Doxycycline, a matrix metalloprotease inhibitor, reduces vascular remodeling and damage after cerebral ischemia in stroke-prone spontaneously hypertensive rats

Paulo W. Pires, Curt T. Rogers, Jonathon L. McClain, Hannah S. Garver, Gregory D. Fink, and Anne M. Dorrance

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan

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Doxycycline, a matrix metalloprotease inhibitor, reduces vascular remodeling and damage after cerebral ischemia in stroke-prone spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 301: H87–H97, 2011. First published May 6, 2011; doi:10.1152/ajpheart.01206.2010.—Matrix metalloproteases (MMPs) are a family of zinc peptidases involved in extracellular matrix turnover. There is evidence that increased MMP activity is involved in remodeling of resistance vessels in chronic hypertension. Thus we hypothesized that inhibition of MMP activity with doxycycline (DOX) would attenuate vascular remodeling. Six-week-old male stroke-prone spontaneously hypertensive rats (SHRSP) were treated with DOX (50 mg·kg⁻¹·day⁻¹ in the drinking water) for 6 wk. Untreated SHRSP were controls. Blood pressure was measured by telemetry during the last week. Middle cerebral artery (MCA) and mesenteric resistance arteries (MRA) passive structures were assessed by pressure myography. MMP-2 expression in aortas was measured by Western blot. All results are means ± SE. DOX caused a small increase in mean arterial pressure (SHRSP, 154 ± 1 mmHg; P < 0.004 vs. 150.5 ± 3 mmHg; P < 0.05). Active MMP-2 expression was reduced in aorta from SHRSP + DOX (0.21 ± 0.06 vs. 0.49 ± 0.13 arbitrary units; P < 0.05). In the MCA, at 80 mmHg, DOX treatment increased the lumen (273.2 ± 4.7 vs. 238.3 ± 6.3 μm; P < 0.05) and the outer diameter (321 ± 5.3 vs. 290 ± 7.6 μm; P < 0.05) and reduced the wall-to-lumen ratio (0.09 ± 0.002 vs. 0.11 ± 0.003; P < 0.05). Damage after transient cerebral ischemia (transient MCA occlusion) was reduced in SHRSP + DOX (20.7 ± 4 vs. 45.5 ± 5% of hemisphere infarcted; P < 0.05). In the MRA, at 90 mmHg DOX, reduced wall thickness (29 ± 1 vs. 22 ± 1 μm; P < 0.001) and wall-to-lumen ratio (0.08 ± 0.004 vs. 0.11 ± 0.008; P < 0.05) without changing lumen diameter. These results suggest that MMPs are involved in hypertensive vascular remodeling in both the peripheral and cerebral vasculature and that DOX reduced brain damage after cerebral ischemia.

vascular remodeling; middle cerebral artery; hypertension

PRIMARY PREVENTION OF ISCHEMIC stroke is increasing in importance as a strategy for management of individuals at high risk. Recently published clinical trials (18), such as ONTARGET and TRANSCEND, show that therapies aimed at reducing cardiovascular incidents are a valuable tool for prevention of the first occurrence of myocardial infarction, heart failure, and stroke. In the ONTARGET trial, the beneficial effects observed were not preceded by a reduction in systemic blood pressure. Therefore, it is possible that a major component of the risk for ischemic stroke is not blood pressure itself. The consequences of vascular adaptation to increased intraluminal pressure might provide an additional risk. Hence, prevention of hypertensive vascular remodeling might be a candidate for primary prevention of stroke.

Damage caused by cerebral ischemia is related to the extent of hypertensive remodeling of the middle cerebral artery (MCA; Refs. 13, 14, 38, 42). Remodeling of the resistance vasculature encompasses structural changes that lead to a reduction in the lumen diameter and an increase in the wall thickness and wall-to-lumen ratio (2, 17, 23, 36). Together, these alterations may lead to impairment in the ability of vessels to autoregulate and dilate. In the cerebral vasculature, these impairments might cause a reduction in blood flow and an increase in ischemic damage (13). When one considers attenuation of vascular remodeling as a primary strategy for stroke prevention, it is important to identify agents that have a maximal effect on the cerebral vasculature and little effect on the periphery, to not decrease blood pressure or impair its control.

Vascular remodeling can be caused by smooth muscle cell hypertrophy or hyperplasia, deposition of extracellular matrix (ECM) elements, or a combination of these two factors (17). ECM turnover in the vessel wall is regulated in part by a family of zinc-dependent proteases known as matrix metalloproteinases (MMPs; Refs. 19, 40). Among them, MMP-2 and -9, also known as gelatinases A and B, are responsible for degradation of basement membrane elements (mainly collagen IV and laminin) and digestion of collagen I fibrils (gelatin) (21, 37). Increased MMP-2 activity in vessels has been reported in many models of hypertension and is associated with vascular remodeling (6, 12, 19). Doxycycline (DOX), a tetracycline antibiotic and nonspecific MMP inhibitor, was shown to prevent remodeling in aorta of the 2-kidney, 1-clip model of hypertension (4, 5). However, very little is known about the effects of DOX in the resistance vasculature of hypertensive rats and how those effects relate to end-organ damage. Therefore, we hypothesized that inhibition of MMPs with DOX would attenuate hypertensive remodeling in the cerebral vasculature of stroke-prone spontaneously hypertensive rats (SHRSP) and that the improvement in cerebrovascular structure would reduce damage induced by cerebral ischemia.

MATERIAL AND METHODS

Animals and treatment. Six-week-old male SHRSP were used for the experiments. Rats were split into two groups: one group received DOX in the drinking water for 6 wk [SHRSP + DOX, 50 mg·kg⁻¹·day⁻¹; n = 28: 10 were used for pressure myography, 5 for telemetry, 5 for transient MCA occlusion (tMCAO), and 8 for per-
MANIFEST MCA OCCLUSION (pmCAO). Untreated SHRSP were controls (n = 28; 12 were used for pressure myography, 5 for telemetry, 5 for pmCAO, and 6 for pmCAO). Twelve-week-old normotensive Wistar Kyoto (WKY) rats were randomized into two groups: untreated WKY (n = 4) and WKY + DOX (n = 7). They were used for passive vascular structure studies. These were included in the study to validate remodeling in the SHRSP and to evaluate possible blood pressure-dependent effects of DOX. DOX dosage was chosen based on the results of a pilot experiment using three doses: 25, 50, and 100 mg·kg⁻¹·day⁻¹. In that study, we observed that 50 mg·kg⁻¹·day⁻¹ exerted the best inhibition of MMP-2; thus we performed all further experiments using this dosage. Animals were maintained on a 12:12-h light-dark cycle, with regular chow and water available ad libitum. DOX water was prepared fresh daily. The experimental protocols were approved by the Michigan State University Institutional Animal Care and Use Committee in accordance with the American Physiological Society’s “Guiding Principles in the Care and Use of Animals.”

**Measurement of blood pressure.** Blood pressure was measured by telemetry in SHRSP + DOX and untreated SHRSP during the last week of experiment. The catheter of the telemeter (TA11PA40; Data Sciences International) was inserted into the distal aorta via the femoral artery, and the body of the transmitter was placed subcutaneously. Rats were allowed to recover for 1 week before the beginning of blood pressure recording. Blood pressure and heart rate were measured every 10 min over a 24-h cycle during the last week of treatment (30). Averages of the day and night period were used to calculate the daily blood pressure, and the average for the week is reported.

**MCA structure.** MCA structure was assessed using pressure myography as described previously (42). MCAs were isolated and placed in cold physiological salt solution (PSS; in mmol/l: 141.9 NaCl, 4.7 KCl, 1.7 MgSO₄, 0.5 EDTA, 2.8 CaCl₂, 10.0 HEPES, 1.2 K₂HPO₄, and 5.0 glucose). The first branch-free segment of the MCA most proximal to the circle of Willis was mounted on two glass micropipettes in a pressure myograph (Danish Myo Technology, Aarhus, Denmark). Vessels were bathed with warm oxygenated PSS at an intralumenal pressure of 80 mmHg, and the vessels were allowed to equilibrate for 20% spontaneous tone were discarded (9). Tone was calculated using the following formula: %tone = (1 – (active lumen diameter/passive lumen diameter)) * 100. The vasodilatory ability of MCA was assessed using increasing concentrations of Bradykinin (BK; 10⁻¹⁰ to 10⁻⁸M) added to the bath. The MCA was then washed to baseline, and the vasoconstrictor 5-hydroxytriptamine (5-HT; 10⁻⁹ to 10⁻⁶M) was added to the bath. The MCA was then washed to baseline, and tone generation was assessed by increasing the intraluminal pressures from 3 to 180 mmHg in 20-mmHg increments. MCA was allowed to equilibrate for 5 min at each new pressure before the measurement was taken. MCA passive structure was analyzed in calcium-free PSS containing 2 mM EGTA following the same pressure increments. Lumen diameter, external diameter, and wall thickness at each pressure were measured after a 5-min equilibration. The wall-to-lumen ratio, circumferential wall stress, and wall strain were calculated using previously described methods (3). The elastic modulus (β-coefficient) was calculated from the stress/strain curves for the individual vessels, and these curves were fitted to an exponential model (y = aeᵇx), where β is the slope of the curve: the higher the β-coefficient the stiffer the vessel.

**Mesenteric resistance artery structure.** Passive structure of mesenteric resistance artery (MRA) was analyzed under zero flow and calcium-free conditions as described for the MCA, except that the pressure was raised from 3 to 180 mmHg in 30-mmHg increments.

**MCAO.** For induction of cerebral ischemia, we used the intraluminal suture model developed by Longa et al. (33) as previously described by our laboratory (38, 41). All animals subjected to MCAO had DOX withdrawn 48 h before the procedure to avoid any possible acute effects of DOX in the outcome of cerebral ischemia (DOX half-life in rodents is ~4 h; Ref. 15). Rats were initially anesthetized with isoflurane in an induction chamber, and anesthesia was maintained with 2% isoflurane in oxygen; body temperature was maintained at 37°C. An incision was made in the top of the head to expose the skull for measurement of pial flow by scanning laser Doppler and attachment of a laser Doppler flow probe to measure blood flow to the region supplied by the MCA (5 mm lateral and 1 mm posterior to the bregma). A midline incision was made to expose the carotid artery. The lingual and thyroid arteries were cauterized, and the external carotid and pterygopalatine arteries were tied off with suture. A 3–0 nylon monofilament with a rounded end (Doccol, Redland, CA) was inserted into the common carotid artery. This monofilament was then advanced through the internal carotid artery to block blood flow to the MCA where it branches from the circle of Willis. MCA occlusion was verified by a drop in flow as measured by both scanning laser Doppler and the Doppler flow probe. One set of animals was subject to pmCAO, and after 24 h, rats were anesthetized and decapitated, and the brain was removed, sliced into 2-mm sections, and stained with 2% 2,3,5-triphenyltetrazolium chloride to assess ischemic damage. When using this technique, areas of viable tissue will exhibit a pink color, and areas of nonviable tissue will not develop color. Brains were fixed in 2% paraformaldehyde, and digital images of brain slices were taken. The percentage of infarction was determined by the following equation: %Hemisphere Infarcted = (1 – (VC – VL)/VC) * 100, where VC is the volume of normal tissue in the nonischemic hemisphere and VL is the volume of normal tissue in the ischemic hemisphere (48).

Another set of rats was subjected to transient ischemia (tmCAO). The MCAO was performed as described above, and ischemia was maintained for 1 h followed by 23 h of reperfusion. After that, the brain was removed and measurement of ischemic damage was performed as described above.

**Table 1. Final body weight, heart-to-body weight ratio, kidney-to-body weight ratio, and blood pressure values at the end of 6 wk of treatment**

<table>
<thead>
<tr>
<th></th>
<th>SHRSP</th>
<th>SHRSP + DOX</th>
<th>WKY Rats</th>
<th>WKY Rats + DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight</td>
<td>261 ± 7</td>
<td>266 ± 4</td>
<td>265 ± 5</td>
<td>270 ± 6</td>
</tr>
<tr>
<td>Heart-to-body weight ratio</td>
<td>0.50 ± 0.02*</td>
<td>0.44 ± 0.01†</td>
<td>0.32 ± 0.01</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>Kidney-to-body weight ratio</td>
<td>0.97 ± 0.02*</td>
<td>1.00 ± 0.02*</td>
<td>0.67 ± 0.01</td>
<td>0.73 ± 0.01‡</td>
</tr>
<tr>
<td>Systolic arterial pressure</td>
<td>184 ± 3</td>
<td>193 ± 3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Diastolic arterial pressure</td>
<td>128 ± 2</td>
<td>135 ± 2‡</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>156 ± 3</td>
<td>164 ± 2†</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Heart rate</td>
<td>311 ± 5</td>
<td>321 ± 1‡</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data were analyzed by one-way ANOVA. DOX, doxycycline; N/A, not applicable. *P < 0.05, statistically different from normotensive Wistar Kyoto (WKY) rats; †P < 0.05, statistically different from untreated stroke-prone spontaneously hypertensive rats (SHRSP); ‡P < 0.05, statistically different from untreated WKY rats.
Scanning laser Doppler measurement of pial blood flow. Pial blood flow was measured by scanning laser Doppler (PeriScan PIM 3, Perimed, Stockholm, Sweden). While the rat was under anesthesia, the skull was exposed and cleaned. The scanning laser Doppler was positioned 18 cm above the skull, and pial blood flow was analyzed in both cerebral hemispheres. The wavelength of the laser light is 670–690 nm with a penetrating depth of 0.5–1 mm. A total of 4 consecutive scans were performed at each time point. The time-points were as follows: before surgery, immediately after MCAO, before and after reperfusion (on animals subjected to tMCAO), and immediately before euthanasia. Mean perfusion in each hemisphere was measured using the LDPIwin 3.1 software (Perimed). Pial blood flows in the ischemic and nonischemic hemispheres are expressed as a percentage of preischemic pial blood flow.

Western blot. To validate MMP inhibition with DOX, Western blot analysis of MMP-2 expression was performed. Aortas were excised, cleaned of perivascular adipose tissue and blood, and snap-frozen in liquid nitrogen. Tissue was homogenized using a mortar and pestle in RIPA buffer containing a cocktail of protease inhibitors. The protein concentration in the sample was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Aliquots of aorta supernatants containing 50 μg of protein were resolved by SDS-PAGE and transferred to a PVDF membrane for Western blot analysis. DOX treatment reduced the expression of active-MMP-2, but not pro-MMP-2, in the aorta of stroke-prone spontaneously hypertensive rats (SHRSP). Data are shown as a ratio of MMP-2 expression to β-tubulin expression (loading control). *P < 0.05, Student’s t-test. Number of animals: SHRSP = 4, SHRSP + DOX = 4.

Fig. 1. Doxycycline (DOX) treatment reduced the expression of active-matrix metalloprotease-2 (MMP-2) in the aorta of SHRSP. Aliquots of aorta supernatants containing 50 μg of protein were resolved by SDS-PAGE and transferred to a PVDF membrane for Western blot analysis. DOX treatment reduced the expression of active-MMP-2, but not pro-MMP-2, in the aorta of stroke-prone spontaneously hypertensive rats (SHRSP). A. Data are shown as a ratio of MMP-2 expression to β-tubulin expression (loading control). B: representative images of blots. *P < 0.05, Student’s t-test. Number of animals: SHRSP = 4, SHRSP + DOX = 4.

Fig. 2. DOX treatment did not alter middle cerebral artery (MCA) reactivity to 5-hydroxytryptamine (5-HT; A) and bradykinin (BK; B) and did not alter MCA myogenic response to increases in intralumenal pressure (C). MCAs were maintained at 80 mmHg of intralumenal pressure, and drugs were added to the bath in a cumulative fashion. The vessel was allowed to equilibrate for 10 min at every dose to reach steady state. To assess myogenic tone (C), intralumenal pressure was increased from 3 to 180 mmHg in 20-mmHg increments, and the vessel was allowed to equilibrate for 5 min at each pressure before measurement was taken. Values are means ± SE. Data were analyzed by two-way repeated-measures ANOVA.
loading control. The intensity of the bands was analyzed using ImageJ software and is expressed as a ratio of β-tubulin.

**Gelatin zymography.** Plasma MMP activity was measured by gelatin zymography. Briefly, aliquots of plasma containing ~35 µg of protein were resolved in 8% SDS-PAGE under reducing conditions. The gels were then incubated in 2.5% Triton X-100, three times for 15 min each to allow for protein renaturation. The gels were then washed with 50 mmol/l Tris buffer, pH 7.4, and incubated with Zymogram development buffer (Bio-Rad, Hercules, CA) for 20 h at 37°C. The gels were stained with 0.5% Coomassie blue for 30 min and destained (30% methanol, 30% acetic acid, and 40% water). Areas of gelatino-lytic activity were identified as white bands against a dark background. Gels were scanned using Odyssey Infrared Imaging System (Li-cor Biosciences, Lincoln, NE) for quantification of the area of the bands.

**Thoracic aorta collagen and elastin content.** Total collagen content in the vessel wall was measured using Picrosirius red and polarized light as described previously (28, 29). The data are presented as a ratio between collagen area and wall area. Elastin fibers were identified using an elastin stain kit (Richard-Allan Scientific, Kalamazoo, MI), following the manufacturer’s guidelines. Data are presented as a ratio between area of elastin fibers and wall area.

**Statistics.** All results are represented as means ± SE. Vascular structure data were analyzed by two-way repeated measures ANOVA with a Bonferroni post hoc test. All other data were analyzed using Student’s t-test. A P value ≤0.05 was considered significant.

**Chemicals and supplies.** Unless otherwise stated, all chemicals and supplies were purchased from Sigma Chemical (St. Louis, MO).

**RESULTS**

**General findings.** Final body weight and heart and kidney weights are summarized in Table 1. There were no differences in final body weight in rats from all groups. The heart-to-body weight ratio, an indicator of cardiac hypertrophy, was increased in SHRSP compared with WKY rats ± DOX (P < 0.001), and DOX treatment prevented the cardiac hypertrophy (P = 0.02). The kidney-to-body weight ratio was increased in SHRSP compared with untreated WKY rats; DOX did not attenuate the kidney hypertrophy in SHRSP, but it caused a slight kidney hypertrophy in WKY rats + DOX (P < 0.001).

**Blood pressure measurements.** Heart rate, systolic, diastolic, and mean arterial pressures measured by telemetry are shown in Table 1. Surprisingly, DOX treatment caused a small but significant increase in SHRSP in all the parameters analyzed (P < 0.01).

**Western blot.** To validate MMP inhibition after DOX treatment, we performed Western blots using an antibody specific to MMP-2 in homogenates of SHRSP aorta. As expected, DOX treatment decreased the concentration of active-MMP-2 (P = 0.03) without changing the concentration of pre-MMP-2 (Fig. 1).

**MCA reactivity and tone generation.** Constriction of MCA to 5-HT and dilation to BK were not changed by DOX treatment in SHRSP (Fig. 2, A and B). No changes in myogenic tone generation over a range of intralumenal pressures were observed (Fig. 2C).

**MCA passive structure.** Passive structure of MCA was measured under calcium-free and zero flow conditions. Remodeling was observed in the MCA of SHRSP as a reduction in outer and lumen diameter and an increase in wall thickness and wall-to-lumen ratio compared with normotensive WKY rats. DOX treatment attenuated hypertensive remodeling of the MCA in SHRSP. Outer (A) and lumen diameter (B) were increased in SHRSP + DOX and were not different from Wystar Kyoto (WKY) rats ± DOX. Wall thickness was increased in SHRSP ± DOX compared with WKY rats ± DOX (C). The wall-to-lumen ratio was improved by DOX treatment, even without decrease in wall thickness (D). Measurements were obtained from cannulated MCAs using pressure myography under zero flow and calcium-free conditions. Values are means ± SE. Data were analyzed by two-way repeated-measures ANOVA. *P < 0.001.
rats, treated or not with DOX (Fig. 3, A–D). DOX treatment attenuated MCA remodeling in SHRSP, as shown by an increase in the outer and lumen diameter (Fig. 3, A and B) and the outer and lumen cross-sectional area (CSA; Fig. 4, A and B). DOX treatment also decreased the wall-to-lumen ratio (Fig. 3D) over the range of intraluminal pressures analyzed. The MCA stress was higher in SHRSP + DOX and WKY rats ± DOX at intraluminal pressures >60 mmHg without any statistical differences in vessel strain (Fig. 4, C and D). Interestingly, no differences were observed in vessel distensibility (Fig. 5A) and stiffness (β-coefficient: 8.8 ± 1.6 vs. 8.01 ± 0.5, SHRSP vs. SHRSP + DOX; Fig. 5B). DOX did not alter MCA passive structure in WKY rats.

MRA passive structure. In the SHRSP, MRA remodeling was observed as an increase in wall thickness, wall CSA, and wall-to-lumen ratio compared with normotensive WKY rats ± DOX (Fig. 6, A–C, respectively) without changes in outer (at 90 mmHg: 320 ± 10 vs. 311 ± 20 μm, SHRSP vs. WKY rats) and lumen diameter (at 90 mmHg: 263 ± 10 vs. 280 ± 20 μm, SHRSP vs. WKY rats). DOX treatment prevented the increase in wall thickness and wall CSA, thus leading to a decrease in wall-to-lumen ratio in SHRSP (Fig. 6, A–C, respectively). However, as with the MCA, no differences were observed in vessel distensibility (at 90 mmHg: 73 ± 5 vs. 83 ± 7, SHRSP vs. SHRSP + DOX) or stiffness (β-coefficient: 5.53 ± 0.46 vs. 5.62 ± 0.44, SHRSP vs. SHRSP + DOX). DOX did not change MRA structure in normotensive WKY rats.

Cerebral ischemia. DOX treatment in SHRSP did not change infarct size after pMCAO (57.6 ± 4.1 vs. 57.4 ± 3.7% hemisphere infarcted, SHRSP vs. SHRSP + DOX; Fig. 7A). In addition, postischemic and 24 h postischemic mean pial blood flow was not altered by DOX treatment (Fig. 7B). Interestingly, DOX treatment reduced the brain damage caused by tMCAO in SHRSP. As shown in Fig. 8A, SHRSP + DOX had a 50% reduction in the infarcted area in the brain (45.5 ± 4.7 vs. 20.7 ± 3.8% hemisphere infarcted, SHRSP vs. SHRSP + DOX; P < 0.05). The reduction in infarct was accompanied by an increase in the pial blood flow 23 h after reperfusion in the ischemic hemisphere (Fig. 8B). Pial blood flow was not altered by DOX treatment at any other time point analyzed in the ischemic hemisphere (Fig. 8B). In addition, blood flow was not different between SHRSP and SHRSP + DOX in the nonischemic hemisphere at any time points analyzed (Fig. 8C).

Gelatin zymography. Activity of both the pre- and active forms of MMP-2 in the plasma of the animals subjected to pMCAO (Fig. 7C) or tMCAO (Fig. 8E) was not altered by DOX. Surprisingly, no MMP-9 activity was detected in the plasma of these animals.

Thoracic aorta collagen and elastin content. Collagen content in the thoracic aorta was not changed by DOX treatment (0.064 ± 0.008 vs. 0.060 ± 0.008 area collagen/wall area, SHRSP vs. SHRSP + DOX). Similarly, DOX treatment did not alter elastin content (0.21 ± 0.01 vs. 0.22 ± 0.1, area elastin/wall area, SHRSP vs. SHRSP + DOX). As a consequence, the collagen to elastin ratio was not affected by DOX treatment (0.31 ± 0.05 vs. 0.29 ± 0.10, SHRSP vs. SHRSP + DOX).

Fig. 4. DOX treatment attenuated hypertensive remodeling of the MCA. Outer (A) and lumen (B) cross-sectional area (CSA) were significantly increased in the MCA from SHRSP + DOX compared with untreated SHRSP, and not different from WKY rats ± DOX. Intraluminal stress was higher in WKY rats ± DOX than SHRSP + DOX and untreated SHRSP at intraluminal pressures >60 mmHg (C). SHRSP + DOX showed higher intraluminal stress than untreated SHRSP (C). Strain was not different between the experimental groups (D). Measurements were obtained from MCAs using pressure myography under no-flow and zero calcium conditions. Values are means ± SE. *P < 0.001, by two-way repeated-measures ANOVA.
**DISCUSSION**

Ischemic stroke is the major cause of adult disability in the United States. The only FDA-approved pharmacotherapy for ischemic stroke can be administered to only 4.5% of stroke patients. With very few available options for the treatment of stroke, to reduce stroke risk it seems prudent to identify therapies aimed at primary prevention. We have shown previously that improving MCA structure reduces the damage caused by cerebral ischemia (13, 41, 42). In the present report, we show that chronic DOX treatment attenuates the hypertensive vascular remodeling observed in SHRSP. This was associated with an increase in pial blood flow in the infarcted hemisphere 23 h after reperfusion and decreased damage after tMCAO. Although tetracycline antibiotics are in clinical trials as acute therapies for stroke treatment (16), this is the first study showing beneficial protective effects to the brain and vasculature after chronic DOX treatment. Importantly, the beneficial effects on stroke outcome are not caused by an acute effect of DOX, since it was withdrawn 48 h before the induction of cerebral ischemia.

MMP activity is triggered in the vessel wall by multiple stimuli. Alterations in flow (1) and increases in transmural pressure (7) stimulate MMP-2 and -9 activity in blood vessels. In addition, vasoactive compounds, particularly the components of the renin-angiotensin-aldosterone system, are linked to

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**Fig. 5.** MCA distensibility and stiffness were not changed by DOX treatment. MCA from SHRSP had less distensibility than MCA from WKY rats, and DOX treatment did not change distensibility in either strain (A). Elastic modulus of the MCA (β-coefficient) was not increased in hypertensive animals compared with normotensive WKY rats (B; number of animals: SHRSP = 12, SHRSP + DOX = 9, WKY rats = 4, WKY rats + DOX = 7). Measurements were obtained from cannulated MCAs using pressure myography under zero flow and calcium-free conditions. Values are means ± SE.

**Fig. 6.** Chronic hypertension induces wall hypertrophy in mesenteric resistance artery (MRA), and this was prevented by DOX treatment. MRA wall thickness (A), wall CSA (B), and wall-to-lumen ratio (C) were increased in untreated SHRSP compared with WKY rats ± DOX. DOX treatment attenuated this increase in wall mass in SHRSP, although wall thickness and wall CSA were higher in SHRSP + DOX than WKY rats ± DOX. Measurements were obtained from cannulated MCAs using pressure myography under zero flow and calcium-free conditions. Values are means ± SE. *P < 0.001, two-way repeated-measures ANOVA.

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increased MMP activity. Angiotensin II stimulates MMP-2 and -9 expression in the rat thoracic aorta, independently of pressure (45), and MMP-2 gene expression is increased in aldosterone-induced hypertensive rats (46). We have shown that the renin-angiotensin-aldosterone system is an important factor involved in the MCA remodeling observed in SHRSP (14, 42). It is possible that one of the mechanisms underlying aldosterone-dependent remodeling in the SHRSP is an upregulation of MMP activity.

Many studies show that the MCA from hypertensive animals undergoes inward remodeling, which includes a reduction in lumen diameter (13, 14, 22, 25, 41). This process begins in hypertensive rats early in the development of the disease (26). These changes in MCA structure can be an adaptive mechanism to protect the vessel from increased stress (20) and prolonged vasoconstriction (47) observed during the rise in blood pressure. Independently of the etiology, the rearrangement of the wall components will ultimately lead to a reduction in lumen CSA that may or may not be accompanied by wall hypertrophy. In this scenario, MMPs might play an important role, since they are the primary enzymes involved in ECM turnover within the vessel wall (21). In the SHRSP, the blood pressure rises exponentially between 6 and 12 wk of age (42). During this time, the remodeling process is highly active making this period attractive for interventions aimed to reduce vascular remodeling.

In the present study, we show that treatment of SHRSP with DOX attenuates MCA remodeling, observed as an increase in the MCA outer and lumen diameters, an increase in outer and lumen CSA, and a reduction in wall-to-lumen ratio. Interestingly, vessel stiffness and distensibility were not changed by DOX treatment, suggesting that the alterations in structure observed are not due to changes in ECM composition. This is corroborated by the observation that the collagen and elastic fiber content in the aorta was not changed by DOX treatment despite the reduction in active-MMP-2 expression. We recognize that aortas are neither a resistance nor a cerebral blood vessel. However, they were used in this study to provide a measurement of MMP activity in the same animals used for vascular structure studies. MCA wall thickness was not reduced by DOX treatment. This is in agreement with our previous findings using the mineralocorticoid receptor antagonist spironolac-
tone (42) and potassium supplementation (41). The increase in MCA wall thickness in SHRSP is an adaptive response to the increase in shear stress due to elevated intraluminal pressure. Thus, in DOX-treated SHRSP, the MCA remains protected against increased intraluminal shear stress as result of the slight increase in blood pressure. Importantly, none of the DOX-treated animals showed any evidence of intracerebral hemorrhage, an important consequence of elevation in blood pressure in SHRSP. DOX did not cause any alteration in MCA passive structure in normotensive WKY rats, suggesting that it attenuates vascular remodeling when a stimulus such as increased intraluminal pressure is present.
Interestingly, the remodeling process seems to be different in the MRA compared with the MCA, since DOX had no effect on MRA lumen diameter. The reasons underlying these differences in remodeling behavior are not clear, but structural differences between these vessels might account for the disparity, at least in part. The MCA does not have an external elastic lamina (32), whereas the MRA does. Moreover, the MRA is surrounded by perivascular adipose tissue and the MCA is not. Perivascular adipose tissue is associated with vascular inflammation and reactive oxygen species generation in the vasculature (34), and both processes are linked to remodeling (24, 49). Lastly, the physiological role of the vascular bed in regulation of systemic blood pressure might also account for the differences. The mesenteric bed receives 15–20% of the cardiac output, thus contributing largely to total peripheral resistance (TPR) and blood pressure regulation. Since lumen diameter is a major determinant of TPR (36), it is possible that the lack of increase in lumen diameter is a consequence of the maintenance of TPR to prevent drops in blood pressure. However, to evaluate this we would need to study tone generation and reactivity of these vessels, and this was not possible in the current study. Despite that, the results observed in the MRA are still positive, since we did not observe reduction in blood pressure in SHRSP + DOX.

We (13, 14, 42) and others (39) have shown that MCA passive structure is associated with the extent of damage following cerebral ischemia. During occlusion of the MCA, collateral vessels dilate to supply blood to the ischemic hemisphere in an attempt to reduce tissue hypoxia. In the SHRSP, the ability of the collateral vessels to dilate in response to ischemia is impaired (11). In animals subjected to pMCAO, this vasodilator mechanism is one of the major factors that modulate the extent of cerebral damage. In our experiment, DOX treatment had no effect on pial blow flow after pMCAO. Importantly, tone generation and BK-induced vasodilation in the MCA were not changed by DOX treatment despite the improvement in MCA structure. Thus it is possible that when facing hypoxia the collateral vessels still exhibited impaired vasodilation, resulting in no reduction in infarct size.

Interestingly, DOX treatment reduced brain damage after tMCAO and improved pial blood flow in the ischemic hemisphere 23 h after reperfusion. This improvement could be explained by the fact that the MCA lumen diameter in DOX-treated SHRSP was greatly increased. In this model of cerebral ischemia, the blockage of the MCA was removed after 60 min, allowing reperfusion of the vessel. During reperfusion, the ability of the MCA to generate tone is diminished (8, 10) and vasodilation is augmented (35). Together, these responses might lead to a maximal dilation of the MCA. Blood flow under these conditions might be determined mainly by the lumen diameter of the cerebral vasculature. In fact, pial blood flow in the ischemic hemisphere was greater in SHRSP + DOX 23 h after reperfusion than in untreated SHRSP. Interestingly, in SHRSP with or without DOX, pial blood flow 23 h postreperfusion was less than preischemic blood flow. This could be a direct consequence of impaired vasodilation of the collateral circulation. Other factors such as hypotension and microvascular obstructions could also reduce blood flow. The current study does not discard these possibilities. Importantly, this occlusion of the MCA was similar in both groups, since the pial blood flow immediately after MCAO was not different between groups and we observed a similar drop in blood flow in the MCA territory by laser Doppler. In addition, plasma MMP-2 activity was not different between the groups, suggesting that MMP inhibition was not present 24 h after ischemia.

One caveat to our measurement of blood flow is that it was measured through an intact skull. Therefore, the perfusion units reported are a combination of skull, pial, and cortical blood flow. We opted not to perform a craniotomy to produce as physiologically relevant a stroke as possible. The rats used were 12 wk old and have relatively thin skulls; hence, thinning of their skulls was not necessary. In addition, the raw perfusion in the ischemic core is almost undetectable when analyzed by scanning laser Doppler, suggesting that the contribution of flow from the skull and dura is minimal.

Other mechanisms might account for the reduction in infarct after tMCAO in SHRSP + DOX. Tetracycline antibiotics such as DOX have important anti-inflammatory activities, and this could potentially reduce the damage after ischemia (27). Even though DOX was not present in the animals at the time of ischemia, it is possible that the anti-inflammatory effects are longer lasting. Another important effect is protection of the blood-brain-barrier (BBB). MMPs have been implicated in BBB disruption following transient ischemia (31, 43) and hemorrhagic transformation after reperfusion (44). The potential that BBB breakdown is reduced in SHRSP + DOX warrants further investigation.

In summary, the present study shows that DOX treatment attenuates hypertensive vascular remodeling despite the small increase in blood pressure associated with DOX treatment. Moreover, it reduces the damage caused by tMCAO. Further studies need to be performed to elucidate the underlying mechanisms of these effects.

Ischemic stroke is the major cause of adult disability in the United States and the third leading cause of death. Therapeutic options for patients with cerebral ischemia are few. All the treatments consist of removal of the clot, either pharmacologically or mechanically, and they have intrinsic risks. Hence, therapies aimed at primary prevention of cerebral ischemia...
might become an important tool for management of patients at high risk, such as in the case of hypertensive patients with uncontrolled blood pressure. To this end, development of strategies to attenuate cerebrovascular remodeling would be valuable. In this context, DOX might be useful, since it is a well tolerated and inexpensive tetracycline antibiotic that could be administered chronically.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).


