

# Pregnancy-induced up-regulation of aquaporin-4 protein in brain and its role in eclampsia

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**ABSTRACT** Neurologic complications of eclampsia are thought to be similar to hypertensive encephalopathy in which an acute, excessive elevation in blood pressure causes blood-brain barrier (BBB) disruption and edema formation. Because women who develop eclampsia are in general normotensive and asymptomatic prior to pregnancy, we hypothesized that pregnancy alone predisposes the brain to edema formation by up-regulation of aquaporin 4 (AQP4), a water channel in the brain that has been shown to positively correlate with edema formation. To test this hypothesis, we compared localization (immunohistochemistry), mRNA (RT-PCR), and protein levels (Western analysis) of AQP4 in brains from Sprague Dawley rats that were nonpregnant (NP, proestrous), mid-pregnant (MP, days 9–10), late-pregnant (LP, days 19–20), and postpartum (PP, days 3–4). AQP4 mRNA was detected in the brains of all the animals and was localized primarily around the brain parenchymal blood vessels, strongly implicating its role in BBB function. Western analysis revealed that the major AQP4 band at ~32 kDa was significantly elevated in MP, LP, and PP animals compared with NP by 9-, 22-, and 17-fold, respectively. These results suggest that pregnancy and the postpartum state up-regulate AQP4 protein located around the intraparenchymal blood vessels, a consequence that could promote edema formation when blood pressure is acutely and excessively elevated, as during eclampsia.—Quick, A. M., Cipolla, M. J. Pregnancy-induced up-regulation of aquaporin-4 protein in brain and its role in eclampsia. *FASEB J.* 19, 170–175 (2005)

*Key Words:* aquaporins • blood-brain barrier • AQP4 protein expression

THE AQUAPORINS (AQP) are a family of channel-forming transmembrane proteins that facilitate the movement of water, glycerol, and other solutes across the plasma membrane of cells (1–4). Eleven aquaporin homologs have been identified to date (2–4). Studies of human mutations and animal knockout models for the genes encoding these proteins have revealed that the physiological significance of AQPs is in the regulation of water transport throughout the body (5). For example, mutations in the gene encoding AQP2 protein, which is located in the principal cells of the renal

collecting ducts and regulated by vasopressin, result in reduced water permeability of the ducts and a urinary concentrating deficit (6). In addition, loss of AQP3 along the basolateral membranes of the principal cells of the renal collecting duct in knockout mice results in nephrogenic diabetes insipidus in humans (7).

In the rodent brain, three aquaporins—AQP1, AQP4, and AQP9—have been identified (4, 8–11). AQP1 is localized in the choroid plexus epithelium and is thought to be involved in cerebrospinal fluid (CSF) formation (8). AQP9 is found in cells surrounding the cerebral ventricles; although its function is still unclear, it is postulated to play a role in the production and reabsorption of CSF or in energy metabolism due to its glycerol permeability (4, 11). AQP4, the predominant aquaporin in the brain, is mainly localized in the endfeet of astrocytes surrounding blood vessels, but recently was also found in the vascular endothelium (9, 10, 12, 13). At these locations, AQP4 is thought to facilitate the movement of water between the blood-brain and CSF-brain interfaces (12).

The blood-brain barrier (BBB), comprised of the endothelial cells lining the cerebral vasculature, regulates the paracellular and transcellular passage of molecules and solutes between the cerebral vessels and the brain neuropil (14, 15). While the site of barrier function is at the level of the endothelium, astrocytes are known to induce and maintain permeability properties of the BBB (14, 16, 17). Recent evidence suggests that another potential role of astrocytes is in the actual regulation of water transport across the BBB, mainly because of the highly polarized localization of AQP4 in the perivascular endfeet of astrocytes (4, 18). This concept is supported by numerous studies that have linked increased AQP4 expression to vasogenic brain edema induced by brain tumors, focal ischemia, and brain injury (18, 19–22).

One pathologic condition that may involve AQP4 and edema formation is eclampsia. Eclampsia is a serious complication of pregnancy due to its neurological complications, including headaches, nausea, vomiting, and seizures (23). Eclampsia is thought to be

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similar to hypertensive encephalopathy in which an acute rise in blood pressure causes forced dilation of the cerebral arteries and arterioles, decreased cerebrovascular resistance, and increased pressure on the microcirculation, resulting in disruption of the BBB and vasogenic edema (23–27). Because women who develop eclampsia are in general normotensive and asymptomatic prior to pregnancy, we hypothesized that pregnancy and/or the postpartum state predisposes the brain to edema formation by increasing levels of AQP4. Pregnancy has been shown to up-regulate aquaporin expression in other organs such as the uterus (28, 29), but the regulation of AQP4 expression in the brain during pregnancy has not been studied. To test this hypothesis, immunohistochemistry was used to localize AQP4 protein in the brain of four different groups of Sprague Dawley female rats: nonpregnant (NP, proestrus stage), mid-pregnant (MP, days 9–10), late-pregnant (LP, days 19–20), and postpartum (PP, days 3–4). AQP4 mRNA expression and protein levels in brain were determined using RT-PCR and Western blot analysis, respectively.

### HISTOLOGY AND LOCALIZATION OF AQP4 IN THE BRAIN DURING PREGNANCY

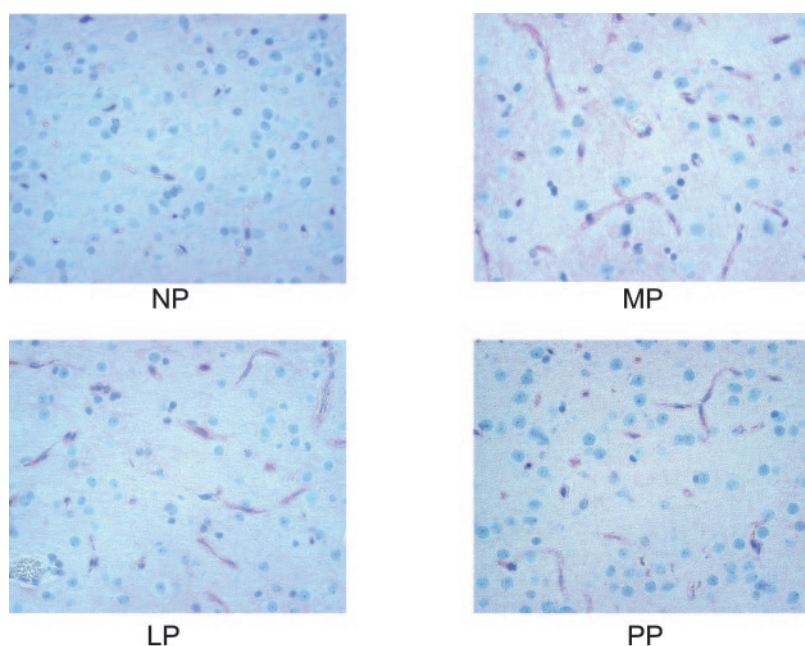
Using an AQP4 antibody (Abcam Inc., Cambridge, MA, USA) (aa sequence 301-318, CIDIDRGDEKKGKDS-SEG) on formalin fixed, paraffin-embedded cerebral cortex tissue, the location of AQP4 was determined in NP, MP, LP, and PP female rats. Deparaffinized and rehydrated brain sections underwent antigen retrieval and blocking before overnight incubation in AQP4 antibody (1:500). Fast red was used to visualize antigen binding. Immunohistochemical analysis (**Fig. 1**) showed that AQP4 was highly localized to the intraparenchymal arterioles and venules but absent from the

vessels of meninges not associated with astrocytes. Parenchymal arterioles and venules were identified by the presence of intraluminal erythrocytes. These findings are consistent with other studies that have shown that AQP4 was localized to the plasma membrane of perivascular astrocytic endfeet and the endothelium of the cerebral blood vessels (12, 13), and strongly implicate a role for AQP4 in regulation of the BBB and the maintenance of water homeostasis from the blood into the brain.

### REGULATION OF AQP4 PROTEIN EXPRESSION IN BRAIN DURING PREGNANCY

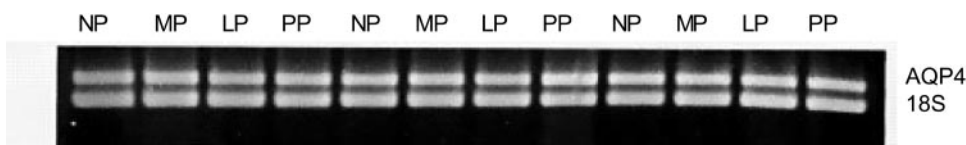
Expression of AQP4 has been shown to be regulated during vasogenic brain edema resulting from human brain tumors, mechanically and chemically induced brain injury, and focal cerebral ischemia (19, 20, 22). During pregnancy, regulation of several aquaporins has been shown to be involved in uterine homeostasis and edema formation (28). For example, pregnancy has been shown to increase AQP2 mRNA and protein expression in the renal collecting ducts of rats, resulting in expansion of extracellular fluid volume and water retention that occurs during normal pregnancy (29). Because pregnancy has been shown to regulate AQP expression in the uterus and kidney, we hypothesized that gestation also increases AQP4 in the brain, a result that could predispose the brain to edema formation under conditions of BBB disruption, such as acute hypertension.

RNA and protein were isolated from the brains of NP, MP, LP, and PP female rats ( $n=3$  per group). Total RNA was isolated from the right hemisphere of the cerebral cortex using TriReagent (Sigma-Aldrich, St. Louis, MO, USA) for RT-PCR. RNA concentration and purity were analyzed with a spectrophotometer (Nano-



**Figure 1.** Photomicrographs of brain sections stained using immunohistochemistry for aquaporin 4 (1:500) from nonpregnant (NP, proestrus), mid-pregnant (MP, days 9–10), late-pregnant (LP, days 19–20), and postpartum (PP, days 3–4) visualized using Fast Red and imaged at 40 $\times$ . Note that AQP4 staining (red) was highly localized around the intraparenchymal blood vessels, identified by the presence of erythrocytes in the lumen.

**Figure 2.** PCR product separated by gel electrophoresis and stained with ethidium bromide. RT-PCR was performed using 25 ng of total RNA to determine the levels of expression of AQP4 mRNA in nonpregnant (NP, proestrous), mid-pregnant (MP, days 9–10), late-pregnant (LP, days 19–20), and postpartum (PP, days 3–4) female rats ( $n=3$  per group). 18S rRNA was coamplified to serve as an endogenous control.



Drop Technologies Inc., Rockland, DE, USA). RT-PCR was performed according to a one-step protocol (Qiagen Inc., Valencia, CA, USA) using 25 ng of total RNA from each sample. AQP4 primers (Qiagen Inc.) were of the following sequence: 5'GCATGAATCCAGCTC-GATCCTTTGG3' (forward) and 5'AATGGGTGGCAG-GAAATCTGAGGC3' (reverse). 18S rRNA (Ambion Inc., Austin, TX, USA) was coamplified in the same reaction tube to serve as an endogenous control. RT-PCR was carried out in a Flexigene thermal cycler (Techne Inc., Burlington, NJ, USA) and amplification was performed for 24 cycles. PCR product and a 100 bp DNA ladder were separated by electrophoresis on a 2% agarose gel in 1× Tris-borate-EDTA buffer. The gel was stained with ethidium bromide and visualized using the Kodak EDAS 290 imaging system.

**Figure 2** shows the AQP4 and 18S bands that were identified on the gel. Note that AQP4 mRNA was detected in all groups of animals. Positive and negative controls for AQP4 mRNA from the cerebellum and liver, respectively, are shown in **Fig. 3**. These controls were chosen because liver contains no AQP4 whereas cerebellum contains high levels (5). To confirm its identity, the PCR product was sequenced and determined to be AQP4 based on a BLAST search (data not shown).

Total protein was isolated from the left hemisphere of the cerebral cortex of the same animals using SDS sample buffer (Invitrogen Corporation, Carlsbad, CA, USA). After homogenization, total protein concentration was determined using Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) and a spectrophotometer (Thermo Electron Corporation, Woburn, MA, USA). Protein (5 μg) from each sample was loaded for electrophoresis into a 4–20% TrisGlycine gel (Invitrogen) and subsequently transferred to an Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After the transfer, the membrane was blocked for 1 h in Aquablock (East Coast Biologics, Inc., North Berwick, ME, USA), then incubated overnight at 4°C in AQP4 polyclonal antibody (Chemicon International, Temecula, CA, USA) diluted 1:1000. The next morning, the membrane in primary antibody was incubated at 37°C for 30 min,

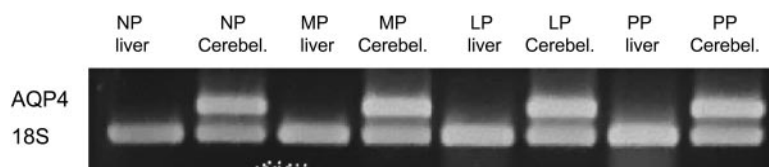
washed three times for 15 min in 1× PBS and TWEEN-20 (PBST), and subsequently incubated for 1 h in secondary goat anti-rabbit antibody diluted 1:3000. After three washes in PBST and one in PBS, the membrane was scanned and densitometric analysis was performed using the Odyssey Infrared Imaging System (Li-Cor Inc., Lincoln, NE, USA).

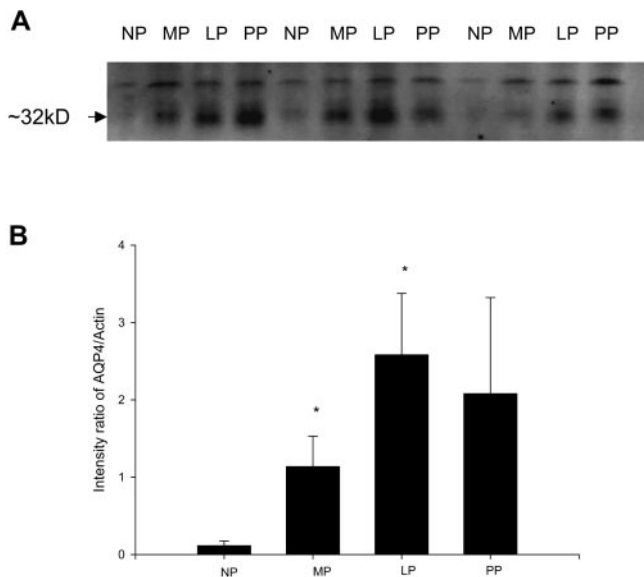
The blot in **Fig. 4A** shows that the AQP4 antibody recognized a major protein band ~32 kDa, most likely corresponding to the highly abundant 32 kDa M23 isoform of AQP4 (10, 31, 32), and a less prominent band at a slightly higher molecular mass. These data are consistent with other experimental findings of a major AQP4 band ~32 kDa and a minor band at ~38 kDa (10, 31, 32). Densitometric analysis was used to quantify AQP4 protein expression levels by determining intensity values for each band relative to β-actin (used as an internal control for lane loading). **Figure 4B** shows the intensity ratio of AQP4 protein for the 32 kDa band in each group. Compared with NP, AQP4 protein increased 9-fold in MP ( $P<0.05$ ), 21-fold in LP ( $P<0.05$ ), and 17-fold in PP animals ( $P>0.05$ ). These data show that in the basal state, pregnant rats have substantially higher levels of AQP4 than nonpregnant rats, with the highest level being found in the late-pregnant females.

### GESTATIONAL REGULATION OF AQP4 IN BRAIN AND ITS ROLE IN EDEMA FORMATION AND ECLAMPSIA

AQP4 in astrocytes is thought to contribute to BBB properties by taking up excess water brought into the brain by disruption of the BBB (18). However, excessive AQP4 may be detrimental and promote edema formation. The involvement of AQP4 in brain edema formation has been demonstrated in studies using knockout mice. For example, one study in which deletion of α-syntrophin, a membrane protein anchoring AQP4 into the perivascular astrocytic endfeet, resulted in loss of AQP4 at these membranes (18). After middle cerebral artery (MCA) occlusion and reperfusion, α-syntrophin knockout mice showed decreased levels of hemi-

**Figure 3.** PCR product from cerebellum and liver separated by gel electrophoresis and stained with ethidium bromide. RT-PCR was performed using 25 ng of total RNA to determine expression of AQP4 mRNA in liver and cerebellar tissue. Liver tissue served as a negative control for AQP4 mRNA and the cerebellum served as a positive control.





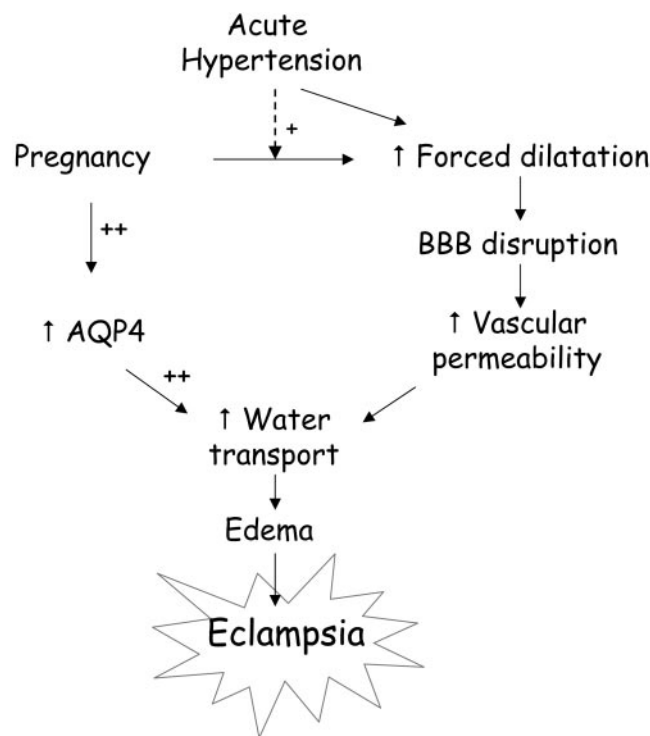
**Figure 4.** A) Western blot showing AQP4 protein expression in nonpregnant (NP, proestrous), mid-pregnant (MP, days 9–10), late-pregnant (LP, days 19–20), and postpartum (PP, days 3–4) female rats ( $n=3$  per group). 5  $\mu$ g of protein from each sample were separated by SDS-PAGE electrophoresis, transferred to a PVDF membrane, and probed with AQP4 antibody. The antibody identified a major band at ~32 kDa, likely corresponding to the abundant M23 isoform. A minor band was seen at ~38 kDa. B) Graph showing the intensity ratio for the 32 kDa AQP4 protein (in relation to  $\beta$ -actin) in brains from nonpregnant (NP, proestrous), mid-pregnant (MP, days 9–10), late-pregnant (LP, days 19–20), and postpartum (PP, days 3–4, PP). Densitometric analysis revealed a substantial increase in AQP4 protein levels in MP, LP, and PP animals by 9- to 22-fold. \* $P < 0.05$ .

spheric enlargement and brain edema compared with wild-type mice, suggesting that the presence of perivascular AQP4 enhanced brain edema formation and is an important component in regulating water transport in the brain under pathologic conditions (18). Another study using AQP4 knockout mice found that after vasogenic edema and BBB disruption from MCA occlusion and focal cerebral ischemia, AQP4<sup>+/+</sup> mice had a higher mortality rate and greater neurological complications than AQP4<sup>-/-</sup> mice. In addition, the AQP4<sup>-/-</sup> mice had significantly less brain edema as measured by the increase in the size of the cerebral hemisphere, suggesting AQP4 promotes edema formation (21).

Another pathologic condition in which AQP4 may be involved is in vasogenic edema formation during eclampsia. The neurologic complications of eclampsia are thought to be similar to hypertensive encephalopathy in which an acute and excessive elevation in mean arterial pressure causes forced dilatation of the cerebral arteries and arterioles, BBB disruption, and edema formation. The presence of increased AQP4 during pregnancy and the postpartum state may promote edema formation once the BBB is disrupted, similar to the AQP4<sup>+/+</sup> mice discussed above. The cerebral arteries of pregnant and postpartum rats have been

found to force dilatate intrinsic myogenic tone at significantly lower pressures than with NP females (33), suggesting that pregnancy alone affects cerebrovascular function that may predispose to forced dilatation and BBB disruption under conditions of acute hypertension. Since this difference was evident only at pressures beyond the autoregulatory range (>150 mmHg), the consequences are likely to be detrimental only when blood pressure is excessively elevated, as during eclampsia. Similarly, it is likely that the higher levels of AQP4 protein in the brain during pregnancy and postpartum do not cause edema under normal conditions, but rather predispose the brain to greater edema formation when a stressor that disrupts the BBB, such as acute hypertension, is introduced. Along these lines, Belfort et. al. showed that pre-eclamptic women with normal blood pressure had abnormally high cerebral perfusion pressure, suggesting that elevated intravascular pressure may cause cerebrovascular injury and the neurologic complications of eclampsia despite normal MAP (e.g., hypertensive encephalopathy) (34). The presence of elevated AQP4 during pregnancy may further contribute to these complications by promoting edema formation.

In summary, we found that AQP4 was localized around the intraparenchymal cerebral blood vessels in



**Figure 5.** Diagram demonstrating the hypothesis that the combination of acute hypertension during pregnancy results in forced dilatation of cerebral arteries and arterioles, blood-brain barrier (BBB) disruption and enhanced vascular permeability. Pregnancy is associated with an increase in aquaporin 4 (AQP4) protein levels, which may enhance water transport across the endothelium into the brain during acute hypertension that leads to edema formation and the neurologic complications of eclampsia.

female rats, strongly implicating its role in BBB function. Western blot analysis demonstrated that AQP4 protein was significantly increased in the brains from pregnant and postpartum rats. Because of the strong link between AQP4 protein and edema formation, we hypothesized that the gestation-induced increase in AQP4 predisposes the brain to edema formation under conditions that promote BBB disruption, such as acute hypertension, leading to the neurologic complications of eclampsia. This hypothesis is summarized in the diagram in Fig. 5. FJ

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