Transient middle cerebral artery occlusion causes different structural, mechanical, and myogenic alterations in normotensive and hypertensive rats

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Submitted 8 February 2007; accepted in final form 27 March 2007

CEREBRAL BLOOD FLOW is maintained constant, independently of blood pressure fluctuations, as a result of autoregulation, a well-established phenomenon caused by a combination of neuronal, myogenic, and metabolic mechanisms (28). Myogenic control, also named myogenic response (5), contributes significantly to autoregulation in the cerebral circulation (13). The myogenic response is characterized by diminishing or increasing vessel diameter after an increase or a decrease of transmural pressure, respectively (36).

The structural and mechanical properties of vessels are also critical to the control of local blood flow. Collagen and elastin are, respectively, less and more distensible components of the vessel wall. Most studies on resistance arteries have focused on the alterations in collagen (21). Nevertheless, elastin is emerging as an important contributor to vascular dimensions and mechanical properties (3). Thus elastin reorganization may be crucial to vessel properties in several diseases.

After ischemia-reperfusion (I/R), the middle cerebral artery (MCA) from normotensive rats shows a decrease in myogenic tone and reactivity (10, 11) as well as an increase in wall stiffness (12) and wall thickness (11). However, the events responsible for altered vascular structure and mechanics after I/R have not been explored.

Chronic hypertension is associated with structural, mechanical, and myogenic changes in resistance vasculature in several animal models and in humans (21, 33). Few studies have reported structural and mechanical changes in MCA of spontaneously hypertensive rats (SHR) (23, 35, 39). Since hypertension is one of the major risk factors of stroke, studies on the contribution of hypertension to myogenic, structural, and mechanical vascular alterations induced by I/R will improve our knowledge of the mechanisms involved in ischemic damage.

In the present study, we analyzed the influence of hypertension on structural, mechanical, and myogenic alterations in MCA induced by I/R, using SHR and its reference control strain, Wistar-Kyoto rats (WKY).

MATERIALS AND METHODS

We used 13- to 15 wk-old male SHR (n = 22) and age-matched male WKY (n = 21) rats (Janvier, Madrid, Spain). Animals were housed under a 12:12-h day-night cycle and had free access to food and water before and after surgery. The research procedures conformed to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publications No. 85-23, Revised 1996), and to guidelines established by Spanish legislation regarding the use of experimental animals (RD 1201/2005). Experiments were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Measurement of blood pressure. Systolic blood pressure and heart rate were measured daily, 1 wk before the experiments, in a group of 13 wk-old conscious SHR (n = 6) and WKY rats (n = 6) by tail cuff, using a Nirepm 645 blood pressure system (Cibertec, Madrid, Spain). Rats were prewarmed to 34°C for 10–15 min before measurements, which were taken between 3:00 and 6:00 PM. Five to six measurements were recorded in 1 h, and the mean of the last three measurements was reported each day per animal. The mean value for the last 2 days before surgery was used.

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Surgical procedures and measurement of infarct volume. Focal brain ischemia was produced by 90-min intraluminal occlusion of the right MCA with reperfusion (24 h), as reported previously (8). Briefly, rats were anesthetized with halothane (4%) and intubated through the trachea for controlled ventilation. Mean arterial pressure was monitored, and body temperature was maintained at 37 ± 0.5°C during surgery. A heat-blunted filament (nylon monofilament 4/0; Sutures Aragó, Barcelona, Spain) was introduced through the carotid artery to the level where the MCA branches out. In addition, the ipsilateral common carotid artery was clamped. After 90 min, the filament was

Fig. 1. Effect of ischemia-reperfusion (I/R) on structural parameters from fully relaxed middle cerebral arteries (MCA) from Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). A and B: vessel diameter-intraluminal pressure. C and D: lumen diameter-intraluminal pressure. E and F: cross-sectional area (CSA)-intraluminal pressure. G and H: wall/lumen ratio-intraluminal pressure. Values are means ± SE; n = 6–8. *P < 0.05; **P < 0.01 vs. sham.
carefully removed and the clip on the ipsilateral common carotid artery was released to allow reperfusion. Following surgery, the rats were allowed to recover spontaneous breathing and were kept in their cages with free access to food and water. Sham-operated animals were used as controls. Rats were killed under halothane (4%) anesthesia at 24 h. The brain was removed and placed in cold Krebs-Henseleit solution (KHS) with the following composition (mM): 112.0 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.1 KH₂PO₄, 1.2 MgSO₄, 25.0 NaHCO₃, and 11.1 glucose. The MCA from both the right (ischemic) and the left (contralateral to ischemia) hemispheres were dissected under a surgical microscope and kept in cold KHS. The brain was then sliced in 2-mm-thick sections, which were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma Chemical, St. Louis, MO) for 10 min at 37°C, followed by overnight fixation with 4% paraformaldehyde (PFA; Sigma Chemical). Infarct volume was measured with an image-analysis system (Imaging Research, St. Catharines, ON, Canada).

Preparation of MCA. The myogenic, structural, and mechanical properties of the MCA were studied with a pressure myograph (Danish Myo Tech, model P100; J. P. Trading, Aarhus, Denmark), as previously described (7). Briefly, vessels were placed on two glass microcannulas and adjusted so that the vessel walls were parallel without stretching. Intraluminal pressure was raised to 140 mmHg, and the artery was unbuckled by adjusting the cannulas. The artery was then set to 70 mmHg pressure and allowed to equilibrate for 30 min at 37°C in KHS gassed with a mixture of 95% O₂ and 5% CO₂. The pressure at the origin was set to 10 mmHg because it is difficult to obtain accurate measures of diameter at lower pressures (4). A pressure-diameter curve was obtained by increasing intraluminal pressure in 20-mmHg steps between 20 and 120 mmHg. Internal and external diameters (DiCa and DeCa) were measured for 3 min at each intraluminal pressure. The artery was then set to 70 mmHg and allowed to equilibrate for 30 min at 37°C in calcium-free KHS (0 Ca²⁺: omitting calcium and adding 10 mM EGTA; Sigma Chemical) gassed with a mixture of 95% O₂ and 5% CO₂. A second pressure-diameter curve was obtained in 0 Ca²⁺-KHS, and Di0Ca and De0Ca were measured. Finally, the artery was set to 70 mmHg in 0 Ca²⁺-KHS, pressure-fixed with 4% PFA in 0.2 M phosphate buffer (pH 7.2–7.4) for 45 min, and stored in PFA (4%) at 4°C until confocal microscopy studies were performed.

Calculation of myogenic, structural, and mechanical parameters. Myogenic response percentages at each pressure were determined from 100 × DiCa/Di0Ca, where DiCa and Di0Ca are the internal diameters measured in active (2.5 mM Ca²⁺-KHS) and passive conditions (0 Ca²⁺-KHS), respectively. Slopes of the myogenic-pressure curves for individual vessels were determined by linear regression.

Wall thickness (WT), cross-sectional area (CSA), and wall/lumen (W/L) ratio were calculated as follows: WT = (De0Ca – Di0Ca)/2; CSA = (π/4) × [(De0Ca)² – (Di0Ca)²]; and W/L = (De0Ca – Di0Ca)/2D0Ca, where De0Ca is the external diameter observed for a given intravascular pressure in passive conditions.

Mechanical parameters were calculated as described by Baumbach and Heistad (4). Circumferential wall strain (ε) was calculated as (Di0Ca – Do0Ca)/Do0Ca, where Do0Ca is the internal diameter at 10 mmHg, measured under relaxed conditions. Circumferential wall stress (τ) was calculated as (P × D0Ca)/2WT, where P is the intraluminal pressure (1 mmHg = 133.4 Nm⁻²) and WT is wall thickness at each intraluminal pressure in 0 Ca²⁺ medium. Elastic

![Fig. 2. Effect of I/R on mechanical properties from fully relaxed MCA from WKY and SHR.](image)
modulus was calculated by fitting stress-strain data to \( \sigma = \sigma_{\text{orig}} \exp (\beta \varepsilon) \), where \( \sigma_{\text{orig}} \) is the stress at the original diameter (10 mmHg). The \( \beta \) value was used as an index of wall stiffness (34).

**Nuclei distribution by confocal microscopy.** Pressured-fixed intact arteries were stained with the nuclear dye Hoechst 33342 (0.01 mg/ml; Sigma Chemical) for 15 min. After washing was completed, arteries were mounted on slides with a well made of silicon spacers to avoid artery deformation. They were visualized with a Leica TCS SP2 (Wetzlar, Germany) confocal system fitted with an inverted microscope and argon and helium-neon laser sources with an oil-immersion lens [\( \times 63; \) excitation (Ex) 351–364 nm and emission (Em) 400–500 nm]. Stacks of serial optical slices (0.5 \( \mu m \) thick) were captured from the adventitia to the lumen of each artery. Individual images of the endothelial layer were also captured. MetaMorph image analysis software (Universal Imaging, Molecular Devices, Downingtown, PA) was used for quantification. The nuclei number was measured in the respective layer CSA (mm\(^2\)) \times 1 mm; total number of adventitial (AC) and smooth muscle cells (SMC) = no. of nuclei per stack \times no. of stacks per artery volume; and total number of endothelial cells (EC) was calculated per luminal surface area (LSA) of 1-mm-long artery, or

\[ \text{LSA} = \pi \left( \frac{\text{diameter}}{2} \right)^2. \]

**Elastin content and organization by confocal microscopy.** The content and organization of elastic fibers in the internal elastic lamina (IEL) were studied in intact pressure-fixed MCA using a Leica TCS SP2 confocal microscope on the basis of the autofluorescent properties of elastin (Ex 488 nm and Em 500–560 nm) (38). Stacks of serial optical sections (0.4 \( \mu m \) thick) were captured from each artery with a \( \times 63 \) oil-immersion objective using the 488-nm line of the confocal microscope. Two stacks of images of several regions were captured in each arterial segment. All the images were taken under identical conditions of zoom (\( \times 3 \)), laser intensity, brightness, and contrast.

Quantitative analysis was performed with MetaMorph image analysis software (Universal Imaging) as described previously (7, 17). From each stack of serial images, individual projections of the IEL were reconstructed, and total fenestrae number and area were measured. Fluorescence intensity values were used as estimate of elastin concentration, as previously described, following the assumption that the concentration of elastin has a linear relationship with fluorescence intensity (6).

**Statistical analysis.** All values are means \( \pm \) SE. The dependence of structural, mechanical, or myogenic properties on ischemia/strain was assessed using two-way analysis of variance (ANOVA) with repeated measures on the pressure factor, and predefined contrasts were included for appropriate comparisons. In the case of one single factor, unpaired Student’s \( t \)-test or one-way ANOVA followed by post hoc Tukey’s test was used for two or more than two groups, respectively. Data analysis was carried out with the SAS/STAT release 9.1 statistical package (SAS Institute, Cary, NC).

**RESULTS**

Systolic blood pressure was substantially higher (\( P < 0.001 \)) in SHR, as expected (WKY: 150.3 \( \pm \) 3.2 mmHg; SHR: 204 \( \pm \) 2 mmHg, \( n = 6 \)), but heart rate was similar in both strains (WKY: 397.1 \( \pm \) 11.8 beats/min; SHR: 384.4 \( \pm \) 8.3 beats/min, \( n = 6 \)). Although the age of the animals was similar, the body weight of WKY (400.9 \( \pm \) 11.4 g, \( n = 21 \)) was slightly greater (\( P < 0.001 \)) than SHR (341.1 \( \pm \) 3.7 g, \( n = 22 \)). Hypertensive rats showed a larger (\( P < 0.001 \)) infarct volume than corre-
sponding normotensive rats at 24 h (WKY: 63.1 ± 10.4 mm³, n = 11; SHR: 283.7 ± 23.7 mm³, n = 12).

Structural, mechanical, and myogenic properties of the MCA. Figure 1 shows the influence of ischemia on structural parameters under fully relaxed conditions (0 Ca²⁺-KHS). In WKY animals, I/R increased the vessel diameter (Fig. 1A), CSA (Fig. 1E), W/L (Fig. 1G), and WT (not shown) of the ischemic MCA compared with sham-operated rats. Nevertheless, lumen diameter (Fig. 1C) was not modified after I/R. Ischemic vessels of WKY rats also showed a decrease in wall stress (Fig. 2A) and stiffness (Fig. 2C), as shown by the diminished β value (sham: 8.18 ± 0.50, n = 6; ischemic: 6.36 ± 0.56, n = 8; P < 0.05) and the rightward shift of the stress-strain relationship. Lumen diameter in active conditions (2.5 mM Ca²⁺-KHS) was enlarged at all the perfusion pressures tested in ischemic vessels from WKY animals (Fig. 3A).

Myogenic response as a function of pressure revealed the extent of the constrictor tone. This parameter is calculated by internal diameter reductions in active relative to passive (0 Ca²⁺-KHS) conditions (Fig. 3, C and D). In ischemic vessels from WKY, the myogenic response (Fig. 3C) was decreased at lower perfusion pressure (10–60 mmHg) but recovered at pressures over 60 mmHg. Analysis of slopes of the myogenic response-pressure curves demonstrated that this parameter increases (P < 0.01) in ischemic (−0.203 ± 0.037, n = 8) compared with sham vessels (−0.045 ± 0.050, n = 6) from WKY, indicating that myogenic reactivity is not lost.

Mechanical (Fig. 2) but not structural (Fig. 1) properties were also altered in contralateral vessels of WKY ischemic rats compared with sham animals. Analysis of the stress-strain relationship showed that the stress-strain curve was shifted to the right (Fig. 2C) and that the β value diminished (sham: 8.18 ± 0.50, n = 6; contralateral: 6.30 ± 0.59, n = 7; P < 0.05) in contralateral compared with sham vessels, thereby indicating an increase in distensibility. Lumen diameter in active conditions (Fig. 3A) was significantly enlarged in contralateral compared with sham vessels. The slope of the myogenic response-pressure curve after I/R (Fig. 3C) was similar in contralateral (−0.085 ± 0.033, n = 7) and sham vessels (−0.045 ± 0.050, n = 6), again indicating that myogenic reactivity is preserved.

Comparison of sham vessels between strains showed that hypertension per se decreased (P < 0.05) lumen diameter and increased (P < 0.01) CSA, W/L (Fig. 1), and WT (not shown). Wall stress was smaller (P < 0.01) but wall stiffness (β value; WKY: 8.18 ± 0.50, n = 6; SHR: 9.64 ± 0.65, n = 7) was similar in SHR and WKY rats (Fig. 2). Furthermore, myogenic response and lumen diameter in active conditions were similar in both strains (Fig. 3).

In SHR, I/R caused a significant decrease (P < 0.05) in CSA (Fig. 1F), W/L (Fig. 1H), and WT (not shown), but vessel (Fig. 1B) and lumen diameters (Fig. 1D) remained unchanged in ischemic vessels compared with sham animals. Wall stress (Fig. 2B) was increased in SHR ischemic vessels. However, wall stiffness (β value; sham: 9.64 ± 0.65, n = 7; ischemic: 7.79 ± 0.67, n = 7; Fig. 2D), lumen diameter in active conditions (Fig. 3B), and myogenic response (Fig. 3D) were not significantly modified in SHR ischemic vessels. Furthermore, none of the structural (Fig. 1), mechanical (Fig. 2), and myogenic (Fig. 3) properties of contralateral vessels from SHR was affected by I/R.

Morphology of the vascular wall. The morphological measurements from intact vessels by confocal microscopy are reported in Fig. 4. I/R increased wall volume (Fig. 4A), adventitial volume (Fig. 4B), and the total number of AC (Fig. 4D) in ischemic vessels from WKY rats. However, media

![Image](image_url)
volume (Fig. 4C) and the number, length, and width of SMC and EC nuclei (results not shown) were not modified in these vessels. In contralateral vessels from WKY rats, I/R also increased wall volume (Fig. 4A), adventitial volume (Fig. 4B) and the total number of AC (Fig. 4D) without modifying the media volume (Fig. 4C) or the number, length, or width of SMC and EC nuclei (results not shown). The morphological alterations in contralateral vessels were less marked than in the ischemic vessels.

Hypertension per se increased wall volume (Fig. 4A), adventitial volume (Fig. 4B), and the total number of AC (Fig. 4D) without modifying media volume (Fig. 4C). In addition, the number, length, and width of SMC and EC nuclei (results not shown) were not modified by hypertension.

In SHR subjected to I/R, wall (Fig. 4A) and adventitial volume (Fig. 4B) were slightly decreased in ischemic compared with sham vessels, but the difference was not statistically significant. Media volume (Fig. 4C), the total number of AC (Fig. 4D), and the number, length, and width of SMC and EC nuclei (results not shown) were not modified in ischemic vessels from this strain. None of the above morphological parameters in SHR contralateral vessels was affected by I/R (Fig. 4).

**Elastin content and organization.** Quantification of the maximal intensity projection of IEL from sham, ischemic, and contralateral vessels showed that the total number of fenestrae was similar in all experimental conditions and strains (Fig. 5B). Ischemic vessels from the two rat strains showed a significant enlargement of fenestrae area (Fig. 5C). Nevertheless, in contralateral vessels, fenestrae area was significantly increased ($P < 0.001$) in WKY but not in SHR ($P = 0.16$) compared with sham vessels. Hypertension per se decreased fenestrae area, but the difference was not significant ($P = 0.06$). Average fluorescence intensity per pixel (results not shown) indicated a similar amount of elastin in all experimental conditions.

**DISCUSSION**

Our results demonstrate that the changes induced by I/R in MCA structure, mechanics, and myogenic behavior differ between SHR and WKY strains. In WKY rats, I/R induced vascular adventitial layer hypertrophy associated with an increase in vessel diameter, CSA, WT, and W/L. Only one study...
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(11) has reported an increase in WT in whole MCA of Sprague-Dawley rats after I/R, but no evidence of the cellular type involved in this hypertrophic response was reported. In the present study, we have demonstrated that the increase of cell number in the adventitial layer is responsible for increased WT in the MCA after I/R. Increasing evidence indicates that the adventitia, traditionally considered a structural support for the blood vessel, is a critical regulator of vessel wall function in health and disease (2, 19, 26, 32, 37) and may be considered the main “injury-sensing tissue” of the vessel wall. Increased fibroblast proliferation/deposition has been described after hypoxia (27). Therefore, a similar phenomenon may participate in the effect observed.

Along the same line of evidence, the decrease in CSA observed in the MCA of SHR after I/R appeared to be coupled to a small decrease in adventitial volume, since media volume and the number of AC were unchanged. Among the most abundant components of the adventitial layer are extracellular matrix proteins such as collagen and elastin. A decreased collagen volume has been reported in cerebral microvessels from baboons after I/R (15). Thus changes in extracellular matrix components may contribute to adventitial volume decrease in MCA of SHR after I/R.

It has been proposed that wall stress is the stimulus for growth, thereby leading to an increase in WT (14). After I/R, wall stress is reduced in WKY and enhanced in SHR. These results correlate with the increase and decrease observed in the CSA, respectively. In SHR, I/R did not modify vessel stiffness. However, this parameter was decreased in ischemic MCA of WKY rats, as shown by the rightward shift of the stress-strain curve and the smaller β value (which reflects arterial stiffness independently of vessel geometry). In contrast, other studies report an increase in vessel stiffness in normotensive Wistar rats after I/R (12). Although we do not have an explanation for the contrasting observations between studies, occlusion time (60 vs. 90 min) or rat strain (Wistar vs. WKY) might account for this discrepancy.

Several studies in rat arteries have demonstrated a relationship between fenestrae size and wall stiffness (3, 7, 17). Thus the increase in IEL fenestrae size observed after I/R might contribute to stiffness reduction. Vascular stiffness is determined mainly by collagen (20) and elastin content/distribution (7). A decrease in collagen volume in cerebral microvessels after I/R has been reported (15). Elastin is an important contributor to vascular dimensions and mechanical properties. Elastase increases the size of IEL fenestrae in small mesenteric arteries (17). Patients with acute cerebral infarction show an increase in polymorphonuclear neutrophil elastase (22). High neutrophil infiltration to the ischemic tissue occurs at 24 h post-I/R (25). Therefore, it is feasible that enhanced elastase levels and/or activity contribute to reduce the elastin content and to enlarge IEL fenestrae of the MCA after I/R in WKY rats. Fenestrae size increases after I/R in SHR, but to a lower extent than in normotensive rats. This milder increase was not related to changes in vessel stiffness in this strain. A structural defect in SHR may later prevent fenestrae from swelling sufficiently to influence vessel stiffness.

An important finding of our study is that in WKY, but not in SHR, I/R induces vasodilatation in ischemic vessels. Consistent with this observation, Cipolla et al. (10) observed a greater lumen diameter in active conditions in normotensive rats. It is known that in cerebral blood vessels, endothelium-derived hyperpolarizing factor plays a crucial role in vasodilatation (16). After I/R, the vasodilatation caused by this factor was enhanced in MCA from normotensive rats (31). Furthermore, the loss of tone after I/R was associated with a decrease in vascular smooth muscle F-actin (10), likely due to an increase of peroxynitrite production (29, 30). Moreover, the observation that I/R induced vasodilatation also in contralateral vessels from this rat strain supports the participation of systemic factor(s) derived from focal ischemia. Consistent with this observation, Cipolla et al. (10) observed a decrease of vascular tone after I/R in contralateral and ischemic MCA from normotensive rats. Cerebral stroke triggers an inflammatory process within the first few hours that has been associated with induced cytokine expression and increases in plasma cytokine levels (9, 1), which may participate in vascular tone. For instance, after I/R in rats, VCAM-1 was induced not only in the ischemic area but also in a remote organ such as the heart (24).

I/R did not alter the structural parameters of contralateral vessels in either rat strain. Nevertheless, the analysis of nuclei distribution revealed an increase in AC number of contralateral vessels, although to a lesser extent than in ischemic vessels, from WKY but not from SHR. Wall stiffness decreased in contralateral vessels from WKY ischemic rats, in concordance with the enlargement of IEL fenestrae observed in these vessels. The changes observed in contralateral vessels suggest the participation of systemic alterations induced by I/R in WKY rats.

The observation that ischemic vessels in WKY, but not in SHR, elicited lower tone (i.e., greater vasodilatation) and enhanced distensibility compared with sham-operated animals indicates a greater capacity for increasing cerebral blood flow during reperfusion in WKY. This finding, together with the hypertension-induced remodeling that diminishes lumen diameter, may contribute to maintenance of a better blood flow in WKY than in SHR, as previously reported (18), and helps to explain the larger infarct volume observed in SHR after I/R.

ACKNOWLEDGMENTS

We thank the Servei de Microscòpia at the Universitat Autònoma de Barcelona, L. Caracuel for assistance with blood pressure measurements, and Dr. R. Rycroft for the English revisions to the article.

GRANTS

This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (SAF2003-1001, SAF2004-06134) and Fondo de Investigaciones Sanitarias (FIS04-1295, FIS04-1104). F. Jiménez-Altayo and C. Justicia have fellowships from Generalitat de Catalunya. S. Rojas and A. Martin have fellowships from Ministerio de Educación y Ciencia and Fondo de Investigaciones Sanitarias, respectively.

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