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Increased vascular smooth muscle cell stiffness: a novel mechanism for aortic stiffness in hypertension

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1Department of Cell Biology and Molecular Medicine, New Jersey Medical School, Rutgers Biomedical and Health Sciences, Newark, New Jersey; 2Department of Biomedical Engineering, New Jersey Institute of Technology, Newark, New Jersey; and 3Dalton Cardiovascular Research Center and Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, Missouri; and 4Department of Medical Physiology, Texas A&M University, College Station, Texas

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Sehgel NL, Zhu Y, Sun Z, Trzeciakowski JP, Hong Z, Hunter WC, Vatner DE, Meininger GA, Vatner SF. Increased vascular smooth muscle cell stiffness: a novel mechanism for aortic stiffness in hypertension. Am J Physiol Heart Circ Physiol 305: H1281–H1287, 2013. First published May 24, 2013; doi:10.1152/ajpheart.00232.2013.—Increased vascular stiffness is fundamental to hypertension, and its complications, including atherosclerosis, suggest that therapy should also be directed at vascular stiffness, rather than just the regulation of peripheral vascular resistance. It is currently held that the underlying mechanisms of vascular stiffness in hypertension only involve the extracellular matrix and endothelium. We hypothesized that increased large-artery stiffness in hypertension is partly due to intrinsic mechanical properties of vascular smooth muscle cells. After confirming increased arterial pressure and aortic stiffness in spontaneously hypertensive rats, we found increased elastic stiffness of aortic smooth muscle cells of spontaneously hypertensive rats compared with Wistar-Kyoto normotensive controls using both an engineered aortic tissue model and atomic force microscopy nanoin indentation. Additionally, we observed different temporal oscillations in the stiffness of vascular smooth muscle cells derived from hypertensive and control rats, suggesting that a dynamic component to cellular elastic stiffness is altered in hypertension. Treatment with inhibitors of vascular smooth muscle cell cytoskeletal proteins reduced vascular smooth muscle cell stiffness from hypertensive and control rats, suggesting their participation in the mechanism. This is the first study demonstrating that stiffness of individual vascular smooth muscle cells mediates vascular stiffness in hypertension, a novel concept, which may elucidate new therapies for hypertension and for vascular stiffness.

Hypertension; vascular stiffness; vascular smooth muscle; atomic force microscopy; pulse wave velocity

Hypertension affects 30% of the adult population in the U.S. (8a) and is responsible for a significant fraction of death and disability from its sequelae, e.g., heart failure, myocardial infarction, stroke, aortic aneurysm, chronic renal disease, and also increased atherosclerosis. Although therapeutic progress has been made over the past several decades, hypertension remains a major health problem. It is well recognized that increased arterial stiffness is sine qua non in the development of systemic hypertension (19, 23), and this by itself amplifies the pathogenesis of hypertension (by increasing the penetration of pressure pulsatility throughout the cardiovascular system) (28). Additionally, based on the results of recent Framingham studies (17), it has been suggested that aortic stiffness precedes hypertension and that it is also an important factor in the development of atherosclerosis. Controlling arterial stiffness has been suggested as a new strategy for pharmacological treatments of hypertension (1, 8, 26, 29, 34).

An understanding of the causes of aortic stiffness is integral to this strategy. Prior studies on vascular stiffness in hypertension have focused on endothelial (27, 31, 33) or extracellular matrix (6, 11, 15, 20) mechanisms. However, less is known about the direct effects of hypertension on the mechanical properties of individual vascular smooth muscle cells (VSMCs). VSMCs are one of the main constituents of the arterial medial layer and the principal targets of pharmacological treatment of hypertension. Previous studies have found hypertensive-induced changes of VSMC orientation and force generation in tissue preparations (2, 3), as well as VSMC proliferation (13), and have associated these with the increase in arterial stiffness (18, 32).

The goal of the present investigation was to test the hypothesis that the intrinsic stiffness of VSMCs themselves contributes to the overall vascular stiffness. There is no direct evidence available to address the contribution of the stiffness of specifically VSMCs to the development of either vascular stiffness or hypertension. One barrier to studying this in hypertension is that the contribution of isolated VSMCs to increased stiffness cannot be measured in intact tissue, in vivo or ex vivo. In the present study we employed two unique techniques, atomic force microscopy (AFM) (38) and a reconstituted aortic tissue model (25), to measure the stiffness of aortic VSMC in vitro. We investigated the potential contribution of increased VSMC stiffness in hypertension and initiated studies into the underlying cellular and molecular mechanisms mediating these changes. Our studies in spontaneously hypertensive rats (SHRs) revealed not only that aortic VSMC stiffness increases but also that it is dynamic and that oscillations in cell stiffness differed in SHRs compared with normotensive Wistar-Kyoto (WKY) rats. Moreover, we found that VSMC stiffness is regulated by cytoskeletal proteins.

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MATERIALS AND METHODS

Animal model. Adult (16–18 wk old) male SHRs and their normotensive controls WKY rats were studied. All animal procedures were done under the approval of the Institutional Animal Care and Use Committee of Rutgers Biomedical and Health Sciences.

Blood pressure measurements. Blood pressure was measured in the conscious state by restraint tail cuff every two days for 1 to 2 wk for the two experimental groups and found to be elevated in SHRs (data not shown). This was confirmed by direct blood pressure measurement (n = 4/group) in the descending thoracic aorta by Millar catheter, after prior intramuscular injection of a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg) anesthetic.

Aortic stiffness measurements in vivo. While under ketamine and xylazine anesthesia, in vivo aortic stiffness was determined by a pulse wave velocity (PWV) technique (5) and measured locally in the descending thoracic aorta by Doppler ultrasound echocardiography. The time between two consecutive Doppler pulses (as demarcated by end-diastolic points on simultaneous EKG recordings) was measured. This was done at proximal and distal points in the descending thoracic aorta of a measured distance apart (Δdistance). The PWV was computed from the following formula: PWV = Δdistance/Δt, where Δt is the difference in propagation time of blood flow between the distal and proximal points in the descending thoracic aorta, as measured by pulse-wave Doppler.

Aortic stiffness measurements ex vivo. Animals were given lethal intraperitoneal injections of pentobarbital sodium (40–60 mg/kg) and euthanized. Aortic ring segments were dissected from the descending thoracic aorta and immersed in ice-cold PBS (0.01 M phosphate and 0.154 M NaCl). First, the ring segments were denuded of the endothelial layer by rubbing the intimal surface with a wire. The ring segments were then subjected to uniaxial tensile stretching after mounting onto wires connected to an isometric force transducer (model 52-9545, Harvard Apparatus, South Natick, MA), to produce stepwise stretches from 2.5–20.0% of their original resting length. The force responses of this series of stress-relaxation tests (2 min each) were recorded using a data acquisition system (NOTOCORD Systems SAS, Croissy-sur-Seine, France). For each stretch, the average baseline and steady-state force values were determined using proprietary software developed in MATLAB (version 7.10.0). The ex vivo aortic stiffness (E) was determined from circumferential stress in the ring segment, and this was calculated from the baseline difference in steady-state force (F) at each stretch level and the cross-sectional area (A) of the ring segment. This was computed based on the formula, \( E = F/L(A\Delta L) \), where \( L \) is the original length of the tissue and \( A\Delta L \) is the stretched length of the tissue. A stress-strain plot was generated from these experiments and used to compute the tangential elastic stiffness from the slope of the curve.

VSMC stiffness measured by the reconstituted tissue model. VSMCs were isolated from the descending thoracic aorta of SHRs and WKY (n = 4/group) rats using enzymatic digestion, as previously described (30). These isolated cells were serially cultured for up to three passages. The primary reason for engineering aortic tissues with VSMCs was isolated from the thoracic aorta and used for AFM experiments. After isolation, they were plated onto glass-bottomed cell culture dishes, and VSMC stiffness was determined by an AFM using a nanoindentation protocol. VSMCs were individually probed through an indentation depth over a range of 100–300 nm in the cortical region using AFM (BioScope), which was coupled to a confocal microscope. Force curves were continuously collected for 2-min durations for several cells to determine the mean VSMC stiffness for each group of cells. The force curves were analyzed using proprietary software NFForceR (registration number TX1-328-659) and MATLAB. The VSMC elastic stiffness was computed from these force curves using a modified Hertz model (9), where the indentation force is computed from Hooke’s law, as previously described (38). The mean of these elastic stiffness values was computed for each cell and then averaged together for each group.

Dynamic stiffness in single VSMC measurement by AFM. VSMCs were also subjected to AFM nanoindentation for 30-min duration to examine the temporal characteristics of the cell stiffness. Force curves were continuously collected during this period and used to determine elastic stiffness, as described above. Time-dependent oscillations in the computed elastic stiffness were observed. These oscillatory stiffness signals were analyzed by Eigenvalue decomposition, and the three major components of the oscillation were isolated and identified, as previously described (38). The frequency, amplitude, and phase (data not shown) characteristics of these three wave components were determined and compared.

VSMC contractile and stiffness response to vasoactive stimulation. In separate experiments, VSMCs were subjected to topographical scanning by AFM. The cell height of a single line scan over the cell nucleus was recorded. The concentration response of cell height and VSMC stiffness to treatment of angiotensin II (10⁻⁹–10⁻⁶ M) was measured.

Protein extraction and Western blot analysis. Isolated VSMCs were homogenized at 4°C in a lysis buffer. Protein extracts were denatured and resolved on SDS-PAGE gels. α-Actin, myosin light chain, phosphorylated myosin light chain, and myosin light chain kinase (MLCK) levels were assayed using specific antibodies, as previously described (25).

Response of VSMC stiffness to pharmacological inhibitors. VSMCs were treated with 10 μM of a highly specific inhibitor of myosin II phosphorylation by MLCK. The mean stiffness of VSMCs was determined by AFM before and after treatment with these pharmacological inhibitors following a 30-min incubation time.

Statistical analysis. Results are presented as means ± SE. The Mann-Whitney U-test was used for two-group comparisons between SHRs and WKY rats. Subsequent parametric statistical tests also achieved significance for each group tested. Comparisons of frequencies and amplitudes were based on the transformed values (i.e., periods and log amplitudes). Multigroup comparisons were done using the Kruskal-Wallis test, followed by post hoc-independent samples t-tests using a Bonferroni correction. For the vasoactive stimulation experiments, dose-response assessment was made by repeated-measures ANOVA, followed by post hoc-paired t-tests using a Bonferroni correction, for both WKY and SHR groups.
tions were done using SPSS 20.0 software. A value of \( P < 0.05 \) was considered significant.

RESULTS

Blood pressure and aortic stiffness are increased in SHRs. Systolic and diastolic aortic pressures, as well as mean arterial pressure and pulse pressure, were found to be elevated in SHRs, compared with WKY rats (Fig. 1A). Additionally, PWV was increased by more than twofold in SHRs (Fig. 1B). Since arterial elastic stiffness is known to be dependent on blood pressure as well as affected by the endothelium in vivo, we also measured aortic stiffness ex vivo in an unpressurized state and free of endothelial cell contribution. Wall stress in the aortic ring segments was computed over a range of strain values, and the mean tangential elastic stiffness, or elastic modulus, was calculated from the relationship between these two measures. Overall, ex vivo aortic wall stress was increased at each strain value by more than 1.5-fold, as well as the tangential elastic stiffness by greater than 1.4-fold in SHRs (Fig. 1C). This indicates that aortic wall stiffness is increased in SHRs, even when the mechanical strains are equivalent. No significant differences in intimal-medial thickness or wall thickness-to-lumen ratio were found. Altogether, these results confirmed that aortic stiffness is increased in hypertension both in vivo and ex vivo, and it is independent of blood pressure and endothelium.

VSMC stiffness is increased in SHRs. To assess how VSMCs may contribute to aortic stiffness, independent of extracellular matrix changes and hyperplasia often found in hypertension, we engineered a reconstituted aortic tissue model (25), whereby the cell density and ECM protein content were controlled. This was measured for the total reconstituted tissue construct and also after treatment with CD, which thereby effectively eliminated the cellular component of stiffness. Both the total tissue stiffness and the cellular contribution were elevated in SHRs (Fig. 2, A–D) by 1.5-fold and greater than fourfold, respectively. These data indicate that the VSMC stiffness contribution to tissue stiffness is increased in hypertension.

Furthermore, we measured the stiffness of individual VSMCs by AFM with a nanoindentation protocol. VSMC stiffness was found to be increased in aortic VSMCs from SHRs compared with WKY rats (Fig. 2, E and F) by twofold. Therefore, increased stiffness in VSMCs was confirmed at both of tissue and single cell levels.

![Fig. 1](http://ajpheart.physiology.org/)

**Fig. 1.** Arterial pressure and aortic stiffness are increased in spontaneously hypertensive rats (SHRs) compared with Wistar-Kyoto (WKY) rats. **A:** arterial pressure in WKY and SHRs. SBP, systolic aortic blood pressure; DBP, diastolic aortic blood pressure; MBP, mean aortic blood pressure; PP, pulse pressure. **B:** in vivo aortic stiffness, determined by pulse wave velocity, in WKY and SHRs. **C:** ex vivo aortic stiffness, determined from stress and strain measurements during tensile testing of aortic ring segments. Tangential elastic stiffness is shown. *\( P < 0.05 \); **\( P < 0.01 \). Data are given as means \( \pm \) SE; \( n = 4 \) per group.

![Fig. 2](http://ajpheart.physiology.org/)

**Fig. 2.** Vascular smooth muscle cell (VSMC) stiffness is increased in SHRs compared with WKY rats. An example of the force recording generated from the reconstituted tissue model before and after cytochalasin D (CD) treatment using WKY (A) and SHR (B) VSMCs. **C:** average stiffness of reconstituted aortic tissues using VSMCs from WKY and SHRs (\( n = 7 \) for each group). **D:** cellular stiffness, determined after treatment with CD, for WKY and SHRs. **E:** distribution of force as a function of indentation in WKY and SHR VSMCs measured by atomic force microscopy. **F:** computed elastic stiffness of individual VSMCs, as determined by atomic force microscopy measurements, in WKY and SHRs (\( n = 30 \) from 4 rats for each group). Data are given as means \( \pm \) SE. *\( P < 0.05 \).
Dynamic changes in VSMC stiffness. Prolonged, continuous measurement of elasticity in single VSMCs revealed dynamic temporal variations in cell stiffness, as shown for representative waveforms from SHRs and WKY rats (Fig. 3A). Spectral analysis showed frequency was generally lower and amplitude greater in SHRs, compared with WKY rats, as shown for a representative case (Fig. 3B). The frequency of the first wave component was found to be significantly decreased in SHRs (Fig. 3C). Additionally, the amplitudes of the each of the three wave components were increased in SHRs (Fig. 3D). Altogether, these data indicate a general pattern of slower, larger oscillations in SHRs and faster, smaller oscillations in WKY rats. Furthermore, these observations uniquely suggest that there are dynamic cytoskeletal mechanisms occurring in the cell cortex that underlie differences in VSMC stiffness and that these mechanisms are inherently altered in SHRs, compared with WKY rats. These data demonstrate that the contribution of VSMC to the vascular stiffness may be caused by novel dynamic elements.

Contractile proteins mediate VSMC stiffness. We sought to determine the molecular mechanisms underlying altered VSMC stiffness. We investigated whether mediators of the VSMC contractile process would have effects on VSMC stiffness. We measured the actin and phosphorylated myosin protein content in VSMCs from SHRs and WKY rats by Western blot analysis. Expression of actin, phosphorylated myosin light chain, and MLCK were found to be increased in SHRs, compared with WKY rats (Fig. 4, A–C). No significant difference was found in the expression of basal unphosphorylated myosin light chain (Fig. 4D). Treatment with a MLCK inhibitor ML-7 dramatically decreased baseline VSMC stiffness, greater than fourfold and sevenfold in WKY and SHRs, respectively, and even eliminated the difference between WKY and SHRs (Fig. 4E). Additionally, treatment with CD decisively reduced VSMC stiffness by greater than 10- and 23-fold in WKY and SHRs, respectively, and also eliminated the difference between SHRs and WKY rats (Fig. 4E). This suggests that cytoskeletal proteins involved in the VSMC contraction, the cross-bridging components myosin and actin and/or cytoskeletal remodeling, partially determine the increase in VSMC stiffness.

Contractile response of VSMCs. To assess a contractile response of the VSMC, we treated VSMCs with angiotensin II and observed an increase in cell height from single AFM line scans over the cell from cell edge to cell edge spanning the central/nuclear region of the cell. The cell height was recorded from these line scans using contact mode AFM topography scanning. Representative traces of the concentration-response relationship of cell height to angiotensin II stimulation showed that VSMC height progressively increased with increasing concentrations of angiotensin II (Fig. 5A). The mean cell height was significantly increased in both WKY and SHR groups of VSMCs (Fig. 5B). Correspondingly, in separate experiments, we also found that VSMC stiffness was also increased in a concentration-dependent manner in response to angiotensin II stimulation (Fig. 5C).

Fig. 3. Real-time oscillations in VSMC stiffness from SHRs and WKY rats. A: oscillations were detected after prolonged measurement of VSMC stiffness in both SHRs and WKY rats, as shown for representative cases. B: spectral analysis of these oscillations showed that frequency was generally increased, whereas frequency was generally decreased in SHRs compared with WKY rats, as shown for representative cases. C: mean frequency was significantly decreased (first component). D: amplitude was significantly increased (all 3 components) in SHRs. *P < 0.05 and **P < 0.01, compared with WKY rats in post hoc comparisons. Column graph data are given as means ± SE.
DISCUSSION

Our studies in SHRs have found increased elastic stiffness of VSMCs that occurs in accordance with increased overall aortic stiffness. Our findings are based on the results of two in vitro methods. The strength of the engineered tissue model is that it allows us to directly assess a metric related to cell stiffness separate from the stiffness of the matrix, and it is a three-dimensional culture model as opposed to a two-dimensional system. In the second method, we determined the stiffness of individual VSMCs by using an AFM nanoindentation protocol. This method is highly sensitive, and measurements can be made on individual cells. The AFM indents the cell 200–400 nm and thus primarily represents a measurement of local cell cortical stiffness. The stiffness in this region is likely highly influenced by actin stress fibers and cell cortical cytoskeletal structures underlying the cell membrane. With this method we were able to detect temporal oscillations in VSMC stiffness in both SHRs and WKY rats. These findings suggest an additional novel finding that there are dynamic components to this increased stiffness, which are altered in hypertension. The stiffness is affected by inhibitors of proteins related to contractile function in VSMCs (e.g., actin, MLCK), which is further supported by the increased expression of these proteins that were found in SHRs, compared with WKY rats, suggesting that contractile and cytoskeletal proteins play an important role in determining VSMC stiffness. This may be driven by the increased expression of actin, as well as MLCK, thereby also increasing the amount of phosphorylation of MLC. It will be an important future direction to further understand the cell signaling mechanisms underlying VSMC stiffness.

Fig. 4. Cytoskeletal protein inhibitors alter mean VSMC stiffness. Expression of cytoskeletal proteins actin (A), phosphorylated myosin light chain (p-MLC; B), myosin light chain kinase (MLCK; C), and MLC (D) in SHRs, compared with WKY rats. E: inhibition targeted at contractile response elements [actin with CD, and MLCK with 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7)] eliminated the baseline VSMC stiffness difference between SHRs and WKY rats. *P < 0.05 and **P < 0.01, compared with WKY rats. Data are from 4 rats per group and are given as means ± SE.

Fig. 5. Contractile response to vasoactive stimulation. A: cell height was found to progressively increase in response to vasoactive stimulation by angiotensin II, as shown for representative cases. B: mean changes in height were increased in groups of WKY and SHR cells (n = 12 cells/group). C: mean VSMC stiffness in both WKY and SHRs (n = 12 cells/group) was also found to be progressively increase under this stimulation. *P < 0.05, *P < 0.05, **P < 0.01, compared with untreated controls of the same groups in post hoc comparisons. Column data are represented as means ± SE.
Our findings of increased VSMC stiffness may be a consequence of either the higher blood pressure or an innately increased vascular stiffness found in the SHRs, if not both. An important future consideration will be to distinguish between these contributions by examination of other models of hypertension. We note that our values of VSMC stiffness derived from the normotensive WKY rats were comparable with those found for the Sprague-Dawley rat (10 kPa) (14), and Fisher rat (15 kPa). Additionally, determining the temporal sequence of the stiffness changes in the vasculature and individual cells as hypertension develops and prevails during aging may be provide further insight into the underlying mechanism and its contributions to the disease process. Finally, it will be of interest to know whether VSMC stiffness also changes in the resistance arteries during hypertension.

There are always limitations to experiments conducted in vitro. To reduce the potential impact of these limitations, we used two in vitro methods that corroborated each other and also the in vivo data. In the first in vitro method, the mechanical contribution of VSMCs was determined in an engineered reconstituted aortic tissue. One limitation of in vitro work is the use of passaged aortic smooth muscle cells for the engineered aortic tissue model. The primary reason for engineering the aortic tissue with cultured cells, as opposed to primary cells, is because of the high cell density needed for the tissues (1 million per ml of collagen tissue gel). Moreover, this also gave us better control over the type and uniformity of the cells we were adding to the tissue gel. It is important to note that despite the use of passaged cells, the engineered tissues using cells from SHRs were significantly different from the tissues generated using the WKY cells.

A potential concern for the assessment of VSMC stiffness in the AFM experiments is that the cells may not exhibit a fully contractile phenotype identical to the in vivo state. We used primary cells, following an overnight incubation, to allow the cells to attach to the substrate and adhere. They were not dividing at this point. Previously, Hong et al. (14) had found that primary cells isolated and handled in this fashion continue to demonstrate contractile responses to agonists such as angiotensin II and adenosine. In our experiments, we were also able to demonstrate a contractile response to angiotensin II in WKY and SHR cells (Fig. 5, A and B). In other unpublished studies, we also find the cells responsive to nitric oxide. Thus, while it is possible that these cells may not have the same contractile ability as in vivo, in our cultured conditions, they continue to retain some contractile properties.

This is significant because, at the present, it appears that the stiffness of VSMCs, a distinct mechanical property, may be related to the contractile state. In the AFM studies of isolated cells, our assessment of contractile response to angiotensin II found a simultaneous increase in VSMC stiffness (Fig. 5C). Furthermore, the treatment with ML-7, which would be expected to eliminate VSMC tone, normalized the stiffness between SHRs and WKY rats. Thus it appears that the increase in stiffness of the SHRs contains an active component that is dependent on actin cross-bridge cycling. It is also known that agonists that enhance VSMC tone also affect actin polymerization and cytoskeletal reorganization (12, 16, 21, 35, 36). It may be that the contractile tone and passive stiffness properties of the cytoskeleton are mechanistically linked. Our in vitro experiments were performed at as close to physiological conditions as possible, and thus the measurements reflect both the innate stiffness and the influence of contractile tone on the stiffness properties of VSMCs.

It is also possible that in vivo VSMC stiffness is influenced by underlying changes in the extracellular matrix. McDaniel et al. (22) found that VSMC phenotype was sensitive to underlying collagen fibril stiffness, when manipulated in vitro. Additionally, altered cellular properties may also result from the connectivity between cells and the extracellular environment. Bezie et al. (7) found SHR VSMCs have a greater portion of cell surface connected to the elastin lamellae than those from WKY rats.

The measurement of stiffness of individual cells is a relatively new metric for evaluating pathological changes in cells, and the significance of observed differences is still being defined. Recent evidence exists implicating cellular stiffness with disease states, e.g., cancer (10, 24) and anemia (37). This study is the first to demonstrate differences in VSMC stiffness in hypertension.

REFERENCES


AUTHOR CONTRIBUTIONS