Augmented Vascular Smooth Muscle Cell Stiffness and Adhesion When Hypertension Is Superimposed on Aging

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Abstract—Hypertension and aging are both recognized to increase aortic stiffness, but their interactions are not completely understood. Most previous studies have attributed increased aortic stiffness to changes in extracellular matrix proteins that alter the mechanical properties of the vascular wall. Alternatively, we hypothesized that a significant component of increased vascular stiffness in hypertension is due to changes in the mechanical and adhesive properties of vascular smooth muscle cells, and that aging would augment the contribution from vascular smooth muscle cells when compared with the extracellular matrix. Accordingly, we studied aortic stiffness in young (16-week-old) and old (64-week-old) spontaneously hypertensive rats and Wistar–Kyoto wild-type controls. Systolic and pulse pressures were significantly increased in young spontaneously hypertensive rats when compared with young Wistar–Kyoto rats, and these continued to rise in old spontaneously hypertensive rats when compared with age-matched controls. Excised aortic ring segments exhibited significantly greater elastic moduli in both young and old spontaneously hypertensive rats versus Wistar–Kyoto rats. were isolated from the thoracic aorta, and stiffness and adhesion to fibronectin were measured by atomic force microscopy. Hypertension increased both vascular smooth muscle cell stiffness and vascular smooth muscle cell adhesion, and these increases were both augmented with aging. By contrast, hypertension did not affect histological measures of aortic collagen and elastin, which were predominantly changed by aging. These findings support the concept that stiffness and adhesive properties of vascular smooth muscle cells are novel mechanisms contributing to the increased aortic stiffness occurring with hypertension superimposed on aging. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.04456)

Key Words: aorta ■ collagen ■ elastin ■ fibronectins ■ focal adhesions ■ microscopy, atomic force ■ vascular stiffness

In the United States, the residual lifetime risk of developing hypertension is >90% in both men and women.1 Long-term sustained hypertension increases morbidity and mortality from myocardial infarction, stroke, atherosclerosis, and renal failure. Large-artery stiffening is comorbid with hypertension,2 but is also accelerated in patients with uncontrolled blood pressure.3 However, increased aortic stiffness is also characteristic of the normal aging process.4 Clinically, elderly hypertensive patients harbor stiffness levels above that of age-matched normotensive older patients,5 suggesting an additive or synergistic effect of hypertension and aging. However, the pathogenesis of aortic stiffening in hypertension, especially as it progresses with age, is not completely understood.

Old spontaneously hypertensive rats (SHR) are the most commonly studied model of aging hypertension, with several reports of advancing aortic pressure5–8 and accelerated changes in either vascular stiffness or decreased vascular compliance in the aging SHR.9–11 The most predominant mechanism thought to underlie changes in vascular mechanical properties is an increase in collagen deposition and a breakdown of elastin. It is generally agreed that these changes are seen with vascular aging and can contribute to increases in vascular stiffness.12 However, these changes are not consistently increased with hypertension,12–14 with several reports actually showing decreases in vascular collagen.15,16 Furthermore, extracellular matrix (ECM) changes alone do not provide a mechanistic explanation for the acceleration, per se, of vascular stiffness seen in hypertensive aging.

Despite these inconsistencies, few alternative structural and cellular mechanisms have been considered. Bézie and colleagues have previously speculated that the connections between vascular smooth muscle cells (VSMCs) and the ECM may play a role in vascular stiffness in hypertension. They have reported an increased VSMC surface occupied by dense plaques,17 and increased expression of fibronectin and α5 integrin12 within the abdominal aortas of SHR, when compared
Stiffness and Adhesion Properties of Isolated VSMCs

VSMCs were obtained by enzymatic digestion of thoracic aortic tissue, as described previously and were plated onto glass-bottomed cell culture dishes. Primary (non-passaged) VSMCs were studied after <1 week in culture, and stiffness and adhesion properties of individual VSMCs were determined by atomic force microscopy (AFM) nanoindentation. A microcantilever tip was mounted to an AFM (Bruker BioScope Catalyst) that was coupled to a confocal microscope (Olympus IX81). In experiments to study adhesion properties, the microcantilever tip was functionalized by coating the tip with fibronectin (0.5 mg/mL) to promote the formation of VSMC adhesion attachments on contact with the cell surface. Individual VSMCs were probed at 0.5 Hz approach–retraction cycle resulting in a cell indentation depth of 100 to 300 nm. For each cell, force curves were continuously collected for four minutes. The force signals were analyzed using proprietary software, NForceR (copyright 2006; Registration Number TXu1-328-659), and a custom MATLAB script. The approach curve was used to compute the stiffness of VSMCs using a modified Hertz model, where the indentation force was computed from Hooke’s law, as described previously. The presence of VSMC adhesions to the fibronectin functionalized cantilever tip was computationally identified from the retraction curves. For each retraction curve, the number of adhesions and the force required to rupture all adhesions were assessed. For each group of rats, measurements of stiffness, adhesion events, and adhesion force were obtained from averages of 60 cells from 3 animals per group.

Histological Analysis of the Thoracic Aorta

Freshly dissected segments of the thoracic aorta were fixed in neutral buffered 10% formalin solution and subsequently embedded into paraffin blocks. Aortic cross-section slices were cut and mounted onto glass slides. Masson’s Trichrome staining was used to visualize the overall aortic morphology and architecture. Tissue dimensions were determined in images using digital calipers. Picosiris red staining under circularly polarized light was used to visualize collagen, and Van Gieson’s staining was used to visualize elastin within the aortic wall. Collagen and elastin contents were determined within each sampled field (15 fields per ring) by direct pixel quantification using custom MATLAB image analysis scripts. These contents were compared for the entire tissue ring by proportionating the sampled field area to the total tissue area. Collagen and elastin densities were computed by normalizing the content within a field to the underlying area of tissue within that field.

Statistical Analysis

The results are presented as means±SEM. Comparisons among the 4 groups were determined by 2×2 multifactorial ANOVA, with factors defined as hypertension (SHR and WKY) and age (16- and 64-week-old), and the interaction term determined the additive effect of these 2 factors. Selected post hoc comparisons were done by independent sample t tests using a Bonferroni correction and are displayed in graphical figures. Computations were performed using SPSS 20.0 software. A value of \( P<0.05 \) was considered significant.

Results

Aortic Pressure Increases in SHR

Measurement of thoracic aortic pressures in both young and old animals revealed significantly elevated pressures within SHR (Figure 1). ANOVA results were significant for the SHR strain, as evaluated for systolic, diastolic, mean aortic, and pulse pressures (\( P<0.001 \) for all) when compared with age-matched WKY. Aortic pressure was unchanged by age in WKY, as verified by post hoc comparison, whereas it continued to increase from young to old in SHR. Importantly, a significant interaction between hypertension and age was found.
for systolic ($P<0.05$) and pulse pressure ($P<0.001$), suggesting a more than additive, or synergistic, effect of hypertension and age in SHR.

Vascular and VSMC Stiffness Increases With Hypertension and Aging

Vascular stiffness was determined by tensile testing of excised aortic rings. The circumferential stress was determined across a range of stretched lengths, and the mechanical strain across this range was used to generate stress–strain curves, as shown for representative rings from each animal group (Figure 2A). The tangential elastic stiffness was evaluated from the stress–strain relationship, within a physiological range of vessel diameter (2.5–3.5 mm). When compared to young WKY, tangential elastic stiffness was increased by 2-fold in the young SHR and also the aging WKY, but rose by 4-fold in old SHR animals (Figure 2B). Significant differences were found by ANOVA between SHR and WKY ($P<0.001$) and between the age groups ($P<0.001$). The comparisons again showed a significant interaction between hypertension and age ($P<0.01$).

The mechanical properties of individual aortic VSMCs were evaluated by AFM nanoindentation for young and old SHR and WKY. The deflection signal from the AFM cantilever was recorded as it approached the surface of the cell, made contact, and indent the cell as shown in representative force curves (Figure 2C). This deflection signal was calibrated against the cantilever sensitivity and used to determine the stiffness (elastic modulus) of the VSMCs. Stiffness of SHR VSMCs roughly doubled with simple aging and increased by >3-fold from young WKY baseline in hypertensive aging. Both factors of SHR strain ($P<0.0001$) and age...
Adhesion Properties of Individual VSMCs Increases With Hypertension and Age

The adhesive properties of the VSMCs were determined from adhesions that occurred between the cell and the fibronectin functionalized AFM probe tip. As the probe was retracted from the cell surface, a cell–probe adhesive interaction was detected as downward deflection of the AFM cantilever followed by abrupt upward steps in the deflection signal, which represented rupture of an adhesion. This is shown in representative force curves (Figure 3A). The numbers of adhesion events were determined from each retraction curve for every approach–retraction cycle that was obtained during a 4-minute indentation experiment. Aging in WKY and SHR significantly increased the number of adhesions with the AFM probe. However, the increase in adhesion was much more pronounced in old SHR when compared with either young SHR or WKY (Figure 3B). Both factors of hypertension (P<0.001) and aging (P<0.001) and their interaction (P<0.001) were significant.

The summed force required to rupture all adhesions in a given retraction curve was increased by hypertension and aging (Figure 3C). In young animals, this sum increased by 93% in the SHR when compared with age-matched WKY; in old animals, it was increased by 104% in the SHR when compared with age-matched WKY. Both factors hypertension (P<0.001) and aging (P<0.001) and their interaction (P<0.001) were significant.

Aortic ECM Remodeling in the Presence of Aging and Hypertension

ECM composition and remodeling of the aortic wall from these groups were determined from Masson’s Trichrome, Picrosirus Red, and Van Gieson’s staining of the aortic tissue rings. Morphometric comparisons between the 4 groups were assessed by Masson’s Trichrome staining (Figure 4A). The aorta from the SHR had 5% greater lumen diameter (Figure 4B) and 19% increase in medial layer thickness (Figure 4C) in young animals when compared with WKY. In old animals, the SHR had a 15% greater lumen diameter (Figure 4B) and a 42% increase in medial thickness (Figure 4C) when compared with WKY. The ratio of the medial layer thickness:lumen diameter (Figure 4D), a measure of mechanical load, experienced at the region of VSMCs, was also increased by 12% in young SHR and by 25% in old SHR when compared with age-matched WKY. Both factors, hypertension and aging, were significant (P<0.001 and P<0.01), with no significant interaction. In addition, the medial cross-sectional area (Figure 4E) was significantly increased in old SHR by 64% when compared with that in age-matched WKY and by 74% when compared with that in young SHR. Both factors, hypertension and aging, were significant (P<0.001 and P<0.001), with no significant interaction.

Collagen fibers were visualized under circularly polarized light (Figure 5A), and the content of collagen was determined within sampled image fields (Figure 5B) and computed for the whole tissue cross-section (Figure 5C). Collagen content significantly increased with age in both WKY and SHR (Figure 5B and 5C). However, there were no differences in total collagen content between WKY and SHR for the young or old groups (Figure 5B and 5C). It was noted that the density of collagen (ie, normalizing collagen content to area) was decreased in young SHR by 40% when compared with that in young WKY, and decreased by 30% in old SHR (Figure 5D) when compared with that in old WKY. For both measures of collagen content and density, only the factor of aging was significant (P<0.001). Within individual image fields, we observe visibly thinner elastin fibers in old animals (Figure 5E) and decreased content (Figure 5F); although for the overall ring cross-sections, the content was significantly increased with age (Figure 5G) in both SHR and WKY. Elastin density decreased with age (Figure 5H) in both SHR and WKY (38% and 54%, respectively). However, comparison of elastin...
content and density did not reveal any significant differences between the SHR and WKY. Only the factor of aging significantly (P<0.001) affected elastin content and density.

Discussion
The major finding in this investigation is that the observed amplification of aortic pressure and vascular stiffness when hypertension is superimposed on aging parallels and correlates with the increase in VSMC stiffness and adhesion properties, and that this increase in vascular stiffness is not mediated by the content or density of collagen and elastin. This finding contrasts with other studies of aging hypertension that have ascribed accelerated vascular mechanical changes to the ECM.7,9

We found that vascular collagen is rarefied (especially between lamina) in the SHR and this is likely due to, in part, hypertrophy of the VSMCs, particularly in the old animals. As shown in Figure 5, we found that the amount of collagen in the thoracic aorta of SHR is unchanged when compared with that of WKY, at either young or old ages. Some previous studies of the SHR have also similarly concluded that hypertension does not increase the absolute collagen content6,15,16 or collagen concentrations6,10,22 in the thoracic aorta. Similar observations and findings have been made in the abdominal aorta,12,23 mesenteric,16,24 and cerebral34 arteries. However, it is noted that in other studies of the SHR, contradictory results have been reported for the thoracic aorta5,14,15 and mesenteric artery.26 In fact, of the numerous studies surveyed on the effect of hypertension, per se, on vascular collagen, inconsistency seems to be the common feature of the collective studies. Furthermore, conflicting trends in vascular collagen content have also been noted within other hypertension models, such as renal clip models27–30 and deoxycorticosterone acetate (DOCA)–salt models.31,32

Numerous reasons may exist for discrepancies among these studies. For example, there are differences in quantification methodologies (histological versus biochemical), as well as the variable ways that samples are reported and normalized (e.g., weight versus length of the tissue, protein content versus density, medial versus total wall region, quantification from image field versus total ring). Other reasons may be innate differences in the hypertension model, stage of hypertension development, the vessel studied and the age at the time of sampling. A time-course study of an aortic coarctation model of hypertension by Hu et al.33 reported that the concentration of collagen in the aorta was only increased at 2 and 4 weeks post surgery, and that by week 6 the concentration had returned to the levels of the sham control, suggesting that remodeling of ECM may be dynamic. Thus, it is possible that acute induction of hypertension, either surgically or by short-term infusions of vasopressor agents, only temporarily alters the levels of certain ECM proteins as an acute adaptive response that is followed by deadaptation of these changes. It is worth noting that such animal models are often euthanized for tissue measurements within a few weeks of the induction of the hypertension, and therefore, may only record these early dynamic changes in the remodeling of the vessels, instead of long-term effects. By contrast, the SHR is a model that develops and adapts to hypertension gradually, which may affect the temporal nature of its remodeling process. In our experiments, we have chosen to sample SHR at a young age (after hypertension was fully established) and also at an older age to assess longer term vascular remodeling events. Of relevance to the observations reported in our present investigation, studies of human essential hypertension that assessed aortic collagen postmortem found that the collagen content was unchanged35,36 with hypertension or had a similar concentration36 compared with age-matched normotensive patients.

Our findings do not support changes in content of collagen and elastin as an explanation for increased stiffness in hypertension. However, other types of ECM remodeling, such as changes in ECM protein type, cross-linking, glycation, and structural or architectural rearrangement, may contribute to altering the mechanical properties of the
vascular wall. Although the directionality of the changes in vascular collagen associated with hypertension is controversial, it is widely accepted that vascular collagen does increase with aging. It may be that vascular collagen plays a larger role in increasing vascular stiffness as part of the aging process than it does in hypertension. To define these relationships ultimately, we suggest that it is important to distinguish between the overlapping and the unique cellular mechanisms underlying vascular stiffness in hypertension and aging.

We postulate that VSMCs play a much larger role in hypertensive vascular stiffness than previously considered. In the SHR, we observe increased medial thickness (Figure 4), which is particularly striking within the old SHR animals and may be attributable to VSMC hypertrophy. Hypertrophy of VSMCs is commonly observed in hypertension and may also contribute to increases in VSMC stiffness. Furthermore, in the SHR, we observe increases in the underlying increased mechanical load in the medial layer (Figure 4D). VSMCs undergo major physiological adaptations to hypertension and thus we propose they may represent a larger contribution to the mechanical characteristics of the aortic wall than has been recognized. Already, we know that VSMCs readily adjust behaviors, such as proliferation, differentiation, and migration, in response to mechanical stimuli. Furthermore, the inherent phenotypic plasticity of VSMCs is a well-recognized major contributor to the progression of several vascular diseases, including atherosclerosis and aging-related pathology.

Our unique finding that the stiffness of individual VSMCs are augmented by hypertension (Figure 2D), and that cellular stiffness continues to rise in older hypertensive animals, suggests that increased VSMC stiffness is an important part of the disease process and plays a critical role in the stiffening of the process taking place in the vascular wall. Of course, it is possible that inherent genetic differences between SHR and WKY may introduce variables in addition to hypertension, and that the blood pressure dependencies of stiffness and adhesion have not been completely defined by our studies. However, we note that the aging WKY, which do not undergo any change in blood pressure, do show an increase in VSMC stiffness and adhesion that correlate with overall vascular stiffening. This supports the conclusion that intrinsic changes in VSMC mechanical and adhesive properties are associated with vascular stiffening. Our studies of young hypertensive rats and aging monkeys also support this concept.
Furthermore, it was observed that the adhesive properties of VSMCs are also increased by hypertension (Figure 3B and 3D). A stiffer cell combined with a larger number of cell adhesions to the ECM would allow the cellular cytoskeletal elements to play a greater role in influencing overall mechanical characteristics of the vascular wall. Importantly, the observed increase in attachment force of the SHR VSMC cytoskeletal connections could exert greater forces on ECM proteins. It is reasonable to envision that the larger pulling forces from these adhesive connections may by themselves contribute to stiffness of the vessel. Indeed the augmentation of adhesion force within the old SHR VSMCs correlates with the accelerated vascular stiffness. This finding of increased adhesion force to fibronectin is also significant in light of several reports of increased presence of fibronectin and α5 integrin within the vessels of several hypertension models: SHR, DOCA-salt, angiotensin II–infusion, as well as recently described nonhypertensive vascular stiffness models. Furthermore, treatment with antihypertensive drugs has been reported to decrease vascular fibronectin levels. Although many of these studies have supported the notion that cell focal adhesions to fibronectin may be correlated with vascular stiffness, our measurements are the first to confirm using biophysical assessment tools that stiffness and increased attachment to fibronectin occur and are modified in the aging hypertensive model. This strongly supports the hypothesis that cell stiffness and adhesion should be considered as alternative therapeutic targets for antihypertensive therapy.

In addition, it is important to note that the adhesive properties of VSMCs are related to VSMC stiffness. It was observed that similar values of VSMC stiffness are obtained in AFM experiments with and without adhesion (fibronectin-coated versus uncoated cantilever probes). Therefore, the presence of fibronectin on the probe was not influencing the stiffness of the VSMC. Conversely, the VSMC stiffness level may be influencing the adhesion properties, as recently published work supports a mechanistic link between stiffness and adhesion. Hong et al demonstrated that the treatment of VSMCs with the vasoactive agents angiotensin II and adenosine, respectively, increase and decrease VSMC stiffness and adhesion in a coordinated manner. It may be that the interplay of these 2 mechanisms, VSMC stiffness and VSMC adhesion, contributes to the interaction of hypertension and aging in accelerating vascular stiffness.

In summary, the current investigation is the first to identify that the stiffness and adhesion properties of VSMCs strongly correlate with increased vascular stiffness within an aging hypertension model. Additional studies are needed to better understand the underlying mechanisms that increase VSMC stiffness in hypertension better, and also how VSMC stiffness enhances VSMC adhesion to ECM proteins. These mechanisms, VSMC stiffness and VSMC adhesion, may elucidate potential therapeutic targets that directly control vascular stiffness in hypertension.

Perspectives

Whereas much of the previous work in this field has focused on changes in the ECM (collagen deposition and cross-linking, elastin breakdown) and endothelial damage (impaired nitric oxide release), this study demonstrates that the intrinsic mechanical properties and adhesive behavior of VSMCs are associated with vascular stiffness in hypertension and aging. Furthermore, the interplay between VSMC stiffness and VSMC adhesion may be structurally responsible for the acceleration of vascular stiffness in hypertensive aging. Increased VSMC stiffness and increased VSMC adhesion are emerging and novel concepts that provide new possibilities for elucidating mechanisms involved in hypertension, and these may guide us toward therapeutic targets for vascular stiffness.

References


**Novelty and Significance**

**What Is New?**

- The intrinsic stiffness and adhesion properties of vascular smooth muscle cells (VSMCs) are increased in hypertension, and these may contribute to accentuated aortic stiffness observed with age in this disease.
- Increased VSMC stiffness and adhesion are emerging and novel concepts in the understanding of aortic stiffness in cardiovascular disease.
- These concepts contrast with our previous understanding of aortic stiffness, which has largely focused on extracellular matrix and endothelial cell changes.

**What Is Relevant?**

- Hypertension is a major health problem in the United States.
- The novel mechanisms of VSMC stiffness and VSMC adhesion suggest the potential for new pharmacological targets to control hypertension and aortic stiffness, directed at VSMCs themselves.

**Summary**

Our findings in the spontaneously hypertensive rats have revealed that aortic VSMC stiffness and VSMC adhesion are increased in hypertension and aging, and that superimposing these 2 conditions leads to significant interaction. This supports the novel hypothesis that the mechanical properties of VSMCs contribute to aortic stiffness in hypertension. Conversely, data in this study demonstrate that collagen and elastin content do not correlate with the increase in vascular stiffness. The mechanisms underlying VSMC stiffness and VSMC adhesion may provide new therapeutic targets and strategies for selectively targeting aortic stiffness in hypertension.
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