Recent Evidence for an Involvement of Rho-Kinase in Cerebral Vascular Disease

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**Background and Purpose**—The small G protein rhoA and its downstream effector rho-kinase are both expressed in vascular cells and are involved in several cellular processes. One of these processes is the regulation of the phosphorylation state of myosin light chain in vascular muscle and thus, the development of force. Recently, considerable evidence for increased activity of this pathway in cerebral and noncerebral vessels has been reported in several cardiovascular diseases associated with increased vascular tone.

**Summary of Review**—The main aim of this brief review is to summarize current evidence for the involvement of rhoA/rho-kinase signaling in dysfunction of the cerebral circulation in disease states, such as cerebral vasospasm, hypertension, diabetes, and ischemic brain injury. We will also briefly consider the novel hypothesis that augmented activity of endothelial rho-kinase decreases nitric oxide production and contributes to increased vascular tone in disease and the possibility of this action being a key therapeutic target of statins (inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase) in cerebral and noncerebral arteries.

**Conclusions**—Considerable evidence indicates that rhoA/rho-kinase activity is commonly increased in cerebral vascular disease, not only in vascular muscle, but also in the endothelium and possibly in inflammatory cells and neurons. *(Stroke. 2006;37:2174-2180.)*

**Key Words:** cerebral arteries □ cerebrovascular disorders □ endothelium □ muscle, smooth □ phospholipids □ statins

Contractility of vascular muscle is governed by myosin light chain (MLC) phosphorylation. MLC is phosphorylated by Ca\(^{2+}\)/calmodulin-dependent MLC-kinase and dephosphorylated by MLC phosphatase (MLCP). Modulation of MLC can thus occur independently of alterations in cytosolic Ca\(^{2+}\) concentration (Ca\(^{2+}\) sensitization) through signaling pathways that regulate MLCP activity, such as the rhoA–rho-kinase mechanism. When activated by rhoA, rho-kinase inhibits MLCP activity by phosphorylation of its (regulatory) myosin-binding subunit1 (Figure 1). In both peripheral (for a review, see Somlyo and Somlyo2) and cerebral3–10 circulations, rhoA/rho-kinase appears to be involved in the regulation of basal tone, as well as in mediating responses to vasoconstrictor agonists. The reader is also directed to several other reviews on the role of vascular rhoA/rho-kinase in cardiovascular diseases3-11-19 and in the central nervous system (CNS).20

**Rho G Proteins**

The rho family of low-molecular-weight G proteins, which belongs to the Ras superfamily, can be divided into several different classes (for a review, see Aspenstrom21). The rho subfamily consists of 3 members: rhoA (which mediates known vascular effects and is expressed in cerebral vascular tissue22–25), rhoB, and rhoC. Evidence for the involvement of rhoA in Ca\(^{2+}\) sensitization in permeabilized ovine cerebral arteries has been shown, wherein C3 exotoxin (a rho inactivator) inhibited GTP\(\gamma\)S-induced increases in tension.26 RhoA is also expressed in noncerebral vascular tissue,27,28 where it is involved in Ca\(^{2+}\) sensitization.3 The activity of rhoA is regulated by 3 classes of enzymes: guanine-nucleotide exchange factors (GEFs), which facilitate the exchange of GDP for GTP, thus rendering rho active; GTPase-activating proteins (GAPs), which regulate inactivation of rho by accelerating intrinsic GTPase activity and converting rho back to its GDP-bound form; and GDP dissociation inhibitors (GDIs), which inhibit the dissociation of GDP bound to rho21 (Figure 1).

**Rho-Kinase**

This ubiquitously expressed Ca\(^{2+}\)-sensitizing effector of rho is a serine-threonine kinase, consisting of a rho-binding domain, and is activated by GTP-bound rhoA. Rho-kinase has 2 isoforms; ROK\(\alpha\)/ROCKII and ROK\(\beta\)/ROCKI,29 with both isoforms expressed in cerebral vascular muscle,22,25 Activated rho-kinase phosphorylates the myosin-binding...
Genetically Altered Mice, Dominant-Negative Mutants, and siRNA

Pharmacological inhibition of rho-kinase has been useful in establishing the role of the enzyme in blood vessels. However, such an approach has limitations, such as being unable to elucidate the rho-kinase isoform that mediates functional alterations in disease states. Very recently, ROCKI−/− mice have been studied to specifically investigate the role of ROCKI in cardiac fibrosis and the downstream effects of hyperglycemia. Such an approach will no doubt be useful to determine the importance of rho-kinase isoforms in cerebrovascular disease, eg, in experimentally induced models of hypertension.

Dominant-negative mutants have not yet been used to study rhoA/rho-kinase in cerebral vessels; however, such an approach has been used to study both rhoA and rho-kinase in cultured endothelial cells and intact peripheral arteries. These approaches, as well as the use of rhoA-specific small interfering (si) RNA, will avoid the uncertainty of nonspecific effects associated with pharmacological inhibitors such as Y-27632 and HA1077.

Involvement of RhoA/Rho-Kinase in Cerebral Myogenic Tone

Myogenic tone, an inherent property of smooth muscle, is characterized by pressure-induced vasoconstriction and is thus important for the development of basal vascular tone. RhoA/rho-kinase appears to be a major determinant of vascular smooth muscle contractility; hence, this pathway may contribute to the myogenic response. Consistent with this, rhoA in vascular smooth muscle can be strongly activated by stretch. Pressure-induced constriction of the middle cerebral artery is inhibited by Y-27632 and Y-27632 relaxes posterior cerebral artery segments after pressure-induced constriction, implicating rho-kinase in the cerebral myogenic response. Another study suggested rho-kinase to be important in maintaining basal tone of the posterior cerebral artery, whereas it may have played a minimal role in pressure-induced constriction. In vivo, Y-27632 causes concentration-dependent dilatation of cerebral arteries and arterioles. Such findings generally implicate the rhoA/rho-kinase pathway as a signaling mechanism of major importance underlying the myogenic response, and hence, basal vascular tone in the cerebral circulation.

Interestingly, a lower rho-kinase function in cerebral arteries of females compared with males is consistent with the lower myogenic tone in female compared with male cerebral arteries. Lower rho-kinase function in the cerebral vessels of females appears to be dependent on endogenous estrogen, which inhibits rho-kinase expression in cultured vascular cells, and this effect appears unrelated to nitric oxide synthase activity. Estrogen may suppress rho-kinase activity via activation of Rnd1 and consequently, GAP, leading to decreased levels of GTP-bound rhoA, rho-kinase activity, and contractile tone.

Vascular Disease States

Cerebral Vasospasm

Increased contractility of vascular muscle, which may, at least in part, be the result of increased rhoA/rho-kinase

Methods Used to Study RhoA/Rho-Kinase

Pharmacological Inhibitors of RhoA/Rho-Kinase

Y-27632 and HA1077 (also known as fasudil) exhibit enhanced selectivity for rho-kinase over numerous other kinases, eg, with IC50 values for inhibition of ROCKII in the nanomolar and low micromolar range, respectively. Both of these cell-permeable compounds inhibit rho-kinase by competing with ATP for binding. Since the initial discoveries of Y-27632 and HA1077 as rho-kinase inhibitors, other inhibitors of this enzyme have been synthesized (discussed recently); however, in vascular studies, Y-27632 and fasudil are by far the most widely used. Although the inhibitor of rhoA, C3 exoenzyme, has also been used in vivo and intact vessel preparations, in its purified form C3 exoenzyme does not possess cell surface binding or translocation components; hence, it is often used in alternative approaches, such as cell membrane permeabilization, to introduce it into intact cells (discussed in Sah et al).
activity, is now thought to contribute to the severe arterial narrowing observed in cerebral vasospasm after subarachnoid hemorrhage (SAH).\textsuperscript{52} After SAH, rho-kinase activity in the basilar artery is increased.\textsuperscript{53} Phosphorylation of myosin binding subunit (MBS; thus decreasing MLCP activity) and MLC are also increased after SAH, and they can be decreased acutely by treatment with Y-27632.\textsuperscript{53} Importantly, Y-27632 prevented narrowing of the basilar artery after SAH,\textsuperscript{54} thereby providing functional evidence for increased rho-kinase activity after SAH. Intravenous injection of HA1077 also inhibits constriction of the basilar artery after SAH.\textsuperscript{55} Furthermore, mRNA expression of both rhoA and rho-kinase is reported to be increased in the basilar artery after SAH.\textsuperscript{25}

Oxyhemoglobin is a proposed mediator of cerebral vasospasm, and the prolonged cerebral artery constriction elicited by oxyhemoglobin is even more pronounced after SAH, a mechanism that involves rhoA/rho-kinase activation.\textsuperscript{56} Y-27632 and HA1077 both inhibit oxyhemoglobin-induced constriction of the rabbit basilar artery, and oxyhemoglobin-induced translocation of rho to the membrane is blocked by an inhibitor of rho prenylation.\textsuperscript{56} Endothelin-1, also implicated as a mediator of cerebral vasospasm, potentiates both rhoA translocation and vasoconstriction by oxyhemoglobin, at least the latter effect being inhibited by Y-27632 and HA1077.\textsuperscript{57} Furthermore, Y-27632 reversed constriction by endothelin-1 and prevented Ca\textsuperscript{2+} sensitization induced by endothelin-1,\textsuperscript{58} consistent with the possibility that endothelin-1 contributes to cerebral vasospasm via rhoA/ rho-kinase activation. Lysophospholipids, such as sphingosine-1-phosphate and sphingosylphosphorylcholine, have also been implicated in the pathogenesis of cerebral vasospasm and constrict cerebral arteries by activating rho-kinase.\textsuperscript{7,8}

Fasudil is now used clinically in Japan for the treatment of cerebral vasospasm after SAH,\textsuperscript{16} and although fasudil has beneficial effects, it has not eliminated vasospasm development. After oral absorption, fasudil is metabolized to hydroxyfasudil, a more selective rho-kinase inhibitor than fasudil,\textsuperscript{59} and this may actually be the active compound. Thus, although it seems unlikely that any single spasmyogen contributes solely to cerebral vasospasm after SAH, the recognition that several major candidates indeed elicit vasoconstriction by increasing rhoA/rho-kinase activity and that a rho-kinase inhibitor has benefit in the clinical context provide good evidence that activation of this pathway is involved (Figure 2).

### Chronic Hypertension

A disease in which myogenic tone of cerebral arteries is enhanced, chronic hypertension is associated with changes in both vascular function (eg, impaired NO production attributable to endothelial dysfunction) and structure (eg, hypertrophy, which contributes to thickening of the vessel wall and reduction in lumen size). To our knowledge, no study has addressed whether rhoA/rho-kinase contributes to the altered structure of cerebral vessels in hypertension. Few studies have addressed whether rhoA/rho-kinase function is increased in the cerebral circulation during hypertension. Enhanced dilator responses of the basilar artery to Y-27632 in vivo in both genetic and pharmacological models of chronic hypertension\textsuperscript{5} suggest an increase in cerebral artery rho-kinase function in hypertension (Figure 2). Similarly, pressure-dependent development of myogenic tone of the posterior cerebral artery is inhibited by Y-27632 to a greater extent in spontaneously hypertensive (SHR) versus Wistar-Kyoto rats (WKY).\textsuperscript{44} These findings suggest an important role for rho-kinase in increased myogenic tone of cerebral arteries during hypertension and are supported by others reporting greater responses to Y-27632 in the basilar artery of SHR versus WKY.\textsuperscript{46,47} Complementary molecular evidence showing increased rhoA/rho-kinase expression is yet to be reported in the cerebral circulation, but it has been demonstrated in various models of hypertension in other vascular beds.\textsuperscript{27,28,60}

As well as inhibiting MLCP activity, rhoA activation can induce actin polymerization,\textsuperscript{61} a process that is involved in the myogenic response.\textsuperscript{43,62} Thus, increased rhoA activity in hypertension resulting in increased myogenic tone may occur as a result of inhibition of MLCP activity and/or activation of actin polymerization. The contribution of actin polymerization to remodeling of smooth muscle may be important in rhoA-induced structural alterations in cerebral vessels in hypertension, although this is yet to be investigated.

Regarding the important question of cause and effect, it remains to be clarified whether increased vascular rho-kinase activity can be a cause of hypertension or whether rho-kinase activity is increased only as a consequence of hypertension. Clearly, rho-kinase activity seems to increase in cerebral and noncerebral vessels after induction of experimental hypertension.\textsuperscript{3,27,28,33} Furthermore, in the aorta from several models of genetic and pharmacologically induced hypertension, GTP-bound rhoA is increased relative to that in normotensive controls, although rhoA, ROK\textgreek{a}, and ROK\textgreek{b} total expression is unaltered.\textsuperscript{28} On the other hand, rhoA expression is increased in the aorta from SHR relative to WKY at 4 weeks of age, ie, before development of hypertension in SHR.\textsuperscript{27} Similarly, hydroxyfasudil (an active metabolite of fasudil) inhibits agonist-induced mesenteric vasoconstriction in 4-week-
old SHR but not WKY, both findings together probably indicate that the increased activity and expression of vascular rhoA/rho-kinase can also be independent of or precede the development of genetic hypertension.

**Diabetes and Aging**

A few studies have investigated whether diabetes is associated with increased cerebral vascular rhoA/rho-kinase activity. In the basilar artery from streptozotocin-injected (ie, type 1 diabetic) rats, levels of rhoA mRNA and membrane-bound rhoA protein were found to be greater than in controls. Functional data showing enhanced dilator responses to Y-27632 in cerebral arterioles of type 2 diabetic compared with control mice also suggest that rhoA/rho-kinase activity is increased in diabetes (Figure 2). By contrast, dilator responses of the basilar artery in vivo to Y-27632 were similar in Zucker lean and Zucker obese rats, suggesting that rho-kinase activity is unaffected by insulin resistance, which is a characteristic of type 2 diabetes. Thus, whether or not rhoA/rho-kinase activity is altered may be dependent on the pathologies produced by different diabetic models.

In the adult rat basilar artery, rhoA mRNA expression and membrane-bound rhoA protein have been reported to increase with age, from 2 to 19 months of age, with these increases occurring both in endothelial and smooth muscle layers. Thus, given the evidence that endothelial rho-kinase activity can attenuate NO generation by endothelial NOS and thus increase smooth muscle contractility (see following section) and that dilator responses of cerebral vessels to endothelium-dependent agonists are impaired with aging, enhanced endothelial rhoA/rho-kinase activity could perhaps contribute to the endothelial dysfunction in aging (Figures 2 and 3).

**Brain Ischemic Injury and Inflammation**

Because rho-kinase is ubiquitously expressed, it is conceivable that activation of this pathway in nonvascular cells also indirectly contributes to brain injury associated with cerebral vascular disease. For example, fasudil was shown to protect against neuronal cell death in the gerbil brain after bilateral occlusion of the common carotid arteries. Beneficial actions of rhoA/rho-kinase inhibition in neurons after brain injury likely involve promotion of neurite outgrowth, because rhoA activity prevents neurite initiation and induces neurite retraction, with mechanism(s) involving several rho proteins and rho-kinase substrates in neurons, including MLCP (discussed in Govek et al). In various rat models of stroke, fasudil and hydroxyfasudil improve blood flow to ischemic brain regions, improve neurological function, decrease infarct volume, prevent neutrophil accumulation, and protect against ischemia-induced neuronal cell loss. Neutrophil accumulation may be detrimental to the ischemic brain by adhering to endothelial cells or releasing pathological mediators such as proteases or superoxide anion. Indeed, involvement of rhoA/rho-kinase in controlling the migration of human neutrophils likely involves phosphorylation of MLC in neutrophils and may or may not involve actin polymerization also. In a model of middle cerebral artery occlusion, mice treated with the rho inhibitor, C3 exotoxin, had smaller cerebral infarcts. Thus, in ischemic brain injury, the target site(s) of the beneficial effect of rhoA/rho-kinase inhibition may be in vascular and/or nonvascular cells. However, it is interesting to note that a recent clinical study reported rho-kinase activity in polymorphonuclear leukocytes to be increased in patients after acute ischemic stroke. Thus, a new concept is that rhoA/rho-kinase activity, in invading inflammatory cells or even neurons, may also contribute to ischemic brain injury.

Cerebral endothelial cells and their tight linking junctions compose the blood-brain barrier (BBB), which limits access of blood-borne molecules into the brain. RhoA/rho-kinase signaling may be a key mechanism in altered BBB permeability in response to stimuli such as monocyte chemoattractant protein-1, which in cultured mouse brain endothelial cells induced functional, morphological, and biochemical changes in endothelial permeability, effects that were prevented by C3 exoenzyme, Y-27632, or a rho-dominant negative mutant. Furthermore, stimuli such as protease-activated receptor-1 activators may enter the brain as a result of increased BBB permeability and induce astrogliosis via activation of rho-kinase. Because astrogliosis is a feature of acute and chronic neurodegenerative diseases that are characterized by an inflammatory component, rhoA/rho-kinase may contribute to brain inflammation after breakdown of the BBB in disorders of the CNS. However, an emerging hypothesis concerns the importance of actin polymerization in modulating BBB permeability. Because rhoA may stimulate actin polymerization, it is conceivable that rhoA activity is important in maintaining the structural integrity of the BBB. Clearly, the role of rhoA/rho-kinase regarding effects on BBB permeability requires further investigation, although it may modulate or contribute to BBB permeability.

**Endothelium, RhoA/Rho-Kinase, and Statins**

To date, the contribution of rhoA/rho-kinase to the control of vascular tone has mostly been considered with respect to increased signaling activity in vascular muscle. However,
Recent studies (so far, mostly in noncerebral vessels) suggest that augmented activity of the rhoA/rho-kinase signaling pathway in endothelial cells may also contribute to the enhanced vascular contractility observed during disease. In the endothelium, phosphatidylinositol 3-kinase (PI3K) enhances the protein kinase Akt, which phosphorylates and activates endothelial NOS, resulting in NO production. In cultured endothelial cells, rho-kinase inhibition is reported to increase Akt phosphorylation and nitrite (a stable product of NO) production, an effect blocked by PI3K inhibition. These findings are in support of functional data that phenylephrine-induced vasoconstriction is inhibited by Y-27632 in an endothelial-, PI3K-, and NOS-dependent manner. Hence, endothelial rho-kinase may normally inhibit NO production via PI3K inhibition even in healthy arteries, but this effect could conceivably be exacerbated in disease states (Figure 3). Moreover, in humans, increases in forearm blood flow in response to acetylcholine, which are impaired in cigarette smokers, are improved by fasudil treatment, consistent with a clinical link between increased rho-kinase activity and endothelial dysfunction. Thus, endothelial and smooth muscle cell rhoA/rhoA-kinase appears to exert procontractile effects through distinct mechanisms. It remains to be determined whether rhoA/rho-kinase-mediated inhibition of endothelial NO production is important in the cerebral circulation, where NO is a major modulator of vascular tone, or indeed whether expression or activity of cerebral endothelial rhoA/rho-kinase is increased during cardiovascular disease.

The beneficial cardiovascular effects of statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are now well known to extend beyond their cholesterol-lowering activity. For example, in noncerebral vessels, statins can modulate constrictor responses of endothelium-denuded and especially of endothelium-intact vessels without lowering plasma cholesterol, they possess antihypertensive effects, and they improve endothelium-dependent vascular relaxation. In blocking cholesterol biosynthesis, statins also prevent formation of isoprenoid intermediates, including geranylgeranyl pyrophosphate, required for the geranylgeranylation of rhoA. Importantly, the isoprenylation of rho is a prerequisite for rho activation, facilitating its interaction with the plasma membrane where GDP-GTP exchange is thought to occur. By preventing this membrane interaction, statins inactivate rhoA, leading to increased endothelial Akt phosphorylation, endothelial NOS expression and activity, and increased endothelial NO production. Interestingly, reduction of cerebral infarct volume (after middle cerebral artery occlusion) by statins is reported to be augmented even in healthy arteries, but this effect could conceivably be exacerbated in disease states.

Conclusions

Increasing evidence indicates that cerebral vascular rhoA/rho-kinase activity is augmented in several disease states.

Thus far, most attention has focused on rhoA/rho-kinase signaling in vascular muscle. However, a new concept is that increased endothelial rhoA/rho-kinase activity may also contribute to exaggerated vasoconstriction during hypertension, diabetes, and aging, all of which are strongly associated with endothelial dysfunction in the cerebral circulation. Furthermore, it is possible that rhoA/rho-kinase activity in invading inflammatory cells, or even neurons, contributes to ischemic brain injury.

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Disclosures

None.

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