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REVIEW ARTICLE

Brain Aquaporin 4 in Hyperammonemia

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KEY WORDS
Liver failure; Hyperammonemia; Cytotoxic edema; Vasogenic edema; Aquaporin-4; Hepatic encephalopathy; Cerebral edema.

Abstract
In liver failure, congenital enzymopathies of the urea cycle, and other disorders, ammonia may not be properly detoxified and thus hyperammonemia ensues. Hyperammonemia is considered one of the main factors leading to cerebral edema and related consequences (increased intracranial pressure, brain herniation, and death). Cerebral edema is a critical component of neurological status impairment in patients with hyperammonemia and hepatic encephalopathy (HE). Although cerebral edema is generally classified as cytotoxic (cellular) and vasogenic (extracellular), both components often coexist in the same patient. Both types of edema can occur in acute hyperammonemia and liver failure with cytotoxic edema being a consequence of astrocytes swelling and vasogenic edema, mainly due to blood-brain barrier (BBB) disruption. It is well known that hyperammonemia is a crucial factor in astrocytes swelling and increased BBB permeability; however, the molecular mechanisms by which ammonia causes these alterations are not completely understood. Aquaporins (water channels) are one of the main pathways leading to water influx into the brain and efflux from the brain; consequently, it is conceivable brain aquaporins disturbances are involved in the pathophysiology of cerebral edema in hyperammonemia and HE. This review summarizes brain aquaporins main functions and distribution, and particularly, the aquaporin 4 (AQP-4) alterations induced under hyperammonemia and acute liver failure (ALF).

PALABRAS CLAVE
Hiperamonemia; Edema citóxico; Edema vasógeno; Acuaporina 4; Encefalopatía hepática; Edema cerebral.

Acuaporina cerebral 4 e hiperamonemia

Resumen
En situaciones de insuficiencia hepática, defectos congénitos de las enzimas del ciclo de la urea y otras alteraciones, el amonio no puede eliminarse correctamente y aparece un cuadro de hiperamonemia. La hiperamonemia se considera uno de los factores principales que conduce a edema cerebral y sus consecuencias (aumento de la presión intracraneal, herniación cerebral y muerte). El edema cerebral es una complicación importante que contribuye al deterioro neurológico de los pacientes con hiperamonemia

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y encefalopatía hepática (EH). Aunque por lo general el edema cerebral se clasifica en citotóxico y vasógeno, muchas veces estos dos tipos coexisten en el mismo trastorno. En hiperamonemia y EH ocurren ambos: el edema citóxico se atribuye al hinchamiento de los astrocitos y el vasógeno se debe a la rotura de la barrera hematoencefálica. Se sabe que la hiperamonemia es un factor crucial en la inducción del hinchamiento de los astrocitos y el aumento de la permeabilidad de la barrera hematoencefálica, pero los mecanismos moleculares por los causa estos efectos aún no se esclarecen. Los canales de agua llamados acuaporinas constituyen una de las principales vías de entrada y salida del agua en el cerebro; por tanto, es lógico pensar que alteraciones de estas proteínas participan en la patogénesis del edema cerebral en hiperamonemia y EH. En esta revisión se pretende resumir tanto las funciones como la distribución de las acuaporinas en el cerebro y, en particular, analizar las alteraciones de la acuaporina 4 en hiperamonemia y encefalopatía hepática.

Introduction

Acute hyperammonemia- and hepatic encephalopathy (HE)-associated brain edema occurs following acute liver failure (ALF) resulting from drug hepatotoxicity, viral hepatitis, and other acute liver conditions, and has a very high mortality.[1,2] It has been proposed that brain herniation resulting from cerebral edema and increased intracranial pressure are important steps in the pathogenic mechanisms in ALF and ammonia intoxication.[3-6] Based on edema pathophysiology, Klatzo[7] first differentiated brain edema in two subtypes, namely cytotoxic edema and vasogenic edema. In cytotoxic edema (also called cellular edema), water accumulation occurs inside the cells, primarily astrocytes, whereas in vasogenic edema water accumulates in the extracellular space of brain parenchyma. Cytotoxic edema results primarily from the impairment of osmotic gradients across cell membranes. Such impairment results from several causes including lack of oxygen and cellular metabolism disruption, which on turn decreases ion-pump function resulting in intracellular osmoles accumulation.[8,9] As water moves between fluid compartments following hydrostatic and osmotic gradients, intracellular ions accumulation leads to free water influx into the cells and cell volume expansion. Cytoxotic edema is seen with various intoxications (dinitrophenol, triethyltin, hexachlorophene, isoniazid), Reye's syndrome, severe hypothermia, early ischemia, encephalopathy, early stroke or hypoxia, cardiac arrest, pseudotumor cerebri, and cerebral toxins.

Vasogenic (extracellular) edema is primarily caused by BBB disruption leading to a subsequent leakage of intravascular fluid (containing water and other plasma constituents) into the extracellular space of the brain parenchyma.[8] Water movement across brain capillaries endothelium is determined by the balance of hydrostatic and osmotic forces across the capillary wall. Under normal conditions, BBB restricts osmoles movement across the vascular endothelium and water flux into brain parenchyma is mainly driven by hydrostatic forces and ion co-trasportation. This movement is counteracted by osmotic gradients, which leads to minimal net fluid absorption by brain parenchyma. When BBB permeability is increased, vascular fluid goes into the brain extracellular space. In such case, fluid flows into the parenchyma along hydrostatic gradients with no opposing osmotic forces leading to net fluid accumulation in the brain extracellular space. This type of edema is seen in case of tumors, neuroinflammation, later stages of cerebral ischemia and hypertensive encephalopathy, and following brain trauma.

In many pathologies in which cerebral edema occurs, either both types of edema coexist[7] or one form tends to predominate over the other, which depends on the underlying lesion and the stage of disease progression. For instance, cytotoxic edema appears in the early phase of brain ischemia while subsequently also vasogenic edema takes place. In acute HE and hyperammonemia both types of edema have been shown and often coexist.[10-14] Exposition to pathophysiological levels of ammonia (5 mM), a level found in the brain in some experimental models of HE,[3] ammonia causes a significant increase in cell volume in primary astrocyte cultures[15,16] as well as in glioma cell lines.[17] Similarly, swelling of astrocytes has been observed in brain slices treated with ammonia.[18] Also endothelial cells culture respond to high ammonia levels with alterations of proteins of the tight junctions such as downregulation of claudin-12 and other molecular alterations.[19-21]

Aquaporins Distribution in the Central Nervous System

Water homeostasis in the brain is of crucial importance for both physiological and pathological mechanisms. Cerebral edema is a serious finding in many central nervous system (CNS) pathologies such as brain trauma, infarction, inflammation, infection, and tumors as well as in HE, a neurological complication that may ensue following liver failure. Recent data suggest that the proteins called aquaporins act (not exclusively though; see later) as water channels that regulate water movement in the brain.[22-26] Over 10 of these proteins have been identified and seven of them are expressed in the rodent brain. AQP-1, 4 and 9 are the aquaporins mainly expressed in the brain and will be discussed separately in this review. Aquaporins 3, 5, 8, and 11 have also been identified in the rat brain although at very low densities and their role remains to be investigated.[27]

Aquaporins 1, 4 and 9 and Regulation of Brain Water Homeostasis

Aquaporin 1

AQP-1 is mainly expressed in the choroid plexus of the lateral ventricles and probably positively modulates cerebrospinal fluid (CSF) secretion;[28-30] in fact, AQP-1 KO mice display decreased CSF production and thus reduced intracranial pressure.[30] AQP-1 is also expressed in the ependymal lining and in the human hippocampus.[29,31,32] Expression of AQP-1 declines with aging[33] and in patients with Alzheimer's disease[34] hence contributing to the slow fluid turnover rate observed in these situations.

Aquaporin 4

AQP-4 is one of the most selective water channels amongst all aquaporins. Immunocytochemistry studies have shown that astrocytes but not CNS neurons express AQP-4.[35,36] As opposed to most of the aquaporins that have been studied, AQP-4 is not inhibited by mercuric ions.

Immunogold technique studies revealed that AQP-4 in astrocytes is rich in plasma membranes facing capillaries and the pia matter.[35] In addition, AQP-4 is highly expressed in astrocytes in the glial limiting border facing the cortical and ventricular surfaces, as well as in the basolateral membrane of the ventricular ependyma.[37]

In osmosensitive organs such as the magnocellular hypothalamic nuclei, AQP-4 channels are expressed on astrocytic plasma membranes facing both capillaries and magnocellular neurons and in other sites not facing the brain surface or capillaries.[35,38] The specificity and intensity of AQP-4 staining in osmosensitive brain regions suggest that AQP-4 channels allow plasma osmotic pressure variations to be transferred from blood to osmosensitive neurons. Furthermore, high blood vessel density is another common feature of these nuclei, which contributes to plasma osmolarity detection.[38] Low AQP-4 levels occur also at plasma membranes that unsheathe neuronal processes such as the glutamatergic synapses between parallel fibres and Purkinje cell spines in the cerebellum.[35,38,39] Although AQP-4 is expressed at low densities by astrocytic plasma membranes that abut on brain neuropil, it should be pointed out that this latter localization has a larger distribution area as compared to astrocytic plasma membranes that abuts on capillaries and pial arteries. Non-endfeet membranes show different levels of expression depending on the brain region. Strong AQP-4 labelling of non-endfeet membranes is prominent in the more dorsal parts of the cortex and in the granule cell layer of the cerebellum. AQP-4 is also expressed in lower levels by endothelial cells of the brain.[36,40,41] AQP-4 is expressed both abluminally and adluminally in endothelial cells.[26,36,41]

AQP-4 is also expressed in the basolateral membrane of ependymocytes.[35] The brain regions of the rodent brain that show the highest level of AQP-4 protein include the cerebellum, the magnocellular hypothalamic nuclei, the hippocampus, the neocortex, and the medial habenular nucleus.[23,42]

Aquaporin 9

AQP-9 is permeable to water and other solutes such as ammonia, urea, and glycerol.[43] However, the osmotic water coefficient varies between reports and it seems that water permeability through this aquaporin is less important than in pure water channels such as AQP-4.[44] Except for one report,[45] experimental data demonstrate that AQP-9 is also permeable to glycerol, urea, polyols such as mannitol and sorbitol,[46,47] purines (adenine), pyrimidines (uracil and the chemotherapeutic agent 5-fluorouracil), and monocarboxylates such as lactate and beta-hydroxybutyrate mainly in their protonated forms.[46] AQP-9 is expressed in astrocytic processes and cell bodies[46,48-50] but, in contrast to AQP-4, it is not polarized on astrocytic endfeet.[39] This aquaporin is also expressed in other cell types: endothelial cells of subpial vessels[49] and, unlike AQP-1 and AQP-4, AQP-9 is expressed in some neurons (see below).[49] Two studies from different research groups[50,51] demonstrated the expression of AQP-9 protein in tanycytes in the border of the third ventricle in the mediobasal hypothalamus and the subfornical organ,two osmosensitive brain regions. Interestingly, these glial cells do not express AQP-4. This difference suggests that AQP-9 is involved in the water movement between the CSF and the brain parenchyma in these hypothalamic structures. AQP-9 immunoreactivity was observed in astrocytes of the white matter (corpus callosum)[48,49] and in the Bergmann glial cells of the cerebellum.[49] In the mouse brain, AQP-9 is also expressed in astrocytes from the septum, the hippocampus, the dorsomedial hypothalamic nucleus, and the arcuate nucleus.[48]

Other Physiological Functions of Aquaporins

AQP-1

Although AQP-1 is permeable to other solutes and gases (NH_3 and CO_2),[52] few studies have addressed its relevance in additional processes occurring in CNS other than regulation of water movement, particularly in CSF secretion.[28-30] AQP1 expression is enhanced in reactive astrocytes accumulating in brain lesions in Creutzfeldt-Jakob disease and multiple sclerosis, which suggests a role for AQP1-expressing astrocytes in brain water homeostasis under some pathological conditions.[53,54]

AQP-4

The subcellular compartmentation of AQP-4 overlaps with that of the potassium channels Kir4.1; such channels are involved in the spatial buffering of K+, which increases in the extracellular space after the action potential occurs. In animals that lack perivascular AQP-4, K+ clearance is delayed and K+ buffering is positively related to the changes of the extracellular space volume.[39,55] The role of AQP-4 in buffering the extracellular K+ is confirmed by the fact that AQP-4 KO mice display a delayed potassium reuptake during electrical activity and thus ability to develop seizures is increased.[56] Alterations of AQP-4 and Kir4.1 genes have been shown to be interrelated in patients with

temporal lobe epilepsy.[57] Another physiological role for AQP-4 is the modulation of cell adhesion.[58] The high level of AQP-4 protein in the glia lamellae of the magnocellular nuclei of the hypothalamus should facilitate the adhesion between astrocyte processes.[58] In this situation, AQP-4 should not be involved in water diffusion but rather in cell adhesion between astrocytes and possibly to endothelial and muscle cells in the perivascular compartment. This finding is supported by the fact that proper astrocytic AQP-4 localization depends on the presence of basal lamina proteins such as agrin, alpha-dextro glycan, and laminin.[59,60]

AQP-9

AQP-9 is a member of the so called aquaglyceroporins that facilitate the diffusion of water and several solutes such as glycerol, urea, and monocarboxylates. Regarding this aquaporin, the role of water homeostasis regulation should be revised because catecholaminergic neurons are not directly involved in the regulation of systemic osmotic pressure but in energy balance.[61,62] Supporting this notion, AQP-9 is expressed not only in plasma membrane of cells but also in the mitochondria of astrocytes and dopaminergic neurons.[63] It has been postulated that the presence of AQP-4 facilitates the bidirectional diffusion of glycerol and monocarboxylates such as lactate which serve as energy substrates for neurons (AQP-9 would promote the influx of such solutes) and astrocytes (AQP-9 would promote the efflux of glycerol and lactate).[64-66] In addition, some catecholaminergic neurons are sensitive to changes in glucose concentration[49] and are activated by lactate and glycerol.[62,67]

AQP-9 could also be involved in the regulation of monocarboxylate through the BBB. Future studies should show whether AQP-9 plays a role in energy balance as a glycerol-lactate channel.

Aquaporins, Hyperammonemia, and Hepatic Encephalopathy

AQP-1

Although AQP-1 expressed in Xenopus oocytes is permeable to ammonia[52,68] and abundantly distributed in the choroid plexus, it seems that its amount (either mRNA or protein) is not affected in the brain of patients experiencing ALF and hyperammonemia. A preliminary report by Eefsen et al[69] showed that the protein and mRNA content of AQP-1 is not altered in mice with ALF and hyperammonemia induced by injecting the hepatotoxin D-galactosamine and endotoxin lypopolysaccharide (LPS), and by infusing intravenously (i.v.) ammonia acetate solution. No studies have yet evaluated the role of AQP-1 in the mediation of swelling neither in cultured astrocytes nor in endothelial cells exposed to ammonia. In addition, no differences were found in chronic liver failure regarding urinary excretion of AQP-1 between healthy subjects and patients with cirrhosis with or without ascites, suggesting AQP-1 may not play a significant role in the progressive dysregulation of peripheral extracellular fluid volume in cirrhosis.[70] However, one unexplored possibility is that the AQP-1 regulation (through phoshorylation or subcellular localization) rather than the total protein amount might be altered in hyperammonemia and hepatic failure.

Aquaporin-4 in Astrocytes Cultured under Hyperammonemia

Cultured astrocytes exposed to ammonia show many of the pathological, metabolic, and neurochemical changes observed in human HE.[6] However, extrapolation of such findings to *in vivo* situation should be done with caution because of the cellular/molecular differences between cultured cells and their *in situ* counterparts. Exposure of cultured astrocytes to 5 mM NH_4Cl led to an increased protein expression of AQP-4, which was correlated with the development of astrocyte swelling.[71-73] AQP-4 increase is observed 10 hours after ammonia treatment, and interestingly, 2 hours before the onset of astrocyte swelling. Such increase is observed for up to 48 hours.[71] The molecular mechanism by which exposure to ammonia increases AQP-4 levels in cultured astrocytes is not known. Oxidative stress is known to upregulate both gene and protein expression of AQP-4 in cultured astrocytes.[74] Since it is well documented that oxidative stress plays a major role in ammonia neurotoxicity,[75] it has been demonstrated that oxidative stress caused by ammonia may be responsible for AQP-4 upregulation.[76]

AQP-4 in Animal Models of Hyperammonemia and Hepatic Encephalopathy

While the role of AQP-4 has been strongly implicated in brain edema associated with various neurological conditions, its involvement in brain edema in acute hyperammonemia and HE due to ALF is not well documented. Until now, only three reports have demonstrated an increased AQP-4 expression in experimental models of ALF. In a preliminary report, Margulies et al.[77] noted an increased brain AQP-4 protein expression in rats whose liver had been devascularized, and such an increase was correlated with the onset of brain edema. Similarly, Yang et al.[78] reported an AQP-4 increase in a rat model of ALF, caused by the hepatotoxin thioacetamide. Another report (although preliminary) by Eefsen et al.[69] has shown that AQP-4 levels were increased in the cerebral cortex in mice with ALF and hyperammonemia through D-galactosamine plus LPS injection and i.v. ammonium acetate solution infusion. In contrast to the above mentioned studies regarding a situation of acute hyperammonemia, in a more chronic animal model of hyperammonemia due to a defect in a urea cycle enzyme (ornithine transcarbamaylase) it was demonstrated that hyperammonemia (18 hours) significantly reduced the expression of AQP-4 in astrocytes isolated *in situ* from mice cerebral cortex.[79] The effects of hyperammonemia differ depending on reached ammonia concentrations and length of hyperammonemia exposure.[80] For example, acute and chronic

hyperammonemia induce different effects on NMDA-type glutamatergic receptors. Excessive activation of the NMDA receptors and of associated signal transduction pathways plays a main role in acute ammonia toxicity and ammonia-induced death. In contrast, in chronic hyperammonemia, levels of ammonia are not high enough to induce death and this seems to allow the induction of an adaptive response, which results in a reduced function of some signal transduction pathways associated to NMDA receptors. Consequently, it is clear that both acute (high levels) and moderate chronic hyperammonemia may affect the same processes differently, through different mechanisms, and can result in different (even opposite) effects on the same processes such as regulation of brain AQP-4.**Aquaporin 9 and Ammonia**

AQP-9 has been shown to be permeable to ammonia at least when expressed in Xenopus oocytes.[43,81] It was speculated that ammonia-permeable aquaporins might constitute a link between metabolism and volume control. In addition to water, human AQP-9 are permeable to ammonia and urea. In humans, these aquaporins supplement ammonia transportation of Rhesus (Rh) proteins and urea transporters (UTs) and least in periphery. The mechanism by which ammonia is transported by aquaporins is not fully understood. A comparison of transport equations, models, and experimental data shows that ammonia is transported in its neutral form (NH_3).[43] The ammonia-permeable aquaporins differ from other aquaporins by having a less restrictive aromatic/arginine region; an exclusively water-permeable aquaporin can be transformed into an ammonia-permeable aquaporin by single point mutations in this region. However, no studies have been conducted yet about the expression and function of AQP-9 in cultured astrocytes or brain from experimental animals with liver failure or hyperammonemia due to other pathologies.

Conclusions and Future Directions

Water homeostasis is critical for the maintenance of normal cell volume[82] as well as for optimal cellular metabolic activities.[83] Brain aquaporins 1, 4 and 9 regulate water homeostasis in the brain.

Except for one preliminary report,[69] no studies (either *in vitro* or *ex-vivo*) have been conducted about hyperammonemia and HE effects on AQP-1 and AQP-9 content, regulation, or their contribution to the entry of ammonia into the brain. In contrast to AQP-1 and AQP-9, more experimental data are available about the effect of hyperammonemia and HE on AQP-4. Cultured astrocytes exposed to ammonia display cell swelling and AQP-4 increase.[71-73] According to these *in vitro* studies, AQP-4 is also increased in the brain of rodents animal with ALF leading to acute hyperammonemia.[69,77,78] In contrast, in an animal model of urea cycle disturbance and hyperammonemia (resulting from an arginine-free diet), AQP-4 content was significantly decreased in cerebral cortex astrocytes.[79] The reason for these discrepancies in different models of acute hyperammonemia is not known but in the case of ALF, other metabolic abnormalities induced by liver failure might play a role; alternatively, different effects on AQP-4 could depend on the level of hyperammonemia reached in the brain in different animal models. AQP-4 role in the modulation of cytotoxic and vasogenic edema is different; in the latter, AQP-4 promotes cytotoxic edema and also allows water clearance.[24,84] Another interesting point that remains to be investigated is the net contribution of AQP-4 in cytotoxic and/or vasogenic edema under hyperammonemia and HE. Pharmacological treatments that modulate brain aquaporins expression and function through channel gating, subcellular distribution, phosphorylation, protein-protein interactions, and orthogonal array formation[85,86] might represent new therapeutic targets to treat cerebral edema in hyperammonemia and HE.

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