Relative contribution of Rho kinase and protein kinase C to myogenic tone in rat cerebral arteries in hypertension

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Arterial smooth muscle constriction in response to, i.e., myogenic tone, may involve calcium-dependent and calcium-sensitization mechanisms. Calcium sensitization in vascular smooth muscle is regulated by kinases such as PKC and Rho kinase, and activity of these kinases is known to be altered in cardiovascular disorders. In the present study, we evaluated the relative contribution of PKC and Rho kinase to myogenic tone in cerebral arteries in hypertension. Myogenic tone and arterial wall calcium in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) were measured simultaneously, and the effect of PKC and Rho kinase inhibitors on myogenic tone was evaluated. SHR arteries showed significantly greater myogenic tone than WKY arteries. Pressure/wall tension-arterial wall calcium curves showed a hyperbolic relation in WKY rats, but the curves for SHR arteries were parabolic. Myogenic tone was decreased by the Rho kinase inhibitors Y-27632 and HA-1077, with a significantly greater effect in SHR than in WKY arteries. Reduction in myogenic tone produced by the PKC inhibitor bisindolylmaleimide I in WKY and SHR arteries was significantly less than that produced by Rho kinase inhibition. The pressure-dependent increase in myogenic tone was significantly decreased by Y-27632, and the decrease was markedly greater than that produced by bisindolylmaleimide I in SHR arteries. In WKY arteries, the pressure-dependent increase in myogenic tone was decreased to a similar extent by Y-27632 and bisindolylmaleimide I. These results suggest greater myogenic tone with increased calcium sensitization in SHR arteries, largely because of Rho kinase activation, with a minor contribution of PKC activation.

spontaneously hypertensive rats; cerebral arteries; myogenic tone

VASCULAR SMOOTH MUSCLE CONTRACTION is regulated by electromechanical and pharmacomechanical coupling mechanisms, resulting in increased intracellular calcium with and without membrane depolarization, respectively. Calcium imaging combined with force/tension measurements has shown evidence of the dissociation between force and intracellular calcium in intact pressurized arteries. The extent of myosin light chain (MLC) phosphorylation or force of contraction induced by an agonist is greater than that caused by equal amounts of calcium increased by depolarization, a phenomenon termed calcium sensitization (26).

Different studies proposed that, in vascular smooth muscle, calcium sensitization produced by contractile agonists involves decreased MLC phosphatase activity mediated by protein kinase C (PKC) or Rho-A-dependent kinase, i.e., Rho kinase. PKC modulates calcium sensitivity through downregulation of MLC phosphatase or phosphorylation of caldesmon and calponin (10, 17), whereas Rho kinase inhibits MLC phosphatase activity by phosphorylation of the regulatory subunit of the enzyme (6). Reduced MLC phosphatase activity leads to increased MLC phosphorylation without a change in intracellular calcium or MLC kinase activity.

Hypertension, a risk factor for the incidence of stroke (4), is characterized by increased vascular smooth muscle contraction, increased peripheral vascular resistance, and structural remodeling of arteries, with arterial hyper- and hyporesponsiveness to endogenous vasoconstrictors and vasodilators, respectively. Cerebral resistance arteries, by virtue of their ability to constrict in response to intraluminal pressure, i.e., myogenic tone, protect the brain from the potential hemorrhage associated with hypertension. Autoregulation in cerebral arteries may operate at a higher level in hypertension (27, 28), presumably because of increased myogenic tone.

Signal transduction mechanisms involved in development of myogenic tone have not been completely elucidated. Different studies showed evidence for involvement of depolarization-induced calcium influx (15), activation of phospholipase C (20), and calcium sensitization (16, 18, 30). Mechanisms underlying calcium sensitization in pressure-induced activation may involve PKC (7, 9) as well as the RhoA/Rho kinase pathway (19, 21). The arterial hyperreactivity to agonists and pressure stimulus in hypertension may involve calcium-dependent and calcium-sensitizing mechanisms. In this study, we evaluated the contribution of two different calcium-sensitization mechanisms to the myogenic tone of cerebral arteries in hypertension.

The focus of the present study was to evaluate 1) myogenic tone and wall calcium in cerebral arteries from spontaneously hypertensive rats (SHR) and normotensive control Wistar-Kyoto (WKY) rats and 2) the relative contribution of PKC and Rho kinase pathways in the myogenic tone of cerebral arteries from SHR and WKY rats. Effects of the structurally different Rho kinase inhibitors Y-27632 and HA-1077 and the structurally different PKC inhibitors chelerythrine chloride and bisindolylmaleimide I on myogenic tone were evaluated.

METHODS

SHR and WKY normotensive controls, 25–30 wk of age, were used in the present study (Charles River). Systolic blood pressure was measured by the tail cuff method. Animal procedures have been
reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida.

Preparation of cerebral arteries and measurement of arterial diameter. Rats were anesthetized with pentobarbital sodium (160 mg/kg ip) and killed by decapitation. The brain was removed and placed in an ice-cold oxygenated physiological cerebrospinal fluid (PCSF; for composition, see Drugs, chemicals, and solutions). Posterior cerebral arteries were isolated and mounted in an arteriograph, as described elsewhere (11), using a pressure servo-null system (Living Systems, Burlington, VT). Diameter was measured with a calibrated video-caliper system. The arteries were slowly pressurized to 70 mmHg under no-flow conditions and warmed to 37°C during superfusion (3 ml/min) with PCSF bubbled with 21% O2-5% CO2-74% N2 (pH 7.3–7.4 in the bath). All experiments were done in endothelium-intact arteries.

Experimental protocol. After ~30 min of equilibration, the arteries showed stable myogenic tone. The viability of pressurized arteries was checked by exposure to 16 and 60 mmol/l KCl, which produce receptor-independent relaxation and contraction, respectively. Concentration-response curves were obtained by cumulative addition of drugs to the superfusate. In this protocol, arteries were exposed to each concentration of the different pharmacological agents for ≥8 min, and the plateau effect at each concentration was observed by ≤6 min of exposure. The passive diameter in calcium-free PCSF was obtained at the conclusion of the experiments. Pressure-diameter curves were obtained by increasing pressure from 10 to 199 mmHg in the presence of PCSF and calcium-free PCSF. In some experiments, pressure-diameter curves were obtained at 1–150 mmHg in the presence of PCSF and PCSF containing Y-27632 (1 μmol/l) or bisindolylmaleimide I (3 μmol/l). Myogenic tone was calculated as follows:

\[ \text{myogenic tone (\%)} = \frac{(D_p - D_0)}{D_p} \times 100 \]

where \( D_p \) is diameter of the arterial segment with active myogenic tone and \( D_0 \) is passive diameter in the presence of calcium-free PCSF at a given intraluminal pressure.

Calcium imaging using fura-2 in isolated arteries. Arteries with stable myogenic tone were loaded with the calcium-sensitive dye fura 2. The arteries were allowed to return to room temperature for 15 min, and then superfusion was stopped. PCSF was removed from the bath and replaced with PCSF containing 5 μmol/l fura-2 AM in DMSO and 2 μg/ml Pluronic F-127. After 30 min, the fura 2 loading solution was washed out at room temperature, and the temperature was returned to 37°C. All arteries were allowed to recover myogenic tone completely before beginning the experiment. Internal diameter was monitored using a video caliper before and after acquisition of calcium images.

Fura-2 ratio images were obtained using a computer-controlled monochromatic excitation light source (Polychrome II, TILL Photonics, Martinsried, Germany) and a cooled charge-coupled device camera with exposure control (SensiCam, TILL Photonics). During excitation at 340 or 380 nm, emission at 510 nm was collected by the camera for 300 ms. A 340-nm to 380-nm ratio image was generated after background subtraction, and one or several areas of interest were used to quantify the ratios after each treatment. To minimize photo-bleaching and photodamage, calcium images were obtained only when arterial diameter was at steady state after each treatment. Pressure-dependent changes in diameter and arterial wall calcium were measured simultaneously. The increase in fura 2 ratio with pressure is expressed as a percentage of that produced by 60 mmol/l KCl at an intraluminal pressure of 70 mmHg.

Data analysis and statistics. Values are means ± SE; \( n \) indicates the number of independent experiments, which equals the number of animals used for experimentation. Means were compared by Student’s \( t \)-test, and concentration-response curves and pressure/wall tension-diameter-to-fura-2 ratio curves were compared by two-way ANOVA using Prism 3.0 (GraphPad Software, San Diego, CA). \( P < 0.05 \) was considered statistically significant.

Drugs, chemicals, and solutions. Y-27632 was purchased from Tocris Cookson (Ellisville, MO), chelerythrine chloride from Calbiochem (San Diego, CA), HA-1077 and bisindolylmaleimide I from Sigma (St. Louis, MO), and fura 2-AM from Molecular Probes (Eugene, OR). Stock solutions (10 mmol/l) of Y-27632 and HA-1077 were prepared in distilled water, and stock solutions of chelerythrine chloride and bisindolylmaleimide I were prepared in DMSO. The composition of PCSF was as follows (mmol/l): 120 NaCl, 3 KCl, 24 NaHCO3, 1.2 NaH2PO4·H2O, 2.5 CaCl2, 1.2 MgSO4·7H2O, and 4 glucose. PCSF with 16 and 60 mmol/l KCl was prepared by replacing NaCl with an equimolar quantity of KCl. Calcium-free PCSF was prepared by replacing CaCl2 with an equimolar quantity of MgSO4·7H2O with 2 mmol/l EGTA.

RESULTS

Systolic blood pressure was significantly higher in arteries from SHR than in arteries from age-matched WKY rats: 184 ± 2 (\( n = 19 \)) and 128 ± 2 (\( n = 20 \)) mmHg (\( P < 0.05 \)). All arteries developed myogenic tone at 70 mmHg: myogenic tone was 30 ± 1% in WKY rats (\( n = 20 \)) and 37 ± 1% in SHR (\( P < 0.01, n = 19 \)). Active diameter was 93 ± 3 μm in WKY rats and 85 ± 3 μm in SHR, and passive diameter was 153 ± 11 μm in WKY rats and 128 ± 6 μm in SHR. No significant difference was observed in passive diameter between the two groups. Elevation of potassium to 60 mmol/l KCl increased constriction, with no difference between strains: 184 ± 7% in WKY rats (\( n = 8 \)) and 165 ± 9% in SHR (\( n = 10 \)). Similarly, arterial wall calcium (fura-2 ratio) increased to a similar extent in response to 60 mmol/l KCl: 2.14 ± 0.10 in WKY rats (\( n = 6 \)) and 2.12 ± 0.06 in SHR (\( n = 4 \)).

Pressure-dependent changes in myogenic tone and arterial wall calcium. Figure 1 shows the myogenic tone developed in WKY and SHR arteries at intraluminal pressures of 10–199 mmHg. In arteries from both strains, 10-mmHg increments in intravascular pressure increased myogenic tone up to 60 mmHg, and myogenic tone was maintained at almost the same level at up to ~150 mmHg. Myogenic tone then tended to decrease or arteries tended to lose resistance and dilate gradually (Fig. 1) at >150 mmHg. Pressure-myogenic tone curves in WKY and SHR arteries were significantly different (\( P < 0.01 \), two-way ANOVA).

Fig. 1. Myogenic tone developed in posterior cerebral arteries from Wistar-Kyoto (WKY, \( n = 6 \)) normotensive rats and spontaneously hypertensive rats (SHR, \( n = 6 \)) at intraluminal pressures of 10–199 mmHg. Pressure-myogenic tone curves were significantly different (\( P < 0.01 \), two-way ANOVA).
Elevation of intraluminal pressure from 10 to 150 mmHg increased arterial wall calcium in WKY rats; this increase was maintained at almost the same level from 150 to 199 mmHg (Fig. 2A). At low (≤30 mmHg) pressure, arterial wall calcium was significantly greater in SHR than in WKY rats (P < 0.05, Student’s t-test at 10, 20, and 30 mmHg). At 50–150 mmHg, where active myogenic tone was observed, the wall calcium of SHR arteries was maintained at nearly the same level, but further elevation of pressure actually resulted in a decrease (Fig. 2A). Pressure-arterial wall calcium curves in WKY and SHR arteries were significantly different (P < 0.001, two-way ANOVA). Because wall tension may represent the stimulus that triggers myogenic tone, we explored the relation between wall tension and arterial wall calcium (Fig. 2B), which was hyperbolic in WKY rats but parabolic in SHR.

Effect of PKC inhibitors on myogenic tone in cerebral arteries. A concentration-dependent decrease in myogenic tone was not observed with chelerythrine chloride (10 nmol/l–3 μmol/l) in WKY and SHR arteries (Fig. 3A). At 3 μmol/l, the maximum decrease in myogenic tone was 12 ± 5% (n = 4) and 35 ± 11% (n = 5) in WKY and SHR arteries (not significantly different). Bisindolylmaleimide I (10 nmol/l–3 μmol/l) produced a concentration-dependent decrease in myo-

Fig. 2. A: pressure-arterial wall calcium relationship in posterior cerebral arteries from WKY rats and SHR at intraluminal pressures of 10–199 mmHg. Fura 2 ratios are a measure of arterial wall calcium and expressed as percentage of response to 60 mmol/l KCl. Pressure-arterial wall calcium curves in WKY and SHR arteries were significantly different (P < 0.001, two-way ANOVA). B: data in A plotted by replacing pressures with wall tension developed at different pressures. Curves show hyperbolic (WKY) and parabolic (SHR) relationships between wall tension and arterial wall calcium.

Fig. 3. Effect of PKC inhibitors chelerythrine chloride (A) and bisindolylmaleimide I (B) on myogenic tone of posterior cerebral arteries from WKY rats and SHR at intraluminal pressure of 70 mmHg. Maximum decrease observed in SHR arteries by bisindolylmaleimide I was significantly different (P < 0.05, n = 5) from that observed in WKY arteries. C: decrease in myogenic tone produced by 3 μmol/l bisindolylmaleimide I at intraluminal pressure of 10–150 mmHg in WKY and SHR arteries. Decrease is significantly greater in SHR than in WKY arteries (n = 5, P < 0.05, two-way ANOVA).
genic tone in SHR arteries; in WKY arteries, myogenic tone was affected only at 3 μmol/l (Fig. 3B). The maximum decrease in myogenic tone was significantly higher in SHR than in WKY arteries: 51 ± 9% vs. 24 ± 8% (n = 5, P < 0.05). At 10 μmol/l, chelerythrine chloride and bisindolylmaleimide I irreversibly abolished myogenic tone in WKY and SHR arteries.

The pressure-dependent increase in myogenic tone in WKY and SHR arteries was examined in the absence and presence of 3 μmol/l bisindolylmaleimide I (Fig. 3C). Development of myogenic tone was decreased by 10–20% at 40–150 mmHg in the presence of this PKC inhibitor in WKY and SHR arteries. Myogenic tone was more sensitive to this PKC inhibitor in SHR than in WKY arteries, and the decrease in myogenic tone observed at different pressure points in SHR arteries was significantly higher (P < 0.01, two-way ANOVA; Fig. 3C).

Effect of Rho kinase inhibitors on myogenic tone in cerebral arteries. Y-27632 concentration dependently (1 nmol/l–3 μmol/l) reduced myogenic tone in WKY and SHR arteries pressurized at 70 mmHg. The maximum decrease in myogenic tone was significantly greater in SHR than in WKY arteries: 80 ± 8% vs. 45 ± 3% (n = 5, P < 0.004; Fig. 4A). Similar results were observed with HA-1077. Myogenic tone was decreased concentration dependently by HA-1077 (1 nmol/ l–30 μmol/l), with a maximum decrease of 73 ± 6% (n = 5) in SHR arteries, which is significantly higher (P < 0.001) than that observed in WKY arteries (35 ± 5%, n = 5; Fig. 4B). Effects of Y-27632 and HA-1077 in these arteries were readily reversible.

The pressure-dependent increase in myogenic tone in WKY and SHR arteries (Fig. 4C) was examined in the absence and presence of 1 μmol/l Y-27632. Development of myogenic tone was decreased with an increase in pressure in WKY and SHR arteries at 20–150 mmHg, with the latter showing higher sensitivity. The decrease in development of myogenic tone in WKY arteries was <10% throughout the pressure range examined. In SHR arteries, the decrease in development of myogenic tone was 15–25% at 10–150 mmHg, which was significantly higher than that observed in WKY arteries (P < 0.001, two-way ANOVA; Fig. 4C). Pressure-myogenic tone curves in SHR arteries in the presence of Y-27632 were significantly different from those in the presence of bisindolylmaleimide I (P < 0.05, 2-way ANOVA).

DISCUSSION

The present study confirms that myogenic tone in rat cerebral arteries operates at a higher level in hypertension. We further provide the first evidence for dissociation of the calcium-myogenic tone relation, implying increased calcium sensitization in cerebral arteries in hypertension. Furthermore, this study provides evidence for a greater involvement and contribution of the RhoA/Rho kinase pathway than of PKC in the elevated myogenic tone of cerebral arteries in hypertension.

Pressure-mediated autoregulation in cerebral arteries in hypertension. The present study shows clear differences in the pressure-dependent constriction of cerebral arteries from normotensive and hypertensive rats at 10–199 mmHg. Higher myogenic tone was observed in hypertensive arteries at 10–130 mmHg than in normotensive arteries. In agreement with the present study, Smeda et al. (24) showed increased myo-

Fig. 4. Effect of Rho kinase inhibitors Y-27632 (A) and HA-1077 (B) on myogenic tone of posterior cerebral arteries from WKY rats and SHR at intraluminal pressure of 70 mmHg. Maximum decrease induced by both inhibitors was significantly higher in SHR than in WKY arteries (P < 0.01, n = 5). C: decrease in myogenic tone produced by 1 μmol/l Y-27632 at 10–150 mmHg intraluminal pressure in WKY and SHR arteries. Decrease was significantly greater in SHR than in WKY arteries (n = 5, P < 0.001, two-way ANOVA).
myogenic tone was <20%, with SHR arteries showing significantly higher sensitivity.

Earlier studies in arteries from normotensive rats that provided evidence for pressure-induced activation of PKC used PKC activators (7) and inhibitors (1, 14, 18). Studies with activators can provide evidence only for a permissive role of PKC, rather than pressure-induced activation of PKC. Different PKC inhibitors show concentration-dependent nonspecific effects in functional studies. Chelerythrine chloride at 10 \(\mu\)mol/l completely reduced myogenic tone in rat skeletal muscle arterioles, whereas no effect was observed at lower concentrations (1, 18). In afferent arterioles, this inhibitor showed similar blockade of constriction by phorbol ester at 1 and 10 \(\mu\)mol/l, whereas sensitivity of myogenic tone was different at these two concentrations (14), indicating that the effect of higher concentrations on myogenic tone was not necessarily due to PKC inhibition. In the present study, <10 \(\mu\)mol/l did not affect myogenic tone, whereas 10 \(\mu\)mol/l showed nonspecific effects. Furthermore, in cell-based bioassays, chelerythrine chloride did not inhibit PKC, whereas bisindolylmaleimide I derivatives were potent and selective inhibitors (3). In the present study, bisindolylmaleimide I showed a minimal effect on myogenic tone in WKY arteries but a significantly greater effect in SHR arteries, suggesting a minor role for PKC in basal myogenic tone that is increased in hypertension. The effects of PKC inhibition were significantly less than those obtained with Rho kinase inhibitors (see below).

Y-27632 and HA-1077 have been used in different studies, and they have been considered selective inhibitors of Rho kinase I and II; IC\(_{50}\) values for these two compounds for Rho kinase II inhibition were 0.8 \(\mu\)mol/l and 1.9 \(\mu\)mol/l, respectively, with 10- to 50-fold selectivity over other kinases (3, 29).

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In the present study, these two inhibitors produced a concentration-dependent decrease in myogenic tone in WKY and SHR arteries, with a significantly greater decrease in SHR arteries. The decrease in pressure-dependent development of myogenic tone by Y-27632 in WKY arteries is minimal and similar to that produced by bisindolylmaleimide I, but in SHR arteries the decrease is significantly greater (~25%) and is also greater than that produced by bisindolylmaleimide I in SHR arteries. These results suggest that in normotensive arteries the Rho kinase-mediated calcium sensitization is involved in maintenance of basal myogenic tone in cerebral arteries and, to a minor extent, in pressure-dependent development. In hypertension, pressure-dependent activation of this enzyme is greater, with an increased contribution to the maintenance of myogenic tone. The increased calcium sensitization observed in SHR arteries is largely due to an increased Rho kinase activation with a minor contribution from PKC.

Our results are consistent with findings from earlier studies. Y-27632 produced a persistent fall in blood pressure in SHR, renal hypertensive rats, and deoxycorticosterone acetate-salt rats, whereas its effects in WKY rats were minimal or transient (29). Wire-mounted serotonin-precontracted mesenteric arteries from mineralocorticoid-hypertensive rats showed greater relaxation to Y-27632 (31). Increased Rho kinase activation, but not PKC, was also shown by a cranial window approach in cerebral arteries from hypertensive rats (2, 12) and by a biochemical approach in aorta from different rat models of hypertension (23). Evidence for an increased Rho kinase activation in coronary and cerebral vasospasm was also reported (13,
REFERENCES


