BRIEF REVIEW

Endothelial Targets in Stroke
Translating Animal Models to Human

Anuska V. Andjelkovic, Jianming Xiang, Svetlana M. Stamatovic, Ya Hua, Guohua Xi, Michael M. Wang, Richard F. Keep

ABSTRACT: Cerebral ischemia (stroke) induces injury to the cerebral endothelium that may contribute to parenchymal injury and worsen outcome. This review focuses on current preclinical studies examining how to prevent ischemia-induced endothelial dysfunction. It particularly focuses on targets at the endothelium itself. Those include endothelial tight junctions, transcytosis, endothelial cell death, and adhesion molecule expression. It also examines how such studies are being translated to the clinic, especially as adjunct therapies for preventing intracerebral hemorrhage during reperfusion of the ischemic brain. Identification of endothelial targets may prove valuable in a search for combination therapies that would specifically protect different cell types in ischemia.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: blood-brain barrier ■ permeability ■ reperfusion ■ tight junctions ■ transcytosis

Globally, ≈5.5 million people die annually of ischemic or hemorrhagic stroke. Animal stroke models have provided insight into the complex pathophysiological mechanisms of underlying brain injury after cerebral ischemia. However, failures in translating preclinical results on neuroprotectants to the clinic has led to questions over the utility of such models. It should be noted, however, that early reperfusion efficacy was shown in animal ischemic stroke, as was the specific benefit of tPA (tissue-type plasminogen activator; alteplase)-induced reperfusion. While there have been failed clinical trials targeting cerebrovascular mechanisms in acute ischemic stroke (AIS), it is still unclear how well animal studies might inform trials with endothelial targets. This review examines recent preclinical studies targeting alterations in the cerebrovasculature in ischemic stroke, particularly focusing on the cerebral endothelium. It discusses the role, progress, difficulties, and opportunities related to that target and translation of those studies to the clinic.

CHALLENGES IN STROKE MODELING WITH PARTICULAR REFERENCE TO THE CEREBRAL ENDOTHELIUM

Multiple concerns have been raised over animal models of human stroke leading to STAIR (Stroke Treatment Academic Industry Roundtable) recommendations for preclinical ischemic stroke studies. While some issues are addressable, such as the inclusion of studies in animals with relevant comorbidities and the inclusion of gyrencephalic models, others are more difficult (eg, absolute lifespan and brain size). In this latter group are species differences in gene and protein expression that may impact stroke. Although the genome is largely conserved between mammals, there are human-specific genes, a significant portion of which are involved in inflammatory and injury responses, raising the question of whether these genes, particularly those expressed at the cerebral endothelium/neurovascular unit (NVU), alter the impact of stroke in humans. It would be informative to conduct stroke experiments in humanized mice that express these human-specific proteins.

Correspondence to: Richard F. Keep, PhD, Department of Neurosurgery, University of Michigan, 5018 BSRB, 109 Zina Pitcher Pl, Ann Arbor, MI 48109. Email rkeep@umich.edu
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There are also species differences in cerebrovascular mRNA and protein expression. For example, there are differences in ATP-binding cassette transporter family mRNA expression that affect the penetration of therapeutics across the blood-brain barrier (BBB). Similarly, there are species differences in cerebrovascular protein expression. For example, while periaxin is present in multiple tissues across species, in brain endothelial cells it is only present in humans where it functions to modulate inflammatory gene expression and BBB permeability. Overall, the extent to which species differences in cerebrovascular gene, mRNA, and protein expression/activity impact stroke merits investigation. Use of cell-specific knockdowns and protein overexpression (including human) studies should provide insight into the importance of variations in particular genes and their protein expression.

Two other modeling issues relate to reperfusion and the endothelium. Thrombectomy results in a more abrupt restoration of blood flow than tPA potentially impacting reperfusion-induced endothelial dysfunction. While there are multiple animal models examining tPA, cerebral vessel size in animals has precluded examining thrombectomy devices. It has been suggested that transient middle cerebral artery occlusion by intraluminal suture might be a thrombectomy model but that requires validation. The second issue is related to the duration of ischemia before reperfusion which impacts endothelial function. Because of injury severity, short middle cerebral artery occlusion times (≤60 minutes) are typically used in mice. This is very different from the current therapeutic time window for reperfusion in patients. While using mice has many advantages, it is very important that results are replicated in gyrencephalic species with long reperfusion times.

### Nonstandard Abbreviations and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| AIS          | acute ischemic stroke |
| APC          | activated protein C |
| BBB          | blood-brain barrier |
| GAMES-RP     | Glyburide Advantage in Malignant Edema and Stroke |
| ICH          | intracerebral hemorrhage |
| NVU          | neurovascular unit |
| PAR          | protease activated receptor |
| PARP         | poly(ADP-ribose) polymerase |
| PDGF         | platelet-derived growth factor |
| ROCK         | Rho-kinase |
| Sur1         | sulfonyleurea receptor 1 |
| TJ           | tight junction |
| tPA          | tissue-type plasminogen activator |
| Trpm4        | transient receptor potential melatonin 4 |

### Highlights

- Cerebral ischemia causes endothelial dysfunction including tight junction disruption, increased transcytosis and a change to a proinflammatory phenotype.
- Endothelial dysfunction contributes to parenchymal damage and neurological deficits.
- Endothelial cell dysfunction is currently being targeted in multiple clinical trials to limit cerebral hemorrhage after tPA (tissue-type plasminogen activator)-induced reperfusion in stroke patients.
- A combination of protectants targeting the cerebral endothelium and the brain parenchyma should be considered for future trials in stroke.

### ROLE OF CEREBRAL ENDOTHELIAL DYSFUNCTION IN STROKE

Cerebral endothelial dysfunction may contribute to stroke-induced brain injury (Figure 1). This review does not address how disease-related endothelial dysfunction may initiate stroke. As in other endothelial beds, the cerebral endothelium regulates blood flow, secreting vasodilators/vasostrictors, and provides a nonthrombogenic surface and stroke alters those functions. For example, ischemia impairs endothelium-dependent vasodilation, limiting blood flow restoration, and induces a prothrombogenic and proinflammatory endothelial phenotype. That may promote thrombus formation, emboli and leukocyte adhesion, and reduce blood flow.

The cerebral endothelium also forms the BBB. It limits brain entry of many compounds through the presence of endothelial tight junctions (TJs), efflux transporters, and limited transcytosis. Stroke causes BBB dysfunction, including hyperpermeability leading to vasogenic brain edema. It induces TJ disruption, allowing hydrostatically driven water flow across the capillary, and affects ion transport and increases transcytosis, both of which may contribute to altered fluid movement.

The BBB also excludes potentially neurotoxic compounds from brain. Ischemia-induced BBB dysfunction may allow their accumulation, for example, prothrombin/thrombin and fibrinogen/fibrin accumulation may cause neuroinflammation and injury. Such changes can form a positive feedback loop with extravasated compounds themselves causing BBB dysfunction. Extreme barrier disruption will result in intracerebral hemorrhage (ICH) as in primary ICH and AIS-induced hemorrhagic transformation. Hemorrhagic transformation limits the use of tPA for AIS.

Under normal conditions, cerebral endothelial cells express low levels of leukocyte adhesion molecules (eg, ICAM-1 [intercellular adhesion molecule] and VCAM-1...
[vascular cell adhesion molecule]), but these are markedly increased after ischemia promoting leukocyte diapedesis. While the initial inflammatory response after stroke is microglial activation, leukocyte extravasation plays an important role in brain injury and recovery after ischemia.

Stroke-induced endothelial dysfunction may be direct or indirect (primary/secondary injury). Whether endothelial injury induces parenchymal injury or vice versa is an important issue for therapeutic targeting. Greater use of endothelial-specific mutants or endothelial-specific targeting is clarifying that issue.

TARGETING ENDOTHELIAL DYSFUNCTION

Permanent and transient ischemia both cause endothelial dysfunction either through direct effects on the endothelial cell or indirect effects on perivascular cells/components, the NVU. The NVU is composed of pericytes, astrocytes, perivascular macrophages, smooth muscle cells, neurons, and their basement membranes. Each component regulates endothelial function and is regulated by the endothelium. Thus, 2 approaches to limit ischemia-induced brain endothelial dysfunction are to target signaling cascades in the NVU that lead to dysfunction or to target endothelial mechanisms impacted by those cascades, that is, endothelial cell death, TJ changes, enhanced transcytosis, altered ion transporter/channel expression/activity, and increased expression of molecules involved in leukocyte trafficking.

Much attention has focused on targeting signaling cascades. For example, there have been many studies examining the role of inflammation and oxidative stress in ischemia- and trauma-induced parenchymal injury that have also examined BBB dysfunction. In terms of directly targeting end effector mechanisms, there is debate about the relative role of TJ disruption, enhanced transcytosis and increased endothelial cell death in BBB hyperpermeability after cerebral ischemia (eg). This may vary with time, permeability marker, and model.

TARGETING ENDOTHELIAL TJ

TJs are comprised of transmembrane proteins (eg, claudin-5, -3, -12, occludin, junctional adhesion molecule-A) occluding the paracellular cleft, cytoplasmic plaque scaffolding proteins (eg, ZO-1 [zonula occludens]) and the actin cytoskeleton that provide TJ physical support. Ischemia impacts TJ proteins at different levels: structure (eg, via protein phosphorylation), distribution (eg, via internalization), and expression (eg, via degradation), contributing to TJ disruption. For example, claudin-5, occludin, and ZO-1, undergo phosphorylation and other post-translational modifications in ischemia and in inflammatory conditions. Such modifications affect protein:protein interactions within the TJ (eg, via protein phosphorylation), distribution (eg, via internalization), and potential lysosomal degradation. There is evidence that ROCK (Rho-kinase) is an important regulator of TJ protein phosphorylation and a ROCK inhibitor, fasudil, reduces BBB disruption after ischemia. Fasudil is already used clinically in Japan and China to limit vasospasm and it is being investigated for cerebrovascular protection and reducing hemorrhage in cerebral cavernous malformations. Further studies on AIS are warranted. There is also evidence that KD205, a specific ROCK-2 inhibitor, is more effective than fasudil, a nonselective ROCK inhibitor, in preventing BBB disruption due to oxygen glucose deprivation in vitro and that it reduces infarct size after middle cerebral artery occlusion in mice. This drug is in clinical trials for other disease states and merits further investigation in stroke.

Many studies, but not all, show decreased TJ protein expression after cerebral ischemia due to reduced transcription or enhanced protein degradation. A broad range of approaches have blunted claudin-5 downregulation...
after cerebral ischemia, for example, an AMPA receptor antagonist (perampanel), doxycycline, progesterone, estrogen receptor activation, integrin blockade, and a microRNA-150 antagonist (reviewed in). Whether these effects are directly on the endothelial TJs is largely uncertain.

Junctional adhesion molecule-A is unusual as inflammatory conditions cause a relocation from the TJ to the endothelial apical membrane where it can act as a leukocyte adhesion molecule. A peptide blocker of this TJ protein reduced leukocyte extravasation after transient cerebral ischemia in mice. Ischemia also induces brain endothelial claudin-1 expression. Its appearance long-term after stroke induces TJ destabilization and BBB leakage. A peptide that causes claudin-1 internalization and degradation blocks BBB hyperpermeability and improves functional outcomes after ischemia in mice.

Actin cytoskeleton changes, including stress fiber formation, have important ramifications for TJ function. Thus, enhancing actin depolymerizing factor or blocking actin polymerization with heat shock protein 27 specifically in endothelial cells reduced ischemia-induced BBB damage. Those approaches also attenuated behavioral deficits, suggesting endothelial injury causes secondary parenchymal damage.

TARGETING ENDOTHELIAL TRANSCYTOSIS

Cerebral ischemia causes a marked increase in endothelial caveolae and vesicles, and there is evidence that enhanced transcytosis plays a role in BBB hyperpermeability. However, it should be noted that different groups have found disparate effects of deleting caveolin-1 on ischemia-induced BBB hyperpermeability (see review). There are also concerns over targeting caveolin-1 as an AIS therapy. Caveolin-1 knockout mice have larger infarcts with caveolin-1 promoting angiogenesis and astrogliosis after ischemia. This highlights the need for endothelial-specific targets or methods to specifically target the endothelium therapeutically.

TARGETING ENDOTHELIAL DEGENERATION

Ischemia also induces endothelial cell degeneration and this has been postulated to be a predominant mechanism underlying BBB hyperpermeability although there is a question over whether endothelial cell death would result in flow cessation, limiting barrier permeability. In neurons, ischemic cell death is linked to insufficient ATP production, ion gradient failure and increased Ca2+-dependent protease activity that degrade vital cell components, and the same likely happens at the cerebral endothelium.

Ischemia and ischemia/reperfusion also impact mitochondrial function leading to free radical production that attacks cellular proteins, lipids, and nucleic acids. Injury is not limited to the cerebral endothelium and damage to cellular and acellular (eg, basement membrane) NVU components may deprive endothelial cells of important signals for BBB function. The precise mechanisms underlying ischemia-induced endothelial cell degeneration need to be elucidated to identify potential therapeutic targets.

TARGETING ENDOTHELIAL ION TRANSPORT

Cerebral ischemia also impacts endothelial ion transport and ion channels. In parenchyma, ischemia increases in intracellular Na+ and Ca2+ and extracellular K+. There is a net Na+ gain and K+ loss in brain, but the former exceeds the latter causing a net gain in cations (and Cl−) and an influx of water into brain (edema). There has been interest in regulating BBB ion transport to reduce edema formation. Initial studies examined endothelial Na+/K+-ATPase, which is stimulated due to increased extracellular K+. Recently, interest has focused on endothelial Na+ transport via Na+/K+/Cl− and Na+/H+exchange and a nonselective cation channel, the Sur1 (sulfonylurea receptor 1)-Trpm4 (transient receptor potential melastatin 4) channel. Stroke upregulates that channel, including in endothelial cells, and it can be inhibited by glyburide. This led to the GAMES-RP (Glyburide Advantage in Malignant Edema and Stroke) phase 2 trial (URL: http://www.clinicaltrials.gov. Unique identifier: NCT01794182). While that trial did not meet its primary end point, modified Rankin scale, it significantly reduced mid-line shift.

TARGETING INFLAMMATORY MECHANISMS AT THE ENDOTHELIUM

There has been a long history of targeting endothelial leukocyte adhesion/diapedesis to limit ischemia-induced injury. However, early trials blocking neutrophil entry failed to improve stroke outcomes (reviewed in). More recently, there has been a Phase 2 trial of natalizumab (URL: http://www.clinicaltrials.gov. Unique identifier: NCT01955707), a monoclonal antibody blocking leukocyte-endothelium interaction by targeting α4 integrin. It failed to reduce infarct size in AIS (primary end point), although there was some evidence of benefit in neurological deficits at 30 days.

While there is substantial preclinical evidence that extravasated leukocytes can be detrimental in stroke, some leukocytes may be beneficial; for example, Treg and B cell lymphocytes. Similarly, macrophage subpopulations (M2) participate in tissue repair and even
neutrophils, long considered detrimental, may have a beneficial subpopulation (N2). Therapeutic leukocyte modulation may require precise modulation of specific subtypes at particular times after stroke. In addition, evidence indicates the brain endothelial cell extracellular matrix is a rate-limiting step in leukocyte extravasation and may be a therapeutic target.

**ENDOTHELIAL TARGETING AS AN ADJUNCT FOR REPERFUSION THERAPY**

The risk of symptomatic ICH limits the use of tPA for AIS driving the development of adjunct therapies to reduce that risk. A wide range of agents/approaches has been tested preclinically to examine whether they can reduce ICH and BBB disruption after tPA-induced reperfusion. Several approaches have shown efficacy in multiple studies including targeting free radicals, enhancing oxygen delivery, using minocycline, cilostazol, the PARP (poly(ADP-ribose) polymerase) inhibitor PJ34, and the ROCK inhibitor fasudil. As noted above, ROCK regulates TJ protein phosphorylation and function.

Figure 2. Preclinical studies have indicated that inflammation, free radicals, and neurovascular unit (NVU) PDGF (platelet-derived growth factor)-CC. Blocking PDGF signaling with imatinib reduces BBB disruption and ICH induced by tPA-induced reperfusion after ischemia. That work resulted in a phase 2 clinical trial of imatinib in AIS patients undergoing thrombolysis and there is a current phase 3 trial as an adjunct therapy for AIS patients undergoing reperfusion therapy (tPA and thrombolysis; URL: http://www.clinicaltrials.gov. Unique identifier: NCT03639922).

Edaravone is used in Japan to treat AIS and preclinical studies indicate it reduces tPA-induced hemorrhagic transformation. Because edaravone and tPA are used in Japan, AIS patients may be treated with both drugs. In a registry study, Yamaguchi et al compared symptomatic ICH incidence in edaravone+tPA and tPA treated patients. Results were inconclusive, with edaravone+tPA treatment showing a significant reduction in hemorrhage or a trend towards a reduction in hemorrhage depending on the definition of symptomatic ICH. A small retrospective Chinese study compared ICA treated stroke patients with and without edaravone. Hemorrhagic transformation (but not symptomatic ICH) was less with cotreatment. Overall, the potential of edaravone for reducing tPA-induced ICH merits further investigation.

Fingolimod is an immune modulator approved for use in multiple sclerosis patients. In a pilot study, patients given fingolimod+tPA had smaller hemorrhages than patients given ICA alone.

**CONCLUSIONS**

While many neuroprotectants are efficacious in preclinical ischemic stroke models, none have successfully translated to the clinic raising skepticism over preclinical stroke models. This review has attempted to highlight the usefulness of preclinical studies in identifying potential endothelial-targeted therapeutic approaches for AIS, as well as potential pitfalls. Combination therapies (reperfusion+endothelial protectant) are already undergoing clinical translation. Currently, most clinical trials have focused on neuronal or whole brain (eg, inflammation and oxidative stress) targets, without success. There may be a case to make for combining therapies targeting...
injury in specific cell types (eg, a neuronal and an endothelial protectant).

**ARTICLE INFORMATION**

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Affiliations

From the Departments of Neurosurgery (A.V.A., J.K., Y.H., G.X., R.F.K.), Pathology (A.V.A., S.M.S.), Neurology (M.M.W.), and Molecular and Integrative Physiology (M.M.W., R.F.K.), University of Michigan, Ann Arbor; and Department of Veterans Affairs, Neurology Service, VA Ann Arbor Healthcare System, MI.

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Disclosures

None.

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