In spontaneously hypertensive rats alterations in aortic wall properties precede development of hypertension

AD W. VAN GORP, DORETTE S. VAN INGEN SCHENAU, ARNOLD P. G. HOEKS, HARRY A. J. STRUIJKER BOUDIER, J G. R. DE MEY, and ROBERT S. RENEMAN. In spontaneously hypertensive rats alterations in aortic wall properties precede development of hypertension. Am J Physiol Heart Circ Physiol 278: H1241–H1247, 2000.—In hypertension arterial wall properties do not necessarily depend on increased blood pressure alone. The present study investigates the relationship between the development of hypertension and thoracic aortic wall properties in 1.5-, 3-, and 6-mo-old spontaneously hypertensive rats (SHR); Wistar-Kyoto rats (WKY) served as controls. During ketamine-xylazine anesthesia, compliance and distensibility were assessed by means of a noninvasive ultrasound technique combined with invasive blood pressure measurements. Morphometric measurements provided in vivo media cross-sectional area and thickness, allowing the calculation of the incremental elastic modulus. Extracellular matrix protein contents were determined as well. Blood pressure was not significantly different in 1.5-mo-old SHR and WKY, but compliance and distensibility were significantly lower in SHR. Incremental elastic modulus was not significantly different between SHR and WKY at this age. Media thickness and media cross-sectional area were significantly larger in SHR than in WKY, but there was no consistent difference in collagen density and content between the strains. Blood pressure was significantly higher in 3- and 6-mo-old SHR than in WKY, and compliance was significantly lower in SHR. The findings in this study show that in SHR, in which hypertension develops over weeks, alterations in functional aortic wall properties precede the development of hypertension. The decrease in compliance and distensibility at a young age most likely results from media hypertrophy rather than a change in intrinsic elastic properties.

METHODS

Study design. The experiments were performed in 1.5-, 3-, and 6-mo-old male WKY and SHR of theOkamoto-Aoki strain (local inbred strains; Central Animal Facilities, Maastricht University, Maastricht, The Netherlands). Each group consisted of 12 animals. The rats were housed in individual cages, maintained on a 12-h light:12-h dark cycle, fed at libitum (Hope Farms, Woerden, The Netherlands), and had free access to tap water. The experimental protocols were approved by the local Institutional Animal Care and Use Committee.

Arterial blood pressure was measured with a heparinized saline-filled catheter (OD: 0.5 mm), inserted up to the renal bifurcation from a femoral artery, connected to an external pressure transducer (CP-01, Century Technology, Inglewood, CA). The frequency response of the system was 50 Hz. The catheter was implanted with the rats under ether anesthesia, guided under the skin, and exteriorized at the base of the carotid artery are significantly reduced compared with age-matched control subjects, whereas the difference in blood pressure between these patients and control subjects is only small (41, 42). In addition, in these patients different parts of the carotid artery bifurcation are affected differently, as far as the reduction of distensibility is concerned, whereas these parts are exposed to the same mean blood pressure (41). Especially the latter observation indicates that in hypertension changes in artery wall properties may occur independent of blood pressure.

The present study was conducted to find support for this hypothesis. The experiments were performed on spontaneously hypertensive rats (SHR), because in our genetic strain, as in others (5, 21, 34), hypertension develops gradually over weeks, making it possible to study structural and functional alterations of the arterial wall compared with those of normotensive Wistar-Kyoto rats (WKY), if any, at normal blood pressures. Besides, SHR have been regarded as a model for primary hypertension in humans (35). Arterial blood pressure, arterial wall distensibility and compliance, incremental elastic modulus, and media cross-sectional area were determined in 1.5-, 3-, and 6-mo-old SHR and WKY. In addition, a number of extracellular matrix (ECM) components, which have previously been shown to play a role in the elastic properties of the vessel wall (6, 8), were determined. We hypothesized that part of the pressure-independent changes in vessel wall properties may be due to alterations in ECM components.

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Elastic properties of arteries are, among others, dependent on the operating blood pressure. To obtain near-equivalent blood pressure conditions, the animals were anesthetized with ketamine-xylazine (10:50 mg/ml; 100 ml/100 g body wt), which lowered the diastolic blood pressure in SHR to the level in WKY, the blood-pressure of which was only marginally affected by anesthesia (see RESULTS). Blood pressure was measured simultaneously with the diameter of the thoracic aorta and the displacement of its walls during the cardiac cycle, using an ultrasonic wall-tracking device (see below). Under anesthesia, measurements were started after blood pressure and heart rate had stabilized (average 20 min). Two observers performed three recordings of 2.5 s in each animal. The data obtained during these sessions were averaged. Experiments were terminated by exsanguination of the anesthetized animals, after which the thoracic aorta was isolated for subsequent histological examination.

Ultrasound assessment of aorta diameter and wall displacement. The aortic diameter and the diameter changes during the cardiac cycle were measured by means of a conventional B-mode ultrasound system (B = brightness) (Pie 480, 7.5 MHz linear array) attached to a vessel wall-tracking system (WTS, Pie Medical, Maastricht, The Netherlands) as described in detail before (38). The probe was positioned on the left side of the sternum, 10 mm above the diaphragm. The B-mode was used to visualize the thoracic aorta, whereafter an M-line (M = motion) was selected. The ultrasound system was switched to M-mode, and ultrasound was emitted and received along the selected line.

The received radio frequency (RF) signals were amplified and captured and temporarily stored by a data acquisition system, whereupon the RF signals were transferred to a personal computer. The first RF signal captured was visualized on the monitor screen to identify manually the anterior and posterior walls by means of two cursors. Subsequently, the position of these cursors was used by the tracking system to follow the position of the anterior and posterior walls and to calculate with a response time of 10 ms the displacements of these walls over time. The difference in the displacements between these walls reflects the change in diameter (distension). The minimal distance between the markers provides the end-diastolic diameter (D_{dia}). The WTS allows assessment of artery wall displacements with a resolution of a few micrometers (15). For the thoracic aorta of rats, the intrasession variability for D_{dia} varies between 3.3 and 6.5%, and the inter-session variability for this parameter is 4.6%. For the distension, intrasession variability varies between 7.9 and 11% and the intersession variability is 9.2%. There is no difference between observers (38).

Relationship between lumen diameter and pressure. Because the WTS and the intraaortic catheter exhibit different frequency characteristics, analysis of the relationship between aorta lumen caliber and intraaortic pressure was limited to the situation at end diastole (D_{dia} and P_{a dia}) and to the maximal changes in pressure (ΔP) and diameter (ΔD) during the cardiac cycle. The values attained for D_{dia} and ΔD were converted to lumen areas and changes thereof (A_{dia} and ΔA), assuming circular cross-section of the vessel. With the additional reasonable assumption that vessel segment length remains constant during the cardiac cycle (29), the absolute and the relative change in aortic lumen area during the cardiac cycle were calculated and expressed per unit of pressure, providing information about the cross-sectional compliance and distensibility of the vessel, respectively (14–16, 30). Below (A/ΔP) and (ΔA/ΔA)ΔP will be referred to as the compliance coefficient (CC) and the distensibility coefficient (DC), respectively.

Aortic wall structure. A segment of the thoracic aorta of 10 mm (L), halfway between the heart and diaphragm, was marked and subsequently removed from the animal. Adhering fat and connective tissue were removed, after which the ex vivo length of the segment (L_e) was measured under a stereomicroscope with a precision of ±5 μm. The segment was fixed in 4% neutral buffered formaldehyde and embedded in paraffin, after which 4-μm thick cross-sections were obtained. These sections were stained with Lawson's solution, the first step in the Von Giesson staining, which highlights elastic laminae. The cross-sectional area of the media was determined using an axioplan microscope (Zeiss), final magnification ×25, equipped with a standard CCD camera (Stemmer, Germany) (2). After identification of the internal and external laminae by setting cursors, the images were digitized and analyzed with commercial software (JAVA, Jandell Scientific, Corte Madera, CA). The area enclosed by the external and internal elastic laminae was considered to represent the ex vivo cross-sectional area of the media (CSA_m). This was converted to in vivo media cross-sectional area (CSA_i) by the following formula

\[
CSA_m = (L_e/L) \cdot CSA_i
\]

in which it was assumed that aortic wall volume remains constant after isolation and fixation and in which L, and L_e refer to the length of the thoracic aorta before and after isolation, respectively.

Media thickness (MT) in diastole was calculated from diastolic diameter (D_{dia}) and from CSA_i, using the formula

\[
CSA_i = \pi(D_{dia}/2 + MT)^2 - \pi(D_{dia}/2)^2
\]

which can be rewritten as

\[
MT = -(D_{dia}/2) + \left[ (D_{dia}/2)^2 + CSA_i/\pi \right]^{1/2}
\]

In these calculations it is assumed that the cross-section of the aorta is circular in situ.

Measurements of mechanical properties and dimensions were combined to calculate the incremental elastic modulus of Young's modulus (E_{inc}) at anesthetized aortic pressure

\[
E_{inc} = D_{dia}/(MT \cdot DC)
\]

Collagen content. Paraffin-embedded, 4-μm-thick cross-sections were also used to determine collagen area. The sections were stained with Sirius Red solution, which highlights collagen (18). The duration of staining was the same in all experiments. The collagen-occupied area, enclosed by the external and internal elastic laminae, representing the media collagen area, was determined planimetrically using the software for the assessment of media thickness as described above. The data are presented as media collagen density, i.e., the collagen volume as approximately a percentage of the total tissue volume in the section.

Biochemical determination of collagen content of the adventitia and media was performed as described by Chiarelli et al. (4). To facilitate separation of adventitia and media, aortas were incubated for 24 h in phosphate-buffered saline (PBS) at 4°C. The media and adventitia were mechanically separated from each other under a dissection microscope. The medium was dried under vacuum, and the dry weight was measured. Afterward, the medium was incubated in a 1% SDS solution in PBS for 24 h at 4°C. Incubation in 1% SDS extracts the noncross-linked collagen from the aorta. The supernatant was collected and stored at −70°C, and the pellet was...
incubated in a cyanogen bromide solution in 70% formic acid for 24 h at room temperature. Cyanogen bromide cleaves proteins at the methionine sites. Because elastin does not and collagen does contain methionine, cross-linked collagen can be extracted from the aortic media by cyanogen bromide. The pellet, containing elastin, and both supernatants, containing noncross-linked and cross-linked collagen, respectively, were dried under vacuum and hydrolyzed in 6 N HCl at 120°C. The collagen content was determined by the amount of hydroxyproline residues. The hydroxyproline residues were oxidized with choramine T, which reacts with p-dimethylaminobenzaldehyde. In the different fractions the colored compound produced was measured photospectrometrically at 558 nm. Hydroxyproline represents between 10.5 and 11.5% of the amino acid residues in collagen (4). The data are presented as milligram of hydroxyproline per gram dry weight normalized for the cross-sectional area of the media.

Statistics. In each animal six consecutive 2.5-s simultaneous recordings of pressure, internal lumen diameter, and distension were obtained, and characteristics were calculated for each individual cardiac cycle. Mean values were calculated for each recording session. These values were then averaged for each individual animal. Eventually a group mean was obtained for each strain-age group.

The data are presented as means ± SD. Comparisons between age groups and strains were performed with the two-way ANOVA extended with a Tukey post hoc test for statistically significant differences.

RESULTS

Between 1.5, 3, and 6 mo of age, body weight increased from 149 ± 15 g (means ± SD) to 435 ± 15 g in WKY and from 156 ± 16 g to 385 ± 18 g in SHR, whereas blood pressure, determined under conscious freely moving conditions, increased in SHR but only slightly in WKY. In the latter strain conscious diastolic pressure was slightly lower at 3 mo of age than at 1.5 mo and 6 mo of age (Fig. 1A). At 1.5 mo of age, body weight and blood pressure were not significantly different between WKY and SHR, whereas in both other age groups body weights were significantly lower (3 mo: 314 ± 27 g in WKY and 276 ± 21 g in SHR; 6 mo: 435 ± 15 g in WKY and 385 ± 18 g in SHR) and blood pressures were significantly higher (Fig. 1, A and B) in SHR than in WKY.

Anesthesia with ketamine-xylazine significantly lowered diastolic blood pressure in the 1.5-mo-old WKY as well as in 1.5, 3-, and 6-mo-old SHR (Fig. 1C). In the 3- and 6-mo-old WKY, diastolic blood pressure was not affected by this type of anesthesia. During anesthesia the original difference in diastolic blood pressure between SHR and WKY was abolished at 6 mo of age (Fig. 1C), as was the difference in pulse pressure at 3 mo of age (data not shown). At 1.5 mo and 3 mo of age, diastolic blood pressure was slightly lower in SHR than in WKY (Fig. 1C). Heart rate decreased between 1.5 and 3 mo of age from 355 ± 105 to 240 ± 25 beats/min in WKY and from 319 ± 25 to 256 ± 17 beats/min in SHR and was not significantly different between 3 and 6 mo of age in either strain. At 1.5, 3, and 6 mo of age, there was no significant difference in heart rate between SHR and WKY.

Aortic wall properties determined at about equivalent pressures in 1.5-, 3-, and 6-mo-old WKY and SHR are presented in Fig. 2. Compliance did not change between 1.5 and 6 mo of age in either strain. In all three age groups, compliance was significantly lower in SHR than in WKY (Fig. 2A). Between 1.5 and 3 mo of age, aortic distensibility decreased significantly in both strains. At 1.5 mo of age, distensibility was significantly lower in SHR than in WKY. There were no significant differences in distensibility between SHR and WKY at 3 and 6 mo of age (Fig. 2B). Incremental elastic modulus (Einc) increased significantly between 1.5 and 6 mo of age in both strains. At 3 mo of age, Einc was significantly smaller in SHR than in WKY (Fig. 2C).

In both SHR and WKY, Ddi measured between 1.5 and 6 mo of age (Fig. 3A). At 1.5 mo of age there was no significant difference in Ddi between SHR and WKY. At 3 and 6 mo of age, however, Ddi measured at compa-
rable $P_{\text{dia}}$ was significantly smaller in SHR than in WKY (Fig. 3A).

Between 1.5 and 6 mo of age, $\text{CSA}_i$ increased in both strains. At 1.5 mo of age $\text{CSA}_i$ was significantly larger in SHR than in WKY, but at 3 and 6 mo of age no significant differences in $\text{CSA}_i$ could be detected between both strains (Fig. 3B). MT did not significantly change between 1.5 mo of age in either strain. At 1.5 mo of age MT was significantly larger in SHR compared with WKY but not at 3 and 6 mo of age (Fig. 3C).

There were no significant changes in media collagen density as determined with morphometry on Sirius Red-stained sections between 1.5 and 6 mo of age in either strain (Table 1). The collagen densities did not differ significantly between SHR and WKY at 1.5, 3, or 6 mo of age (Table 1). In the media there were no significant changes in hydroxyproline fraction in the noncross-linked collagen residue between 1.5- and 6-mo-old SHR (Fig. 4A). In WKY, hydroxyproline fraction of the noncross-linked collagen residue was significantly larger at 1.5 mo compared with 3- and 6-mo-old WKY (Fig. 4A). At 1.5 mo of age hydroxyproline fraction of the noncross-linked collagen residue was significantly higher in WKY compared with SHR (Fig. 4A). In the

<table>
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<tr>
<th>Months in Age</th>
<th>15</th>
<th>3</th>
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<tbody>
<tr>
<td>WKY</td>
<td>12.1 ± 2.8</td>
<td>9.9 ± 3.3</td>
<td>9.9 ± 3.2</td>
</tr>
<tr>
<td>SHR</td>
<td>13.7 ± 5.2</td>
<td>16.1 ± 5.2</td>
<td>13.3 ± 3.9</td>
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Values are means ± SD (in %); $n = 12$ rats. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
The findings in the present study show that in SHR, in which hypertension develops gradually over weeks, alterations in aortic wall properties precede the development of hypertension. Distensibility and compliance of the thoracic aorta are lower in 1.5-mo-old SHR than in 1.5-mo-old WKY, whereas conscious systolic and diastolic blood pressures are not significantly different between these strains. The reduced distensibility and compliance most likely results from media hypertrophy rather than a change in intrinsic elastic properties. This conclusion is based on the findings that at the age of 1.5 mo, the incremental elastic modulus, a measure of elasticity that is independent of wall geometry and mass (29), is not significantly different between SHR and WKY, whereas the media is thicker and the media cross-sectional area is larger in SHR. This assumption is supported by the finding that cross-linked collagen content, a determinant of artery wall elasticity (6, 8, 17), is not significantly different between SHR and WKY. From the present study it cannot be concluded whether media hypertrophy in SHR developed after birth or was already present during fetal life. The reduced compliance of the thoracic aorta in SHR at 1.5 mo of age may contribute to the increase in systolic blood pressure at an older age in this strain, but one should keep in mind that changes in arteries with a diameter of <100 µm play a more important role in the development of systemic hypertension.

We deliberately performed the experiments during ketamine-xylazine anesthesia to be able to assess dynamic arterial wall properties at near-equivalent blood pressures. Xylazine is well known for its $\alpha_2$-adrenergic agonistic properties. The compound thereby 1) inhibits the activity of the vasomotor center in the central nervous system (43), 2) reduces peripheral adrenergic neurotransmission by a prejunctional inhibitory action (40), and 3) dilates the aorta through a local endothelium-dependent mechanism (39). After the administration of ketamine-xylazine, circulating catecholamine levels were reduced by 80–90% and, unlike in conscious restrained WKY, prazosin and sodium nitroprusside failed to dilate the aorta in vivo (unpublished results). Ketamine-xylazine anesthesia likely reduces the active component at arterial compliance, which is small under normal circumstances, in both SHR and WKY. That the blood pressure-lowering effect of ketamine-xylazine was far more marked in SHR than WKY is compatible with the hyperactivity of the sympathetic nervous system in SHR (7, 34). The slightly lower diastolic blood pressure in SHR than in WKY following ketamine-xylazine anesthesia at 1.5 and 3 mo of age does not affect the main conclusions of our study, because this difference in blood pressure leads to an underestimation of the differences in compliance and distensibility between the two strains.

The finding in the present study that in SHR hypertension does not develop in the first 6 wk of life is in agreement with the study of Christensen et al. (5) but at variance with the studies of others (10, 21). These
differences in the onset of hypertension development could be explained by intrastrain differences in SHR used by the various investigators and by different methods of blood pressure measurement. In their early work Okamoto and Aoki (26) referred to three general stages in spontaneous hypertension in the rat. The first stage was referred to as “prehypertensive” period, encompassing the first 40–50 days of life. At this stage blood pressure is not or only slightly increased, compared with WKY, but left ventricular mass is already significantly increased (10, 28). Also observed at this stage of life is hypertrophy throughout the whole vascular tree, on both the arterial and the venous side, including the thoracic aorta (10). These data and the data in the present study suggest that in SHR the early changes in arterial wall properties are not necessarily related to an increase in blood pressure. In other studies, however, left ventricular hypertrophy was found to follow an increase in blood pressure (12), and arterial wall hypertrophy was found to occur concomitantly with the rise in blood pressure (27).

The mechanism responsible for the increase in media mass in SHR is as yet unknown. From the findings in the literature the following mechanisms may be considered. Media hypertrophy could be the result of enhanced activity of the sympathetic nervous system, which is known to be present before the onset of blood pressure elevation (19). Moreover, in SHR sympathetic innervation density of the vasculature is increased (13), whereas the response to norepinephrine is enhanced already during the prehypertensive phase (20, 31). This overall increase in activation of and sensitivity to the sympathetic nervous system could induce hypertrophy of smooth muscle cells by its growth-promoting effect (1, 7). A possible role for the renin-angiotensin system cannot be excluded because Saavedra et al. (32) described an increase in angiotensin-converting enzyme in the aorta of prehypertensive SHR compared with WKY. Angiotensin II has a growth-inducing activity on vascular smooth muscle cells in cell cultures (9) and on large and small arteries even at subpressor doses (2, 3, 11). Inhibition of this system is associated with a reduction in medial thickness (44). There is increasing evidence that the vascular hypertrophic effects of the sympathetic system and of the renin angiotensin system may be intimately related (36).

During the state of elevated blood pressure (3 and 6 mo of age), at near-equivalent blood pressure, compliance of the thoracic aorta was significantly lower in SHR than in WKY, but no significant difference in media cross-sectional area and distensibility of the thoracic aorta could be observed between the two strains at 3 and 6 mo of age. Therefore, at this age differences in compliance are most likely caused by the significantly smaller diastolic diameters at near-equivalent pressures in SHR than in WKY. The observation that cross-sectional compliance does not change significantly with age in both SHR and WKY, despite a significant decrease in distensibility and loss of elasticity as indicated by the increase in incremental elastic modulus, indicates that cross-sectional compliance is the regulated parameter. It has been proposed that compliance is kept constant as possible with age by an increase in diameter at equivalent pressure (22, 29).

At the age of 1.5 mo, the reduced distensibility and compliance of the thoracic aorta in SHR, compared with WKY, has to be attributed to media hypertrophy due to an increase in muscle mass in SHR rather than a difference in wall structure and composition. The latter is indicated by the absence of a difference in total and cross-linked collagen content of the thoracic aorta. The finding at the age of 1.5 mo that the noncross-linked collagen content of the thoracic aortic wall is lower in SHR than in WKY may indicate that the rate of collagen turnover is lower in SHR. The noncross-linked collagen molecules do not have tight interactions with the existing extracellular matrix and, hence, are not able to possess tensile strength. Therefore, the noncross-linked collagen fraction will have very little or no influence on the mechanical properties of the aortic wall (37).

The loss of distensibility and elastic properties of the thoracic aorta, as indicated by the increase in elastic modules, with age in both WKY and SHR has likely to be ascribed to an increase in muscle mass, because the increase in media cross-sectional area with age is not associated with a change in total and cross-linked collagen content of the aortic wall. The finding in the present study that the total collagen content of the thoracic aortic wall as measured with collagenase is not increased in SHR is at variance with the observations of other investigators (23). This discrepancy could be explained by the significantly higher blood pressure levels in their study than in our study (17). It cannot be excluded that the tremendous variation in the individual collagen values (Fig. 4, A and B) contributes to this discrepancy.

In conclusion, the findings in the present study show that in SHR, alterations in functional properties of the thoracic aortic wall precede the development of hypertension. The reduction of distensibility and compliance before blood pressure increases most likely results from media hypertrophy rather than a change in intrinsic elastic properties and the composition of the wall.

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