Fenestrations of the Carotid Internal Elastic Lamina and Structural Adaptation in Stroke-Prone Spontaneously Hypertensive Rats

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Abstract—Our aim was to determine the structural factors that determine the mechanical adaptation of the carotid arterial wall in stroke-prone hypertensive rats (SHRSP). Distensibility-pressure and elastic modulus–stress curves assessed by in vivo echo-tracking measurements indicated a reduction in arterial stiffness in 13-week-old SHRSP compared with Wistar-Kyoto rats (WKY). Elastin and collagen contents determined biochemically were not different between SHRSP and WKY. Confocal microscopy showed that the mean area of fenestrations and fraction of area occupied by fenestrations of the internal elastic lamina (IEL) were smaller in SHRSP than in WKY, which indicated a reduction in stress-concentration effects within the IEL. Immunohistologic staining of EIIIA fibronectin isoform and total fibronectin (also as determined by Western blot) was greater in SHRSP, which suggested increased cell-matrix interactions. We suggest that these structural modifications of the vascular wall play a synergistic role in the mechanical adaptation to a high level of stress in SHRSP. (Hypertension. 2001;37:1101-1107.)

Key Words: arteries ■ elastin ■ lamina, internal, elastic ■ fenestrations ■ fibronectin ■ remodeling ■ hypertension, experimental

Spontaneously hypertensive rats of the stroke-prone sub-strain (SHRSP) are considered to be a good model for severe hypertension associated with increased cerebrovascular fragility.1 In elastic arteries, recent study has demonstrated that distensibility of the carotid artery is increased in SHRSP compared with that in Wistar-Kyoto rats (WKY) for a given arterial pressure level (AP).2 This finding suggests a mechanical adaptation of the arterial wall, which indicates qualitative or quantitative changes in arterial components.

Elastin plays a major role in determining mechanical properties of the vascular wall. Elastic lamellae of large arteries were fenestrated, as well illustrated by electron microscopy.3–5 More recently, confocal microscopy has shown that an enlargement of these fenestrations in the internal elastic lamina (IEL) during development contributes greatly to vascular wall remodeling induced by the increase in blood flow.6 The influence of these fenestrations may be explained by stress-concentration phenomena; enlarged fenestrations concentrate stresses in the immediately adjacent tissue, which induces vessel development.4,7 In chronic hypertension, mean circumferential wall stress is most often increased, despite the development of arterial wall hypertrophy.5,10 Consequently, an adaptive response that was able to limit stress-concentration effects in the IEL would be a reduction in size and total area of fenestrations in the IEL.

We have suggested that stress-induced activation of the muscle cell, which causes enhanced synthesis of the adhesion protein fibronectin (FN) in SHR, is such a response.11 By increasing cell-matrix attachment sites, the accumulation of FN may alter distribution of wall stress within the arterial wall and play an important role in regulation of elastic properties during chronic hypertension.

Thus, in the present study, our objectives were (1) to determine the intrinsic elastic properties of the arterial wall by evaluating the relationship between elastic modulus and circumferential wall stress, (2) to quantify the number and surface area of fenestrations in IEL by use of laser scanning confocal microscopy (LSCM), (3) to evaluate immunohistologic staining of FN, and (4) to compare these parameters in SHRSP and WKY.

We report a lower intrinsic stiffness of the wall, an accumulation of FN in the media, and a marked reduction in the size and fraction of area occupied by fenestrations in the IEL of carotid artery in SHRSP compared with WKY. We suggest that these structural changes are involved in the mechanical adaptation of the vascular wall in SHRSP.

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Methods

Animals

The study was performed in 13-week-old male SHRSP and WKY (n=22 per strain). SHRSP and WKY were obtained from the Glasgow colonies, which have been inbred since 1991 in the Department of Medicine and Therapeutics (University of Glasgow, UK). All animals were fed standard rat chow (AO4, UAR Villenomson-sur-Orge). All procedures were in accordance with institutional guidelines for animal experimentation.

The rats were divided into 3 groups. In the first group, we determined in vivo carotid artery stiffness and FN content. In the second group, we determined aortic elastin and collagen contents. In the third group, we studied fenestrations of the IEL.

Determination of Carotid Artery Stiffness

We simultaneously recorded arterial diameter (left common carotid artery) and blood pressure (right common carotid artery) in pentobarbital-anesthetized rats and determined arterial distensibility, incremental elastic modulus (£Einc), and circumferential wall stress (σ) as previously described. The distensibility-AP curve characterizes the behavior of the whole arterial wall, whereas the £Einc-stress curve characterizes the intrinsic elastic properties of the wall material. Internal arterial diameter, D, was measured with an ultrasonic echo-tracking device (NIUS-01, Asulab SA). The relationship between the AP, P, and the lumen cross-sectional area, Alcs, was fitted with the model of Langewouters et al by use of an arc tangent function and 3 optimal fit parameters (α, β, and γ):

\[ A_{lcs} = \frac{PD^2}{4} \left[ \frac{\pi}{2} + \tan^{-1}\left( \frac{P - \beta}{\gamma} \right) \right] \]

Arterial cross-sectional distensibility (S), σ, and £Einc are given by the following equations:

\[ S(P) = \frac{2A_{lcs}}{A_{lcs} - \Delta A_{lcs}} \left( \frac{P}{2} \right) \]

\[ \sigma = \frac{2A_{lcs} \times P}{A_{lcs}} \]

\[ £E_{inc} = \frac{3}{5} \left( \frac{A_{lcs}}{A_{lcs} - \Delta A_{lcs}} \right) \]

where Alcs is medial cross-sectional area (MCSA).

All parameters are computed within the physiological pressure range of each animal. Fixation is well known to modify the geometry of the arterial wall. Usually MCSA is measured after formaldehyde fixation under pressure. In our experiments, MCSA is measured in samples that underwent immunohistologic studies that are incompatible with formaldehyde. We used freeze-dried paraffin-embedded sections that maintain reactivity of tissue antigens better than do cryostat sections. In a preliminary study, we verified that the ratio of MCSA measured after freeze-drying to MCSA measured after formaldehyde fixation was similar (0.6) in animals from both strains. Values of MCSA were corrected by this factor for the calculation of £Einc-stress curves.

Quantification of Aortic Elastin and Collagen

Thoracic aortas were frozen in liquid nitrogen and stored at −80°C. Biochemical analysis was performed on the descending thoracic aorta after the aortic arch was discarded. Under a dissecting microscope, each aortic segment was cleaned of periadventitial tissue and opened longitudinally, and its length was recorded by use of a grid in the eyepiece. After removal of the adventitia, the elastin, collagen, and cell proteins were quantified on individual aortic medias without homogenization as previously described. Briefly, after delipidation and recording of dry weight, cell proteins are extracted with 0.1% SDS and extracellular proteins other than elastin are solubilized with hot 0.1N NaOH. The residue, elastin, is quantified by weighing, and the collagen present in the NaOH is quantified by determining hydroxyproline after hydrolysis. Results are expressed as percentage dry weight of aorta and as milligrams per centimeter of aorta.

FN Expression

For FN immunohistologic staining, 5-μm-thick freeze-dried paraffin-embedded sections of common carotid artery were used as previously described. We used the indirect immunoperoxidase technique. Briefly, samples were treated with mouse anti-FN antibodies (total FN, clone P1H11, Valbiotech; EIIIA isofrom, clone IST-9, Sera-Laboratory). After 3 washes in TBS, the biotinylated anti-mouse antibody (kit LSAB2, Dako Laboratories) was added. After 3 washes in TBS, the slides were incubated with streptavidin-peroxidase complex. The presence of peroxidase was revealed after incubation with diaminobenzidine. Controls were performed by omission of the first or second antibody.

To confirm the results of the immunohistochemistry experiments, a Western blot analysis of FN was performed following standard techniques described previously. Arterial tissue was extracted from the thoracic aorta and the carotid artery of SHRSP (n=4) and WKY (n=4). Total protein content was determined by the Bradford technique. Equal amounts (100 μg) of the denatured proteins were loaded per lane, separated on a 4% to 15% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were incubated with a mouse anti-human monoclonal antibody to all FN isoforms using a dilution of 1:1000 (Valbiotech). Subsequent analysis used an anti-mouse IgG peroxidase complex diluted at 1:5000 as a second antibody; chemiluminescence emitted from luminol oxidized by peroxidase was used as a detection method (enhanced chemiluminescence Western blotting detection system, Amersham).

Laser Scanning Confocal Microscopy

We used an Odyssey LSCM fitted with a UV and an argon-ion laser (Nanor Innova Instruments) coupled to a Nikon Optiphot microscope with a ×40 water-immersion objective (Nikon NA 1.15). Meta- morph software (Universal Imaging Corporation) was used for image acquisition and morphometric analysis as previously described. Arteries were pressurized in vitro with 10% formaldehyde-saline solution for 1 hour with a pressure-perfusion myograph (Living Systems), as previously described, at 100 or 170 mm Hg. All carotid arteries were incubated in vitro for 30 minutes with PSS that contained the nuclear dye Hoescht 33342 (0.01 mg/mL; Sigma Chemical Co). After arteries were washed out several times in PSS, the extremities of the arteries were discarded. Two pieces of the vessel were prepared from the central part of each artery opened longitudinally. Sections were mounted on a slide, endothelial side facing up, and were used to study (1) endothelial and smooth muscle cells (SMC) and (2) IEL fenestrations.

Lamellae are mainly composed of elastin, which has autofluorescent properties in the band of 500/560-nm wavelength and can be detected with the 488/515-nm wavelength of the LSCM. From each vessel, 10 images were captured for analysis. To avoid biased data or regions damaged by manipulation, the 10 regions were chosen on the basis of the presence of an intact endothelial layer, as follows. First, the endothelial nuclei, stained with Hoescht 33342, were outlined with the UV filter of the LSCM and the image captured. The filter then was changed to 488/515 nm and the image of the IEL below also captured. Finally, the filter was changed again to 364/400 nm and the first layer of SMC focused and captured.

Two values of fixation pressure (100 and 170 mm Hg) were chosen to evaluate the influence of pressure on fenestra size and number. Metamorph software was used to quantify the number and area of fenestrations in the images and fraction of wall area occupied by fenestrations.

Statistical Analysis

The different mechanical arterial parameters (£Einc and wall stress) of SHRSP were compared with those of WKY rats at mean AP (MAP). To compare £Einc-stress curves in SHRSP and WKY, we calculated...
the area between $E_{\text{inc}}$ axis and $E_{\text{inc}}$-stress curve within the range of $E_{\text{inc}}$ common to both groups (600 to 1400 kPa).

All values were averaged and expressed as mean±SEM. Unpaired Student’s t tests were performed to compare SHRSP to WKY. Differences were considered significant for values of $P<0.05$.

Results

Mechanical Properties of the Carotid Artery

Hypertensive rats had significantly lower body weight than did normotensive WKY. The Table and Figure 1 show the in vivo comparison of hemodynamic and carotid arterial parameters between the 2 strains. MAP and pulse pressure were significantly higher in SHRSP, with no change in heart rate.

For each group, diameter-AP and distensibility-AP curves were obtained within the common AP range of all rats. Figure 1 shows no overlapping of AP values between the 2 groups. Therefore, the diameter-AP and distensibility-AP curves of SHRSP were shifted to the right compared with those of WKY. SHRSP had significantly smaller diameter than WKY over the diastolic-systolic range (the systolic diameter of SHRSP was significantly lower than the diastolic diameter of WKY). The mean distensibility (calculated at each individual MAP) was significantly lower in SHRSP.

Figure 1c shows that the $E_{\text{inc}}$-stress curve of SHRSP was significantly shifted rightwards (45-kPa mean shift), which indicates decreased stiffness of the material composing the arterial wall in SHRSP. The increase in $E_{\text{inc}}$ at MAP of SHRSP compared with WKY is explained by the parallel increase in wall stress at MAP (Table).

Aortic Elastin and Collagen Contents

Figure 2 shows the elastin content in the media of the thoracic aorta for the 2 groups. When elastin is expressed as milligrams per centimeter aorta, SHRSP have higher elastin content compared with WKY. When elastin is expressed as a percentage of aortic dry weight, no significant difference is seen between the 2 strains, which indicates that the absolute increase in elastin in SHRSP is mainly related to the increase in aortic wall mass rather than a specific increase in elastin. However, the ratio of elastin to collagen was significantly higher in SHRSP compared with WKY because of a small relative decrease in collagen content in SHRSP.

LSCM Study of Elastic Lamella Structure

Figure 3 shows fenestrations of the IEL after fixation at 100 and 170 mm Hg in WKY and SHRSP. The IELs of WKY and SHRSP were perforated with elliptical fenestrations. In WKY, number of fenestrations per field was increased when the carotid arteries were fixed at 170 compared with 100 mm Hg, whereas this number was decreased in SHRSP fixed at 170 compared with 100 mm Hg. Mean area of fenestrations was lower in WKY after fixation at 170 compared with 100 mm Hg, whereas in SHRSP, this parameter was not significantly modified by fixation pressure. The fraction of area occupied by fenestrations tended to be smaller but was not significantly different in WKY and SHRSP fixed at 170 mm Hg versus control arteries fixed at 100 mm Hg.

Mean area and fraction of area occupied by fenestrations were always smaller in SHRSP than WKY, even when
carotid arteries from SHRSP fixed at 100 mm Hg were compared with WKY fixed at 170 mm Hg.

In the 2 strains, SMC nuclei located in layers next to endothelium were uniformly oriented perpendicular to the longitudinal axis of the vessel. No vascular smooth muscle disorganization existed in the media of SHRSP compared with WKY. Endothelial cell orientation was also normal in the 2 strains.

**FN Immunostaining and Western Blot**

In the carotid artery of SHRSP, the surface labeled positively for total FN and cellular EIIIA FN was significantly increased (by 2-fold and 4-fold, respectively) compared with WKY (Figure 4). Total aortic and carotid FN assessed by Western blot analysis was higher in SHRSP than in WKY (Figure 4).

**Discussion**

The aim of the present study was to determine in vivo mechanical properties of the carotid artery wall, FN expression, and the structure of IEL by use of LSCM in SHRSP and WKY. The new findings reported in SHRSP compared with WKY indicate that the vascular wall of SHRSP is adapted to a high level of wall stress through several mechanisms: (A) lower intrinsic stiffness of the arterial wall material, (B) reduction of stress-concentration phenomena in the IEL, and (C) increase in the amount of adhesion molecules within the media.

In the present study, distensibility-AP and $E_{inc}$-stress curves of the carotid artery were studied with a high-resolution echo-tracking device as previously described in rats. Determination of $E_{inc}$ and wall stress required determination of the MCSA. In the present study, MCSA was measured in freeze-dried tissue. Avoiding tissue fixation allowed the determination for each animal of both the $E_{inc}$-stress curve and immunohistologic staining for FN. Parallel to the present study, we have shown that measurement of MCSA was dependent on the technique used for preparation of the tissue. In both strains of rats, we have observed a ratio of 0.6 for MCSA after freeze-drying to MCSA after in vivo fixation. These modifications of MCSA were taken into account in the calculation of $E_{inc}$ and circumferential wall stress.

Arterial distensibility calculated at mean AP was significantly lower in SHRSP than in WKY. Due to the absence of any common AP range between the 2 groups, no direct comparison of distensibility at the same level of AP can be made between SHRSP and WKY. However, distensibility-AP curves of SHRSP were shifted upward compared with WKY. This confirms one of the results reported by Zanchi et al., who showed a similar shift of the in vivo distensibility of the carotid artery in halothane-anesthetized SHRSP compared with WKY. Whereas arterial distensibility evaluates the elastic properties of the artery as a hollow structure, $E_{inc}$ evaluates the elastic properties of the wall material independently of geometry. Because the spatial arrangement of wall materials is dependent on the level of circumferential wall stress, we compared $E_{inc}$ of SHRSP and WKY within a common range of circumferential wall stress. One of the main findings of the present study is that the $E_{inc}$-stress curve of SHRSP, within a common range of $E_{inc}$, was significantly shifted rightward compared with that of WKY: in SHRSP, identical values of $E_{inc}$ are obtained for higher levels of wall stress. This result indicates a higher elasticity of the wall material in SHRSP than in WKY. A decreased wall stiffness has been previously observed in vitro in aorta of SHRSP by applying a high level of longitudinal stress. As previously demonstrated in hypertensive humans and in SHR, the higher $E_{inc}$ in SHRSP is explained only by the higher level of circumferential stress. Despite a higher elasticity, mean $E_{inc}$ of the SHRSP is increased compared with WKY, which indicates a partial mechanical adaptation of the arterial wall in the former. The higher elasticity of the wall material contributes to a limiting of the increase in wall stiffness that results from the increase in wall stress. In the absence of such adaptive phenomena, we can estimate that the increase in $E_{inc}$ would exceed values compatible with cohesion of the vascular wall.

Elastin is a crucial determinant of mechanical properties in the large arteries. The organization of elastin in the remodeled vascular wall of hypertensive rats is still unclear. In accordance with other studies, relative elastin content was not significantly increased in SHRSP. The small absolute increase in elastin and the increase in the elastin/collagen ratio may have contributed to the increase in arterial elasticity as previously suggested in cerebral arterioles of SHRSP. However, we hypothesize that qualitative changes in elastic lamella structure may have occurred and that these changes may play a role in mechanical adaptation of the vascular wall in SHRSP. Elastin content is concentrated in cylindrical, fenestrated membranes, the most prominent of which is the IEL. The present study provides the first in situ visualization of fenestrations of the IEL in rat carotid arteries and especially in spontaneously hypertensive rats. Such visualization was possible because of the use of LSCM, which provides a rapid and accurate new method for determining the morphology of the elastic network of whole carotid arteries in fixed conditions.
An important finding of the present study was the decrease in the size of fenestrations and in fraction of area occupied by fenestrations in SHRSP compared with WKY. To determine the effect of pressure fixation on fenestra size and number, we studied fenestra morphology in WKY and SHRSP carotid arteries at 2 different fixation pressures: 100 and 170 mm Hg. In WKY carotid arteries, a significant decrease in fenestra size and an increase in number were observed at 170 mm Hg versus arteries pressurized at 100 mm Hg. In SHRSP arteries, no change in fenestra size and a decrease in fenestra number were observed with increasing distending pressure.

Fenestrae are defined by intensity of autofluorescent fibers below a definite threshold and by a minimal size in the same plane of focus below the endothelial layer. When the IEL is stretched at 170 mm Hg, more autofluorescent elastic fibers are discernible in the plane of focus. Therefore, large fenestrations appear to be fragmented and some of the small fenestrations disappear because they are below the minimal size for detection. This can explain the diverging evolution of fenestrations as distending pressure increases in WKY and SHRSP; ie, fragmentation of large fenestrations in WKY (decrease in size and increase in number) and lack of detection of some of the small fenestrations in SHRSP (decrease in number).

Whatever the pressure of fixation, size of fenestrations and especially fraction of tissue occupied by fenestrations were markedly reduced in SHRSP compared with WKY. In terms of mechanical properties, fenestrations in the IEL induce stress-concentration phenomena: in solid structures under tension, stresses are more elevated in the vicinity of holes, especially large holes. In the IEL, recent results have demonstrated that stress in the vicinity of the fenestrations may be many times higher than mean stress. Thus, any enlargement of fenestrations would lead to stresses...
that exceed the maximal circumferential stress acceptable and may cause rupture of the IEL leading to structural fragilization of the arterial wall. This theory has been implicated in the formation of microaneurysms at the bifurcation of human cerebral arteries. Therefore, the decrease in size of fenestrations observed in SHRSP could deter an excessive stress concentration in the IEL and thus represents an additional adaptive mechanism against increased mean wall stress.

In the present study, both total FN and EIIIA FN determined by immunohistochemistry were significantly increased in SHRSP compared with WKY. This increase is confirmed for total FN by Western blot analysis. This result has been previously demonstrated in the vascular wall of SHR but not in SHRSP. In addition, a previous study has shown that aortic FN mRNA measured by Northern blot analysis is increased in SHRSP compared with WKY. These observations together suggest that FN mRNA levels correlate with protein levels. We have suggested that activation of SMCs through the increase in wall stress was responsible for enhanced synthesis of FN. Accumulation of FN may exert a favorable mechanical effect in SHRSP: by increasing the cell-matrix interactions, FN may contribute to protect the wall components of SHRSP against mechanical deterioration (for instance, rupture of elastin fibers) through an increase in maximum acceptable circumferential wall stress.

Figure 4. FN protein analysis: immunostaining of sections of common carotid arteries of an SHRSP and a WKY with antibodies to total FN and EIIIA FN, showing that total FN and EIIIA FN staining were significantly increased in SHRSP (n=5) vs WKY (n=6). Bar=25 μm; *P<0.05. Bottom, Western blot showing a higher level of total FN in thoracic aorta and carotid artery of SHRSP vs WKY. First line represents purified rat plasma FN used as a control.
In conclusion, these results indicate that SHRSP have a lower intrinsic stiffness in arterial wall material compared with the WKY and are capable of resisting increased AP and increased circumferential wall stress by a limitation of stress-concentration phenomena and accumulation of adhesion molecules within the media.

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