Hyperglycemia Exacerbates Intracerebral Hemorrhage via the Downregulation of Aquaporin-4
Temporal Assessment With Magnetic Resonance Imaging

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Background and Purpose—Intracerebral hemorrhage (ICH) is associated with high mortality and neurological deficits, and concurrent hyperglycemia usually worsens clinical outcomes. Aquaporin-4 (AQP-4) is important in cerebral water movement. Our aim was to investigate the role of AQP-4 in hyperglycemic ICH.

Methods—Hyperglycemia was induced by intraperitoneal injection of streptozotocin (STZ; 60 mg/kg) in adult Sprague–Dawley male rats. ICH was induced by stereotaxic infusion of collagenase/heparin into the right striatum. One set of rats was repeatedly monitored by MRI at 1, 4, and 7 days after ICH induction so as to acquire information on the formation of hematoma and edema. Another set of rats was killed and brains were examined for differences in the degree of hemorrhage and edema, water content, blood–brain barrier destruction, and AQP-4 expression.

Results—Hyperglycemia ICH rats exhibited increased brain water content, more severe blood–brain barrier destruction, and greater vasogenic edema as seen on diffusion-weighted MRI. Significant downregulation of AQP-4 was observed in STZ-treated rats after ICH as compared with non–STZ-treated rats. Apoptosis was greater on day 1 after ICH in STZ-treated rats.

Conclusions—The expression of AQP-4 in the brain is downregulated in hyperglycemic rats as compared with normoglycemic rats after ICH. This change is accompanied by increased vasogenic brain edema and more severe blood–brain barrier destruction. (Stroke. 2013;44:00-00.)

Key Words: aquaporin-4 ■ hyperglycemia ■ intracerebral hemorrhage ■ MRI

Intracerebral hemorrhage (ICH) accounts for 15% of all strokes in developed countries and is associated with significant mortality and poor prognosis caused by hematoma enlargement and subsequent perihematomal edema. Cerebral edema can be life-threatening because of the lack of effective treatments. Recent therapeutic strategies for ICH have been focused on modulating the imbalance of cerebral water after the onset of stroke. Proteins that are responsible for cerebral edema after ICH and facilitate water transport across biomembranes represent attractive therapeutic targets.

Aquaporins (AQP) are a family of molecular water channels that regulate water movement across the plasma membrane and have been identified in many organ systems. Several AQP have been found to be expressed in regions of the central nervous system. AQP-4 is abundantly expressed in the brain, particularly at tissue–water interfaces, such as the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier. AQP-4 has been shown to be involved in fast water transport in cultured astroglial cells and proposed to have a role in ischemia-induced cerebral edema. Depletion of AQP-4 aggravates vasogenic brain edema caused by a fluid leakage, and overexpression of AQP-4 has been shown to exacerbate cytotoxic brain swelling in transgenic animals. Numerous other studies using animal models have shown changes of AQP-4 expression after brain injury.

Patients with diabetes mellitus or hyperglycemia who experience ICH have been shown to have increased mortality and poorer neurological outcomes. High blood glucose concentration has been reported to be associated with increased...
brain edema and level of apoptosis in perihematomal regions after ICH.\textsuperscript{20} The purpose of this study was to investigate changes in hematoma formation, AQP-4 expression, and neurological outcomes after ICH associated with hyperglycemia in a rat model of ICH.

Materials and Methods

Animals
All animal experimental protocols were in compliance with the regulations of the Animal Welfare Committees of the Academic Sinica, Taipei, Taiwan. Rats were housed in triplets in plastic cages with free access to food and water. The housing environment was specific pathogen free with a 12-/12-hour light/dark cycle and controlled humidity and temperature. A total of 240 male Sprague–Dawley rats weighing 300 to 350 g were used for the studies.

Hyperglycemia and the ICH Rat Model
Hyperglycemia was induced by intraperitoneal injection with streptozotocin (STZ; Sigma, St. Louis, MO) at 60 mg/kg for 3 days before induction of ICH. Blood glucose was measured from the tail vein by OneTouch Ultra Test Strips (LifeScan Milpitas, CA) and calibrated according to plasma glucose levels. Hyperglycemia was defined as a blood glucose level from 300 to 400 mg/dL during the experiment. Experimental ICH was induced as described previously.\textsuperscript{21} Briefly, isoflurane-anesthetized rats were placed in a stereotaxic frame (David Kopf Instruments Tujunga, CA). A burr hole was made and a 30-gauge Hamilton syringe needle was inserted into the striatum (3.5 mm right lateral to the midline, 0.5 mm anterior to bregma, and 5.5 mm in depth below the skull). After intrastriatal infusion of 1-\(\text{L}^+\) (3.5 mm right lateral to the midline, 0.5 mm anterior to bregma, and 5.5 mm in depth below the skull). After intrastriatal infusion of 1-\(\text{L}^+\) saline containing 0.14 U collagenase and 1.4 U heparin IV (Sigma) and 2 mL/kg: Sigma) filtered through a 0.22-m filter. The animals were given an intravenous injection of Evans blue solution (6% in saline, 1 mg/mL) and then killed with pentobarbital at designated time points and brains were harvested immediately, fixed with 4% phosphate-buffered paraformaldehyde, and sectioned coronally through the visible needle entry site. Serial slices of 1 mm in thickness both anterior and posterior to the needle entry site were obtained. Perihematomal samples were 2-mm\(^2\) brain slices taken from the striatal tissue adjacent to hematoma.

BBB Disruption
Under anesthesia, 48 rats (24 STZ-treated, 24 non–STZ-treated) were given an intravenous injection of Evans blue solution (6% in saline, 2 mL/kg: Sigma) filtered through a 0.22-m filter. The animals were killed with pentobarbital at designated time points and brains were removed and examined. The stained regions of the brain indicated the dispersion of Evans blue after leakage through the disrupted BBB. Harvested brain were weighted and homogenized in a 10-fold volume of 100% trichloroacetic acid solution. The supernatant was obtained by centrifugation and diluted 4-fold with ethanol. Evans blue dye was quantified by its fluorescence intensity, which was determined by a microscope fluorescence reader (excitation 620 nm and emission 680 nm). Calculations were based on the concentration of dye extravasation.

Perihematoma Identification
The perihematoma area was defined as described previously.\textsuperscript{22} In brief, animals were anesthetized and decapitated. Brains were harvested immediately, fixed with 4% phosphate-buffered paraformaldehyde, and sectioned coronally through the visible needle entry site. Perihematomal samples were 2-mm\(^2\) brain slices taken from the striatal tissue adjacent to hematoma.

Measurement of Brain Water Content
Twelve brains (6 STZ-treated and 6 non–STZ-treated) were harvested on day 1 after ICH with removal of the cerebellum and brain stem. The brains were divided into 2 hemispheres along the midline, and the wet weight of the hemispheres was measured. The hemispheres were then incubated at 100°C for 24 hours, and the dry weight was measured. Water content was calculated as (wet weight−dry weight)/(wet weight)\times100.

Neurobehavioral and Plasma Osmolality Testing
Six rats from each group (STZ-treated and non–STZ-treated) were selected, and the bilateral grip test was used to quantify neurological function before and at 1, 4, and 7 days after ICH induction with protocols previously described.\textsuperscript{23} Briefly, the test measures the strength (in g) to hold onto a steel grip-bar (Dyadic Systems, Osaka, Tokyo, Model DPS-5R: range, 0-5 kgf) with forepaws. Plasma osmolality was quantitatively determined using a Micro-Osmometer (Advanced Instruments) according to the manufacturer’s instructions.

MRI Studies
MRI experiments were performed on a 4.7T spectrometer (Biospec 47/40, Bruker, Germany) with an active shielding gradient of 200 mT/m in 80 \(\mu\)s. A birdcage coil with an internal diameter of 72 mm was used for radiofrequency excitation, and a 4-cm active radiofrequency-decoupling quadrature surface coil, placed directly over the head of the animal, was used for signal detection. Six rats from each group (STZ-treated and non–STZ-treated) were maintained under anesthesia with 1% isoflurane in O\(_2\) at a flow rate of 1 L/min. The anesthetized animals were immobilized using a custom-designed head holder inside the MRI magnet to minimize motion artifacts. The body temperature was maintained at 37°C using a warm-water blanket, and the respiratory rate was maintained at 40 to 50 breaths/min by adjusting the isoflurane flow rate.

Multislice axial T2-weighted imaging (T2WI) and diffusion-weighted imaging (DWI) were performed at baseline and days 1, 4, and 7 after ICH. T2WI used a fast spin-echo sequence with repetition time=5100 ms, effective echo time=70 ms, echo train length=8, and number of excitations=4; DWI used a Stejskal–Tanner spin-echo sequence with repetition time/echo time=1500/34 ms, number of excitations=4, diffusion gradient duration=7 ms, diffusion gradient separation=15 ms, and b values of 1100\(\mu\)m\(^2\) applied along the x direction. The x direction represented the readout direction, which was the left to right direction. All MRIs were obtained using a field of view of 3.0 cm, a slice thickness of 1.0 mm, and a 256x256 matrix. Isolurane anesthesia with 100% O\(_2\) tends to render a hyperoxic condition, where molecular oxygen may contribute to signal changes in T1, T2, and T2* contrast.\textsuperscript{24} However, because of its relatively low T2 relaxivity, the effects of molecular oxygen on the T2WI can be considered negligible.\textsuperscript{25}

Quantification of MR Signals
MR data analysis was performed using Para Vision ( Bruker Medical, Ettlingen, Germany) and MR Vision (MR Vision Co., Menlo Park, CA). To eliminate rater variability, 2 well-trained imaging analysts who were blind to the conditions performed the analysis. The analyzed region of interest was the hematoma region. The region of interest was manually traced and the mean signal intensity was measured. The value was then normalized as a ratio to that of the contralateral striatum. The signal ratios of the region of interest were compared between groups across the 7-day time course.

Immunofluorescence Staining
Six rats from each group (STZ-treated and non–STZ-treated) were killed on days 1, 4, and 7 after ICH. Brain sections were incubated with a rabbit polyclonal antibody anti-AQP-4 (1:500; Chemicon International, Temecula, CA) at 37°C for 4 hours followed by incubation with Alexa Fluor 568-nm–coupled secondary goat antirabbit antibody (1:200; Vector Laboratories, Burlingame, CA) at room temperature for 1 hour. Sections were mounted with antifading medium, Vectashield containing 46-diamidino-2-phenylindole (Vector). AQP-4–positive cells were enumerated in triplicate samples at a \(\times400\) magnification using a calibrated grid. All intact tissue was examined for each treatment, and counts were expressed as cells/0.1 mm\(^2\) of tissue.
Western Blotting
Six rats from each group (STZ-treated and non–STZ-treated) were killed on days 1, 4, and 7 after ICH. Brain tissues were homogenized and subjected to a 12% SDS-PAGE. The proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking, the blotting membranes were incubated with a rabbit polyclonal antibody anti-AQP-4 (1:500; Chemicon International) for 2 hours at room temperature and then with biotinylated goat anti-rabbit IgG (1:200, Vector) for 1 hour at room temperature. The proteins were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, ON, Canada). The intensity of blots was quantified using the Leica Image Processing and Analysis System (Gallen, Switzerland). β-Actin was used as an internal control.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP-Biotin Nick End Labeling Assay
Six rats from each group (STZ and non-STZ) were killed on days 1, 4, and 7 after ICH. Apoptotic cells were detected in situ using terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling staining as previously described.26 In brief, serial 1-mm-thick sections were cut on a cryostat and mounted on superfrost/plus slides (Menzel-Glaser, Braunschweig, Germany). Apoptosis was defined as cells with terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling–positive nuclei that were condensed and fragmented, as assayed by 4',6-diamidino-2 phenylindole (Molecular Probes; 1:2,000 dilutions). The number of apoptotic transplanted cells was expressed as a percentage of the total number of nuclei counted, with at least 25,000 nuclei for each condition.

Statistics Analysis
Data are presented as means±SE. Statistical analysis was performed by Student t test or 2-way ANOVA followed by paired t test. P value of <0.05 indicated statistical significance.

Results
Characterization of Diabetic Rats With ICH
Figure 1 shows that the glucose levels of STZ-treated and non–STZ-treated rats were 354.7±16.9 and 92.8±13.4 mg/dL (P<0.001), respectively, and the significant elevation was maintained throughout the 7 days of the study. In addition, the STZ-treated rats also exhibited higher blood osmolarity than the non–STZ-treated group before ICH induction (458.7±12.3 versus 342±10.9 mOsmo/L; P=0.0001). Serum sodium and potassium concentrations, body weight, and body temperature were not significantly different between groups (data not shown).

MRI Findings
Figure 4A shows that, on T2WI, the hematomas in the 2 groups seemed similarly homogenous. On DWI, the STZ-treated group exhibited heterogeneous signal intensities as compared with the non–STZ-treated group. In Figure 4B, significantly lower DWI signal ratios in the hematoma were detected in the STZ-treated group during the 7 days (Figure 4B). Signal changes on T2WI were not different between the 2 groups (Figure 4C).

AQP-4 Expression
Immunofluorescent staining and the quantitative analysis (Figure 5A) showed that AQP-4 expression was significantly lower in the STZ-treated group on days 1 and 4 after ICH, but no difference was noted on day 7. Consistently, Western blotting (Figure 5B) showed AQP-4 expression was significantly lower in the STZ-treated group on days 1 and 4 after ICH, but no difference was noted on day 7. Reverse transcriptase-polymerase chain reaction (Figure 5C) showed that cerebral expression of AQP-4 was significantly lower in the STZ-treated group on day 1, but not difference was noted on days 4 and 7 after ICH.

Cell Apoptosis
The number of terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling–positive cells in STZ-treated rats on day 1 was significantly greater than in non–STZ-treated rats, but no difference was noted on days 4 and 7 (Figure 6).

Discussion
Perihematoma cerebral edema is a major complication of ICH, and hyperglycemia has been shown to exacerbate...
perihematoma edema and contribute to cell death after ICH, leading to worse outcomes. AQP-4 plays a critical role in the maintenance of water homeostasis in the brain, and its expression is under a sophisticated regulation system that responds to stimuli such as changes in osmolarity and the release of glutamate. The present study demonstrated that AQP-4 expression in the brain was downregulated in hyperglycemic ICH. These findings are consistent with previous studies in which diabetes mellitus was associated with alteration of AQP-4 expression in retinocytes and renal cells. The relationship between alterations in AQP-4 expression and different neurological outcomes related to cerebral edema suggests a connection between brain water content and severity of BBB destruction.

The perivascular endfeet of astrocytes that play a key role in cerebral water channels contain a high density of orthogonal arrays of particles containing AQP-4. The orthogonal arrays of particles/AQP-4 polarities of astrocytes correlate with the expression of agrin, a heparin sulfate proteoglycan on the basal lamina, which is closely related to BBB integrity and is required for segregation of AQP-4. When the BBB is disrupted after an ICH, agrin and orthogonal arrays of particles/AQP-4 are released, resulting in upregulation of AQP-4. Agrin is downregulated in hyperglycemia, thus increased BBB destruction, less AQP-4 production, and more severe cellular breakdown associated with necrosis would be expected as compared with a normoglycemic state.

Figure 2. Forelimb grip strength performance. More severe functional impairment was seen in streptozotocin (STZ)-treated rats after intracerebral hemorrhage (ICH).

Figure 3. Blood–brain barrier (BBB) disruption as determined by Evans blue dye injection. Quantification of Evans blue in brain tissue showed more severe BBB destruction in the streptozotocin (STZ)-treated group as time progressed.
DWI and T2WI used in combination are sensitive to differentiate between the cytotoxic and the vasogenic forms of edematous lesions. Vasogenic edema is associated with high or pseudonormal DWI and high T2 signals at the lesion site. On days 1 and 4 after normoglycemic ICH, the injury seemed high or pseudonormal on DWI and high on T2WI. This indicates

**Figure 4.** In vivo changes in hematoma formation and edema by MRI. A, On diffusion-weighted imaging (DWI), the hematomas in the streptozotocin (STZ)-treated group were more heterogeneous in appearance compared with the non-STZ-treated group. On T2-weighted imaging (T2WI), the hematomas were similarly homogenous between the 2 groups. B, The hematomas of the STZ-treated group showed a significantly lower signal ratio on DWI during the 7 days. C, No difference of signal ratio on T2WI was observed between the 2 groups.

**Figure 5.** Aquaporin-4 (AQP-4) expressions. A, Immunofluorescent staining showed AQP-4 expression was significantly lower in the streptozotocin (STZ)-treated group on days 1 and 4 after ICH, but no difference was noted on day 7. B, Western blotting showed AQP-4 expression was significantly lower in the STZ-treated group on days 1 and 4 after ICH, but no difference was noted on day 7. C, Reverse transcriptase-polymerase chain reaction (RT-PCR) showed that cerebral expression of AQP-4 was significantly lower in the STZ-treated group on day 1, but not difference was noted on days 4 and 7 after intracerebral hemorrhage.
that vasogenic edema was ongoing. On day 7, the DWI signal of the injury site dropped further, whereas the T2WI signal returned to a level similar to that of the unaffected contralateral site. The further DWI signal reduction reflected the loss of physical barriers to diffusion and thus might be associated with severe cellular breakdown. In the meantime, the water content of the injured site was likely resolved toward normal levels given signal pattern of T2WI between days 4 and 7.

With respect to hyperglycemic ICH, on day 1 the injury was in the vasogenic edema phase as reflected by pseudo-normal DWI and high T2WI signals. But unlike the normoglycemic condition, hyperglycemia accelerated the ICH toward the necrotic stage. As a result, the injury signal seemed much lower on DWI at days 4 and 7 as compared with the normoglycemic ICH. This implies that hyperglycemic is associated with increased severity of cellular breakdown and necrosis.

As revealed by both DWI and T2WI, the hematoma formed under the hyperglycemic condition may be associated with accelerated cellular breakdown from the phase of vasogenic edema. This edematous/necrotic change may be caused by dysfunction of water exchange, transport, production, and reabsorption in the area. A previous study documented that AQP-4 is crucial for fluid clearance in vasogenic brain edema, suggesting that modulation of AQP-4 may be a novel therapeutic option for the treatment of vasogenic brain edema. Acetazolamide was used for the treatment of high altitude mountain sickness and cerebral edema and may be effective because of its anti-AQP-4 properties. Although further experiments are needed to confirm the connection between AQP-4 modulation and brain tissue damage in hyperglycemia ICH, our results indicate that it is likely that the vasogenic edema of ICH in hyperglycemia involves the altered regulation of AQP-4.

Temporal differences were noted in the data from the mRNA, protein, imaging, and behavior experiments. On day 1, the entire cascade was affected by hyperglycemia and ICH. On day 4, all but mRNA were still compromised. On day 7, the behavioral and brain imaging changes were still present. The results seem to suggest that hyperglycemia and ICH resulted in brief AQP-4 mRNA downregulation, transiently reduced protein expression, and relatively sustained effects at the behavioral and tissue levels. These changes associated with the signaling cascades involving the mRNA transcripts, proteins, brain systems, and behaviors reflect the particular modulatory mechanism of AQP-4 in the scenario of hyperglycemic ICH on the basis our experimental model.

AQP-4 inhibition has been proposed as a means of neuroprotection. However, the role of AQP-4 in the water homeostasis of brain diseases is very complicated. AQP-4 has been reported to be upregulated in some pathologies, but downregulated in others. The time window, magnitude, and directions of changes in AQP-4 transcription and protein expression vary with the associated brain damage and progression of the insult. Suitable regimens of AQP-4 modulation remain to be established in the complex scenario of hyperglycemic ICH.

Conclusions

In summary, the expression of AQP-4 in the brain is down-regulated in hyperglycemic rats as compared with the normoglycemic rats after ICH. This change is accompanied by
accelerated cellular breakdown from the phase of vasogenic edema and more severe BBB destruction. This finding may be exploited for reducing injury resulting from ICH.

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Disclosures

None.

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