure (DBP) were significantly elevated in SHR compared to WKY rats, but were progressively lowered by treatment with CCG-100602, achieving a significant difference as early as the 10th day after initiating treatment. Direct aortic pressure was also detected by an invasive Millar catheter at the end of the 2-week treatment with CCG-100602 or vehicle control (Figure 6C). A significant increase in aortic pressure was observed in untreated SHR compared with normotensive WKY rats, including SAP, DAP, and PP. These elevated blood pressures in SHR were significantly reduced by CCG-100602 at the end of the 2-week treatment period (Figure 6D), consistent with the systemic blood pressures measured by tail cuff. Notably, we found that, compared to vehicle control, treatment with CCG-100602 resulted in a larger reduction in SBP (26.8%) than in DBP (16.5%), yielding a dramatic 48.4% reduction in PP in SHR (Figure 6E). Importantly, consistent with the observations of aortic stiffness (Figure 5B–G), CCG-100602 had no significant effects on systemic or aortic blood pressure in WKY rats.

4. Discussion

Although recent studies demonstrated that the increased intrinsic stiffness in VSMCs from TA is a key contributor to the development of aortic stiffening,^{7–9} the characteristics of VSMC mechanical properties in other arterial regions have not been identified. An important finding in the present study is the heterogeneity of mechanical properties in VSMCs between the large aorta and downstream distal arteries in the SHR disease model, which has not been reported previously. This finding brings to light a new concept that intrinsic VSMC mechanical properties in large arteries (TA), but not in distal arteries (RA), are crucial contributors in the pathogenesis of aortic stiffening in hypertension.

In particular, the apparent elastic modulus of VSMCs isolated from TA increased from about 5 kPa in WKY to about 7 kPa in SHR. This compares to our *in vivo* estimated aortic Young's modulus values of 5 kPa and 15 kPa for WKY and SHR, respectively, with reported elastic modulus

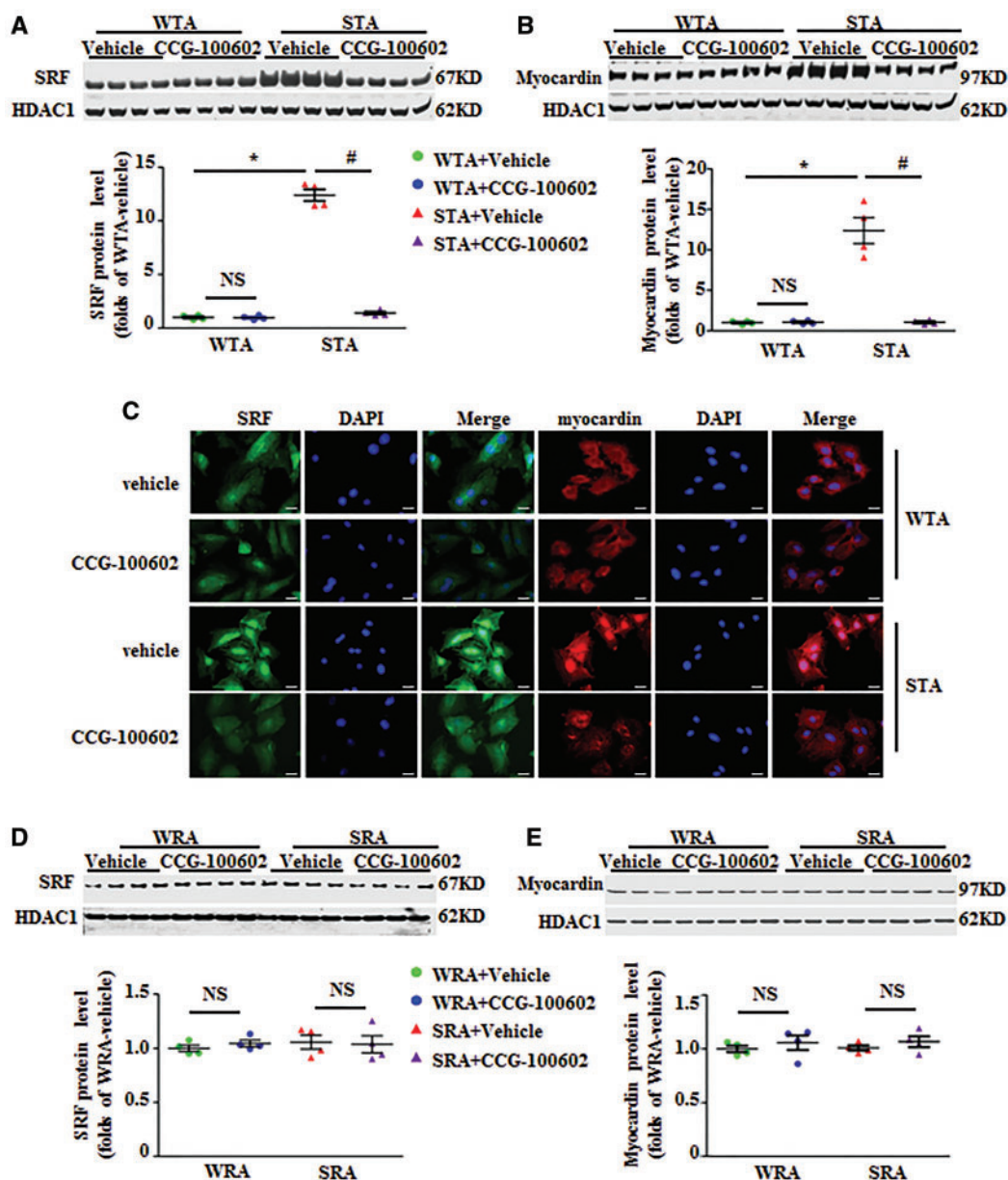


Figure 3 CCG-100602 inhibits the expression of SRF and myocardin in TA VSMCs but not in RA VSMCs from SHR. Protein levels of SRF (A) and myocardin (B) in nuclear fraction of VMSCs from WKY TA (WTA) and SHR TA (STA) upon the treatment of CCG-100602 or vehicle (DMSO). (C) Immunostaining of SRF and myocardin in single VSMC. Scale bar: 50 μ m. (D and E): Protein levels of SRF (D) and myocardin (E) in nuclear fraction in VMSCs from WKY RA (WRA) and SHR RA (SRA) upon the treatment of CCG-100602 or vehicle (DMSO). HDAC1 was used as a loading control of nuclear fraction. Data are shown as mean \pm SEM, * P < 0.01 vs. WTA; # P < 0.01 vs. corresponding vehicle. NS: no significant difference. All, n = 4 rats/group. Two-way ANOVA was used for panels A, B, D, and E.

values in the range of 5–50 kPa for various cardiovascular tissues probed by AFM indentation.²¹ The increase in both cell and tissue stiffness with hypertension supports the usefulness of VSMC intrinsic elastic modulus as an in vitro assay relevant to the pathophysiology. Furthermore, the smaller effect on isolated cell stiffness suggests alterations in VSMC modulus might impact the intact vessel tissue stiffness not only through direct mechanical contributions, but may also involve changes in VSMC mechanobiology. This, combined with the distinct regional variations of intrinsic VSMC mechanical properties in SHR, motivated further mechanistic investigation. Our data revealed that the variation of SRF/

myocardin expression between TA and RA was highly correlated to the heterogeneity of VSMC mechanical properties in SHR, and the resultant alterations of downstream target genes associated with VSMC stiffness. These findings suggested that the altered SRF/myocardin signalling may be responsible for regional differences in VSMC mechanical properties in SHR. SRF is a master transcription factor that regulates smooth muscle genes and is essential for orchestrating actin cytoskeleton and vessel function.^{11,12} SRF regulates the target genes via interactions with tissue-specific transcriptional cofactors, e.g. myocardin, megakaryoblastic leukaemia-1 (MKL1), and MKL2. While MKL1 and 2 are broadly

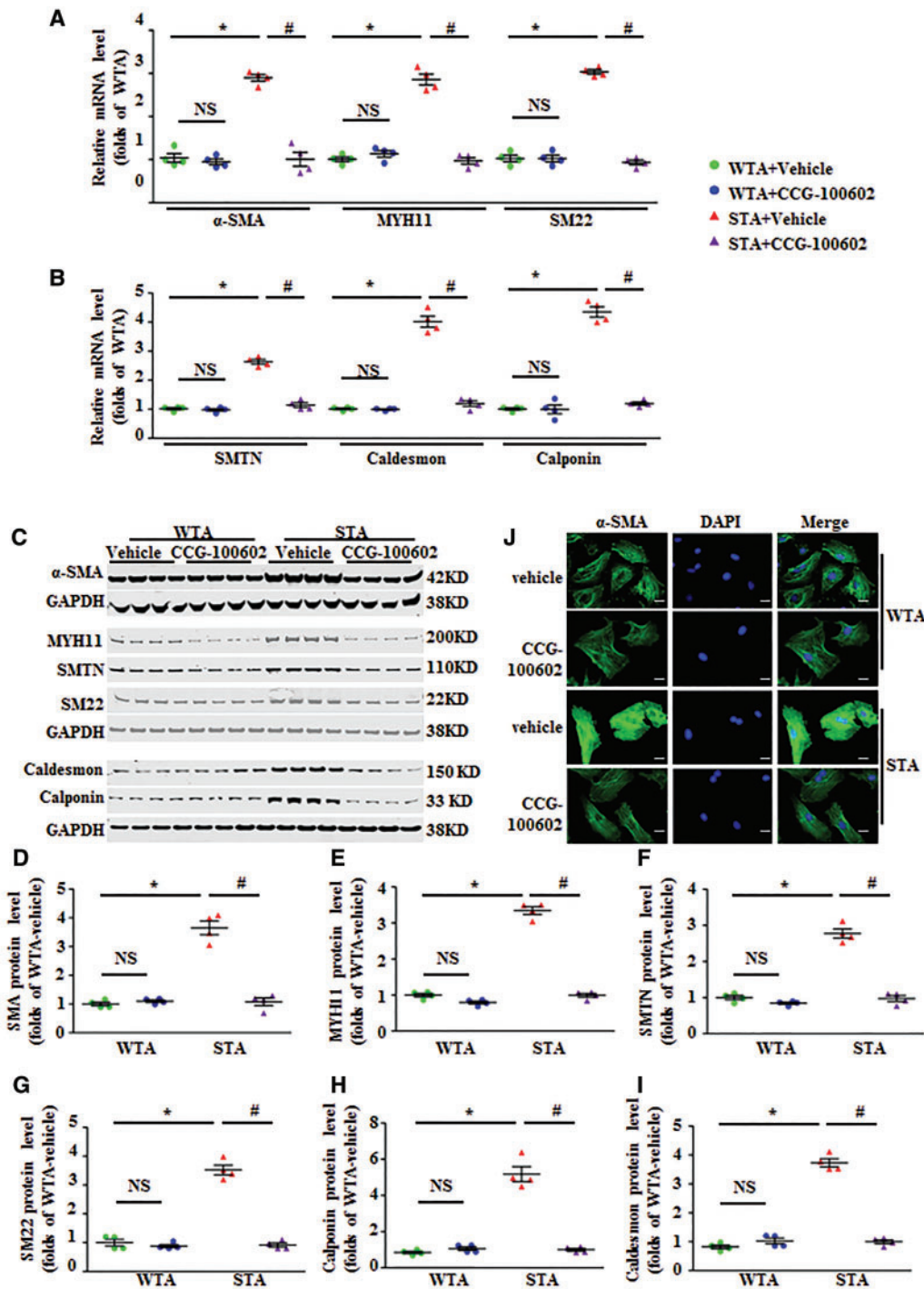


Figure 4 Inhibition of SRF/myocardin attenuates the expression of stiffness-associated proteins in SHR TA VSMCs. (A) and (B): The relative mRNA levels of target genes in VSMCs upon the treatments. (C). Western blots of corresponding proteins. GAPDH was used as a loading control of total cell lysis. D to I: Protein levels of corresponding genes in panel A and B in total cell lysates upon the treatments. Data are shown as mean \pm SEM, * P < 0.01 vs. WTA; # P < 0.01 vs. corresponding vehicle. NS: no significant difference. All, n = 4 rats/group. Two-way ANOVA was used for panels (A, B and D–I). (J) Immunostaining of α -SMA in VSMCs. Scale bar: 50 μ m.

expressed, myocardin is specifically expressed in cardiac and smooth muscle cells.^{12,22} A decreased stiffness in large arteries was recently reported in a mouse with a smooth muscle-specific genetic deletion of SRF.¹³ These studies, together with our findings, suggest that SRF/

myocardin may be involved in the regulation of aortic stiffness in hypertension.

Based on the observed localization of hypertension-related changes in aortic VSMCs, we further investigated the underlying mechanism of

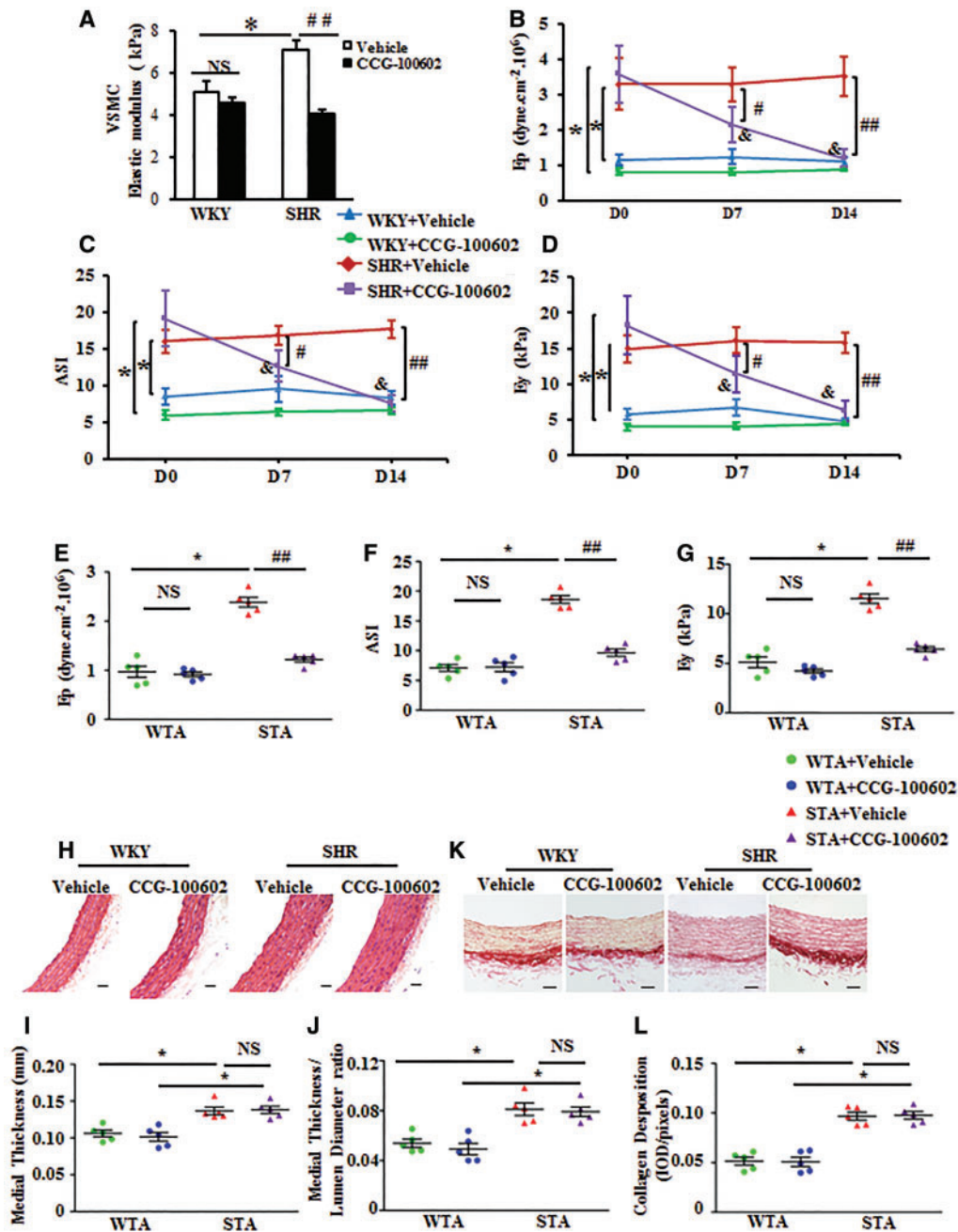


Figure 5 Inhibition of SRF/myocardin signaling selectively ameliorates the stiffening not only in VSMCs but also in aortic wall in SHR. (A) Average apparent elastic modulus (E_{ap}) of TA VSMCs from AFM indentation upon the treatments. $N = 5-12$ cells/group from *four different animals/group*. (B–D) Aortic stiffness in SHR and WKY rats *in vivo* evaluated non-invasively before (D0) or at day 7 (D7) and day 14 (D14) after the initiation of the treatments, reflected by the change of pressure-strain modulus (E_p) (B), arterial stiffness index (ASI) (C) and Young's modulus (E_y) (D). (E–G) Aortic stiffness in SHR and WKY rats *in vivo* evaluated by aortic pressure at day 14 after the initiation of the treatments. (H–J) Thickness of medial layer of aortic wall and the ratio of lumen diameter. Scale bar: 50 μm . (K–L) Collagen deposition in medial layer of aortic wall, quantified in terms of integrated optical density (IOD). Scale bar: 50 μm . $n = 5$ rats/group for panels B to G, I to J and L. H and K was one example from *five animals/group*. Data are shown as mean \pm SEM. * $P < 0.01$ vs. corresponding WKY; # $P < 0.05$, ## $P < 0.01$ vs. corresponding vehicle. NS: no significant difference. Two-way ANOVA test was used for panels A–G, I, J, and L for group comparisons. Repeated measure ANOVA test was used for panels B–D for comparison among time points. & $P < 0.01$ vs. D0.

VSMC stiffening with a focus on the TA of SHR. Our findings establish a link between activation of SRF/myocardin signalling and the increase of cell elastic modulus in VSMCs from the stiffened aorta. We observed not only a significant increase of both SRF and myocardin expression in

the stiffer SHR TA VSMCs, but also a remarkable increase of SRF activity as evidenced by increased reprogramming of its downstream target genes. Moreover, we found that inhibition of SRF/myocardin with a specific inhibitor, CCG-100602, not only dramatically reduced SRF/

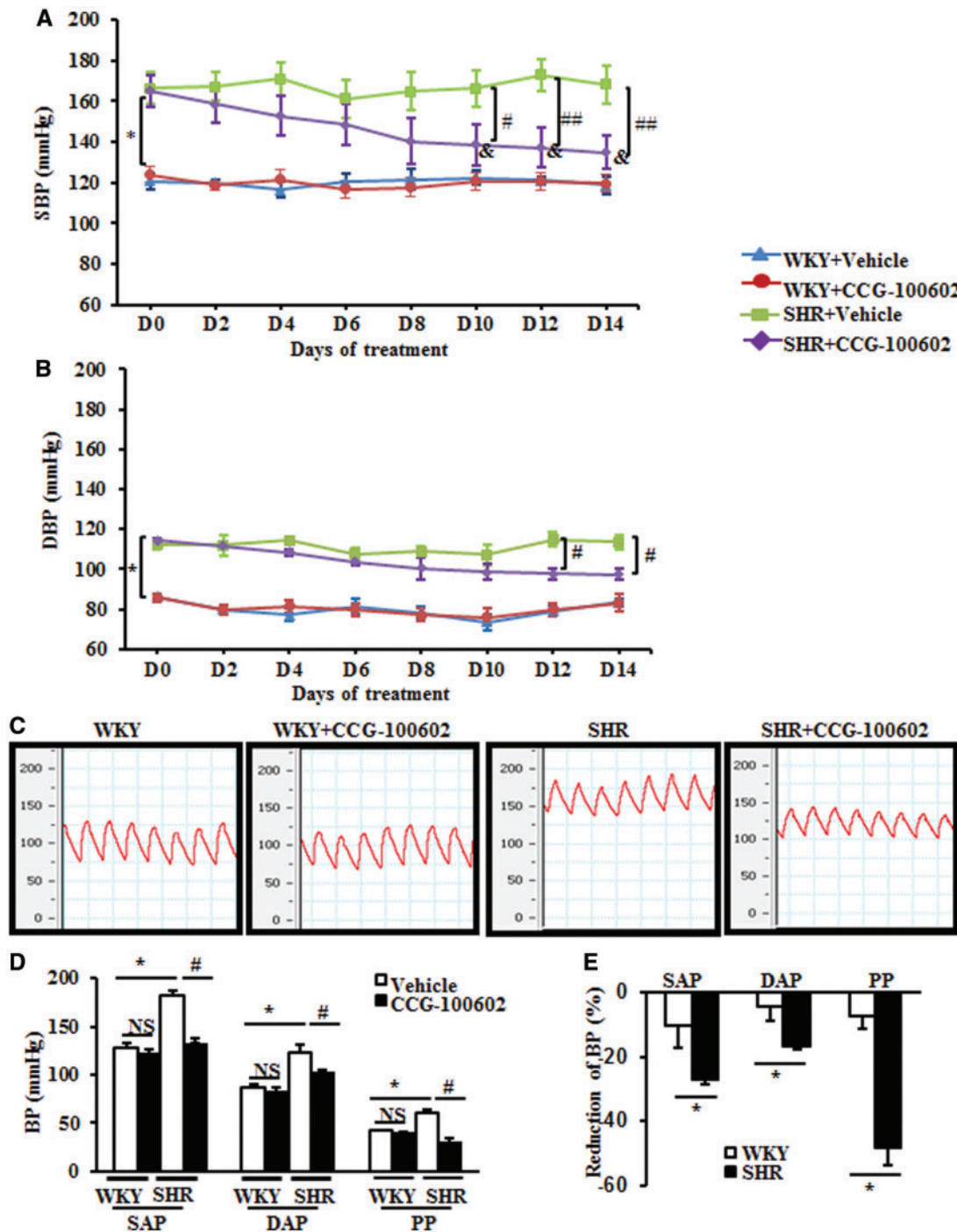


Figure 6 Inhibitor of SRF/myocardin signaling selectively reduces blood pressure in SHR. (A and B) Time dependent changes of systemic blood pressure SBP (A) and DBP (B) at conscious status. (C) Representative waveform of aortic blood pressure by a Millar catheter. (D) Aortic blood pressure after two week treatments. (E). Reduction rates of blood pressures vs. vehicle control. $n = 5$ rats/group for A, B, D and E. C was an example from five animals/group. Data are shown as mean \pm SEM, * $P < 0.01$ vs. corresponding WKY; # $P < 0.05$, ## $P < 0.01$ vs. corresponding vehicle. NS: no significance. Two-way ANOVA was used for panels A, B, and D, and one-way ANOVA was used for panel E. Repeated measure ANOVA test was used for panels A and B for comparison among time points. $^{\&}P < 0.05$ vs. D0.

myocardin activity in aortic VSMCs from SHR, attenuating the transcription of its downstream stiffness-associated genes, but also significantly decreased the intrinsic stiffness of VSMCs from SHR TA. CCG-100602 is a second-generation of specific SRF/myocardin inhibitors developed

for improved selectivity, potency, and attenuated cytotoxicity relative to its parent compound CCG-1423.^{13,23,24} The site of inhibition in the pathway by CCG-100602 is not precisely defined, yet its inhibition on RhoA/C-mediated SRF-driven luciferase expression in PC-3 prostate

cancer cells has been reported.^{25,26} A recent study also showed that CCG-100602 dramatically repressed both matrix stiffness and transforming growth factor beta-mediated fibrogenesis.²³ In the present study, we showed a new effect of CCG-100602 on the suppression of VSMC stiffening selectively in the TA of SHR but not in WKY cells nor in RA VSMCs, indicating a unique role in regulating cellular mechanical properties in hypertension. Our cumulative findings suggest SRF/myocardin signalling acts as a crucial mediator of VSMC stiffening in SHR TA.

Furthermore, this study also demonstrated that the reduction of VSMC stiffness serves as a promising therapeutic target to correct aortic stiffening and high blood pressure in hypertension. It has been shown that increased VSMC stiffness is a major contributor to aortic stiffening;^{7,8} however, there is no previous direct evidence that intervention to reduce VSMC stiffness can also result in a decrease of aortic stiffness or blood pressure *in vivo*. Our data showed that inhibition of SRF/myocardin by CCG-100602 not only reduced the VSMC stiffness *in vitro*, but also reduced aortic wall stiffness selectively in SHR but not in WKY rats. Aortic wall stiffness can also be influenced by remodelling of the wall structure, such as collagen content and wall thickness.⁵ Indeed, the observed increases in medial thickness and thickness/diameter ratio in unloaded aortas from SHR rats by histology (Figure 5H–J) are consistent with a remodelling response that would act to normalize aortic wall stress under the elevated hemodynamic load measured by echography *in vivo*. However, our data demonstrated that short-term treatment with CCG-100602 did not alter these structural parameters, suggesting they did not play a major role in causing the observed reduction of aortic stiffening in SHR TA. Thus, the reduction of aortic VSMC stiffening by CCG-100602 is thought to be a likely contributor to the correction of aortic vessel stiffness in this model of hypertension.

Although the association between aortic stiffness and hypertension is well established, whether aortic stiffening is a cause or a consequence of hypertension remains controversial. It has been shown in hypertensive patients and animal models that aortic stiffening precedes a reduction of nitric oxide function and an increase in blood pressure.^{27–29} These findings strongly suggested that targeting the reduction of aortic stiffness might serve as a preventative strategy for the development of hypertension and its attendant complications. Our data showed that inhibition of SRF/myocardin signalling by CCG-100602 not only reduced aortic stiffness but also significantly lowered blood pressure in SHRs. While changes in BP can confound measurements of vessel stiffness index, however, our data showed that aortic stiffness was significantly lower in inhibitor-treated SHRs compared to vehicle control SHRs by day 7 post-treatment even though BP was not statistically different between the two groups until at least day 10. Thus, the aortic stiffness reduction preceded the reduction of blood pressure in SHR, suggesting the recovery of aortic stiffness in CCG-treated vessels is not likely an artefact of the reduction in blood pressure, and may even be a contributing factor. In addition, inhibition of SRF/myocardin induced an earlier and greater reduction in SBP than DBP compared to vehicle control in SHR; this disproportionate reduction in blood pressure in our model also supports that aortic stiffness is a primary target of the inhibition of SRF/myocardin signalling.

Importantly, CCG-100602 treatment selectively corrected pathological alterations in the hypertensive aorta, without adversely affecting the normotensive control rats, suggesting the off-target effects for treating hypertension may be minimal. Thus, selective inhibition of SRF/myocardin signalling, e.g. by CCG-100602 treatment, provides a new avenue for the development of safer therapies for hypertension. Despite the phenomenon of selective effects on disease condition also being

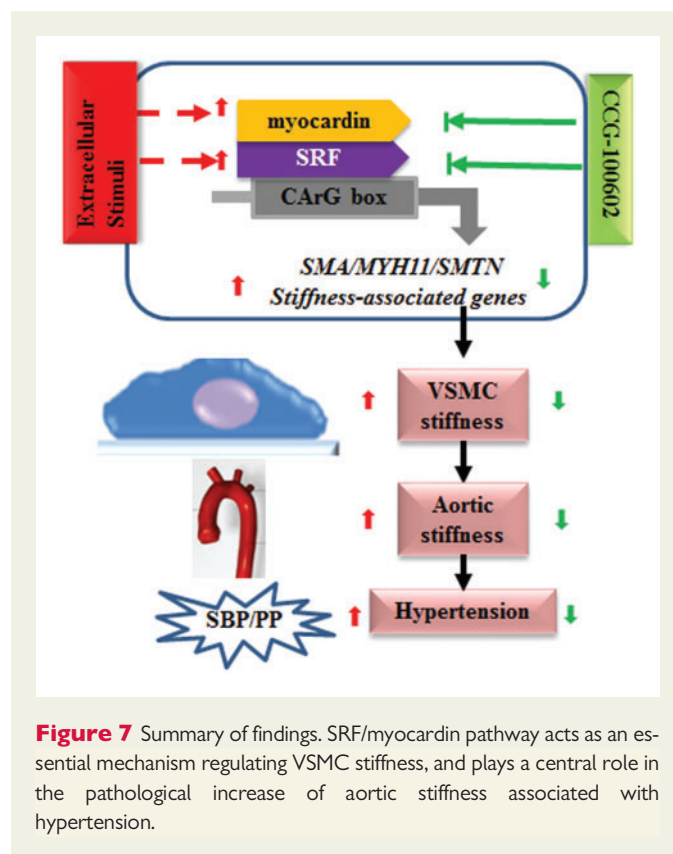


Figure 7 Summary of findings. SRF/myocardin pathway acts as an essential mechanism regulating VSMC stiffness, and plays a central role in the pathological increase of aortic stiffness associated with hypertension.

observed in other studies using different drugs,^{30–32} the mechanism is not clear yet. One possible explanation is that under the normal condition like in WKY rats, the regulators, e.g. endogenous activator or inhibitor, that govern the expression and activity of SRF remain balanced at a low level and in a stable status, which results in a desensitization to exogenous inhibitor (CCG-100602). Under diseased conditions like hypertension, the activator of SRF in VSMCs becomes stimulated, resulting in an imbalance between activator and inhibitor, which dramatically increases the binding affinity to exogenous inhibitor. Another possibility is that the regulatory mechanism of SRF in the pathological state differs from that in physiological conditions. Our results will stimulate further investigation for a detailed mechanistic understanding of selective effects of the inhibitor and the role of the SRF/myocardin pathway in both physiological and pathological conditions. Furthermore, substrate properties are known to impact cell function, and VSMC stiffness may be altered when cells are cultured on materials with different mechanical, structural or molecular attributes, which represents an interesting topic for future investigations.

In summary, as illustrated in Figure 7, our study demonstrates that the SRF/myocardin pathway acts as an essential mechanism regulating VSMC stiffness, and plays a central role in the pathological increase of aortic stiffness associated with hypertension. Pharmacological inhibition of this pathway by CCG-100602 specifically rectifies the increased intrinsic stiffness of isolated VSMCs and intact aorta in the SHR model, with negligible effects on normotensive controls or smaller muscular arteries, thus providing a potentially attractive target for pharmacological treatment of hypertension. This study also has implications for developing novel therapies for aortic stiffness in aging and debilitating age-related vascular diseases, such as obesity, diabetes mellitus, and kidney disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

Funding

This work was supported by the National Institutes of Health (1R01 HL115195-01 to H.Q., S10 RR027609 to K.D.C.); and the National Natural Science Fund of China (81100087 and 81570261 to N.Z).

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