Small GTPase RhoA and Its Effector Rho Kinase Mediate Oxygen Glucose Deprivation-Evoked In Vitro Cerebral Barrier Dysfunction

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Background and Purpose—Enhanced vascular permeability attributable to disruption of blood–brain barrier results in the development of cerebral edema after stroke. Using an in vitro model of the brain barrier composed of human brain microvascular endothelial cells and human astrocytes, this study explored whether small GTPase RhoA and its effector protein Rho kinase were involved in permeability changes mediated by oxygen-glucose deprivation (OGD), key pathological phenomena during ischemic stroke.

Methods and Results—OGD increased RhoA and Rho kinase protein expressions in human brain microvascular endothelial cells and human astrocytes while increasing or unaffected that of endothelial nitric oxide synthase in respective cells. Reperfusion attenuated the expression and activity of RhoA and Rho kinase in both cell types compared to their counterparts exposed to equal periods of OGD alone while selectively increasing human brain microvascular endothelial cells endothelial nitric oxide synthase protein levels. OGD compromised the barrier integrity as confirmed by decreases in transendothelial electric resistance and concomitant increases in flux of permeability markers sodium fluorescein and Evan’s blue albumin across cocultures. Transfection of cells with constitutively active RhoA also increased flux and reduced transendothelial electric resistance, whereas inactivation of RhoA by anti-RhoA Ig electroporation exerted opposite effects. In vitro cerebral barrier dysfunction was accompanied by myosin light chain overphosphorylation and stress fiber formation. Reperfusion and treatments with a Rho kinase inhibitor Y-27632 significantly attenuated barrier breakdown without profoundly altering actin structure.

Conclusions—Increased RhoA/Rho kinase/myosin light chain pathway activity coupled with changes in actin cytoskeleton account for OGD-induced endothelial barrier breakdown. (Stroke. 2010;41:2056-2063.)

Key Words: cerebral barrier ■ ischemic stroke ■ RhoA ■ Rho kinase

The endothelium covers the entire inner surface of all blood vessels and forms barriers to regulate vascular permeability. One such barrier is the blood–brain barrier (BBB), which stops the passage of substances from the blood into the central nervous system. Ischemic stroke develops through an interference with blood supply to the central nervous system and is characterized by anoxia and aglycemia.1 Restoration of blood flow after ischemia places ischemic organs at risk for further organ damage. The brain edema associated with BBB damage serves as an example to this type of damage and constitutes the leading cause of mortality after stroke, whereas the mechanisms that account for barrier damage remain largely unknown.1,2

The BBB consists of capillary basement membranes, brain microvascular endothelial cells (BMEC), and astrocyte endfeet. The “restraining” ability of the BBB is in part attributed to the absence of fenestrae and presence of a continuous ring of tight junctions restricting paracellular diffusion,3 implying that phenotypic endothelial changes may affect barrier permeability. Astrocytes cover the outer surface of microvascular basement membranes and contribute to formation of the BBB. BMEC express BBB-specific proteins only when cocultured with astrocytes.3

Small GTPases, particularly RhoA, play pivotal roles in many cellular processes, including the regulation of endothelial integrity and permeability.4,5 Active RhoA binds to its effector protein Rho kinase, promoting stress fiber formation, evoking myosin light chain (MLC) phosphorylation, and reducing nitric oxide (NO) bioavailability.6–8 Because MLC destabilizes cell junctions to increase interendothelial cell permeability, and because nitric oxide is critical for the maintenance of endothelial integrity, the inhibition of Rho/Rho kinase pathway may enhance microvascular endothelial function and suppress barrier permeability.

This study aimed to investigate whether any changes exist in RhoA, Rho kinase, and endothelial nitric oxide synthase (eNOS) protein expression/activity and actin cytoskeleton organization in human BMEC exposed to oxygen-glucose deprivation.
deprivation (OGD) vs normal conditions. It also comparatively investigated the impact of these phenomena on the time course of barrier disintegration/integration using a cell culture model of human cerebral barrier with particular reference to intracellular RhoA activity and MLC phosphorylation.

Materials and Methods

Cell Culture
Human BMEC (HBMEC) and human astrocytes were purchased from TCS Cellworks and grown to subconfluence before exposure to OGD (94.95% N₂, 0.05% O₂, 5% CO₂) or normoxic conditions (75% N₂, 20% O₂, 5% CO₂ plus 5 mmol/L D-glucose) for 4 or 20 hours. OGD experiments were performed in multigas incubator (MCO-18M, Sanyo UK) in which O₂ levels were checked using an oxygen fyrite test kit (LEEC). The contact coculture model, established as previously described, was used throughout the studies.7

BBB Experiments
The fluxes of permeability markers sodium fluorescein and Evan’s blue-labeled albumin were measured across cocultures. Briefly, confluent inserts were transferred to fresh 12-well plates containing 2 mL Hank’s balanced salt solution in the basolateral compartments. In the apical chambers, culture medium was replaced by 500 μL Hank balanced salt solution containing sodium fluorescein (10 μg/mL; molecular weight, 376 Da) or Evan’s blue-labeled albumin (165 μg/mL; molecular weight, 67 kDa). Both luminal and abluminal samples were taken at 20, 40, and 60 minutes after treatment to determine the concentrations of markers using a fluorescent plate reader and a multiscan plate reader, respectively. Flux across cell-free inserts was determined and transport was calculated as μL of luminal compartment volume from which the tracer is completely...
BBB integrity was also assessed by transendothelial electric resistance (TEER) measurements as previously described.7

**Rho Activation Assay**

Rho activation was measured using a kit (Millipore). Briefly, HBMEC lysates were incubated with rhotekin Rho-binding peptide immobilized on agarose. Activated GTP-Rho bound to rhotekin-agarose was detected by immunoblotting with anti-Rho antibody (Santa Cruz).

**Immunoblotting**

Immunoblottings were performed as previously described when monoclonal primary antibodies for RhoA, Rho kinase (Autogen Bioclear), or eNOS (Transduction Laboratories) were used.7

**Urea Gel Electrophoresis**

HBMEC were lysed in 10% trichloroacetic acid plus 0.05% dithiothreitol solution. The lysates were centrifuged and the pellets were resuspended in urea buffer containing 6.7 mol/L urea, 20 mmol/L Tris base, 22 mmol/L glycine, 9 mmol/L dithiothreitol, 0.004% bromophenol blue, and 1% Triton X-100. Proteins were separated on a native gel and MLCs were subsequently visualized by immunoblotting using anti-MLC2 or antiphospho-Thr18/Ser19 MLC2 antibody (Autogen Bioclear).

**Immunocytochemistry**

HBMEC were grown to ~60% confluence on cover slips before fixing and permeabilizing in 4% paraformaldehyde/phosphate-buffered saline and 0.05% Triton X-100/phosphate-buffered saline, respectively. Cells were then incubated with Rhodamine-labeled phalloidin (5 U/mL) for 30 minutes before mounting cover slips onto slides with distyrene-plasticizer-xylene mountants to view by fluorescence microscopy.

**Nitrite Detection**

Nitrite levels, an index of nitric oxide generation, were measured by Griess reaction as previously reported.7

**Transfection Experiments**

HBMEC (≈5×10⁶) were resuspended in 100 µL of media without supplements or antibiotics and stored on ice in a prechilled sterile cuvette. Constitutively active human recombinant His-RhoA L63 (0.2 mg/mL; Bioquote) or anti-RhoA Ig (0.2 mg/mL; Autogen Bioclear) were added to the cell suspensions before electroporation at 1.8 kV (Equibio Easyject Prima). Electroporated cells were resuspended in prewarmed supplemented media and allowed to recover at 37°C for 4 hours. Similar procedures without His-RhoA or anti-RhoA Ig served as electroporation controls. Cellular viabilities were measured by Trypan blue exclusion after 4 hours and overnight incubations.7

**Statistical Analysis**

Results are presented as mean±SD. Statistical analyses were performed using the nonparametric Mann-Whitney U test and P<0.05 was considered significant.

**Results**

**Effects of OGD on Protein Expression and Enzyme Activities**

OGD produced time-dependent increases in HBMEC RhoA/Rho kinase protein expressions and activity that were diminished by reperfusion compared to matching OGD treatments only. Although RhoA overactivation evoked further increases in these parameters, its inhibition through an anti-RhoA Ig...
electroporation dramatically attenuated protein expressions and Rho kinase activity (Figure 1A–C).

OGD caused a selective but insignificant decrease in eNOS protein expression after 4 hours of insult while decreasing nitrite levels in a time-dependent fashion. Both eNOS protein and nitrite levels were enhanced after reperfusion, with nitrite levels remaining significantly lower than those obtained in normoxic cells. Significant decreases were observed in both parameters in HBMEC transfected His-RhoA L63 (Figure 2A,B).

In human astrocytes, OGD did not affect eNOS protein expression while increasing RhoA/Rho kinase protein expressions and Rho kinase activity after only 20 hours of insult. Although these increases were subsided by reperfusion, they remained significantly higher compared to normoxic cells (Figure 3A–D).

**Effects of RhoA/Rho Kinase on BBB Integrity**
OGD evoked time-dependent decreases in TEER, which were significantly elevated by reperfusion compared to cells exposed to equal periods of OGD alone. Transfection of HBMEC with His-RhoA L63 compromised barrier integrity under both normoxic and experimental conditions that were specifically attenuated by Y-27632 in OGD-exposed groups, suggesting a protective role to this compound under pathological conditions. Suppression of RhoA activity led to BBB disruption under normoxic conditions while increasing TEER in cells subjected to OGD or OGD/reperfusion (Figure 4A).

In support of these findings, OGD produced time-dependent increases in the flux of low-molecular-weight (sodium fluorescein) and high-molecular-weight (Evan blue-labeled albumin) permeability markers across cocultures that were diminished by reperfusion (Figures 4B, 4C and 5). RhoA overactivity increased Evan’s blue-labeled albumin and sodium fluorescein flux across all experimental conditions when the addition of Y-27632 to cocultures abolished these increases under OGD conditions. Suppression of RhoA activity increased and decreased paracellular permeability under normoxic and OGD conditions, respectively. Studies investigating the effects of electroporation on the BBB function revealed no significant differences between basal and electroporation groups (Figure 4A–C).

**Effects of OGD on Actin Cytoskeleton and MLC Phosphorylation**
HBMEC grown in normoxia displayed marginal actin band distribution, whereas OGD led to formation of stress fibers and morphological changes characterized with cuboidal or elongated appearance. Cells subjected to reperfusion after OGD appeared to have both marginal and diffuse staining coupled with presence of actin stress fibers. Although RhoA overactivity led to formation of thick stress fibers similar to those observed in OGD cells, its inhibition eradicated all phenotypic changes and produced morphologies resembling those seen in normal HBMEC (Figure 6A).

OGD enhanced both total and Thr18/Ser19 MLC2 phosphorylation rates in HBMEC that were not greatly affected by reperfusion, significantly increased by RhoA overactivation, and radically reduced by RhoA inhibition (Figure 6B,C).
Cellular Viability

No significant variations were observed in viability of cells cultured under similar conditions. Because of the electroporation process reducing cell viability by 20±3%, the number of cells after this procedure was increased by 30% to allow all experimental groups to reach confluence simultaneously.

Discussion

Breakdown of the BBB, associated with formation of brain edema, constitutes one of the leading causes of mortality after ischemic strokes. Despite a consensus on endothelial cell dysfunction, the underlying mechanism of this defect during ischemic injury remains largely unknown. Using an in vitro model of the human cerebral barrier consisting of HBMEC and human astrocytes, the current study explored the involvement of RhoA/Rho kinase pathway in OGD-evoked barrier impairment and showed significant increases in protein expressions in OGD-exposed vs normoxia-exposed HBMEC. Similar increases in RhoA protein levels previously have been reported in stroke brain sections and in various cellular components of rodent cerebrovasculature after exposure to global ischemia. In the latter study, suppression of neuronal RhoA levels via hyperbaric oxygen confirms the ability of ischemia to regulate GTPase expression.

Consistent with increases in Rho kinase protein levels, cells exposed to OGD also possessed higher levels of Rho kinase activity. Suppression of Rho kinase activity in mice with fasudil or Y-27632, specific Rho kinase inhibitors, before transient middle cerebral artery occlusion decreased infarct volume and kinase activity by ~50% and substantiated the relevance of Rho kinase overactivity to ischemic cerebral damage. As an inhibitor of Ca²⁺-dependent protein kinases (pKC and pKA) and an intracellular Ca²⁺ antagonist, it is possible that fasudil may attenuate Ca²⁺/calmodulin-

Figure 4. Transendothelial electric resistance (A) and sodium fluorescein (B) and Evan's blue-labeled albumin (C) flux across human brain microvascular endothelial cells-astrocyte cocultures. Data are expressed as mean±SD from 6 different experiments. *P<0.05 compared to normoxia in the same group. #P<0.05 compared to corresponding treatment in the basal group.
dependent MLC kinase activity to reduce MLC phosphorylation and infarct volume. However, given the selection of relatively specific concentrations of fasudil for Rho kinase, the protective effects observed, at least in the aforementioned study, are less likely to be attributable to inhibition of these secondary mechanisms. Other mechanisms including vasodilatation through activation of eNOS are likely to contribute to beneficial effects of Rho kinase inhibitors. In eNOS−/− mice, Rho kinase inhibitors exerted no benefits on cerebral infarct size.

The present study demonstrates that OGD decreases HBMEC nitrite levels while causing a selective increase in eNOS protein expression after 20 hours of insult. Despite upregulation of both parameters after reperfusion, nitrite levels remained less than those observed in normoxic cells, possibly because of Rho kinase-mediated attenuation of eNOS activity. Time-dependent regulation of eNOS protein was previously documented in an in vivo study in which extended (≥24 hours) but not shorter (≤6 hours) periods of ischemia yielded consistent increases. Considering the short half-life of eNOS protein in endothelial cells (81±0.1 minutes) and its quick regulation by physiopathological elements such as apolipoprotein A-I and high-density lipoprotein, in which the latter, eg, increases in protein half-life by ~3.5-fold, the specific effects of OGD on HBMEC eNOS expression warrants further investigation.

In addition to endothelial cells, astrocytes also contribute to BBB formation and show some resistance to ischemic damage, possibly attributing to their high intracellular glycogen content and low energy demand to maintain ATP and transmembrane ion concentrations. By revealing similar eNOS and increased RhoA/Rho kinase protein expressions only after long-term exposure to OGD, this study provides some in vitro evidence for the so-called astrocyte resistance to ischemia and supports the results of a previous study reporting similar levels of astrocyte eNOS immunoreactivity of a gerbil forebrain ischemia after 6 to 168 hours of reperfusion.

Several mechanisms including overt openings in paracellular pathways may compromise endothelial barrier function in the presence of OGD. TEER and flux of permeability markers across HBMEC–human astrocytes cocultures were measured to assess the barrier function, which showed OGD-evoked significant time-dependent decreases in TEER, and increases in flux, which were somewhat normalized by reperfusion, thereby implying a physical breakdown and a partial recovery of barrier function by OGD and reperfusion, respectively.
To determine the actual relevance of RhoA to endothelial barrier integrity, HBMEC were transfected with anti-RhoA Ig or constitutively active His-RhoA L63 in that the former increased TEER and decreased paracellular permeability specifically in pathological conditions, whereas the latter exerted the opposite effects. Taken together, these findings verified the importance of RhoA in maintaining barrier integrity and showed a requirement for baseline levels to preserve normal cellular activity.\textsuperscript{17} Attenuation of OGD-induced hyperpermeability by Y-27632 lent further support to the involvement of RhoA/Rho kinase pathway in barrier disruption.\textsuperscript{18} Given that Y-27632 inhibits only protein kinase C–related protein kinase 2 with similar efficacy as Rho kinase, the inhibitions of other kinases to the observed effects may not be significant.\textsuperscript{19}

The changes in cell morphology may also contribute to OGD-induced barrier failure. Experiments examining putative structural differences in microfilaments, a key component of cytoskeleton,\textsuperscript{20} showed that HBMEC grown under normoxia possessed a marginal actin filament distribution, whereas those exposed to OGD had an elongated and cuboidal appearance and had stress fibers traversing the cells. Reperfusion led to more diffuse cytosolic staining of actin filaments, along with an intense presence of stress fibers and some marginal staining. Transfection of HBMEC with His-RhoA or anti-RhoA Ig induced thick stress fiber formation and preserved normal phenotype, respectively. The cortical actin bands play a pivotal role in the prevention of paracellular flux by participating in formation and stabilization of intercellular junctions. Once formed, stress fibers can generate a tensile centripetal force to pull junctional proteins inward to break-up junctions and create intercellular gaps. Several mechanisms, including activation of vascular endothelial growth factor and transcriptional factors HIF-1 and NF-κB, have been implicated in OGD-mediated and ischemia-mediated junctional disruptions.\textsuperscript{21,24}

Enhanced phosphorylation of MLC triggered by RhoA/Rho kinase activity may also cause destabilization of interendothelial cell junctions.\textsuperscript{4,5} Although activation of RhoA in HBMEC via His-RhoA transfection increased total and diphospho-MLC2 phosphorylations (at Thr18/Ser19 residues) possibly through inhibition of myosin phosphatase or activation of MLC kinase,\textsuperscript{22,23} its inhibition via anti-RhoA Ig dramatically decreased both phosphorylations. By simultaneously investigating the changes in cell morphology with MLC2 phosphorylation levels, this study has provided some evidence for the existence of a correlation between the degree of actin stress fiber formation and the extent of MLC phosphorylation. Given that activation of Rho kinase leads to phosphorylation of the ezrin-radixin-moesin proteins and that these, in turn, provide a regulated linkage between membrane proteins and the cortical cytoskeleton, this mechanism warrants further investigation.\textsuperscript{24,25}

There are some limitations to this study. It is known that although brain edema continues to develop during the reper-
fusion in in vivo situations, consistent barrier-protective effects have been observed during reoxygenation phase of our in vitro study. Although the reasons for this dichotomy is at present unknown, the reoxygenation period used in this study (20 hours) may be long enough for the synthesis of nascent proteins to close interendothelial cellular gaps to attenuate OGD-evoked barrier damage. Therefore, it is crucial to study the effects of shorter periods of reoxygenation on eNOS, RhoA, Rho kinase protein expressions and activities, and on in vitro barrier function. It also remains essential to confirm the results presented in this study in in vivo settings by using an animal model of ischemic stroke.

**Conclusion**

In conclusion, OGD compromises the structural and functional capacities of an in vitro model of human cerebral barrier through activation of RhoA/Rho kinase pathway and consequent induction of MLC overphosphorylation.

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**Disclosure**

None.

**References**

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