Heightened aberrant deposition of hard-wearing elastin in conduit arteries of prehypertensive SHR is associated with increased stiffness and inward remodeling

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Arribas SM, Briones AM, Bellingham C, González MC, Salaices M, Liu K, Wang Y, Hinek A. Heightened aberrant deposition of hard-wearing elastin in conduit arteries of prehypertensive SHR is associated with increased stiffness and inward remodeling. Am J Physiol Heart Circ Physiol 295: H2299–H2307, 2008. First published October 10, 2008; doi:10.1152/ajpheart.00155.2008.—Elastin is a major component of conduit arteries and a key determinant of vascular viscoelastic properties. Aberrant organization of elastic lamellae has been reported in resistance vessels from spontaneously hypertensive rats (SHR) before the development of hypertension. Hence, we have characterized the content and organization of elastic lamellae in conduit vessels of neonatal SHR in detail, comparing the carotid arteries from 1-wk-old SHR with those from Wistar-Kyoto (WKY) and Sprague Dawley (SD) rats. The general structure and mechanics were studied by pressure myography, and the internal elastic lamina organization was determined by confocal microscopy. Cyanide bromide-insoluble elastin scaffolds were also prepared from 1-mo-old SHR and WKY aortas to assess their weight, amino acid composition, three-dimensional lamellar organization, and mechanical characteristics. Carotid arteries from 1-wk-old SHR exhibited narrower lumen and greater intrinsic stiffness than those from their WKY and SD counterparts. These aberrations were associated with heightened elastin content and with a striking reduction in the size of the fenestrae present in the elastic lamellae. The elastic scaffolds isolated from SHR aortas also exhibited increased relative weight and stiffness, as well as the presence of peculiar trabeculae inside the fenestra that reduced their size. We suggest that the excessive and aberrant elastin deposited in SHR vessels during perinatal development alters their mechanical properties. Such abnormalities are likely to compromise vessel expansion during a critical period of growth and, at later stages, they could compromise hemodynamic function and participate in the development of systemic hypertension.

remodeling; hypertension; elastic lamellae; fenestrae; spontaneously hypertensive rats

UP TO 20% OF INDIVIDUALS LIVING in industrialized countries are diagnosed with systemic arterial hypertension, a major risk factor for the development of deleterious cardiovascular diseases. In the majority of cases, the development of high blood pressure is of unknown etiology, which is known as essential hypertension. The two widely recognized hallmarks of essential hypertension are the narrowing of resistance arteries, which increases peripheral resistance (25, 34), and the stiffening of conduit arteries (33) that compromises vascular compliance. Both these alterations contribute to the permanent cardiovascular complications in hypertensive patients (31). Despite decades of extensive study, our understanding of the pathological mechanisms leading to these vascular abnormalities is still incomplete. Moreover, a fundamental question remains as to whether structural and mechanical abnormalities trigger the initial elevation of blood pressure or whether they simply develop as a consequence of hypertension.

Despite the widely accepted fact that hypertension is associated with alterations to the extracellular matrix (ECM) in the arterial walls, most mechanistic explanations have been linked to the aberrant deposition of collagen fibers. Hence, the alterations to vascular elastic fibers and lamellae associated with the pathological mechanisms underlying systemic hypertension have not been adequately addressed. It has been demonstrated that the initial increase in mechanical stress imposed on vascular smooth muscle cells (VSMC) by high blood pressure could trigger the abnormal deposition of elastin (19) and that chronic hypertension contributes to the loss of arterial wall resilience (27). On the other hand, it has also been proposed that early aberrations in the deposition of elastic fibers, the most durable elements of arterial matrix (30, 35), might be key elements in the pathophysiology of hypertension (2, 7, 10). Indeed, defective elastogenesis, due to either genetic abnormalities of elastic fiber elements or to altered hemodynamic or nutritional environments during fetal development and infancy, may be associated with reduced vascular compliance and hypertension (for review see Ref. 2). A large bulk of our understanding of the mechanism of hypertension has been accumulated from studies of spontaneously hypertensive rats (SHR) and stroke-prone SHR. We have previously demonstrated that both conduit and resistance arteries in these strains display abnormal elastic fiber content and organization, generating a denser network of elastic fibers in the adventitia and a more compact internal elastic lamina (IEL) (4, 5, 37). Moreover, in resistance arteries these alterations are associated with vascular stiffening, and they can even be observed in 1-mo-old rats before the development of hypertension (15).

Based on these data and the fact that elastic fiber synthesis is restricted to the fetal period and early postnatal life, we hypothesized that perinatal aberrations in the content and/or
organization of elastic fibers might constitute a crucial element in vascular remodeling that contributes to the development of systemic hypertension in adulthood. Thus the present study aimed to gain insight into the possible importance of early alterations to elastic fibers in the development of conduit artery stiffening and narrowing. Hence, we studied the gross structure and mechanical properties of carotid arteries from 1-wk-old SHR and compared them with arteries derived from two normotensive strains: Wistar-Kyoto (WKY) and Sprague Dawley (SD) rats. We paid particular attention to the morphological properties of their fenestrated IEL. In addition, cyanide bromide-insoluble elastin scaffolds were prepared from 1-mo-old SHR and WKY aortas to assess their relative weight, amino acid composition, the three-dimensional organization of their elastic lamellae, and their mechanical characteristics.

**METHODS**

**Animals.** In this study, 8 ± 2-day (1 wk old) SHR and WKY and SD male rats weighing 9–11 g were used, as well as 30 ± 2-day (1 mo old) SHR and WKY male rats weighing 75–80 g. All the animals were bred at the Animal House facilities of the Universidad Autónoma de Madrid. All experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals published by National Institutes of Health (Publication No. 85-23, Revised 1996) and with current Spanish legislation (RD 1201/2005) and approved by the Animal Care and Use Committee of Universidad Autónoma de Madrid.

**Pressure myography.** Carotid artery segments from 1-wk-old SHR and WKY and SD rats and from 1-mo-old SHR and WKY animals were used. The pressure-diameter curves were assessed in calcium-free conditions with a pressure myograph (Danish Myo-Tech, Model P100; J.P. Trading I/S, Aarhus, Denmark) as described previously (5). The internal and external diameters were measured from the images captured with Metamorph image analysis software (Universal Imaging), and the stress-strain relationship and $\beta$-values were calculated in segments from 1-wk-old rats as described previously (5). The segments from 1-mo-old rats were too thick to detect the internal diameter at low pressures from the pressure myograph, and only the external diameters could be measured across the entire range of intraluminal pressures. At the end of the experiment, the segments were pressure-fixed with 4% paraformaldehyde at 37°C at the systolic blood pressure of the rat, according to previous studies (40 mmHg for 1-wk-old rats and 90 mmHg for 1-mo-old rats) (15), and they were stored for confocal microscopy analysis.

**Confocal microscopy.** The organization of the elastic lamellae was assessed in intact pressure-fixed carotid arteries and nonpressurized aortas by fluorescent laser scanning confocal microscopy (Leica TCS SP2) as described previously (5, 15). The arterial segments were mounted in a well constructed with silicon spacers on a glass slide that was of sufficient depth to avoid vessel compression and that was covered with a
coverslip. The intact segments were visualized at a wavelength of the 488/515 nm, and single images of the lumen were captured with a ×20 objective focusing on the midpoint of the artery. Scanning of the entire vessel wall and the assessment of fenestrated elastic lamella were also performed with a ×63 oil immersion objective (a video showing all the lamellae of a rat carotid artery is provided as supplemental material; all supplemental material is published with the online version of this article). The images of the IEL were the clearest to study detailed organization of their fenestrae. Serial optical sections (0.5 μm) of each IEL were captured, and the autofluorescence intensity values (relevant to the elastin content), as well as the average area of the fenestrae, were quantified by morphometry with Metamorph image analysis software as described previously (5, 15).

Purification and analysis of aortic elastin. The thoracic aorta from 1-mo-old WKY and SHR was measured, weighed, and digested in cyanide bromide-formic acid (11) to purify the mature (covalently cross-linked) elastin from each aorta. The resulting three-dimensional scaffolds of concentric fenestrated lamellae connected with elastic fibers were weighed (the purity of which was confirmed by amino acid analysis; see Table 1) (39), and the relative elastin scaffold weight-to-segment weight ratio before digestion was calculated for each artery. Thereafter, one ring of each scaffold was used to assess the number and organization of the lamellae by scanning electron microscopy (SEM) (17) and a second one was used in mechanical tests.

Mechanical testing. The rings of parallel elastin scaffolds derived from SHR and WKY rats were mounted with wire loops, clamped into test grips of a Mach-1 stretch test apparatus (Biosyntech Montreal, QC, Canada), and mechanically tested in phosphate-buffered saline (pH 7.3) at 22°C as previously described (22). Elastin scaffold rings were exposed to an initial load of 0.1 g before they were stretched to the point of rupture. After normalization for the specimen’s volume, load extension data were converted to stress-strain data using the formulae for engineering stress and strain, and the elastic modulus was presented as the slope of the stress-strain relationship (3).

Statistical analysis. Statistical analyses were performed with GraphPad Prism 4, and the results are expressed as the means ± SE; n denotes the number of animals used in each experiment. The dependency of vascular structure or mechanics on rat strain or age and intraluminal pressure was studied by two-way ANOVA, followed by a Bonferroni correction for multiple comparisons. For specific comparisons of two means, the Student’s t-test was used.

RESULTS

Carotid arteries from neonatal SHR are stiffer and narrower than those from their age-matched WKY and SD counterparts. The internal and external carotid artery diameters were significantly smaller in the physiological pressure range in 1-wk-old

![Fig. 2. A: extended focus images reconstructed from serial optical sections of the internal elastic lamina (IEL) of carotid arteries from 1-wk-old SHR and WKY and SD rats (top) and from 1-mo-old SHR and WKY rats (bottom). Optical sections were obtained by confocal microscopy (×63 objective, zoom 4; scale bar = 8 μm). B: average area of the fenestrae and fluorescence intensity values in the IEL obtained from the same arteries. *P < 0.05 compared with age-matched SHR; + P < 0.05 compared with 1-wk-old rats of the same strain; # P < 0.05 compared with age-matched WKY rats. The number of rats is shown in brackets.](image)
SHR compared with their age-matched WKY and SD counterparts (Fig. 1A). No significant difference was observed between SD and WKY rats. Likewise, when measured by confocal microscopy from segments pressure-fixed at 40 mmHg, the internal diameter of the carotid artery followed a similar trend and the arteries from 1-wk-old SHR had a significantly smaller diameter (306.2 ± 13 μm; n = 7) than the WKY (364.0 ± 12 μm; n = 8; P < 0.05) and SD (372.0 ± 9 μm; n = 7; P < 0.05) rats.

At 1 mo of age the results obtained were similar and the external diameter of SHR carotid arteries was significantly smaller at all intraluminal pressures than that of WKY age-matched counterparts (Fig. 1B). The internal diameters from segments pressure-fixed at 90 mmHg and measured in confocal microscopy images were also significantly smaller in SHR (600.1 ± 20 μm; n = 6) than in age-matched WKY rats (721.5 ± 10.3 μm; n = 6; P < 0.01).

Moreover, the stress-strain relationship of carotid artery segments from 1-wk-old SHR was shifted to the left, and the β-values were significantly higher than those from WKY and SD age-matched counterparts. In addition, significantly smaller β-values were obtained from SD compared with WKY rats (Fig. 1C).

The IEL of carotid arteries from neonatal SHR have smaller fenestrae. The carotid arteries from 1-wk-old SHR that were distended at physiological pressures exhibited smaller fenestrae associated with heightened elastin-derived fluorescence intensity values in the IEL than the age-matched WKY and SD rats. The average size of the IEL in the fenestrae from carotid arteries was larger in SD than in WKY rats while the values of elastin-derived fluorescence intensity were similar in both these normotensive strains (Fig. 2). Moreover, in SHR the rate of progressive enlargement of the fenestra during the first postnatal month was much smaller than that of WKY rats. Furthermore, during the 3-wk period of postnatal life examined, the intensity of autofluorescence of the elastic lamellae only increased in SHR vessels, reflecting the significant gain in their net elastin content (Fig. 2).

Elastin scaffolds isolated from SHR aortas have higher elastin content and an abnormal structure. The relative average weight of the elastin frameworks purified from aorta segments from 1-mo-old SHR was significantly higher than those from their WKY counterparts (Fig. 3A). Specimens from both SHR and WKY animals contained fenestrated lamella of a similar thickness, while the SHR lamellae appeared straighter and more rigid than the wavy lamellae from WKY rats (Fig. 3B). Despite the fact that images obtained from some specimens suggested additional elastic lamellae in SHR aortas, the statistical analysis did not reveal a significant difference in the number lamellae between SHR (8.8 ± 0.8; n = 6) and WKY rats (8.0 ± 0.7; n = 5).

Fig. 3. A: elastin weight relative to segment weight before digestion with cyanide bromide in formic acid of aortas from 1-mo-old SHR and WKY rats. *P < 0.05 compared with age-matched SHR. The number of rats is shown in brackets. B: representative scanning electron microscopy micrographs of the lamellar organization of elastin frameworks isolated from 1-mo-old SHR and WKY aortas. Scale bar = 10 μm.
The amino acid composition of those isolated scaffolds indicated that WKY and SHR aortas contained very similar profiles, characteristic of mature elastin (Table 1). However, the SEM images acquired from the IEL of elastin frameworks of SHR revealed an abnormal organization of their fenestrae, which were characterized by the presence of multiple trabecular interconnections that caused a significant decrease in their area. Such trabecular interconnections were scarce or absent in the fenestrae of aortas from WKY rats (Fig. 4A). The presence of these structures was confirmed by confocal microscopy in the IEL from freshly dissected nonpressurized aortas (Fig. 4B) and carotid arteries (data not shown), excluding the possible introduction of artifacts generated during the isolation of the elastin framework. It was also noteworthy that the IEL of aortas from SHR displayed more intense elastin-derived autofluorescence, indicating a higher content of this protein (Fig. 4B).

Elastic frameworks isolated from SHR aortas are stiffer and less resistant than those from their WKY-derived counterparts. Mechanical tests of the elastin frameworks isolated from 1-month-old SHR aortas showed a larger Young’s Elastic modulus and an increased maximum strain before rupture than those from their age-matched WKY counterparts (Fig. 5).

DISCUSSION

The data presented in this study indicate that the significant increase in the net elastin content and the aberrant organization of elastic membranes in arteries of neonatal SHR may be associated with the compromised mechanical performance of these arteries, even in the prehypertensive period. The abnormal elastin organization detected in SHR after the first week of life seems to contribute to the growing stiffness and narrowing of conduit arteries. These early alterations might compromise hemodynamic function and participate in the development of systemic hypertension later in life.

Table 1. Amino acid composition of elastin purified from SHR and WKY aortas

<table>
<thead>
<tr>
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<th>SHR Aorta</th>
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<th>WKY Aorta</th>
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<tbody>
<tr>
<td></td>
<td>Nanomoles</td>
<td>Res Weight</td>
<td>Nanomoles</td>
</tr>
<tr>
<td>ASP</td>
<td>0.66</td>
<td>115.1</td>
<td>0.67</td>
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<tr>
<td>GLU</td>
<td>1.45</td>
<td>129.1</td>
<td>1.52</td>
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<td>HYP</td>
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<td>1.09</td>
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<td>SER</td>
<td>1.58</td>
<td>87.1</td>
<td>1.57</td>
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<tr>
<td>GLY</td>
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<td>32.08</td>
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<td>0.43</td>
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<tr>
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<td>THR</td>
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<td>ALA</td>
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</tr>
<tr>
<td>LYS</td>
<td>0.30</td>
<td>128.2</td>
<td>0.26</td>
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The elastin scaffolds obtained by purifying spontaneously hypertensive rat (SHR) and Wistar-Kyoto rat (WKY) aortas with cyanide bromide in formic acid were additionally digested with protease K, and the cleavage products were subjected to the phase protein sequencing after separation by C-18 reversed-phase HPLC. Res, residue.

The ECM produced by arterial VSMC is responsible for the passive biomechanical properties of the vessels, providing them with mechanical strength, resilience, and compressibility. Therefore, aberrations in the content and spatial arrangement of ECM elements are likely to compromise vascular performance. In fact, increased vascular ECM is usually associated with hypertension with abnormal mechanics. Although the development of vascular stiffening in hypertension has always been linked to excessive deposition of collagen in the arterial walls (18, 28, 32), much less attention has been paid to the alterations in elastic fibers. There are two important aspects to be taken into consideration regarding the vascular elastic element, particularly abundant in conduit arteries. First, elastic fibers are key elements that are responsible for the periodic resilience of arterial walls, which guarantees the uninterrupted delivery of blood from the heart to organs and tissues (23). Finally, normal elastogenesis only occurs during fetal and early postnatal development and, thereafter, elastic fibers only exhibit minor turnover being considered the most durable elements of the ECM (30, 35). Based on these two facts, we hypothesized that alterations to the elastic fibers and lamellae in conduit vessels during pre- or perinatal development will probably have a significant impact on the mechanical properties and structure of arterial walls, which might subsequently compromise hemodynamic function.

Both conduit and resistance arteries of adult SHR exhibit an abnormal organization of the IEL, with smaller fenestrae and more compact elastic fiber distribution (4, 5, 37). In resistance arteries, these alterations to elastic fibers are evident within the first 30 days of life, before the development of high blood pressure in this strain (15), although not as early as the changes reported here in conduit arteries. Indeed, conduit vessels from 1-wk-old SHR already contain significantly smaller fenestrae in the IEL compared with age-matched normotensive WKY and SD strains. The detection of these differences between SHR and SD rat arteries excludes the possibility that such phenomenon might be due to genetic variations between the SHR and WKY strain, a strain that, although frequently used as normotensive counterpart, exhibits important genetic variation between substrains (19% in WKY and 11% in SHR) (20, 40).

In the carotid arteries from 1-wk-old SHR, the elastin content was also significantly higher than in WKY and SD counterparts, as reflected by the elastin autofluorescence intensity (16), and it further increased by the age of 1 mo. This data was also confirmed in the most reliable assay, measuring relative weights of cyanide bromide-insoluble elastin frameworks isolated from WKY and SHR aortas. Hypertension can stimulate elastin synthesis by generating direct mechanical pressure on VSMC (19). However, SHR have a normal blood pressure in the first month of postnatal life (7, 15, 41, 43). Therefore, the increased elastin content in the arteries of neonatal SHR cannot be attributed to the effect of systemic hypertension. However, the influence of other local stimuli that may affect individual VSMC cannot be excluded at this time. For example, young SHR exhibit higher interstitial levels of norepinephrine (NE) (8) and higher levels of circulating epinephrine (42), as well as an increased contractile response of VSMC (1, 36). Thus it is possible that the mechanical stretch of individual VSMC induced by catecholamines in prehypertensive SHR could act as a stimulus for elastin synthesis, especially since treatment with NE induces increased elastin...
accumulation in rats (12). Interestingly, the increase in elastin content induced by NE was also associated with an elevation in VSMC number (12); VSMC from SHR display an abnormal cell cycle (38). Moreover, we recently observed that VSMC derived from neonatal SHR display fewer terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling-positive cells than WKY counterparts (unpublished data). Hence, it is conceivable that the net accumulation of elastin observed in conduit arteries from prehypertensive SHR might be linked to a decrease in the proliferation-to-apoptosis ratio, which may maintain the fetal behavior of VSMC and extend their active period of elastin production.

Although differences between SHR and WKY elastin have been previously reported (14, 29), we did not detect any significant differences in the amino acid composition of cyanide bromide-insoluble elastin isolated from WKY and SHR.
aortas. Our standard amino acid analysis did not assay cross-linking amino acids (desmosine and isodesmosine). However, the similar low levels of free lysine detected in the elastin isolated from both strains indirectly indicate an equivalent level of cyanide bromide-resistant desmosines (engaging 3 or 4 lysines). We postulate that the differences in amino acid composition of elastin isolated from SHR and normotensive strains reported previously could be related to impurities generated by older methods of elastin isolation (29).

Our detailed scanning microscopy analysis of the isolated elastin frameworks demonstrated that SHR fenestrae were filled with peculiar trabeculae also made of elastin, whereas in WKY arteries most of the fenestrae appeared to be empty. At this moment, we can only speculate on the mechanism responsible for the formation of these unique structures that obscure the fenestrae in the elastic lamina of SHR arteries. Although some of them might derive from branches connecting adjacent elastic lamellae, the majority of these trabeculae merges with the edges of the fenestrae and probably constitutes unique remnants of the solid elastin that could not be removed by normal remodeling. Regardless of their origin, these elastin remnants obscure the lumen of individual fenestrae and contribute to the significant decrease in total fenestrae area in SHR arteries.

The data presented here suggest that the augmented elastin content in young SHR, which results in more compact lamellae and fenestrae filled with trabeculae, contributes to the observed increase in carotid artery stiffness. The apparent paradox that excess elastin results in material stiffening, as demonstrated by the mechanical tests on purified elastin scaffolds, can be explained by the presence of trabeculae inside the fenestrae that effectively reduce their size, probably contributing to the mechanical impairment. Thus the present study in carotid arteries from SD, WKY, and SHR demonstrates an inverse

Fig. 5. Representative stress-strain curves of the elastic frameworks obtained from 1-mo-old SHR and WKY rat aortas. Y, young elastic modulus; Stressmax and Strainmax, maximum stress or strain before rupture, respectively.

Fig. 6. Schematic diagram explaining the effect of the trabeculae inside the fenestrae on the mechanical properties of the tissue. In a composite formed by an elastic matrix, the existence of randomly distributed voids modifies the mechanical properties of the material. It has been demonstrated that the larger the volumetric ratio (volume of fenestrae/volume of elastin) the smaller the elastic modulus (Ref. 26). In analogy, we suggest that the presence of trabeculae inside SHR fenestrae would increase material stiffness, limiting the expansion of fenestrae upon pressure and reducing the effective lumen size.
association between the area of fenestrae and $\beta$-values (a parameter indicative of intrinsic material stiffness) and confirms our previous data in small arteries (16). Moreover, there is evidence that the elastic modulus (also indicative of material stiffness) of a composite formed by an elastic matrix containing voids (in our case elastic lamellae containing fenestrae) is inversely proportional to the volumetric ratio (volume of void to volume of elastin) (26). That would mean that larger fenestrae volume with no trabeculae provide better elasticity than fenestrae filled with material, even if such material were elastic. Since collagen content is not increased in the SHR conduit (43) and resistance arteries (15) before hypertension develops, we assume that aberrant elastin plays a key role in the generation of the abnormal mechanical features observed in our studies of neonatal SHR. The abnormal elastin distribution in SHR lamellae also seems to add fragility to the structure, provoking rupture with less strain. This fragility is in accordance with previous studies demonstrating that mechanical strength decreased in 1-mo-old stroke-prone SHR (24).

Previous studies demonstrated that the steady enlargement of fenestrations, and not constant elastin synthesis, is responsible for the systematic circumferential and longitudinal growth of vessels during normal development (13, 44). Elastin removal by local enzymatic elastolysis constitutes a crucial part of normal developmental remodeling, permitting the steady enlargement of the fenestrae (44). Hence, we suggest that by imposing a mechanical limitation on the lamina, the aberrant fenestrae probably compromise vessel expansion during a critical period of vessel growth. Accordingly, they could participate in the early development of vessel narrowing in SHR, particularly in conduit vessels that exhibit several fenestrated lamellae. A schematic diagram explaining the relationship between abnormal fenestrae, material stiffness, and lumen growth is shown in Fig. 6.

Finally, we conclude that the heightened and aberrant initial deposition of arterial elastin, the most durable element of arterial ECM, in neonatal SHR probably constitutes an early mechanism that, through a mechanical impairment and in conjunction with other known alterations, contributes to the development of high blood pressure in SHR. Since the abnormal organization of elastin in arteries from young SHR does not occur in vessels from nongenetic models of hypertension (6), we speculate that a unique genetic program of SHR (triggering primary aberrant assembly of elastic lamellae and/or lack of normal elastin remodeling) compromises the mechanical properties of the arterial walls and eventually contributes to the development of hypertension.

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