Published in final edited form as:

Expert Rev Neurother. 2009 August ; 9(8): 1171–1186. doi:10.1586/ern.09.70.

Targeting eNOS and beyond: Emerging heterogeneity of the role of endothelial Rho proteins in stroke protection

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Summary

Currently available modalities for the treatment of acute ischemic stroke are aimed to preserve or augment cerebral blood flow (CBF). Experimental evidence suggests that statins, which show 25–30% reduction of stroke incidence in clinical trials, confer stroke protection by upregulation of eNOS and increasing CBF. The upregulation of eNOS by statins is mediated by inhibition of small GTP-binding protein RhoA. Our recent study uncovered a unique role for a Rho-family member Rac1 in stroke protection. Rac1 in endothelium does not affect CBF. Instead, inhibition of endothelial Rac1 leads to broad upregulation of genes relevant to neurovascular protection. Intriguingly, inhibition of endothelial Rac1 enhances neuronal cell survival through endothelium-derived neurotrophic factors including artemin. This review discusses the emerging therapeutic opportunities to target the neurovascular signaling beyond the blood-brain barrier, with special emphasis on the novel role of endothelial Rac1 in stroke protection.

Keywords

eNOS; statins; GTP-binding protein; RhoA; Rho kinase; neurovascular unit; neurotrophic factors; blood-brain barrier; Rac1; vascular endothelium

Overview

Ischemic stroke is the second or third leading cause of death in developed countries. To date, nearly 300 clinical trials concerned with acute treatment of ischemic stroke have been registered: most of the completed trials, however, have been disappointing except the modest benefit with the use of tissue plasminogen activator (tPA) [1,2]. In addition, although some agents like statins show proven efficacy in stroke prevention, the magnitude of this effect is unsatisfactory. Therefore, a critical need exists for a deeper understanding of the stroke pathophysiology for new therapeutic paradigms.

The disease process of stroke is multifactorial. It is noteworthy, however, that all modalities currently available for the treatment or prophylaxis of ischemic stroke, such as antiplatelet, antithrombotic, and thrombolytic drugs, are aimed to preserve or restore cerebral blood flow (CBF); this clearly underscores the importance of vascular function in the treatment of stroke. On the other hand, the limited efficacy of therapies targeting CBF has necessitated additional approaches for direct neuroprotection. Energy depletion in ischemia initiates a cascade of

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events: excitotoxicity, oxidative stress, proteolysis and inflammation, which ultimately lead to cell demise [3]. Many promising experimental treatments targeting these processes have shown little effect in clinical trials. There are several sources for this failure, including the poor penetration of the agents across the blood-brain barrier (BBB) [4]. Remarkable advance has been recently made that may ultimately lead to circumventing BBB and facilitate direct neuroprotection. In this review, we will discuss the recent progress in neuroprotective modalities that target vascular endothelium to prevent or treat ischemic stroke, with special emphasis on the emerging heterogeneous roles of Rho proteins, RhoA and Rac1, in these processes.

Targeting eNOS for stroke protection

Role of NO synthases in stroke: insights from knockout mice

An important endogenous mediator of CBF and cerebrovascular protection is endothelium-derived nitric oxide (NO), which is synthesized by endothelial NO synthase (eNOS or NOS3) through oxidative conversion of L-arginine to L-citrulline [5,6]. NO formed by the vascular endothelium within nanomolar range diffuses to the adjacent cells such as vascular smooth muscle, platelets and leucocytes, activates soluble guanylate cyclase, and increases intracellular cGMP, which in turn mediates many of the beneficial effects of NO. These include vasodilation, antithrombotic, anti-inflammatory and antiproliferative effects.

In the brain, two other isoforms of NOS have been characterized. Neurons produce NO mostly by activation of neuronal NOS (nNOS or NOS1), which is constitutively expressed in these cells [7]. Glial cells (astrocytes, microglia and macrophages) synthesize NO through inducible NOS (iNOS or NOS2) expression by distinct stimuli [8]. Substantial evidence suggests that NO can exert both protective and deleterious effects depending on factors such as the temporal stage after the onset of ischemic brain injury and the NOS isoform and cellular source of NO. Immediately after brain ischemia, NO release from eNOS is protective by promoting vasodilation and by inhibiting microvascular aggregation and adhesion; however, after ischemia develops, NO produced by overactivation of nNOS and, later, NO release by de novo expression of iNOS contributes to the brain damage [9].

The differential roles of NOS isoforms in stroke have been revealed by the development of the knockout mice for the respective isoform for the past two decades. Following ischemia, NO levels in the brain rise several orders of magnitude, from baseline nanomolar levels to stimulated micromolar levels [10]. nNOS and iNOS knockout mice reveal that these NOS isoforms mediate this increase at early and late stages post-ischemia, since the knockout mice do not show it [11,12]. When subjected to a middle cerebral artery occlusion (MCAo) model of ischemia, iNOS and nNOS knockout mice develop significantly smaller infarct sizes and have better neurological outcome than wild-type mice. nNOS and iNOS may contribute to neuronal toxicity in several ways, including reaction with superoxide to form peroxynitrite, activation of poly-ADP ribose synthase resulting in depletion of cellular energy stores, and direct inhibition of mitochondrial respiration [13–15].

In contrast, eNOS knockout mice subjected to the MCAo model develop larger infarct sizes compared to wild-type mice [16]. Laser Doppler flowmetry and temporal correlation mapping shows that eNOS knockout mice have significantly reduced blood flow than do wild-type mice [17]. This confirms that eNOS normally serves to vasodilate and preserve blood flow in the setting of ischemia; in its absence, inability to preserve blood flow contributes to the enlarged infarct sizes observed in eNOS knockout mice. Additionally, enhancing NO production by administration of the eNOS substrate L-arginine increases regional CBF in the ischemic territory and confers protection from stroke [18]. This suggests that augmenting eNOS activity protects the ischemic brain, probably by maintaining sufficient CBF.

Therapeutic strategies that enhance eNOS for the prevention and treatment of stroke

The activity of eNOS is regulated by complex, multiple mechanisms. eNOS is a constitutively expressed enzyme; however, its protein abundance can be dynamically altered by transcriptional and post-transcriptional mechanisms, and serve as a major determinant of eNOS activity [19,20]. In addition, eNOS enzymatic activity is post-translationally regulated by the availability of the substrate L-arginine, cofactors FAD, FMN and BH₄, and intracellular Ca²⁺/calmodulin level [21]. Post-translational regulation of eNOS further involves phosphorylation, protein-protein interaction and intracellular localization. eNOS is phosphorylated at multiple sites including serine 1177 and threonine 495. Importantly, eNOS serine 1177 is phosphorylated by protein kinase B/Akt, and mediates the rapid activation of eNOS and NO generation by fluid shear stress and growth factors such as VEGF [22,23]. eNOS is localized to caveolae by N-terminal fatty acid modifications-myristoylation and palmitoylation, as well as through interaction with heat shock protein hsp90 and caveolins. While caveolin-1 and caveolin-3 bind to eNOS and inhibit its activity, hsp90 enhances eNOS activity [24].

Recently, various experimental modalities have shown capacities to alleviate tissue injury in rodent models of acute ischemic stroke. Evidence shows that many of the modalities exert their effects by increasing expression or activity, or both, of eNOS (Table 1).

1) Statins (HMG-CoA reductase inhibitors) and inhibitors of RhoA/ROCK pathway—

Statins are potent cholesterol-lowering drugs through inhibition of HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway for cholesterol biosynthesis in the hepatic and non-hepatic cells [25]. In addition, statins exert a number of pleiotropic vasculoprotective effects that include improvement of endothelial function, increased NO bioavailability, antioxidative and anti-inflammatory properties [26]. Indeed, in rodent models of stroke, statins augment CBF and confer significant protection partly via mechanisms related to the upregulation of eNOS [27–30]. Many pleiotropic effects of statins are beneficial, and most of them are because of HMG-CoA reductase inhibition. Inhibiting HMG-CoA not only blocks the synthesis of cholesterol but also a number of isoprenoid intermediates, which themselves have important biologic functions. Isoprenoids include geranylpyrophosphate (GPP), farnesylpyrophosphate (FPP), geranylgeranylpyrophosphate (GGPP), and squalene. Particularly, FPP and GGPP serve as important lipid attachments for the posttranslational modification of proteins including heterotrimeric G-proteins and small GTP-binding proteins. Isoprenylation converts small GTPase from a cytosolic (inactive) state to a membrane-bound (active) state.

Although there is evidence that reduction of cholesterol levels by any intervention indirectly increases eNOS expression [31], it is important to note that statins are able to directly upregulate eNOS independent of cholesterol levels [32,33]. There is considerable evidence suggesting that the direct upregulation of eNOS by statins is at least in part mediated through inhibition of small GTPase RhoA by intracellular depletion of GGPP [34]. Importantly, Botulinum toxin C3 (RhoA inhibitor), fasudil (inhibitor of ROCK, the downstream effector of RhoA) and cytochalasin D (inhibitor of actin polymerization) all upregulated eNOS mRNA in cultured endothelial cells by increasing eNOS mRNA half-life, and not eNOS gene transcription [34, 35]. RhoA/ROCK pathway is an established regulator of actin cytoskeletal organization. Therefore, statins upregulate eNOS, at least in part, by stabilizing eNOS mRNA through inhibition of RhoA/ROCK/actin polymerization cascade. This mechanism most likely plays a major role in the neuroprotective effects of statins in rodent models of stroke. Administration of statins, C3 exoenzyme, cytochalasin D or fasudil increased CBF and conferred stroke protection in wild-type mice, but not in eNOS knockout mice [34,35].

In addition to this prophylactic effect, the therapeutic benefit of statins extends to a 3-h post-stroke treatment paradigm [29]. This may involve mechanisms in which statins rapidly activate eNOS within minutes to hours, since upregulation of eNOS expression takes hours to days. Indeed, statins acutely activate the pathway involving phosphoinositide 3-kinase (PI-3K) and protein kinase B/Akt [36,37]. In line with this evidence, pravastatin acutely increases NO production and improves endothelium-dependent vasodilation in a PI-3K and Akt-dependent manner. The rapid activation of PI3-K and Akt by statins may possibly involve inhibition of RhoA and ROCK pathway, since fasudil has been shown to acutely activate Akt [38]. Administration of ROCK inhibitors fasudil and Y-27632 immediately prior to or after MCAo increased CBF in an eNOS-dependent manner [39]. The activation of eNOS by statins, ROCK inhibitors, and Akt is most likely mediated through phosphorylation of eNOS at serine 1177 (serine 1179 in bovine eNOS), the major phosphorylation site by Akt. The significance of eNOS phosphorylation at this residue in vascular reactivity and stroke protection has been recently tested in an elegant study using transgenic mouse models [40]. Transgenic mice expressing a phosphomimetic (S1179D) form of eNOS showed greater vascular reactivity, developed less severe strokes, and had improved CBF in a MCAo model than mice expressing an unphosphorylatable (S1179A) form. This result underlines the important contribution of eNOS serine 1177/1179 phosphorylation to the regulation of CBF during ischemia.

In addition to the eNOS upregulation and activation, the pleiotropic effects of statins also involve antithrombotic, antioxidative, and anti-inflammatory properties. In a mouse model of embolic stroke, atorvastatin reduced the infarct size through increased expression of tPA, but not eNOS [41]. Moreover, in rat embolic MCAo, atorvastatin enhanced the neuroprotective effect of exogenous tPA, when co-administered 4 hours after stroke, in an eNOS-independent fashion [42]. Conversely, in a mouse filament MCAo model, atorvastatin exerted eNOS-dependent stroke protection. Exogenous tPA therapy often causes aggravation of infarct size in a filament MCAo model associated with downregulation of eNOS. Rosuvastatin mitigates tPA-induced worsening of stroke [43]. Interestingly, however, this protective effect of rosuvastatin was eNOS-independent. In ob/ob mice, the mouse model of insulin resistance, the infarct size was larger than wild-type mice following MCA occlusion. Rosuvastatin decreased the infarct size of ob/ob mice, but did not change the CBF nor eNOS expression. Instead, the stroke-protective effect of rosuvastatin in ob/ob mice seemed to be exerted through inhibition of the induction of intercellular adhesion molecule-1 (ICAM-1) expression after ischemia. Taken together, the involvement of eNOS in statin-mediated neuroprotection may be variable between different disease models and settings.

Despite the fact that cholesterol is not an established risk factor for stroke, large-scale clinical trials have shown that statins may reduce stroke incidence by 29–32% [44]. However, further analysis is needed to address the effect of statins in the secondary prevention in stroke patients. Small prospective analyses indicate that statins might improve stroke outcome in humans [45]. Furthermore, discontinuation of statin therapy may provoke a withdrawal effect and increase the incidence of cardiovascular events [46,47]. These apparently beneficial effects of statins are likely cholesterol-independent and may be exerted through eNOS-dependent mechanisms as have been proven in animal experimental models.

2) Non-statin agents/modalities with stroke-protective properties by enhancing eNOS—Endothelium-derived NO diffuses into adjacent cells, such as platelets and vascular smooth muscle cells (VSMCs), binds to and activates soluble guanylyl cyclases. The resultant increase in cGMP inhibits platelet aggregation and relaxes VSMC, thereby leading to the preservation and augmentation of tissue blood flow. Intracellular cGMP is degraded by phosphodiesterases (PDEs). Recent study demonstrated that dipyridamole (DP), a PDE inhibitor used clinically as an anti-platelet drug, in combination with statins shows additive stroke protection by increasing CBF in the mouse filament MCAo model [48](Table 1).

Experimental evidence is now accumulating to show that, in addition to statins and ROCK inhibitors, various agents and modalities protect against stroke through upregulation of eNOS expression. This underscores the importance of eNOS and endothelial-derived NO in the regulation of CBF and stroke protection. Pretreatment for 14 days with angiotensin II type I (AT1) receptor blocker candesartan ameliorated the infarct size after MCAo of stroke-prone spontaneously hypertensive rats [49]. Increasing evidence suggests that AT1R blockade activates angiotensin II signaling through AT2 receptor, which in turn increases the expression of eNOS, promotes vasodilation and augments tissue perfusion [50]. Peroxisome proliferators-activated receptor- γ (PPAR- γ) agonists, such as pioglitazone and rosiglitazone, have been shown to confer potent protection against brain ischemia. The neuroprotective effects of PPAR- γ agonists have been claimed to be due to their antioxidative and anti-inflammatory properties, through suppression of the gene induction of COX-2, iNOS, TNF- α and NF- κ B [51]. One report recently showed that rosiglitazone upregulates eNOS and thereby increases angiogenesis post-ischemia [52]. Beneficial effects of the polyphenol resveratrol in red wine have been reported in an animal model of cerebral ischemia. A recent study demonstrated that the stroke protection by resveratrol is mediated through upregulation of eNOS [53]. Many studies have shown that voluntary physical activity increases the expression and activity of eNOS in the vasculature. Exercise significantly improved endothelium-dependent vasodilation, augmented CBF, and protected against tissue injury in the mouse MCAo [54]. These effects were not observed in eNOS knockout mice, suggesting that eNOS upregulation is the main mechanism of stroke protection by exercise. Physical activity also increased eNOS expression and led to NO-dependent reduction in thrombotic tendency in the brain of stroke-prone spontaneously hypertensive rats [55]. In addition, physical training increases the number of circulating endothelial progenitor cells (EPCs), increases angiogenesis post-ischemia, and improves long-term outcome following brain ischemia [56,57]. These effects are at least partly dependent on the upregulation of eNOS by exercise.

Steroid hormones, such as corticosteroids, estrogen and thyroid hormone, directly activate eNOS and thereby protect from ischemia-reperfusion injury. Although many cellular responses to these steroid hormones involve the transcriptional modulation of target genes by the glucocorticoid receptor (GR), estrogen receptor (ER), and thyroid hormone receptor (TR), a rapid non-nuclear effect leads to eNOS activation [58–61]. The ligand-bound steroid hormone receptors directly associate with the p85 subunit of PI-3 kinase, which in turn triggers the activation of PI-3K/Akt cascade and phosphorylates eNOS at serine 1177. In mice, acute administration of pharmacological concentrations of corticosteroids, as well as thyroid hormone, rapidly increases eNOS activity and augments CBF following transient MCAo. Infarct size was significantly attenuated, while the protective effects were completely abolished by receptor antagonists or by inhibitors for PI-3K or eNOS, and were absent in eNOS knockout mice. The genomic effects of GR and TR will potentially lead to unfavorable conditions for stroke treatment, ie, hyperglycemia, immune suppression and hyper-hemodynamic status. Therefore, specific drugs would need to be developed that selectively mediate the nontranscriptional effects of the steroid hormones.

Emerging pleiotropism of eNOS functions in brain ischemia

Recently, eNOS has been shown to be indispensable for neovascularization and recruitment of EPC following tissue ischemia [62,63]. EPCs are circulating stem cells derived from the bone marrow and contribute to improving endothelial function and to new vessel formation. Post-ischemic brain regeneration requires angiogenesis. Indeed, therapeutic modalities that enhance eNOS, such as statins, rosiglitazone and physical activity, increase the brain angiogenesis following ischemic stroke [52,57]. In humans, pre-stroke statin use was correlated with higher collaterals in angiography in acute stroke patients [64]. Interestingly, An intriguing study recently demonstrated that eNOS knockout mice were not only defective in post-ischemic

angiogenesis, but also in neurogenesis, in part due to reduced expression of brain-derived neurotrophic factor (BDNF), not VEGF [65]. This indicates that eNOS is a critical mediator of neurogenesis by regulating BDNF expression in the brain. Together, these findings suggest that therapeutic eNOS induction may be beneficial for inducing angiogenesis to reduce ischemic injury and enhance post-ischemic neuroregeneration.

eNOS and human stroke

Although compelling data from animal studies suggest that eNOS, in contrast to nNOS and iNOS, plays a protective role during brain ischemia, there is no direct evidence that it is the case in humans. Nonetheless, one might argue that most modalities that diminish stroke risk may actually do so through eNOS induction. For example, statin treatment, regular exercise, and moderate consumption of red wine have all shown association with reduced risk of stroke in large cohorts [44,66,67]. In this context, it may be worth noting that a large-scale, randomized clinical trial [Efficacy of Nitric Oxide in Stroke (ENOS) trial] is underway to address the effect of 7-day transdermal glyceryl trinitrate after acute ischemic stroke in patients with hypertensive medications [68].

Extensive epidemiological studies have investigated the association of three common polymorphisms in the eNOS gene [T786C (promoter), intron4ab, and G894T (exon 7)], which are known to confer predisposition to coronary vasospasm, with human ischemic stroke. To date, the findings have been equivocal [69,70]. However, some studies show positive correlation for each of the above mutation with ischemic stroke. Clearly, further studies with a larger number of samples are needed for future implications in the treatment and prevention of human stroke. Interestingly, recent studies showed strong correlation of T786 polymorphism with delayed cerebral vasospasm following aneurysmal subarachnoid hemorrhage (aSAH) [71,72]. Evidence shows that deficiency in endothelial-derived NO is associated with aSAH. Consistent with this human data, simvastatin upregulated eNOS, ameliorated neurological deficit, and increased MCA diameter after endovascular perforation of the right anterior cerebral artery in mice.

Targeting the neurovascular unit for stroke protection

As supported by the substantial evidence outlined above, there is little debate that eNOS-mediated CBF is the major target for stroke protection. However, this strategy is promising but apparently limited in efficacy: for example, the 20 to 30% stroke prevention by statins in clinical trials may indicate that 70 to 80% of stroke incidents cannot be prevented by targeting CBF alone. Therefore, novel therapeutic paradigms are critically needed that enable more extensive salvage of brain cells in ischemia.

Cerebral ischemia triggers a complex series of biochemical and molecular mechanisms that impairs the neurologic functions through breakdown of cellular integrity mediated by excitotoxicity, oxidative stress and apoptosis [3]. Although much progress has been made in dissecting the molecular pathways of these processes, clinically effective stroke treatments remain elusive. This picture argues powerfully that, for stroke therapy, the conventional concept of single drug intervention cannot be effective, and ultimately, strategies must look beyond the single cell or pathway for a more integrative answer to brain damage after ischemic injury [73].

The neurovascular unit

The brain cell components, ie, neurons, astrocytes, oligodendrocytes, microglia, and the microvascular endothelial cells and pericytes comprising the cerebral vasculature, are located in close proximity to each other within 25 μm [74,75]. There is a growing recognition that

integrated brain function and dysfunction arise from the complex interactions between an anatomical and functional network of these cells. This functional unit, which consists of the neurons, astrocytes, and endothelial cells, along with an extracellular matrix, has been termed the “neurovascular unit” [74,76]. In addition to the physical association within the microenvironment, these cells are functionally coupled via reciprocal cross talk and positive and negative feedback mechanisms (Figure 1). Accordingly, treatment modalities that target the neurovascular unit may ultimately yield more therapeutically beneficial results for neuroprotection. Indeed, most major therapeutic treatments achieved in the past decade for CNS disorders have been largely targeted to preserve the neuron, but clinical trials that ensued have failed to show benefit. There seems to be several sources for this failure. One possibility is that therapies aimed at neuronal survival may fail to support other cell types. Thus, in many current research endeavors, there is an effort to investigate the neurovascular unit as a therapeutic target from the functional perspective.

Functional crosstalk within the neurovascular unit includes hemodynamic coupling between neuronal firing and vascular response, which allows for matching blood supply to metabolic demand by neurons. Astrocytes and endothelial cells cooperate to form the integrity of the blood brain barrier (BBB). In addition, the three cell types provide trophic signals to each other via matrix-cell and cell-cell interactions (Figure 1).

Thearpeutic potential of exogenous vs. endogenous neurotrophic factors to enhance neuronal cell survival

The strategy of neuroprotection with neurotrophic factors has been always prioritized in the research of new therapies against acute stroke [77]. To date, more than 30 neurotrophic factors have been identified, and at least nine of them have shown neuroprotective properties in rodent models of MCA occlusion, when delivered locally by surgical procedures (Table 2). Glial cell line-derived neurotrophic factor (GDNF), a member of the TGF- β superfamily, is thought to be most potent among neurotrophic factors for the survival of cultured neurons [78]. Furthermore, GDNF has been shown to confer neuroprotection in animal models of Parkinson’s disease in addition to ischemic stroke [79,80]. The stroke protection by GDNF is mediated through inhibition of neuronal cell apoptotic death [81]. Despite the promising results in animal studies, the clinical trials in humans have shown inconsistent results. Although there may be multiple underlying reasons for this, the BBB is an outstanding issue [77]. GDNF, like other large molecule drugs, does not cross the BBB. Due to the BBB problem, prior human clinical trials with recombinant GDNF delivered the protein to brain via invasive trans-cranial routes, such as intra-cerebroventricular injection [82,83]. However, these approaches were still limited by poor penetration, short half-life and adverse effects of the neurotrophic factors. On the other hand, the BBB is disrupted following brain ischemia, which may allow for the drug entry into brain parenchyma; however, this occurs only after several hours of the stroke onset. Because the therapeutic time window for neuroprotective agents is less than 6 hours, the neurotrophic factors need to access the target before this BBB breakdown.

Accumulating evidence suggests that vascular endothelial cells provide paracrine survival signals to neurons, as evidenced by non-contiguous co-culture assays in vitro [84,85]. In addition, it has been demonstrated that neuronal stem cells requires signals from adjacent endothelial cells to execute neuronal differentiation [86]. Therefore, it is likely that in the adult brain, endothelial cells are capable of promoting neuronal survival by producing neurotrophic cues within the neurovascular unit. Exploitation of endothelial signals could be an alternative neurotrophic approach for neuosalvage therapy in ischemic stroke.

Blood-brain barrier as a key target for neurosalvage therapy in brain ischemia

In the neurovascular unit, endothelium, astrocytic endfeet, and basal lamina cooperate to form and maintain the BBB phenotype [75]. In addition, endothelial intercellular junctions, such as tight junctions and adherence junctions, are critical for the integrity of BBB [87]. Although BBB hampers delivery of neuroprotective agents as mentioned above, protection of the BBB integrity is crucial to prevent ischemic brain damage in stroke. Following ischemia and reperfusion, rapid increase in endothelial permeability and the production of reactive oxygen species (ROS) occur [88]. Subsequently, increased ROS is thought to activate proteases such as matrix metalloproteinase (MMP)-2 and MMP-9, which in turn degrades basement membrane and deteriorates the BBB. In late phases, endothelial cell death ensues and further worsens the barrier function. This sequence of events cause transudation of plasma components into brain parenchyma and lead to vasogenic edema. Movement of molecules from blood to brain across the damaged endothelium is thought to be important in the general development of neuronal damage [89]. Perturbation of neurovascular matrix disrupts the cell-matrix and cell-cell trophic signaling within the neurovascular unit and is thus detrimental to the neuronal cell survival. In agreement, inhibitors or neutralizing antibody against MMPs protects against edema formation and reduces infarct size in the MCAo model [90].

Given the evidence for involvement of BBB damage as an early event in many neurological conditions, there is a growing shift in emphasis from the rescue of neurons to the treatment of the BBB, which may eventuate in the prevention of secondary neuronal damage. Indeed, recent evidence suggests that several neuroprotective agents or modalities, such as dexamethazone, calcium channel blockers, local brain hypothermia, and pan-caspase inhibitors, are thought to exert brain protection, at least in part, through prevention of BBB damage [75].

Targeting endothelial Rac1 for stroke protection

The endothelial functions involved in the regulation of BBB property include permeability, ROS production, cell-cell and cell-matrix adhesion, and cell survival. Rac1, a member of the Rho-family small GTP-binding proteins, has been shown to play a critical role in all of these endothelial functions [91]. Therefore, we hypothesized that Rac1 in endothelial cells plays a role in the BBB regulation and neurovascular protection in ischemic mouse brain. To address this hypothesis, we generated endothelial-specific *Rac1* null mice by crossing transgenic mice expressing Cre recombinase under the control of Tie2 enhancer/promoter with mice harboring Rac1 floxed allele. As described previously, Tie2-Cre *Rac1*^{flox/flox} genotype resulted in embryonic demise due to the failure in angiogenesis in development [92,93]. Accordingly, Tie2-Cre *Rac1*^{flox/+} (EC-*Rac1*^{+/-}) mice were used for analysis with *Rac1*^{flox/+} mice as control. Primary endothelial cells (ECs) derived from EC-*Rac1*^{+/-} mice showed 50% reduction in Rac1 protein level compared to control.

To investigate the effect of EC *Rac1* haploinsufficiency on stroke outcome, EC-*Rac1*^{+/-} mice were subjected to transient MCAo (Figure 2) [94]. EC-*Rac1*^{+/-} mice showed 37% reduction in infarct size and 63% reduction in edema volume as compared to control (Figure 2, A to C). In contrast, the absolute CBF reduction in the ischemic hemisphere at the end of ischemia was similar between EC-*Rac1*^{+/-} and control mice (Figure 2D). This suggests that EC Rac1 haploinsufficiency conferred protection against neuronal death and edema formation independent of CBF.

To investigate the mechanisms for the neuroprotection in EC-*Rac1*^{+/-} mice, we conducted genome-wide microarray analysis to examine the differential gene expression between *Rac1*^{+/-} and *Rac1*^{+/+} ECs. *Rac1*^{+/-} ECs showed upregulation of gene clusters that contained 3 important categories relevant to neurovascular protection: (1) stress response genes, (2) cell adhesion and extracellular matrix (ECM) genes, and (3) growth factor genes.

(1) Stress response genes—The stress response genes included *Gpx3*, which encodes glutathione peroxidase 3 (Gpx3), an extracellular scavenger of ROS that catalyzes the reduction of hydrogen peroxide and lipid peroxides [95]. Decreased Gpx3 activity is associated with familial child stroke [96]. Upregulated stress response genes also included those encoding three heat shock proteins (HSPs), crystallin aB, hsp25, and hsp70, which protect cells from apoptosis under various stress conditions [97–99]. Consistent with these expression profiles, *Rac1*^{+/-} ECs showed decreased ROS formation in response to hypoxia/reoxygenation (H/R). In addition, H/R-induced apoptotic cell death was diminished in *Rac1*^{+/-} ECs compared to *Rac1*^{+/+} ECs.

(2) Cell adhesion and extracellular matrix (ECM) genes—The “ECM genes” showing increased expression in *Rac1*^{+/-} ECs included a subset that encodes basement membrane (BM) constituents. These BM constituents comprised structural (collagens [*Col2a1*, *Col8a1*], fibronectin [*Fnl1*] and regulatory proteins (thrombospondin 2 [*Thbs2*], osteopontin [*Spp1*]). Additionally, a weak but coordinate increase in expression was noted for genes encoding perlecan (*Hspg2* [heparan sulfate proteoglycan 2]), collagens (*Col1a1*, *Col4a1*, *Col4a2*), thrombospondin 1 (*Thbs1*), and SPARC (secreted acidic cysteine rich glycoprotein)(*Sparc*). Many of these proteins enhance collagen fibrillogenesis and matrix-cell adhesion, processes that stabilize the BM [100]. Specifically, thrombospondin 1 and 2 bind to and inhibit MMP-2, a constitutively expressed protease that initiates the degradation of BM early in MCAo [88, 100,101].

The “cell adhesion gene” cluster also included genes encoding components of adherence junctions (protocadherin 10), desmosomes (desmocollin 3), and tight junctions (claudin 3) [75]. Consistent with the increased abundance of BM and intercellular adhesion proteins in *Rac1*^{+/-} ECs, *Rac1*^{+/-} EC monolayers showed highly resistant phenotype against cell dispersion by trypsin digestion. Furthermore, the paracellular permeability of *Rac1*^{+/-} EC monolayers against a macromolecule HRP was significantly lower than that of *Rac1*^{+/+} ECs.

(3) Growth factor genes—The expression of genes encoding 13 growth factors was upregulated in *Rac1*^{+/-} ECs. Four of these growth factors (artemin, FGF-5, BMP-6, TGF- α) are endowed with potential neurotrophic capacity [81,102–105]. The genes encoding these factors are expressed basally or inducibly in vascular endothelium and in the central nervous system (CNS). In the EC-*Rac1*^{+/-} mouse whole brain, artemin (4.5-fold) and FGF-5 (1.4-fold) were significantly upregulated as compared to control. To address the significance of the upregulation of these neurotrophic factors, we isolated ECs from EC-*Rac1*^{+/-} and control mouse brain (mouse brain ECs, MBEC), and conducted non-contiguous co-culture with primary mouse cortical neurons (Figure 3A). Following 2-day co-culture, the neurons were challenged with 2-hr oxygen and glucose deprivation (OGD) followed by 22-hr reoxygenation. Administration of recombinant artemin (with blank inserts) elicited a dose-dependent inhibition of neuronal apoptosis, determined by the fraction of cells containing cleaved caspase 3 (Figure 3B). Endothelial co-culture attenuated neuronal apoptosis by 23% with control MBEC and by 52% with EC-*Rac1*^{+/-} MBEC, indicating that EC secretion of neurotrophic factors is increased by *Rac1* haploinsufficiency. Next, we tested the effect of artemin-neutralizing antibody or control IgG in neuron-EC co-cultures. Artemin-neutralizing antibodies had little effect on neuroprotection by control MBEC, but reduced neuroprotection by EC-*Rac1*^{+/-} MBEC by half. These results suggest that the potentiation of EC-derived neurotrophic activity by *Rac1* haploinsufficiency depends in part on increased production of artemin. The residual anti-apoptotic activity that persists in the presence of artemin-neutralizing antibody implicates additional neurotrophic factors in the neurotrophic effects of EC-*Rac1*^{+/-} MBEC.

Inhibition of endothelial Rac1 promotes vascular integrity and neuronal cell survival

We have shown that *Rac1* haploinsufficiency in ECs is neuroprotective through the release of neurotrophic factors. The neuroprotective mechanism is due, in part, to increased release of artemin from haploinsufficient *Rac1* ECs. Artemin is a member of the GDNF family of ligands (GFLs) that is expressed in various neural and non-neural tissues, including vascular walls (smooth muscle cells and ECs) [102,106,107]. GFLs exert anti-apoptotic signals on neurons by binding to a receptor complex composed of GDNF receptor α (GFR α) and RET (Rearranged during transfection) proto-oncogene [108]. The principal receptor for artemin is GFR α 3, which is expressed mainly in non-neural tissues and in the peripheral nervous system [109]. Studies with mice lacking artemin uncovered its role as a vascular-derived guidance factor for sympathetic neuron axonal projections [106]. In the CNS, artemin is expressed in low abundance during development and adulthood in distinct regions, such as the basal ganglia and thalamus, whereas GFR α 3 expression is almost non-existent [102,109]. Despite the low abundance and restricted distribution of endogenous artemin in the CNS, administration of exogenous artemin potently promotes the survival of neurons from various CNS regions including midbrain dopaminergic neurons, cortical GABAergic neurons, and hippocampal CA1 neurons [110–112]. Reportedly, artemin can also signal through GFR α 1, which is the principal receptor for GDNF and shows widespread, ischemia-inducible CNS expression and neuroprotective capacity in MCAo [81,102,105]. Therefore, we consider that the reduction in infarct size in EC-*Rac1*^{+/-} mice was likely exerted through EC-derived artemin acting through GFR α 1 system.

Our data suggest that EC Rac1 haploinsufficiency is neuroprotective through two mechanisms: It enhances vascular barrier integrity and independently promotes neuronal survival (Figure 4). Accumulating evidence indicates that targeting a single pathological process does not suffice for neuroprotection and points to the importance of combination therapies [73]. In this regard, the multiple neuroprotective mechanisms potentially elaborated by *Rac1*^{+/-} ECs may be beneficial. Considering the well-established roles of matrix-cell interaction in cell survival, increased expression of BM components may not only enhance BBB integrity, but also could directly contribute to preventing neuronal cell death. Indeed, fibronectin provides neuroprotection in mice MCAo [113]. Conversely, the neurotrophic factors upregulated in *Rac1*^{+/-} ECs also possess gliotrophic functions that could enhance the vascular barrier [75]. For example, FGF-5 regulates the astrocyte endfeet coverage of blood vessels and enhances BBB function [114]. Furthermore, it is becoming increasingly clear that cytokines that are ineffective in isolation can become neurotrophically active when acting together [115]. The small but coordinate upregulation of various growth factors in *Rac1*^{+/-} ECs conforms to this notion, and may underlie the neuroprotection seen in MCAo.

Because EC represent a highly drug-accessible brain compartment, targeting endothelial Rac1 may provide a therapeutically feasible approach for intervention of the crosstalk between ECM and cellular components within the neurovascular unit. The therapeutic time window of neurotrophic factor therapy for acute ischemic stroke is typically within 1 hr of reperfusion [81]. Furthermore, BM disruption commences within hours of the stroke onset [88]. Therefore, prophylactic inhibition of EC Rac1 may be beneficial for effective neuroprotection. However, Rac1 deletion in ECs could also lead to decreased angiogenic response. It remains to be determined, therefore, what the effect of sustained inhibition of endothelial Rac1 is on neurogenesis, which is coupled to angiogenesis within the neurovascular niche [86,116].

Emerging therapeutic strategies to modulate neurovascular signaling beyond BBB

A key challenge in the development of drugs for CNS diseases is their transport across the BBB. Our study represents recent endeavor aiming to directly intervene the cell-matrix signaling in the neurovascular unit. Recently developed modalities including ours commonly target the vascular endothelium and show the potential to target neuronal cells beyond the BBB (Table 3). These approaches can be classified into two categories.

(1) Conjugation of targeted agents to allow receptor-mediated trans-endothelial transport

Kumar and colleagues [117] developed a method to target small-interfering RNA (siRNA) to the CNS, providing a safe and non-invasive approach for the delivery of this new therapeutic modality, and possibly other therapeutic molecules, across the BBB. To facilitate the transport of siRNA across the BBB, the authors took advantage of a strategy that enables the rabies virus to enter neuronal cells and spread throughout the brain. A short (29 amino acid) peptide derived from the rabies virus glycoprotein (RVG) was shown to act as a “key” to the CNS, by specifically binding to the nicotinic acetylcholine receptor (AChR) present on neurons and the vascular endothelium of the BBB, allowing transvascular delivery, probably by receptor-mediated transcytosis. When siRNA was bound to this peptide and intravenously administered to mice, the uptake of the siRNA was brain-specific. Importantly, antiviral siRNA bound to RVG peptide was able to protect mice from fatal infection with Japanese encephalitis virus. Spencer and Verma [118] adopted a similar approach in which the proteins of interest were fused to low-density lipoprotein receptor-binding domain of the apolipoprotein B, which allowed for receptor-mediated transcytosis across the EC layer. Intraperitoneal injection of lentiviruses coding for these fusion proteins successfully delivered the targeted proteins to neurons and astrocytes in the mouse brain. As noted above, substantial information has been accumulating with regards to the molecular targets at multiple steps of tissue damage associated with brain ischemia. Despite the vast knowledge, drug discovery and evaluation *in vivo* have been hampered by the BBB, as already mentioned; therefore, the advent of these innovative approaches that enable targeted delivery of siRNA and recombinant proteins to CNS will powerfully delineate the therapeutic potential of otherwise undeliverable molecules.

(2) Exploitation of endogenous EC signals

Our data [94] have shown that specific inhibition of endothelial Rac1 confers neuroprotection through coordinated upregulation of genes comprising stress response genes, cell adhesion/ECM genes, and neurotrophic factors. Through this mechanism, *Rac1* haploinsufficient ECs conferred neuroprotection by promoting BBB integrity and humoral factor-mediated direct neuroprotection. As noted above, endothelium is located at the interface between the circulating blood flow and brain parenchyma, and thus serves as a pharmacologically-targetable compartment in the neurovascular unit; therefore, manipulation of EC signaling is a feasible approach for trans-vascular intervention of neurovascular signaling. In addition, as compared with the single drug-based therapeutic approaches, the coordinated mobilization of various genes by EC *Rac1* inhibition may enable targeting multiple pathways, which in concert may be beneficial for stroke protection. More importantly, this approach conceivably utilizes the endogenous protective mechanisms by endothelium, which may be of further advantage for improved therapy. Lastly, given the importance of the BBB integrity in neurological disease outcome, dual modulation of the vascular integrity and direct neuroprotection by EC *Rac1* inhibition would add more therapeutic benefit.

Guo and colleagues [119] demonstrated that activation of $\beta 1$ integrin and integrin-linked kinase (ILK) signaling enhances EC-derived production of brain-derived neurotrophic factor and protects cultured neurons from apoptotic death. Noncytotoxic levels of oxidative stress disrupts

ILK signaling and reduces endothelial levels of neuroprotective BDNF. Although this signaling needs further validation using in vivo models, it provides another molecular target for EC-mediated neuroprotection. It is anticipated that further studies will increase knowledge about key EC signals for EC-derived neurotrophic activity, and its coordination with EC-mediated vascular functions such as endothelial permeability.

Expert commentary

In this review, we have summarized evidence showing that therapies that primarily aim to increase or preserve the CBF and improve vascular function have been the central strategy for stroke prevention and treatment. Experimental evidence has suggested that endothelium-derived NO bioavailability and eNOS function are key mediators to increase CBF, and that the stroke-protective effect of statins is dependent on eNOS upregulation. Large clinical trials substantiate claims that statins are promising drugs for stroke prophylaxis; long-term statin administration reduces stroke risk for both first and secondary events by 25–30%. At this point, it is important to address whether early treatment with statins after a cerebrovascular event reduces morbidity and mortality after several months. This hypothesis has been tested in the prospective FASTER trial, but the trial was stopped early due to a failure to recruit patients at the prespecified minimum enrolment rate. The trial was unable to provide evidence of benefit of simvastatin in this setting. This aggressive prevention approach merits further study.

It is important to note that there will be an even greater need for acute stroke treatments, as shifting demographics and an ageing population demand better acute therapy as well as better outcomes after stroke. One might argue that the benefit of combined prophylactic or treatment regimens with statins might be more than additive for stroke, as has been estimated for heart attack. In this context, given the experimental evidence that multi-factorial therapeutic approaches targeting distinct pathways or mechanisms may be beneficial, and building upon the accumulating repertoire of the neurotrophic agents showing effects in animal studies, it is reasonable to assume that direct neuroprotective strategies may be one answer to complement CBF-enhancing therapies for better stroke prevention and outcome. Meanwhile, more than 160 clinical trials of neuroprotective therapies for ischemic stroke have been initiated with overall disappointing results [2]. For example, a randomized multicenter trial for FGF-2, a potent neuroprotectant in animals, has been implemented with 286 patient enrollment, but failed to show benefit [120]. It is important to note that, although neurotrophic factors cannot cross the intact BBB, recombinant FGF-2 was administered intravenously in this study. Therefore, it is unlikely that the negative results from this study design preclude the therapeutic potential of this neurotrophic agent shown in animal models of stroke [121]. In this context, the recent advance in the development of modalities that circumvent the BBB may ultimately lead to reviewing the therapeutic efficacy of many neurotrophic factors.

Five-year view

As we have emphasized in this review, it is becoming increasingly clear that cell-cell signaling within the entire neurovascular unit is a fundamental requirement for function and dysfunction in brain. Therefore, it is logical to propose that focusing on a single intracellular pathway or cell type might not suffice, and that neuroprotection, glioprotection, and vasculoprotection must be considered to coordinate blood flow, the BBB, and cell survival signals and to prevent the ischemic brain damage. Of note, there is growing interest in the versatility of endothelial cells. Indeed, the recent recognition that paracrine signals from endothelium could have a global role in tissue differentiation and function indicates the broad applicability of exploiting EC signals for therapies targeting adjacent parenchymal tissues [122]. This is a rapidly evolving field, and new mediators and signaling mechanisms in endothelial cells, in addition to Rac1 and ILK, are expected to emerge at an amazing pace. The expected increase in the knowledge

of EC signaling will fuel the development and refinement of modalities that enhance EC-mediated neurovascular protection. Importantly, it is expected that considerable research effort will focus on the regulation of neuro-restorative therapy in the recovery phase of stroke. This will involve the stimulation of the brain's endogenous repair mechanisms. It is important to note that neurogenesis and angiogenesis are tightly coupled in the context of the neurovascular niche [116]. Therefore, there is a possible scenario where intervention of EC signaling will be chosen for the control of neuro-regeneration.

Lastly, this review has presented a picture in which the two prominent Rho family members, RhoA and Rac1, play quite distinct roles in EC signaling and uniquely contribute to stroke protection. Inhibition of RhoA or its downstream effector ROCK upregulates eNOS and promotes the increase of CBF. On the other hand, inhibition of Rac1 leads to endothelial barrier protection and increased neurotrophic factor production. Hence, dual blockade of RhoA/ROCK (with fasudil/Y-27632) and Rac1 (with NSC23766) [123] would improve CBF and neurovascular unit function, and could exert more than additive neuroprotective effects. It would be of interest to explore this opportunity in animal models of stroke. [124–130]

Key issues

- Many of the experimental modalities that confer stroke protection in rodent models, such as statins, exert their effects by increasing expression or activity, or both, of eNOS and thereby augmenting cerebral blood flow.
- The direct upregulation of eNOS by statins is at least in part mediated through inhibition of small GTPase RhoA by intracellular depletion of geranylgeranylpyrophosphate.
- Administration of Rho-associated kinase (ROCK) inhibitor increases cerebral blood flow and confers stroke protection in an eNOS-dependent manner.
- Clinical trials have shown that long-term administration of statins reduce stroke incidence by 30%. Statins may also improve stroke outcome in humans. It is controversial whether statin therapy initiated after cerebrovascular events improves short-term outcome.
- The neurons, astrocytes, and endothelial cells form an anatomical and functional compartment termed the “neurovascular unit”. Cell components in this unit cooperate to regulate blood flow, the blood-brain barrier and provide survival signals. Treatment modalities that target the neurovascular unit may yield more therapeutically beneficial results for neuroprotection than targeting single cell types or pathways.
- Neurotrophic factors, when administered exogenously, show potent neuroprotection only through invasive delivery because they cannot penetrate the BBB. Exploitation of endogenous neurotrophic activity from ECs in the neurovascular unit would be an alternative strategy.
- The disruption of the BBB following ischemia/reperfusion causes transudation of plasma components and develops brain edema. There is growing shift in emphasis from the rescue of neurons to the treatment of the BBB to prevent secondary neuronal damage.
- Endothelial-specific haploinsufficiency of small GTPase Rac1 leads to blood flow-independent stroke protection in mouse MCA occlusion. Rac1 haploinsufficient ECs show upregulation of 3 categories of genes relevant to neurovascular protection: “stress response genes”, “cell adhesion/extracellular matrix genes” and “growth factor genes”.

- The neuroprotection in endothelial Rac1 haploinsufficient mice is mediated by 2 mechanisms: protection of the endothelial barrier property and direct neuroprotection by upregulation of EC-derived neurotrophic factors. Half of the humoral neurotrophic activity by Rac1 haploinsufficient ECs is accounted for by a GDNF member, artemin.
- Therapeutic intervention of the neurovascular signaling beyond the BBB may become feasible by the recently developed approaches. These include conjugation of siRNA and proteins to moieties that allow receptor-mediated trans-endothelial transport.

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Figure 1. The neurovascular unit
Based on fundamental concepts outlined in Ref. ⁷⁴.

Figure 2. Haploinsufficiency of endothelial *Rac1* in mice leads to neuroprotection

EC-*Rac1*^{+/-} and control mice underwent 2-hr MCAo followed by 22-hr reperfusion. (A) Representative brain sections stained with 2,3,5-triphenyltetrazolium chloride visualizing the infarct area. (B, C) Infarct (B) and edema (C) volumes of the ischemic hemisphere. (D) Change in cerebral blood flow (CBF) in the ischemic hemisphere at the end of 2-hr MCAo. (B–D) Values are % of the non-ischemic hemisphere and shown as means ± SEM of 7–10 mice (*, *P* < 0.05, ANOVA). (E) Expression profiling of *Rac1*^{+/-} ECs. Differential mRNA expression between *Rac1*^{+/-} and *Rac1*^{+/+} mouse ECs was explored using microarray analysis. Functional annotation clustering of the upregulated genes is shown. Reproduced from Ref. ⁹⁴.

Figure 3. *Rac1*^{+/−} ECs show enhanced neurotrophic activity through paracrine mechanisms (A) Schematic of EC-neuron co-culture. (B) Cortical neurons isolated from fetal mouse brains (~E16) were co-cultured with EC-*Rac1*^{+/−} or control MBEC in the presence of artemin-neutralizing antibody, control IgG, or vehicle in the lower chamber. Control neurons were treated with blank inserts and recombinant artemin proteins. Bar graphs show the quantification of the cleaved caspase 3-positive fractions. Values are means $\pm$ SEM (*, $P < 0.05$; **, $P < 0.01$; ANOVA). Reproduced from Ref. ⁹⁴

Figure 4. A proposed model of neuroprotection through targeting endothelial Rac1
 Reproduced from Ref. ⁹⁴.

Table 1

Agents and modalities that confer stroke protection by enhancing eNOS/cGMP pathway.

Agents/Modalities	Ref.
Prophylaxis: eNOS upregulation through mRNA stabilization by inhibition of RhoA/ROCK pathway	
Statins	27
ROCK inhibitor	35
Prophylaxis: eNOS upregulation through undetermined mechanisms	
Angiotensin II type 1 receptor blocker	49
Peroxisome proliferator-activated receptor- γ agonists	52
Resveratrol	53
Exercise	54
Prophylaxis: cGMP increase through inhibition of phosphodiesterase dipyridamole	48
Acute treatment: eNOS activation through S1177 phosphorylation by activation of PI-3 kinase/Akt pathway	
Statins	36
Corticosteroids	59
Estrogen	60
Thyroid hormone	61

Table 2

Growth factors with neuroprotective potential in a rodent model of MCA occlusion.

Growth factors	Ref.
Brain-derived neurotrophic factor (BDNF)	124
Glial cell line-derived neurotrophic factor (GDNF)	79
Transforming growth factor-b (TGFb)	125
Bone morphogenetic protein-6 (BMP6)	126
Bone morphogenetic protein-7 (BMP7)	127
Fibroblast growth factor-2 (FGF2)	121
Fibroblast growth factor-2 (FGF13)	128
Fibroblast growth factor-2 (FGF18)	129
Transforming growth factor-a (TGFa)	130

Table 3

Therapeutic approaches to modulate the neurovascular signaling by targeting the vascular endothelium.

Therapeutic modalities	Ref.
Targeted delivery of therapeutics across the blood-brain barrier	
Targeted protein fused to the LDLR-binding domain of apolipoprotein B	118
Targeted siRNA bound to a RVG-9R peptide derived from neurotropic virus	117
Exploitation of endothelial cell signals	
Activation of integrin-linked kinase enhances endothelial secretion of BDNF	119
Inhibition of Rac1 enhances endothelial integrity and neurotrophic factor secretion	94