Effect of Secondary Insults upon Aquaporin-4 Water Channels following Experimental Cortical Contusion in Rats

Keisuke Taya, Christina R. Marmarou, Kenji Okuno, Ruth Prieto, and Anthony Marmarou

Abstract

Although secondary insults of hypoxia and hypotension (HH) are generally considered to cause fulminant brain edema in traumatic brain injury (TBI), the combined effect of TBI with HH on brain edema and specifically the expression of aquaporin-4 (AQP4) have not been fully elucidated. The goal of this study was to document the effect of secondary insults on brain water, AQP4 expression, electrolytes, and blood–brain barrier (BBB) permeability during the acute stage of edema development. We measured brain water content and electrolytes (series 1); BBB permeability based on Evans blue (EB) dye extravasation (series 2); and AQP4 expression using immunoblotting (series 3) at 1 h and 5 h following cortical contusion injury (CCI). Secondary insults significantly worsened BBB function at 5 h post injury. Moreover, a significant reduction of upregulation on AQP4 expression was observed in trauma, coupled with a mild secondary insult of hypoxia hypotension. These findings indicate that a secondary insult following CCI at 5 h post injury worsens brain edema, disrupts ionic homeostasis, and blunts the normal upregulation of AQP4 that occurs after trauma, suggesting that the blunting of AQP4 may contribute to the detrimental effects of secondary insults.

Key words: aquaporin-4; blood–brain barrier; brain edema; controlled cortical impact; secondary insult; traumatic brain injury

Introduction

BRAIN EDEMA, THE INFILTRATION and accumulation of excess water in the brain, is a common and serious consequence of severe traumatic brain injury (TBI) often associated with a poor neurological outcome (Ghajar, 2000). In many cases, TBI is frequently accompanied by respiratory problems, resulting in secondary insults such as hypoxia and hypotension (Chestnut, 1995), and it is generally considered that following an initial trauma, the injured brain is highly vulnerable to secondary brain injury. Thirty years ago, Miller and associates (1978) reported that hypoxia and hypotension are adversely related to poor outcome following TBI in patients. Analysis of clinical data from the Traumatic Coma Data Bank revealed that hypoxia and hypotension, of which incidences were 45.6% and 34.6% respectively, were independently associated with significant increases in morbidity and mortality (Chestnut et al., 1993). In addition, they reported that the combined influences of hypoxia and hypotension were greater than their individual influences. Most recently, the IMPACT (International Mission on Prognosis and Analysis of Clinical Trials in TBI) study has confirmed that patients with both hypoxia and hypotension have poorer

outcomes than those with either insult alone following TBI (McHugh et al., 2007).

Currently, acute clinical intervention to ameliorate the effects of primary brain injury is limited and relates mostly to surgical procedures directed toward relieving mass effect. Consequently, the management goals of severe TBI are primarily directed toward preventing secondary injuries. Several experimental methods have been used to study the effect of secondary insults following severe TBI in producing a similar pathobiological sequence of events experienced by head-injured patients; however, the underlying mechanisms are not well defined. The identification of the precise pathogenesis that leads from secondary insults to secondary brain damage may provide a means of improving neurological outcome in the clinical setting.

Previous experimental reports assessing the effects of secondary insults have focused on evaluating the resulting histopathological neuronal damage (cell damage and death) and neurological outcome. Numerous studies have reported a worsening of neuronal damage and neurological outcome following a secondary insult (Bramlett et al., 1999; Clark et al., 1997). Among these investigations, a few focused on the early stage of edema following experimental TBI combined with a

secondary insult (Ishige et al., 1987a; Van Putten et al., 2005). In order to identify the pathophysiological mechanism inherent in edema formation, our laboratory has utilized and characterized an animal model of TBI coupled with hypoxia and hypotension (Barzó et al., 1996; Ito et al., 1996; Tavazzi et al., 2005; Yamamoto et al., 1999). In focal injury, our studies of controlled cortical impact (CCI) have suggested that the role of vasogenic edema may have been overemphasized in the past, and that traumatic brain edema is a combination of both vasogenic and cytotoxic edema, with the cellular component predominating (Barzó et al., 1997).

With regard to cellular edema, what are the mechanisms leading to increased water in the brain? Recently, many groups have proposed that aquaporin-4 (AQP4) plays a significant role in the pathophysiology of brain edema (Agre and Kozono, 2003; Kleindienst et al., 2006a; Manley et al., 2000). Aquaporins (AQPs), a family of water channel proteins that comprises at least 13 members in mammals, mediate rapid trans-membrane movement of water, and one member of this family, AQP4, is abundantly expressed in brain astrocytes and ependymal cells, the cells facing capillaries and the pia mater (Verkman, 2002). Manley and associates (2000) provided compelling evidence that AQP4 deletion protected mice from brain swelling in two models of primarily cytotoxic edema: water intoxication and permanent focal cerebral ischemia. Conversely, in a vasogenic edema model, Papadopoulos and associates (2004) found that AQP4-null mice had more vasogenic brain edema compared with wild-type mice, suggesting that AQP4 channels were involved in the clearance process of vasogenic edema. In earlier studies, Ishige and associates (1987a), utilizing brain-tissue specific-gravity measurements, reported that a much more widespread and severe brain edema developed in rats with fluid percussion impact injury and a secondary insult of hypoxia. Although a few reports in the last two decades have described water and electrolytes after TBI and hypoxia in the FPI model (Katayama et al., 1989), the effect of secondary insults and AQP4 on early edema formation in the CCI model has not been studied. Given the changes in brain water and electrolytes after injury, coupled with hypoxia and hypotension, we hypothesized that the effect of secondary insults in exacerbating brain edema may be mediated through the change of abundant AQP4 water channels in the injured brain.

The purpose of this study was to quantify and characterize the early stage of brain-edema formation, when early secondary insults of hypoxia and hypotension were superimposed with focal cortical contusion in rats. Accordingly, our experiments focused on the effect and possible mechanisms of secondary insults in the injured brain by assessing brain water content and electrolytes (series 1), blood-brain barrier (BBB) permeability based on Evans blue (EB) dye extravasation (series 2), and aquaporin-4 (AQP4) expression using immunoblotting (series 3) at 5 h post injury. To the best of our knowledge, this is the first report to investigate AQP4 expression following TBI, coupled with secondary insults of hypoxia and hypotension, in rats.

Methods

Animals

All experimental procedures involving animals were approved by the Virginia Commonwealth University (VCU)

Institutional Animal Care and Use Committee (IACUC), and were conducted in accordance with the recommendations provided in the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals. Rats were housed at $21 \pm 1^\circ\text{C}$ with 60% humidity, 12 h light/12 h dark cycles, and given pellet food and water *ad libitum*.

Experimental protocol

A total of 122 adult male Sprague-Dawley rats (Harlan, IN) weighing 350–430 g were divided into three experimental series (Table 1): brain water content and electrolytes (series 1, $n = 44$); quantitative analysis of BBB integrity (series 2, $n = 42$); and aquaporin-4 (AQP4) protein expression (series 3, $n = 36$). All rats were then randomly assigned into the following experimental groups: (1) sham-operated group (sham); (2) hypoxia and hypotension alone group (HH alone); (3) CCI alone group (CCI alone); and (4) CCI plus hypoxia and hypotension group (CCI + HH). All animals were assessed at 1 h and 5 h after injury.

Controlled cortical impact injury and surgical procedure

A well-established CCI injury model as previously described was used to cause TBI in the animals (Dixon et al., 1991). Rats were initially anesthetized with isoflurane (4.0%), intubated, and then artificially ventilated with a gas mixture of N_2O (67%), O_2 (33%) and isoflurane (0.4–2.0%). Rectal temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ using a heat lamp. Catheters (P.E.50, Becton Dickinson & Company, Sparks, MD) were placed into the femoral artery and femoral vein. Mean arterial blood pressure (mABP), arterial blood gas levels (pH, PaCO_2 , and PaO_2), and body temperature (BT) were monitored and recorded continuously using a data-acquisition system (ADInstruments, Colorado Springs, CO). Animals were placed on a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and secured using two ear bars and an incisor bar. A midline scalp incision was made, the skin and periosteum retracted from the skull surface, and a 10-mm-diameter craniotomy was made midway between the bregma and lambda on the right side, 1 mm lateral to the midline. Injury was produced using a pneumatic impactor bolt (10 mm, bevelled tip) mounted at an angle of 10° from the vertical plane. A single impact at a velocity of 6 m/sec with a deformation depth of 3.0 mm was delivered to the parietal cortex. After injury, the removed skull section was immediately replaced and sealed with bone wax and the incision was closed.

Secondary insult of hypoxia and hypotension

Secondary insults of hypoxia and hypotension (HH) were induced immediately following CCI for a period of 30 min as previously described (Yamamoto et al., 1999). Hypoxia was induced by reducing inspired oxygen (FiO_2) to 12%, and hypotension was produced by increasing the isoflurane level to 2% above baseline levels while increasing the inspired nitrous oxide to 88%. Reduction of PaO_2 and mABP to 40 mm Hg was maintained for the duration (30 min) of the secondary insults.

Brain edema measurement (series 1)

Percentage brain-water content was determined by using the wet and dry weight method. Animals were killed by de-

TABLE 1. EXPERIMENTAL GROUPS

Series	Sacrifice time	Group name	Animals n	Injury	Secondary insult (30 min)
Series 1: Brain water and electrolytes					
	1 h post-injury	Sham	5	Non-injured	—
		HH alone	5	Non-injured	+
		CCI alone	6	Injured	—
		CCI + HH	6	Injured	+
	5 h post-injury	Sham	5	Non-injured	—
		HH alone	5	Non-injured	+
		CCI alone	6	Injured	—
		CCI + HH	6	Injured	+
Series 2: BBB integrity					
	1 h post-injury	Sham	4	Non-injured	—
		HH alone	5	Non-injured	+
		CCI alone	6	Injured	—
		CCI + HH	6	Injured	+
	5 h post-injury	Sham	4	Non-injured	—
		HH alone	5	Non-injured	+
		CCI alone	6	Injured	—
		CCI + HH	6	Injured	+
Series 3: AQP4 expression					
	1 h post-injury	Sham	3	Non-injured	—
		HH alone	3	Non-injured	+
		CCI alone	6	Injured	—
		CCI + HH	6	Injured	+
	5 h post-injury	Sham	3	Non-injured	—
		HH alone	3	Non-injured	+
		CCI alone	6	Injured	—
		CCI + HH	6	Injured	+

HH, hypoxia and hypotension; CCI, controlled cortical impact injury; BBB, blood-brain barrier; AQP4, aquaporin-4.

TABLE 2. PHYSIOLOGICAL DATA AT BASELINE, 10, 25 MIN AND 1 H AFTER TRAUMA

Group	BT (°C)	mABP (mm Hg)	pH	PaCO ₂ (mm Hg)	PaO ₂ (mm Hg)	Plasma Na (mEq/L)
Sham (<i>n</i> = 24)						
Baseline	37.1 ± 0.0	87.5 ± 1.4	7.43 ± 0.01	37.5 ± 1.0	158.9 ± 3.2	139.6 ± 0.5
10 min	37.0 ± 0.0	88.0 ± 1.4	7.43 ± 0.01	37.0 ± 1.0	160.9 ± 4.2	140.1 ± 0.7
25 min	37.0 ± 0.0	87.2 ± 2.0	7.43 ± 0.01	37.6 ± 1.0	156.9 ± 4.1	139.9 ± 0.5
1 h	37.0 ± 0.1	88.6 ± 1.6	7.44 ± 0.01	36.8 ± 1.1	160.7 ± 3.4	140.1 ± 0.5
HH alone (<i>n</i> = 26)						
Baseline	37.0 ± 0.0	89.3 ± 1.5	7.42 ± 0.01	37.4 ± 1.1	159.0 ± 5.4	140.5 ± 0.5
10 min	37.0 ± 0.1	43.4 ± 1.3 ^{a,b,c}	7.38 ± 0.01 ^{a,b,c}	39.3 ± 1.4	39.8 ± 2.5 ^{a,b,c}	140.7 ± 0.6
25 min	37.0 ± 0.1	41.2 ± 1.2 ^{a,b,c}	7.33 ± 0.02 ^{a,b,c}	37.7 ± 1.2	40.5 ± 1.8 ^{a,b,c}	139.7 ± 0.6
1 h	37.0 ± 0.1	90.2 ± 3.2	7.38 ± 0.01 ^{a,b,c}	37.9 ± 0.9	160.8 ± 6.5	140.7 ± 0.5
CCI alone (<i>n</i> = 36)						
Baseline	36.9 ± 0.1	88.9 ± 1.4	7.42 ± 0.01	37.3 ± 1.2	156.1 ± 3.9	139.9 ± 0.9
10 min	36.9 ± 0.1	89.1 ± 1.6	7.41 ± 0.01	38.4 ± 1.2	156.3 ± 4.0	138.7 ± 0.8
25 min	36.9 ± 0.1	89.5 ± 1.3	7.42 ± 0.01	37.7 ± 1.6	153.1 ± 4.5	140.0 ± 0.5
1 h	37.0 ± 0.0	88.3 ± 1.5	7.43 ± 0.01	37.8 ± 0.9	158.5 ± 3.6	139.5 ± 0.8
CCI + HH (<i>n</i> = 36)						
Baseline	37.0 ± 0.1	88.6 ± 1.0	7.42 ± 0.01	36.5 ± 1.0	155.5 ± 2.9	139.4 ± 0.7
10 min	37.0 ± 0.1	42.9 ± 1.0 ^{a,b,c}	7.34 ± 0.01 ^{a,b,c}	37.4 ± 1.3	38.3 ± 1.3 ^{a,b,c}	139.5 ± 0.7
25 min	37.0 ± 0.1	39.8 ± 0.9 ^{a,b,c}	7.26 ± 0.02 ^{a,b,c}	36.8 ± 1.4	40.2 ± 1.4 ^{a,b,c}	139.8 ± 0.9
1 h	37.0 ± 0.0	89.7 ± 1.7	7.36 ± 0.01 ^{a,b,c}	36.2 ± 0.9	155.6 ± 3.7	139.0 ± 0.7

BT, body temperature; mABP, mean arterial blood pressure. Values are expressed as mean ± SEM.

Data was analyzed by unpaired *t* test. No significant differences were found between sham and CCI alone groups.

^a*p* < 0.05 versus CCI alone.

^b*p* < 0.05 versus sham.

^c*p* < 0.05 versus baseline.

capitation. The skull was removed, the intact dura excised, and the brains were quickly removed. The cerebellums were discarded, the right and left hemispheres were separated along the anatomic midline, and the wet weight of each hemisphere was measured. The tissues were completely dried in an oven at 95°C for 5 days, and the dry weight of each hemisphere was recorded. The percentage water content (% water) was calculated for each hemisphere as follows:

$$\% \text{water} = [(wet\ weight - dry\ weight) / wet\ weight] \times 100.$$

To measure the brain sodium and potassium concentrations, the dried samples were desiccated by heating for 24 h at 400°C. The resulting ash was extracted with distilled water and the concentrations of sodium and potassium were determined using a flame photometer (943; Instrument Laboratory, Milan, Italy) with cesium as an internal standard (Fukui et al., 2003).

Evaluation of blood–brain barrier integrity (series 2)

The integrity of the BBB was evaluated by assessing the extravasation of EB dye as described previously (Fukui et al., 2003). Briefly, 2% solution of EB dye (E515; Fisher Scientific, Fairlawn, NJ) in 0.9% NaCl was administered intravenously over 1 min in a dose of 7 mL/kg, and then allowed to circulate for 30 min prior to sacrifice. At 1 h and 5 h post injury, a thoracotomy was performed after an overdose of isoflurane (5%). Therefore, in the 1-h post-injury groups, EB dye was admin-

istered at 30 min post injury, whereas in the 5-h group, EB dye was administered at 4.5 h post injury. Brains were perfused with approximately 700 mL of saline via a catheter inserted into the left ventricle of the heart, exposed, and the intact dura incised and rapidly removed. The cerebrum of the brain was cut with a blade into the right and left hemispheres along the anatomic midline. Each hemispheric section was placed separately in a precisely measured volume (2 mL) of formamide and allowed to soak for 48 h at room temperature. The supernatant solution was transferred to a microcuvette, and the absorbance of each solution was measured against a pure formamide standard at 625 nm using a Shimadzu UV 1601 Spectrophotometer (Shimadzu Instruments, Columbia, MD). Data was expressed as the relative absorbance (unit/g wet weight) and compared to normal values obtained from sham rats that were intravenously injected with EB solution for 30 min and sacrificed at 5 h without any injury or treatment (sham group in series 2, Table 1).

Immunoblotting for aquaporin-4 (series 3)

For western blotting, animals were killed by decapitation, the skull dissected, the dura excised, and the cerebral tissue immediately separated into injured (right) and noninjured (left) hemispheres, excluding the most rostral and caudal sections from further analysis. The obtained injured and noninjured hemispheres were homogenized on ice with a tissue homogenizer in radioimmunoprecipitation (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium *n*-dodecyl sulfate (SDS), 1% sodium deoxycholate, pH 7.2) containing proteolysis inhibitors (AEBSF 5 mM, Aproti-

FIG. 1. Effects of secondary insults on brain-edema formation following experimental cortical contusion. At 1 h and 5 h after injury, both CCI alone and CCI + HH groups showed significantly higher water content in the injured hemisphere when compared to the sham and HH alone groups. Comparison of the CCI alone and CCI + HH groups at 1 h showed no significant change in water content. At 5 h post-injury, comparison of the CCI alone and CCI + HH groups showed a statistically significant increase in water content. There was no significant difference between the sham and HH alone groups in both injured (right) hemisphere and noninjured (left) hemisphere at 1 h and 5 h. Data are expressed as mean $\pm$ standard error of mean (SEM) and were analyzed by one-way ANOVA followed by Tukey's post-hoc test. (* $p < 0.001$ compared with the sham and HH alone groups, $\dagger p < 0.004$ compared with the CCI alone group.)

FIG. 2. Effects of secondary insults on brain sodium concentrations following experimental cortical contusion. At 1 h and 5 h after injury, both CCI alone and CCI + HH groups showed significantly higher brain sodium concentrations in the injured hemisphere than either the sham or HH alone group. (* $p < 0.025$ compared with the sham and HH alone groups). The CCI + HH group showed significantly increased brain sodium concentrations in the injured (right) hemisphere at 5 h post-injury ($\dagger p < 0.025$ compared with the CCI alone group). There was no significant difference between the sham and HH alone groups in both the injured (right) hemisphere and noninjured (left) hemisphere at 1 h or 5 h post-injury. Data are expressed as mean $\pm$ standard error of mean (SEM) and were analyzed by one-way ANOVA followed by Tukey's post-hoc test.

FIG. 3. Effects of secondary insults on brain potassium concentrations following experimental cortical contusion. At 1 h post injury, both the CCI alone and CCI + HH groups showed a lower brain potassium level in the injured (right) hemisphere than did both the sham and HH alone groups, although this difference was not statistically significant. At 5 h post injury, the brain potassium level in the injured hemisphere was significantly lower in the CCI + HH group than in the both the sham and HH alone groups (* $p < 0.03$ compared with the sham group, † $p < 0.03$ compared with the HH group). There was no significant difference among the sham, HH alone, and CCI alone groups in the injured hemisphere. Data are expressed as mean $\pm$ standard error of mean (SEM) and were analyzed by one-way ANOVA followed by Tukey's post-hoc test.

nin 1.5 mM, E-64 Protease Inhibitor 10 μ M, Leupeptin 10 μ M, EDTA 5 mM). Homogenates were centrifuged at 13500g at 4°C for 30 min to obtain supernatants and remove nuclei and mitochondria. The protein concentration of each supernatant was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) and samples were adjusted to the same concentrations (0.6 μ g/ μ L) using a sample buffer (Invitrogen, Carlsbad, CA). Protein (15 μ g) from each sample was loaded for electrophoresis into 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA), and subsequently transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA). After the transfer, membranes were blocked for 45 min at room temperature in Tris-buffered saline plus Tween-20 (TBS-T) (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) with 3% milk powder, then incubated overnight at 4°C in the same buffer with a mouse monoclonal antibody against AQP4 (Abcam, Inc., Cambridge, MA) diluted 1:750. The membrane was then incubated at room temperature for 20 min in primary antibody, washed three times for 10 min in TBS, blocked for 30 min, and subsequently incubated for 2 h in TBS-T plus 3% milk with a horseradish peroxidase (HRP) conjugated goat anti-mouse (Rockland Gilbertsville, PA) diluted 1:5000. After two washes in TBS-T and three in TBS, immunodetection of AQP4 proteins was accomplished using an enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK). Densitometric analysis was used to quantify AQP4 protein expression levels by determining intensity values for each band relative to cyclophilin-A (used as an internal control for lane loading).

Statistical analysis

Data are expressed as mean $\pm$ standard error of mean (SEM). Physiological values were analyzed using a Student's unpaired two-tailed t test. Series 1, 2, and 3 were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Values of $p < 0.05$ were considered significant. Analysis was performed using SPSS statistical software (V13.0; SPSS, Inc., Chicago, IL).

Results

Physiological values

Physiological measurements of PaO₂, PaCO₂, mABP, pH, plasma sodium level, and body temperature (BT) values before, during, and after 30 min of secondary insults are summarized in Table 2. In the CCI alone group, mABP transiently increased at the moment of the impact, but returned to baseline values within 10 min. During induction of the secondary insult, both the CCI + HH and the HH alone groups had mean PaO₂ values of 39.3 ± 1.4 and 40.2 ± 2.2 mm Hg respectively. These numbers fell well within the range for hypoxia (PaO₂ = 35–45 mm Hg) that has been reported in other studies (Ishige et al., 1987b; Yamamoto et al., 1999). Similarly, mABP dropped in both the CCI + HH and the HH alone groups to averages of 41.4 ± 1.0 and 42.3 ± 1.3 mm Hg during the secondary insults. In the CCI + HH and HH alone groups, post-traumatic pH, PaO₂, and mABP were significantly decreased compared with other groups. Physiological measurements for the CCI alone and the sham groups were not statistically significant.

Brain-edema measurement (series 1)

Brain water content. CCI resulted in increased water content when compared to sham values at 1 h and 5 h post injury (Fig. 1). Importantly, secondary insults of hypoxia and hypotension exacerbated brain-edema formation in the injured hemisphere at only 5 h after injury following CCI.

At 1 h after injury, both the CCI alone ($79.96 \pm 0.17\%$, $n = 6$, $p < 0.001$) and the CCI + HH ($79.67 \pm 0.11\%$, $n = 6$, $p < 0.001$) groups showed significantly higher water content in the injured hemisphere than did both the sham ($78.24 \pm 0.17\%$, $n = 5$) and the HH alone ($78.22 \pm 0.12\%$, $n = 5$) groups. However, there was no significant difference between the CCI alone and CCI + HH groups in the injured hemisphere at this time point ($p = 0.456$).

At 5 h after injury, brain-water content in the injured hemisphere was significantly higher in the CCI + HH group ($81.44 \pm 0.25\%$, $n = 6$, $p < 0.004$) than in the CCI alone group ($80.44 \pm 0.17\%$, $n = 6$). There was no significant difference between the sham and HH alone groups in both hemispheres at the 1- and 5-h time point. Therefore, secondary insults of hypoxia and hypotension adversely affected brain-edema formation in CCI rats.

Brain sodium level. Secondary insults of hypoxia and hypotension increased brain sodium concentration in the injured hemisphere 5 h after injury following CCI consistent with increased water content (Fig. 2).

At 1 h after injury, both the CCI alone (241 ± 7 mEq/kg dry weight, $n = 6$, $p < 0.002$) and the CCI + HH (229 ± 13 mEq/kg

FIG. 4. Demonstrable findings of the extravasation of Evans blue (EB) dye following experimental cortical contusion combined with a secondary insult. **(A)** EB was not observed in the sham or HH only groups at 1 or 5 h. At 1 h post injury, extravasation of EB was principally observed in the restricted area around the contusion in the CCI alone and CCI + HH groups compared with the sham and HH alone groups. At 5 h post-injury, EB was also observed in the CCI + HH and CCI alone groups compared with sham and HH alone groups. At 5 h post injury, EB was distributed bilaterally and observed in the injured and noninjured hemisphere in the CCI + HH group compared with the CCI alone group. **(B)** Semi-quantitative analysis of the extravasation of EB dye following the experimental cortical contusion combined with secondary insults. Compared with the sham and HH groups, the CCI alone and CCI + HH groups showed significant higher EB dye extravasation in the injured hemisphere at 1 h (* $p < 0.001$ compared with the sham and HH groups). At 1 h after injury, the relative absorbance of EB dye was higher in the CCI + HH group than the CCI alone group, although the difference was not statistically significant ($p = 0.053$). Compared with the sham and HH groups, the CCI + HH group showed significantly higher EB dye extravasation in the injured hemisphere at 5 h (* $p < 0.001$ compared with the sham and HH groups). At 5 h after injury, EB dye extravasation was significantly higher in the CCI + HH group than in the CCI alone group in the injured hemisphere († $p = 0.001$ compared with CCI alone group). EB in the noninjured hemisphere showed higher values in the CCI + HH group than in the CCI alone group, although this difference was not statistically significant ($p = 0.091$). There were no significant differences between the sham and HH alone groups at both 1 h and 5 h post injury. Data are expressed as mean $\pm$ standard error of mean (SEM) and were analyzed by ANOVA followed by Tukey's post-hoc test.

FIG. 5. Western blot analysis of aquaporin 4 (AQP4) expression at 1 h and 5 h post injury in the noninjured (left) and injured (right) hemispheres following experimental cortical contusion combined with secondary insults. (A) Representative Western blot analysis demonstrating AQP4 expression at 1 h post injury in the noninjured (left) and injured (right) hemispheres. Cyclophilin-A was used as an internal control for lane loading. (B) Immunoblot demonstrating AQP4 expression at 5 h post injury in the noninjured (left) and injured (right) hemispheres. Cyclophilin-A was used as an internal control for lane loading. (C) Bar-chart graph illustrates quantitative evaluation of densitometric values for AQP4 protein expression levels normalized against cyclophilin-A at 1 h and 5 h post injury in the noninjured (left) and injured (right) hemispheres. At 1 h and 5 h post injury, in both the CCI alone and the CCI + HH groups, a significant increase in AQP4 expression was observed in the injured (right) hemisphere when compared to that in the noninjured (left) hemisphere of the sham and HH alone groups at 1 h and 5 h post injury (* $p < 0.001$ compared with the sham and HH groups). At 1 h and 5 h post injury, comparison of AQP4 expression between the CCI only and CCI + HH groups showed a significant decrease in the injury-induced upregulation of AQP4 expression ($\dagger p < 0.05$ compared with the CCI alone group). Data are expressed as mean $\pm$ standard error of mean (SEM) and were analyzed by one-way ANOVA followed by Tukey's post-hoc test.

dry weight, $n = 6$, $p < 0.002$) groups showed significantly higher brain sodium levels in the injured hemisphere than did both the sham (170 ± 8 mEq/kg dry weight, $n = 5$) and the HH alone (168 ± 5 mEq/kg dry weight, $n = 5$) groups. There was no significant difference between the CCI alone and CCI + HH groups in the injured hemisphere at this time point ($p = 0.784$).

At 5 h after injury, brain sodium level in the injured hemisphere was significantly higher in the CCI + HH group (302 ± 22 mEq/kg dry weight, $n = 6$, $p < 0.03$) than in the CCI alone group (238 ± 11 mEq/kg dry weight, $n = 6$). There was no significant difference between the sham and HH alone groups in both hemispheres at 1 h and 5 h post injury. Therefore, TBI coupled with secondary insults of hypoxia and hypotension adversely affected brain sodium levels in CCI rats.

Brain potassium level. At 1 h post injury, both the CCI alone (326 ± 10 mEq/kg dry weight, $n = 6$) and the CCI + HH (309 ± 18 mEq/kg dry weight, $n = 6$) groups showed a lower brain potassium level in the injured hemisphere than did both the sham (352 ± 21 mEq/kg dry weight, $n = 5$) and the HH alone (359 ± 17 mEq/kg dry weight, $n = 5$) groups, although the difference was not statistically significant (Fig. 3).

At 5 h post injury, the brain potassium level in the injured hemisphere was significantly lower in the CCI + HH group (290 ± 17 mEq/kg dry weight, $n = 6$, $p < 0.03$) than in both the sham (353 ± 10 mEq/kg dry weight, $n = 5$) and the HH alone (354 ± 16 mEq/kg dry weight, $n = 5$) groups. There was no significant difference between the sham and HH alone groups in the right hemisphere ($p = 1.000$). Also, there was no significant difference among the CCI alone (326 ± 12 mEq/kg dry weight, $n = 6$), HH alone, and sham groups in the right hemisphere (respectively, $p = 0.539$, $p = 0.571$). Hence, secondary insults reduced brain potassium concentration in the injured hemisphere 5 h after injury.

Blood-brain barrier integrity (series 2)

We questioned the role of the BBB in edema formation. Figure 4A shows the extravasation of EB dye after the experimental CCI. Extravasation of the dye was principally observed in the restricted area around the contusion. At 5 h post injury, BBB leakage was observed in the CCI + HH and CCI alone groups compared with the sham and HH alone groups. However, the barrier leakage was more pronounced in both hemispheres in the CCI + HH group.

Semi-quantitative analysis revealed that secondary insults worsen BBB function assessed by EB dye extravasation (Fig. 4B). At 1 h after injury, the relative absorbance of EB dye was higher in the CCI + HH group (1.489 ± 0.197 unit/g wet weight, $n = 6$) than the CCI alone group (0.991 ± 0.119 unit/g wet weight, $n = 6$), although not statistically significant ($p = 0.053$). At 5 h after injury, the extravasation was significantly higher in the CCI + HH group (0.783 ± 0.156 unit/g wet weight, $n = 6$, $p < 0.01$) than the CCI alone group (0.335 ± 0.025 unit/g wet weight, $n = 6$) in the injured hemisphere. On the other hand, in the noninjured hemisphere, relative absorbance demonstrated higher values in the CCI + HH group (0.549 ± 0.255 unit/g wet weight, $n = 6$) than in the CCI alone group (0.053 ± 0.008 unit/g wet weight, $n = 6$), although the difference was not statistically significant ($p = 0.091$). Overall, there was no significant difference in

relative absorbance between the sham and HH alone groups at the 1- and 5-h time points. In contrast, a secondary insult combined with TBI exacerbated barrier permeability.

Aquaporin-4 expression (series 3)

AQP4 protein expression level was assessed by Western blot analysis in the injured hemisphere and in the noninjured hemisphere in the sham, HH alone, CCI alone, and CCI + HH groups. The most rostral and caudal sections were excluded from this analysis.

A representative immunoblot demonstrating AQP4 expression levels is shown in Figure 5A, B. Quantitative evaluation (Fig. 5C) showed in both the CCI alone and CCI + HH groups a significant increase of AQP4 expression in the injured (right) hemisphere when compared to that in the noninjured (left) hemisphere of the sham and HH alone groups at both 1 h and 5 h time points. Thus, we found that AQP4 protein expression was strongly upregulated in the injured brain. There was no significant difference between the sham and HH alone groups at 1 h and 5 h post injury. However, a significant decrease of upregulated AQP4 expression in the injured hemisphere was found in the CCI + HH group [1.423 ± 0.099 (1 h) $n = 6$, 1.435 ± 0.288 (5 h) $n = 6$] when compared with the CCI alone group [2.271 ± 0.183 (1 h) $n = 6$, 2.918 ± 0.289 (5 h) $n = 6$] at both 1 h and 5 h ($p < 0.002$, $p < 0.004$ respectively). Therefore, we observed that secondary insults decrease AQP4 expression despite the upregulation observed in TBI alone.

Discussion

The results of the present study clearly show that early post-traumatic secondary insults of hypoxia and hypotension exacerbate brain-edema formation at an early stage following CCI. These results are consistent with early studies in fluid percussion injury (FPI), which have shown that a secondary insult causes widespread brain edema (Ishige et al., 1988). Our laboratory has also demonstrated post-traumatic increases in brain edema and rapidly rising ICP, which can also contribute to secondary damage in the injured brain (Ito et al., 1996). Taking these findings together, worsened neurological damage and a poorer outcome are associated with secondary insults due to brain-edema formation exacerbated by early hypoxia and hypotension. We should emphasize that the main objective of the current series of studies was not to tease out the differential effect of either hypoxic or hypotensive events that contribute to morbidity and mortality following TBI. Rather, our intent was to utilize a model that most replicates the condition of a subset of patients that present with the worst case: the combination of hypoxia and hypotension following TBI. It is this combination that results in maximal production of edema, and the concomitant response of AQP4 under these severe conditions was important to understand more fully the role that AQP4 plays under these conditions.

Effect of hypoxia and hypotension on brain edema

Interestingly, we revealed that PaO₂ and mABP of 40 mm Hg for 30 min in the absence of TBI did not exacerbate brain-edema formation assessed 1 h and 5 h post injury, since there were no significant differences between the sham and HH alone groups on brain water content. These findings are similar to those of other investigations, which showed that a

PaO₂ of 40 mm Hg for 30 min in the absence of TBI did not produce ischemic neuronal changes or worsen neurological function (Ishige et al., 1988). Similarly, in normal rats, severe hypoxemia produced by a PaO₂ of 20 mm Hg for 20 min did not result in morphologic changes, increases in extracellular glutamate (Pearigen et al., 1996), or induction of the 72-kDa heat shock protein, which is known as a sensitive indicator of cellular stress in ischemia (Gonzalez et al., 1989). In summary, in the absence of trauma, our results are similar to other studies, and we show negligible effects on brain edema with a 30-min insult of hypoxia and hypotension.

Effects of secondary insults on tissue sodium and potassium

Conceptually, it is accepted that water and sodium tend to coexist and interchange in the physiologic and pathologic state (Gotoh et al., 1985). In the present studies, we found that hypoxia and hypotension after TBI significantly increased brain sodium concentrations in the injured hemisphere, though secondary insults without TBI had little effect on tissue sodium. In fact, as we expected, changes in brain sodium levels showed a time course parallel to that of the water content. This finding is consistent with previous reports showing a similar tendency between the water and sodium content at various periods (Gotoh et al., 1985; Kleindienst et al., 2006b). Furthermore, hypoxia and hypotension after TBI decreased brain potassium concentrations in the injured hemisphere, though secondary insults alone showed little effect. Therefore, we confirmed that secondary insults of hypoxia and hypotension following TBI exacerbate ionic dysfunction, whereas PaO₂ and mABP of 40 mm Hg for 30 min in the absence of TBI was not sufficient to aggravate ionic homeostasis. Katayama and associates (1989) reported that secondary insults exacerbate ionic dysfunction in the FPI model. These studies suggested that post-traumatic hypoxia and hypotension can produce significant detrimental secondary damage on brain edema and ionic function to the injured brain, even though it is not injurious on its own. These results are consistent with our studies. Thus, it reaffirms the notion that the injured brain is highly vulnerable to secondary brain injuries and edema, as well as ionic dysfunction, which can be exacerbated by post-traumatic hypoxia and hypotension.

The Na⁺-K⁺ ATPase ion pumps are responsible for maintaining ionic balance in glial cells and neurons. A decrease in energy supply to the brain results in a decrease in Na⁺-K⁺ ATPase activity. Consequently, it is conceivable that in an energy crisis, glial cells and neurons are unable to pump accumulated intracellular Na⁺ and cannot maintain ionic gradients (Ates et al., 2007). In the absence of brain trauma, approximately 90% of mammalian oxygen consumption is used by mitochondria, of which approximately 80% of oxygen consumption is coupled to ATP synthesis. Of this amount, ATP production is used by protein synthesis (25–30%), Na⁺-K⁺ ATPase (19–28%), Ca²⁺ ATPase (4–8%), actinomyosin ATPase (2–8%), glucogeneogenesis (7–10%), and ureagenesis (3%), with mRNA and substrate cycling also making significant contributions (Rolfe and Brown, 1997). In keeping with our previous studies (Tavazzi et al., 2005), we have recently stated that hypoxia and hypotension after TBI results in a disturbance of energy and neuron-specific metabolites, such as adenosine triphosphate (ATP) and *N*-acetylaspartate

(NAA). Following TBI, our laboratory showed that hypoxia and hypotension after injury strongly reduced ATP and NAA at 2, 6, 24, and 48 h. Although the exact nature of the pathophysiological mechanisms that contribute to deleterious effects of secondary insults are not fully elucidated, adding secondary insults of hypoxia and hypotension clearly worsens the energy metabolic crisis. Recent evidence provided confirmation that secondary hypoxemia exacerbates the FPI-induced disruptions of the energy metabolism in rats (Bauman et al., 2005). We posit that concomitant or progressive energy depletion during secondary hypoxia and hypotension related to mitochondrial impairment may propagate Na^+/K^+ ATPase failure and may aggravate brain-edema formation and ionic function.

Effect of secondary insults on blood–brain barrier function

In the present experiment, we utilized the CCI model to induce an experimental cortical contusion in rats, thereby leading to an increase in the BBB permeability as reported previously (Fukui et al., 2003) and demonstrated in the present study. We also found that secondary insults of hypoxia and hypotension exacerbated BBB permeability, delayed the recovery of BBB function, and promoted widespread BBB permeability. However, the BBB remained intact in the HH alone group. The extravasation of dye at 1 h post injury was highest in the CCI plus HH group, and this difference compared to the CCI alone group closely approached significance ($p = 0.053$). At 5 h post injury, the extravasation of dye with the CCI plus HH group was significantly higher than the CCI alone group ($p < 0.01$). Taking these findings together, it is reasonable to conclude that a secondary insult worsens BBB permeability, which reaches significance at 5 h post injury. Hypoxia after FPI delays the recovery of the BBB and contributes to the vascular pathogenesis of brain injury (Tanno et al., 1992). Post-traumatic secondary insults prolong the time of BBB dysfunction utilizing the impact-acceleration model of closed head injury combined with hypoxia and hypotension (Barzó et al., 1996). The fact that hypoxia and hypotension influence the status of the BBB after injury suggests that secondary insults are either directly injurious to the barrier, or a component of BBB repair after injury may be energy dependent. The present results, showing the relationship between early edema formation and BBB permeability to EB dye, lead us to consider that post-traumatic hypoxia and hypotension mainly contribute to BBB dysfunction and consequently worsen early vasogenic edema formation in CCI.

Effect of secondary insults on aquaporin-4

Water can cross cell membranes through different pathways: specific water channels (aquaporins), the lipid bilayer, and/or ion-water co-transport proteins (Zeuthen et al., 1997). Specifically, AQP4, the most abundant water channel in the central nervous system (CNS), has been reported to play a crucial role in cerebral water balance because of the anatomical and cellular localization on the membrane of astrocytic foot processes opposed to the brain capillaries, pia, and ependymal epithelium. Recently, AQP4 expression in water homeostasis during TBI and ischemic brain-edema development has been described, although controversial results have been obtained (Ke et al., 2001; Kiening et al., 2002; Neal et al.,

2007; Sun et al., 2003; Taniguchi et al., 2000). Several groups have reported that AQP4 expression is increased at the injury site in TBI, and is upregulated following stroke or water intoxication in rodents (Neal et al., 2007; Sun et al., 2003; Taniguchi et al., 2000). To the contrary, a few groups have also reported that AQP4 expression is reduced in the contusion model (Ke et al., 2001; Kiening et al., 2002). Our finding is consistent with the observed upregulation of AQP4 expression in the injured hemisphere and implicates *de novo* protein biosynthesis as a result of immunoactivity, though the precise mechanisms of AQP4 regulation have not completely been identified.

Influence of aquaporin-4 on brain edema

In the cellular edema model, strong evidence was presented that cellular edema is markedly reduced in AQP4-null mice (Manley et al., 2000). On the other hand, in the vasogenic edema model, Papadopoulos and associates (2004) reported that AQP4-null mice had more vasogenic brain edema compared with wild-type mice. In the latter case, one may conclude that a reduction of AQP4 prevents extracellular water from exiting the brain. However, it is equally possible that the increased water was simply due to an increased permeability in AQP4-null mice. In the experiments of this report, adding a secondary insult reduced AQP4 expression, and this was coupled with increased water content similar to the findings of Papadopoulos. Again, it may also be argued that the increased water content found in our studies was a result of increased barrier permeability exacerbated by the secondary insult. Thus, when barrier permeability is altered, it is difficult to isolate the precise role of AQP4 in brain edema. A partial answer is provided by our studies utilizing the infusion model of edema, where barrier properties remain intact and extracellular water is increased (Gulsen, 2006). In these studies, AQP4 was reduced pharmacologically and the clearance of edema was not affected (Gulsen, 2006). If, indeed, AQP4 pathways do not enhance the clearance of vasogenic edema, then we must conclude that the increased water found in our studies in the presence of reduced AQP4 was due to the inability of *cellular* water to exit. It is also noted that a similar downregulation of AQP4 occurs with a secondary insult at 1 h post injury compared to injury alone. In this case, as the insult was 30 min in duration, the water content, although increased compared to the sham group, was limited to the edema that developed within a 30-min period and thus does not reflect the magnitude of change seen at 5 h.

Studies in animals and head-injured patients have shown that the predominant edema in TBI is cellular and not vasogenic (Marmarou, 2006). Thus, taking these findings together, we reason there are at least two factors leading to increased water in our experiments: first, the exacerbation of edema due to increased barrier permeability; and second, the inability of *cellular* water to exit as a result of reduced AQP4 expression.

Effect of secondary insults upon aquaporin-4

The present study revealed that secondary insults of hypoxia and hypotension significantly downregulated AQP4 protein expression compared with TBI alone. These findings are consistent with previous *in vitro* studies where hypoxia was shown to downregulate protein and mRNA for AQP4 in astrocytic cultures (Fujita et al., 2003). Our study is the first to

describe changes in AQP4 expression after CCI combined with hypoxia and hypotension in rats. We found that water was increased as AQP4 was downregulated. This finding might be interpreted that the reduction of AQP4 expression by secondary insults contributes to the retardation of vasogenic edema resolution. The present BBB study would also support an increase in vasogenic edema, as secondary insults of hypoxia and hypotension increased BBB permeability assessed by EB dye extravasation. However, the fact that trauma in this CCI model induced a strong upregulation of AQP4 expression in the injured hemisphere compared with sham-operated animals sets the stage for more water entry into cells, and would support the opposing view that the increase in edema resulting from a secondary insult may be predominantly cellular. Moreover, studies by our laboratory (Gulsen et al., 2006) indicate that downregulation of AQP4 does not affect resolution of extracellular water. How do these results impact upon strategies for treating brain swelling? Under a profound BBB breakdown, techniques to enhance AQP4 levels may be beneficial. In contrast, with a predominantly cellular edema, AQP4 inhibition may be a novel therapeutic strategy for the treatment of brain edema. Further studies are required to investigate more thoroughly the precise mechanism of secondary insults on AQP4, and determine what type of intervention is most effective following TBI with HH in patients.

Conclusion

In conclusion, a secondary insult following CCI worsens brain edema, disrupts ionic homeostasis, and blunts the normal upregulation of AQP4 that occurs following trauma. The net increase in brain water can be attributed to a greater contribution of vasogenic edema by a compromised BBB and/or a retardation of clearance of both cellular and vasogenic edema components, which we suspect occurs by a downregulation of AQP4.

Acknowledgments

We would like to thank Caroline Dermer for her technical assistance in the preparation of this manuscript. This research was supported by grants RO1 NS12587, RO1 NS19235 from the National Institutes of Health, Bethesda, MD; RFP 07-302 from the Commonwealth Neurotrauma Initiative Trust Fund, Richmond, VA; and the Beverly and Wallace Hudson Research Fund. The contents are the sole responsibility of the authors and do not necessarily represent the official views of the Fund or the Advisory Board.

Author Disclosure Statement

No competing financial interests exist.

References

- Agre, P., and Kozono, D. (2003). Aquaporin water channels: Molecular mechanisms for human diseases. *FEBS Lett.* 555, 72–78.
- Ates, O., Cayli, S., Gurses, I., Yucel, N., Altinoz, E., Iraz, M., Kocak, A., and Yologlu, S. (2007). Do sodium channel blockers have neuroprotective effect after onset of ischemic insult? *Neurol. Res.* 29, 317–323.
- Barzó, P., Marmarou, A., Fatouros, P., Corwin, F., and Dunbar, J. (1996). Magnetic resonance imaging –Monitored acute blood–brain barrier changes in experimental traumatic brain injury. *J. Neurosurg.* 85, 1113–1121.
- Barzó, P., Marmarou, A., Fatouros, P., Hayasaki, K., and Corwin, F. (1997). Contribution of vasogenic and cellular edema to traumatic brain swelling measured by diffusion-weighted imaging. *J. Neurosurg.* 87, 900–907.
- Bauman, R.A., Widholm, J., and Long, J.B. (2005). Secondary hypoxia exacerbates acute disruptions of energy metabolism in rats resulting from fluid percussion injury. *Behav. Brain Res.* 7, 25–33.
- Bramlett, H.M., Dietrich, W.D., and Green, E.J. (1999). Secondary hypoxia following moderate fluid percussion brain injury in rats exacerbates sensorimotor and cognitive deficits. *J. Neurotrauma* 16, 1035–1047.
- Chestnut, R.M. (1995). Secondary brain insults after head injury: Clinical perspectives. *New Horiz.* 3, 366–375.
- Chestnut, R.M., Marshall, L.F., Klauber, M.R., Blunt, B.A., Baldwin, N., Eisenberg, H.M., Jane, J.A., Marmarou, A., and Foulkes, M.A. (1993). The role of secondary brain injury in determining outcome from severe head injury. *J. Trauma* 34, 216–222.
- Clark, R.S., Kochanek, P.M., Dixon, C.E., Chen, M., Marion, D.W., Heineman, S., DeKosky, S.T., and Graham, S.H. (1997). Early neuropathologic effects of mild or moderate hypoxemia after controlled cortical impact injury in rats. *J. Neurotrauma* 14, 179–189.
- Dixon, C.E., Clifton, G.L., Lighthall, J.W., Yaghamai, A.A., and Hayes, R.L. (1991). A controlled cortical impact model of traumatic brain injury in the rat. *J. Neurosci. Methods* 39, 253–262.
- Fujita, Y., Yamamoto, N., Sobue, K., Inagaki, M., Ito, H., Arima, H., Morishima, T., Takeuchi, A., Tsuda, T., Katsuya, H., and Asai, K. (2003). Effect of mild hypothermia on the expression of aquaporin family in cultured rat astrocytes under hypoxic condition. *Neurosci. Res.* 47, 437–444.
- Fukui, S., Fazzina, G., Amorini, A.M., Dunbar, J.G., and Marmarou, A. (2003). Differential effects of atrial natriuretic peptide on the brain water and sodium after experimental cortical contusion in the rat. *J. Cereb. Blood Flow Metab.* 23, 1212–1218.
- Ghajar, J. (2000). Traumatic brain injury. *Lancet* 356, 923–929.
- Gonzalez, M.F., Shiraishi, K., Hishanaga, K., Sagar, S.M., Mandabach, M., and Sharp, F.R. (1989). Heat shock proteins as markers of neuronal injury. *Brain Res. Mol. Brain Res.* 6, 93–100.
- Gotoh, O., Asano, T., Koide, T., and Takakura, K. (1985). Ischemic brain edema following occlusion of the middle cerebral artery in the rat. I: The time courses of the brain water, sodium and potassium contents and blood–brain barrier permeability to 125I-albumin. *Stroke* 16, 101–109.
- Gulsen, S., Ozisik, P., Okuno, K., and Marmarou, A. (2006). The effect of AQP4 suppression in resolution of extracellular edema. *J. Neurotrauma* 23, 1040.
- Ishige, N., Pitts, L.H., Berry, I., Carlson, S.G., Nishimura, M.C., Moseley, M.E., and Weinstein, P.R. (1987a). The effect of hypoxia on traumatic head injury in rats: Alterations in neurologic function, brain edema, and cerebral blood flow. *J. Cereb. Blood Flow Metab.* 7, 759–767.
- Ishige, N., Pitts, L.H., Hashimoto, T., Nishimura, M.C., and Bartkowski, H.M. (1987b). Effect of hypoxia on traumatic brain injury in rats: Part 1. Changes in neurologic function, electroencephalograms, and histopathology. *Neurosurgery* 20, 848–853.
- Ishige, N., Pitts, L.H., Berry, I., Nishimura, M.C., and James T.L. (1988). Effects of hypovolemic hypotension on high-energy

- phosphate metabolism of traumatized brain in rats. *J. Neurosurg.* 68, 129–136.
- Ito, J., Marmarou, A., Barzo, P., Fatouros, P., and Corwin, F. (1996). Characterization of edema by diffusion-weighted imaging in experimental traumatic brain injury. *J. Neurosurg.* 84, 97–103.
- Katayama, Y., Tamura, T., Becker, D.P., and Kayamata, T. (1989). Traumatic brain injury facilitates potassium flux during secondary ischemic insult. *Neurotrauma Soc.* 6, 200(abst).
- Ke, C., Poon, W.S., Ng, H.K., Pang, J.C., and Chan, Y. (2001). Heterogeneous responses of aquaporin-4 in oedema formation in a replicated severe traumatic brain injury model in rats. *Neurosci. Lett.* 23, 21–24.
- Kiening, K.L., van Landeghem, F.K., Schreiber, S., Thomale, U.W., von Deimling, A., Unterberg, A.W., and Stover, J.F. (2002). Decreased hemispheric aquaporin-4 is linked to evolving brain edema following controlled cortical impact injury in rats. *Neurosci. Lett.* 17, 105–108.
- Kleindienst, A., Fazzina, G., Amorini, A.M., Dunbar, J.G., Glisson, R., and Marmarou, A. (2006a). Modulation of AQP4 expression by the protein kinase C activator, phorbol myristate acetate, decreases ischemia-induced brain edema. *Acta Neurochir. Suppl.* 96, 393–397.
- Kleindienst, A., Fazzina, G., Dunbar, J.G., Glisson, R., and Marmarou, A. (2006b). Protective effect of the V1a receptor antagonist SR49059 on brain edema formation following middle cerebral artery occlusion in the rat. *Acta Neurochir. Suppl.* 96, 303–306.
- Manley, G.T., Fujimura, M., Ma, T., Noshita, N., Filiz, F., Bollen, A.W., Chan, P., and Verkman, A.S. (2000). Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat. Med.* 6, 159–163.
- Marmarou, A., Signoretti, S., Fatouros, P.P., Portella, G., Aygok, G.A., and Bullock, M.R. (2006). Predominance of cellular edema in traumatic brain swelling in patients with severe head injuries. *J. Neurosurg.* 104 (5), 720–730.
- McHugh, G.S., Engel, D.C., Butcher, I., Steyerberg, E.W., Lu, J., Mushkudiani, N., Hernández, A.V., Marmarou, A., Maas, A.I., and Murray, G.D. (2007). Prognostic value of secondary insults in traumatic brain injury: Results from the IMPACT study. *J. Neurotrauma* 24, 287–293.
- Miller, J.D., Sweet, R.C., Narayan, R., and Becker, D.P. (1978). Early insults to the injured brain. *JAMA* 240, 439–442.
- Neal, C.J., Lee, E.Y., Gyorgy, A., Ecklund, J.M., Agoston, D.V., and Ling, G.S. (2007). Effect of penetrating brain injury on aquaporin-4 expression using a rat model. *J. Neurotrauma* 24, 1609–1617.
- Papadopoulos, M.C., Manley, G.T., Krishna, S., and Verkman, A.S. (2004). Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. *FASEB J.* 18, 1291–1293.
- Pearigen, P., Gwinn, R., and Simon, R.P. (1996). The effects in vivo of hypoxia on brain injury. *Brain Res.* 725, N2, 184–191.
- Rolfe, D.F., and Brown, G.C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* 77, 731–758 Review.
- Sun, M.C., Honey, C.R., Berk, C., Wong, N.L., and Tsui, J.K. (2003). Regulation of aquaporin-4 in a traumatic brain injury model in rats. *J. Neurosurg.* 98, 565–569.
- Taniguchi, M., Yamashita, T., Kumura, E., Tamatani, M., Kobayashi, A., Yokawa, T., Maruno, M., Kato, A., Ohnishi, T., Kohmura, E., Tohyama, M., and Yoshimine, T. (2000). Induction of aquaporin-4 water channel mRNA after focal cerebral ischemia in rat. *Brain Res. Mol. Brain Res.* 31, 131–137.
- Tanno, H., Nockels, R.P., Pitts, L.H., and Noble, L.J. (1992). Breakdown of the blood–brain barrier after fluid percussion brain injury in the rat: Part 2: Effect of hypoxia on permeability to plasma proteins. *J. Neurotrauma* 9, 335–347.
- Tavazzi, B., Signoretti, S., Lazzarino, G., Amorini, A.M., Delfini, R., Cimatti, M., Marmarou, A., and Vagnozzi, R. (2005). Cerebral oxidative stress and depression of energy metabolism correlate with severity of diffuse brain injury in rats. *Neurosurgery* 56, 582–589.
- Van Putten, H.P., Bouwhuis, M.G., Muizelaar, J.P., Lyeth, B.G., and Berman, R.F. (2005). Diffusion-weighted imaging of edema following traumatic brain injury in rats: Effects of secondary hypoxia. *J. Neurotrauma* 22, 857–872.
- Verkman, A.S. (2002). Physiological importance of aquaporin water channels. *Ann. Med.* 34, 192–200.
- Yamamoto, M., Marmarou, C.R., Stiefel, M.F., Beaumont, A., and Marmarou, A. (1999). Neuroprotective effect of hypothermia on neuronal injury in diffuse traumatic brain injury coupled with hypoxia and hypotension. *J. Neurotrauma* 16, 487–500.
- Zeuthen, T., Meinild, A.K., Klaerke, D.A., Loo, D.D., Wright, E.M., Belhage, B., and Litman, T. (1997). Water transport by the Na⁺/glucose cotransporter under isotonic conditions. *Biol. Cell* 89, 307–312.

Address correspondence to:

Anthony Marmarou, Ph.D.

Department of Neurosurgery

Virginia Commonwealth University

1101 East Marshall Street, Box 980508, Sanger 9-036

Richmond, VA 23298-0508

E-mail: marmarou@vcu.edu