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Rho kinase as a target for cerebral vascular disorders

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Abstract

The development of novel pharmaceutical treatments for disorders of the cerebral vasculature is a serious unmet medical need. These vascular disorders are typified by a disruption in the delicate Rho signaling equilibrium within the blood vessel wall. In particular, Rho kinase overactivation in the smooth muscle and endothelial layers of the vessel wall results in cytoskeletal modifications that lead to reduced vascular integrity and abnormal vascular growth. Rho kinase is thus a promising target for the treatment of cerebral vascular disorders. Indeed, preclinical studies indicate that Rho kinase inhibition may reduce the formation/growth/rupture of both intracranial aneurysms and cerebral cavernous malformations.

The cerebral vasculature facilitates delivery of oxygenated, nutrient-rich blood to the tissues of the brain and the subsequent removal of deoxygenated blood and metabolic byproducts. Weakening or defects in cerebral blood vessels can lead to intracranial hemorrhage and subsequent cerebral vasospasm or ischemia. Mortality after more serious hemorrhagic events, such as **subarachnoid hemorrhage**, is as high as 50% and more than a third of survivors suffer major neurological deficits [1,2].

Recent advances in imaging techniques permit increasingly reliable identification of cerebral vascular disorders prior to vascular leakage or rupture [3,4]. However, prophylactic or ameliorating pharmaceutical options are lacking and the risks of invasive surgical management may outweigh the risk of hemorrhage from an untreated disorder [5]. The development of drug-based treatments for cerebral vascular disorders is thus an area that requires serious attention.

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Identifying promising molecular targets sets the basis for the development of effective therapeutics to manage and prevent cerebral vascular disorders. Target identification is aided by the fact that vascular disorders with different disease pathologies are often typified by similar mechanical and signaling changes within the blood vessel. In particular, many vascular disorders are characterized by an overactivation of the Rho family of GTPases and downstream Rho kinases within the endothelial cells and smooth muscle cells of the vessel wall [6,7]. Overactivation of the Rho signaling pathway within these cells leads to the mechanical hallmarks of vessel leakage and rupture: reduced endothelial barrier function, increased invasion of inflammatory cells, abnormal remodeling and vasoconstriction. Preclinical studies suggest that inhibition of Rho kinase may reverse these vascular abnormalities and restore vascular integrity.

To highlight the promise of Rho kinase as a therapeutic target for cerebral vascular defects, this review will explore the role of the Rho pathway in the formation, growth, leakage/rupture and potential treatment of two distinct vascular disorders: intracranial aneurysms and cerebral cavernous malformations. An emerging theme is that the delicate balance between activation and inactivation of the Rho signaling pathway is likely to control the dynamic cytoskeletal rearrangements that underlie the equilibrium between vascular integrity versus vessel leakage and rupture.

Neurovascular unit & blood–brain barrier

The properties of blood vessels differ throughout the body. In the brain, the blood vessels form part of a neurovascular unit specialized in the regulation of blood–brain transport and hemodynamic neurovascular coupling [8]. The neurovascular unit is an integrated system of vascular endothelial cells, neighboring **pericytes** and smooth muscles cells, circulating blood cells, the extracellular matrix, glia and microglia, astrocytic end feet and nerve terminals [9]. Disruption of the neurovascular unit contributes to many different neurological diseases [8,10].

The endothelial cell portion of this unit and adjacent astrocyte feet form a semipermeable structure called the **blood–brain barrier** that regulates entry into the brain from the vasculature. Paracellular transport across the endothelial cells of this barrier occurs through intercellular junctions; the integrity of these junctions is thus key for barrier preservation [11]. Interendothelial junctional complexes consist of both tight junctions and adherens junctions, structures composed of distinct transmembrane proteins and cytoplasmic plaque proteins that control intercellular transport and adhesion [12,13]. Tight junction transmembrane proteins include occludin, claudins and junctional adhesion molecules [10]. Adherens junctions consist of transmembrane cadherens and cytoplasmic members of the catenin family [13]. In the capillaries of the brain vasculature, the gap junctions between pericytes and endothelial cells are also important for maintaining vascular integrity: an absence of pericytes results in vascular leakage [14] and interference with pericyte-endothelial adhesion *in vivo* results in hemorrhage [15]. These pericyte-endothelial gap junctions contain N-cadherin, beta-catenin and a variety of extracellular matrix proteins such as fibronectin and the chondroitin sulfate proteoglycan NG2 [16].

Structure of cerebral blood vessels

Within the vascular portion of the neurovascular unit, the vessel wall structure is very similar to that of comparable vessels throughout the body. In particular, the wall of a cerebral artery or vein consists of three layers:

- The intima: the innermost layer, the intima, consists of a monolayer of endothelial cells attached to a basement membrane composed of collagen and laminin. Endothelial cells secrete a number of mediators including nitric oxide (NO) that regulate vascular tone;
- The media: the middle layer, the media, consists of vascular smooth muscle cells (VSMCs) embedded in a matrix of elastin, collagen and proteoglycans. VSMCs secrete growth factors that are important in vascular remodeling;
- The adventitia: the outermost layer, the adventitia, consists of fibroblasts embedded in a matrix of collagen and elastin.

Cerebral capillaries have a less complex structure: these small vessels are formed from a simple ring of endothelial cells held together by **interendothelial junctions** and embedded in a basal lamina. However, capillaries are also closely associated with smooth muscle-like cells in the basal lamina called pericytes that wrap long processes around capillaries and make direct contact with the endothelial cells of the capillary wall through 'peg and hole' contacts and gap junctions [16,17].

Rho signaling

Rho family proteins (RhoA, RhoB and RhoC) are part of the Ras superfamily of guanosine triphosphate hydrolase enzymes (GTPases). These GTPases cycle between inactive (GDP-bound) and active (GTP-bound) forms. Active Rho GTPase binds to and activates the downstream effector Rho kinase. Rho kinase is an approximately 160 kD serine-threonine kinase with an N-terminal kinase domain, middle coiled-coil domain with a Rho-interacting interface and C-terminal zinc-finger-like motif and pleckstrin homology domain [18,19]. Two Rho kinase isoforms have been identified (ROCK1 and ROCK2): ROCK2 is more highly expressed in the central nervous system [20] and has been suggested to be the dominant Rho kinase in endothelial cells [21,22].

Rho kinase phosphorylates a range of substrates to regulate cytoskeleton-based cellular processes, including stress fiber formation, focal adhesion formation, neurite retraction, cellular motility, and contraction [23,24]. A key underlying mechanism is the fact that Rho kinase activation modulates the calcium sensitivity of myosin regulatory light chain phosphorylation via inhibition of myosin light chain phosphatase (MLCP) [25]. In particular, Rho kinase phosphorylates myosin phosphatase-targeting subunit isoform 1 (MYPT1), a myosin binding subunit of MLCP, at Thr696, Ser854 and Thr853 [26,27]. MYPT1 phosphorylation reduces MLCP activity and so results in a higher overall phosphorylation state of the myosin II population and thus a higher contractile activity level [28]. Rho kinase also directly phosphorylates smooth muscle myosin II and nonmuscle myosin II *in vitro* [25,29], and phosphorylates CPI-17, a small protein that when

phosphorylated binds to and inhibits MLCP [30]. All of these factors facilitate actin-activated myosin ATPase activity and so induce contraction of the intracellular actin cytoskeleton.

Rho kinase also regulates the formation of the tight junctions and adherens junctions that are important for regulating permeability through endothelial cell monolayers. The tight junction proteins occludin and claudin are phosphorylated by Rho kinase to diminish barrier function [31–33]. Adherens junctions on brain endothelial cells are important for generation of tension in cell monolayers [34] and activation of Rho kinase disrupts adherens junction structure [35]. Overactivation of Rho kinase in endothelial cells causes leakiness by disruption of cell–cell junctions.

Rho kinase inhibitors such as Y-27632 and Fasudil have been widely used to explore the role of Rho signaling in the function of vascular endothelial and smooth muscle cells. Y-27632 and Fasudil are nonisoform-specific Rho kinase inhibitors that competitively inhibit the ATP-binding site of both ROCK1 and ROCK2 [36,37]. At higher concentrations, Fasudil has been shown to inhibit other kinases, such as protein kinase C-related protein kinase 2 (PRK2), mitogen- and stress-activated kinase (MSK1), and protein kinase A (PKA) [38,39]. The potential off-target effects of Y-27632 similarly include PRK2 inhibition, and, at higher concentrations, MSK1 inhibition [38]. The overall Rho kinase specificity of Fasudil and Y-27632 is a key consideration when analyzing Rho kinase inhibition studies with these commonly used compounds; the potential for off-target effects reflects the importance of maintaining careful dosing levels in experimentation. Newer Rho kinase inhibitors are in development as clinical candidates for various diseases, although no Rho kinase inhibitors have been approved for any indication in the United States.

Based on promising preclinical results, Rho kinase has been suggested as a target for a range of disorders in both the central nervous system (CNS) and the cardiovascular system [40–42]. In the CNS, Rho kinase directly affects neurons in disorders such as neuropathic pain [43], multiple sclerosis [44], spinal cord injury [45,46] and focal cerebral ischemia [47]. In the cardiovascular system, Rho kinase is involved in the regulation of blood vessel wall integrity/tone in diseases such as hypertension [40,48] and atherosclerosis [49]. Given its background as a common target in disorders of the central nervous system and cardiovascular system, Rho kinase is a promising potential target for defects of the cerebral vasculature, such as cerebral cavernous malformations and intracranial aneurysms.

Cerebral cavernous malformations & Rho signaling

Cerebral cavernous malformations (CCMs, also ‘cavernous angioma’ or ‘cavernoma’) are multilobed clusters of grossly dilated and thin-walled capillaries in the brain and spinal cord found in approximately 0.3–0.9% of the population [50–53]. These hyper-permeable vascular lesions consist of a single layer of endothelium with altered subendothelial extracellular matrix and no intervening brain parenchyma [54]. CCM lesions range in size from a few millimeters to several centimeters and are susceptible to chronic leakage or large hemorrhagic events [55].

CCM lesions can occur due to sporadic or germline loss-of-function mutations in one of three well-characterized genes: *CCM1* (KRIT1), *CCM2* (malcavernin, MGC4607), or *CCM3* (PDCD10) [50]. Malformation development requires a two-hit molecular mechanism for pathogenesis; the second hit may be chemical, rather than genetic, such as exposure to cytokines in response to stress or inflammation [56,57]. Resulting loss of any one of the three CCM proteins can produce the typical lesion phenotype, though *CCM3* mutation often results in a more severe phenotype [58,59]. Patients with CCM lesions usually manifest with headache, epilepsy or hemorrhagic stroke at a mean age of 34 years [60]. The location and number of lesions in a given individual determines the severity of the overall effect, but the existence of even one lesion may predispose a patient to seizures, stroke and neurological deficits [61]. Familial mutations are autosomal dominant and distinguished by the presence of multiple lesions, an earlier age of onset and a more aggressive overall phenotype [56,62,63].

The CCM lesion endothelium bears the typical characteristics of increased vascular permeability [64–66]. Vascular walls are abnormally thin and dilated and ultrastructural analysis reveals ruptures in the endothelium indicating physical breakage between cells or loss of junctional integrity [67]. Tissue from subjects treated for CCM lesions reveals an absence of tight junctions [68]. The endothelial wall is detached from nearby extracellular matrix, suggesting loss of focal adhesions [67]. Decreased numbers of smooth muscle-like pericytes are observed in association with the capillary walls, and visible pericytes show an abnormal morphology [66,67].

At present, there are no approved drugs to prevent the formation, growth and leakage of cerebral cavernous malformations, nor are there any treatments to shrink formed malformations. The only management option that can be fully curative is surgical resection to remove the lesion [69]. However, the complications of this surgery include permanent or transient neurological morbidity and risk of serious systemic infection [70,71]. Given the risk-benefit considerations of surgical resection, identified lesions are typically monitored by MRI for signs of lesion expansion and hemorrhage and a resection is conducted only when the surgeon feels that the complications of the untreated lesion (e.g., intractable seizures, progressive neurological deficit) outweigh the surgical risks [72]. Stereotactic radiosurgery may be used if a lesion is both serious and inaccessible by conventional resection, but the benefit to risk ratio is still controversial [68,73,74].

Rho signaling & CCM formation/growth

The formation and growth of the dilated and clustered phenotype characteristic of the CCM malformation appears to arise from endothelial instability during vascular remodeling [56]. In particular, *CCM1/CCM2/CCM3* protein loss results in impaired **angiogenesis**, as indicated by defects in vessel-like tube formation and loss of endothelial cell invasion of the extracellular matrix [57,75].

On a molecular level, altered remodeling stems directly from the overactivation of Rho signaling in endothelial cells upon *CCM1/CCM2/CCM3* knockdown. Wildtype CCM proteins play a critical role in the downregulation of Rho signaling: *CCM2* has been shown to bind the ubiquitin ligase Smurf1 and localize Smurf1 in a manner that facilitates Rho

degradation [76]. The resulting overactivation of the Rho signaling pathway upon CCM protein knockdown would be expected to disrupt the dynamic process of blood vessel formation/repair, as Rho signaling directly regulates the cytoskeletal changes underlying endothelial remodeling and migration during angiogenesis and Rho kinase overactivation is associated with pathological angiogenesis [77–79]. Indeed, the specific endothelial abnormalities underlying the dilation/multilobed appearance of the CCM lesion have been tied experimentally to overactivation of Rho signaling [57]. Treatment with Rho or Rho kinase inhibitors can rescue these endothelial abnormalities and restore vascular stability [57,75]. Thus CCM lesion growth/repair is intimately linked to a disruption in the Rho equilibrium underlying abnormal vascular remodeling, and inhibition of Rho signaling represents a potent mechanism for preventing or slowing lesion formation/expansion.

Rho signaling & CCM hemorrhage/leakage

Hemorrhage from CCM lesions stems from a disruption in endothelial barrier integrity characterized by increased intracellular actin stress fibers and decreased interendothelial junctions [54,57,80]. Both of these hallmarks of vascular leakage are characteristic of dysregulated Rho signaling. Rho overactivation increases intraendothelial contractility via overactivation of Rho kinase, which leads to the inactivation of myosin light chain phosphatase and so activation of myosin II and the formation of intracellular stress fibers [81]. When the centripetal tension from the contraction of these stress fibers outbalances the adhesive forces at cell–cell junctions, endothelial cells contract and pull apart from one another, forming gaps in the endothelial barrier [82,83]. An imbalance in the Rho signaling equilibrium also increases permeability at interendothelial junctions [84,85]; Rho signaling regulates the formation of tight and adherens junctions [86] and Rho kinase overactivation has been shown to promote the adherens junction disruption and tight junction opening necessary for blood leakage through the endothelial barrier [12,33,87,88].

Studies of the inherited mutations leading to CCM formation have been extremely helpful in clarifying that the hemorrhage-inducing disruption in endothelial barrier integrity is indeed caused directly by the overactivation of Rho signaling in the lesion endothelium upon loss of CCM protein function. Early work *in vitro* and in *Ccm2*-heterozygous mice revealed that RhoA was overactivated in *Ccm2*-depleted endothelial cells and that inhibition of Rho signaling could rescue the increased intracellular actin stress fibers, decreased interendothelial junctions and increased permeability characteristic of CCM2 loss, both *in vitro* and *in vivo* [57]. Further work demonstrated that Rho kinase inhibition could rescue the Rho signaling overactivation and resulting endothelial dysregulation arising from loss of CCM1, CCM2, or CCM3 [75]. Additional *in vivo* studies demonstrated that *Ccm1*^{+/-} and *Ccm2*^{+/-} heterozygous mice showed increased Rho activation and Rho kinase activity and resulting impairments in cerebral vessel barrier function that could be reversed by treatment with the Rho kinase inhibitor Fasudil [54]. As Rho also regulates the junctions formed between endothelial cells and pericytes, the Rho overactivation stimulated by CCM1/CCM2/CCM3 protein depletion may also decrease vascular integrity by causing the pericyte depletion associated with CCM lesions. Importantly, recent work indicates that the *same* mutations in *Ccm1/Ccm2/Ccm3* genes and the same resulting changes to the Rho signaling pathway underlie the formation of spontaneous (nonfamilial) CCM lesions [89].

Rho kinase as a target for cerebral cavernous malformations

The clear link between CCM pathogenesis and Rho overactivation indicates that a drug targeting the Rho pathway could serve as a prophylactic in families with the known autosomal dominant *CCM* mutations or a treatment to prevent growth and hemorrhage of existing lesions. In addition, this clear Rho-based mechanism underlying a cerebral vascular pathogenesis nicely illustrates the more general observation that abnormalities in Rho signaling may disrupt vascular endothelial cells and so increase susceptibility to hemorrhage (Figure 1).

The potential role for Rho kinase inhibition in the treatment of cerebral vascular malformations is supported by preclinical studies. The effect of oral administration of Fasudil on CCM lesion development was tested in a transgenic mouse line with a defect in the *Ccm1* gene [90]. Fasudil treatment significantly reduced the number of CCM lesions formed per animal in comparison with placebo. In addition, the maximal diameter of formed multicavernous CCM lesions was significantly smaller in the Fasudil group than placebo, and phenotypic studies indicated decreased leakage by the CCM lesions in Fasudil-treated animals (reduced local thrombus, reduction of the extravascular iron deposition typical of chronic hemorrhage). This preclinical demonstration that Fasudil can reduce both the formation and pathological progression of CCM lesions reinforces the promise that a pharmacological Rho kinase inhibitor might serve both as a prophylactic to prevent CCM formation and a treatment to reduce the growth and leakage of formed CCM lesions.

Intracranial aneurysms & Rho signaling

An intracranial aneurysm is a localized dilation of a cerebral artery. The most common form is an intracranial saccular aneurysm, which manifests as a balloon-like outpocketing in the arterial wall up to 30 mm in diameter [91]. An individual aneurysm generally enlarges from an initial smaller (5–10 mm) outpocketing over months or years and larger aneurysms are more likely to rupture [91]. Intracranial aneurysms most commonly occur at the apex of an arterial bifurcation at or near the Circle of Willis, a central network of arteries at the base of the brain [92]. Histopathological examination of aneurysm walls reveals a thinning of the adventitia, media and intima, with endothelial disruptions and sparse smooth muscle cells [92–94].

Intracranial aneurysms are present in 2–5% of the population [95], more frequently in women [96]. More than one third of individuals with one aneurysm will develop multiple additional aneurysms [97]. The majority of aneurysms remain dormant and may stay asymptomatic for years, but 0.7% rupture [98]. This rupture can take the form of a temporary leak from the artery or a more serious hemorrhage from a complete opening of the arterial wall [91]. Either form of rupture can be life-threatening and rupture-induced hemorrhage often leads to irreversible neurological sequelae. Indeed, ruptured aneurysms are the leading cause of nontraumatic subarachnoid hemorrhage and cerebral vasospasm [99].

Pharmaceutical options to prevent formation, growth, or rupture of intracranial aneurysms are lacking. The standard of care for intracranial aneurysms involves physically isolating the

aneurysm from its parent artery by: surgical clipping (securing of the aneurysm neck with a metal clip) or, increasingly; endovascular coiling (the insertion of platinum coils into the aneurysm) [100]. Complications include intraoperative aneurysm rupture leading to hemorrhage and stroke/hematoma, systemic infection, mechanical vasospasm and thromboembolism [101,102]. Given the lack of treatment options and the fact that the risks of surgical coiling/clipping may outweigh the natural risk of rupture, preemptive screening for aneurysms is controversial.

Increased familial incidence of aneurysm suggests a genetic component to aneurysmal formation, though a clear genetic/mechanistic pathway has not been determined [103,104]. The formation and growth of intracranial aneurysms is currently considered to be a complex and multifactorial process that stems from a variety of environmental and genetic factors. Cerebral arteries are structurally susceptible to force-induced weakening (e.g., they have sparse medial elastin, lack supporting perivascular tissue and have structural irregularities at the apex of bifurcation [105,106]) and the biomechanical forces on the vessel walls from systemic hypertension and intravessel blood flow are viewed as key factors in aneurysm growth and rupture. Inflammatory processes are also implicated in pathogenesis; leukocyte infiltration and subsequent degrading of the arterial wall seem to contribute to both growth and rupture. On a mechanistic level, each of these key etiologic factors may contribute to a vascular defect by effecting an imbalance of Rho signaling in the endothelial or smooth muscle cells of the arterial wall (Figure 1). The following sections will review each pathogenic factor in more detail, with an emphasis on downstream modifications of Rho signaling.

Arterial hypertension & Rho signaling

Systemic arterial hypertension contributes to the development and eventual rupture of saccular aneurysms [91,107]. Experimental induction of hypertension induces saccular aneurysm formation in animals with carotid artery ligation [108–112] and the degree of blood pressure elevation correlates significantly with the number of aneurysms induced [113]. Indeed, subjects with ruptured intracranial aneurysms are twice as likely to have hypertension as the normal population [107] and the presence of an intracranial aneurysm correlates positively with systemic arterial hypertension in general [114]. The role of hypertension in aneurysm pathogenesis is generally viewed as a multifactorial process that involves weakening of the arterial wall due to increased hemodynamic stress.

It has long been known that Rho kinase is substantially involved in the functional and structural alterations of hypertensive blood vessels, and that Rho kinase inhibitors can reverse hypertension in hypertensive rats [115]. The molecular mechanisms whereby Rho affects blood vessel tension are also well known: Rho kinase is a key determinant of smooth muscle calcium sensitivity, and Rho kinase inhibitors have been shown to induce relaxation of smooth muscle via inhibition of calcium sensitivity [116]. In particular, within the smooth muscle cells of the blood vessel wall, the contractile activity of myosin proteins is activated by phosphorylation of their regulatory light chain subunit by the calcium-calmodulin-dependent myosin light chain kinase and other kinases [117]. This activation is countered by dephosphorylation of the myosin regulatory light chain by myosin light chain phosphatase

(MLCP), and the overall level of muscle cell contractility is set by an equilibrium between these two activities. Rho kinase activation in smooth muscle cells inhibits MLCP activity and increases phosphorylation of smooth muscle myosin, tipping the balance toward actomyosin-mediated contraction within the smooth muscle layer of the arterial wall. In addition, Rho kinase activation directly inhibits vasodilation: activation of Rho kinase within the endothelial cells of the arterial wall inhibits release of vasoactive factors such as nitric oxide that relax vascular smooth muscle [118]. These observations combine to suggest that Rho kinase inhibition could block the arterial hypertension that contributes to aneurysm development and rupture.

The potential for Rho kinase inhibition to mediate hypertension-induced aneurysm formation is further evidenced by the direct role played by Rho kinase in cerebral pressure autoregulation. Cerebral arteries are known to maintain a constant blood flow despite increases/decreases in systemic arterial pressure [119]. A multifactorial mechanism underlies this autoregulatory process, including an interplay between the effects of chemical stimuli (e.g., arterial carbon dioxide tension), metabolic byproducts (e.g., adenosine, O₂, K⁺), sympathetic/parasympathetic nervous activity and the myogenic constriction/dilation of arterial smooth muscle cells in response to elevated/decreased blood pressure [119–121]. Importantly, the myogenic aspects of cerebral autoregulation in conditions of high pressure stem from a lowered smooth muscle membrane potential and subsequent influx of calcium in response to changes in vascular wall tension [122–124]. These myogenic changes are mediated by Rho kinase, and inhibition of Rho kinase with Y-27632 or Fasudil can inhibit pressure-induced increases in intracellular calcium and myogenic vessel constriction [125,126]. This suggests that inhibition of Rho kinase would also directly reduce the autoregulatory vasoconstrictive response that contributes to the hypertension-induced hemodynamic stresses underlying aneurysm formation.

Hemodynamic shear stress & Rho signaling

The frictional shear stress on cerebral arterial walls from blood flow through the cerebral vasculature is implicated in the pathogenesis of intracranial aneurysms [127,128]. It is well known that sustained hemodynamic stresses can result in flow-induced outward vascular remodeling [129]. Intracranial aneurysms are often found in areas with altered hemodynamic stresses (e.g., arterial junctions and bifurcations) [130,131], and remodeling triggered by abnormal shear stress may underlie aneurysm development [91,132]. Experimental aneurysm induction studies frequently report hemodynamic shear stress as a critical factor in the formation and rupture of saccular aneurysms [111,133] and hemodynamic parameters can be used to stratify the risk of rupture in unruptured intracranial aneurysms [134].

The translation of mechanical stress signals to vascular remodeling changes occurs via the inner endothelial layer of arterial vessels [129,135]. Endothelial cell detection of fluid shear strain rates stems from sensation of changes in drag force by the cytoskeletal components of interendothelial adhesions and focal adhesions [136,137]. These mechanotransducers activate signaling pathways important to vascular structure, including the Rho signaling pathway [138,139]. Rho signaling then regulates the endothelial proliferation underlying

vascular growth and remodeling [140]: Rho activation within endothelial cells of the blood vessel wall disrupts proliferation by increasing stress fiber formation and cellular contractility [141]. Consistent with this role, Rho kinase inhibition can attenuate high flow induced arterial remodeling in rats [142]. In addition, genome-wide association studies of intracranial aneurysm have identified several gene products correlated with determining cell cycle progression that could affect proliferation of progenitor cells, including one with Rho-GAP domains [143]. This suggests overall that the stress-induced chronic vascular remodeling that contributes to aneurysm formation may also be prevented by Rho kinase inhibition.

Hemodynamic shear stress can also induce injury to the endothelial wall of the artery that promotes macrophage invasion [109,144], and the wall degradation resulting from this invasion can result in aneurysm pathogenesis.

Inflammation & Rho signaling

Inflammation has long been viewed as a pathogenic process in the origin/growth and rupture of saccular aneurysms [92,132,145]. Observational studies commonly report the presence of inflammatory cells throughout the wall of intracranial aneurysms, including macrophages/monocytes and T-lymphocytes [132,145] and ultrastructural analyses also reveal leukocyte infiltration into the aneurysmal wall [146]. This infiltration is associated with wall weakening: damage/loss of smooth muscle cells and collagen fibers, degradation of the elastic lamina by leukocyte-secreted lytic enzymes and degradation of the extracellular matrix via overactivation of metalloproteinases by proinflammatory cytokines [28,132,145,146]. Existence and extent of leukocyte infiltration is positively correlated with risk of aneurysmal rupture [146,147].

Leukocyte migration from blood vessels into tissue is regulated closely by Rho-mediated events in the endothelial cells of the arterial wall [148]. In particular, leukocyte interactions with endothelial cells stimulate intraendothelial Rho signaling, inducing stress fiber formation and contractility that leads to gap formation in the endothelial layer [149]. Rho activation may also augment inflammation by inducing proinflammatory mediators in monocytes [28]. Indeed, Rho inhibition with C3 toxins results in reduced monocyte transmigration in tissue culture models [150] and *in vivo* cell-permeable C3 blocks transmigration of neutrophils in neurotrauma [151]. These results combine to suggest that downregulation of the Rho signaling pathway may also reduce the inflammatory infiltration and resulting arterial wall damage that contributes to aneurysm pathogenesis.

Rho kinase as a target for intracranial aneurysms

Since Rho signaling is implicated in the generation of intracranial aneurysms, Rho and related proteins are potential targets for prophylactic treatments in families with elevated risk of aneurysm development. In addition, since Rho signaling is implicated in the processes of growth and wall decay that increase risk of aneurysmal rupture, the Rho signaling pathway may also be a target for rupture prevention in identified aneurysms and potentially the long-awaited alternative to surgical coiling or clipping.

Preclinical studies also provide some support for the specific application of Rho kinase inhibition as a therapeutic treatment for intracranial aneurysms. In particular, oral administration of Fasudil as a prophylactic treatment for aneurysm formation was tested in a renal hypertension rat model of saccular aneurysm. Fasudil treatment was reported to reduce the formation of saccular aneurysms at cerebral artery junctions [108]. Additional preclinical work with intracranial aneurysm models will be required to fully assess Rho kinase inhibition as a treatment to reduce the progression and rupture of formed saccular aneurysms. However, relevant studies indicating that Rho kinase inhibition can reduce the severity of abdominal aortic aneurysms support an additional role for Rho kinase inhibition as a treatment to forestall aneurysm progression and rupture [152,153].

Safety considerations: Rho kinase target

The Rho kinase inhibitor Fasudil has been used in Japan as an intravenous treatment for the cerebral vasospasm that often accompanies aneurysm-induced subarachnoid hemorrhage since its approval in 1995 [154,155]. Initial clinical testing of Rho kinase inhibition for cerebral vasospasm in the 1990s stemmed from preclinical observation of the vasodilatory properties of Rho kinase inhibition discussed above. Post-marketing surveillance of treated subjects supports the Phase III trial findings that Fasudil is well-tolerated as a treatment for cerebral vasospasm, with treated subjects showing no significant difference from placebo in rates of intracranial hemorrhage and hypotension [156]. This indicates that systemic Rho kinase inhibition could potentially be used therapeutically in a patient population with severe vascular defects. The lack of drug-related serious adverse events during the Phase I/IIa clinical trial of the Rho antagonist Cethrin for acute spinal cord injury reinforces the premise that carefully targeted inhibition of the Rho signaling pathway has the potential to be safely used as a treatment for neurological disorders [157].

Selection of Rho kinase inhibitors for evaluation as CCM/aneurysm therapeutics

Given the promise of Rho kinase as a target for both CCMs and intracranial aneurysms, it is relevant to review the current Rho kinase inhibitors with potential for development as a therapeutic for these indications. In the past 15 years, there have been more than 100 filed patent applications targeting Rho kinase inhibition [158]. For example, a series of soft Rho kinase inhibitors designed based on classic Rho kinase inhibitor scaffolds (e.g., the 5-aminoindazole scaffold, the 5-aminoisoquinoline scaffold, the Y-series pyridines) has recently undergone preliminary evaluation in other Rho kinase-related indications [159–163]. Rho kinase inhibitors with new binding modes developed based on new scaffolds (e.g., a 4,7-dihydrotetrazolo[1,5-a]pyrimidine scaffold, a 3-aminomethyl-substituted benzamide scaffold) have also been found to have moderate to high potency for Rho kinase inhibition [163–167].

Any new potential therapeutic for CCMs and intracranial aneurysms must be evaluated for both efficacy and safety. Since the Rho signaling equilibrium in the central nervous system is both delicate and dynamic, and the independent functional roles of ROCK1 versus ROCK2 are increasingly evidenced (e.g., [168–171]), it would be reasonable to begin a

therapeutic evaluation with preclinical testing of the safety/efficacy of Rho kinase inhibitors with varying potencies and varying ROCK1 versus ROCK2 selectivities. The patented Rho kinase inhibitor BA-1049 [172] is one inhibitor that shows potential for testing in these indications, due to its increased selectivity for Rock2 (the isoform that is more highly expressed in the central nervous system [20] and has been suggested to be dominant in endothelial cells [21,22]). BA-1049 was the lead compound selected from a rational design program involving screening of novel inhibitors for Rock2 inhibition, neuronal cell penetration and ability to promote neurite outgrowth.

Recent work demonstrating the impact of 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors ('statins') on cerebral aneurysms demonstrates the importance of considering the selectivity of a potential therapeutic for specific Rho kinase isoforms versus other kinases in the cerebral vasculature. Statins exhibit pleiotropic effects including a weak, nonisoform-specific Rho kinase inhibition that stems from an effect on RhoA prenylation, a critical early step in RhoA/Rho kinase pathway activation [173,174]. Based on this Rho kinase inhibitory activity, one might suggest statins as a potential therapeutic for CCMs and intracranial aneurysms (indeed a current trial of statins in individuals with CCMs is ongoing based on such suggestions [175–177]). However, statin use has actually been associated with the rupture of intracranial aneurysms [178], an effect likely mediated by the pleiotropic impact of statins on kinases other than Rho kinase. Indeed the risks of statin use as a treatment for cerebral vascular disorders due to the effects of statins on all prenylation-dependent pathways has recently been highlighted by Eisa-Begyi *et al.* [179]; the authors note that statins are competitive inhibitors of a rate-limiting enzyme involved in the prenylation of more than 100 cell signaling proteins, including members of the Rho, Rac and CDC42 families. Statins thus provide a nice case study illustrating the need for selection of therapeutics with selectivity for the Rho kinase isoforms for use in preclinical safety and efficacy testing for CCMs and intracranial aneurysms.

Conclusion & future perspective

Disruption of the delicate Rho signaling equilibrium within the cells of the cerebral blood vessel walls is a common factor underlying the growth and development of cerebral vascular lesions. Though the cause of this disruption can vary between different vascular disorders (and even between different variants of the same vascular disorder), the resulting impact of Rho-related cytoskeletal remodeling on vascular integrity and growth is often quite similar. Rho kinase inhibition is thus a potential target for pharmacological treatment/prevention of cerebral vascular defects. This is supported by promising preclinical studies of Rho kinase inhibition as a treatment for intracranial aneurysms and cerebral cavernous malformations. The development of an effective and safe Rho kinase-based treatment to reduce the formation/growth/rupture of cerebral cavernous malformations or intracranial aneurysms presents challenges in drug development, but a successful drug could vastly improve the standard of care for these cerebral vascular defects.

Key terms

Subarachnoid hemorrhage	Leakage of blood from the vasculature into the areas of the central nervous system normally filled with cerebrospinal fluid. More than 80% of cases of subarachnoid hemorrhage are caused by the rupture of an intracranial aneurysm
Pericytes	Smooth muscle-like cells that wrap long processes around capillaries and make direct contact with the endothelial cells of the capillary wall via gap junctions. Pericytes can regulate blood flow in capillary beds by producing vasoconstriction and vasodilation in response to vasoactive peptides, and are thought to regulate the formation of new capillaries
Blood–brain barrier	Tightly joined monolayer of endothelial cells (supported by surrounding astrocytes, pericytes and microglia) that regulates passage from the cerebral vasculature into surrounding tissue
Interendothelial junction	Paracellular transport across the endothelial cells of the cerebral vasculature occurs through intercellular junctions, including tight junctions and adherens junctions. Tight junction transmembrane proteins include occludin, claudins and junctional adhesion molecules. Adherens junctions consist of transmembrane cadherens and cytoplasmic members of the catenin family
Angiogenesis	Growth of new vessels from existing vessels, the process that underlies expansion and remodeling of the cerebral vasculature. Insufficient or abnormal angiogenesis is a pathological factor in many disorders, such as stroke, myocardial infarction and pulmonary hypotension

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Executive summary

- Pharmaceutical treatment for cerebral vascular defects such as intracranial aneurysms and cerebral cavernous malformations (CCMs) is an unmet medical need.
- Overactivation of Rho signaling within the endothelial and smooth muscle cells of the vessel wall appears to play a pathogenic role in the vascular dysfunction underlying development of both aneurysms and CCMs.
- Inhibition of Rho kinase activity might be used to restore vascular integrity and prevent abnormal vascular growth.
- Inhibition of Rho kinase activity decreases the frequency of formation of intracranial aneurysms in rats and the rate of formation/growth of CCMs in mice, and might be a useful strategy to treat human aneurysms and CCMs.

Figure 1. Overactivation of Rho signaling leads to a disruption in the integrity of the cerebral vasculature

The overactivation of Rho kinase causes cytoskeletal changes within the endothelial and smooth muscle layers of the cerebral vessel walls that disrupt vascular integrity. In particular, stress fiber formation within endothelial cells upon Rho activation leads to cell contraction and thus gap formation in the endothelial barrier; this is exacerbated by the disruption of interendothelial junctions upon Rho overactivation. Stress fiber formation within smooth muscle cells and a reduction in the secretion of vasoactive factors combine to cause vascular contraction and resulting hypertension/hemodynamic shear stress. Abnormalities in endothelial remodeling lead to the formation of atypical vascular structures more prone to leakage or rupture. Rho kinase activation also increases invasion by inflammatory leukocytes and resulting wall degradation, and may result in depletion of the smooth muscle-like pericytes that provide structural support to the capillary wall. These Rho kinase-based disruptions in vascular integrity appear to underlie the formation/growth/rupture of aneurysms in cerebral arteries (left side of figure) and cerebral cavernous malformations in cerebral capillaries (right side of figure).