Acute Vascular Disruption and Aquaporin 4 Loss
After Stroke

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Background and Purpose—Ischemic protection has been demonstrated by a decrease in stroke-infarct size in transgenic mice with deficient Aquaporin 4 (AQP4) expression. However, it is not known whether AQP4 is rapidly reduced during acute stroke in animals with normal AQP4 phenotype, which may provide a potential self-protective mechanism.

Methods—Adult male rats underwent transient occlusion of the middle cerebral artery (tMCAo) for 1 to 8 hours followed by reperfusion for 30 minutes. Protein and mRNA expression of AQP4 and glial fibrillary acidic protein (GFAP) were determined by Western blot and rtPCR. Fluorescence quantitation was obtained with laser scanning cytometry (LSC) for Cy5-tagged immunoreactivity along with fluorescent signals from pathological uptake of plasma-borne high-molecular-weight fluorescein-dextran. Cell death was assessed with in vivo Propidium Iodide (PI) nucleus labeling.

Results—In the ischemic hemisphere in tissue sections, patches of fluorescein-dextran uptake were overlapped with sites of focal loss of AQP4 immunoreactivity after tMCAo of 1 to 8 hours duration. However, the average levels of AQP4 protein and mRNA, determined in homogenates of whole striatum, were not significantly reduced after 8 hours of tMCAo. Tissue section cytometry (LSC) of immunoreactivity in scan areas with high densities of fluorescein-dextran uptake demonstrated reductions in AQP4, but not in IgG or GFAP, after tMCAo of 2 hours or longer. Scan areas with low densities of fluorescein-dextran did not lose AQP4. There was sparse astrocyte cell death as only 1.7±0.85% (mean, SD) of DAPI labeled cells were PI- and GFAP-labeled after 8 hours of tMCAo.

Conclusions—During acute tMCAo, a rapid loss of AQP4 immunoreactivity from viable astrocytes can occur. However, AQP4 loss is spatially selective and occurs primarily in regions of vascular damage. (Stroke. 2009;40:2182-2190.)

Key Words: Aquaporin 4 ■ blood brain barrier breakdown ■ stroke

Water balance in the brain is regulated primarily by Aquaporin 4 (AQP4) channels, which are concentrated in perivascular astrocytic end-feet1 and belong to the multi-gene family of Aquaporins.2,3 Little is known about the kinetics of AQP4 turnover or the physiological basis for maintained AQP4 expression in astrocyte endfeet. The demonstration, in transgenic mice, that reduced4,5 or abrogated6,7 AQP4 function increases resistance to brain edema arising from stroke in a permanent ischemia model8–10 has supported the suggestion that AQP4 levels may serve to gate evolving edema. Importantly, the protective anti-edema effects observed in transgenic mice are demonstrated in the context of a preexisting reduction of AQP4 before the stroke onset. In normal animals, if AQP4 serves a gating role it would be predicted that its levels should change rapidly to impact the earliest phases of stroke-evoked edema.

It is well known that brain injury dysregulates AQP4 channel expression11,12 with model-dependent results of both up- and downregulation. Brain injury from experimental stroke has been associated with upregulation of tissue AQP4 protein, mRNA, and immunoreactivity in both stroke core and infarct in a tMCAo model of 30 minutes occlusion followed by 1 hour of reperfusion in adult mice.13 Experimental tMCAo in mice has also been linked to fluctuating up- and downregulation of AQP4 in a model with 90 minutes of vessel occlusion followed by 1 to 12 hours of reperfusion in adult mice.14 In this latter study, AQP4 dysregulation converted to a stable and significant reduction only after 24 hours of reperfusion in the stroke core while the penumbra demonstrated a restoration of AQP4.14

It remains unclear whether there are conditions that trigger a rapid, possibly self-protective, reduction of brain AQP4 in the acute phase of ischemia, defined here as less than 24 hours postsinsult. The perivascular concentration of astrocytic AQP4 suggests a hypothesis that severe vascular injury may interconnect with the regulation of AQP4 channels. An approach to test the hypothesis of a linkage between vascular damage and rapid ischemic changes in AQP4 expression is to determine the effect, on AQP4, of modulating vascular damage through increased duration of ischemic insult.15,19–21 The resultant vascular damage that is severe can be monitored on the basis of the presence of pathological endothelial cell
permeability to high-molecular-weight fluorescein-dextran (2 MDa).15–18 To study ischemic alterations in AQP4 that are rapid, brain reperfusion can be limited to short epochs, because even 30 minutes of reperfusion captures acute cytotoxic edema in astrocytes22 after cardiac arrest. The present study asks if a tMCAo model, with varying occlusion-durations and with only 30 minutes of reperfusion, uncovers a capacity for rapid AQP4 loss in ischemic brain regions with severe vascular damage.

Methods

Surgery for tMCAo
All procedures were performed with approval of the VAMC San Diego IACUC. We used 45 male Sprague-Dawley rats (290 to 305 gm) in this study. Rats were anesthetized with isoflurane (1 to 2% in oxygen: nitrous oxide 30:70), and this was followed by tail-vein injection of high-molecular weight fluorescein-dextran (2MDa; Sigma; 0.3 mL of a 5% [wt/vol] solution).

For tMCAo, the left common carotid artery was threaded with a 4 to 0 nylon suture (Ethilon, Animal Health) that was blunted to a diameter between 300 and 310 μm using a microforge (Narishige). The suture was advanced 17.5 to 18.0 mm from the bifurcation point to cover the origin of the MCA. Occlusion durations varied from 1, 2, 4, or 8 hours, and reperfusion time was fixed at 30 minutes. Transcardial perfusion and tissue fixation was performed as previously described.17,18

Immunocytochemistry
Brain sections were cut into 50-μm sections and then immunostained18 with rabbit polyclonal anti-Aquaporin 4 (Chemicon or Millipore), rabbit polyclonal anti-GFAP (Sigma), or with biotinylated universal secondary antibody for rat IgG staining (Vector). Nonbiotinylated primary antibody incubations were followed by incubation in biotinylated antirabbit secondary antibody (Vector). For fluorescent localization of biotinylated antibodies, sections were incubated overnight in Cy5-conjugated strepavidin (Jackson Immunoresearch). Fluorescent immunostained sections were washed in PBS and then mounted on slides and cover-slipped with Pro-Long Antifade mountant (Molecular Probes). Background staining was assessed in sections processed without primary antibody.

Western Blots
After barbiturate overdose at the end of 8 hours of tMCAo and 30 minutes of reperfusion, rats were exsanguinated with cold saline and whole striata were dissected from the ischemic and contralateral hemispheres and snap frozen in liquid nitrogen (n=4 rats). Tissue samples were homogenized in a standard lysis buffer23 that included 1% SDS. Insoluble material was pelleted in two runs at 14 000g and resultant lysates were fractionated on a NuPage 4% to 12% Bis-Tris gradient gel (Invitrogen) followed by transfer to a nylon membrane (Life Sciences). Immunoblotting was performed first with anti AQP4 antibody (1:500 in tween buffered saline [TBS-T] with 1% milk). After washing, the membranes were incubated in antirabbit antibody (1:2000 in TBS-T with 5% milk). Immunoreactive protein was visualized with Western C (BioRad) for chemiluminescence. Scanning densitometry and analysis was obtained with a Versidoc 4000 (BioRad). Blots were stripped twice for reprobing first with anti-GFP (1:5000 in TBS-T and 5% milk) and then with antibeta actin antibody (1:5000 in TBS-T and 5% milk, Sigma-Aldrich). Secondary antimonouse antibody for GFP and for beta actin was provided by Cell Signaling Technologies and used at a concentration of 1:5000 in TBS-T and 5% milk. Lane loading differences were controlled for by normalization to the corresponding actin signals for each sample.

RNA Extraction and Real-Time RT-PCR
RNA was isolated from frozen tissue samples of ischemic cortex, striatum, and contralateral uninjured homologous control tissue after 8 hours of tMCAo using the RNasy Mini Kit (Qiagen) according to manufacturer’s instructions. RNA was reverse transcribed to cDNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems) using random hexamer primers. Real-time PCR analysis from samples from each rat (n=7) was run in triplicate and performed using the Opticon DNA Engine 2 (MJ Research) and the Power SYBR Green PCR kit (Applied Biosystems) using 1.5 μL of cDNA template in a 25 μL reaction. Results were analyzed with the Opticon 2 Software using the comparative Ct method, as described.24 Data were expressed as 2–ΔΔCt for the experimental gene of interest normalized against the housekeeping gene GAPDH and presented as fold change versus contralateral control samples. The primers were designed using the Primer Express software (Applied Biosystems) following the guidelines suggested in the Primer Express applications-based primer design manual. The following primers were used:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CGCGGCTGAGGAGAGTTCG 3′</td>
<td>TGCAAACTTGGACCATCCA 3′</td>
</tr>
<tr>
<td>Aquaporin4</td>
<td>Fwd 5′ TGTCGCCAGGTCACCTTTAC 3′</td>
<td>Rev 5′ GCTGTCAGCTTTGCTGAAG 3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Fwd 5′ CTCTACCAAGCGGCAAGTCAA 3′</td>
<td>Rev 5′ GCCTCCTGAAGATGTTGAT 3′</td>
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Imaging and Quantitative Analysis
Digitized fluorescent images were acquired with an Olympus BX50 Microscope fitted with a CompuCyte laser scanning cytometry (LSC) acquisition system (Olympus of America). Fluorescein fluorescence, from excitation with a focused argon laser (488 nm), was collected through an emission filter with a bandwidth of 530±30 nm. Cy5 fluorescence, from excitation with a focused helium-neon laser (633 nm), was collected through an emission filter with a bandwidth of 675±50 nm. Fluorescein and Cy5 gates were set for background levels that were determined from negative control tissue sections. Section fluorescence was digitized from 40-μm diameter scan areas that were tiled across the whole section (eg, Figure 4A2 and 4A3). The thresholded and integrated fluorescence signal intensities for both fluorescein and Cy5 determined whether a particular scan area was classified as backround, or single or double labeled. This is illustrated by the scattergrams created by the Wincyte software (Figure 4A4). For example, the scattergram in Figure 4A4 provides a frequency of double labeled scan areas (1047, or 3% of the total number of scan areas) from the digitization of the section illustrated in Figure 4A1 and 4A3; then, a whole section double label index was calculated from the ratio of double labeled scan areas (upper right quadrant) to the number of all scan areas with dextran signal (sum of upper and lower right quadrants: Figure 4A4) to normalize the double labeling frequency. Laser scanning cytometry was also used to compare total AQP4 in operator-defined subregions of interest that typically covered 300 scan areas. The operator-defined subregions were placed in areas with high levels of fluorescein-dextran label and in areas with low levels of fluorescein-dextran label (boxes in Figure 4A3). For striatal analyses, data were obtained from 2 high-label subregions and from 2 to 4 low-label subregions per section in 1 to 2 sections per rat. In ischemic neocortex, the analysis of the AQP4 staining was restricted to 8 hours of tMCAo cases and included selection of 1 high leakage subregion and 1 to 2 low leakage subregions in 1 to 2 sections per rat. Brain sections selected for striatal or neocortex analysis originated from levels that spanned from 0.1 mm anterior to −0.3 mm posterior to Bregma.25

In Vivo Propidium Iodide Labeling
Rats were subjected to 8 hours of tMCAo (n=3) and propidium iodide (0.3 mL of a 2 mg/mL solution) was injected via tail vein for the last 2 hours of occlusion (n=2) or for the last 4 hours of occlusion (n=1). All rats were reperfused for 30 minutes and then killed with a barbiturate overdose followed by transcardial perfusion with saline and then buffered paraformaldehyde fixative. The brains were removed, cryoprotected in 30% sucrose, and cryostat sectioned at 15 μm. Immunostaining for GFP was performed after antigen retrieval27 with visualization either with cy3-avidin or with use of Alexa-conjugated GFP primary antibody (Invitrogen). Slides were coverslipped with Prolong-Gold mounting containing DAPI for...
nuclear visualization (Invitrogen). Data were collected on an Olympus scanning confocal microscope with a 60× oil objective. For each case 15 to 20 fields were examined in each of 2 sections from PI-positive regions, previously identified in ischemic striatum at lower magnification (10× objective), for a total count of 1848 DAPI-positive cells.

Two-Photon Microscopy
Fluorescent images of high-molecular-weight fluorescein-dextran leakage and of the Cy5 tagged AQP4 fluorescence in 50-μm-thick sections were acquired with a 2-photon microscope of local design. Volume rendered images of the vessel fluorescence was performed with using VOX2 (public domain software) and with MatLab (The Mathworks). Spectral separation of these images was performed by subtracting the crossover fluorescein signal from the image observed in the Cy5 channel.

Statistical Analyses
The double label index (double-positive counts normalized to total fluorescein-dextran in individual sections) was assessed for differences as a function of occlusion duration using 1-way ANOVA. To assess the significance of differences between the high and low dextran subregions over different occlusion durations, 1-way ANOVA was used with Tukey’s procedure for testing differences posthoc. Plotted immunocytochemical data were expressed as mean±2SE (standard errors). The paired t test was used to assess the significance of differences of mean values, in neocortex, in the total fluorescence in high and low leakage subregions after 8 hours of tMCAo. Differences between means of GFAP and AQP4 mRNA in cortex and striatum relative to the contralateral side were assessed by ANOVA. Data from Western blots and rtPCR were plotted as mean±1SE.

Results
Acute tMCAo of 1 Hour Resulted in Patchy Reductions in AQP4 Immunoreactivity
Patches of tissue marked by pathological uptake of high-molecular-weight fluorescein-dextran overlapped regions with AQP4 staining deficiencies (Figure 1). There was negligible fluorescein-dextran uptake on the contralateral side of the brain, supplied by the nonoccluded middle cerebral artery (Figure 1). A macroscopic spatial correspondence between AQP4 immunostaining deficiencies and fluorescein-dextran tagged tissue uptake was further observed after 2, 4, and 8 hours of tMCAo (Figure 2). Regions of reduced AQP4 staining and of emerging fluorescein-dextran labeling expanded

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Figure 1. Immunostaining of AQP4 after 1 hour of tMCAo. Panels on left are scans of a single section respectively imaged for Cy5-AQP4 immunoreactivity (top left), for pathological fluorescein-dextran uptake (middle left); the bottom left panel shows the merged image. Middle panels are views of the section that are converted to gray scale and inverted for clarity. Right panels show areas at arrows at higher magnification. The pale area that lacks Aquaporin 4 staining overlies a dense patch of high-molecular weight fluorescein-dextran uptake.

Figure 2. Whole brain sections from cases with tMCAo of 1, 2, 4, or 8 hours duration imaged for AQP4 immunoreactivity (left panels) and for fluorescein-dextran uptake (right panels). The striatum in the ischemic hemisphere shows pale areas with reduced AQP4. Fluorescein images demonstrate overlapping areas with high-molecular-weight fluorescein-dextran uptake. In the ischemic neocortex, after 8 hours of tMCAo, periodic discontinuities emerge in the AQP4 immunoreactivity along with patches of fluorescein-dextran leakage. The contralateral cortex retains a normal, continuous AQP4 staining pattern. Scale bar=1 mm.
changes in AQP4 expression, we segmented immunoreactiv-
ity provides a tool to stratify spatially microheterogeneous
tissue homogenates may arise from a microheterogeneity of loss
ischemic striata (Figure 3B; see also supplemental Figure II). This lack of significant change in AQP4 protein or mRNA in ischemic striata and cortices was significantly greater than levels on the contralateral side (ANOVA, P<0.005). C, High-resolution images of fluorescein-dextran labeling (red) in a microvessel after tMCAo of 8 hours. Rotated view (labeled X–Z) shows vessel in cross-section; note intensely fluorescent vessel walls labeled with fluorescein-dextran uptake, unlabeled vessel lumen, and perivascular AQP4. Images illustrate heterogeneity of perivascular AQP4 expression in single sections of striata.

To test whether stroke-induced fluorescein-dextran labeling provides a tool to stratify spatially microheterogeneous changes in AQP4 expression, we segmented immunoreactiv-
y according to the presence of concomitant uptake and tissue-labeling by high-molecular-weight fluorescein-dextran. Laser scanning cytometry analysis of whole brain sections (Figure 4A1 through 4A5) was used to probe scan areas of 40-μm diameter. These scan areas were rastered across whole brain sections (Figure 4A2). Consideration of the number of scan areas with fluorescein-dextran label in whole brain sections demonstrates a significant increase in number after 4 or 8 hours of tMCAo relative to 1 hour tMCAo (Figure 4A5). This is consistent with the macroscopic data illustrated in Figure 2.

The fluorescein-dextran positive scan areas were further assessed for the presence or absence of Cy5-tagged immunolabel. This was quantified as a double-label index (Figure 4F) which reflects a section by section normalization of the number of double-labeled scan areas to the total number of fluorescein-dextran scan areas per section. The double-label index of scan areas with both AQP4 (Cy5) and fluorescein-dextran label after 2 hours tMCAo (P<0.02 for 1 hour versus 2 hour tMCAo, ANOVA with the Dunnett procedure). Further AQP4 reductions were observed with longer tMCAo (P<0.001 for 1 hour versus 4 hour or 8 hour tMCAo). However, even at 8 hours tMCAo, double-labeled scan areas were present, consistent with high resolution microscopic examination (see Figure 3C). These data indicate that stratification of AQP4 staining according to proximity to stroke-induced high-molecular-weight fluorescein-dextran uptake permits quantitation of AQP4 staining loss in response to acute stroke.
Figure 4. The use of laser scanning cytometry (LSC) to compare the effects of increased occlusion duration on the frequency of scan areas that are colabeled by fluorescein-dextran and immunoreactivity to AQP4, IgG or GFAP. A1 and A2 illustrate a brain section that shows fluorescein-dextran labeling in ischemic hemisphere after 8 hours of tMCAo in a photomicrograph (A1) and in a companion LSC-generated digitization of this section (A2). A3. Example of 40-μm-diameter scan areas used to quantify fluorescence. This panel illustrates positive scan areas which result from vascular uptake of high-molecular-weight fluorescein-dextran from a case after 2 hours of tMCAo. A4. LSC generated scattergram with data from scan areas rastered across an entire section. Each quadrant provides the percent area for 1 of 4 different types of counts: 1, The upper left quadrant demonstrates single-labeled Cy5 immunoreactivity; 2, The upper right quadrant demonstrates counting regions that are double positive for fluorescein-dextran and Cy5; 3, The lower right quadrant demonstrates counting regions single-labeled with fluorescein-dextran; 4, The lower left quadrant demonstrates counting regions with only background levels of signal. The double labeling indices for whole sections shown in graphs in panel F were determined from the percent of total scan areas per section that are double positive (in the upper right quadrant), and this value is then normalized to the summed percent of scan areas with fluorescein-dextran label (obtained from the upper and lower right quadrants). A5, Changes in the density of scan areas with fluorescein-dextran label as a function of occlusion duration of tMCAo. The number of cases that were assayed at individual occlusion durations was: 1 hour tMCAo, n=5; 2 hour tMCAo, n=6; 4 hour tMCAo n=5; 8 hour tMCAo, n=5. Different sections from each brain, at the level of the anterior commissure, were immunostained with AQP4, IgG, and GFAP. From 1 to 2 sections per stained series were LSC assayed for fluorescein-dextran uptake so that the dextran density index shown in panel A5 represents an average value across 16 to 19 sections per occlusion duration. The dextran density index is the total number of fluorescein-dextran labeled scan areas divided by the total number of scan areas for that brain section. The areal density of fluorescein-dextran uptake increases significantly with occlusion duration (1 vs 4 hours; \( P<0.05 \) and 1 vs 8 hours; \( P<0.001 \): ANOVA followed by the Tukey test). B, Images of immunostaining merged with fluorescein-dextran label in a 50-μm section from 1 brain after 2 hours of tMCAo. Sections are within a 1-mm-thick block. B1, Fluorescein-dextran uptake concentrates among segments of microvessels. B2, Higher magnification of inset (white box in B1) shows heterogeneity in label intensity in affected vessels of varying caliber. C1, Fluorescent merged image of fluorescein-dextran labeled vasculature (green with yellow-green borders) and AQP4 immunoreactivity (red). C2, The inset panel shows overlap of vessels marked by fluorescein-dextran uptake within the vessel (green), and by perivascular AQP4 which is yellow near vessels with fluorescein-dextran leakage or red near vessels that are unlabeled by fluorescein-dextran. D1, Merged images of fluorescein-dextran labeled vasculature and IgG leakage (red). D2, High magnification of merged image demonstrates nearly complete overlap of fluorescein-dextran fluorescence and Cy5-IgG immunoreactivity (yellow). This section is the same section shown in panels B1 and B2 where the fluorescein-dextran fluorescence appears green. Fluorescein-dextran labeled vessels are also surrounded by...
The double-label index for immunoreactivity to biotinylated IgG was determined as a technical control. This allowed evaluation of whether the reduction of the AQP4 double-label index represented a nonspecific effect of ischemic brain swelling because swelling alone could reduce the density of AQP4-positive scan areas. As focal cerebral ischemia triggers leakage to endogenous blood-borne molecules such as IgG, we predicted that counting regions labeled with fluorescein-dextran should also contain IgG immunoreactivity. Immuno-reactivity to IgG, reported by Cy5, resulted in label of brain parenchyma in areas with blood vessels that were marked by fluorescein-dextran uptake (Figure 4D1, 4D2, and 4F). However, in contrast to AQP4, the double-label index for IgG and fluorescein-dextran showed no significant change with increased tMCAo duration (Figure 4F). The stability of the IgG and fluorescein-dextran double-labeling index indicates that dextran leakage and IgG extravasation increase in parallel with longer durations of tMCAo. Thus, the reduction in AQP4 immunoreactivity, proximate to uptake of high-molecular-weight fluorescein-dextran by hyperpermeable vessels, is not a result of brain swelling.

To determine whether the decline in the AQP4 double-label index generalized to an independent marker for astrocytes, we determined the double-label index for GFAP (Figure 4E1 and 4E2). GFAP staining intensity was especially dense in regions of fluorescein-dextran label and also near the lateral ventricle (Figure 4E1 and 4E2). The double labeling index for GFAP and fluorescein-dextran did not change significantly with tMCAo duration (Figure 4F). Therefore, the expansion of the uptake of high-molecular-weight fluorescein-dextran occurred among populations of astrocytes with simultaneously increased GFAP staining and reduced AQP4 immunoreactivity.

Ischemic regions with low levels of fluorescein-dextran uptake showed less dysregulation of AQP4 immunoreactivity. This is illustrated in Figure 5A, which shows similar levels of AQP4 staining in the nonischemic striatum and in the ischemic striatum in fields that span across areas with low levels of fluorescein-dextran uptake. AQP4 loss was evident in fields that span across areas with high levels of fluorescein-dextran uptake and in fields that span across areas with low levels of fluorescein-dextran uptake. The double-label index for IgG and fluorescein-dextran showed no significant change with increased tMCAo duration (Figure 4F).
high to low levels of dextran leakage. Immunoreactivity for AQP4 in areas with low levels of dextran leakage was qualitatively similar in ischemic and contralateral striatum after tMCAo of 1 or 4 hours duration. After 8 hours of tMCAo, AQP4 immunoreactivity was more heterogeneous in ischemic areas with low levels of dextran, as there were patches of increased and reduced staining (Figure 5A).

To test the qualitative impression of an apparent stability in the AQP4 immunoreactivity in ischemic regions with low levels of fluorescein-dextran signal, the total AQP4 staining density was determined in operator-defined subregions that were segmented according to fluorescein-dextran density. The analyzed regions typically encompassed 300 scan areas (for examples of operator-defined subregions see boxes in the fluorescein-dextran rich and poor areas on the ischemic side and in the fluorescein-dextran poor area on the contralateral side of the digitized section in Figure 4A3). Scanned subregions with high or low densities of fluorescein-dextran label were identified for all durations of tMCAo (Figure 5B). The AQP4 staining density in such subregions was also measured (Figure 5C). In subregions with low fluorescein-dextran the total Aquaporin 4 immunoreactivity showed no significant change over increasing durations of occlusion (Figure 5C). In contrast, in subregions with a high density of fluorescein-dextran, the total AQP4 immunoreactivity declined with increasing occlusion duration (Figure 5C, white bars; ANOVA was used with the Tukey procedure for posthoc comparisons, F = 3.78, P = 0.018).

Aquaporin 4 staining was additionally disrupted in ischemic neocortex after 8 hours of tMCAo (Figure 2; see also supplemental Figure 1). Subregion quantitation of AQP4 staining density, in 8 hour tMCAo ischemic neocortex, showed significant reductions of AQP4 in high–dextran leakage subregions relative to low-leakage regions (9.2 ± 3% versus 17.4 ± 1.8%, mean ± SEM; n = 5 rats; t test, P = 0.033). Thus regions with low uptake of high-molecular-weight fluorescein-dextran in ische-

Figure 6. Cell death induced by acute stroke was identified by in vivo uptake of circulating Propidium Iodide. A series of images of 1 field are shown. Panels A1 and A2 show DAPI stained nuclei in ischemic striatum (in A1) and the corresponding distribution of PI nuclear uptake consequent to loss of plasmalemma permeability barriers in dying cells (in A2). Merged image (white arrowhead) shows white halo at site of overlap of DAPI and PI. Panels B1 and B2 show the GFAP immunoreactivity of a cell colabeled by PI (arrowhead) and in an adjacent PI negative cell (double arrow). C. Pie chart of proportion of cells (DAPI-positive) which are colabeled with propidium iodide or GFAP. The blow-up chart on the right delineates the proportion of propidium iodide-positive cells which are colabeled by GFAP (in tan sector). Data are based on 2 sections per rat brain (n = 3) in fields imaged at 60 × in the ischemic striatum. The number of DAPI cells counted per rat brain was 594, 566, and 678.

Discussion

We sought to determine whether ischemic dysregulation of astrocytic AQP4 could occur rapidly in response to experimental stroke in animals with normal AQP4 phenotype. Immunocytochemical data provided qualitative evidence that even 1 hour of tMCAo results in patchy staining loss of AQP4; the heterogeneity of this astrocyte response is corroborated by the absence of detectible reductions of AQP4 protein or mRNA in whole striatal homogenates. Early focal reductions of AQP4 in regions with pathological uptake of fluorescein-dextran were quantified with laser scanning cytometry, between 1 and 2 hours of tMCAo. Increased duration of tMCAo, to occlusions of 4 hours,
resulted in both an expansion of vascular damage, as evidenced by the spread in high-molecular-weight fluorescein-dextran uptake, and also in a further reduction in the AQ4P-fluorescein-dextran double labeling index. The fall in the double-labeling index was selective for AQ4P immunoreactivity because the double-label index for IgG and GFAP remained stable with increased occlusion duration. The decline in AQ4P was also selective for scan areas with high levels of fluorescein-dextran tissue label because AQ4P was not significantly altered in clusters of scan areas with sparse fluorescein-dextran label. Taken together our findings suggest that stroke can cause rapid losses in AQ4P staining in those ischemic astrocytes where ischemic injury has been sufficiently severe to allow pathological passage of high-molecular-weight fluorescein-dextran across ischemic vasculature.

Our demonstration of rapid changes in AQ4P during the acute phase of stroke extends previous experimental studies and human pathology data, where AQ4P loss has been observed after longer poststroke survival periods. The chronic AQ4P loss observed in human stroke infarcts has been ascribed to associated astrocyte cell death. However, in light of the present study it is also possible that the persistent loss of AQ4P actually originated as an early rapid response of astrocytes during acute injury, as our data indicates that AQ4P loss occurs in the face of retained GFAP expression and in advance of widespread ischemic death. The notion that AQ4P loss represents an active rapid physiological response in viable astrocytes is supported by recent work documenting a reversibility of AQ4P loss in the stroke penumbra of adult mice. In addition, the seminal observations of the extraordinarily rapid disappearance of membrane particle assemblies, later identified as astrocytic perivascular AQ4P channels, in response to circulatory arrest further argues that the loss of AQ4P after ischemic stroke can represent an active astrocyte response.

The results should be interpreted in light of the limitation of examination of tMCAo occlusions of 1-hour durations or longer. The 1-hour occlusion time was chosen because shorter occlusion durations failed to yield consistent labeling of ischemic vasculature or parenchyma by circulating high-molecular-weight fluorescein-dextran, and our LSC stratification depends on the presence of fluorescein-dextran labeling of scan areas. Thus hyperacute effects of ischemia on AQ4P expression in viable astrocytes is supported by recent work documenting a reversibility of AQ4P loss in the stroke penumbra of adult mice. In addition, the seminal observations of the extraordinarily rapid disappearance of membrane particle assemblies, later identified as astrocytic perivascular AQ4P channels, in response to circulatory arrest further argues that the loss of AQ4P after ischemic stroke can represent an active astrocyte response.

Our data contrast with other experimental models in which blood-brain barrier breakdown has been shown to provoke acute upregulation of AQ4P. Sudden blood-brain barrier breakdown and inflammation from intraparenchymal injection of lipopolysaccharide (LPS) into the substantia nigra of female rats results in significant increases in the number of AQ4P mRNA-positive cells after 6 hours of injury. Furthermore, in vitro studies demonstrate that administration of IL-1, a mediator of edema after ischemia, upregulates AQ4P mRNA in rat astrocytes in vitro and consequent to intracerebroventricular administration in rat brain in vivo. This supports a role for vascular leakage as a potential mediator of upregulation of AQ4P after brain injury. On the other hand, LPS injection into the chick optic tectum results in reductions of AQ4P immunoreactive protein in perivascular astrocytic endfeet. Experimental studies with stroke models in mice have also yielded contradictory results concerning the effects of stroke on the regulation of AQ4P. For example, mice that were subjected to 30-minute occlusion duration tMCAo demonstrated significant increases in immunoreactivity in both the ischemic core and in the surrounding penumbra at 1 and 48 hours of reperfusion. However, a separate study of stroke in mice demonstrated reductions in AQ4P immunoreactivity in perivascular astrocytic glial endfeet in ischemic striatum after 24 hours of reperfusion. Of interest, the MCA occlusions in the latter study that resulted in reductions in AQ4P immunoreactivity, were 1 hour longer than the MCA occlusions that resulted in stroke-evoked increases in AQ4P levels. Thus the loss of AQ4P after stroke may require a critical duration or severity of ischemic injury.

Little is known about the molecular mechanisms that regulate expression of AQ4P in normal or in injured astrocytes. AQ4P promoter regions have been identified with binding activity for transcription factors that are upregulated by ischemia and hypoxia (reviewed in Simard et al). Nondisease conditions such as pregnancy also upregulate the expression of astrocyte AQ4P. On the other hand other types of brain injury that do not involve widespread ischemia, such as hypertensive encephalopathy, have been shown to result in reductions in perivascular AQ4P immunoreactivity.

Regulation of AQ4P mRNA at subacute (48-hour survival times) has been linked to antecedent alterations in levels of targeted microRNA sequences. However, there are also precedents for a role for AQP channel regulation processes that are independent of changes at the level of transcription; for example, AQP2 surface-expression and function in the kidney is rapidly inactivated by channel ubiquitination followed by endocytosis in response to channel phosphorylation. Furthermore, endocytic regulation of AQ4P channels expression can be extremely rapid as histamine stimulation provokes rapid AQ4P internalization and reduced water transport as early as 20 minutes after stimulation in a gastric parietal cell line. In conclusion, the demonstration of rapid but selective loss of astrocytic AQ4P suggests that brain astrocytes may possess the cellular machinery for a self-protective response to early ischemia; however, the localization of this effect to regions that are supplied by blood vessels that have been severely damaged by stroke may indicate that there is an injury threshold for this effect.

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Disclosures

None.

References


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Beth Friedman, Christian Schachtrup, Philbert S. Tsai, Andy Y. Shih, Katerina Akassoglou, David Kleinfeld and Patrick D. Lyden

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