Sustained Mitochondrial Functioning in Cerebral Arteries after Transient Ischemic Stress in the Rat: A potential target for therapies.

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Running Head: Mitochondrial Influences after tMCAO

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Objective: To determine whether mitochondrial function in the cerebral vasculature is maintained after transient middle cerebral artery occlusion (tMCAO) in rats.

Approach and results: Sprague-Dawley rats were exposed to 90 min tMCAO followed by 4 or 48 h reperfusion. Middle cerebral arteries (MCAs) from ischemic (IPSI) and non-ischemic (CONTRA) sides were compared with CONTROL MCAs from sham rats. We determined: (1) vasoreactivity to diazoxide (DZ), a mitochondrial ATP activated potassium channel opener, acetylcholine (Ach), bradykinin (BK), serotonin (5-HT), and sodium nitroprusside (SNP); (2) levels of mitochondrial and non-mitochondrial proteins and mitochondrial DNA (mtDNA); and (3) vascular levels of tetramethylrhodamine-ethyl-ester, an indicator of mitochondrial membrane potential. All dilator responses including DZ were intact 4 h post-tMCAO. Dilator responses to Ach, BK, and SNP were reduced in IPSI at 48 h compared with CONTRA, but DZ responses were comparable with CONTROL. Surprisingly, CONTRA responses to Ach, BK, and 5-HT were reduced compared with CONTROL at 48 h. IPSI vasodilation to DZ at 48 h was eliminated by endothelial denudation and endothelial nitric oxide synthase (eNOS) inhibition, but was only reduced in CONTROL. Mitochondrial proteins, phosphorylated eNOS, mtDNA, and mitochondrial membrane potential were higher in IPSI compared with CONTRA MCAs.

Conclusions: Contrary to conventional wisdom, mitochondria remain functional for at least 48 h following severe ischemic stress in MCAs, and DZ-induced dilation is preserved due to maintained mitochondrial mass, probably in the endothelium, and eNOS signaling. Our findings support the concept that functioning vascular mitochondria are an unexpected target for novel stroke therapies.
**Key words:** diazoxide, endothelium, middle cerebral artery occlusion, mitochondrial depolarization, vasodilation, experimental strokes
INTRODUCTION

Ischemic stroke is a leading cause of human morbidity and mortality (34).

Currently, thrombolytic or surgical therapies are the only accepted treatments for occlusive stroke but clot removal is not prescribed for many patients because the time window for administration is severely limited (14). Until recently, thrombolytic or surgical interventions were only medically endorsed if initiated within three hours following the occurrence of a stroke (18, 19). New studies, especially those from Europe, indicate that the therapeutic window of clot resolution can be extended to four and one-half hours or more after the onset of stroke, but increasing cerebral vascular dysfunction and neurological damage may reduce beneficial results when initiated at a later time (1, 11, 31, 39). Therefore, new therapies for these later time periods are urgently needed for vulnerable stroke patients. Recently, limited information from several sources indicate that, contrary to currently held beliefs, significant mitochondrial function remains intact following ischemic stress and thus mitochondria may provide a novel, but yet unexploited, therapeutic target (28, 38). However, no direct in vivo assessment of vascular mitochondrial function has yet been conducted in the cerebral vasculature following ischemic stress.

Previous studies have shown that selective activation of mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels located on the inner mitochondrial membrane by agents such as diazoxide (DZ) reduces brain infarct volume, preserves the responsiveness of large cerebral arteries, and protects the blood-brain barrier (BBB) when given prior to transient global ischemia or transient middle cerebral artery occlusion (tMCAO) (6, 12, 13, 26). In addition, activation of mitoK$_{ATP}$ protects cultured
endothelium, neurons, and astroglia against cell death following transient oxygen
glucose deprivation (OGD) (4, 17, 20, 24, 32). Finally, recent studies have also
indicated that pharmacological activation of mitochondria after tMCAO or OGD is as
effective as pretreatment, thereby suggesting that significant mitochondrial functionality
is present even following severe anoxic/ischemic stress (5). One limitation of in vivo
studies is that the location of cellular protection cannot be known with certainty since the
neurovascular unit represents many cell types including neurons, astroglia, microglia,
and vascular cells. Nonetheless, several recent studies have demonstrated that the
cerebral vasculature, especially the endothelium, plays a critical role in determining
neurological outcome following stroke by maintaining appropriate blood flow and the
BBB (10, 15). However, no studies have directly examined mitochondrial function in the
cerebral vasculature after tMCAO or other experimental models of stroke. Under normal
conditions, we and others have shown that mitochondrial dependent vasodilation of
large cerebral arteries has both endothelial and vascular smooth muscle (VSM)
components which contribute to the overall, integrated vascular response (23, 42). Thus
increased nitric oxide production by endothelium augments intrinsic relaxation of VSM
of cerebral arteries following application of DZ.

We hypothesized that mitochondrial function is maintained after severe ischemic
stress in cerebral arteries following tMCAO and represents a previously unappreciated
therapeutic target which potentially can protect the cerebral vasculature and the brain.
Specifcally, we investigated: (1) the effects of tMCAO on the responses of isolated
middle cerebral arteries (MCAs) to mitochondrial activation as well as to other general,
non-mitochondrial-based vasoactive stimuli; (2) the relative contribution of endothelium
and VSM to the integrated cerebral vascular response to mitochondrial activation following tMCAO; and (3) the effects of tMCAO on the expression of mitochondrial and non-mitochondrial proteins and mitochondrial biogenesis in MCAs. We chose to use DZ because of its selectivity to mitochondria, widespread use and tolerance, proven effectiveness for treatment of non-neurological diseases in people, and its ability to cross the BBB (4, 6, 16, 37). We studied cerebral arteries 48 h after tMCAO, at a time when the brain infarct is fully developed, but also examined the arterial responses at 4 h when post-tMCAO hemodynamics had stabilized.

**MATERIALS AND METHODS**

**Animals**

Eight to ten week old, male, Sprague-Dawley rats (SD) were obtained from Charles River Laboratories (Wilmington, MA) (n=124). Animals were housed and cared for according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Tulane University in compliance with NIH guidelines. Animal care was provided by the Department of Comparative Medicine. The rats were given soft food and water *ad libitum*.

**Transient Middle Cerebral Artery Occlusion (tMCAO)**

Rats were randomly assigned to two groups: a CONTROL (sham operated) or an operated group, in which the cerebral vasculature was exposed to tMCAO (36). Rats were subjected to a modified Longa intraluminal filament method to induce ischemia by the occlusion of one of the MCAs. We performed a 90 min period of ischemia under
ketamine-xylazine anesthesia consisting of 100 mg/ml Ketamine HCl (KetaVed, St. Joseph, MO) and 20 mg/ml AnaSed Xylazine (Santa Cruz, Dallas, TX) followed by 48 h reperfusion without anesthesia. During surgery, supplemental oxygen was given, ointment was used to maintain eye lubrication, and a rectal temperature probe and heating pad were used to monitor and maintain body temperature at normal levels. Using an operating microscope for visualization, a midline incision was made in the neck. The right common carotid artery (CCA), external carotid artery (ECA), and the internal carotid artery (ICA) were isolated from the surrounding tissue from the bifurcation to the base of the skull. A 5-0 silk suture was used to ligate the ECA and the lingual, maxillary, and occipital artery branches were dissected and coagulated. Afterward, a 5-0 silk suture was placed around the CCA to prevent bleeding. To occlude the MCA, a rubber-coated silicone monofilament (Doccol Corporation, Sharon, MA), 3 cm in length and 0.35 or 0.37 mm in diameter, was inserted through the small incision on the CCA into the ICA and into the Circle of Willis, effectively occluding the right MCA. A vascular clamp was used to fix the filament in the vessel to prevent bleeding and the incision was covered with sterile, 0.9% saline soaked gauze. After 90 min of ischemia, the intraluminal filament was removed, the incision was closed using a wound clip (Fine Science Tools, Foster City, CA), and rats were allowed to recover for 4 or 48 h with free access to soft food and water. Buprenorphine (0.05 mg/kg) was available for post-operative analgesia if needed. The tissue dehydrogenase marker 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Aldrich. St. Louis, MO) was used to visualize and document the volume of infarcted brain after isolation of the MCAs. The reaction between the TTC-salt and dehydrogenase enzyme resulted in red staining of viable
tissue, whereas the infarcted tissue resulted in a lack of staining due to the absence of enzyme activity with which the TTC could react. After isolation of the MCAs, 2.0 mm thick coronal sections were acquired using a rat brain slicer matrix (Zivic, Pittsburgh, PA). The brain slices were placed in a beaker containing a pre-warmed (37°C) solution of 2% TTC dissolved in 0.9% saline which was placed in a 37°C water bath for 20 min. A scan was taken of the slices and the infarct size was determined using ImageJ Software (NIH). The infarct volumes were established by averaging the infarcted area of the slices and were expressed as a percentage of each hemispheric volume (% infarction). In the CONTROL group, rats underwent a sham operation with the same length of anesthesia as the operated group with a midline neck incision performed, but without filament insertion (8).

**Isolated, Pressurized Artery Technique**

We have used this method previously (23). Under 5% isoflurane (VetOne, Boise, ID) induced anesthesia, rats were decapitated and brains were removed and transferred to ice-cold 4°C oxygenated (20 % O₂, 5 % CO₂, 75 % N₂) Ca²⁺ Krebs solution (mmol/L: 110 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 KH₂PO₄, 5.0 glucose, and 24.0 NaHCO₃) at 7.4 pH. Microsurgery instruments (Fine Science Tools, Foster City, CA) and a Nikon SMZ1000 operating microscope were used for the isolation of MCAs in 4°C Ca²⁺ Krebs solution. An isolated section of MCA (<1 mm in length) was transferred into a self-heated, 37°C organ chamber (Living Systems Instrumentation, St. Albans, VT) containing two glass micropipettes filled with Ca²⁺ Krebs solution and cannulated on both ends. One of the micropipettes was closed and the other was connected with
silicone tubing to a pressure servo control system (Living Systems Instrumentation, St. Albans, VT) and the temperature was maintained at 37°C. Changes in arteriolar diameter were continuously measured and recorded by a video microscope (Nikon Eclipse TS100, Sony CCD camera, VDA-10 Living Systems Instrumentation Video Dimension Analyzer, LabView 2.0, and HP computer). Spontaneous myogenic tone developed in response to 70 mmHg intraluminal pressure during a one hour equilibration period.

Endothelium dependent responses of the MCAs were tested using 10 µmol/L of acetylcholine (Ach) (Sigma Aldrich, St. Louis, MO) and 10 µmol/L of bradykinin (BK) (Calbiochem, San Diego, CA), and smooth muscle dependent functions were characterized using 10 µmol/L of serotonin (5-HT) (Sigma Aldrich, St. Louis, MO) and 10 µmol/L of sodium nitroprusside (SNP) (Sigma Aldrich, St. Louis, MO). After the addition of each agent, the Ca\(^{2+}\) Krebs in the organ chamber was allowed to equilibrate for 15 min.

Vascular responses to 10, 50, and 100 µmol/L DZ (Sigma Aldrich, St. Louis, MO) were determined on intact and endothelium denuded MCAs. Endothelial denudation was induced by injecting 1 ml of air through the artery lumen. To investigate the different signal transduction pathways, diazoxide (DZ) was applied in the absence and presence of the non-selective nitric oxide synthase (NOS) inhibitor, \(N_\omega\)-Nitro-L-arginine methyl ester hydrochloride (L-NAME) at 100 µmol/L for 30 min (Sigma Aldrich, St. Louis, MO), and the specific mitoK\(_{\text{ATP}}\) channel inhibitor, 5-hydroxydecanoate (5-HD), at 1 mmol/L.
Western blot

We performed Western blot analysis on isolated MCAs (23), (40). Proteins were extracted by homogenizing the MCAs using a Radnoti glass tissue grinder in 4°C NP40 lysis buffer (Invitrogen, Frederick, MD) containing a proteinase and phosphatase inhibitor, both at 5 μL/mL (Sigma Aldrich, St. Louis, MO). The homogenate was centrifuged at 1,000 g for 10 min; the supernatant was used for the gel electrophoresis and Bradford protein assay to determine the protein concentration of the sample. The proteins were separated by 4% - 20% SDS-PAGE gradient gel and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) in Tris-buffered saline and 1% Tween-20 (TBST) (Sigma Aldrich, St. Louis, MO) for 1 h at room temperature, then washed three times with TBST. The membranes were incubated overnight at 4ºC with the primary antibodies in 1% BSA-TBST blocking solution for the following mitochondrial proteins: ATP synthase, Complex V subunit α at 1:500 dilution (#459240, Invitrogen, Frederick, MD), total dynamin related protein -1 (DRP-1) at 1:1000 dilution (#611112, BD Transduction, San Jose, CA) and its Ser^{616} phosphorylated form (pDRP-1) at 1:1000 dilution (#3455, Cell Signaling Technology, Danvers, MA), voltage-dependent anion channel (VDAC) at 1:1000 dilution (#4866, Cell Signaling Technology, Danvers, MA). The same incubation conditions were applied for the following non-mitochondrial proteins: total endothelial NOS (eNOS) at 1:500 dilution (#610297, BD Transduction Laboratories, San Jose, CA) and its Ser^{1177} phosphorylated form (peNOS) at 1:500 dilution (#9571, Cell Signaling Technology, Danvers, MA), and the loading control β-actin at 1:5000 dilution (#A5441, Sigma Aldrich, St. Louis, MO). The membranes were
washed 3 times and incubated with their respective horseradish peroxidase conjugated secondary anti goat-rabbit IgG at 1:5000 dilution (Cell Signaling Technology, Danvers, MA) or mouse IgG at 1:5000 (Santa Cruz, Dallas, TX) in 1% BSA-TBST for 2 h at room temperature. Chemiluminescence (LumiGLO, Gaithersburg, MD) and autoradiography were used to visualize the final reaction. The optical density of each band was quantified and normalized to β-actin immunoband intensity using ImageJ Software (% intensity).

Mitochondrial DNA (mtDNA) Quantification
DNA was harvested by homogenizing the isolated MCAs in 200 μL Nuclei Lysis Solution using a DNA extraction kit (Promega, Madison, WI). The levels of mtDNA were measured by using the mitochondrial Cytochrome b gene (MT-CYB, Rn03296746_s1), which was normalized to the nuclear heat shock protein 70 gene (Hspa 1a, Rn00583013_s1) (Applied Biosystems) (40). Triplicates of the samples were run using the following protocol: one cycle at 95°C for 15.05 min, 45 cycles at 95°C for 15 s and at 60°C for 1 min. The gene expression levels were quantified using the ΔΔCT method and the mtDNA data were normalized to nuclear DNA and expressed as mean ± s.e.m.

Mitochondrial Membrane Potential (ΔΨm)
We investigated the ΔΨm using tetramethylrhodamine-ethyl-ester dye (TMRE) with excitation at 543 nm and emission at >565 nm (Invitrogen, Frederick, MD), which accumulates in the active, negatively charged mitochondria via the strong electrochemical gradient, resulting in a red fluorescence (22). Arteries were loaded with
100 nmol/L TMRE for 30 min followed by a 15 min washing period, then treated with the vehicle control dimethyl sulfoxide (DMSO), 100 µmol/L of DZ, or 500 nmol/L of carbonyl-cyanide-4-trifluoromethoxy-phenyldrazone (FCCP) (Sigma Aldrich, St. Louis, MO). FCCP is an ionophore uncoupler of oxidative phosphorylation, which eliminates the mitochondrial membrane potential and therefore diminishes mitochondrial TMRE staining. Images were acquired before and after the treatments using a Zeiss confocal microscope. ImageJ software was used to analyze the recorded fluorescence Z stack images and the intensity of fluorescence was determined and expressed as relative fluorescence units (RFUs). The change in TMRE fluorescence was expressed as percent decrease in control RFU after DZ/DMSO/FCCP treatments (% RFU).

**Data analysis and statistics**

All data were expressed as mean ± s.e.m and analyzed using one-way analysis of variance for repeated measures (ANOVA) and the Tukey post hoc test. A p<0.05 was considered as statistically significant. “n” indicates the number of arteries.

**RESULTS**

**Effectiveness of tMCAO**

All animals used for the stroke experiments had well defined areas of infarcted tissue on the ischemic side, ipsilateral (IPSI) to the tMCAO without obvious parenchymal damage on the non-ischemic, contralateral (CONTRA) side after 48 h. Specifically, the tMCAO produced an infarction which covered 60.4±2.7% (n=33, p<0.05, data not shown) of the IPSI hemisphere and which involved both the cortical
and subcortical areas. Non-ischemia (CONTROL) animals that received a sham operation showed no evidence of neurological impairment or loss of TTC staining.

**MCAs 4 h after tMCAO**

Internal diameters of pressurized IPSI MCAs were 272±9 μm and 252±11 μm for CONTRA MCAs (n=10 in each group) compared with CONTROL (231±6 μm; n=22) arteries. Both IPSI and CONTRA arteries generated similar myogenic tone (IPSI: 38±6 %, CONTRA: 44±4 %, p=NS) (Figure 1D).

There were no significant differences among responses in IPSI and CONTRA arteries to 10 μM of Ach (IPSI: 15±5 μmol/L; CONTRA: 13±4 μmol/L, p=NS), BK (IPSI: 51±10 μm; CONTRA: 45±18 μm, p=NS), SNP (IPSI: 100±27 μm; CONTRA: 76±20 μm, p=NS), and 5-HT (IPSI: -88±10 μm; CONTRA: -92±17 μm, p=NS) (Figure 1A-C, E).

Although dilator responses to IPSI and CONTRA MCAs in response to Ach and BK tended to be lower than 48 h CONTROL responses, these were not significant.

Dilator responses of MCAs to DZ with intact endothelium were similar following tMCAO in both groups (IPSI: 10±5 μm, 23±4 μm, and 54±6 μm; CONTRA: 2±3 μm, 18±5 μm, and 43±6 μm; respectively, for 10, 50, and 100 μmol/L of DZ, p=NS) (data not shown).

Denudation of endothelium decreased DZ induced dilation on the stroke side in response to 50 and 100 μmol/L of DZ (IPSI: 4±3 μm, 1±1 μm, and 4±2 μm; CONTRA: 1±0 μm, 8±1 μm, and 21±5 μm; respectively, for 10, 50, and 100 μmol/L of DZ, p<0.05).

In the presence of L-NAME, DZ induced responses of IPSI arteries were decreased to 10 and 50 μmol/L of DZ and to 100 μmol/L of DZ in CONTRA arteries (IPSI: -3±3 μm,
2±2 μm, and 46±10 μm; CONTRA: 1±4 μm, 11±4 μm, and 25±9 μm; respectively, for 10, 50, and 100 μmol/L of DZ, p<0.05) (data not shown).

Of the proteins examined, we detected a significant difference (p<0.05) only in the phosphorylated dynamin related fission protein-1 (pDRP-1) levels between IPSI and CONTRA MCAs which were more than two-fold higher in IPSI (data not shown). We did not determine mtDNA levels or TMRE staining at 4 h.

**MCAs 48 h after tMCAO**

The diameters of IPSI MCAs were larger when initially visualized following cannulation compared with CONTRA and CONTROL MCAs (250±4 μm vs. 233±6 μm and 231±6 μm, respectively) (n=22 in each group). After pressurization to 70 mmHg and a 60 min equilibration period, the IPSI MCAs developed less myogenic tone than the CONTRA and CONTROL MCAs (29±3 % vs. 35±2 % and 39±3 %, respectively, p<0.05) (n=22 in each group) (Figure 1D).

In IPSI MCAs, the responses to Ach, BK, 5-HT, and SNP (3±2 μm, 27±4 μm, -35±5 μm, and 37±3 μm, respectively, n=22, p<0.05) were significantly decreased compared with the CONTRA or CONTROL responses (Figure 1A-C,E). In addition, the CONTRA MCAs responses (Ach: 11±2 μm, BK: 41±6 μm, 5-HT: -62±6 μm, SNP: 76±7 μm, n=22, p<0.05) were significantly decreased compared with CONTROL (Ach: 21±3 μm, BK: 82±7 μm, 5-HT: -85±10 μm, SNP: 80±8 μm, n=22) for all of the drugs administered except SNP.

Despite the overall reduction of responsiveness of the IPSI MCAs to other drugs, DZ elicited a significantly larger vasodilation in IPSI MCAs to all doses compared with
CONTRA MCAs and the IPSI MCA responses to 50 and 100 μmol/L of DZ were similar to those of CONTROL MCAs (IPSI: 8±2 μm, 23±3 μm, 39±5 μm; CONTRA: -5±2 μm, 6±2 μm, 20±3 μm; CONTROL: -2±4 μm, 21±6 μm, 43±7 μm, for 10, 50, and 100 μmol/L of DZ, respectively, n=22 in each group, p<0.05) (Figure 2A). Reduced vasodilation was observed in the CONTRA MCAs at 50 and 100 μmol/L of DZ compared with CONTROL arteries. Diazoxide-induced responses to 10, 50, and 100 μmol/L were decreased in the presence of 1 mmol/L of 5-hydroxydecanoate in IPSI MCAs (4±6 μm, 4±11 μm, and -6±13 μm, respectively, n=3, p<0.05), demonstrating the involvement of mitoK<sub>ATP</sub> channels (data not shown).

Responses to Ach and BK were eliminated following denudation of the endothelium (data not shown). The endothelium denuded MCAs had a diminished vasodilation in response to all doses of DZ in IPSI arteries and for 50 and 100 μmol/L of DZ in CONTRA MCAs. However denudation did not fully eliminate dilation in the CONTROL group (IPSI: -4±3 μm, 2±3 μm, 0±3 μm; CONTRA: -3±1 μm, -2±2 μm, 8±3 μm; CONTROL: 6±5 μm, 11±4 μm, 16±4 μm; for 10, 50, and 100 μmol/L of DZ, respectively; n=11 in each group, p<0.05) (Figure 2B).

Co-treatment of L-NAME with DZ to endothelium intact arteries resulted in a significantly reduced vasodilation in IPSI and CONTROL MCAs to 50 and 100 μmol/L of DZ, although there was no significant decrease in the responses of the CONTRA MCAs to DZ (IPSI: 5±3 μm, 7±3 μm, 20±6 μm; CONTRA: -3±2 μm, 1±3 μm, 13±5 μm; CONTROL: -2±4 μm, 2±2 μm, 19±4 μm; for 10, 50, and 100 μmol/L of DZ, respectively; n=11 in each group, p<0.05) (Figure 2C).
All mitochondrial protein levels examined were significantly (p<0.05) increased in the IPSI MCAs including voltage dependent anion channel (VDAC) (77±20 %), Complex V subunit α (227±22 %), DRP-1 (309±24 %), and pDRP-1 (131±40 %) when compared with the CONTRA (VDAC: 33±5 %, Complex V: 119±19 %, DRP-1: 101±11 %, and pDRP-1: 44±13 %) and CONTROL (VDAC: 55±3 %, Complex V: 96±12 %, DRP-1: 103±10 %, and pDRP-1: 34±10 %) arteries (Figure 3A-D). Levels of total eNOS (IPSI: 106±22 %, CONTRA: 97±19 %, CONTROL: 82±15 %) were unchanged (Figure 4E), however, peNOS expression level was significantly increased in IPSI (peNOS: 73±22 %, p<0.05) compared with CONTRA (peNOS: 21±6) and CONTROL (peNOS: 27±4 %) MCAs (Figure 3F). In each group we used 33 MCAs to perform the Western blot experiments.

The basal IPSI TMRE intensity (65±0.5 %, p<0.05, n=4) in a standard field of observation following tMCAO was increased compared with CONTRA intensity (56±0.4 %, n=4). Furthermore, DZ significantly decreased the TMRE intensity in all groups (IPSI: 48±0.3 %, CONTRA: 54±0.4 %, and CONTROL: 86±0.8 %, p<0.05) indicating mitochondrial membrane depolarization but the decrease in TMRE intensity was significantly greater for the IPSI compared with CONTRA arteries (Figure 4A-B).

Ischemia-reperfusion resulted in a significantly increased mtDNA to nuclear DNA ratio in the IPSI (102±16 %, p<0.05) compared with CONTRA MCAs (44±2 %) (n=20 in
each group) normalized to the CONTROL MCAs (Figure 5). However there was no
significant difference between the IPSI and CONTROL groups. The mtDNA to nuclear
DNA ratio in CONTRA MCAs was significantly decreased compared with CONTROL
MCAs (100%) (Figure 5).

DISCUSSION

There are several new findings from this study. First, contrary to generally held
expectations, we found that mitochondrial-dependent cerebral arterial dilation to DZ was
intact and comparable to CONTROL values on the IPSI side following tMCAO at both 4
and 48 h. Thus, despite a prolonged period of ischemia, overall mitochondrial
responses in cerebral arteries were preserved whereas other, non-mitochondrial dilator
responses, especially at 48 h, were severely impaired. Second, vascular endothelium
appeared to play a dominant role in the preserved mitochondrial derived vasodilation on
the IPSI side at 48 h. Third, preserved MCA dilation to DZ at 48 h on the IPSI side was
associated with strong evidence of maintained or increased mitochondrial mass as well
as preserved mitochondrial depolarization to DZ, as shown by TMRE measurements.
An unexpected finding was that cerebral vascular responsiveness in MCAs on the side
opposite the ischemia was substantially impaired at 48 h, indicating that cerebral
vascular dysfunction extends beyond the ischemic zone. We also found that the
CONTRA MCAs showed decreased responsiveness to all of the agents examined at 48
h when compared with CONTROL arteries. Thus, even in cerebral arteries distant from
previously occluded arteries, vascular dysfunction was present, thereby identifying the
serious limitation of the practice of only comparing IPSI and CONTRA MCAs as in some
stroke studies. These results clearly illustrate the diversity of responses to ischemic stress that occur in different segments of the cerebral vasculature and provide very strong support for the concept that mitochondria, probably specific to the endothelium, represent a novel therapeutic target for treatment of stroke patients, especially when clot resolution is delayed beyond the conventional 3 h.

The relatively large cerebral arteries, including the MCAs, play an important role in maintaining cerebral blood flow and contributing to changes in myogenic activity to diverse stimuli under normal and pathological conditions (3, 30). We found that under basal conditions, the IPSI MCAs had a significantly increased vascular diameter compared with the CONTRA and CONTROL MCAs and a significantly impaired myogenic response 48 h after tMCAO to 70 mmHg intravascular pressure. At this juncture, severe vascular dysfunction in the form of reduced MCA responsiveness occurred to several dilator or constrictor agents on the IPSI side. Our results are generally, but not totally, in agreement with those of previous investigators who showed variable effects on cerebral vascular responsiveness to various stimuli at various times after tMCAO (7-9, 27, 29). The variability of reported results is probably due to the use of different techniques, species, and protocols. For example, we show that despite the same duration of tMCAO, dilator and constrictor responses of IPSI MCAs to all examined stimuli were largely intact at 4 h but severely impaired at 48 h.

We appear to be among the first to show prominent impaired vasodilator and vasoconstrictor responses of CONTRA MCAs that were never occluded compared with CONTROL MCAs from normal rats. Our finding suggests that transient ischemic stress produces endothelial and VSM dysfunction even in cerebral arteries which are
somewhat distant from occluded arteries. During our review of the literature, we found only limited reports of cerebral vascular dysfunction on the CONTRA side following tMCAO since responses in other studies were not normally compared to sham operated, CONTROL arteries. The reasons for the relative vascular dysfunction on the CONTRA side are unclear at this time and deserve systematic investigation since similar effects may be present in non-occluded arteries in people suffering from a stroke and thus may affect the extent of functional vascular and neuronal recovery.

We elicited endothelium dependent dilator responses using Ach or BK and the relaxation responses of MCAs to these agents were greatly reduced 48 h following tMCAO on the IPSI side as were endothelium independent dilator and constrictor responses to SNP and 5-HT, respectively. Therefore, we expected IPSI MCA responses to DZ to be similarly impaired since dilation to this stimulus was previously found to be due to contributions from both endothelium and VSM (23). In addition, in an earlier report we found that dilation to DZ was impaired in another disease condition: insulin resistance (22). Consistent with our previous studies in normal animals, DZ is a potent vasodilator in CONTROL MCAs and endothelium denudation and L-NAME treatment attenuated but did not block dilation of cerebral arteries to DZ (12, 23). Surprisingly, DZ-induced dilation in IPSI MCAs was maintained at similar levels as CONTROL arteries at not only 4 h, but also 48 h after tMCAO, rather than as dual contributions from VSM and endothelium to DZ, as seen in CONTROL arteries and reported previously (16). Thus, the removal of endothelium virtually eliminated MCA dilation to DZ at all doses examined. Similarly, inhibition of endothelium-dependent dilator stimuli by the administration of a NOS inhibitor caused a significant decrease in the responses of
MCAs to DZ. Taken together, the data imply that the endothelium is the primary determinant of retained or enhanced dilation to DZ following IR, but also indicate that its role is complex. For example, L-NAME administration might inhibit both coupled and uncoupled eNOS, such that both dilator (NO) and constrictor (superoxide anion) influences from endothelium may be altered. In the IPSI MCAs at 4 h after tMCAO, there is a tendency for enhanced dilation to DZ with L-NAME treatment, possibly implicating a role of uncoupled eNOS during this time period. Nonetheless, an increase in peNOS in IPSI MCAs provides further support for an augmented role of endothelium in mediating DZ-induced dilation following tMCAO and may also demonstrate the basis for an amplification of mitochondrial-initiated signaling. The endothelium is the most accessible component of the cerebral circulation for the systemic administration of drugs and other agents such as stem cells and thus is a potential target for new stroke therapies (15, 41).

Supporting our vascular reactivity studies with DZ, we found evidence of maintained or increased mitochondrial mass in IPSI arteries after tMCAO. First, the expression level of the highly conserved VDAC, one of the major proteins in mitochondrial mediated apoptosis, was increased in the IPSI compared with the CONTRA and CONTROL MCAs. The VDAC is located in the outer mitochondrial membrane that provides transport pathways for respiratory chain substrates required for oxidative phosphorylation (21, 25, 33, 44). Our results are supported by results from other studies (33, 44). Furthermore, we found that the protein levels of the Complex V subunit α, located on the inner mitochondrial membrane, were significantly increased following ischemia-reperfusion in IPSI MCAs compared with CONTROL and CONTRA
arteries. Complex V produces ATP from ADP via phosphorylation in the presence of
the respiratory chain’s electron transport generated proton gradient across the inner
mitochondrial membrane (35). These findings concerning VDAC and Complex V
subunit $\alpha$ are in agreement with our previous findings on rat primary cortical neurons
following OGD (40). However, there was no difference between the protein levels in the
CONTRA and control groups. One possible cause of the increased ATP synthase
expression may be a required amplified function in order to restore oxidative
phosphorylation, which is a time-dependent mechanism. The increased levels of VDAC
and Complex V support the concept that total mitochondrial mass is increased after
transient ischemia due to mitochondrial biogenesis. Corresponding with evidence of
increased mitochondrial mass were elevated levels of fission proteins DRP-1 and
pDRP-1 in IPSI MCAs (2, 40). Our second level of evidence of maintained or increased
mitochondrial mass in IPSI arteries after tMCAO is that mtDNA levels were higher in the
IPSI compared with CONTRA MCAs. Similarly, Yin et al. (43) showed increased mtDNA
and number of mitochondria in the brain 24 h after hypoxic-ischemic brain injury. We
have shown similar findings in cultured neurons following OGD (40). Third, we observed
a greater intensity of the mitochondrial specific dye TMRE fluorescence in IPSI
compared with CONTRA MCAs. Evaluation of TMRE responses to DZ indicated a
greater decrease in the IPSI mitochondrial membrane potential compared with
CONTRA arteries, thereby providing information independent of vascular
responsiveness that the mitochondria retained normal functionality. However, DZ
caused a similar decrease in the IPSI and CONTROL membrane potentials, which
reflects our vascular data where DZ induced vasodilation is similar in IPSI and
CONTROL MCAs. The greater amount of TMRE staining in CONTROL than in IPSI MCAs may indicate that although mitochondrial proteins and perhaps mitochondrial numbers are augmented following tMCAO, all mitochondria, especially in VSM, might not be fully functioning. Nonetheless, increased biosynthesis of mitochondria, as demonstrated by increased mitochondrial proteins and mtDNA as well as the remodeling protein pDRP-1, together with enhanced levels of activated eNOS, appears to be sufficient for preservation of MCA responses to DZ 48 h after tMCAO.

To our knowledge, this is the first study to report preserved mitochondria derived vascular responses in the ischemic side and diminished responses in the contralateral side in MCAs after 90 min of ischemia and 4 and 48 h of reperfusion. Our results support the concept that the endothelium exerts a dominant compensatory role in maintaining mitochondrial function after prolonged, transient cerebral ischemia. Although the underlying mechanisms are not totally clear, these results may contribute to a greater understanding of the ongoing changes after ischemic stress as well as aid in the development of potential therapies to help people suffering from strokes. Thus, targeting mitochondria in the immediate post-ischemic period may not only improve cerebral blood flow, but may also protect the neurovascular unit from further damage and cell death and thereby decrease stroke related morbidity and mortality. In addition, our finding that both previously occluded as well as non-occluded arteries were affected differently by tMCAO indicates that distinct therapeutic approaches are needed to preserve cerebral vascular function following strokes.
ACKNOWLEDGMENTS

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Disclosures: None.
REFERENCES


Figure 1. Vascular reactivity at 4 and 48 h following tMCAO. CONTROL MCAs showed prominent dilation to Ach (A), BK (B) and SNP (C) as well as constriction to 70 mmHg intraluminal pressure (D) and 5-HT (E). Transient MCAO did not significantly affect these responses at 4 h in either IPSI or CONTRA MCAs (n=10 in each group). However, dilator response to Ach, BK, and SNP as well as constrictor responses were significantly decreased in IPSI arteries at 48 h compared to either CONTROL or CONTRA arteries. CONTRA MCAs also showed decreased dilation to Ach and BK as well as 5-HT compared to CONTROL arteries. p<0.05, * 48 h IPSI vs. 48 h CONTRA, † 48 h IPSI vs. CONTROL, ‡ 48 h CONTRA vs. CONTROL (n=22 in each group). Data are expressed as mean±s.e.m.

Figure 2. Mitochondrial activation at 48 h following tMCAO of MCAs with intact endothelium, after endothelium denudation, and in the presence of nitric oxide synthase inhibitor, L-NAME. (A) Diazoxide caused a dose-dependent dilation in CONTROL arteries. Diazoxide-induced dilation was significantly enhanced in 48 h IPSI arteries compared with 48 h CONTRA. The 48 h CONTRA responses were significantly decreased to 50 and 100 µM of DZ compared with CONTROL. p<0.05, * 48 h IPSI vs. 48 h CONTRA, † 48 h IPSI vs. CONTROL, ‡ 48 h CONTRA vs. CONTROL (n=22 in each group). (B) Denudation of endothelium eliminated the 48 h IPSI MCAs responses to all DZ concentrations. The 48 h CONTRA group also showed significantly decreased responses to 50 and 100 µM of DZ. Denudation of endothelium significantly decreased only the 100 µM of DZ induced dilation in the CONTROL group. p<0.05, * intact
endothelium vs. endothelium denuded, (n=11). Data are expressed as mean±s.e.m. (C)

Administration of L-NAME decreased the DZ induced dilation in all groups, p<0.05, *
intact endothelium vs. in the presence of L-NAME (n=11). Data are expressed as
mean±s.e.m.

Figure 3. Protein expression 48 h following tMCAO. Representative Western blots
and summary data of: (A) 32 kDa VDAC, (B) 46.9 kDa Complex V subunit α, (C) the
82.8 kDa mitochondrial fission protein DRP-1, (D) pDRP-1, (E) 140 kDa total eNOS,
and (F) peNOS. Histograms showing that all of the mitochondrial proteins and peNOS
levels were significantly increased in 48 h IPSI MCAs compared with 48 h CONTRA and
CONTROL. p<0.05, * 48 h IPSI vs. 48 h CONTRA, † 48 h IPSI vs. CONTROL, ‡ 48 h
CONTRA vs. CONTROL (n=33 in each group). Data are expressed as mean±s.e.m.

Figure 4. TMRE staining 48 h following tMCAO. (A) Representative images of TMRE
loaded MCA sections and (B) summary data showing treatments with DMSO (vehicle),
DZ, and FCCP. Ischemia-reperfusion resulted in a decrease in the TMRE staining
compared with CONTROL MCAs. However, the TMRE staining in IPSI MCAs was
significantly increased compared with CONTRA arteries. Diazoxide depolarized the
mitochondria producing a significant decrease in the TMRE fluorescence, which had a
similar magnitude in IPSI compared with CONTROL. Although statistically significant,
the decrease in TMRE fluorescence in the CONTRA MCAs was small. p<0.05, * 48 h
IPSI vs. 48 h CONTRA, † 48 h IPSI vs. CONTROL, ‡ 48 h CONTRA vs. CONTROL, §
DZ treatment vs. baseline (n=4). Data are expressed as mean±s.e.m.
Figure 5. Increased mtDNA 48 h following tMCAO. Graph showing a significantly larger mtDNA expression in IPSI MCAs following tMCAO compared with CONTRA MCAs. Levels of mtDNA were similar in the IPSI and CONTROL MCAs p<0.05, * 48 h IPSI vs. 48 h CONTRA, ‡ 48 h CONTRA vs. CONTROL (n=20 in each group). Data are expressed as mean±s.e.m.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

A

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B

- **CONTROL**
- **48 h CONTRA**
- **48 h IPSI**

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Figure 4.
Figure 5.