TRPC-mediated actin-myosin contraction is critical for BBB disruption following hypoxic stress

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Hicks K, O’Neil RG, Dubinsky WS, Brown RC. TRPC-mediated actin-myosin contraction is critical for BBB disruption following hypoxic stress. Am J Physiol Cell Physiol 298: C1583–C1593, 2010. First published February 17, 2010; doi:10.1152/ajpcell.00458.2009.—Hypoxia-induced disruption of the blood-brain barrier (BBB) is the result of many different mechanisms, including alterations to the cytoskeleton. In this study, we identified actin-binding proteins involved in cytoskeletal dynamics with quantitative proteomics and assessed changes in subcellular localization of two proteins involved in actin polymerization [vasodilator-stimulated phosphoprotein (VASP)] and cytoskeleton-plasma membrane cross-linking (moesin). We found significant redistribution of both VASP and moesin to the cytoskeletal and membrane fractions of BBB endothelial cells after 1-h hypoxic stress. We also investigated activation of actin-myosin contraction through assessment of phosphorylated myosin light chain (pMLC) with confocal microscopy. Hypoxia caused a rapid and transient increase in pMLC. Blocking MLC phosphorylation through inhibition of myosin light chain kinase (MLCK) with ML-7 prevented hypoxia-induced BBB disruption and relocation of the tight junction protein ZO-1. Finally, we implicate the transient receptor potential (TRPC) family of channels in mediating these events since blockade of TRPC channels and the associated calcium influx with SKF-96365 prevents hypoxia-induced permeability changes and the phosphorylation of MLCK needed for actin-myosin contraction. These data suggest that hypoxic stress triggers alterations to cytoskeletal structure that contribute to BBB disruption and that calcium influx through TRPC channels contributes to these events.

Breakdown of the blood-brain barrier (BBB) contributes to edema formation, infarct size, and brain damage following ischemic stroke (3, 10, 36, 39). There are many factors contributing to BBB disruption in ischemia, including generation of oxygen radicals (24, 56, 58), nitric oxide (22, 42), production of vascular endothelial growth factor (67, 69), and changes in intracellular calcium (5, 26, 35). Under normal conditions, the integrity of the BBB is maintained by tight junction complexes between adjacent brain capillary endothelial cells (2). Changes in BBB permeability are correlated with changes in tight junction structure from ischemic stroke (33, 45, 51, 59). After hypoxia, this increased permeability is associated with disruptions in the subcellular localization of the tight junction proteins zonula occludens-1 (ZO-1) (43) and occludin (5).

A major contributing factor to disruption of the BBB after hypoxia is contraction of the actin-myosin cytoskeleton (23). A recent study demonstrated that inhibition of actin-myosin contraction protected the BBB after hypoxic stress (34); hypoxia-induced BBB disruption was prevented by inhibition of myosin light chain kinase (MLCK), by inhibition of NADPH-oxidase, or by chelation of intracellular calcium. These protective effects were correlated with a decrease in the amount of phosphorylated myosin light chain (pMLC) detected by immunofluorescence. However, this study did not investigate the effects of these protective treatments on tight junction structure, which is critical to BBB functional integrity. Furthermore, the reported increases in permeability were detected by measuring changes in transendothelial electrical resistance, which may represent a very small change in barrier tightness and not translate to increased permeability of solutes larger than ions. Activation of MLCK requires an increase in intracellular calcium and subsequent binding of calcium to calmodulin (50). Pathways for calcium entry in BBB endothelial cells have not been well characterized but may include various transporters (30, 38) and members of the transient receptor potential (TRPC) family of cation-permeable channels. There are seven families of TRP channels, three of which have been found in BBB endothelial cells (4). Of these families, the TRPC family is the best candidate for mediating calcium influx following hypoxic stress; the Drosophila TRPC homolog trp is activated by anoxia (1), and hypoxic stress can increase the expression of the TRPC4 channel isoform in human pulmonary artery endothelial cells (14). Furthermore, lung endothelial cells from TRPC4-knockout mice show blunted responses to disrupting stimuli, implicating TRPC-mediated calcium influx in barrier disruption (60).

We hypothesized that calcium influx through TRPC channels on BBB endothelial cells contributes to MLCK activation, MLC phosphorylation, and alterations in BBB permeability following hypoxic stress. We utilized quantitative proteomics to investigate relative expression of cytoskeleton-associated proteins after hypoxia, including MLCK, MLC, actin, moesin, and vasodilator-stimulated phosphoprotein (VASP). We investigated changes in the subcellular localization of actin, moesin, and VASP as well as the effect of MLCK inhibition on MLC phosphorylation, BBB permeability, and tight junction structure after hypoxic stress. Finally, we used SKF-96365, an inhibitor of the cation-permeable TRPC channels, to block calcium influx through these channels and to prevent MLC phosphorylation and BBB disruption after hypoxia.

Materials and Methods

Chemicals and antibodies. DMEM, fetal bovine serum, penicillin-streptomycin, TRIZol, tetramethylrhodamine-wheat germ agglutinin (Molecular Probes), and mouse anti-GAPDH were from Invitrogen (Carlsbad, CA). Mouse anti-actin was from Sigma (St. Louis, MO). The iTRAQ isobaric tag system was from Applied Biosystems (Foster City, CA). Complete MINI EDTA-free protease inhibitors were from Roche Applied Science (Indianapolis, IN). Rabbit anti-VASP, anti-moesin, and anti-pMLC were from Cell Signaling Technologies.
Proteomics. Differential expression of proteins in cells exposed to hypoxia (1% O2, 1, 3, or 6 h) was determined by mass spectrometry (MS) using the iTRAQ system of isotopic labeling for the identification and quantitation of proteins (20, 21, 68). In brief, 100-μg protein mixtures from each treatment group (control, 1, 3, and 6 h hypoxia) were precipitated with acetone. The precipitate was denatured and the mixtures from each treatment group (control, 1, 3, and 6 h hypoxia) were labeled with isobaric tags (iTRAQ system), and relative expression of proteins each time it was performed, was quite variable in the degree of change seen between data sets. Therefore we also used in-cell Western blot (ICWB) analysis to confirm changes seen in our proteomic data sets (data not shown). Actin levels as detected by iTRAQ were slightly elevated after 1 h of hypoxia, then fell at 3 and 6 h. Moesin, vasodilator-stimulated phosphoprotein (VASP), and myosin light chain (MLC) were unchanged in iTRAQ analysis. Data are presented as means ± SE for 3 iTRAQ experiments. A significant change was only found for actin levels after 1 h of hypoxia by one-way analysis of variance. *P < 0.05, ***P < 0.001 vs. control.

Table 1. Expression of proteins involved in cytoskeletal contraction and/or actin remodeling following hypoxic stress

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession No.</th>
<th>Average Expression (1 h)</th>
<th>Average Expression (3 h)</th>
<th>Average Expression (6 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Actinin</td>
<td>P62737</td>
<td>1.45 ± 0.11†</td>
<td>0.74 ± 0.03*</td>
<td>0.93 ± 0.04</td>
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<tr>
<td>α1-Actinin</td>
<td>Q77PR4</td>
<td>1.25 ± 0.28</td>
<td>0.89 ± 0.05</td>
<td>1.25 ± 0.28</td>
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<td>α4-Actinin</td>
<td>P57780</td>
<td>0.90 ± 0.04</td>
<td>0.90 ± 0.09</td>
<td>0.90 ± 0.03</td>
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<tr>
<td>ARP3</td>
<td>Q99Y9</td>
<td>1.33 ± 0.34</td>
<td>1.09 ± 0.36</td>
<td>1.03 ± 0.35</td>
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<tr>
<td>Collin-1</td>
<td>P168760</td>
<td>0.95 ± 0.03</td>
<td>0.99 ± 0.07</td>
<td>0.93 ± 0.05</td>
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<td>Moesin</td>
<td>P26041</td>
<td>0.98 ± 0.03</td>
<td>1.01 ± 0.03</td>
<td>0.99 ± 0.04</td>
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<tr>
<td>MHC9</td>
<td>Q8VDD5</td>
<td>1.08 ± 0.27</td>
<td>0.94 ± 0.13</td>
<td>0.94 ± 0.17</td>
</tr>
<tr>
<td>MHC10</td>
<td>Q61879</td>
<td>0.97 ± 0.34</td>
<td>0.79 ± 0.08</td>
<td>0.84 ± 0.18</td>
</tr>
<tr>
<td>MLC6</td>
<td>Q60605</td>
<td>1.10 ± 0.26</td>
<td>0.94 ± 0.16</td>
<td>0.95 ± 0.13</td>
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<tr>
<td>MLC9</td>
<td>Q9CQ19</td>
<td>1.85 ± 0.66</td>
<td>1.30 ± 0.36</td>
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<tr>
<td>Paxillin</td>
<td>Q8V136</td>
<td>0.97 ± 0.03</td>
<td>0.77 ± 0.05</td>
<td>0.94 ± 0.29</td>
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<tr>
<td>Protilin-1</td>
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<td>1.17 ± 0.23</td>
<td>1.20 ± 0.16</td>
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<td>RhoA</td>
<td>Q8XU0</td>
<td>0.89 ± 0.07</td>
<td>0.88 ± 0.05</td>
<td>0.88 ± 0.07</td>
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<tr>
<td>ROCK</td>
<td>P70356</td>
<td>0.88 ± 0.04</td>
<td>0.82 ± 0.16</td>
<td>0.84 ± 0.14</td>
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<tr>
<td>α-Spectrin</td>
<td>P16546</td>
<td>1.14 ± 0.13</td>
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<td>β-Spectrin</td>
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<tr>
<td>Talin-1</td>
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<td>0.94 ± 0.08</td>
<td>0.93 ± 0.07</td>
<td>0.94 ± 0.07</td>
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<td>Thymosin-β4</td>
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<td>1.48 ± 0.49</td>
<td>1.42 ± 0.37</td>
<td>1.14 ± 0.22</td>
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<td>Tropomyosin4</td>
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<td>0.94 ± 0.06</td>
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<td>0.98 ± 0.02</td>
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<td>VASP</td>
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<td>1.12 ± 0.23</td>
<td>0.96 ± 0.09</td>
<td>1.17 ± 0.11</td>
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<td>Vinculin</td>
<td>Q6Q277</td>
<td>0.98 ± 0.01</td>
<td>0.98 ± 0.02</td>
<td>0.95 ± 0.004</td>
</tr>
<tr>
<td>Zyxin</td>
<td>Q62523</td>
<td>0.87 ± 0.07</td>
<td>0.86 ± 0.14</td>
<td>0.87 ± 0.10</td>
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</tbody>
</table>

Data are means ± SE for 3 separate labeling experiments. Protein extracts were labeled with isobaric tags (iTRAQ system), and relative expression (normalized to control expression) was determined. ARP3, actin-related protein 3; MHC, myosin heavy chain; MLC, myosin light chain; RhoA, Ras homolog gene family A; ROCK, Rho kinase; VASP, vasodilator-stimulated phosphoprotein. *P < 0.05, †P < 0.001 vs. control.
with methyl methane-thiosulfonate, and trypsin was added to the mixture to a protein-to-trypsin ratio of 10:1 to generate a protein digest. The mixture was incubated overnight at 37°C, and the protein digests were labeled by mixing with the appropriate iTRAQ reagent. Desalted and concentrated peptide mixtures were quantified and identified by nano-LC tandem MS (MS/MS) on a QSTAR Elite mass spectrometer (ABS Sciex Instruments) operating in positive ion mode. Peptides were loaded on a 75-μm × 10-cm, 3-μm fused silica C18 capillary column, followed by mobile phase elution: buffer A (0.1% formic acid in 2% acetonitrile, 98% Milli-Q water) and buffer B (0.1% formic acid in 98% acetonitrile, 2% Milli-Q water). The peptides were eluted in a gradient from 2% buffer B to 30% buffer B over 180 min at a flow rate of 250 nl/min. The LC eluent was directed to a NanoES source for electrospray ionization (ESI)/MS/MS analysis. With information-dependent acquisition, peptides were selected for collision-induced dissociation by alternating between an MS (1 s) survey scan and MS/MS (3 s) scans. Accumulated MS/MS spectra were analyzed by ProteinPilot software (Applied Biosystems, Foster City, CA) with the SwissProt fasta database for protein identification. The ProGroup reports were generated with a 95% confidence level for protein identification. Proteins that were consistently altered from experiment to experiment were investigated further.

Subcellular fractionation and immunoblotting. Differential detergent fractionation was used to isolate enriched fractions of cytoplasm, membranes, and cytoskeleton (11) from cells exposed to control conditions (normoxia) or hypoxic stress (1% O2) for the indicated periods of time. In brief, after being washed in ice-cold PBS, confluent monolayers of bEnd3 cells were incubated with digitonin-EDTA extraction buffer (in mM: 10 PIPES pH 6.8, 300 sucrose, 100 NaCl, 3.0 MgCl2, and 5.0 EDTA, with 0.01% digitonin) for 10 min on ice with gentle agitation. The supernatant, containing cytoplasmic proteins, was removed and spun to pellet any detached cells. Resulting supernatant was stored at −80°C (cytosolic fraction). The remaining cellular material in the dish was then extracted with Triton X-100-EDTA extraction buffer (in mM: 10 PIPES pH 7.4, 300 sucrose, 100 NaCl, 3.0 MgCl2, and 3.0 EDTA, with 0.5% Triton X-100) for 10 min on ice with agitation. The supernatant containing the membrane/organelle fraction was removed, and any detached cells were pelleted and removed. Supernatant was stored at −80°C until further use (membrane fraction).

Cellular material still remaining on the plate after the digitonin-EDTA extraction were then extracted with Tween 40-deoxycholate extraction buffer (in mM: 10 PIPES pH 7.4, 10 NaCl, and 1.0 MgCl2, with 1.0% Tween 40 and 0.5% deoxycholate) to isolate nuclear proteins. Plates were incubated with buffer for 10 min on ice with vigorous agitation, the supernatant was removed and spun, and the resulting cell-free supernatant was stored at −80°C (nuclear fraction).

Finally, the insoluble cytoskeletal fraction was extracted by scraping the remaining cellular debris into 6 M urea buffer (in mM: 6,000 urea, 10 Tris, 1 DTT, 5 MgCl2, 5 EGTA, and 150 NaCl pH 8.0). All

![Graphs showing VASP and moesin expression in cytoplasm, membrane, and cytoskeleton fractions](http://ajpcell.physiology.org/)

Fig. 2. Subcellular localization of moesin and VASP is altered after exposure to hypoxic stress. We used differential detergent fractionation and conventional immunoblotting to assess the distribution of VASP and moesin after exposure to hypoxia (see MATERIALS AND METHODS). VASP levels in the cytoplasm were not significantly altered from normoxic levels after either 1- or 6-h hypoxia, although there was a significant decrease in cytoplasmic VASP between the 1-h and 6-h hypoxia groups (**P < 0.01 vs. 1-h hypoxia). VASP associated with the plasma membrane and the insoluble cytoskeletal fraction was significantly increased after 1-h hypoxia (**P < 0.001 and *P < 0.05, respectively) and dropped back to control levels at 6 h. Similarly, cytoplasmic levels of moesin were not altered, but levels of moesin associated with the membrane and cytoskeleton were significantly increased at 1 h (**P < 0.01 and ***P < 0.001, respectively). The 6-h levels were similar to control values. These data suggest an early and robust alteration in blood-brain barrier (BBB) endothelial cell cytoskeletal dynamics and structure that may contribute to barrier disruption. Data are presented as means ± SE for 3 fractionation studies.
BBB CYTOSKELETAL CONTRACTION POSTHYPOXIA REQUIRES TRPC

phospho-Myosin light chain (pMLC)  WGA-TR  Merged (plus nuclei)

Control (normoxia)

15 min hypoxia

30 min hypoxia

![Graph showing pMLC fluorescence/membrane fluorescence over time: Control, 15 min, 30 min, 60 min, 180 min, 360 min.](http://ajpcell.physiology.org/)

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fractions were suspended in 6 M urea buffer before protein quantification. The relative proportion of proteins in each enriched fraction was ~35–40% cytosolic, 30–35% membrane, 5–10% nuclear, and 15–25% cytoskeletal. Protease inhibitors were included in all extraction buffers and final 6 M urea buffer used for resuspending protein pellets.

Protein samples (10–20 μg) were separated by electrophoresis on 4–20% gels at 125 V for 75–90 min. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and were incubated with primary antibody in Odyssey blocking buffer overnight at 4°C. IRDye-conjugated secondary antibody (1:2,000–1:10,000) was applied for 30 min–1 h at room temperature. Protein bands were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification of band density was performed with Scion Image software [National Institutes of Health (NIH), Bethesda, MD].

Western blotting. We used both conventional and in-cell (ICWB) Western blotting to determine changes in protein expression. For ICWB, cells were grown in 96-well plates and processed according to the manufacturer’s instructions (LI-COR Biosciences). In brief, after exposure to hypoxic stress (1% O₂) for the indicated periods of time, cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature. Cells were washed and permeabilized before blocking and incubation with primary antibodies (anti-VASP, anti-moesin, anti-MLC, 1:500–1:1,000 dilution; anti-actin and anti-GAPDH, 1:1,000) in Odyssey blocking buffer overnight at 4°C. IRDye-conjugated secondary antibody (1:2,000–1:10,000) was applied for 1 h at room temperature. Plates were scanned with the Odyssey Infrared Imaging System. Fluorescent intensities for two channels (1 for protein of interest, 1 for GAPDH normalization) were corrected for background (wells with antibody but no cells), and fluorescence for proteins of interest was normalized using GAPDH fluorescence as a measure of cell density.

For conventional Western blotting, protein samples were run on 4–20% SDS-PAGE gels, transferred to PVDF membrane, and blocked with Odyssey blocking buffer. Primary antibody (1:500–1:1,000 dilution) was incubated with the membranes overnight at 4°C, and IRDye-conjugated secondary antibody (1:2,000–1:10,000) was applied for 1 h at room temperature. Membranes were scanned and quantified with Scion Image software (NIH). Confocal microscopy. bEnd3 cells were grown to confluence on permeable Transwell filters or glass slides. After treatment, cells were incubated in tetramethylrhodamine-wheat germ agglutinin (1:200) for 30–40 min at 4°C to label cell membranes. Cells were then fixed in 3.7% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.1% Triton X-100, cells were incubated with anti-ZO-1 (1:200) in PBS with 10% donkey serum for blocking. Cells were washed, incubated with Cy2-labeled donkey anti-rabbit or antimouse secondary antibody, and mounted in antifade medium containing DAPI nuclear counterstain. Fixed cells were imaged with a Zeiss LSM 510 META confocal microscope with a ×63 oil immersion objective in the multiscanning mode. Excitation wavelengths were set at 488 nm (argon laser) and 543 nm (HeNe laser), and emission wavelengths were 505–530 nm and >560 nm for Cy2 and tetramethylrhodamine, respectively. pMLC confocal images were analyzed with Adobe Photoshop. Pixel intensities in the green (pMLC) and red (tetramethylrhodamine–wheat germ agglutinin, membrane) channels were determined for each individual slice in each stack. The total green channel intensity per stack was normalized to the total red channel intensity, and three stacks were averaged for each treatment.

Permeability. Paracellular solute permeability studies were performed with [¹⁴C]sucrose to determine paracellular diffusion across confluent bEnd3 monolayers. [¹⁴C]sucrose is a relatively impermeant marker at the BBB because of its size (mol wt 342); it is largely impermeant across the tight junctions between endothelial cells under normal conditions but will diffuse across the BBB when cell-cell junctions are disrupted. Apical-to-basolateral diffusion was determined by dividing the picoforms of radioactive marker appearing in the receiver chamber by the time in minutes (6, 7). The apparent permeability coefficient was calculated with the equation Pm (cm²/min) = [{volume/(SA × CD)} × (CR/time)], where volume is the volume of medium in the receiving chamber, SA is the specific activity of the radioactive marker, CD is the initial donor concentration of radioactive marker, and CR is the concentration of the radioactive marker in the receiving chamber at a specific time.

Statistics. All data are expressed as means ± SE. Statistical analysis was performed with Sigma Stat 2.03 (SPSS, Chicago, IL), with a significance level set at *P < 0.05. Data were analyzed with one-way analysis of variance (ANOVA) or Student’s t-test, with post hoc tests as appropriate.

RESULTS

Global changes in BBB proteome following hypoxic stress. To identify broad changes in BBB protein expression after hypoxic stress, we used iTRAQ labeling and MS/MS to identify proteins up- or downregulated after 1, 3, or 6 h of hypoxic stress (1% O₂). This experiment was performed three times; 367 proteins were affected in all three experiments, and the resulting fold changes were averaged. Those proteins that were actin-binding and cytoskeleton-associated proteins were compiled (Table 1). We found that, in particular, proteins involved in cytoskeletal contraction [Ras homolog gene family A (RhoA), Rho kinase (ROCK), myosin], anchoring of the cytoskeleton to the plasma membrane (moesin, α-actinin), and actin filament polymerization (VASP, profilin, cofillin-1) were altered by hypoxic stress. However, given the variability between separate experiments, the only protein that was significantly affected was actin, which was significantly upregulated at 1 h (*P < 0.001) and significantly downregulated after 3 h of hypoxia (∗∗∗P < 0.001). This suggested that dynamic regulation of the cytoskeleton could be important in mediating BBB function after hypoxic stress. We chose to focus on one protein from each of the three groups described above (myosin, moesin, and VASP) as well as actin in further studies (Fig. 1).

While the iTRAQ procedure was useful for identifying potential proteins of interest, substantial variability in the degree of change in protein expression was seen from experiment to experiment. We utilized ICWB analysis (13) to confirm the lack of changes in protein expression seen for VASP, moesin, MLC, and actin after hypoxic stress (data not shown). Overall no significant differences were found with either approach in the expression of actin (iTRAQ: *P = 0.914209, ICWB: *P = 0.137590), moesin (iTRAQ: *P = 0.859688, ICWB: *P = 0.234690, VASP (iTRAQ: *P = 0.979841, ICWB: *P = 0.803693), or MLC (iTRAQ: *P = 0.988782, ICWB: *P = 0.229830) in our treatment groups.

Changes in subcellular distribution of moesin and VASP. Since there was no significant change in total protein expres-
sion of moesin or VASP, we used sequential differential detergent fractionation (11) to isolate subcellular fractions from normoxic and hypoxic monolayers to determine whether there were any alterations in the subcellular localization of these proteins. Isolated protein fractions were subjected to conventional Western blot analysis. We utilized 1, 3, and 6 h of hypoxic stress in these studies. The 6 h time point is the condition under which we have found the most consistent and reproducible degree of barrier disruption, but we chose earlier time points to investigate changes that might occur before the functional end point of increased sucrose permeability. There was no significant change in VASP levels in the cytoplasm after hypoxic stress. However, both the membrane and cytoskeletal fractions had increased amounts of VASP after 1 h of hypoxia (Fig. 2, \( P < 0.001 \) and \( P < 0.05 \), respectively); this returned to control levels after 6 h of hypoxic stress. The only change seen after 6 h of hypoxia was a significantly lower distribution of VASP in the cytoplasm in cells exposed to 6-h hypoxic stress compared with those exposed to 1-h hypoxia (Fig. 2, \( P < 0.01 \) vs. 1-h hypoxic stress).

The cytoplasmic distribution of moesin was not significantly increased after 1 h of hypoxic stress but was significantly increased at this time point in both the membrane and insoluble cytoskeletal fractions (Fig. 2, \( P < 0.01 \) and \( P < 0.001 \) vs. normoxic control, respectively). There was no significant alteration in the total protein levels of either VASP or moesin by iTRAQ or ICWB analysis. This therefore suggests subcellular redistribution of these proteins in response to hypoxia within the first hour of hypoxic stress. Taken together, the data in Figs. 2 and 3 suggest an involvement of the actin cytoskeletal polymerization machinery early in hypoxic stress responses; however, these changes have been resolved by the time there is measurable, reproducible disruption of barrier function.

Phosphorylation of myosin light chain after hypoxia. Contraction of the actin-myosin cytoskeleton has been implicated in regulation of BBB function (23). We assessed MLC phosphorylation after hypoxic stress for 15–360 min in our system, using immunofluorescence and confocal microscopy. Confocal microscopy analysis revealed a significant increase in MLC phosphorylation \( [F_{5,17} = 48.976, P < 0.001, 1\text{-way ANOVA}] \) after 15 min of hypoxia (Fig. 3A). This phosphorylation disappeared at 30 min of hypoxia and did not increase significantly for the duration of the hypoxic stress period (Fig. 3B). This indicates a rapid and transient activation of endothelial cell contraction, potentially contributing to later BBB disruption.

Inhibition of actin-myosin contraction protects BBB function after hypoxic stress. To determine the role of actin-myosin contraction in BBB disruption following hypoxia, we treated bEnd3 cells with 1 \( \mu \)M ML-7, a specific MLCK inhibitor, and assessed paracellular permeability, using \([1\text{C}]\text{glucose} \). We found that 6 h of hypoxic stress increased BBB permeability in bEnd3 cells (Fig. 4, \( P < 0.05 \), similar to the disruption seen previously in primary cultures of BBB endothelial cells (7). This disruption was significantly inhibited by treatment with 1 \( \mu \)M ML-7 (1\text{-way ANOVA}, \( F_{5,51} = 3.601, P = 0.020 \)), while ML-7 under normoxic conditions had no significant effect on barrier function. These results indicated that actin-myosin contraction plays a role in BBB disruption after hypoxic stress.

**DISCUSSION**

Stroke causes a disruption of the BBB that contributes to neuronal damage via the development of edema and swelling (28, 57). This barrier disruption is due to the activation of protein kinase C (PKC), leading to phosphorylation of myosin light chain (MLC) and subsequent contraction of the actin-myosin cytoskeleton. Previous studies have shown that PKC activation is a key player in BBB disruption, contributing to increased paracellular permeability. However, the exact mechanisms by which PKC triggers this process are not fully understood.

**Figure 4.** Inhibition of myosin light chain kinase (MLCK) protects against hypoxia-induced BBB disruption. Cells were incubated with and without the MLCK inhibitor ML-7 (1 \( \mu \)M) and exposed to 6-h hypoxia, after which paracellular permeability was assessed as described in text. Hypoxia caused a significant increase in paracellular permeability \( (*P < 0.05 \text{ vs. normoxic control}) \) that was blocked by incubation with ML-7. There was no significant effect of ML-7 on basal permeability. Data are presented as means ± SE for 10–14 measurements.

**Inhibition of actin-myosin contraction protects tight junction structure.** If hypoxia disrupts paracellular BBB permeability, there should be concomitant changes in tight junction structure. We assessed the subcellular localization of ZO-1, as an indicator of tight junction integrity, in bEnd3 cells exposed to hypoxic stress with and without MLCK inhibition. Hypoxia disrupted ZO-1 distribution, leading to largely cytoplasmic staining (Fig. 5) and an apparent decrease in cell-cell contact, reflective of cytoskeletal contraction. This redistribution was completely prevented by MLCK inhibition. These results indicate that actin-myosin contraction contributes to disruption of the tight junction, potentially by removing ZO-1 from the tight junction via its interaction with actin.

**Inhibition of TRPC channels prevents barrier disruption and MLC phosphorylation after hypoxia.** Activation of MLCK is dependent on \( \text{Ca}^{2+} \)-calmodulin binding to the enzyme (62). In previous studies we have shown (4) the presence of functional TRPC and TRPV cation-permeable channels in brain endothelial cells, and we hypothesize that hypoxia-induced calcium influx through these channels contributes to actin-myosin contraction and BBB disruption. Permeability experiments indicate that treatment with a TRPC channel inhibitor, SKF-96365, prevents BBB disruption following 6-h hypoxia in a dose-dependent manner (Fig. 6A, \( P < 0.01 \text{ vs. normoxic control} \), \( P < 0.05 \text{ vs. hypoxia} \)). The protection of barrier function was correlated with a decrease in pMLC at the point of peak pMLC fluorescence in control cells (Fig. 6B, \( P < 0.01 \text{ vs. hypoxic control} \) in cells treated with 1 \( \mu \)M SKF-96365. This indicates that blockade of TRPC channels can prevent both MLC phosphorylation and barrier disruption, presumably by blocking the \( 
\text{Ca}^{2+} \) influx needed to trigger \( \text{Ca}^{2+} \)-calmodulin-mediated activation of MLCK (19) and cytoskeletal contraction.
numerous cellular events, including hypoxia-inducible factor-1α (HIF-1α) (67), production of nitric oxide (42), and calcium influx (6, 26). In this study, we investigated global changes in the BBB endothelial cell proteome after exposure to hypoxic stress to identify proteins potentially contributing to BBB disruption following stroke, using quantitative analysis of a pool of total proteins. This enabled us to identify proteins that were potentially important in mediating BBB endothelial cell responses to hypoxic stress. Our data indicated that hypoxia activates signaling pathways involved in dynamic remodeling of the cytoskeleton, leading to alterations in cell morphology, tight junction structure, and BBB function. We found that two proteins, particularly involved in cytoskeletal dynamics and morphology, were affected by hypoxic stress: moesin and VASP.

Moesin is a member of the ezrin/radixin/moesin (ERM) family of actin-binding proteins and links actin filaments to the plasma membrane (41). Inactive (unphosphorylated) moesin is present in the cytoplasm but translocates to the plasma membrane upon activation by phosphorylation (66). Moesin can be phosphorylated by multiple kinases, including PKC-θ (54), p38 MAPK (31), and RhoA (44). Our data showed that the overall expression of moesin did not change during 6-h hypoxic stress, but subcellular localization of the protein shifted toward the membrane and cytoskeletal fractions. This suggested activation, an increase in binding to F-actin, and an increase in membrane-cytoskeletal linkage. The exact signaling cascade responsible for the putative phosphorylation of moesin in BBB endothelial cells is unknown, but previous studies found activation of p38 MAPK (29, 53) and PKC-θ (16). RhoA is also activated during periods of acute hypoxia, leading to stress fiber formation and increased tight junction permeability (27, 65).

VASP is a member of the Ena/VASP family of proteins and is involved in actin polymerization (32), as well as in the maintenance of endothelial barriers (17). There are several

Fig. 5. Inhibition of actin-myosin contraction protects tight junction structure. Zonula occludens 1 (ZO-1) is a tight junction accessory protein that links actin filaments to the plasma membrane at tight junctions (61). After 6-h hypoxic stress, ZO-1 immunofluorescence moves away from the cell-cell border into the cytoplasm, indicating disruption of the BBB. However, in cells treated with 1 μM ML-7, a MLCK inhibitor, ZO-1 localization at the plasma membrane is preserved after 6-h hypoxia. Representative fluorescent images are shown (×63 objective).
domains within the protein that mediate its role in actin filament formation: the Ena/VASP homology (EVH)1 domain targets VASP to specific sites within the cell, including focal adhesions and tight junctions; the central proline-rich domain binds profilin, another actin-binding protein; and the EVH2 domain binds both G- and F-actin. When VASP is phosphorylated at Ser157 by PKA and/or PKG (12, 64), it localizes to cell-cell junctions in endothelial cells and can be colocalized with ZO-1, a tight junction accessory protein (12, 37). Our data indicated that VASP levels increased in the membrane and cytoskeletal fractions after 1-h hypoxia, supporting the idea that there is an early dynamic change in the actin cytoskeleton. This mobilization is likely to be mediated through multiple pathways, including PKG (15, 63), MLCK (34), and RhoA/ROCK (27).

Hypoxia can also trigger cytoskeletal contraction, which is controlled by the phosphorylation state of MLC. This phosphorylation state is controlled by the calcium-calmodulin-independent MLCK and by MLC phosphatase (MLCP) working in opposition (Fig. 7). MLCP, in turn, can be inhibited by activation of RhoA and ROCK, leading to an increase in MLC phosphorylation and actin-myosin contraction. In our study, confocal microscopy indicated peak phosphorylation of MLC after 15 min of hypoxia, which returned rapidly to baseline levels. Phosphorylation of MLC and actin-myosin contraction leads to changes in cell morphology and, in BBB endothelial cells, disruption of barrier function (18). Blocking MLC phosphorylation by inhibiting MLCK protected barrier function and tight junction structure after hypoxia, implicating cytoskeletal contraction in the disruption of the tight junction (see Fig. 7). It should be noted, however, that the studies presented here utilized bEnd3 cells, an immortalized mouse brain endothelial cell line that may not respond in the same fashion as primary cultures of BBB endothelial cells. Future experiments with primary cell cultures will confirm the involvement of the proteins we have identified in this study.

While activation of the actin cytoskeleton contractile machinery is a reasonable hypothesis for a mechanism contributing to BBB disruption after hypoxia, presumably via calcium-calmodulin activation of MLCK, the proteins mediating calcium influx after hypoxia are not well understood. We hypothesized that members of the TRPC family might be involved. TRPC channels are expressed in BBB endothelial cells (4) and can be activated by hypoxic stress (14, 40). Furthermore, inhibiting TRPC channels prevents both phosphorylation of MLC and barrier disruption (Fig. 6). This directly links activation of TRPC channels, subsequent calcium influx, and barrier disruption through an actin-myosin contraction mechanism that contributes to morphological changes in the cells and pulls apart cell-cell junctions. This calcium influx may also contribute to changes in moesin and VASP via the p38 MAPK (9) or RhoA (27) pathways and PKG activation (53), respectively.

Fig. 6. Inhibition of transient receptor (TRP)C channels prevents BBB disruption and MLC phosphorylation following hypoxic stress. A: treatment with SKF-96365 (0.1, 1.0, and 10 μM), an inhibitor of TRPC channels, dose-dependently blocked BBB disruption following 6-h hypoxia (***P < 0.01 vs. control, *P < 0.05 vs. hypoxia). B: treatment with 1 μM SKF-96365 also significantly reduced pMLC immunofluorescence following hypoxia (***P < 0.01 vs. hypoxia), indicating prevention of MLCK activation and/or RhoA/ROCK mediated inhibition of MLC phosphatase (MLCP). Data are presented as means ± SE for 8–10 measurements (A) or 3 confocal stacks (B).

Fig. 7. Pathways implicated in cytoskeletal regulation are altered after hypoxia. Isobaric protein labeling followed by LC tandem mass spectrometry (MS/MS) identified a number of proteins that were up- or downregulated by hypoxia in BBB endothelial cells. We are particularly interested in proteins involved in actin filament polymerization (VASP, profiling, cofilin-1), actin-myosin contraction [MLC, MLCK, Ras homolog gene family A (RhoA)/Rho kinase (ROCK)], and anchoring of actin filaments to the plasma membrane (moesin, α-catenin). Arrowheads indicate activation/phosphorylation, while blocked arrows indicate inhibition. α-Act, α-actinin; α-cat, α-catenin; β-cat, β-catenin; Ca+++, calcium; CaM, calmodulin; Cof-1, cofilin-1; γ-cat, γ-catenin; MHC, myosin heavy chain; PFN, profilin.
This is, as far as we know, the first direct link from TRPC channels to BBB functional integrity in a stroke model. The involvement of moesin and VASP in this scenario is not clear as yet, although TRPC4 has been shown to interact with EBP50, another member of the ERM family of proteins that contains moesin (48), and with VASP itself (64). We can hypothesize a system in which activation of TRPC channels under conditions of hypoxic stress leads to calcium influx and activation of MLCK. MLCK then phosphorylates pMLC, leading to cytoskeletal contraction and stress on cell-cell junctions. In addition, this calcium influx can activate RhoA, leading to phosphorylation of moesin and its translocation from the cytoplasm to the cell membrane, linking actin filaments to membrane domains. RhoA may also feed back onto TRPC channels to BBB functional integrity in a stroke model. The involvement of moesin and VASP in this scenario is not clear as yet.

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