Mitochondrial Crisis in Cerebrovascular Endothelial Cells Opens the Blood–Brain Barrier

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Background and Purpose—The blood–brain barrier (BBB) is a selectively permeable cerebrovascular endothelial barrier that maintains homeostasis between the periphery and the central nervous system. BBB disruption is a consequence of ischemic stroke and BBB permeability can be altered by infection/inflammation, but the complex cellular and molecular changes that result in this BBB alteration need to be elucidated to determine mechanisms.


Results—We report here that lipopolysaccharide worsens ischemic stroke outcome and increases BBB permeability after transient middle cerebral artery occlusion in mice. Furthermore, we elucidate a novel mechanism that compromised mitochondrial function accounts for increased BBB permeability as evidenced by: lipopolysaccharide-induced reductions in oxidative phosphorylation and subunit expression of respiratory chain complexes in cerebrovascular endothelial cells, a compromised BBB permeability induced by pharmacological inhibition of mitochondrial function in cerebrovascular endothelial cells in vitro and in an in vivo animal model, and worsened stroke outcomes in transient middle cerebral artery occlusion mice after inhibition of mitochondrial function.

Conclusions—We concluded that mitochondria are key players in BBB permeability. These novel findings suggest a potential new therapeutic strategy for ischemic stroke by endothelial cell mitochondrial regulation. (Stroke. 2015;46:1681-1689. DOI: 10.1161/STROKEAHA.115.009099.)

Key Words: blood-brain barrier ■ mitochondria ■ stroke

The blood–brain barrier (BBB) is a highly specialized vascular interface that maintains homeostasis in brain by separating the blood compartment from the central nervous system. Disruption of cerebrovascular endothelial cells not only allows entry of unwanted solutes into brain but also disrupts the normal central nervous system entry route of critical nutrients.1-3 Disruption or dysfunction of the BBB has been observed in cerebrovascular diseases and neurodegenerative disorders, such as stroke, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and epilepsy.1

Stroke is the second leading cause of death and the leading cause of disability worldwide.4 It is estimated that 30% to 40% of all strokes occur during or recently after an acute infection.5 Acute infections initiate rapid inflammation, and poststroke infections worsen outcomes in patients6 and in animal models.7 However, the mechanisms of this exacerbation by infection on acute stroke outcome are not completely understood.

Ischemia and reperfusion events result in complex cellular and molecular changes that further need to be elucidated. To date, studies have provided evidence that the release of oxidants, proteolytic enzymes, and inflammatory cytokines alter BBB permeability. The BBB excludes the majority of bacteria; however, similar to ischemia and reperfusion, inflammation induced by bacteria alters BBB permeability. Lipopolysaccharide has been shown to disrupt the BBB in vivo and in vitro; however, how lipopolysaccharide exerts its effects on the BBB is under debate.8 Previous studies found that a lipopolysaccharide challenge results in a larger infarct volume,7 impairs survival and disrupts BBB9 in experimental...
stroke models, through mechanisms that are proposed to involve cytokines. In the present study, we provide the first evidence that the exacerbation of stroke outcome by a bacterial infection mimics is because of a novel mechanism of compromised cerebrovascular endothelial mitochondrial function. Potential deleterious mitochondrial responses to ischemia have been observed, such as rapid changes in ATP, the release of cytochrome c, and the induction of the mitochondrial permeability transition. It is apparent that mitochondria play a pivotal role during ischemia; however, the contributions of mitochondrial dysfunction in non-neuronal cells and the interactions between these cells and neurons are poorly understood. In addition, the involvement of mitochondria in ischemic damage has not been fully elucidated. We show that pharmacological disruption of endothelial mitochondrial function recapitulates all aspects of the lipopolysaccharide exacerbation of stroke, including disruption of the BBB, and worsening of stroke outcome. This discovery could provide a previously unknown mitochondrial-dependent mechanism for acute stroke damage that may offer new therapeutic directions for the treatment of acute stroke.

**Methods**

**Mice**

All procedures were conducted according to the criteria approved by the Institutional Animal Care and Use Committees at the West Virginia University (WVU). C57/BL6J male mice (3–4 months old, 25-30 g; Jackson Laboratories) were used for all studies.

**Anesthesia**

All surgical anesthesia was induced with 4% to 5% isofluorane until the animal showed no response to a toe pinch and was maintained with 1% to 2% isofluorane via face-mask in O₂-enriched air.

**Randomization and Blinding of the Animal Experiments**

To assign pretreatments of mice, we numbered the animals and applied a simple randomization by using excel-generated random numbers. To avoid biases, we also assured that different pretreatments were performed on the same day. The experimenters were blinded to the pretreatments and data analysis.

**Drug Administration**

In a subgroup of mice, lipopolysaccharide (Escherichia coli 055:B5, 100 μg/kg; Sigma, Saint Louis, MO) dissolved in saline (B. Braun Medical Inc, Irvine, CA) was injected intraperitoneally 30 minutes before transient middle cerebral artery occlusion (tMCAO) or sham surgery. In another subgroup of mice, rotenone (1 mg/kg; Sigma) was administered intraperitoneally 30 minutes before MCAO. In the epi-dural application (EA) model, rotenone (2 mg/mL, 2 μL) was topically applied to the epidural membrane. An equal volume of saline was administered to control mice.

**Ischemic Model and Sham Surgery**

We performed focal cerebral ischemia for 30 or 60 minutes by occlusion of the right middle cerebral artery with a 0.6 mm monofilament suture (Doccol, Sharon, MA). We used laser Doppler flowmetry (Moor Instruments, United Kingdom) to detect regional cerebral blood flow and confirm a successful occlusion (>70% decrease in flow). Rectal body temperature was maintained at 37±0.5°C during surgery. Mice were euthanized at several time points as indicated in the text.

**Neurological Deficits**

Neurological deficit was determined daily before and after tMCAO according to a 0- to 5-point scale neurological score system as published.12

**EA Model**

We induced anesthesia with 4% isofluorane and maintained with 1% isofluorane. We placed the animal in a prone position under a stereo dissecting microscope and made an incision down the midline of the head and retracted the skin then removed the fascia from the skull. Using a sharp blade, we thinned an area of the skull =0.3 to 0.5 mm in diameter, divided the thinned area into several segments, performed a craniotomy by gently removing the thinned skull segments, applied drug or vehicle, and closed the incision.

**Exclusion Criteria for Animal Experiments**

The exclusion criteria for tMCAO were as follows: (1) regional cerebral blood flow decreases <70% during occlusion as detected by laser Doppler flowmetry; (2) surgery time lasts >30 minutes; (3) no neurological deficits 3 hours after MCAO (neurological score 0); (4) no infarction in the MCA territory on 2,3,5-triphenyltetrazolium chloride (TTC) staining; (5) subarachnoid hemorrhage on postmortem examination; and (6) substantial ambient temperature change in the animal facility. Animals that died before the planned time of assessments were postmortem examined for subarachnoid hemorrhage, and the mortality was recorded. The exclusion criteria for EA model were as follows: (1) damaged epidural membrane; (2) bleeding from the epidural membrane; (3) the leakage of cerebrospinal fluid from the epidural membrane; (4) body temperature >39.5°C within 3 hours post surgery; and (5) death.

In this study, 7 mice were excluded: 2 mice (1 vehicle and 1 lipopolysaccharide pretreatment) because of subarachnoid hemorrhage, 4 mice (2 vehicle and 2 lipopolysaccharide pretreatment) because the animal facility air conditioner broke down on the day that the experiments were performed, 1 mouse (pretreated with rotenone) because laser Doppler flow did not reach 70% reduction during the occlusion. The animal numbers included in the results are 18 vehicle and 23 lipopolysaccharide for 30 minutes tMCAO mice (4 lipopolysaccharide pretreated mice died), 12 vehicle and 12 rotenone for 60 minutes tMCAO mice, 5 vehicle and 5 rotenone for EA mice. No animals were excluded in EA model.

**Analysis of Brain Infarct Volume**

Mice were euthanized with isofluorane. We removed the brains and cut 2-mm coronal sections with a mouse brain matrix. We stained the sections with 2% TTC (Sigma, Saint Louis, MO) in phosphate buffer solution (PBS) at 37°C for 30 minutes then fixed the tissue in 10% formalin phosphate buffer for digital photograph. We analyzed the digitized image of each brain section using a computerized image analysis software (Imaged, National Institutes of Health) in a double-blinded manner. To minimize the effect of brain edema, the volume was expressed as a percentage of contralateral cortex, striatum, and total hemisphere.

**BBB Permeability Assay In Vivo**

The permeability of the BBB was determined by measuring the penetration of Evans blue (Sigma) in the brain tissue. Evans blue (2% in saline; 4 mL/kg body weight) was administered intravenously via the tail vein 1 hour before measurement. The anesthetized animals were perfused transcardially with saline before sampling. Each sample was weighed and homogenized with 400 μL PBS, then precipitated by 50% trichloroacetic acid overnight. The samples were centrifuged for 30 minutes at 10000 rpm to pellet brain tissue. Absorption of the supernatant was measured at a wavelength of 610 nm with a plate reader (BioTek, Winooski, VT). The extravasation of Evans blue was quantified as microgram/gram brain tissue with an Evans blue standardized curve.
Isolation of Immune Cells in the Blood, Spleen, and Brain

Blood was collected via cardiac puncture, then brain and spleen were sampled after transcardial perfusion with saline. Peripheral blood mononuclear cells and splenocytes were prepared using red cell lysis buffer (eBioscience, San Diego, CA). For brain immune cells, the forebrain was dissected from the cerebellum and suspended in RPMI-1640 medium (Corning, Pittsburgh, PA). The suspension was digested with type IV collagenase (1 mg/mL; MP Biomedicalals, Solon, OH) and DNase I (50 μg/mL; Roche Diagnostics, Indianapolis, IN) at 37°C for 45 minutes, then immune cells were isolated by 37% to 70% Percoll (GE Healthcare, Piscataway, NJ) density gradient centrifugation (10 min) and collected from the interface. Single-cell suspension was washed with staining buffer (PBS containing 0.1% Na3 and 2% FCS) and stained with CD45 (30-F11; eBioscience), CD11b (M1/70; eBioscience), and Gr1 (IA8; BD Bioscience). Propidium iodide (2 μg/mL; Sigma) was used to exclude dead cells. Appropriate isotype control antibodies were applied to set quadrants for calculating the percentage of positive cells. Data were acquired on fluorescence-activated cell sorter Calibur (BD) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Cell Culture

The bEnd.3 cell line (CRL-2299 from ATCC, Manassas, VA) was originally derived from mouse brain cortex endothelial cells and confirmed by the observed major phenotypic features of the BBB.13 Passages 25 to 30 were used in the study. The bEnd.3 cells were routinely grown in high glucose Dulbecco modified Eagle medium (DMEM, ATCC) supplemented with 10% FCS and 1% penicillin/streptomycin (Hyclone, South Logan, UT) at 37°C in 5% CO2 humid atmosphere. Mouse primary brain microvascular endothelial cells (B129-7023; Cell Biologics, Chicago, IL) were routinely grown in complete endothelial cell medium (M1168; Cell Biologics) at 37°C in 5% CO2 humid atmosphere.

BBB Permeability Assay In Vitro

Permeability assays were performed in triplicate as follows: 1.5x105 endothelial cells were grown on transwell inserts (pore size, 0.4 μm; diameter, 6.5 mm; Millipore, Darmstadt, Germany) for 2 days. 250 μg/mL fluorescein isothiocyanate (FITC)-labeled dextran FD-70 (70 kDa; Sigma) was added to the apical side of the filters, and the medium in the basolateral compartment was sampled every 15 minutes for 2 hours. The FD-70 permeability through the cultured endothelial monolayer was determined directly by analysis of the apparent permeability coefficient (Papp).14 The concentration of FD-70 was determined with an FD-70 standard curve on plate reader (Ex. 490 nm, Em. 515 nm). Papp (cm/s) was calculated.

Oxygen Consumption

Oxygen consumption rate was measured at 37°C using an XF96e extracellular analyzer (Seahorse Bioscience, North Billerica, MA) according to the manufacturer’s instructions. Briefly, the bEnd.3 cells or primary endothelial cells were seeded into Seahorse Bioscience XF96e cell culture plates (16,000 cells/well) in 80-μL medium and allowed to adhere and grow overnight in the 37°C humidified incubator with 5% CO2. Then the cells were supplied with 80-μL medium and allowed to adhere and grow overnight in the 37°C humidified incubator. Single-cell suspension was washed with staining buffer (PBS containing 0.1% Na3 and 2% FCS) and stained with CD45 (30-F11; eBioscience), CD11b (M1/70; eBioscience), and Gr1 (IA8; BD Bioscience). Propidium iodide (2 μg/mL; Sigma) was used to exclude dead cells. Appropriate isotype control antibodies were applied to set quadrants for calculating the percentage of positive cells. Data were acquired on fluorescence-activated cell sorter Calibur (BD) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Detect Respiratory Chain Complex I–IV by Flow Cytometry

We cultured cells in 6-well plates and treated cells with lipopolysaccharide (1 μg/mL) for 48 hours, then we washed cells and performed intracellular staining with an intracellular staining set (cat. 72-5775; eBioscience). We stained cells with anti–rabbit-NADH-dehydrogenase-ubiquinone-1-alpha-subcomplex-assembly-factor-1 (NDUFAF1; sc-292085, 1:100; Santa Cruz), anti–mouse-NADH-dehydrogenase-ubiquinone-1-subunit-C2 (NDUFC2; sc-393771, 1:100; Santa Cruz), anti–mouse-NADH-dehydrogenase-ubiquinone-iron-sulfur-protein-2 (NDUFS2; sc-390596, 1:100; Santa Cruz), anti–rabbit-succinate-dehydrogenase (SDH; cat.11998, 1:100; Cell Signaling, Beverly, MA), anti–rabbit-cytochrome c (Cyc; cat.4260, 1:100; Cell Signaling), anti–mouse-cytochrome c oxidase (COX IV; cat. 4850, 1:100, Cell Signaling) antibodies for 30 minutes and labeled the cells with a proper second antibody, PE–anti-rabbit (Cat.8885, 1:100; Cell Signaling) or PE–anti-mouse (Cat.8887, 1:500; Cell Signaling). We acquired data on BD Calibur flow cytometry and analyzed mean fluorescence intensity by Flowjo software.

Immunohistochemistry Staining

To perform the immunohistochemistry staining of mitochondria and MyD88, the bEnd.3 cells were cultured on cover slips, stained with mitotracker (Life technologies, Grand Island, NY) for 30 minutes, washed with PBS then fixed with 2% paraformaldehyde (PFA; Polysciences, Inc) for 10 minutes at 37°C. The cells were blocked with 5% goat serum staining buffer and stained with anti–rabbit-MyD88 (1:200; Abcam, Cambridge, MA) overnight, then washed with PBS, and stained with goat-anti-rabbit-FITC (Life Technologies) for 2 hours. The cells were further washed with PBS and mounted on glass slides using prolong gold antifade reagent (Life Technologies). The slides were photographed with confocal LSM 510 microscope Zeiss (Zeiss, Oberkochen, Germany) using software ZEN 2012.

Statistical Analysis

Statistical analysis was performed with Prism 5 software (Graphpad software, La Jolla, CA). Differences between groups were analyzed by the unpaired Student t test, 1-way ANOVA or 2-way ANOVA as indicated in the figure legends.

Results

Lipopolysaccharide Increases Cerebral Infarction and BBB Permeability in Stroke

To test the effects of a bacterial infection mimic on stroke outcome, we performed intraperitoneal injections of lipopolysaccharide (100 μg/kg) or saline 30 minutes before a 30-minute tMCAO in mice (Figure 1A). Lipopolysaccharide challenge significantly increased infarct volume in the cortex (P=0.04), striatum (P=0.009), and total hemisphere (P=0.04) after 30-minute tMCAO followed by 48-hour reperfusion (Figure 1B and 1C).

However, the time course of poststroke BBB leakage, whether biphasic or progressive, remains subject to debate.15,16 Using Evans Blue as a tracer, our recent work demonstrated biphasic peaks of BBB openings at 6 hours and 72 hours but not at 24 hours or 48 hours reperfusion after 1-hour tMCAO.17 The 6-hour-peak of BBB opening may suggest the early
inflammatory infiltration and brain edema in acute stroke. Thus, to determine whether the acute infection challenge regulates cerebrovascular response to stroke, we compared the effects of lipopolysaccharide versus saline on Evans blue extravasation at 6-hour reperfusion after tMCAO (Figure 2A). Six hours after tMCAO (30-minute occlusion), mice were perfused and isolated brains were divided into ipsilateral and contralateral hemispheres, and each hemisphere was analyzed separately for Evans blue extravasation. Lipopolysaccharide significantly increased Evans blue extravasation in the ipsilateral hemisphere after tMCAO compared with control mice (Figure 2B), indicating that lipopolysaccharide enhances cerebrovascular permeability after stroke.

**Lipopolysaccharide Increases Neutrophil Infiltration Into the Injured Brain in Stroke**

Stroke and acute infections elicit inflammatory responses in the injured brain that are mediated by multiple factors. Neutrophils are among the first peripheral cells to infiltrate ischemic brain tissue within 30 minutes to a few hours after focal cerebral ischemia, and neutrophil infiltration is thought to contribute to acute phase damage in stroke. To assess brain leukocyte invasion, we measured neutrophils in brain and peripheral tissues by flow cytometry. Neutrophil accumulation in the ipsilateral and contralateral brain hemispheres was significantly greater in the lipopolysaccharide group than in saline control (n=5; P<0.05) at 6-hour tMCAO post reperfusion (Figure 2C). Concurrently, fewer neutrophils and greater lymphocytes were detected in the peripheral blood and spleen, but monocytes did not differ between vehicle- and lipopolysaccharide-treated mice (Figure IA and IC in the online-only Data Supplement). The neutrophil:lymphocyte ratio was significantly decreased in the blood (n=5; P=0.02) and the spleen (n=5; P=0.004) of lipopolysaccharide-treated mice (Figure IB and ID in the online-only Data Supplement). tMCAO mice with lipopolysaccharide exhibited worsened neurological deficits compared to saline controls (n=18; LPS, n=23). Data are expressed as mean±SD; 1-way ANOVA followed by post hoc Tukey test was used for multiple group comparison and Student t test was used for 2-group statistical analysis. (**P<0.01; ***P<0.001; ****P<0.0001.)
Lipopolysaccharide Impairs Mitochondrial Oxidative Phosphorylation in Cerebrovascular Endothelial Cells

To determine whether the lipopolysaccharide effects in stroke are caused by direct effects on cerebrovascular endothelial cells, we used a cultured cerebrovascular endothelial cell (cCVEC) model. Immunohistochemical staining showed that CD31 (an endothelial cell marker) and TLR4 (a lipopolysaccharide receptor) colocalize in the brain vasculature (Figure IIA in the online-only Data Supplement), closely resembling the expression of CD31 and TLR4 on cCVECs (Figure IIB in the online-only Data Supplement). This indicates that cCVECs have critical components for an in vitro model to investigate lipopolysaccharide effects on cerebrovascular endothelial cells.

The brain has a high energy demand and elevated mitochondrial content resulting in it being vulnerable to reductions in oxidative phosphorylation. Given the expression of TLR4 on the brain vascular endothelial cells, we determined whether lipopolysaccharide would directly affect oxidative phosphorylation and mitochondrial capacity in CVECs. Using Mitotracker Red for visualization of mitochondria, we detected MyD88, an adaptor protein involved in TLR4 signaling, broadly distributed but also colocalized with mitochondria in cCVECs (Figure 3A), suggesting that lipopolysaccharide-induced signaling pathways are linked to mitochondria. To directly evaluate the mitochondrial function in cCVECs affected by lipopolysaccharide, a bioenergetic assay was used to examine cellular energetic oxygen consumption rates. Basal oxygen consumption rate did not significantly differ in cCVECs challenged by lipopolysaccharide for 24 hours, but maximal respiration and spare capacity were significantly reduced in cCVECs cultured with 0.1 to 100 μg/mL lipopolysaccharide (Figure 3B; Figure IIIA in the online-only Data Supplement). Similarly, in primary cerebrovascular endothelial cells, an lipopolysaccharide challenge for 24 hours resulted in a decrease in maximal respiration and spare capacity to 100 μg/mL (Figure 3C; Figure IIIB in the online-only Data Supplement). However, reduced oxidative phosphorylation was not associated with cell death or cell viability in cCVECs or primary CVECs, as evidenced by propidium iodide staining (Figure IVA and IVB in the online-only Data Supplement), calcein AM staining (Figure IVC and IVD in the online-only Data Supplement) for 24 hours in the exception of high dose of lipopolysaccharide (100 μg/mL). In view of the lipopolysaccharide effects on mitochondrial respiration, respiratory chain complex I proteins (NADH dehydrogenase ubiquinone subunits: NDUFAS1, NDUFBS2, and NDUFAS2), complex II protein (SDH), complex III protein (Cyc), and complex IV protein (COX IV) were examined after a 1-μg/mL lipopolysaccharide challenge for 48 hours, and flow cytometry confirmed that lipopolysaccharide decreases the expression of complex I (NDUFS2 and NDUFAS2), complex III (Cyc), and complex IV (COX IV; Figure 3D) proteins. Together, these results strongly suggest that oxidative phosphorylation of CVECs is compromised with lipopolysaccharide exposure, and this effect does not induce endothelial cell death. As such, we asked whether mitochondrial activity affects endothelial cell function.

Impaired Mitochondria Disrupt CVEC Tight Junction and Increase BBB Permeability

It is known that lipopolysaccharide impairs the BBB permeability both in vitro and in vivo at doses that are thought to be caused by inflammatory mediators such as cytokines, but little is known about the role of mitochondria in BBB integrity. Using a pharmacological strategy to manipulate mitochondrial respiration, we first demonstrated that inhibition of respiratory chain complex I with rotenone (Figure 4A), uncoupling of electron flow from ATP production with FCCP (Figure 4B), or inhibition of complex V with oligomycin (Figure 4C) rapidly increased FITC-dextran 70 permeability in a cCVEC monolayer transwell system in vitro. Immunocytochemical analysis revealed that the normally well-defined, linear cell–cell junctions were disrupted when oxidative phosphorylation was inhibited by mitochondrial inhibitors (Figure 4D). Both increased permeability and cell–cell junction disruption caused by the inhibition of oxidative phosphorylation occurred in the absence of cell death as evidenced by propidium iodide staining (Figure VA in the online-only Data Supplement) and calcein AM staining (Figure VB in the online-only Data Supplement). These data suggest for the first time that mitochondria play a key role in maintaining BBB integrity in vitro.

To determine whether inhibition of mitochondria affects the BBB permeability in vivo, we developed an epidural application model for central nervous system drug administration in mice (Figure 5A–5D). This model permits local drug delivery without traumatic brain injury (Figure 5C), thus allowing assessment of BBB permeability. Rotenone (2 μL, 2 mg/mL dissolved in saline) was applied to the epidural surface, and BBB permeability was evaluated by Evans blue quantification after 6 hours. Mice treated with rotenone showed significantly higher Evans blue extravasation than vehicle controls (Figure 5D). These data support the hypothesis that mitochondria are critical for the regulation of cerebrovascular permeability in vivo.

Inhibition of Mitochondrial Function Worsens Stroke Severity in Mice

If inhibition of mitochondrial function increases BBB permeability, the same perturbation would be expected to affect infarct size and stroke severity. When we treated mice with rotenone before tMCAO (Figure 6A), increased BBB permeability (Figure 6B), increased infarct volumes (Figure 6C), and worsened neurological deficits (Figure 6D) were observed. Thus, the lipopolysaccharide-induced exacerbation of stroke outcome most likely is because of the deleterious effect of mitochondrial inhibition.
Acute systemic infection is a risk factor or trigger for human stroke and is associated with worsened clinical outcome. Although much is known about the factors that worsen stroke severity in clinical subjects and experimental stroke models, less is understood about their mechanisms. Our data are the first to demonstrate that a bacterial infection mimic acts with ischemic challenge to markedly exacerbate stroke damage via a mitochondrial-dependent mechanism. This new finding could also provide an explanation for the association of infections with severity of brain damage and BBB dysfunction from stroke: the induction of hypometabolism in cerebrovascular endothelial cell mitochondria.

Mitochondrial dysfunction is increasingly recognized as an accomplice in vascular diseases and ischemic stroke. More recently, it has been shown that components of the TLR4 signaling pathways, MyD88 and TRAF6, are linked with mitochondria and affect oxidative phosphorylation. The expression of MyD88 in mitochondria of endothelial cells (Figure 3A) supports that mitochondrial function can be affected by lipopolysaccharide (Figure 3B and 3C). It is notable that no significant effect of lipopolysaccharide is seen on cCVEC basal oxygen consumption and ATP-linked oxygen consumption on treatment with oligomycin (Figure 3B; Figure III in the online-only Data Supplement), indicating that lipopolysaccharide does not significantly change basal oxygen demand in mitochondria after 24 hours of treatment with lipopolysaccharide. However, the loss of maximal respiration and spare capacity (Figure 3B; Figure III in the online-only Data Supplement), suggesting that MyD88 expresses on mitochondria of cCVECs. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Results are representative of 4 independent experiments. Images were taken under ×63 objective. Scale bars, 10 μm. 

**Discussion**

Acute systemic infection is a risk factor or trigger for human stroke and is associated with worsened clinical outcome. Although much is known about the factors that worsen stroke severity in clinical subjects and experimental stroke models, less is understood about their mechanisms. Our data are the first to demonstrate that a bacterial infection mimic acts with ischemic challenge to markedly exacerbate stroke damage via a mitochondrial-dependent mechanism. This new finding could also provide an explanation for the association of infections with severity of brain damage and BBB dysfunction from stroke: the induction of hypometabolism in cerebrovascular endothelial cell mitochondria.
III in the online-only Data Supplement) demonstrates that mitochondrial capacity is reduced by lipopolysaccharide and thus the effects exacerbate the mitochondrial impairment by ischemia. Cellular respiration, mitochondrial biogenesis, and mitochondrial function require coordinated expression of proteins encoded by nuclear and mitochondrial genes including mitochondrial complex I–IV. The reduced expression of respiratory chain complex I, III, and IV proteins in cCVECs by lipopolysaccharide provides further evidence that lipopolysaccharide compromises the endothelial mitochondria function (Figure 3D).

The 30-minute tMCAO model mimics a mild ischemic event that results in a small infarct (Figure 1C) and does not cause visible BBB disruption at 6-hour reperfusion (Figure 2B). The bacterial infection mimic challenges before tMCAO increased BBB permeability (Figure 2B) and exacerbates infarct volume (Figure 1C). There are inconsistent data for lipopolysaccharide- or cytokine-induced BBB

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**Figure 4.** Pharmacological inhibition of mitochondria increases blood–brain barrier permeability in vitro. Fluorescein isothiocyanate (FITC)-Dextran-70 permeability after addition of 20-μmol/L rotenone (A), 20-μmol/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; B) and 20-μmol/L oligomycin (C) vs vehicle to cultured cerebrovascular endothelial cells (cCVECs). Data are presented as both real-time rate of permeability (2-way ANOVA followed by post hoc Dunnett test) and calculated apparent permeability coefficient (P_{app}; Student t test). n=3 per group. **P<0.01; ***P<0.001; ****P<0.0001. D, Confocal fluorescence images of cCVEC confluent monolayers after treatment with 20-μmol/L rotenone, 20-μmol/L FCCP, and 20-μmol/L oligomycin vs vehicle for 2 hours. The immunohistochemistry staining of ZO-1 (red) was performed for tight junctions. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, blue). Mitochondrial inhibitors apparently disrupted tight junctions and resulted in gaps between cells (white arrows).

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**Figure 5.** Pharmacological inhibition of mitochondria increases blood–brain barrier permeability in vivo. A, Graph depicting the epidural application (EA) model. B, Exposure of epidural membrane: vessels were visible under surgical microscope. C, 2,3,5-Triphenyltetrazolium chloride (TTC) staining in the EA model showing no brain injury. D, Evans blue accumulation was visible in the brain of rotenone applied mice (red arrows) in EA model and quantitative analysis of Evans blue extravasation (μg/g brain tissue) in individual brain. n=5 per group; 1-way ANOVA followed by post hoc Tukey test. Data are expressed as mean±SD; **P<0.01.
Figure 6. Impairment of mitochondria function exacerbates stroke outcomes. A, Scheme of experimental design and workflow. Rotenone (1 mg/kg, IP) or vehicle (saline, IP) was administered 30 minutes before right transient middle cerebral artery occlusion (tMCAO; 60-minute occlusion), blood–brain barrier (BBB) permeability was evaluated at 6-hour reperfusion and infarct volume was measured at 24-hour reperfusion. B, Representative brain coronal sections for Evans blue accumulation and quantitative analysis of Evans Blue extravasation (μg/g brain tissue) in contralateral (left) and ipsilateral (right) hemispheres. Vehicle, n=4; rotenone, n=4. One-way data are analyzed with ANOVA followed by post hoc Tukey test (*P<0.05). C, Representative 2,3,5-triphenyltetrazolium chloride–stained coronal sections used to analyze infarct of tMCAO mice and quantitative analysis of infarct volumes. Mice treated with rotenone had significant larger infarct volume than vehicle group, in cortex, striatum, and total hemisphere. Rotenone (1 mg/kg, IP) or vehicle (saline, IP) was administered 30 minutes before right tMCAO (1-hour occlusion) and 24-hour reperfusion was performed. Vehicle, n=8; rotenone, n=8. Data are analyzed with Student t test (**P<0.01; ***P<0.001; ****P<0.0001). D, Rotenone worsened neurological deficits at 6 and 24 hours after tMCAO. Vehicle, n=12; rotenone, n=12. Data are analyzed with Student t test (****P<0.0001).

We observed that lipopolysaccharide-induced inhibition of mitochondria exacerbates infarct volume and opens BBB in tMCAO mice (Figures 1 and 2), but that mitochondria inhibition in cCVECs and primary CVECs does not affect cell viability in 24 hours (Figure IV in the online-only Data Supplement). This suggests that the functional impairment of endothelial mitochondria by lipopolysaccharide may be reversible, and an effective intervention could be designed to prevent/restore BBB during strokes. However, our data also indicated that a high dose of lipopolysaccharide (100 μg/mL) induced cell death and reduced cell viability in cCVECs (Figure IV in the online-only Data Supplement), and extension of lipopolysaccharide exposure for 48 hours induced cell death in both cCVECs and primary CVECs (Figure IVA–IVD in the online-only Data Supplement). Therefore, the effective mitochondrial directed intervention may be limited by the severity or the length of the infectious exposure to rescue BBB damage.

Leukocytosis and cytokine and chemokine storm are features of the acute-phase reaction in systemic infections and in acute stroke. It is becoming increasingly apparent that inflammation can disturb cell energy metabolism. Our recent study found that neuronal mitochondrial function was rapidly and profoundly decreased by tumor necrosis factor-α resulting in neuronal cell death.27 Another study demonstrated that tumor necrosis factor-α elevated oxygen consumption rate in endothelial cells.28 The present study did not address the complex lipopolysaccharide-induced effects on mitochondria; however, our novel findings refreshed the traditional idea of infection–inflammation responses and provided a new explanation for the lipopolysaccharide effects on BBB dysfunction, a direct mitochondrial-dependent mechanism.

The application of rotenone on the epidural surface argues that its effect on BBB opening is by compromised mitochondria in vascular endothelial cells (Figure 5). Rotenone exposure is associated with clinical features of parkinsonism in

dysfunction9 that may be because of complex mechanisms. McColl et al7 demonstrated an interleukin-1–dependent mechanism of stroke exacerbation using the same lipopolysaccharide-tMCAO model. Others have demonstrated that lipopolysaccharide alters transporters or enzymes involved in BBB maintenance.22 Our observation that pharmacological inhibition of mitochondria disrupts BBB integrity and increases BBB permeability in vitro (Figure 4) and in vivo (Figure 5) strengthens the argument that BBB permeability is increased by lipopolysaccharide after stroke (Figure 2) through a direct mitochondrial mechanism resulting in a larger infarct volume and worsening neurological deficit.

Increased BBB permeability is positively correlated with massive vasogenic and cytotoxic edema post stroke23,24 and elevated neutrophils in the ischemic area, which contribute to infarct expansion25 and poor clinical outcome.26 Our observation of neutropenia and low neutrophil:lymphocyte ratio may be a consequence of BBB damage that causes rapid migration of neutrophils into damaged tissue. Brain infiltration of neutrophils could also account for secondary lesion growth in acute stroke. It is becoming increasingly apparent that inflammation can disturb cell energy metabolism. Our recent study found that neuronal mitochondrial function was rapidly and profoundly decreased by tumor necrosis factor-α resulting in neuronal cell death.27 Another study demonstrated that tumor necrosis factor-α elevated oxygen consumption rate in endothelial cells.28 The present study did not address the complex lipopolysaccharide-induced effects on mitochondria; however, our novel findings refreshed the traditional idea of infection–inflammation responses and provided a new explanation for the lipopolysaccharide effects on BBB dysfunction, a direct mitochondrial-dependent mechanism.

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Humans\textsuperscript{29} and broadly used to induce Parkinson’s disease-like symptoms in animal models\textsuperscript{30} as well. This suggests that the mitochondrial-dependent BBB opening may be involved not only in the progress of stroke but also in other neurodegenerative disorders such as Parkinson’s disease and Alzheimer’s disease.

In summary, the present study describes a previously unknown mechanism of infection-induced direct mitochondrial dysfunction in cerebrovascular endothelial cells, which compromises BBB permeability and exacerbates acute stroke outcomes. Moreover, our observations argue that maintenance of mitochondrial function is critical to the integrity of the BBB. The data also suggest a translational significance: maintenance of brain endothelial cell mitochondrial function can improve the acute outcome of stroke and perhaps other neurodegenerative diseases.

Acknowledgments

We thank Dr Linda Van Eldik (University of Kentucky) and Dr Paul Lockman (West Virginia University) for their critical prereview of the article. We acknowledge Dr Jeffrey Wimsatt and Dr Matthew Kessler (West Virginia University) for their critical prereview of the article. We acknowledge Dr Jeffrey Wimsatt and Dr Matthew Kessler for their advice on Institutional Animal Care and Use Committees protocol.

Sources of Funding

This work was supported by National Institutes of Health (NIH: P20 GM109098, P01 AG027956, U54 GM104942, and T32 GM081741). Imaging experiments were performed at the West Virginia University (WVU) Microscope Imaging Facility supported by the Mary Babb Randolph Cancer Center (MBRCC) and NIH (P20 RR016440, P30 RR032138/GM103488, and P20 RR016477). Flow cytometry was performed at the WVU Flow Cytometry Core Facility supported by MBRCC and NIH (P30 RR032138/GM103488 and GM103434).

Disclosures

None.

References


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Stroke. 2015;46:1681-1689; originally published online April 28, 2015;
doi: 10.1161/STROKEAHA.115.009099
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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Data Supplement (unedited) at:
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Mitochondrial Crisis in Cerebrovascular Endothelial Cells Opens the Blood-Brain Barrier

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This PDF file contains
Supplemental Materials and Methods
Supplemental Figures I-V and Figure Legends
Supplemental Materials and Methods:

Cell viability assay
We performed the cell viability assay simultaneously with the oxygen consumption assay. We seeded cells in 96 black well plates and treated with LPS. We added PI (2 µg/ml) to evaluate cell death on plate reader (Ex. 535 nm and Em. 617 nm), washed with PBS, added Calcein AM (Life technology) and stained for 30 minutes to measure cell viability (Ex. 490 nm and Em. 520 nm).

Immunohistochemistry staining
To obtain brain samples for immunohistochemistry assays, animals were anesthetized with isofluorane and perfused with PBS followed by 10% formalin PBS. Brains were removed, post-fixed overnight in 10% formalin PBS at 4°C, cryoprotected in graded sucrose solutions, and embedded in Tissue-Tek OCT (VWR Scientific). Coronal sections (20 µm) were cut through, blocked with 5% goat serum staining buffer and stained with rabbit anti-mouse TLR4 (1:100, Abcam, Cambridge, Massachusetts) and rat anti-mouse CD31 (1:200, Abcam) overnight, and then washed with PBS and stained with goat-anti-rabbit-FITC and goat-anti-rat-PE antibodies (Life technologies, Grand Island, New York) for 2 hours. The sections were further washed with PBS and mounted on glass slides using prolong gold anti-fade reagent (Life technologies).

To perform the immunohistochemistry staining on bEnd.3 cells, the cells were cultured on cover slips and fixed with 4% cold paraformaldehyde (PFA, Polysciences, Inc.) followed by blocking, staining, washing and mounting, as outlined above. The slides or sections were photographed with confocal LSM 510 microscope Zeiss (Zeiss, Oberkochen, Germany) using software ZEN 2012.

Statistical Analysis
Statistical analysis was performed with Prism 5 software (Graphpad software, La Jolla, California). Differences between groups were analyzed by the unpaired Student’s t-test or one way ANOVA as indicated in the figure legends.
Supplemental Figures:

Supplemental Figure I. LPS significantly decreases neutrophils and increases lymphocytes in peripheral blood and spleen after stroke. Analysis of neutrophils, monocytes and lymphocytes in blood (A) and spleen (C). Representative FACS data of blood and spleen showing percentage of Gr1+ polymorphonuclear neutrophils (PMNs), CD11b+Gr1- monocytes, and CD11b- Gr1- lymphocytes gated in CD45+ white blood cells (WBCs). Neutrophil-to-lymphocyte ratio (NLR) in blood (B) and spleen (D). Mice pre-treated with LPS had significantly fewer neutrophils but more lymphocytes in the blood and spleen than vehicle group after 30 min tMCAO and 6 hours reperfusion. Data are expressed as mean ± S.D.; n = 5 per group; Student t-test was used for significant analysis, *, P < 0.05; **, P < 0.01.
Supplemental Figure II. LPS receptor, TLR4, is expressed by endothelial cells in brain tissue and in culture. (A) Sections from normal mouse brains stained with antibodies to TLR4 (purple) show TLR4 staining in association with CD31 (green), a specific marker for endothelial cells. (B) Micrographs showing TLR4 (purple) expression in cultured cerebrovascular endothelial cells (CCVECs, bEnd.3 cell line). Nuclei were stained with DAPI (blue). Results are representative from four independent experiments. Images were taken under 20× objective. Scale bars, 20 μm.
Supplemental Figure III. The raw data of oxygen consumption rate determined by the Seahorse XF96e analyzer. (A) cultured cerebrovascular endothelial cells (cCVECs, bEnd.3 cell line) were incubated with various concentrations of LPS for 24 hours then oxygen consumption rate (OCR) was determined by the Seahorse XF96e analyzer. (B) Primary cerebrovascular endothelial cells (pCVECs, originally from adult mouse brain) were incubated with various concentrations of LPS for 24 hours then OCR was determined by the Seahorse XF96e analyzer. The mitochondrial inhibitors: oligomycin, FCCP, and rotenone plus antimycin A were sequentially injected after measurement points 3, 6, and 9 as indicated. Data are presented as mean ± S.D. at each measurement.
Supplemental Figure IV. LPS and cell viability in cultured cerebrovascular endothelial cells and primary cerebrovascular endothelial cells. Evaluation of LPS on cell death by propidium iodide (PI) staining. cCVECs (A) or pCVECs (B) were cultured with LPS (0.01 - 100 µg/ml) for 24 and 48 hours then stained with PI (2 µg/ml). A microplate reader was immediately used to determine the mean fluorescent intensity of PI staining. Effect of LPS on cell viability of cCVECs (C) or pCVECs (D) by calcein-AM staining. After PI staining, cells were washed with PBS and incubated with calcein-AM (5 µM) for 30 minutes. A microplate reader was used to determine the mean fluorescent intensity of Calcein AM staining. Data are presented as mean ± S.D., analyzed by One-way ANOVA followed by post-hoc Tukey’s test. LPS at 100 µg/ml significantly induced cell death and reduced cell viability in cCVECs after 24 hours culture. *, P<0.05, **, P < 0.01; ****, P < 0.0001.
Supplemental Figure V. Mitochondria inhibitors and cell viability in cultured cerebrovascular endothelial cells at 2 hours. (A) Evaluation of mitochondria inhibitors: rotenone, FCCP and oligomycin on cell death by propidium iodide (PI) staining in cCVECs. Confluent cCVEC monolayer were treated with rotenone, FCCP or ologomycin (5 - 80 µM) for 2 hours then stained with PI (2 µg/ml). A microplate reader was immediately used to determine the mean fluorescent intensity of PI staining. (B) Effects of mitochondria inhibitors on cell viability by calcein-AM staining. After PI staining, cells were washed with PBS and incubated with calcein-AM (5 µM) for 30 minutes. Mean fluorescence intensity was determined using a microplate reader. Data are presented as mean ± S.D., analyzed by One-way ANOVA followed by post-hoc Tukey’s test. Rotenone at 80 µM significantly reduced cell viability after 2 hours treatment. Data are presented as mean ± S.D., analyzed by One-way ANOVA followed by post-hoc Tukey’s test. *, P<0.05.