Evidence That Rho-Kinase Activity Contributes to Cerebral Vascular Tone In Vivo and Is Enhanced During Chronic Hypertension
Comparison With Protein Kinase C

Sophocles Chrissobolis, Christopher G. Sobey

Abstract—The small G protein Rho and its target Rho-kinase may participate in the mechanisms underlying vascular contractile tone via inhibition of myosin light chain phosphatase. The present study has tested the hypothesis that Rho-kinase activity normally contributes to cerebral vascular tone in vivo, and that this effect is augmented during chronic hypertension. Comparative studies also examined the role of protein kinase C (PKC) in regulation of cerebral artery tone. Two Rho-kinase inhibitors, Y-27632 (0.1 to 100 μmol/L) and HA1077 (1 to 10 μmol/L), caused marked concentration-dependent increases in basilar artery diameter of anesthetized normotensive rats (Sprague-Dawley and Wistar-Kyoto [WKY] strains), as measured using a cranial window approach. By comparison, the selective PKC inhibitors calphostin C (0.01 to 0.5 μmol/L) and Ro 31-8220 (5 μmol/L) had little or no effect on basilar artery diameter. Vasodilator responses to Y-27632 were unaffected by PKC inhibition or activation. In two models of chronic hypertension (spontaneously hypertensive rats and WKY rats treated with N-nitro-L-arginine methyl ester for 4 weeks), Y-27632 elicited cerebral vasodilator responses that were significantly greater than in control WKY rats (P<0.05), indicating that the chronically hypertensive state and not genetic factors contributed to the increased responses to Rho-kinase inhibition. PKC inhibition had no significant effect on basilar artery diameter in chronically hypertensive rats. These data suggest that Rho-kinase, but not PKC, activity contributes substantially to cerebral artery tone in vivo, and this effect is augmented in the cerebral circulation during chronic hypertension. (Circ Res. 2001;88:774-779.)

Key Words: cerebral artery ■ hypertension ■ protein kinase C ■ Rho-kinase

A major determinant of vascular tone is the degree of phosphorylation of myosin light chain (MLC) in vascular muscle. This process can be enhanced in the presence of even low intracellular Ca2+ concentrations, such as under basal conditions in vivo, through inhibition of MLC-phosphatase (ie, Ca2+ sensitization). It is now recognized that the small G protein Rho and its target Rho-kinase can participate in this mechanism of vascular contraction by inhibiting MLC-phosphatase.1 A rapidly accumulating body of literature now documents the important role of the Rho/Rho-kinase–MLC-phosphatase inhibition pathway in a variety of vascular and nonvascular cell types.2

Rho is known to be activated by numerous trimeric G proteins, and Rho/Rho-kinase is therefore expected to play a fundamental role in vascular signal transduction during responses to vasoconstrictor agonists3 and also stretch.3 The use of bacterial exotoxin C3, which deactivates Rho, provided recent indirect evidence that activation of Rho (and thus potentially Rho-kinase) modulates Ca2+ sensitivity in permeabilized cerebral arteries in vitro,4 but the role of Rho-kinase in regulating intact cerebral vessel tone in vivo is not known, nor are effects of cerebrovascular disease states on Rho-kinase function.

Chronic hypertension, a major risk factor for stroke, is reportedly associated with increased myogenic tone of cerebral5 and noncerebral6,7 arteries. Significant functional changes develop in cerebral arteries during hypertension, including enhanced constriction and impaired dilatation,8 and are accompanied by structural changes (hypertrophy and remodeling) within the vascular wall.9 Because Rho-kinase may participate in both hypertrophy/reorganization of actin cytoskeleton10 and enhanced vascular tone,11 increased Rho-kinase activity could contribute significantly to the structural and functional changes in cerebral arteries during hypertension. The present study has tested the hypothesis that Rho-kinase function normally plays an important role in regulating cerebrovascular tone in vivo, and that this contribution is increased in two models of chronic hypertension. For comparison, we have also examined the role of protein kinase C (PKC), another kinase proposed to be involved in Ca2+ sensitization and regulation of cerebral vascular tone.12,13
Materials and Methods

Procedures used in these experiments were approved by the University of Melbourne Animal Experimentation Ethics Committee. Experiments were performed in 3- to 6-month-old male Sprague-Dawley rats (SD, 432 ± 14 g, n = 42), 5- to 12-month-old male Wistar Kyoto (WKY, 432 ± 7 g, n = 20), and spontaneously hypertensive rats (SHR, 436 ± 11 g, n = 10) (Animal Resource Centre, Western Australia).

Experimental Protocol

Rats were anesthetized with pentobarbital sodium (50 mg/kg IP) supplemented at 10 to 20 mg · kg−1 · h−1 IV. A tracheostomy was performed for mechanical ventilation with room air and supplemental oxygen. A femoral artery catheter was used to measure arterial pressure and to obtain arterial blood, and a femoral vein was cannulated for injection of supplemental anesthetic. Arterial blood gases and pH were maintained at normal levels for the duration of the experiment (pH 7.36 ± 0.01; Pco2 = 37 ± 1 mm Hg; P02 = 169 ± 5 mm Hg). Body temperature was monitored continuously using a rectal probe and was maintained at 37°C to 38°C with a heating pad. The rat was then placed in a head holder in a supine position. The larynx and esophagus were retracted rostrally and laterally and the musculature covering the basiocciptal bone removed. A craniotomy was then performed over the ventral brain stem and the dura and arachnoid were incised to expose the basilar artery. The cranial window was superfused at 3 mL/min with artificial cerebrospinal fluid (CSF; composition [mmol/L]: Na+ 154.8, Cl− 136.1, HCO3− 22.9, K+ 2.95, Ca2+ 1.71, Mg2+ 0.65, and d-glucose 3.69) at 37°C to 38°C. When sampled from the cranial window, CSF gases and pH were as follows: pH 7.38 ± 0.01, Pco2 = 35 ± 1 mm Hg, and P02 = 125 ± 3 mm Hg. Diameter of the basilar artery was monitored using a microscope equipped with a TV camera coupled to a video monitor and was continuously measured using a computer-based tracking program (Diamtrak).

Studies in SD Rats

The basilar artery was allowed to stabilize for 30 minutes after the preparation of the cranial window before responses were obtained to topical application of vasoactive agents. The following drugs were tested: Y-27632 (1 to 100 μmol/L) and HA1077 (1 to 10 μmol/L)—both Rho-kinase inhibitors; phorbol 12,13-dibutyrate (0.1 μmol/L)—a PKC activator; calphostin C (0.01 to 0.5 μmol/L) and Ro 31-8220 (5 μmol/L)—both PKC inhibitors; acetylcholine (1 to 10 μmol/L)—an endothelium-dependent vasodilator; and sodium nitroprusside (0.01 to 1 μmol/L)—a nitric oxide donor. Drugs, diluted in artificial CSF, were then superfused over the cranial window in cumulatively increasing concentrations. Diameter of the basilar artery was recorded under basal conditions and when vessel diameter was stable during application of each agonist. After vessel diameter had returned to the control level, an additional 15- to 30-minute recovery period was allowed before application of another drug. The sequence of application of drugs was randomized. No more than 4 vasoactive agents were tested in each animal.

Effectiveness of the PKC inhibitors calphostin C (0.1 μmol/L, n = 6) or Ro 31-8220 (n = 5) was evaluated by testing the basilar artery response to the PKC activator phorbol 12,13-dibutyrate (0.1 μmol/L) after a 20-minute pretreatment with the inhibitor. The possibility that PKC activation or inhibition affects the vasodilator responses of the basilar artery to Rho-kinase inhibition was examined in rats in which responses to Y-27632 (1 μmol/L) were recorded in the presence of phorbol 12,13-dibutyrate (0.1 μmol/L, n = 5) or Ro 31-8220 (5 μmol/L, n = 5), respectively. For comparison, control responses to phorbol 12,13-dibutyrate (n = 12) or Y-27632 (1 μmol/L, n = 16) were measured in separate groups of rats. A relatively low concentration of Y-27632 (1 μmol/L) was chosen for these studies to utilize the high selectivity of this compound for inhibition of Rho-kinase versus PKC.12,14

Studies in WKY and SHR Rats

Responses to Y-27632, calphostin C, sodium nitroprusside, and acetylcholine were compared in WKY and SHR. The main purpose of these experiments was to determine whether vasodilator responses to Y-27632 and calphostin C were altered during chronic hypertension.

Studies in N-Nitro-L-Arginine Methyl Ester–Treated WKY Rats

WKY rats (n = 6) were treated for 4 weeks with N-nitro-L-arginine methyl ester (L-NNAME) in their drinking water (50 mg L-NNAME/100 mL). This dose of L-NNAME equated to ~30 mg/kg per day. On the day of experimentation, concentration-dependent vasodilator responses to Y-27632 and acetylcholine were tested. The purpose of these experiments was to determine whether vasodilator responses to Y-27632 were altered in a second nongenetic model of chronic hypertension.

Drugs

Acetylcholine chloride, sodium nitroprusside, and L-NNAME were obtained from Sigma Chemical Co. HA1077 (1-[5-isoquinolenesulfonyl]homopiperazine), calphostin C, Ro 31-8220, and phorbol 12,13-dibutyrate were obtained from Calbiochem. Y-27632 (R-[1]-trans-N-[4-pyridyl]-L-[1]-aminomethyl)-cyclohexancarboxamide) was generously provided by the Pharmaceutical Research Division, Welfide Corporation (Osaka, Japan). Calphostin C, Ro 31-8220, and phorbol 12,13-dibutyrate were dissolved in DMSO and diluted in saline. All other drugs were dissolved and diluted in saline. At the final concentration used, DMSO alone (<0.5%) had no effect on basilar artery diameter.

Statistics

Vascular responses are presented as percent change in diameter of the basilar artery compared with baseline. Data are expressed as mean ± SE. Comparisons were made using Student’s paired or unpaired t tests or ANOVA, as appropriate. A value of P < 0.05 was considered significant.

Results

SD Rats

In SD rats, arterial blood pressure averaged 125 ± 2 mm Hg (n = 42). Basilar artery diameter averaged 214 ± 5 μm (n = 42) under control conditions. Arterial pressure was not affected by application of any vasoactive substance to the cranial window (data not shown).
Figure 2. A, Change in basilar artery diameter of SD rats in response to the PKC inhibitors calphostin C (0.1 μmol/L; n=12, baseline diameter=206±13 μm) or Ro 31-8220 (5 μmol/L; n=5, baseline diameter=230±9 μm). B, Constrictor effect of phorbol 12,13-dibutyrate (PdB) on the basilar artery in control rats (n=12, baseline diameter=223±10 μm) and in rats pretreated with calphostin C (n=6, baseline diameter=276±24 μm) or Ro 31-8220 (n=5, baseline diameter=222±7 μm). *P<0.05 vs control PdB response. C, Effect of Y-27632 (1 μmol/L) on basilar artery diameter in control rats (open bar, n=16, baseline diameter=211±10 μm) or in rats pretreated with PdB (solid bar, n=5, baseline diameter=154±6 μm) or Ro 31-8220 (striped bar, n=5, baseline diameter=215±12 μm). Values are mean±SE.

Effect of Rho-Kinase Inhibitors on Basilar Artery Diameter
Application of Y-27632 (1 to 100 μmol/L) to the cranial window caused marked concentration-dependent increases in basilar artery diameter (Figures 1A and 1B). In a separate group of rats, HA1077 (1 to 10 μmol/L) elicited cerebral vasodilator responses with a potency similar to that of Y-27632 (Figure 1B).

Effects of PKC
Neither of the PKC inhibitors studied, calphostin C or Ro 31-8220, had a substantial effect on basilar artery diameter. Three concentrations of calphostin C (0.01, 0.1, and 0.5 μmol/L) had the following effects on basilar artery diameter: 2±1% (n=8), 10±6% (n=12), and 0±3% (n=3), respectively. Effects of calphostin C (0.1 μmol/L) and Ro 31-8220 (5 μmol/L) are shown in Figure 2A. Both inhibitors virtually abolished vasoconstrictor responses to the PKC activator phorbol 12,13-dibutyrate (0.1 μmol/L; Figure 2B), confirming that PKC activity was effectively inhibited by both agents. Further, vasodilator responses to Y-27632 were unaffected by either PKC activation or inhibition (Figure 2C).

WKY and SHR Rats
Arterial blood pressure averaged 109±6 mm Hg in WKY (n=14) and 189±4 mm Hg in SHR (n=10; P<0.05 versus WKY). Baseline basilar artery diameter was slightly greater in WKY (242±7 μm) compared with SHR (216±7 μm; P<0.05).

Y-27632 elicited dilator responses of the basilar artery that were greater in SHR versus WKY (P<0.05; Figure 3A). In contrast, calphostin C had no significant effect on basilar artery diameter in either WKY or SHR (Figure 3B). Furthermore, vasodilator responses to sodium nitroprusside were similar in WKY and SHR (Figure 3C), whereas responses to acetylcholine were reduced in SHR (Figure 3D), reflecting normal responsiveness of cerebral vascular muscle but dysfunction of endothelium, respectively, during chronic hypertension.

L-NAME–Treated WKY Rats
Arterial blood pressure averaged 147±6 mm Hg in L-NAME–treated WKY (P<0.05 versus control WKY). Baseline diameter of the basilar artery averaged 212±15 μm in L-NAME–treated WKY rats (P<0.05 versus control WKY diameter). Y-27632 elicited cerebral vasodilator responses in L-NAME–treated
Rho-Kinase Function in Blood Vessels

Because Rho is known to be activated by numerous trimeric G proteins, the Rho/Rho-kinase mechanism of Ca$^{2+}$ sensitization is expected to play a fundamental role in signal transduction during responses to vasoconstrictor agonists.\textsuperscript{2,15} Further, the basal contractile state of cerebral arteries in vivo occurs largely as a response to the intravascular blood pressure,\textsuperscript{13} and it is now known that Rho can be strongly activated by stretch in vascular muscle cells.\textsuperscript{3} Thus, the combined effects of endogenous vasoconstrictor agonists and intravascular pressure in vivo could represent important stimuli for significant Rho-kinase activity in the normal regulation of vascular tone. The present experiments are the first to investigate the function of Rho-kinase in normal or hypertensive cerebral arteries in vivo. As expected, Western blotting confirmed that Rho-kinase (160 kDa), a ubiquitously expressed enzyme, is present in the rat basilar artery (data not shown). Our finding that topical application of two structurally unrelated Rho-kinase inhibitors, Y-27632 and HA1077, to the basilar artery of anesthetized normotensive (SD and WKY) rats produces marked concentration-dependent vasodilatation is consistent with the concept that Rho-kinase activity plays an important role in physiological regulation of cerebral vascular tone in vivo. Nevertheless, it is acknowledged that despite the precautions taken to maintain animals under controlled conditions throughout each experiment, we cannot exclude the possibility that induction of anesthesia, preparation of craniotomy, and other operative and perioperative procedures could potentially increase Rho-kinase activity above levels normally present in an ambulatory animal.

Selectivity of Rho-Kinase Inhibitors

The novel cell-permeant pyridine derivative, Y-27632, is described as a highly selective Rho-kinase inhibitor.\textsuperscript{2,11,14} In comparison to its inhibitory effect on Rho-kinase, the potency of Y-27632 is >200-fold lower as a PKC inhibitor and >1700-fold lower as an MLC-kinase inhibitor.\textsuperscript{11} In the concentration range of $\approx$0.3 to 100 \textmu mol/L, Y-27632 is reported to cause profound relaxation of receptor agonist–contracted vascular rings through inhibition of Rho-kinase.\textsuperscript{11} It therefore seems likely that the vasodilator effects of similar concentrations of Y-27632 observed in the present study were also related to inhibition of Rho-kinase in basilar artery smooth muscle cells. HA1077, also named fasudil, is a vasodilator known to possess several mechanisms of action including inhibition of PKC.\textsuperscript{16} although it is now known to be $\approx$30-fold more potent as a Rho-kinase inhibitor.\textsuperscript{11,14} Of particular relevance to our study is that Y-27632 and HA1077 are reported to have similar potencies as Rho-kinase inhibitors in in vitro assays of enzyme activity and of Ca$^{2+}$ sensitization–induced contraction of intestinal smooth muscle.\textsuperscript{11,14} We also observed remarkably similar potencies of Y-27632 and HA1077 in producing dilatation of the basilar artery (Figure 1B), consistent with the proposal that these effects were due to inhibition of Rho-kinase.

Effect of Hypertension on Rho-Kinase Function in the Cerebral Circulation

Pathological conditions involving an increased level of myogenic tone in cerebral vessels may potentially compromise brain blood flow and contribute to cerebral ischemia and stroke. Chronic hypertension, a major risk factor for stroke, is reportedly associated with increased myogenic tone of cerebral\textsuperscript{5} and noncerebral\textsuperscript{6,7} arteries. An augmented hypotensive effect of intravenous Y-27632 in experimental hypertension has provided evidence for pathological activity of Rho-kinase in chronic hypertension.\textsuperscript{11} However, the effects of hyperten-
sion on Rho-kinase function have not been reported in any specific vascular bed in vivo. Our present findings, that cerebral vasodilator responses to Y-27632 are selectively augmented in the hypertensive SHR and L-NAME-treated WKY rats in comparison to normotensive control WKY rats, indicate that chronic hypertension leads to increased Rho-kinase function in the cerebral circulation. Thus, abnormally high Rho-kinase activity in vascular smooth muscle during hypertension may not only exacerbate the increased blood pressure but could potentially limit physiological increases in cerebral blood flow as a result of increased vascular tone. Analogous findings have recently been reported in experimental models of coronary and cerebral vasospasm, consistent with an emerging general concept that Rho-kinase activity may be abnormally elevated in blood vessels during a number of cardiovascular disease states.

We confirmed previous in vivo findings that in genetically hypertensive rats dilator responses of the basilar artery to acetylcholine are reduced, whereas responses to sodium nitroprusside are preserved. This phenomenon is thought to indicate impaired production and/or activity of endothelium-derived nitric oxide but normal responsiveness of the vascular muscle to nitric oxide during chronic hypertension. Similarly, in WKY rats that were chronically treated with L-NAME, vasodilator responses to acetylcholine were partially (but not fully) impaired. This finding probably indicates that endothelial nitric oxide synthase activity in the basilar artery was submaximally inhibited by the daily L-NAME dose of ~30 mg/kg. Nevertheless, the L-NAME treatment regimen was clearly effective in producing elevated arterial pressure that was associated with an increased cerebral vasodilator response to Y-27632. Thus, vascular Rho-kinase activity appears to be increased as a secondary consequence of hypertension, independent of the primary mechanism contributing to the elevated pressure and of genetic mechanisms.

Role of PKC
PKC activity is potentially important in regulating basal tone and myogenic responses of cerebral arteries. Pharmacological activation of PKC, eg, by using phorbol esters, is known to be a powerful cerebral vasconstrictor stimulus in vitro and in vivo because of increased Ca^{2+} sensitivity of vascular contractile elements. We confirmed that activation of PKC is a strong constrictor stimulus in the basilar artery, in that phorbol 12,13-dibutyrate produced marked vasoconstriction that could be blocked by pretreating the artery with either of two relatively selective PKC inhibitors, calphostin C or Ro 31-8220.

Of particular importance to our study was the finding that calphostin C and Ro 31-8220 each had only a minimal effect on baseline diameter of the basilar artery, suggesting that any normal effect of basal PKC activity on the tone of this cerebral vessel in vivo is very small and apparently much less important than Rho-kinase. Such a conclusion is conceptually consistent with recent findings in isolated and permeabilized ovine cerebral arteries, in which agonist-induced Ca^{2+} sensitization could be prevented by inactivation of Rho (using exotoxin C3) but was not affected by inhibition of PKC. Thus, although activation of PKC and Rho-kinase may both lead to increased vascular tone through Ca^{2+} sensitization, it appears that only the latter mechanism is normally active in the basilar artery under resting conditions. Moreover, the finding that calphostin C had virtually no effect on baseline diameter of the basilar artery in SHR or WKY rats suggests that the influence of PKC activity on cerebral artery tone under basal conditions in vivo remains insignificant during chronic hypertension. Because the vasodilator response to Y-27632 was not affected by PKC activation (using phorbol 12,13-dibutyrate) or inhibition (using Ro 31-8220), it appears that cerebral vascular effects of Rho-kinase are independent of, and unaffected by, PKC activity, consistent with the finding that Y-27632 does not affect PKC-induced Ca^{2+} sensitization in permeabilized pulmonary vascular smooth muscle.

Poorly selective PKC inhibitors (eg, sphingosine, H-7, staurosporine) have also been reported to have little or no effect in vivo on baseline diameter of the basilar artery or of cremaster muscle arterioles, in agreement with our findings using much more selective PKC inhibitors. By contrast, other investigators have reported that staurosporine or U-73122, a nonselective inhibitor of phospholipase C, which also inhibits voltage-operated Ca^{2+} channels with greater potency, can dilate isolated cannulated posterior cerebral arteries, and suggested that PKC activation contributes to cerebrovascular myogenic tone. Thus, the difference between those and the present findings could be related to the differing selectivity of inhibitors used, different vessels studied, or the in vivo versus in vitro conditions.

In summary, the findings of the present study provide the first evidence that Rho-kinase activity normally contributes to the regulation of cerebral vascular tone in vivo. Moreover, Rho-kinase–mediated contractile tone appears to be enhanced in the basilar artery in models of chronic hypertension that are either pharmacologically induced or genetic in origin. In contrast, PKC activity does not appear to contribute significantly to basilar artery tone in either normotensive or hypertensive animals. Thus, the augmented cerebrovascular Rho-kinase activity appears to be a secondary phenomenon and not a primary cause of this disease state. Nevertheless, elevated Rho-kinase activity in the vasculature may represent a harmful positive feedback mechanism that could contribute to the generalized hypertensive state by further increasing vascular resistance. Vascular Rho/Rho-kinase may represent a novel therapeutic target in clinical conditions for which hypertension is a major risk factor, such as stroke and myocardial infarction. Indeed, it has recently been noted that HMG-CoA reductase inhibitors (statins), which inhibit Rho activation as a consequence of inhibiting isoprene formation, appear to have beneficial effects that cannot be accounted for in some patients by the level of reduction of plasma cholesterol. Hence, inactivation of Rho/Rho-kinase function may be an important additional beneficial action of statins in the treatment of numerous cardiovascular diseases. Further exploration of potential opportunities to target this biochemical pathway in cardiovascular therapy will be an important direction for future studies.
Acknowledgments

These experiments were supported by a project grant from the National Health and Medical Research Council of Australia. We are grateful to Dr Phil Marley for helpful advice throughout this study and to the Pharmaceutical Research Division, Welfide Corporation (Osaka, Japan), for generously providing Y-27632.

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_Circ Res._ 2001;88:774-779; originally published online April 13, 2001;
doi: 10.1161/01.RES.0000042472.29330.54
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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