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Short Communication

Expression of Aquaporin-4 Augments Cytotoxic Brain Edema after Traumatic Brain Injury during Acute Ethanol Exposure

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We previously reported that ethanol consumption affects morbidity and mortality after traumatic brain injury (TBI) by accelerating brain edema via oxidative stress after TBI. Aquaporin-4 (AQP4), a water channel, is involved in brain edema formation. In this study, we found that acute ethanol administration increased AQP4 expression after TBI, leading to severe brain edema in rats. Rats were pretreated with ethanol (3 g/kg) or DL-buthionine-(*S*,*R*)-sulfoximine (BSO; 100 mg/kg), an oxidative stressor, before TBI. Acetazolamide, an AQP4 inhibitor, was administered to ethanol-pretreated rats 3 or 12 hours after TBI. Brain edema was increased 24 hours after TBI in both the ethanol- and BSO-pretreated groups. Ethanol pretreatment induced lipid peroxidation 24 hours after TBI. Transcription factors, NF-κB and hypoxia-inducible factor-1α, were activated 3 and 24 hours after TBI in the BSO- and ethanol-pretreated groups, respectively. In the ethanol-pretreated group, AQP4 was accumulated, particularly in astrocyte end feet, 24 hours after TBI. Acetazolamide treatment improved the survival rate to 100% and decreased brain edema and AQP4 in ethanol-pretreated rats. These findings suggest that ethanol induces up-regulation of AQP4, leading to brain edema. The accumulation of AQP4 may play an important role in the augmentation of brain edema after TBI under ethanol consumption. ***(Am J Pathol 2012, 180:17–23; DOI: 10.1016/j.ajpath.2011.09.011)***

Ethanol consumption just before traumatic brain injury (TBI) is well known to aggravate morbidity and mortality.[1–4] On the other hand, other clinical studies[5–7] reported that ethanol consumption had no effect on the mortality of patients with TBI or that it can reduce it. Therefore, the effects of ethanol consumption on TBI remain unclear. Brain edema after TBI aggravates secondary injuries, including delayed hemorrhage, ischemia, and intracranial hypertension, which frequently leads to death.[8] Acute ethanol exposure induces primary rat astrocytes to swell.[9,10] Ethanol-induced swelling of astrocytes may be involved in inducing brain edema after TBI.

In a previous report,[11] we determined that prior ethanol injection exacerbates brain edema after TBI and that antioxidant treatment after TBI reduces the brain edema augmentation to promote survival. These findings suggested that accumulating oxidative stress under ethanol consumption plays a key role in augmentation of brain edema after TBI. Ethanol treatment alone did not induce brain edema.[11] Therefore, it remains unclear why ethanol causes severe brain edema only after TBI. Then, we hypothesized that the pretreatment with another oxidative stressor, such as DL-buthionine-(*S*,*R*)-sulfoximine (BSO), can clarify the role of oxidative stress in developing brain edema.

Aquaporin-4 (AQP4) is a water channel protein strongly expressed in the brain, predominantly in astrocyte foot processes at the borders between the brain parenchyma and major fluid compartments, including cerebrospinal fluid and blood.[12,13] AQP4 has detrimental effects in cytotoxic edema, including hyponatremia, early focal cerebral ischemia,[14] and bacterial meningitis.[15] On the other hand, vasogenic edema was decreased in AQP4-null mice with brain tumors.[16] These findings indi-

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cate that AQP4 plays a pivotal role in brain edema formation.

AQP4 is increased by hypoxic conditions associated with up-regulation of hypoxia-inducible factor-1α (HIF-1α).[17] In addition, the expression of AQP4 in response to IL-1β could be regulated by NF-κB in rat astrocytes.[18] We had observed that NF-κB activation was greatest 24 hours after TBI under ethanol pretreatment[11]; previously, ethanol activated NF-κB in the rat brain[19] and astrocytes.[20]

We hypothesized that brain edema due to TBI is significantly potentiated by acute ethanol exposure, which triggers signaling via transcription factors (NF-κB and HIF-1α) and leads to increased expression of edema-producing AQP4. To test this hypothesis, we determined whether ethanol augments the expression of AQP4 after TBI in the rat and evaluated whether brain edema was reduced by AQP4 inhibition, using acetazolamide (AZA), a well known carbonic anhydrase inhibitor, also recently identified as an inhibitor of AQP4.[21,22]

Materials and Methods

Animals

Male Wistar/ST rats (aged 8 to 9 weeks; weight, 270 to 300 g; Sankyo Labo Service Corporation Inc., Sapporo, Japan) were used in the present study. They were housed in cages and kept at 24°C with a normal 12-hour light–12-hour dark schedule (lights on at 7 AM). They had free access to food and water until 24 hours before TBI and were fasted overnight in the experiments. All experimental procedures were approved by the Animal Care and Use Committee of Sapporo Medical University School of Medicine (approval number 08–126). At 1 hour before TBI, rats were i.p. administered 10 mL/kg saline (saline group), 3 g/kg body weight ethanol (ethanol group), or 100 mg/kg body weight BSO (BSO group). Each group consisted of four to six animals. Dead animals were removed from analyses, except for survival analysis. Brain samples were obtained 3, 6, or 24 hours after TBI. AZA (Sigma-Aldrich, St. Louis, MO), an AQP4 inhibitor, was administered i.p. as doses of 10 mg/kg body weight (10 mL/kg), 3 hours (AZA3 group) or 12 hours (AZA12 group) after TBI under ethanol exposure (4 or 13 hours, respectively, after ethanol treatment).

TBI Formation

TBI was performed as previously described.[11] Briefly, each rat was i.p. injected with ketamine (75 mg/kg) and xylazine (10 mg/kg) and then placed in a stereotaxic frame (Narishige Scientific Instrument Lab, Tokyo, Japan) by fixing the ears. The midline scalp incision was performed, and the periosteum was removed from the skull surface. A 7-mm craniotomy was performed by drilling the right parietal bone, 1 mm right-lateral from the midline. A vertical Plexiglas tube that contained a 30-g steel weight was placed onto the rat's head using a stand. The weight was held in place 1 m above the end of the Plexiglas tube by a stopper. Before dropping the weight, it was manually lowered onto the brain surface to ensure precise TBI. After removing the weight, a trauma device was precisely lowered into the brain to a 1.5-mm depth using a stereotaxic manipulator. The weight was then dropped, achieving a final velocity of 4 m/second. The diameter of the weight surface affecting the brain was 4 mm. Any rebound impact was prevented by pulling on the rope attached to the weight. After TBI, the scalp was closed by sutures, with no remodeling of the skull bone for the prevention of increased intracranial pressure.

Estimation of Brain Edema by MRI

Under anesthesia with ketamine (75 mg/kg) and xylazine (10 mg/kg), magnetic resonance imaging (MRI) was performed using a 7-T, 18-cm-bore superconducting magnet (Oxford Magnet Technologies, Oxfordshire, UK) interfaced to a UNITY INOVA console (Oxford Instruments Inc., Oxfordshire, UK; and Varian, Palo Alto, CA), as previously described.[11] Each T_2-weighted image (T_2WI) was produced from a coronal section (1.0 mm thick) with a gap (0.5 mm thick) using a 30 $\times$ 30-mm field of view, a repetition time of 3000 milliseconds, an echo time of 37 milliseconds, and a *b* value of 0; reconstruction images were produced by a 256 $\times$ 256 image matrix. Diffusion-weighted images (DWIs) were produced with the same parameters used for T_2WIs, except with a *b* value of 1000. The MRIs were obtained 3, 6, or 24 hours after TBI. In this study, high-intensity areas in T_2WIs and DWIs, with a signal intensity 1.25 times higher than the contralateral brain area, were recognized as brain edema and brain contusion areas and areas of cytotoxic edema, respectively. A brain edema lesion, including the brain contusion area, was calculated from T_2WIs and DWIs using Scion Image (Scion Corporation, Frederick, MD).

Histopathological and IHC Findings

The brain was quickly extracted and fixed for 3 days in 10% neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, and 1.5% methanol, pH 7.0. Brains were cut into sections (5 μm thick) using a microtome and mounted on glass slides. All sections were stained with H&E. Immunoreactivity was visualized using the streptavidin-biotin method [Histofine Simple-stain MAX-PO (M) kit; Nichirei, Tokyo]. After washing the sections with PBS, they were treated with 3% (v/v) hydrogen peroxide in methanol for 10 minutes to inactivate endogenous peroxidase. They were then incubated with 1% nonfat milk, followed by incubation with rabbit polyclonal anti-AQP4 antibody (sc-20812; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:200 in PBS containing 1% nonfat milk. The sections were then incubated with secondary antibody [anti-rabbit IgG polyclonal antibody (Fab′) conjugated with peroxidase; Nichirei] and, finally, with streptavidin-biotin-peroxidase complex. After washing with PBS, the sections were counterstained with hematoxylin.

Extraction of Proteins in Cytosol and Nucleus

Brain contusion tissue (500 mg) was homogenized using a mechanical tissue homogenizer in TKM buffer, consisting of 0.32 mol/L sucrose; 50 mmol/L Trizma/HCl, pH 7.5; 50 mmol/L Trizma base; 25 mmol/L KCl; 5 mmol/L $MgCl_2$; 1 mmol/L dithiothreitol (DTT); 1 mmol/L Na_3VO_4; and 0.5 mmol/L phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 600 × *g* for 10 minutes, and the supernatant was centrifuged at 7000 × *g* for 10 minutes. The resulting supernatant contained cytosolic proteins. The insoluble material was incubated in 2 mol/L sucrose-TKM buffer, followed by centrifugation at 105,000 × *g* for 1 hour. After removal of the supernatant, the resuspended pellet was incubated in buffer A, consisting of 10 mmol/L HEPES; 2 mmol/L $MgCl_2$; 0.1 mmol/L EDTA; 10% (v/v) glycerol; 1 mmol/L DTT; and 0.5 mmol/L phenylmethylsulfonyl fluoride. This was followed by centrifugation at 15,000 × *g* for 30 seconds. After removal of the supernatant, the pellet was incubated in buffer B, consisting of 50 mmol/L HEPES; 50 mmol/L KCl; 300 mmol/L NaCl; 0.1 mmol/L EDTA; 10% (v/v) glycerol; 1 mmol/L DTT; and 0.5 mmol/L phenylmethylsulfonyl fluoride. This was followed by centrifugation at 15,000 × *g* for 5 minutes. The resulting supernatant contained nuclear proteins. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Oxidative Stress Assessment by GPx and MDA Assays

A glutathione peroxidase (GPx) assay was conducted with 70-μL brain contusion tissue (1.0-mg/mL protein concentration) using the BIOXYTECH GPx-340 TM assay kit (OXIS International, Inc., Beverly Hills, CA). A malondialdehyde (MDA) assay was performed as previously described, with slight modification.[25] The brain contusion tissue was homogenized (100 mg/mL) in 1.15% KCl buffer. The homogenate (0.5 mL) was added to 1 mL reaction mixture, consisting of 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25N HCl. The mixture was heated at 95°C for 15 minutes. After cooling to room temperature, the sample was centrifuged at 1500 × *g* for 10 minutes. The supernatant absorbance was measured at 532 nm using 1,1,3,3–tetraethoxypropane as an external standard. The MDA level was expressed as MDA (in micrograms)/protein (in milligrams).

Immunoblot Analysis

Cytosolic protein extracts (5 μg/lane) were resuspended in TKM buffer and solubilized in equal volumes of two times sample loading buffer, containing 4% SDS; 0.29 mol/L sucrose; 0.125 mol/L Tris-HCl, pH 6.8; 0.004% bromophenol blue; and 0.1 mol/L DTT. Then, the extracts were heated at 95°C for 3 minutes. They were then loaded onto 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated in 5% nonfat milk. As a control, the amount of β-actin protein was determined with an anti–β-actin antibody (072K4872; Sigma-Aldrich, Inc.), diluted 1:1000 in 1× PBS and 0.1% Tween 20 containing 1% nonfat milk. AQP4 was determined by immunoblotting with rabbit polyclonal anti-AQP4 antibody (sc–20812; Santa Cruz Biotechnology, Inc.), diluted 1:1000 in PBS-0.1% Tween 20 containing 1% nonfat milk. Immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) by application of horseradish peroxidase–labeled anti-rabbit IgG antibody and diluted 1:10,000 in PBS-0.1% Tween 20, and protein levels were determined by a VersaDoc 5000 Imaging System (Bio–Rad Laboratories).

EMSA Findings

An electrophoretic mobility shift assay (EMSA) was performed as previously described.[23,24] Briefly, consensus NF-κB and HIF-1α double-stranded oligonucleotides (NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′; and HIF-1α, 5′-GCCCTACGTGCTGTCTCA-3′) were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer Mannheim Corp, Indianapolis, IN). In binding reactions, the labeled oligonucleotide probes were incubated with 5 μg of nuclear protein extract of brain contusion tissue and 1 μg of poly-(dI-dC) in binding buffer [4% (v/v) glycerol; 1 mmol/L $MgCl_2$; 0.5 mmol/L EDTA; 0.5 mmol/L DTT; 50 mmol/L NaCl; and 10 mmol/L Tris-HCl, pH 7.5]. The protein-DNA reactions were loaded onto 7% native polyacrylamide gel in one half times Tris-borate-EDTA buffer and visualized and analyzed with an FLA-3000 fluorescent image analyzer (Fuji Photo Film Co, Ltd, Tokyo).

Statistical Analysis

The results were expressed as the mean ± SE ($n = 4$ independent samples). One-way analysis of variance, with Dunnett's post hoc tests, was performed for comparisons between the saline group and the ethanol or BSO group. One-way analysis of variance, with Scheffé's post hoc tests, was performed for comparisons among the ethanol, AZA3, and AZA12 groups. Differences in survival rates among groups were assessed by log-rank test. $P < 0.05$ was considered statistically significant.

Results

The saline-treated rats all survived for >24 hours after TBI. By contrast, the 24-hour survival rates after TBI in ethanol- and BSO-treated rats were 53% ($P = 0.0064$ versus saline) and 67% ($P = 0.303$ versus saline), respectively.

Brain edema was estimated by *in vivo* MRI 3, 6, and 24 hours after brain contusion. DWI and T_2WI are more effective than other methods, such as measurement of water content by drying the extracted brain and morphological estimation in brain slice, for detecting cytotoxic edema lesions and areas of brain contusion and edema, respectively. At 24 hours, both the ethanol and BSO

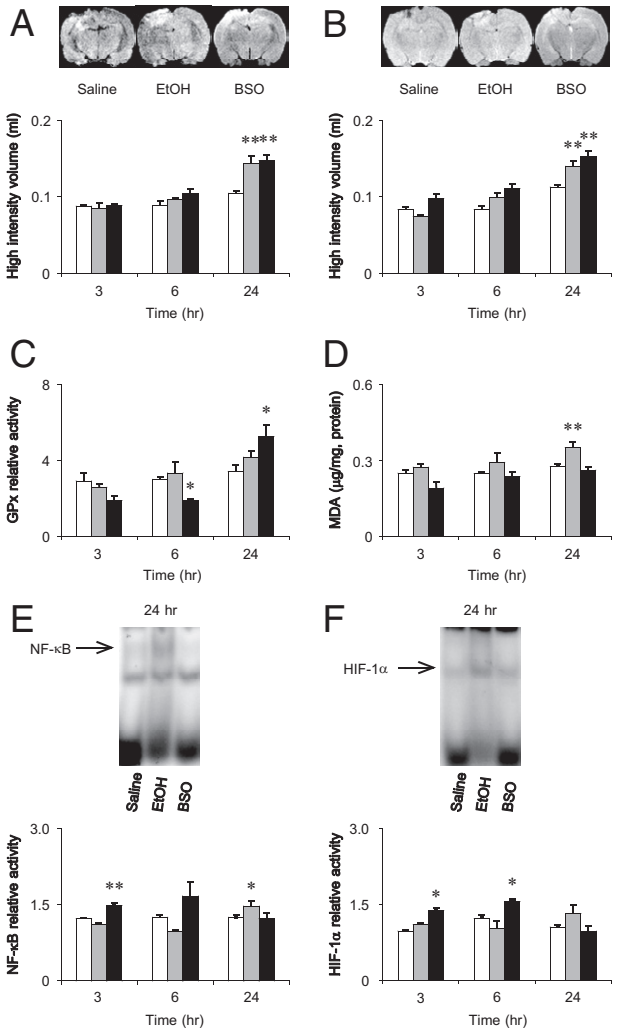

Figure 1. Accumulation of brain edema after TBI by prior ethanol administration and BSO treatment. **A** and **B:** Estimation of brain edema. High-intensity volume in DWIs (**A**) and T_2WIs (**B**) after TBI. **Top panels:** Typical MRIs 24 hours after TBI are shown. Saline, ethanol (EtOH), or BSO was administered i.p. to rats at 1 hour before injury. The high-intensity volumes in both DWIs and T_2WIs were most increased at 24 hours in all groups. Pretreatment with ethanol (3 g/kg body weight) or BSO (100 mg/kg) significantly enhanced the high-intensity volume at 24 hours compared with the saline group. **C** and **D:** Oxidative stress in the pericontusional area of cortex after TBI. Relative GPx (**C**) and MDA (**D**) activities in the pericontusional cortex were measured by spectrophotometry. **E** and **F:** Activation of NF-κB and HIF-1α after TBI. Relative NF-κB (**E**) and HIF-1α (**F**) binding activities in the pericontusional cortex were evaluated by digital image analysis of EMSA signals. **Top panels:** Representative NF-κB (**A**) and HIF-1α (**B**) EMSA results are shown. Data represent the mean ± SE ($n = 4$). White, gray, and black bars represent the saline, ethanol, and BSO groups, respectively. $^*P < 0.05$, $^{**}P < 0.01$ versus the saline group.

groups demonstrated significant increases in brain edema, estimated by both DWI and T_2WI (Figure 1, A and B, respectively). No significant difference in the formation of brain edema was observed between the ethanol and BSO groups.

Figure 1C shows GPx activity in the brain contusion area. GPx catalyzes the reduction of hydroperoxides, including hydrogen peroxides, by reduced glutathione and functions to protect the cell from oxidative damage. BSO pretreatment led to significantly decreased GPx activity at 6 hours after TBI and significantly increased GPx activity at 24 hours after TBI, whereas ethanol pretreatment led to significantly increased GPx activity at 24 hours after TBI. MDA levels were measured as an indicator of lipid peroxidation in the brain. Ethanol pretreatment significantly enhanced MDA levels at 24 hours after TBI compared with control (Figure 1D).

NF-κB and HIF-1α activity in the pericontusional area of cortex was also examined by EMSA (Figure 1, E and F). Ethanol pretreatment resulted in significantly decreased NF-κB activity at 6 hours after TBI but significantly increased NF-κB activity at 24 hours after TBI. By contrast, BSO pretreatment significantly increased NF-κB activity at 6 hours after TBI (Figure 1E). Ethanol pretreatment also increased HIF-1α activity significantly at 3 hours after TBI. BSO pretreatment significantly increased HIF-1α activity at both 3 and 6 hours after TBI.

Figure 2 shows the expression of AQP4 in the augmentation of brain edema. Figure 2A shows histopathological findings in the pericontusional area of cortex. Cell swelling, decreased number of cells, liquid space, and empty space were observed after pretreatment with ethanol or BSO in H&E-stained tissue. AQP4 protein was significantly increased in the pericontusional cortex 24 hours after TBI in the ethanol group and significantly increased 3 hours after TBI in the BSO group (Figure 2, B and C). These phenomena are consistent with changes in NF-κB and HIF-1α activity. Ethanol pretreatment before TBI increased AQP4 expression in the perivascular layer and end feet of glia, particularly astrocytes, more severely than BSO pretreatment.

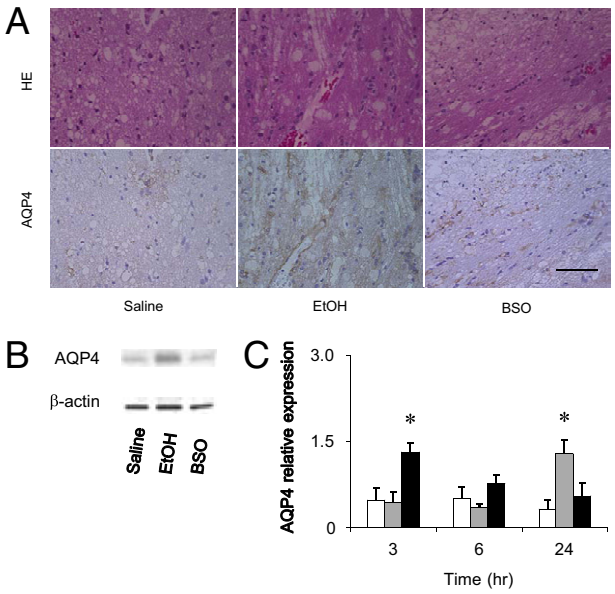

Figure 2. Pericontusional AQP4 expression after TBI. **A:** Histopathological findings in the pericontusional cortex at 24 hours after TBI. **Top panels:** Representative H&E staining of rat pericontusional cortex. **Bottom panels:** Representative AQP4 protein expression determined by IHC with rabbit anti-AQP4 antibody (diluted 1:200) in the pericontusional cortex. AQP4 expression was recognized in the perivascular layer and glia. Scale bar = 50 μm. **B:** Representative AQP4 immunoblots. Rat pericontusional brain was processed for immunoblotting with rabbit polyclonal anti-AQP4 antibody (diluted 1:1000). **C:** AQP4 relative protein expression at 24 hours after TBI under ethanol (EtOH) consumption was normalized to β-actin. Data represent the mean ± SE ($n = 4$). White, gray, and black bars represent the saline, ethanol, and BSO groups, respectively. $^*P < 0.05$ versus the saline group.

The AQP4 inhibitor AZA was administered 3 or 12 hours after TBI (4 or 13 hours after ethanol administration). The administration of AZA at 3 hours after TBI increased the survival rate of the ethanol group from 53% to 75% ($P = 0.2599$ versus ethanol) at 24 hours after TBI. The administration of AZA at 12 hours after TBI restored the survival rate to 100% ($P = 0.0799$ versus ethanol) at 24 hours after TBI. Moreover, the administration of AZA at 12 hours after TBI significantly decreased brain edema at 24 hours after TBI compared with the ethanol and AZA3 groups (Figure 3A). However, the administration of AZA at 3 hours after TBI did not result in any significant difference in brain edema compared with the ethanol group (Figure 3A). The administration of AZA at 3 or 12 hours after TBI resulted in decreased AQP4 protein expression at 24 hours after TBI (Figure 3D). Immunohistochemical (IHC) findings indicated the same pattern of AQP4 inhibition by AZA (Figure 3, B and C). Therefore, the administration of AZA at 12 hours after TBI is more effective from the perspective of reducing brain edema through inhibition of AQP4.

The treatment of saline, ethanol, and BSO in the sham–operated on groups did not cause brain edema or any significant results (data not shown).

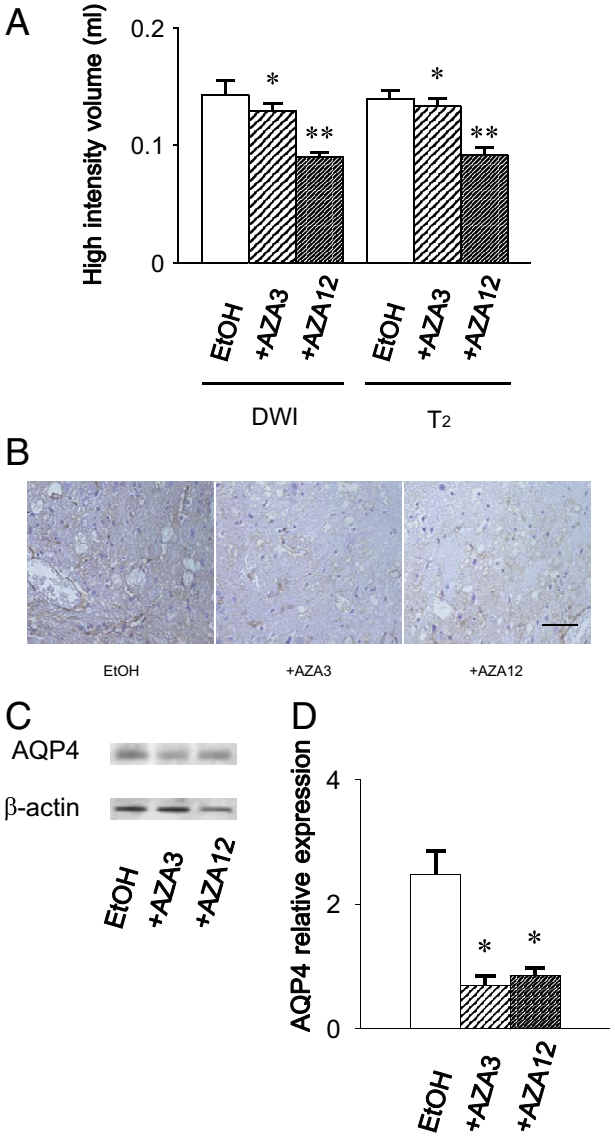

Figure 3. Effects of inhibition of AQP4 on brain edema. **A:** High-intensity volume in DWIs and T_2WIs at 24 hours after TBI. An AQP4 inhibitor, AZA, was administered to rats i.p. at 3 hours (AZA3) or 12 hours (AZA12) after TBI under ethanol (EtOH) consumption. The high-intensity volumes in both DWIs and T_2WIs were decreased in the AZA12 group after TBI under ethanol consumption. Data represent the mean ± SE ($n = 4$). $^*P < 0.01$ versus the saline group; $^{**}P < 0.01$ versus the AZA3 group. **B:** AQP4 histopathological findings in the pericontusional area of cortex at 24 hours after TBI under ethanol consumption with AZA administration at 3 or 12 hours after TBI. AQP4 protein expression was determined by rabbit polyclonal anti-AQP4 antibody immunoreactivity (diluted 1:200) in the pericontusional area of the cortex. AQP4 expression was recognized in the perivascular layer and glia. Scale bar = 50 μm. Representative images of AQP4 and β-actin immunoreactivity within brain contusion areas are shown. **C:** AQP4 relative expression at 24 hours after TBI under ethanol consumption in the ethanol AZA3, and AZA12 groups. Rat pericontusional brain was processed for immunoblotting with rabbit polyclonal anti-AQP4 antibody (diluted 1:1000). **D:** AQP4 relative protein expression at 24 hours after TBI under ethanol consumption was normalized to β-actin. Data represent the mean ± SE ($n = 4$). $^*P < 0.01$ versus the saline group.

Discussion

In the present study, we confirmed that ethanol pretreatment augmented brain edema after TBI in the rat. In addition, we found that ethanol pretreatment increased the expression of AQP4 surrounding the contusion area. Brain edema observed at 24 hours after TBI occurred similarly in both the ethanol and oxidative stress inducer pretreatment groups. Ethanol pretreatment induced lipid peroxidation at 24 hours after TBI but did not activate GPx. By contrast, the oxidative stress inducer BSO increased the activity of GPx but not lipid peroxidation. We observed activation of NF-κB and HIF-1α at 24 hours after TBI in the ethanol-pretreated group and at 3 hours after TBI in the BSO-pretreated group. Therefore, these findings indicate that ethanol pretreatment causes lipid peroxidation but no GPx accumulation 24 hours after TBI, which leads to the activation of NF-κB and HIF-1α. These phenomena may be caused by a swift increase in alcohol metabolism (SIAM).[25] SIAM can occur by the accumulation of oxygen consumption in mitochondria, leading to an increase in the ethanol metabolism rate.[25] On the other hand, brain edema in the contusion area can increase gradually after TBI. This phenomenon may be similar to SIAM. Considering SIAM, two events may occur in the contusion-affected brain: the accumulation of oxygen consumption by ethanol metabolism via CYP2E1 and catalase in brain cells[26] may induce oxidative stress,[27,28] and acute liver injury or disturbance by oxidative stress may induce brain edema by hyponatremia or a high ammonia level.[29] Therefore, such factors may cause brain edema after TBI under ethanol consumption. AQP4 can be co-operated with Na, K-ATPase,[30] Na^+-K^+-$2Cl^-$ cotransporter,[31] and K channel.[32] Therefore, hyponatremia or ammonemia induced by ethanol may cause activation of AQP4.

AQP4 is well-known to shift water through cell membranes under various conditions and is involved in brain edema formation.[12,33] Notably, AQP4 was induced 6 to

12 hours after IL-1β treatment via the NF-κB pathway in astrocytes.[34] NF-κB activity and AQP4 expression were most increased at 24 hours after TBI in the ethanol-pretreated group. These findings indicate that AQP4 may be induced by the NF-κB signaling pathway after TBI under ethanol consumption.

The expression of AQP4 protein was increased at 24 hours after TBI in the ethanol-pretreated group, whereas it was increased at 3 hours after TBI in the BSO-pretreated group. Manley et al[16] observed better survival in AQP4-deficient mice compared with wild-type counterparts in models of brain edema caused by both acute water intoxication and focal ischemic stroke produced with middle cerebral artery occlusion. Therefore, the accumulation of AQP4 protein observed in the present study may be related to the formation of brain edema. AQP4 demonstrated bidirectional function under various pathological conditions.[16] Brain edema is divided into cytotoxic and vasogenic edema.[35] In the present study, brain edema observed in the ethanol-pretreated group was mainly cytotoxic edema (Figure 1). AQP4-knockout mice showed a decrease in cytotoxic brain edema after water intoxication and focal cerebral ischemia,[18] and AQP4-overexpressed mice showed an accelerated progression of cytotoxic brain edema.[36] These findings suggest that AQP4 contributes to the formation of cytotoxic brain edema. Therefore, ethanol pretreatment causes AQP4 accumulation via NF-κB and HIF-1α to induce cytotoxic edema, leading to death. Jamie et al[37] reported that HIF-1α is increased by TBI as early as 1 hour after TBI, persisting for 48 hours. In the present study, ethanol pretreatment increased HIF-1α at 24 hours after TBI, whereas BSO pretreatment increased HIF-1α at 3 hours. The increase of HIF-1α by ethanol was consistent with brain edema augmentation and AQP4 expression. AQP4 is specifically expressed in astrocyte end feet, ependymal cells, and subependymal astrocytes.[38,39] In the present study, AQP4 was markedly expressed at the end feet of glia. This finding is in accordance with our MRI finding of cytotoxic edema induction in DWIs.

AZA, a carbonic anhydrase inhibitor, is also known as an AQP4 inhibitor.[21,22] We used this AQP4 inhibitor to ask whether the inhibition of AQP4 up-regulation could decrease brain edema after TBI under ethanol consumption. When AZA was administered 12 hours after TBI, brain edema was remarkably decreased 24 hours after TBI under ethanol consumption. Recently, brain edema and AQP4 were elevated in the 1-week ethanol-exposed rat brain slices in culture, and the brain edema was reduced by AZA treatment.[40] These findings further confirm that AQP4 plays a crucial role in ethanol-induced brain edema and that inhibition of AQP4 prevents severe brain edema 24 hours after TBI under ethanol consumption.

In conclusion, we found that AQP4 plays a crucial role in brain edema augmentation after TBI under ethanol consumption, suggesting that inhibition of AQP4 may reduce morbidity and mortality in alcohol-intoxicated patients with TBI and in those with ischemic brain injury or brain tumor edema.

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