Ontogeny, Distribution, and Possible Functional Implications of an Unusual Aquaporin, AQP8, in Mouse Liver

Domenico Ferri,[1] Amelia Mazzone,[2] Giuseppa Esterina Liquori,[1] Grazia Cassano,[2] Maria Svelto,[2] and Giuseppe Calamita[2]

Aquaporins are channel proteins widely expressed in nature and known to facilitate the rapid movement of water across numerous cell membranes. A mammalian aquaporin, AQP8, was recently discovered and found to have a very distinct evolutionary pathway. To understand the reason for this divergence, here we define the ontogeny and exact subcellular localization of AQP8 in mouse liver, a representative organ transporting large volumes of water for secretion of bile. Northern blotting showed strong AQP8 expression between fetal day 17 and birth as well as at weaning and thereafter. Interestingly, this pattern was confirmed by immunohistochemistry and coincided both temporally and spatially with that of hepatic glycogen accumulation. As seen by reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry, fasting was accompanied by remarkable down-regulation of hepatic AQP8 that paralleled the expected depletion of glycogen content. The level of hepatic AQP8 returned to be considerable after refeeding. Immunoelectron microscopy confirmed AQP8 in hepatocytes where labeling was over smooth endoplasmic reticulum (SER) membranes adjacent to glycogen granules and in canalicular membranes, subapical vesicles, and some mitochondria. In conclusion, in addition to supporting a role for AQP8 in canalicular water secretion, these findings also suggest an intracellular involvement of AQP8 in preserving cytoplasmic osmolality during glycogen metabolism and in maintaining mitochondrial volume. AQP8 may have evolved separately to feature these intracellular roles as no other known aquaporin shows this specialization. (HEPATOLOGY 2003;38:947-957.)

Aquaporins are a family of proteinaceous channels widely distributed in a range of living organisms from bacteria to humans, where they mediate the osmotic movement of water across biological membranes.[1] Aquaporins are being shown to be involved in physiologic processes of central importance, including secretion and absorption of fluids in the gastrointestinal and reproductive tracts, renal water handling, fluid balance in the lung and brain, maintenance of fluid and ionic homeostases in the inner ear,[2] and preserving corneal transparency.[3] Aquaporins also have pathophysiologic relevance because they have been found in humans to underlie severe forms of nephrogenic diabetes insipidus,[4] defective urinary concentrating,[5] and congenital cataracts,[6] and their involvement in the pathogenesis of many other clinical conditions with altered fluid homeostasis is under intense investigation.

Among the 11 aquaporins (AQP0-AQP10) that have so far been recognized in mammals, AQP8 represents a very distinctive homologue as indicated by the striking divergence of its evolutionary pathway.[7] However, the reasons for this phylogenetic divergence are obscure. AQP8 was recently cloned from rat,[8,9] mouse,[10] and human[11] tissues. By heterologous expression in *Xenopus laevis* oocytes, the rat[8,9] and human[11] AQP8 channels were found to be selectively permeable to water, whereas the mouse homologue showed permeability also to urea.[10]

Abbreviations: mRNA, messenger RNA; E, embryonic day; P, postnatal day; RT-PCR, reverse-transcription polymerase chain reaction; PBS, phosphate-buffered saline; PAS, periodic acid–Schiff; SER, smooth endoplasmic reticulum.

From the [1]Department of Zoology, Laboratory of Histology and Comparative Anatomy, and [2]Department of General and Environmental Physiology, University of Bari, Bari, Italy.

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Address reprint requests to: Giuseppe Calamita, Ph.D., Dipartimento di Fisiologia Generale ed Ambientale, Università degli Studi di Bari, via Amendola 165/A, I-70126 Bari, Italy. E-mail: calamita@biologia.uniba.it; fax: (39) 0805443388.

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We recently cloned and structurally characterized the mouse *Aqp8* gene.[12] Interestingly, besides the presence of 3 distinct messenger RNA (mRNA) start sites and a possible site of alternative splicing, we found the exon-intron organization of *Aqp8* to be very distinct from that of other mammalian AQPs. We also mapped the mouse and human *AQP8* genes on the syntenic chromosomal regions 7F3[12] and 16p12,[13] respectively. AQP8 shows a wide ranging tissue expression being immunodetected in multiple locations of the gastrointestinal tract,[14-18] male and female reproductive systems,[19-21] nervous system,[22] and kidney and airways.[15] Important physiologic roles for AQP8 have been already suggested, including that of facilitating the secretion of canalicular bile water,[14,16,23,24] pancreatic juice,[17] and seminiferous fluid,[19] mediating the osmotic efflux of water out of the spermatid during spermiogenesis,[19] and being involved in the absorption of water both in the small and large intestine.[14] Recently, functional AQP8 has been shown to be inserted into the plasma membrane of isolated rat hepatocytes under glucagon challenge.[24] Interestingly, our recent collaborative work evidenced a pathogenetic contribution of AQP8 to bile secretory dysfunction of cholestatic hepatocytes.[25] Because one of the most typical aspects of AQP8 is its predominant intracellular localization, we did not disregard the possibility that this AQP might also be involved in the homeostatic osmoregulation of the cytoplasm and its vesicle/organelle content.[14] Indeed, there are no doubts that the intracellular localization of AQP8 is critical for its regulation and physiologic function and that assessment of the intracellular membrane systems expressing AQP8 may provide important clues in defining such aspects.

The present study was undertaken to study the developmental regulation and functional localization of AQP8 in liver, an organ of central importance managing considerable volumes of fluid daily. Besides identifying AQP8 in subcellular locations where water channels have never been found, the results reported here provide important information for an understanding of the functional and regulatory features of AQP8 in liver.

Materials and Methods

Animals

Balb/c mice of varying ages starting from embryonic day (E) 14 were obtained from Harlan Italy (San Pietro al Natisone, Italy) either with their actual mothers or with foster mothers. Animals, if not killed earlier, were weaned at the age of 20 postnatal days (P) and fed with a standard diet and water *ad libitum*. For all of the experiments, mice were decapitated after anesthesia and the tissues were collected as described in the following text. For the histochemistry and immunohistochemistry experiments, adult male Wistar rats (250-300 g; Harlan Italy) were fed and watered *ad libitum*.

Northern Blotting and Reverse-Transcription Polymerase Chain Reaction

Livers were removed from killed mice at different ages and frozen in liquid nitrogen. Total RNAs were isolated using the TRIzol extraction kit (Invitrogen, San Diego, CA). RNA samples (10 μg/lane) were electrophoresed through formaldehyde/1% agarose gels, transferred to nylon membranes, and hybridized with a 440–base pair reverse-transcription polymerase chain reaction (RT-PCR) fragment of rat *AQP8* complementary DNA as a probe labeled with α-^{32}P deoxycytidine 5′-triphosphate. Hybridization was performed in a solution of 50% formamide, 5× standard saline citrate, 5× Denhardt's solution, 1% sodium dodecyl sulfate, and 100 μg/mL denatured salmon sperm DNA at 42°C for 20 hours. The membranes were washed twice in 2× standard saline citrate and 0.1% sodium dodecyl sulfate at room temperature or 15 minutes each and once in 0.1× standard saline citrate and 0.1% sodium dodecyl sulfate at 42°C for 30 minutes. Membranes were autoradiographed with intensifying screens for 3 days. Expression of the *AQP8* mRNA was normalized against the expression of the 28S ribosomal RNA.

For experiments designed to study the expression of the *AQP8* mRNA in the liver of fasted and refed male mice, the fasted group (3 male Balb/c mice) was fasted for 48 hours (by keeping water *ad libitum*) before being killed and the livers removed. The refed group had free access to standard laboratory diet for 48 hours after a 48-hour fast. In parallel, the fed group (3 male Balb/c mice deriving from the same pool of littermates from whom the fasted and refed groups originated) was allowed to have free access to standard laboratory diet and tap water before being killed and the livers removed. Total RNAs from the livers were extracted as previously described. The samples of total RNA were submitted to semiquantitative RT-PCR as previously described[14] and the mouse *AQP8* primers mAQP8-2 (5′-ATGTCTGGGGAGCAGACA-3′) and mAQP8-5 (5′-TCAGGTCCTGCAGGAGAT-3′), which led to the amplification of an 882–base pair fragment of DNA. RT-PCR reactions were normalized against the β-actin expression.[14]

Northern blotting and RT-PCR bands were quantified by densitometry by using the NIH image 1.61 program. Densitometry data were calculated as means ± SE of 3 to 5 experiments per time point. P values less than .05 were considered statistically significant.

Antibodies

Antibodies to the C-terminal portion of rat AQP8 were from Alpha Diagnostic International (San Antonio, TX), whereas those directed against an N-terminal peptide of rat AQP8 (CSMDLREIKGKETNMAD) were prepared in our laboratory using standard immunization techniques and affinity purified using the SulfoLink kit (Pierce, Rockford, IL).

Determination of Hepatic Glycogen

Glycogen was extracted from the liver of fasted or fed mice by alkaline extraction.[26] Extracted glycogen was hydrolyzed by treatment with amyloglucosidase at pH 4.7, and the related glucose was measured spectrophotometrically with adenosine triphosphate, oxidized nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate dehydrogenase, and hexokinase.

Histochemistry and Immunohistochemistry

Mice or rats were killed after ether anesthesia, and their livers were quickly removed and processed to be included in Technovit 8100 (Heraeus-Kulzer, Wehrheim, Germany), a hydrophilic resin. Samples were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4, for 4 hours at 4°C. The samples were incubated overnight in PBS added with 6.8% sucrose and then dehydrated with acetone and embedded in Technovit 8100 at 4°C. Before staining, semithin sections were incubated for 5 minutes at 37°C in 0.01% trypsin in 0.1% $CaCl_2$ (pH 7.8).

Histochemistry. Sections were stained with the periodic acid–Schiff (PAS) reaction or with diastase PAS. The diastase digestion was performed by incubating the sections in diastase (α-amylase type VI-B; Sigma Chemical Co., St. Louis, MO) 0.2% in distilled water for 15 minutes at 37°C.

Immunohistochemistry. AQP8 was localized by the peroxidase-antiperoxidase method. Endogenous peroxidase was blocked by 1% H_2O_2 for 10 minutes at room temperature. Sections were then incubated for 5 hours at 37°C with the AQP8 affinity-purified antibodies at a concentration of 5μg/mL in blocking buffer (1% normal goat serum in PBS). Successively, sections were treated for 1 hour at 37°C with goat anti-rabbit immunoglobulin G (Sigma Chemical Co.) diluted 1:100 in blocking buffer and then incubated with peroxidase-antiperoxidase (Sigma Chemical Co.) at a dilution of 1:100 for 1 hour at 37°C. Finally, the immunolabeling was visualized by incubation with 3,3′-diaminobenzidine/H_2O_2 medium for 10 minutes at room temperature.

The immunolabeling was also performed after diastase digestion/PAS reaction to exclude unspecific reactions between glycogen and AQP8 antibodies. Controls were performed using antibodies preadsorbed with immunizing peptides or by omitting the primary antibodies. Images were captured using a photomicroscope (Leica DMRXA, Bannockburn, IL).

Immunoelectron Microscopy

Samples of liver were fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/L PBS at pH 7.4 for 4 hours at 4°C. Some specimens were postfixed in 1% OsO_4 in PBS for 30 minutes at 4°C. Fixed specimens were dehydrated in ethanol and then embedded in Epon (Taab, Reading, England).

For immunoelectron microscopy, ultrathin sections of osmicated samples were oxidized with sodium metaperiodate to restore specific labeling. Both osmicated and nonosmicated sections were treated with 0.05 mol/L glycine in PBS buffer for 15 minutes at room temperature. Grids were incubated for 30 minutes at room temperature with 1% bovine serum albumin in PBS containing 0.2% gelatin and then placed on a drop of AQP8 antibodies (10 μg/mL PBS containing 0.2% gelatin) overnight at 4°C. The grids were then incubated in 1:10 10-nm gold-conjugated anti-rabbit immunoglobulin G (Sigma Chemical Co.) in PBS containing 0.2% gelatin for 1 hour at room temperature and lightly stained with uranyl acetate and lead citrate. Finally, the grids were observed with a Zeiss EM 109 electron microscope (Zeiss, Germany). Immunolabeling controls were performed as in immunohistochemistry.

Results

AQP8 mRNA Analysis in Mouse Developing Liver

Total RNAs from mouse livers at multiple time points during development and adulthood showed a 1.5-kilobase transcript on Northern blots probed with a 440–base pair *AQP8* complementary DNA (Fig. 1A). The 1.5-kilobase size of the *AQP8* mRNA was the same as previously reported in the adult mouse by other investigators.[10] Interestingly, while a faint signal was detected in the livers on E14 to E15, the *AQP8* transcript abruptly increased at E17, reached considerably high levels at E19, 2 days before birth, and then decreased to significantly lower levels at P0, soon after birth. Little *AQP8* mRNA was detected in the liver of mouse pups up to P15, but levels increased significantly at weaning and increased steadily thereafter until maturity (Fig. 1B). Loading of the gels used for Northern blotting analysis was checked by

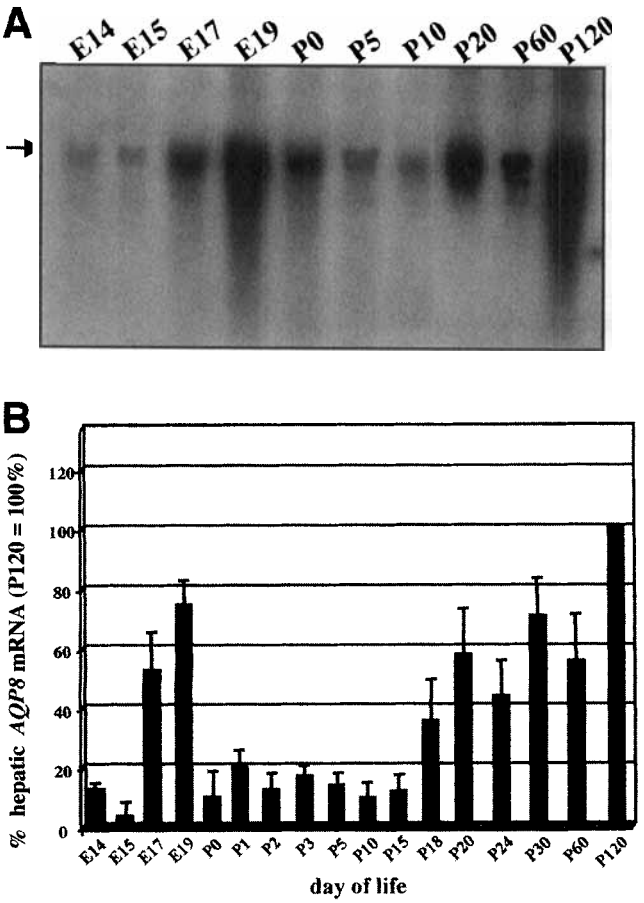

Fig. 1. Northern blotting analysis of the ontogeny of mouse *AQP8*. Ten micrograms of pools of total RNA extracted from the liver of mice at different days of fetal (E14-E19) and postnatal (P0-P120) life was probed with a 440-base pair fragment of the mouse *AQP8* open reading frame and detected by autoradiography. (A) Representative autoradiograph. The expression of *AQP8* (1.5-kilobase band, **arrow**) increases abruptly 4 days before birth (E17), peaks at E19, and decreases to a low level a few hours after birth. The *AQP8* band is once again strong at weaning (P18-P20) and thereafter, reaching its maximal extent at P120. (B) Densitometric analysis. The expression of *AQP8* in developing liver is compared with that measured in the P120 liver (adult liver), which is arbitrarily assumed as 100%. Each histogram represents the mean of at least 3 Northern blotting analyses of pooled total RNAs.

densitometry of the 28S RNA band stained by ethidium bromide (data not shown).

Immunohistochemical Localization of AQP8 in Developing Mouse Liver

The cellular and subcellular distribution of AQP8 in developing mouse liver was assessed by immunohistochemistry by using an affinity-purified immunoglobulin G specific for the C-terminal domain of rat AQP8 (brown staining). Hepatic glycogen was visualized by PAS reaction (red staining). As shown in Fig. 2A, no appreciable AQP8 immunostaining was observed in the liver at day 15 of gestation when the hepatic cords contained many hemopoietic cells, mainly erythroid precursors (bold arrows), some macrophages, and few small hepatocytes (thin arrows). The latter did not contain glycogen, being unreactive to the PAS reaction. At E18 (Fig. 2B), 3 days before birth, the hepatocytes were more numerous and larger those at E15 and few displayed PAS staining (thin arrows and inset) and weak AQP8 reactivity. Megakaryocytes and immature erythropoietic cells (bold arrows) were also present, mainly in the perisinusoidal areas. The AQP8 immunolabeling in hepatocytes became particularly strong at birth (P0) (Fig. 2C, arrows). Higher magnification clearly showed immunoreactive AQP8 over intracellular membranes widely distributed within the hepatocyte cytoplasm (inset). Interestingly, the pattern of cellular and subcellular AQP8 distribution in the liver appeared very similar to the distribution pattern of the glycogen granules (Fig. 2D, arrows and inset). In line with the Northern blotting analysis, AQP8 expression was low in liver on P5 to P15, where the number and staining intensity of immunoreactive hepatocytes appeared significantly diminished (Fig. 2E) compared with the P0 liver. At P5 to P15, low levels of glycogen stores within the hepatocytes were also observed (Fig. 2F). The AQP8 immunoreactivity was once again considerable at weaning (P20), when most hepatocytes showed an increase in intracellular staining (Fig. 2G). Consistent with a spatial and temporal correlation between glycogen accumulation and AQP8 expression, most hepatocytes showed a significant increase in glycogen volume density at P20 (Fig. 2H).

Cellular Distribution of AQP8 in Adult Mouse Liver

Immunohistochemical experiments were also performed to assess the precise cellular and subcellular distribution of AQP8 in the adult mouse liver. The pattern of AQP8 staining in the adult liver (P120) remained unchanged compared with that seen with the developing liver at P20; however, consistent with the Northern blotting studies, a general increase in labeling intensity within the immunoreactive hepatocytes was observed (Fig. 3A). Overall, AQP8 labeling seemed to be more extensive in the periportal and midlobular than the pericentral regions of the hepatic lobule. However, hepatocytes with heavy immunoreactivity were sometimes found lining the centrolobular vein (arrow). No apparent labeling was seen in cholangiocytes (inset, double arrows), the epithelial cells lining the intrahepatic bile ducts, or endothelial or Kupffer cells. The specificity of AQP8 labeling in mouse liver was confirmed by the fact that an identical immunolabeling pattern was obtained by using affinity-purified antibodies developed against the N-terminal domain of

rat AQP8 (Fig. 3B). No staining was observed in control experiments performed omitting the primary antibody (Fig. 3C). In agreement with our preliminary observations,[14] a similar pattern of cellular and subcellular AQP8 distribution was seen in rat liver (Fig. 3D). As in mouse liver, rat cholangiocytes did not show apparent AQP8 reactivity (double arrows).

Spatial Association of AQP8 With Hepatic Glycogen Stores

The spatial and temporal relationship between hepatic glycogen stores and AQP8 evidenced by the ontogenetic studies was better evaluated by histochemistry and immunohistochemistry, immunogold electron microscopy, and semiquantitative analyses of fasted versus fed/refed mice.

In a first series of experiments, control sections of adult mouse liver were submitted to PAS reaction or AQP8 immunolabeling to evidence the presence of glycogen stores (Fig. 4A) and AQP8 (Fig. 4B), respectively. Other sections of the same livers were treated with diastase, an enzyme known to hydrolyze glycogen, and PAS and then immunostained or not with the AQP8 antibodies. The sections in which diastase treatment was followed by AQP8 immunolabeling fully retained the typical pattern of AQP8 reactivity, indicating absence of unspecific reaction between AQP8 antibodies and glycogen (Fig. 4C and D).

The spatial association between glycogen stores and AQP8-containing membranes in mouse liver was also analyzed at an ultrastructural level by immunogold electron microscopy (Fig. 5). In line with the previous immunohistochemical studies, gold particles (Fig. 5A, arrows and inset) were often observed in smooth endoplasmic reticulum (SER) membranes surrounding glycogen stores (Fig. 5A; inset, arrowheads). The functional correlation between hepatic glycogen granules and SER membranes is established evidence because it has been shown that 2 important enzymes regulating glycogen synthesis, glycogen synthase phosphatase and glycogen phosphorylase phosphatase, are associated with SER membranes adjacent to glycogen particles.[27] However, AQP8 was not exclusively localized in SER membranes because gold particles were also frequently observed over the canalicular membrane (Fig. 5B, arrows), intracellular vesicles often located just beneath the canalicular membrane (Fig. 5B, inset), and some mitochondria (Fig. 5C, single and double arrows).

Temporal Correlation of SER AQP8 and Hepatic Glycogen Accumulation

Because fasting leads to hepatic glycogenolysis with consequent depletion of hepatic glycogen deposits,[28] the possible temporal correlation between glycogen accumulation and AQP8 expression was studied by assaying the *AQP8* mRNA in fasted versus fed or refed mice by semiquantitative RT-PCR. Forty-eight hours of fasting led to a nearly 70% decrease in the hepatic level of *AQP8* mRNA, which paralleled the expected reduction (−65%) in hepatic glycogen content (Fig. 6A-C). When mice fasted for 48 hours were refed, levels of AQP8 returned to the fed levels after 48 hours (Fig. 6B). This result was confirmed by light microscopy, by which fed livers showed both AQP8 and glycogen granules (Fig. 7A and B), whereas 48 hour–fasted livers showed a remarkable reduction in AQP8 immunolabeling (Fig. 7C) accompanied by a strong decrease in glycogen stores (Fig. 7D). AQP8 and glycogen were once again expressed after 48 hours of refeeding (Fig. 7E and F).

Discussion

The recent recognition of aquaporin water channels in the hepatobiliary tract[29] is highly instructive, providing important insights into the full knowledge of the molecular mechanisms underlying the hepatocellular hydration state and the movement of water across the hepatobiliary epithelia in bile formation. Here, we have assessed the ontogenetic expression and ultrastructural localization of AQP8 in mouse liver and found a temporal and spatial correlation between hepatic glycogen synthesis and accumulation and AQP8 expression. The results suggest that AQP8 is involved in important processes such as the formation of canalicular bile, glycogen deposition, and mitochondrial volume homeostasis.

One of the most distinctive features of the mouse hepatocyte AQP8 observed in this work is its multiple subcellular distribution with widespread expression in intracellular membrane systems. Indeed, AQP8 immunoreactivity is found in the SER and to a relatively minor extent in canalicular membranes, some intracellular vesicles, and mitochondria. No other characterized AQPs display such a varied pattern of subcellular distribution, making AQP8 unique among aquaporins. This biological property may relate to several other distinctive aspects already reported for this AQP such as (1) the remarkable divergence of its evolutionary pathway,[7,9] (2) the unusual organization and predicted membrane topology of its gene[12,30] and its protein,[8-10] respectively, (3) the presence of at least 3 active mRNA start sites in the mouse *Aqp8* gene,[12] and (4) the existence of at least 3 expressed sequence tags database (dEST) sequences of mouse *AQP8* distinct at their 5′ end (accession nos. BU522621, CA457561, and CA773076).

The presence of AQP8 immunoreactivity in the mouse canalicular membrane and the fact that hepatic *AQP8*

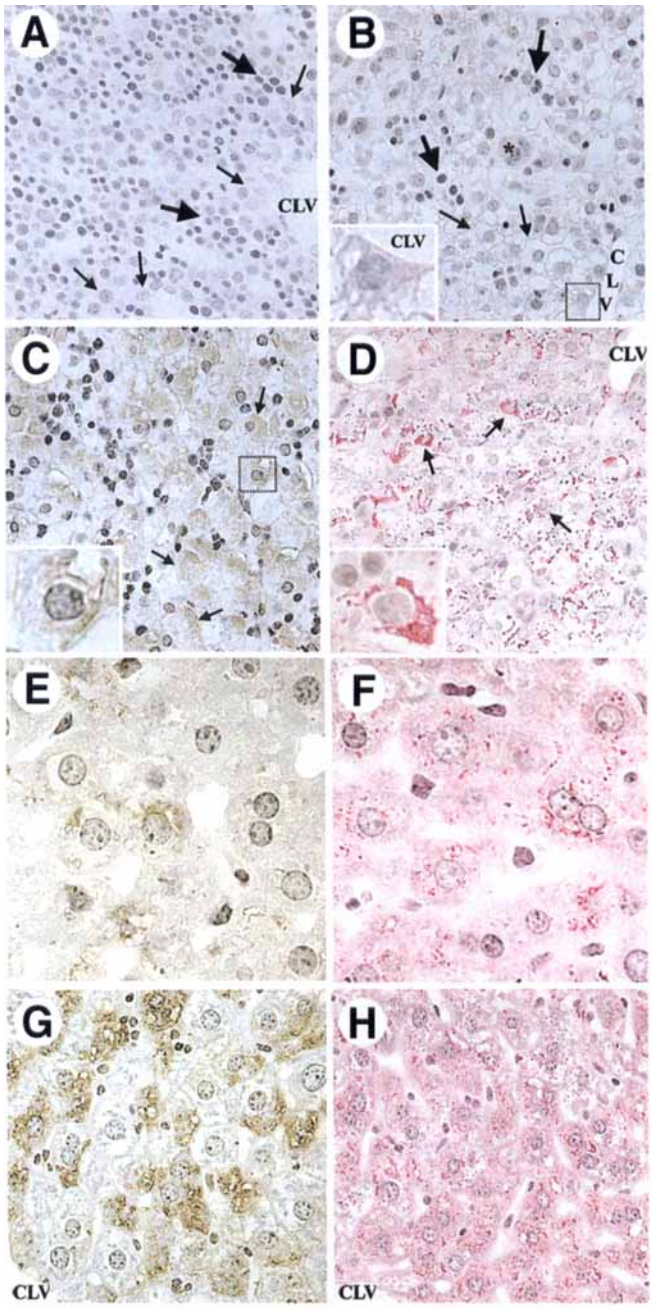

Figure 2.

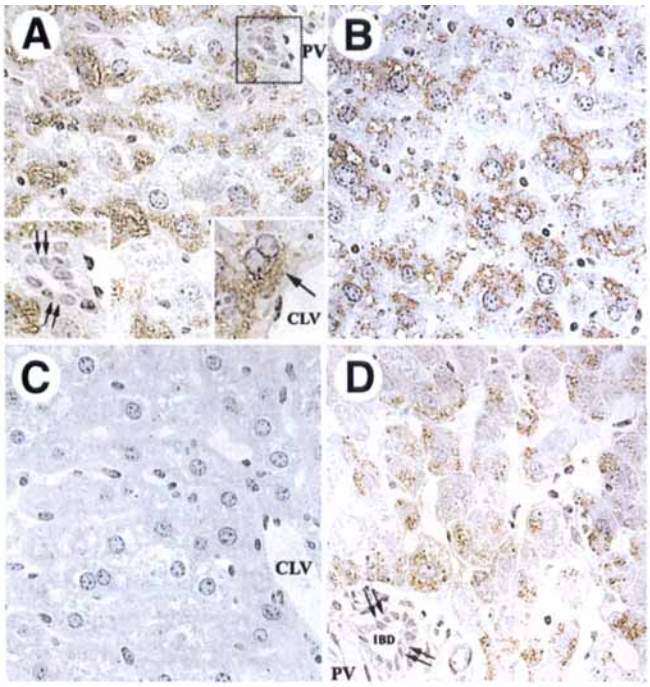

Figure 3.

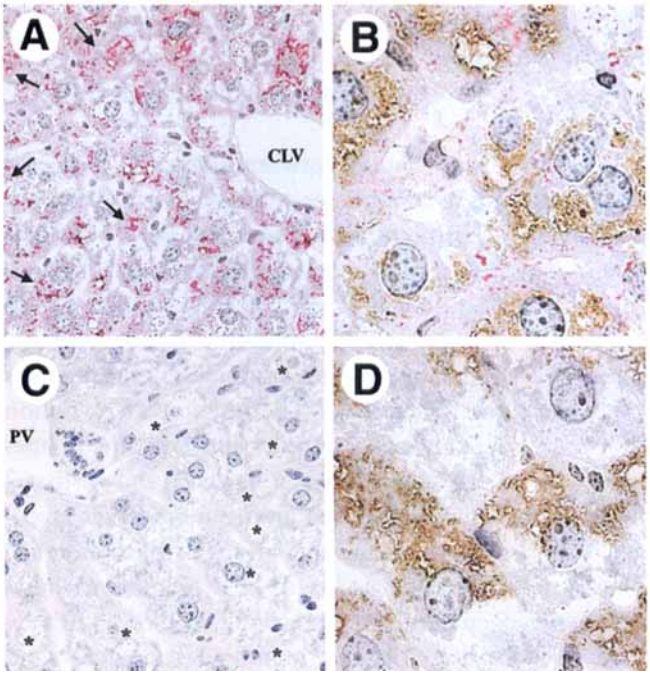

Figure 4.

mRNA and protein expression increase abruptly at the time of weaning when the hepatobiliary transport systems complete their maturation[31,32] suggest that this AQP is involved in canalicular bile formation likely mediating the transcellular movement of bile water. A similar role for AQP8 was recently hypothesized in rat liver by us and other investigators.[14,16,23,24] Hence, it is attractive to speculate that a pool of intracellular AQP8 is inserted exocytotically into the canalicular membrane of the mouse or rat hepatocyte in response to choleretic stimuli. This functional pool of AQP8 would relate to intracellular vesicles being distinct from the other pools residing in SER membranes and mitochondria, respectively. The translocation of the AQP8 channel into the apical membrane

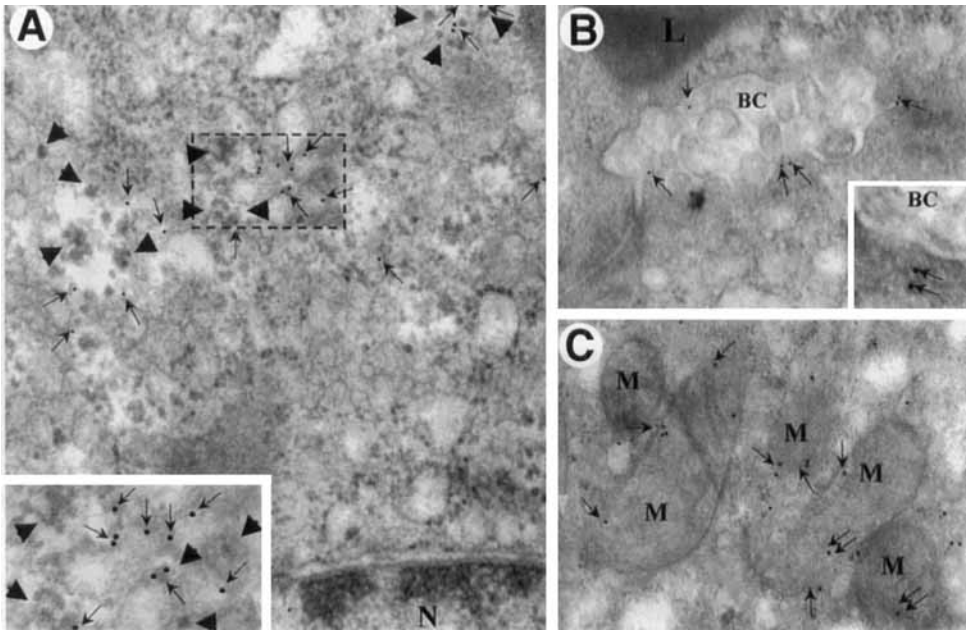

Fig. 5. Ultrastructural localization of AQP8 in mouse liver hepatocytes by immunogold electron microscopy. (A) AQP8 immunogold labeling (**single arrows**; **inset**) is observed in SER membranes adjacent to glycogen particles (**arrowheads**; **inset**). (B and C) Immunoreactive sites are also observed over the canalicular membranes (B, **single arrows**), subapical vesicles (B, **inset**), and some mitochondria (C, **single and double arrows**). Labeled mitochondria show immunoreactivity both on the edges (**single arrows**) as well as cristae membranes (**double arrows**). N, nucleus; L, lysosome; BC, bile canaliculus; M, mitochondrion. (Original magnification: A-C, ×12,000; inset in A, ×22,000; inset in B, ×20,000.

may explain the rapid and selective increase in canalicular osmotic water permeability observed during the active canalicular secretion of cholephilic compounds such as bile salts, glutathione, and inorganic ions.[33] Many canalicular transporters are known to undergo regulated exocytotic insertion into the canalicular membrane.[34] Moreover, aquaporins with regulated recruitment into the apical membrane in response to specific stimuli (hormones) are already known: AQP2,[35,36] AQP1,[37] and AQP5.[38] Mouse (and also rat) hepatocytes also express AQP9, an aquaglyceroporin localized exclusively in the basolateral membrane,[39,40] which may be responsible for the sinusoidal uptake of water. AQP9 has also been suggested to be the route for the hepatic uptake of blood

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Fig. 2. Immunohistochemical localization of AQP8 in mouse developing liver. (A) At day 15 of gestation (E15), the hepatic lobules contain mainly erythroid precursors (**bold arrows**). Hepatocytes (**thin arrows**) are few and small and are apparently unreactive to PAS staining or anti-AQP8 antibodies. (B) Three days before birth (E18), hepatocytes (**thin arrows**) are more numerous and larger than at E15, whereas erythroid precursors (**bold arrows**) are diminished. Some hepatocytes evidence a weak PAS positivity (**inset, red staining**). (C and D) At birth (P0), (C) AQP8 immunolabeling and (D) PAS positivity are intense and extensive in hepatocytes (**arrows**). Both reactivities are apparently restricted to the intracellular compartment (**insets**). (E and F) Five days after birth (P5), both the (E) AQP8 immunolabeling and (F) PAS staining appear significantly diminished compared with the perinatal livers. (G and H) At weaning (P20), the (G) intracellular AQP8 immunolabeling and (H) PAS staining of hepatocytes are once again of remarkable extent. CLV, centrolobular vein; *, megakaryocyte. (A and B) Antibodies anti-AQP8 C-terminus/PAS/Mayer's hematoxylin; (C, E, and G) antibodies anti-AQP8 C-terminus/Mayer's hematoxylin; (D, F, and H) PAS/Mayer's hematoxylin. (Original magnification: A-D, G, and H, ×400; E and F, ×1,000; inset in B, ×1,300; insets in C and D, ×1,500.)

Fig. 3. Cellular distribution of AQP8 in adult mouse and rat liver. (A) In adult mouse liver (P120) incubated with anti-AQP8 C-terminus antibodies, considerable immunoreactivity is noted within the cytoplasmic compartment of most hepatocytes. Overall, labeling appears to be greater in the periportal and midlobular regions than in the pericentral area. However, hepatocytes with remarkable immunoreactivity are often seen lining the centrolobular vein (**right inset, single arrow**). No AQP8 reactivity is observed in the cholangiocytes lining the intrahepatic bile duct (**left inset, double arrows**). (B) The pattern of cellular and subcellular distribution of AQP8 in the mouse hepatic lobule is not changed by using affinity-purified antibodies directed against the N-terminal domain of rat AQP8. (C) Control experiment performed by omitting the primary antibodies shows no staining in mouse liver. (D) Like in mouse liver, the AQP8 expression in adult rat liver appears to be predominantly intracellular and of larger extent in hepatocytes residing in the periportal and intermediate lobular areas. No labeling is observed in the epithelial cells lining the intrahepatic bile ducts (**double arrows**). PV, portal venule; CLV, centrolobular vein; IBD, intrahepatic bile duct. (A and C) Anti-AQP8 C-terminus/Mayer's hematoxylin; (B) anti-AQP8 N-terminus/Mayer's hematoxylin; (D) control staining by omitting the primary antibody in peroxidase-antiperoxidase method. (Original magnification: A-D, ×400; insets in A, ×720.)

Fig. 4. Spatial association of intracellular AQP8 with glycogen stores in the adult mouse. (A) In the adult fed mouse, large quantities of glycogen stores are observed in most periportal and pericentral hepatocytes (**red staining, arrows**) and less frequently in centrolobular hepatocytes. (B) Generally, AQP8 immunolabeling and PAS reactivity show a similar pattern of intracellular distribution in hepatocytes. This spatial correlation is also indicated by the fact that PAS staining of glycogen adjacent to SER membranes is masked by the dehydrogenated diaminobenzidine precipitating over the AQP8 immunoreactive sites. (C) Pretreatment with the glycogen-hydrolyzing enzyme diastase causes digestion of glycogen and consequently total loss of PAS reactivity (**asterisks**). (D) Retaining of typical pattern of AQP8 reactivity after diastase digestion/PAS reaction excludes unspecific reaction between AQP8 antibodies and glycogen. CLV, centrolobular vein; PV, portal venule. (A) PAS/Mayer's hematoxylin; (B) anti-AQP8 C-terminus/PAS/Mayer's hematoxylin; (C) diastase/PAS/Mayer's hematoxylin; (D) diastase/anti-AQP8 C-terminus/PAS/Mayer's hematoxylin. (Original magnification: A and C, ×400; B and D, ×1,000.)

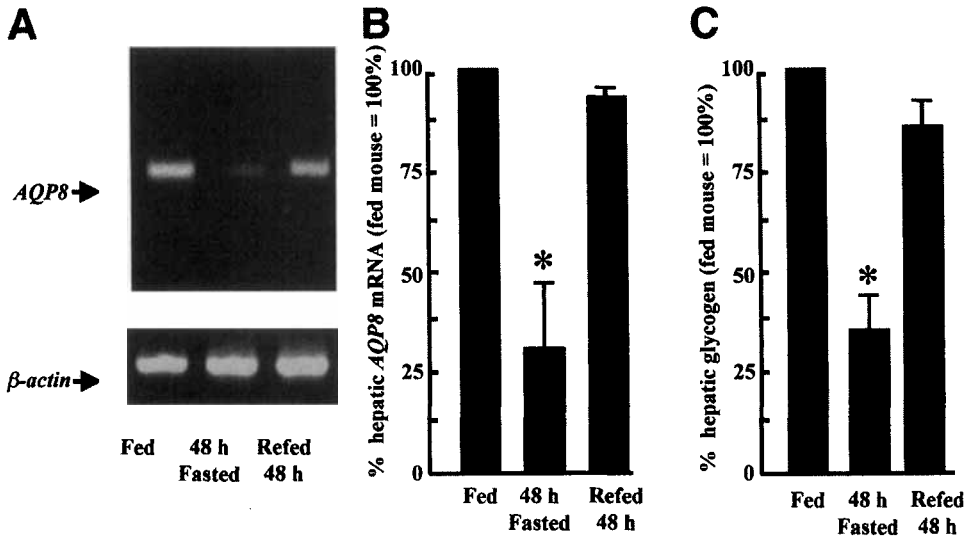

Fig. 6. Effect of fasting and refeeding on expression of *AQP8* mRNA and glycogen in mouse liver. Mice were killed after 48 hours of fasting or after 48 hours of refeeding and the livers removed for semiquantitative RT-PCR assay and determination of glycogen contents, respectively. (A) RT-PCR analysis, representative gel. The expression of *AQP8* (882-base pair band, **arrow**) was normalized against that of β-actin, a housekeeper gene. A striking reduction in the *AQP8* transcript is observed in fasted compared with fed or refed mice. (B) Densitometric analysis of hepatic *AQP8* expression. The expression of *AQP8* in fed mice is assumed arbitrarily as 100%. Fasting leads to a 68.9% ± 14.6% reduction (*$P < .01$) of the hepatic *AQP8* transcript compared with that of fed mice. The *AQP8* mRNA returns to the fed levels after 48 hours of refeeding (97% ± 3.9%). (C) Determination of hepatic glycogen content. The content of glycogen in fed mice is assumed arbitrarily as 100%. As expected, fasting leads to a strong depletion of the glycogen content (−64.6% ± 10.2%; *$P < .01$) whereas refeeding restores the hepatic glycogen deposits. Assays were performed in triplicate. See Materials and Methods for more experimental details.

glycerol,[40,41] a major substrate for hepatic gluconeogenesis, or to be the molecular pathway for the exit of urea produced within the hepatocyte. Huebert et al.[23] recently hypothesized that, under choleresis, water transport across the hepatocyte takes place mainly through AQPs, whereas in the basal (unstimulated) state it is non–channel mediated, occurring through diffusional (*i.e.*, via the lipid bilayer) and paracellular routes.

The close temporal parallel between AQP8 expression and glycogen accumulation in fetal and postnatal mouse hepatocytes is as much striking as surprising and provides interesting clues into the knowledge of the physiologic meaning of AQP8. The remarkable ontogenic peak of expression observed a few days before birth when the bile secretory function is still immature[32] suggests that AQP8 has additional functions than its involvement in canalicular secretion. Interestingly, the peak of fetal AQP8 expression coincides exactly with the known transient accumulation of large quantities of hepatic glycogen rapidly mobilized as glucose to sustain the newborn until the onset of suckling and gluconeogenesis.[32,42-45] The fact that the transcript level of *AQP8* is high at E18 whereas the related protein is weak probably relates to the time shift needed to translate the mRNA that is suddenly accumulated in the cytoplasm on that day of gestation. We previously observed a similar time shift at the onset of AQP8 expression in developing rat testis.[19] The expression of AQP8 and glycogen accumulation are again increased at weaning, likely reflecting the hepatic adaptation to the onset of meal feeding. The involvement of AQP8 in the processes underlying the hepatic storage of blood/glucose in the form of glycogen in mouse is indicated by additional observations in this work. (1) The changes in both the hepatic AQP8 transcript and protein parallel exactly the changes in hepatic glycogen consequent to fasting and refeeding. This does not seem to be a general physiologic mechanism of regulation because AQP9, the other aquaporin expressed in hepatocytes, is up-regulated in fasted liver.[41] (2) AQP8 shows higher expression in the periportal and medial regions of the hepatic lobule, which are the sites where most glycogen is stored. (3) As shown by the immunoelectron microscopy experiments, intracellular AQP8 is mostly expressed in SER membranes restricted to glycogen granules. The hepatic SER and glycogen stores are functionally correlated because of the association of 2 important enzymes regulating glycogen synthesis, glycogen synthase phosphatase and glycogen phosphorylase phosphatase, with SER membranes adjacent to glycogen particles.[27] The precise elucidation of the role of SER AQP8 in the biophysical processes associated with glycogen synthesis and deposition is a matter for further study. However, it is attractive to speculate that the specific function played by AQP8 is to mediate the osmotic movement of water between the

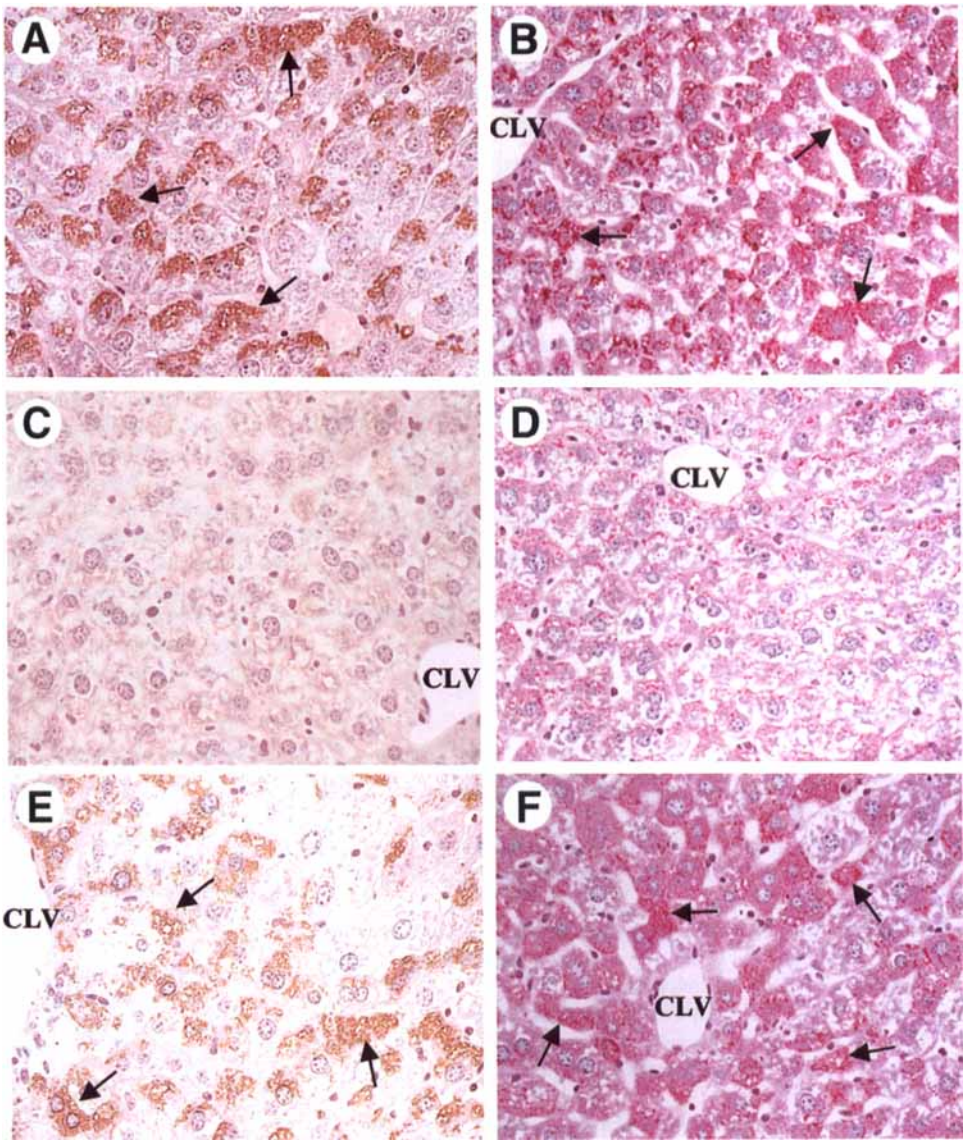

Fig. 7. Immunohistochemical expression of hepatic AQP8 in fed, fasted, and refed mouse. (A) Distribution of AQP8 in fed mouse liver. Remarkable AQP8 labeling is observed in the liver of fed mice. AQP8 reactivity is seen in most hepatocytes where staining is located predominantly within the cell interior (**arrows**). (B) Histochemistry of glycogen in fed liver. A large amount of glycogen granules (**arrows**) are seen in the hepatocytes. (C) Distribution of AQP8 in fasted mouse liver. Very little intracellular AQP8 immunoreactivity is seen in the liver of mice submitted to 48 hours of fasting. (D) Histochemistry of glycogen in fasted liver. As expected, 48 hours of fasting leads to considerable depletion of the glycogen deposits. (E) Immunohistochemistry of AQP8 in the liver of mice refed after 48 hours of fasting. Confirming the mRNA studies, hepatic AQP8 is once again expressed to a considerable extent (**arrows**) in mice refed for 48 hours following 48 hours of fasting. (F) Histochemical staining of hepatic glycogen in refed mouse. As expected, refeeding leads to glycogen reaccumulation within the hepatocyte cytoplasm (**arrows**). CLV, centrolobular vein. (A, C, and E) Anti-AQP8 C-terminus/Mayer's hematoxylin; (B, D, and F) PAS/Mayer's hematoxylin. (Original magnification: A-E, ×400.)

SER lumen and the region of the hepatocyte cytoplasm where newly deposited glycogen occurs. However, an alternative function for SER AQP8 may be its involvement in the movements of water underlying the hepatocellular hydration state, a condition by which canalicular secretion is efficiently controlled in a short-term way.[46] Also, a role for AQP8 in mediating the movement of small solutes across SER membranes cannot be discarded because the permeability of the AQP8 channel is presently not fully assessed.

Like other SER proteins, AQP8 could be regulated transcriptionally by hormones that act on the surface density and composition of the SER membranes involved in glycogen synthesis[47] and breakdown[48] such as glucocorticoids and glucagon, respectively. This possibility is a matter for future studies. Interestingly, the ontogenic expression of hepatic AQP8 also coincides with the hormonally controlled maturation of the mouse hepatocyte SER.[32,49] The mouse liver AQP9 has recently been shown to be regulated at a transcriptional level coordinately by the plasma concentrations of insulin in accordance with the nutritional condition, such as fasting and refeeding. Whether SER AQP8 is regulated by hormones controlling the metabolism of hepatic glucose is an exciting matter for future studies.

The recognition of AQP8 in mitochondria represents a striking finding because aquaporins may represent the molecular pathway underlying the osmotic movement of water across the inner membrane during changes in mitochondrion volume. Hence, AQP8 may be important for mitochondrial function because this organelle must maintain its volume homeostasis to perform oxidative phosphorylation.[50]

In summary, this work defines the ontogenic expression of AQP8 in mouse liver, reports its ultrastructural distribution, and documents a close temporal and spatial

correlation between SER AQP8 and glycogen accumulation in hepatocytes. Data support a developmental regulation of AQP8 in mouse liver and an involvement of AQP8 in canalicular bile secretion and suggest a homeostatic implication of AQP8 in the biophysical processes associated with glycogen synthesis and deposition likely mediating the osmotic movement of water between the SER lumen and cytoplasmic regions where glycogen is accumulated. The striking observation that AQP8 is present in mitochondria provides important insights into knowledge of the molecular mechanism by which mitochondria preserve their volume to ensure oxidative phosphorylation. Because no other aquaporins have been recognized in SER and mitochondria, it is conceivable to speculate that the evolutionary pathway of AQP8 was addressed to perform the above intracellular functions.

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