NADPH Oxidase Plays a Central Role in Blood-Brain Barrier Damage in Experimental Stroke

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Background and Purpose—Cerebral ischemia/reperfusion is associated with reactive oxygen species (ROS) generation, and NADPH oxidases are important sources of ROS. We hypothesized that NADPH oxidases mediate blood-brain barrier (BBB) disruption and contribute to tissue damage in ischemia/reperfusion.

Methods—Ischemia was induced by filament occlusion of the middle cerebral artery in mice for 2 hours followed by reperfusion. BBB permeability was measured by Evans blue extravasation. Monolayer permeability was determined from transendothelial electrical resistance of cultured porcine brain capillary endothelial cells.

Results—BBB permeability was increased in the ischemic hemisphere 1 hour after reperfusion. In NADPH oxidase–knockout (gp91phox−/−) mice, middle cerebral artery occlusion–induced BBB disruption and lesion volume were largely attenuated compared with those in wild-type mice. Inhibition of NADPH oxidase by apocynin prevented BBB damage. In porcine brain capillary endothelial cells, hypoxia/reoxygenation induced translocation of the NADPH oxidase activator Rac-1 to the membrane. In vivo inhibition of Rac-1 by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor atorvastatin or Clostridium difficile lethal toxin B also prevented the ischemia/reperfusion–induced BBB disruption. Stimulation of porcine brain capillary endothelial cells with H2O2 increased permeability, an effect attenuated by inhibition of phosphatidyl inositol 3-kinase or signal–regulated kinase-1/2 or p38 mitogen-activated protein kinase. Inhibition of Rho kinase completely prevented the ROS-induced increase in permeability and the ROS-induced polymerization of the actin cytoskeleton.

Conclusions—Activation of Rac and subsequently of the gp91phox containing NADPH oxidase promotes cerebral ROS formation, which then leads to Rho kinase–mediated endothelial cell contraction and disruption of the BBB. Inhibition of NAPDH oxidase is a promising approach to reduce brain injury after stroke. (Stroke. 2007;38:3000-3006.)

Key Words: endothelium ■ ischemia/reperfusion ■ oxidative stress ■ RhoA ■ statins

Cerebral ischemia and reperfusion (I/R) result in disruption of the blood-brain barrier (BBB) and formation of brain edema. These processes are in part a consequence of increased vascular permeability that results from endothelial cell contraction and disassembly of tight junctions. I/R also increases the formation of reactive oxygen species (ROS)1 and ROS, in turn, are thought to alter BBB integrity2: Incubation of endothelial cells with ROS promotes cellular contraction and increases the permeability of endothelial monolayers.3,4 Moreover, in vivo treatment with superoxide dismutase or antioxidants such as Tempol attenuates vascular leakage after ischemia.5,6 Important sources of ROS in many cells are NADPH oxidases of the Nox family. In leukocytes, the gp91phox (Nox2) containing NADPH oxidase is responsible for the respiratory burst in which the cell generates toxic amounts of superoxide anions. Although expressed at a lower level, the gp91phox containing NADPH oxidase has also been observed to contribute to ROS formation by glia cells, fibroblasts, and vascular endothelial cells.7-11 Indeed, it has previously been observed that genetic deletion of gp91phox confers protection against ischemic stroke in mice12 and that ischemia-induced ROS production is attenuated in the lung in gp91phox−/− mice.13 Given that ROS may disrupt the BBB and that the gp91phox containing NADPH oxidase is an important source of ROS, we hypothesized that genetic deletion or inhibition of this enzyme prevents BBB dysfunction in cerebral I/R.

Materials and Methods

Animal Model of Focal Cerebral Ischemia

All experiments were approved by the local governmental authorities (approval numbers F24/01 and F28/05) and were performed in accordance with animal protection guidelines. Male gp91phox−/−
mice (Jackson Laboratories, Bar Harbor, Me; backcrossed 10 generations into C57BL6 mice and bred at the local animal facility) and wild-type C57BL6 mice (7 to 9 weeks; Charles River Laboratories, Sulzfeld, Germany) were anesthetized with 1.5% isoflurane (Forene; Abbott, Wiesbaden, Germany) in an air-oxygen mixture under spontaneous respiration. Analgesia was established by subcutaneous injection of 0.1 mg/kg body weight (BW) buprenorphine (Temgesic; Essex Pharma, Munich, Germany).

Focal cerebral ischemia was induced by introducing a silicone-coated 8-0 monofilament into the right common carotid artery and advancing it along the internal carotid artery until the tip occluded the proximal stem of the middle cerebral artery (MCA). Regional cerebral blood flow was monitored by laser Doppler flowmetry (PF5010, Perimed, Sweden) with use of a flexible fiberoptic probe fixed to the intact skull above the territory of the right MCA. Rectal temperature was maintained between 37°C and 38°C with a heating pad. Two hours after induction of ischemia, the filament was withdrawn to allow reperfusion.

Treatment Protocol
Pharmacological inhibition of NADPH oxidase was achieved in wild-type mice with apocynin (Fluka 55559) at 0.4, 4, and 40 mg/kg BW given intravenously 1 hour before induction of MCA occlusion and of Rac-1 with Clostridium difficile lethal toxin B (Sigma-C-4102) at 4 μg/kg BW given intravenously 1 hour before MCA occlusion or with atorvastatin (Gödecke AG) at 10 mg/kg BW given intraperitoneally at 48 and 24 hours before ischemia.

Lesion Size and Brain Swelling
Twenty-two hours after reperfusion, brains were removed and cut into 1-mm sections with a brain matrix (RBMI 2000C, ASI Instruments, Warren, Mich). Brain slices were digitized and infarct volumes were quantified with National Institutes of Health Image J software on 2,3,5-triphenyltetrazolium chloride–stained sections. The ratio of the contralateral to the ipsilateral hemisphere section volume was measured by a microplate fluorescence reader (excitation 600 nm, emission 540 nm).

In Vivo BBB Permeability
At the time of reperfusion, 2% Evans blue in normal saline (6 mL/kg BW, ~150 μL) was injected into the tail vein and allowed to circulate for 1 hour. Mice were deeply anesthetized with isoflurane and transcardially perfused with saline and then with Paraflin (1 mg/mL; Sigma-Aldrich, St. Louis, Mo). Brains were removed quickly, and transcardially perfused until colorless fluid was obtained from removal of the meninges and secretory areas, brains from freshly slaughtered pigs were minced and digested stepwise with dispase and collagenase/dispose. Endothelial cells were obtained by terminal Percoll density gradient centrifugation and cultured in M199 with 10% normal calf serum, 100 μg/mL penicillin/streptomycin, and 100 U/mL gentamicin. Cells were passaged on day 2. For transendothelial electrical resistance (TEER) measurements, primary PBCECs were seeded at a density of 100 000 cells per well on rat tail collagen-coated electrical cell substrate impedance sensing 8W10E slides (see subsequent section). For immunofluorescence imaging, cells were plated on collagen-treated microdishes (Ibidi, Munich, Germany). On day 4 after the initial preparation, the culture medium was exchanged for low-serum M199 containing 2% normal calf serum only. Experiments were started on day 6.

Determination of Cerebral Endothelial Barrier Function In Vitro
An electrical cell substrate impedance sensing apparatus (model 1600; Applied Biophysics, Troy, NY) was used to measure TEER in endothelial cell confluent monolayers with 8W10E slides obtained from Ibidi. The effect of H2O2 on TEER was measured in the presence or absence of different inhibitors.

Oxygen Glucose Deprivation and Reoxygenation Studies
On day 6, cell culture medium was exchanged for low-serum (2% normal calf serum) and glucose-free medium equilibrated with N2 on endothelial cells subcultured on microdishes. Cells were then exposed to 1% O2, 5% CO2, and 94% N2 for 8 hours in a 37°C incubator. Normoxic controls received 5.5 mmol/L glucose. For reoxygenation, cells were again placed in a normoxic incubator.

Confocal Microscopy
For oxygen glucose deprivation, the medium was exchanged with glucose-free medium equilibrated with N2. Cells were then exposed to 1% O2, 5% CO2, and 94% N2 for 8 hours in a 37°C incubator. Normoxic controls received 5.5 mmol/L glucose. For reoxygenation, cells were again placed in a normoxic incubator. Experiments were terminated by adding phosphate-buffered paraformaldehyde solution. Cells were permeabilized with Triton X-100 (0.05%) and stained for Rac-1 (Upstate Technology, 1:200), VE-cadherin (Santa Cruz, 1:200), G-actin (Alexa Fluor 488–conjugated DNsase I, Invitrogen) or F-actin (Alexa Fluor 546–conjugated phalloidin, Invitrogen). Primary antibodies were marked with appropriate Alexa-labeled secondary antibodies (1:300, Invitrogen). Antibodies were dissolved in Tris-buffered saline (pH 7.2) containing 3% bovine serum albumin and 0.5% Tween 20, and this solution was also used for blocking. Imaging was performed with a 40× objective mounted on a Zeiss laser scanning confocal microscope (LSM 510 Meta) operated in multitracking mode to prevent interference from the dyes.

Statistical Analysis
Data are presented as mean±SEM. Statistical analysis was performed with an unpaired t test and ANOVA followed by Fisher’s least significant difference test. Differences at P<0.05 were considered statistically significant.

Results
NADPH Oxidase gp91phox−/− Mice Develop Smaller Brain Infarcts
After 2 hours of ischemia and 22 hours of reperfusion, cerebral ischemic damage as determined by 2,3,5-triphenyltetrazolium chloride staining was significantly less in brain hemispheres of gp91phox−/− mice (43±10 mm3) compared with hemispheres from wild-type controls (84±12 mm3, P<0.05). Moreover, brain swelling at 24 hours after ischemia...
was significantly less pronounced in mice lacking functional NADPH oxidase (16±8 vs 45±10 mm³, P<0.05; Figure 1).

**NADPH Oxidase Is Involved in Early BBB Disruption**
Compared with the nonischemic hemisphere, BBB permeability, as determined by Evans blue extravasation, was increased by 130±50% in the ischemic hemisphere within the first hour of reperfusion. Inhibition of NADPH oxidase by apocynin as well as genetic deletion of the large catalytic subunit gp91phox of NADPH oxidase largely prevented the I/R-induced early increase in BBB permeability. The early onset of permeability changes may suggest that activation of a local NADPH oxidase rather than induction of the enzyme underlies I/R BBB damage. Using fluorescein as a marker for permeability, we observed a 241±20% increase in permeability in the ischemic hemisphere within the first hour of reperfusion (n=7). In the apocynin (40 mg/kg BW) group, BBB disruption was 180±7% (n=6). Statistical comparison revealed a trend toward a beneficial effect of apocynin (P=0.07).

**Rac-1 Is Involved in BBB Disruption**
Because activation of the oxidase is mediated by Rac-1, this small GTPase was inhibited by 2 different approaches before I/R. A 48-hour pretreatment with the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor atorvastatin and the irreversible GTPase inhibitor C. difficile lethal toxin B was applied 1 hour before the onset of ischemia. Both compounds prevented the I/R-induced increase in BBB permeability and were equally effective as apocynin (Figure 2). To provide direct evidence for Rac-1 activation, translocation of this small GTPase was studied in cultured PBCECs in response to hypoxia/reoxygenation. Indeed, brief reoxygenation of hypoxic endothelial cells resulted in partial translocation of Rac-1 to the membrane, a process reflecting activation of the protein (Figure 3A).

**ROS Increase PBCEC Monolayer Permeability**
Changes in BBB integrity are linked to several different mechanisms, like disruption of vascular integrity by matrix metalloproteinases, astrocyte apoptosis, and endothelial cell contraction and detachment. Because the present study focused on early changes in BBB permeability, we postulated that this effect was primarily a consequence of endothelial cell contraction and disassembly of tight junctions, which increase paracellular leakage and permeability. To establish a link from ROS production to increased permeability, we determined the effects of H₂O₂ on the TEER of primary PBCECs. H₂O₂ rapidly increased monolayer permeability in a dose-dependent manner (Figure 4) and promoted polymerization of the actin cytoskeleton of PBCECs (Figure 3B). The H₂O₂-induced changes in monolayer permeability were unaffected by apocynin, demonstrating that the NADPH oxidase inhibitor indeed acted upstream of H₂O₂.

**ROS-Induced Increase in PBCEC Monolayer Permeability Involves Rho Kinase**
Using pharmacological inhibitors, we attempted to identify signaling pathways involved in the ROS-induced changes in monolayer permeability. Inhibition of p38 mitogen-activated protein (MAP) kinase or extracellular signal-regulated kinase (ERK) 1/2 was without effect, whereas there was a trend toward attenuated H₂O₂-induced permeability changes by SP600125, an inhibitor of c-Jun N-terminal kinase. Blockade of phosphatidylinositol 3 (PI3) kinase with wortmannin significantly prevented the H₂O₂-induced increase in permeability by ~50%, whereas inhibition of Rho kinase by Y27632 completely blocked the response to H₂O₂ (Figure 4). Y27632 also prevented the H₂O₂-induced increase in TEER (Figures 3A and 3C). Catalase (1000 U/mL) completely prevented the H₂O₂-induced changes in TEER (data not shown).
Discussion

In this study, we observed that genetic deletion of gp91phox prevented early BBB dysfunction and offered protection from brain swelling after stroke. Inhibition of the NAPDH oxidases with apocynin or inhibition of Rac was also effective in maintaining BBB function, as was knockout of gp91phox. In cultured PBCECs, hypoxia/reoxygenation induced the translocation of Rac to the membrane and exposure of the cells to H$_2$O$_2$ induced actin cytoskeleton polymerization via a process involving Rho kinase.

ROS formation after I/R is a well-known phenomenon. The potential enzymatic sources of ROS in I/R such as xanthine oxidase, mitochondria or nitric oxide synthase are discussed controversially. Several reports have suggested that in endothelial cells, ischemia may activate a NADPH-oxidase by a process involving the small GTPase Rac. We have previously reported that endothelial cells express a functionally active gp91phox containing NADPH oxidase, and indeed, several studies have demonstrated that inhibition of NAPDH oxidase limits infarct size in different models. The contribution of the enzyme to BBB regulation, however, has not been studied so far. Using 2 different approaches, genetic deletion of gp91phox and pharmacological inhibition of NADPH oxidase, we clearly demonstrated that the early BBB disruption after I/R involves the gp91phox containing oxidase. This approach, however, did not allow us to identify the cellular origin (ie, leukocytes, endothelial cells, glia, or neurons) of the ROS, which could be generated in an autocrine as well as a paracrine manner to induce endothelial cell contraction.

Although ROS formation has been demonstrated during I/R in a multitude of studies, we did not perform such assays in the present study. The main reason for this omission is that there is still no direct way to determine cerebral oxidative stress in the MCA occlusion model because the tracers do not reach the ischemic tissue, as we experienced with the in vivo application of dihydroethidium (Kahles et al, unpublished observations, 2006). However, because we achieved NADPH oxidase inhibition by several different mechanisms, it is exceedingly unlikely that the results obtained in the present study were a consequence of nonspecific effects of the inhibitory approaches used.

We also demonstrated that early disruption of the BBB could be prevented by blocking NADPH oxidase activation, a
process that critically depends on Rac-1. Indeed, in cultured PBCECs, hypoxia/reoxygenation induced the translocation of Rac to the membrane, and this process is thought to reflect Rac activation.\(^{27,28}\) To demonstrate involvement of this small GTPase in BBB dysfunction, we used 2 different approaches: direct inhibition by \textit{C difficile} lethal toxin B and inhibition of geranylgeranylation\(^ {29}\) with a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (statin).\(^ {30,31}\) These approaches are limited by the fact that both compounds inhibit RhoA equally well, which is an important mediator of cellular contractility. Indeed, inhibition of Rho kinase prevented polymerization of the actin cytoskeleton in cultured PBCECs, as demonstrated by confocal microscopy, and blocked the ROS-induced increase in PBEC monolayer permeability. Moreover, RhoA is also involved in regulating the activity and expression of endothelial nitric oxide synthase,\(^ {32}\) switching the balance from an ROS- to a nitric oxide–dominated environment. It has previously been shown that statins reduce infarct size in the MCA occlusion model in a nitric oxide–dependent manner.\(^ {33}\) These effects require a relatively long treatment with these compounds, and compared with the pronounced effects on BBB integrity observed in the present study, were less marked. Consequently, we assume that the beneficial effects of atorvastatin seen in the present study can only partially be attributed to the inhibition of NADPH oxidase.

TEER measurements were used to model endothelial monolayer permeability. The rate of permeability largely depends on particle size; therefore, TEER has a tendency to overestimate permeability. Moreover, endothelial monolayer cultures are certainly only a basic model of the BBB, as any contribution of astrocytes or microglia is excluded. Despite these limitations, changes in TEER reflect changes in actin polymerization. Thus, we assume that although the magnitude of the changes in permeability was not determined for different particle sizes ex vivo, in this particular case, TEER was a suitable method to extrapolate changes in permeability.

In the present study, we focused on early changes in BBB function after I/R for several reasons. Late BBB disruption is a consequence of inflammation, cell necrosis, the production of tissue-degrading enzymes such as matrix metalloproteinases, and alterations in gene expression; therefore, late BBB disruption represents a very complex scenario.\(^ {34-36}\) In contrast, early disruption of the BBB occurs before the afore-
mentioned effects and is primarily mediated by altered endothelial cell function. In cultured PBCECs, the effects of MAP kinase inhibitors and a PI3 kinase inhibitor on the H$_2$O$_2$-induced increase in permeability were tested. It is well known that PI3 kinase and the MAP kinases are activated by H$_2$O$_2$. Of the compounds studied, only the c-Jun N-terminal inhibitor and the PI3 kinase inhibitor slightly attenuated the H$_2$O$_2$-induced increase in permeability, suggesting that the activation of Rho occurs through either direct radical-induced stimulation of Rho-GEFs (Guanine nucleotide Exchange Factors activate GDP/GTP exchange of RhoA) or other redox-sensitive kinases, such as protein kinase C or phospholipase C. Our observations are in contrast to previous reports on an important function of the ERK 1/2–MAP kinases in ROS-induced increases in endothelial cell permeability. In the present study, monolayer permeability was determined with an electrical method excluding transcellular transport, and much lower concentrations of H$_2$O$_2$ were used, which only elicited transient activation and contraction of the cells but not permanent rigor and cell death. Given the small size of electrons, TEER measurement is a very sensitive method to assess monolayer integrity but does not allow estimation of the permeability of large molecules and cannot mimic the complexity of the BBB. Therefore, we cannot exclude the possibility that many additional effects that occurred in vivo were not detected by this monolayer culture technique.

In conclusion, we have demonstrated that selective inhibition of NADPH oxidase prevents BBB disruption in experimental stroke. The beneficial effects of statins on the BBB, which occur as a consequence of NADPH oxidase inhibition, may also help delineate the positive effects of this class of drugs on stroke outcome in patients. Future studies will be needed to demonstrate the cellular origin of ROS in I/R.

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Disclosures
None.

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