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Temporal alterations in aquaporin and transcription factor HIF1α expression following penetrating ballistic-like brain injury (PBBI)

Casandra M. Cartagena *, Katie L. Phillips, Frank C. Tortella, Jitendra R. Dave, Kara E. Schmid

Brain Trauma Neuroprotection and Neurorestoration Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910, USA

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ABSTRACT

Objectives: Brain edema is a primary factor in the morbidity and mortality of traumatic brain injury (TBI). The various isoforms of aquaporin 4 (AQP4) and aquaporin 9 (AQP9) are important factors influencing edema following TBI. Others have reported that these AQPs are regulated by the transcription factor hypoxia inducible factor (HIF) 1α. Therefore, we examined the temporal alterations in the multiple isoforms of AQP4 and AQP9, and its possible upstream regulation by HIF1α, and evaluated whether different severities of penetrating injury influence these mechanisms.

Methods: In the penetrating ballistic-like brain injury (PBBI) model, a temporary cavity and resultant injury was formed by the rapid inflation/deflation (i.e. <40 ms) of an elastic balloon attached to the end of the custom probe, injuring 10% of total rat brain volume. Tissue from the ipsilateral core and perilesional injury zones was collected. Total RNA was isolated at 4, 12, and 24 h, 3 and 7 days post-injury (sham and PBBI, n = 6 per group). cDNA was synthesized using oligodT primers. Quantitative real time PCR was performed using Taqman expression assays for *aqp4* (recognizing all isoforms), *aqp9*, and *hif1α*. Using separate animals, tissue lysate was collected at 4 and 24 h, 3 and 7 days post-injury and analyzed by immunoblot for protein expression of multiple isoforms of AQP4, the single known isoform of AQP9 and for expression of transcription factor HIF1α (sham, probe only control, and PBBI, n = 8–10 per group).

Results: Global a*qp4* mRNA was decreased at 24 h ($p < 0.01$) with PBBI. Three of the four known protein isoforms of AQP4 were detected, M1 (34 kDa), M23 (32 kDa) and isoform 3 (30 kDa). AQP4 M1 decreased at 3 and 7 days post-injury ($p < 0.001$; $p < 0.01$). AQP4 M23 levels were highly variable with no significant changes. AQP4 isoform 3 levels were decreased 3 days post-PBBI ($p < 0.05$). From 4, 12, and 24 h *aqp9* mRNA levels were decreased with injury ($p < 0.01$, $p < 0.05$, $p < 0.01$) while AQP9 levels were decreased at 3 and 7 days after PBBI ($p < 0.001$, $p < 0.01$). At 12 and 24 h post-PBBI hif1α mRNA levels increased ($p < 0.05$, $p < 0.01$) but at 3 and 7 days mRNA levels decreased ($p < 0.05$, $p < 0.01$). From 24 h and 3 and 7 days HIF1α protein levels were decreased ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$). In comparison to probe control, PBBI led to greater decreases in protein for AQP4 M1 (trend), AQP4 isoform 3 (trend), AQP9 ($p < 0.05$) and HIF1α ($p < 0.05$).

Conclusion: PBBI is characterized by a loss of AQP4 M1, AQP4 isoform 3 and AQP9 at delayed time-points. The severity of the injury (PBBI versus probe control) increased these effects. Therefore, AQP9 and the AQP4 M1 isoform may be regulated by HIF1α, but not AQP4 isoform 3. This delayed loss of aquaporins may markedly reduce the ability of the brain to efflux water, contributing to the protracted edema that is a characteristic following severe penetrating TBI. Factors contributing to edema differ with different types and severities of TBI. For example, cellular based edema is more prominent in diffuse non-penetrating TBI whereas vasogenic edema is more prevalent with TBI involving hemorrhage. Molecular regulation leading to edema will likely also differ, such that treatments which have been suggested for non-hemorrhagic moderate TBI, such as the suppression of aquaporins, may be detrimental in more severe forms of TBI.

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Introduction

Brain edema is a primary factor in the morbidity and mortality of traumatic brain injury (TBI) and in order to control or reduce edema, it may be necessary to understand what factors regulate edema in the injured brain. Some important factors influencing the observed edema following TBI are likely aquaporins. Aquaporins are membrane channels

* Corresponding author.
E-mail addresses: casandra.m.cartagena.civ@mail.mil (C.M. Cartagena), katie.phillips1@us.army.mil (K.L. Phillips), frank.c.tortella.civ@mail.mil (F.C. Tortella), jitendra.r.dave.civ@mail.mil (J.R. Dave), kara.e.schmid.mil@mail.mil (K.E. Schmid).

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Table 1
aqp4 mRNA findings in previous TBI studies.

Reference	Model	N	Time (h)	AQP4 mRNA reported findings	Recognized reference sequences
Ding et al. (2009)	Weight drop with helmet	5	1, 4, 24, 48	Increased in whole brain	NM_012825.3, NM_001142366.1, NM_001270558.1, NM_001270559.1
Ke et al. (2001)	Weight drop	4	24	Decreased at contusion site	

that allow water and other solutes to cross membrane barriers, including the blood brain barrier, and are therefore likely to play a role in regulating edema. Aquaporins in the brain include aquaporin 1, 4 and 9. Aquaporin 1 (AQP1) is expressed primarily at apical membranes of the choroid plexus epithelial cells (Mobasheri and Marples, 2004). Aquaporin 9 (AQP9) is expressed in astrocytes located within the corpus callosum, white matter tracks, as well as endothelial cells in pial vessels and catecholaminergic neurons (Badaut et al., 2004). Importantly, it is also present in astrocytic endfeet at the glia limitans (blood brain barrier) (Badaut et al., 2004) and thus plays a role in fluid influx and efflux between the brain and the periphery. Aquaporin 4 (AQP4) is the most abundant aquaporin in the brain (Zelenina, 2010) and therefore is a likely candidate for regulating edema. Although it is expressed throughout the brain, AQP4 has highest expression at astrocytic endfeet (Zelenina, 2010).

AQP4 has four confirmed isoforms: variant 1/M1 (34 kDa), variant 2/M23 (32 kDa), variant 3 (29 kDa) and variant 4 (25 kDa). AQP4 isoforms 3 and 4 are not well studied, however the isoforms M1 and M23 have both been shown to form functional channels with similar water permeability whether in the form of homotetramers or heterotetramers (Neely et al., 1999). Some differences in these isoforms have been demonstrated, including the greater ability of M23 to assemble square arrays of water channels while M1 formed singlet channels when expressed as homotetramers in cultures (Furman et al., 2003). AQP 1 and AQP9 have only been confirmed by the national center for biotechnology information (NCBI) to have single protein isoforms.

Logically, one proposed option for treating edema in TBI is the inhibition of aquaporins (Ding et al., 2009). Whether this would in fact be beneficial is debatable as AQP4 levels have been show to increase in some TBI studies (Ding et al., 2009; Guo et al., 2006; Neal et al., 2007; Taya et al., 2010) and decrease in others (Ke et al., 2001; Kiening et al., 2002). Of the TBI studies looking at AQP4 by Western blot, each reports a different kiladalton (kD) band size suggesting that the isoforms quantified in those studies are not the same and therefore are not comparable. A summary of these previous studies and their reported finding can be found in Tables 1 and 2.

If edema was to be regulated via aquaporin expression, one mechanism may be to affect presumptive upstream regulators such as hypoxia inducible factor (HIF) 1α. For example, in focal ischemia and moderate TBI, HIF 1α is upregulated locally in brain regions showing signs of hypoxia (Bergeron et al., 1999; Ding et al., 2009; Park et al., 2009). There is some in vivo evidence that both AQP4 and AQP9 may be regulated by HIF1α (Ding et al., 2009), suggesting HIF1α may be an upstream regulator of edema. However, HIF1α regulation after TBI has not been widely studied, except in a weight drop model of contusive TBI where HIF1α, AQP4 and AQP9 levels were reported increased after injury, effects mitigated by indirect inhibition of HIF1α with 2-methoxyestradiol (Ding et al., 2009).

Since there is a lack of consensus in previous studies of aquaporin regulation following TBI, and the causes and profile of edema differ depending on the type of TBI, a detailed study of aquaporin regulation should be examined in specific types of brain injury. Here we investigate the regulation of edema using the model of PBBI. PBBI is well characterized by its extensive volume of the brain injury (10% total brain volume), prevalence of intracerebral hemorrhage, and persistent ICP and edema. Previous studies of PBBI demonstrated that edema significantly increases within 4 h of injury, peaking from 3 to 5 days but continuing through 7 days (Shear et al., 2011). In addition, ICP levels begin to rise within hours of injury and peak at 24 h but remain upregulated through 3 days after injury (Wei et al., 2010). Therefore, we examined the temporal alterations in the multiple isoforms of AQP4 and AQP9, and its possible upstream regulation by HIF1α, and evaluated whether different severities of penetrating injury influence these mechanisms.

Results

AQP4

The relative quantity of *aqp4* mRNA was measured in PBBI injured tissue at 4 h, 12 h, 24 h, 3 days, and 7 days post-injury and compared to sham tissue at the corresponding time point. Transcript for *aqp4* showed a decreasing trend at 4 h post-injury and was significantly decreased at 24 h (t-test, $p < 0.01$). Later time points were similar to sham levels (Fig. 1). These initial mRNA experiments specifically compared sham to the PBBI injury and did not include a probe only control. However when initiating further experiments evaluating protein changes we included a probe control to determine whether severity of injury influenced aquaporin isoform levels post-injury.

Three isoforms of AQP4 were detected by Western blot. Using the 25 and 37 kDa standards and the pixel/mm distance between standards, these bands were estimated to be 34, 32, and 30 kDa in size. These correspond with the variant 1 or M1 isoform (NP_036957.1), the variant 2 or M23 isoform (NP_001135838.1) and the variant 3 (NP_001257487.1) respectively. The forth AQP4 isoform (NP_001257488.1) was not detected (Fig. 2). Currently there are no antibodies that target single AQP4 isoforms due to the very high homology across isoforms (Supplemental Fig. 1). We have therefore chosen to evaluate various AQP4 isoforms by Western blot. We detected three bands corresponding to three of the four known isoforms of AQP4, namely the AQP4 M1 isoform (34 kDa), the AQP4 M23 isoform (32 kDa), and the AQP4 isoform 3 (30 kDa). Detection of these bands was inhibited when the antibody was first blocked with AQP4 peptide (Supplemental Fig. 2A). Protein levels of these three AQP isoforms were measured in probe and PBBI injured tissue at 4 h, 24 h, 3 days and 7 days post-injury and compared to sham tissue at the corresponding time point. Representative blots of sham, probe and PBBI can be seen in Supplemental Fig. 2B. Global protein levels

Table 2
AQP4 protein findings in previous TBI studies.

Reference	Model	N	Time (h)	AQP4 protein reported findings	Method	Reported size (kDa)
Taya et al. (2010)	Unilateral CCI	6	1, 5	Increased ipsilateral hemisphere	Western	28
Guo et al. (2006)	Bilateral CCI	5	72	Increased pericontusional areas	Western	32
Ding et al. (2009)	Weight drop with helmet	5	1, 4, 24, 48	Increased in whole brain	Western	31
Neal et al. (2007)	PBBI	1	24, 72	Increased in ipsilateral cytosol	Western	40
Neal et al. (2007)	PBBI	6	24, 72	Decreased in injury core increased in perilesional area	IHC	Unknown
Kiening et al. (2002)	Unilateral CCI	5	48	Decreased in both hemispheres	Western	30
Ke et al. (2001)	Weight drop	6	4, 24	Decreased at contusion site	IHC	Unknown