Phenotype analysis of aquaporin-8 null mice

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Yang, Baoxue, Yuanlin Song, Dan Zhao, and A. S. Verkman. Phenotype analysis of aquaporin-8 null mice. Am J Physiol Cell Physiol 288: C1161–C1170, 2005. First published January 12, 2005; doi:10.1152/ajpcell.00564.2004.—Aquaporin-8 (AQP8) is a water-transporting protein expressed in organs of the mammalian gastrointestinal tract (saliary gland, liver, pancreas, small intestine, and colon) and in the testes, heart, kidney, and airways. We studied the phenotype of AQP8-null mice, and mice lacking AQP8, together with AQP1 or AQP5. AQP8-knockout mice lacked detectable AQP8 transcript and protein, and had reduced water permeability in plasma membranes from testes. Breeding of AQP8 heterozygous mice yielded AQP8-null mice, whose number, survival, and growth were not different from those of wild-type mice. Organ weight and serum/urine chemistries were similar in wild-type and AQP8-null mice, except for increased testicular weight in the null mice (4.8 ± 0.7 vs. 7.3 ± 0.3 mg/g body wt). Urinary concentrating ability in AQP8-null mice was unimpaired as assessed by urine osmolality (3,590 ± 360 mosmol/kgH2O) and weight loss (22 ± 2%) after 36-h water deprivation; urinary concentrating ability was similarly impaired in AQP1-null mice vs. AQP8/AQP1 double-knockout mice. Agonist-driven fluid secretion in salivary gland was not different in AQP8 vs. wild-type mice (~1 μl/min · g body wt −1) or in AQP5-null mice vs. AQP8/AQP5 double-knockout mice. Closed intestinal loop measurements in vivo indicated unimpaired osmotically driven water transport, active fluid absorption, and cholera toxin-driven fluid secretion in AQP8-null mice. After 21 days on a 50% fat diet, wild-type and AQP8-null mice had similar weight gain (~15 g), with no evidence of steatorrhea or dry skin with reduced glycerol content in AQP3 deficiency (12). Phenotype studies have also revealed the tissue-specific expression of aquaporins without demonstrable physiological import. For example, in the lung, despite substantial reduction of transalveolar osmotic water permeability, AQP1 and AQP5 knockout mice had unimpaired lung function, alveolar fluid absorption, neonatal fluid clearance, and response to lung injury (2, 21, 42). In the gastrointestinal tract, which, next to the kidney, has the highest rate of transepithelial fluid transport, mice lacking AQP4 have normal colonic fluid transport despite AQP4 expression in colonic epithelium (50) and normal gastric acid secretion despite AQP4 expression in gastric crypts (49).

AQP8 has been proposed to be a potentially important water transporter in the gastrointestinal tract. Three groups reported the cloning and functional analysis of AQP8, including Ishibashi et al. (16) and Koyama et al. (18) in the rat and our laboratory (27) in the mouse. Initial Northern blot and RT-PCR analyses indicated strong AQP8 transcript expression in organs of the rat and mouse gastrointestinal tract, including the salivary gland, liver, pancreas, small intestine, and colon. AQP8 transcript was also seen in the testes, kidney, and heart. Subsequent immunolocalization studies from several laboratories reported AQP8 protein expression in salivary gland, liver, pancreas, small intestine, colon, kidney, and testes (4–6, 13, 15, 31, 44). However, available anti-AQP8 antibodies have been poor because AQP8 has few polar residues at its NH2 and COOH termini compared with other aquaporins. In tissue distribution studies, several possible functions of AQP8 have been proposed, including secretion of saliva, bile, and pancreatic fluid, intestinal fluid absorption/secretion, and urinary concentration. If correct, then pharmacological modulation of AQP8 function might be useful in regulating intestinal fluid transport: for example, AQP8 inhibition might reduce intestinal hypersecretion in cholera.

Here, we examine the phenotype of AQP8-null mice using methods established previously to study gastrointestinal, glan- dular, renal, and reproductive phenotypes in mice. Contrary to expectations from the AQP8 expression pattern and from results in transgenic mice lacking other aquaporins, we found only minor phenotype differences between wild-type and AQP8 mice, even after attempting to expose subtle phenotype differences by physiological stresses and codeletion of other aquaporins.

METHODS

AQP8 knockout mice. The targeting vector for homologous recombination consisted of a 4.8-kb genomic AQP8 DNA fragment spanning from exon 1 to part of exon 4 (left arm), and a 3.0-kb fragment spanning parts of exons 4 through 6 (right arm) (Fig. 1A). A 35-bp fragment (GGTGCCTGCAATGAGAAGACAATGGGCCCTTT-AGC) in exon 4 was replaced with a 1.8-kb PpolNeoPbA cassette for

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positive selection, and a PGK-tk cassette was inserted downstream for negative selection. The linearized targeting vector was electroporated into 129/SvJ × 129/Sv-CP embryonic stem cells, and targeted embryonic stem cells were injected into C57Bl/6 blastocysts. Chimeric mice were bred with C57Bl/6 mice to produce F1 heterozygotes. Offspring were genotyped by genomic PCR and Southern blot analysis. Heterozygous founder mice containing the disrupted AQP8 gene (Fig. 1) were mated to produce homozygous AQP8 knockout mice. Offspring were genotyped by genomic PCR and Southern blot analysis for AQP8 gene targeting was confirmed by Southern hybridization, in which 10 µg of genomic DNA were digested with EcoRV, electrophoresed, transferred to a nylon+ membrane (Amersham Pharmacia), and hybridized with a 0.7-kb genomic fragment (indicated in Fig. 1A). For Northern blot analysis, total RNA from mouse tissues was isolated using TRIzol reagent (Invitrogen), mRNA was purified using the Oligotex direct mRNA kit (Qiagen) and resolved on a 1% formaldehyde-agarose denaturing gel (1.5 µg/lane), transferred to a nylon+ membrane, and hybridized at high stringency with a 32P-labeled probe corresponding to the mouse AQP8 cDNA coding sequence.

Reverse transcription PCR. Total RNA from mouse tissues was reverse transcribed with oligo(dT) (SuperScript II preamplification kit; Invitrogen). PCR was carried out using the GeneAmp PCR system 9700 with Taq DNA polymerase (Invitrogen) using the following primers: 5’-ATGTTGGGAGACACAAATGTG-3’ (sense) and 5’-TACCTCGACTTTAGAATTAGGCG-3’ (antisense) for AQP8 and 5’-TATGTCCTTGGTGCTGACC-3’ (sense) and 5’-CAGGTTCA-GACGAGGATT-3’ (antisense) for β-actin as reference. Primer sequences were derived from published sequences with GenBank accession numbers BC010982 (AQP8) and NM007393 (β-actin). PCR products were electrophoresed on a 2% agarose gel.

Immunofluorescence and immunoblot analysis. Testes, liver, kidney, brain, heart, colon, and salivary gland tissue samples were fixed with 4% paraformaldehyde in PBS for 4 h, infiltrated with 30% sucrose in PBS overnight, frozen in optimum cutting temperature compound with liquid nitrogen, and cut into 3-µm-thick sections with a cryostat. Tissues were incubated with 1:500 dilution of rabbit polyclonal serum (Fig. 1A) and probed as indicated in A. C: Northern blot of mouse liver probed with the mouse AQP8 coding sequence. D: immunoblots of mouse submandibular gland, liver, colon, and testes using polyclonal AQP8 antiserum. +/+ , wild type; −/− , null.

Water permeability measurements. Stop-flow measurement of cell and vesicle osmotic water permeability were carried out on a Hi-Tech SF-51 instrument. The kinetics of decreasing cell/vessel volume were measured from the time course of 90° scattered light intensity at 530-nm wavelength. Hepatocytes were isolated using a modification of the two-step liver perfusion method (41). Livers were perfused with Liver Perfusion Medium (35 ml over 10 min) and then Liver Digest Medium (35 ml over 10 min) (GIBCO). Isolated hepatocytes were filtered, washed, and suspended in Williams’ Medium E (GIBCO) containing 5% fetal bovine serum and 2 mM glutamine. For measurement of osmotic water permeability, hepatocytes were suspended in PBS at −106 cells/ml and subjected to a 250 mM inwardly directed gradient of sucrose. In some experiments, hepatocytes were incubated with 100 µM dibutyryl cAMP (Bt2cAMP; Sigma) at 37°C for 10 min before measurements. Osmotic water permeability coefficients (P) were computed from the time course, as described previously (48). For measurements on fractionated membrane vesicles, the liver or testes were homogenized as described above, and a post-nuclear supernatant was mixed with an equal volume of 2.3 M sucrose to obtain a 1.4 M sucrose fraction. The sucrose density gradient consisted of the following: 0.5 ml of 2.0 M sucrose, 1 ml of 1.6 M sucrose, 2 ml of 1.4 M sucrose fraction containing homogenate, 2 ml of 1.2 M sucrose, and 0.5 ml of 0.8 M sucrose. The gradients were centrifuged at 2.5 h at 37,500 rpm in an SW 50.1 rotor, and 1-ml fractions were collected. Membrane pellets were resuspended at −1 mg of protein/ml in PBS and passed 10 times through a 27-gauge needle. In some experiments, 0.3 mM HgCl2 was added to the vesicle suspension before stop-flow experiments.

Urinary concentrating studies. Urine samples were collected by placing mice on a wire mesh platform in a clean glass beaker until spontaneous voiding was observed. In some experiments, urine samples were obtained from the same mice under basal conditions (unrestricted access to food and water) and after 18- and 36-h
deprivation of food and water. Blood samples were collected in heparinized glass tubes by puncture of the periportal venous sinus. Plasma was separated from blood cells by centrifugation. Urine osmolality was measured by freezing-point osmometry (Micro-osmometer; Precision Systems). Urine and plasma chemistries were measured by the University of California, San Francisco, Clinical Chemistry Laboratory.

Salivary gland fluid secretion. The mice were anesthetized with the use of intraperitoneal Avertin (0.01 ml/g, 2.5%). Saliva production was stimulated by subcutaneous injection of pilocarpine (80 mg/kg) as described previously (23). Saliva was collected in preweighed vials during two 5-min intervals using a suction apparatus. Mice were positioned on their sides with their heads slightly downward to facilitate suctioning every 10–15 s.

Intestinal fluid transport. Osmotic water permeability, isosmolar fluid absorption, and cholera toxin-induced fluid secretion were measured in midjejunum and/or colonic loops in vivo. After solid food was withheld for 24 h (5% sucrose in water was given instead), the mice were anesthetized with Avertin (0.01 ml/g, 2.5%). Body temperature was kept at 36–37°C using a heating lamp and a heating pad during anesthesia and measurement. The small intestine and colon were exposed by a midline abdominal incision. Loops of jejunum and/or colon (12–20 mm length) were isolated using 5-0 nylon suture. For measurement of intestinal fluid secretion, in which loops were injected with 0.1 ml of a fluid sample was withdrawn, and the peritoneum and skin were closed with sutures. The intestine was exposed at 5 or 15 min to withdraw fluid samples for measurement of osmolality. For measurement of isosmolar fluid absorption, jejunal and descending colonic loops were created as above and injected with physiological PBS buffer (325 mosmol/kgH2O, pH 7.4, with 0.5% blue dextran). Fluid samples were withdrawn at 20 min and assayed for blue dextran concentration. For measurement of intestinal fluid secretion, 4 µg of cholera toxin (Sigma) in 0.1 ml of PBS were injected into jejunum and ascending colonic loops. The abdomen was sutured and the mice were allowed to recover. After 3 h, the mice were reanesthetized and the loops were removed for determination of loop weight, length, and luminal fluid content (45). A similar protocol was used to measure fluid secretion, in which loops were injected with 0.1 ml of a secretion-activating cocktail (50 µM pilocarpine, 100 µM forskolin, 100 µM IBMX, and 5 mM theophylline, in PBS). After 30 min, the mice were reanesthetized and the loops were removed for determination of luminal fluid content. For measurement of fecal dehydration, fresh feces were collected, and the wet-to-dry weight ratio was determined by overnight oven drying at 80°C.

High-fat diet challenge. Mice were fed a high-fat diet containing 50% animal fat (Bioserve, Frenchtown, NJ) and water ad libitum for 3 wk. Body weight was recorded every 3 days. Plasma triglyceride, cholesterol, lipase, and electrolyte content on normal and high-fat diets were measured. Stool samples were collected immediately after spontaneous defection for analysis of fecal fat content using Sudan VI staining (22).

Sperm count and shape analysis. Cauda epididymides were isolated and minced in 1 ml of PBS. Tissue fragments were removed by filtration through course mesh. Suspended sperm were counted using a hemacytometer. An aliquot of the suspension was smeared on a glass slide, fixed in methanol for 5 min, and stained in eosin for 1 h. One thousand sperm were examined for each mouse at ×400 total magnification. Abnormal sperm were recorded as hookless, banana-like, or amorphous, as described by Wyrobek and Bruce (52).

RESULTS

Figure 1A summarizes the strategy for targeted AQP8 gene disruption. Genomic Southern blot analysis of mouse liver genomic DNA digested with EcoRV and probed as indicated in Fig. 1A showed the predicted >10 kb fragment in wild-type mice, and 5.4 kb in AQP8-knockout mice, with both fragments seen in heterozygous mice (Fig. 1B). Northern blot analysis showed AQP8 transcript in the liver of wild-type and heterozygous mice but not knockout mice (Fig. 1C). Immunoblot analysis revealed a 28-kD band in the liver and testes of wild-type mice, but not in the null mice (Fig. 1D). Bands at higher apparent molecular mass were seen in wild-type mice in the liver and colon (~37 kDa) and in the colon (~70 kDa), with the latter possibly representing an AQP8 dimer. No specific band was found in the submandibular gland.

The tissue distribution of transcript encoding AQP8 was determined by RT-PCR analysis, in which the full-length AQP8 coding sequence was amplified. AQP8 transcript was found in heart, kidney, submandibular gland, liver, small intestine, colon, testes, and epididymis of wild-type mice, but was not found in tissue of the knockout mice (Fig. 2A). AQP8 protein was localized in mouse tissues by immunofluorescence using a rabbit anti-AQP8 antibody raised against an NH2-terminal peptide of the mouse AQP8 sequence. Figure 2B shows specific AQP8 immunostaining in liver, colon, and testes of wild-type mouse (left), with AQP8-null controls shown on the right. Labeling in the liver was seen at the plasma membrane and weakly in intracellular vesicles (top). Specific labeling of the luminal membranes of crypts (arrows) was seen in ascending colon (middle, cross-, and longitudinal sections shown), but not in transverse or descending colon (not shown). In testes, labeling was seen in spermatogenic cells (bottom). Specific labeling was not detected in submandibular gland, kidney, brain, lung/airways, and heart. Also, specific labeling of AQP8 was not detected in any mouse tissue using commercial antibodies (Alpha Diagnostics, San Antonio, TX, and Chemicon, Temecula, CA) raised against the COOH terminus of the rat AQP8 sequence.

There was no significant difference in the weights of wild-type vs. AQP8 knockout mice over the first 7 wk of life (Fig. 3A). The AQP8 knockout mice had grossly normal appearance, activity, and behavior. Genotype analysis of offspring from breeding of AQP8 heterozygous mice at 5 days after birth indicated a nearly 1:2:1 Mendelian distribution (34 wild-type, 73 heterozygous, and 32 knockout mice; Fig. 3B), indicating normal neonatal survival. As summarized in Table 1, serum chemistries were similar in wild-type and AQP8-knockout mice. Also, organ weights were similar, except for significantly greater weight/size of testes in the AQP8-null mice (Table 2 and Fig. 3C, left). Analysis of water content by measurement of wet-to-dry weight ratios showed no significant difference in testes from wild-type [85.2 ± 0.7% (mean ± SE); n = 4] vs. AQP8-null (84.1 ± 1.2%) mice. Histological examination of testes showed no significant difference in the diameter of seminiferous tubules between wild-type and AQP8-null mice; however, the ratio of spermatogenic cells to Sertoli cells in seminiferous tubules was greater in the AQP8-null mice (Fig. 3C, right).

To determine whether AQP8 was functional as a water channel in mouse tissues, osmotic water permeability was measured in membranes from testes and liver by the stopped-flow light-scattering method. Figure 3D (left curves) shows a significant component of rapid osmotic equilibration [half time (t1/2), 228 ± 21 ms by biexponential regression (mean ± SE); 3 preparations], as denoted by the arrow, in a plasma membrane-enriched fraction of testes from wild-type mice. There
was only a single slow component of osmotic equilibration ($t_{1/2}: 1,067 \pm 74$ ms by monoexponential regression) in the same fraction from AQP8-null mice. HgCl$_2$ largely abolished the component of high water permeability in the vesicles from wild-type mice ($t_{1/2}, 985 \pm 69$ ms by monoexponential regression) to that in vesicles from the null mice ($t_{1/2}, 1,126 \pm 81$ ms) (Fig. 3D, right curves), indicating that AQP8 is the major mercury-sensitive water transporter in these membranes.

Osmotic water permeability was also measured in freshly isolated hepatocytes from mouse liver. Water permeability in intact hepatocytes was similar in wild-type vs. AQP8-null mice [$t_{1/2}, 527 \pm 49$ vs. $583 \pm 47$ ms (mean $\pm$ SE); 3 preparations] (Fig. 3E, left top and bottom curves). Also, osmotic water permeability was not different after 10-min incubation of hepatocyte suspensions with 100 $\mu$M Bt$_2$CAMP ($t_{1/2}, 521 \pm 51$ ms, Fig. 3E, left middle curve). Water permeability measurements were also done on membrane fractions from liver homogenates. Osmotic equilibration did not differ significantly in vesicles from AQP8-null ($t_{1/2}, 114 \pm 13$ ms) vs. wild-type ($t_{1/2}, 101 \pm 14$ ms) mice in a plasma membrane-enriched vesicle fraction ($P > 0.05$, Fig. 3E, right curves).

The possible involvement of AQP8 in renal function was assessed by measurement of urinary concentrating function before and after water deprivation. Urine osmolalities did not differ in wild-type vs. AQP8-null mice at baseline or after 36-h water deprivation (Fig. 4A), nor did the reduction in body weight after water deprivation (Fig. 4B). Urine osmolality was also measured in AQP1 knockout and AQP8/AQP1 double-
knockout mice, because a subtle effect of AQP8 might be detected after deletion of the major proximal nephron water channel AQP1. However, urine osmolality was not significantly different in AQP1-null mice vs. mice lacking AQP8 and AQP1 together (Fig. 4).

The possible involvement of AQP8 in salivary gland function was studied by measuring saliva fluid secretion after pilocarpine stimulation. Secretion of saliva was not different in wild-type vs. AQP8-null mice (Fig. 5), but it was significantly reduced in AQP5-null mice as reported previously (23). Because AQP8 and AQP5 are both expressed in salivary gland acinar cells, we generated AQP8/AQP5 double-knockout mice, reasoning as we had for kidney studies that a subtle phenotype difference might be seen in AQP8 deficiency after deletion of the major salivary gland acinar water channel AQP5. However, there was no significant difference in saliva secretion in AQP5-null mice vs. mice lacking AQP8 and AQP5 together (Fig. 5).

A modified closed loop model was used to study the role of AQP8 in fluid transport in small intestine and colon. Osmotically driven water secretion, isosmolar fluid absorption, and choleratoxin-induced active fluid secretion were compared in wild-type vs. AQP8-null mice. Osmotically driven water secretion was measured from luminal fluid osmolalities measured at 5 and 15 min after a hyperosmolar solution (PBS containing 300 mM mannitol) was infused into closed jejunal loops in vivo. Figure 6A shows that osmotic equilibration did not differ significantly in wild-type vs. AQP8-null mice. Apparent transepithelial osmotic water permeability coefficients, computed assuming the jejunum as a smooth cylinder of 1.3 mm inner diameter, were 0.012 and 0.014 cm/s for wild-type and AQP8-null mice, respectively.

Isosmolar fluid absorption was measured from the increase in concentration of a volume marker (blue dextran) at 20 min after infusion of jejunal and descending colonic loops with an isosmolar saline solution. Ascending colonic loops were found to be always secretory, so absorption could not be studied in that segment. Figure 6B shows comparable rates of isosmolar fluid absorption in wild-type vs. AQP8-null mice. Computed fluid absorption rates in jejunum were 2.0 ± 0.4 and 1.7 ± 0.4 μl/min−1·cm−2 luminal surface for wild-type and AQP8-null mice, respectively. In descending colon, the absorption rates were 1.8 ± 0.1 and 2.0 ± 0.4 μl/min−1·cm−2. As a separate...
assessment of fluid absorption in the colon, water content of freshly defecated stool was measured using wet-to-dry weight ratios. Stool water content did not differ significantly [60 ± 3% vs. 59 ± 1% (means ± SE); 10–12 mice] in the wild-type vs. AQP8-null mice.

Cholera toxin-induced fluid secretion was measured from the loop fluid content at 3 h after infusion of cholera toxin as described by Thiggarajah et al. (45). Figure 6C shows robust fluid secretion in jejunum and ascending colon after cholera toxin (top) but no significant difference between wild-type and AQP8-null mice. In another model of agonist-induced fluid secretion, loops were injected with an agonist cocktail to stimulate fluid secretion rapidly and maximally. Over the first 30 min, there was considerable fluid secretion (bottom), although, again, no significant differences were observed in the loops of wild-type vs. AQP8-null mice.

To investigate a possible role for AQP8 in hepatobiliary/pancreatic function, mice were placed on a diet containing 50% fat for 3 wk, a maneuver used previously to detect a subtle fat misprocessing phenotype in AQP1-null mice (22). As summarized in Table 1, body weight in wild-type and AQP8-knockout mice increased similarly. All mice appeared grossly healthy and active. Sudan VI staining of feces from mice on a high-fat diet showed few fat globules in mice of both genotypes, without significant steatorrhea. There were no significant differences in plasma chemistries in wild-type vs. AQP8-null mice, except for modest elevations in plasma triglyceride and cholesterol concentrations in the AQP8-null mice.

Because of the increased testicular weight in AQP8-null mice and the localization of AQP8 to spermatogenic cells in wild-type mice, sperm number and morphology were examined. There was no significant difference in sperm number in cauda epididymides from wild-type mice [11 ± 2 × 10^5 sperm/mouse (mean ± SE); 6 mice] vs. AQP8-null mice [10 ± 2 × 10^5 sperm/mouse (mean ± SE)]. Analysis of sperm morphology showed <0.3% abnormal sperm in wild-type and AQP8-knockout mice.

DISCUSSION

We analyzed the phenotype of AQP8-null mice, focusing on gastrointestinal and other organ systems in which AQP8 is expressed and for which there are adequate methods to identify phenotype differences. The AQP8-null mice lacked detectable AQP8 transcript or protein in all tissues examined, yet had normal appearance, survival, and growth, and their genotype distribution conformed to the expected 1:2:1 Mendelian distribution. Analysis of organ weight and serum chemistries showed no difference between the wild-type and AQP8-deficient mice under control conditions, except for larger testes in the AQP8-null mice.

Table 1. Weight gain and plasma chemistries of mice on a 50% fat diet for 5 wk

<table>
<thead>
<tr>
<th>Organ</th>
<th>Normal (4% Fat) Diet</th>
<th>High-Fat (50%) Diet</th>
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<tbody>
<tr>
<td></td>
<td>++/+</td>
<td>AQP8−/−</td>
</tr>
<tr>
<td></td>
<td>++/+</td>
<td>AQP8−/−</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>8.3 ± 0.5</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>71 ± 9</td>
<td>89 ± 14</td>
</tr>
<tr>
<td>Ca^{2+}, mg/dl</td>
<td>9.2 ± 0.5</td>
<td>8.9 ± 1.7</td>
</tr>
<tr>
<td>K^{+}, mM</td>
<td>4.5 ± 0.3</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Cl^{−}, mM</td>
<td>110 ± 2</td>
<td>113 ± 1</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>105 ± 17</td>
<td>79 ± 16</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>33 ± 7</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>Lipase, U/l</td>
<td>34 ± 3</td>
<td>32 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 mice per group. AQP8, aquaporin 8; ++/+, wild-type; −/−, double knockout. Control samples were from mice that fasted overnight. *P < 0.05 (Student’s t-test).

Table 2. Organ weight in wild-type and AQP8 knockout mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>++/+</th>
<th>AQP8−/−</th>
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<tbody>
<tr>
<td>Brain</td>
<td>14 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Submandibular gland</td>
<td>4.4 ± 0.3</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>Heart</td>
<td>4.3 ± 0.5</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>44 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Kidney</td>
<td>12 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Testis</td>
<td>4.8 ± 0.7</td>
<td>7.3 ± 0.3*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 mice per group. Organ weight (in mg/g body wt) shown as means (4 mice per group). Data are from matched 7-wk-old wild-type mice (26 ± 2 g) and AQP8 knockout mice (26 ± 2 g). *P < 0.01 (Student’s t-test).
expressed at the apical membrane of acinar epithelial cells (11). AQP8-null mice compared with wild-type mice. AQP5 is impaired of maximal agonist-stimulated saliva secretion in absence or immunoblot analysis. Functional analysis showed no could not confirm AQP8 protein identity by immunofluorescence or immunoblot analysis. Here, we found strong AQP8 transcript expression but could not confirm AQP8 protein identity by immunofluorescence or immunoblot analysis. As mentioned in the introduction, AQP8 antibody studies are particularly challenging because of AQP8’s unfavorable amino acid sequence/topography. For this reason, in view of the evidence for transcript expression in mice and the prior reports in rat, we performed functional studies on kidney and salivary gland, despite the negative immunostaining/immunoblot data reported here.

The testes are a major site of AQP8 expression. Immunocytochemistry described previously in rat (6, 8, 17) and here in mouse has indicated AQP8 protein expression in all stages of spermatogenic cells (spermatogonia, spermatocytes, and spermatids). Mercury-sensitive AQP8 water channel function was demonstrated in a plasma membrane-enriched vesicle fraction of testes from wild-type mice. However, the role of AQP8 in male reproductive physiology is unclear because the testes express numerous aquaporins, including AQP1, AQP7, and AQP9 (1, 5, 7). Although the testes in AQP8-null mice were significantly larger than those in wild-type mice, we did not find impaired fertility or abnormalities in sperm count or morphology in the AQP8-null mice. The elevated ratio of spermatogenic cells to Sertoli cells and the normal size of seminiferous tubules in the AQP8-null mice suggest the possibility of an abnormality in sperm development. However, impaired fertility in transgenic mice is rarely seen because of the high intrinsic efficiency and multiple redundant systems for sperm maturation.

Previous studies indicated strong AQP8 transcript expression in the salivary gland (8, 18, 27). Immunocytochemistry done by different laboratories showed various AQP8 protein expression patterns, including localization to the basolateral membrane of acinar epithelial cells (51) and myoepithelial cells (8) in the rat. AQP8 expression was also reported at the apical membrane of rat salivary gland epithelial cell cultures (13). Here, we found strong AQP8 transcript expression but could not confirm AQP8 protein identity by immunofluorescence or immunoblot analysis. Functional analysis showed no impairment of maximal agonist-stimulated saliva secretion in AQP8-null mice compared with wild-type mice. AQP5 is expressed at the apical membrane of acinar epithelial cells (11, 33, 30), and impaired saliva secretion was found in AQP5-null mice (20, 23). In an attempt to identify a subtle phenotype caused by AQP8 deficiency, double-knockout mice lacking AQP8 and AQP5 together were generated by serial breeding of single-knockout mice. Such subtle phenotypes have been identified previously using strategies to reduce erythrocyte water permeability in AQP1/AQP3- vs. AQP1-knockout mice (54) and to reduce urinary concentrating function in AQP3/AQP4- vs. AQP3-knockout mice (24). However, we found no significant further impairment in salivary secretion in AQP8/AQP5 double-knockout mice compared with AQP5 knockout mice. Thus AQP8 does not appear to play an important role in salivary gland fluid secretion in mice.

The liver is a major site of AQP8 transcript expression in rat (8, 14) and mouse (27). Immunocytochemistry in the rat (8, 14) and mouse (9) showed AQP8 protein expression in intracellular vesicles in hepatocytes. It was suggested that AQP8 is involved in the formation of canalicular bile by ATP-dependent secretion of biliary constituents from the sinusoidal blood or the cellular interior into the bile canalicular lumen. Interestingly, the liver has been reported to be the site of expression of several aquaporins, including AQP0, AQP1, AQP4, AQP8, and AQP9 (10, 14, 34, 39), which were proposed to facilitate solute-driven movement of water into the bile canalculus. However, deletion of AQP1, which is expressed in cholangiocytes, did not affect the bile flow and bile salt concentration.
(22). Also, AQP1 was not rate limiting for water movement in mouse cholangiocytes and did not appear to be regulated by cAMP (32). An initial study (55) of water permeability in isolated hepatocytes from rat showed moderate water permeability with $P_f$ of $66 \times 10^{-4}$ cm/s at 37°C; however, follow-up studies from the same group reported a much lower $P_f$ of $<10^{-4}$ cm/s (10). They also reported cAMP agonist induced an almost twofold increase in rat hepatocyte water permeability (10), relocation of intracellular AQP8 to the plasma membrane (14), and a sixfold increase in water permeability of canalicular plasma membrane domains in cAMP-treated rat hepatocytes (29). Here, we found AQP8 immunolocalization in the plasma membranes of hepatocytes with weak intracellular labeling. However, osmotic water permeability in freshly isolated hepatocytes from wild-type mice was low ($P_f$ of $6 \times 10^{-4}$ cm/s) and did not increase after addition of cAMP agonists or decrease in AQP8 deficiency. These results provide the evidence against constitutive or cAMP-regulated AQP8 water permeability in hepatocytes in mice. Of note, rat and mouse AQP8 do not contain consensus sequences for phosphorylation by protein kinase A or C (16, 18, 27).

The AQP8-null mice were stressed by a high-fat diet to expose potentially subtle defects in hepatobiliary function, as we had done previously to characterize dietary fat misprocessing in AQP1-null mice (22). Weight gain in wild-type and AQP8-null mice on a high-fat diet was similar, and the AQP8-null mice did not develop steatorrhea or abnormalities in serum lipid profile, liver function tests, or pancreatic enzymes. AQP8 thus does not appear to have an essential role in hepatobiliary/pancreatic function, although a more definitive conclusion will require direct assessment of the secretion rate and composition of bile and pancreatic fluid. The only significant difference between wild-type and AQP8-null mice on a high-fat diet was mildly elevated plasma triglyceride and cholesterol concentrations in the AQP8-null mice, but the etiology and physiological significance of this finding remain unclear.

Fluid transport in small intestine and colon plays a critical role in body fluid balance. The expression of at least six aquaporins in the small intestine and colon has been reported: 1) AQP1 in endothelia of lacteals of small intestine and microvascular endothelia throughout the intestine (19); 2) AQP3 protein at the basolateral membrane of the epithelial cells lining the villus tip of the small intestine and colon in rat (38); 3) AQP4 at the basolateral membrane of colonic surface epithelium (50); 4) AQP5 in the apical membrane of secretory cells in duodenal glands (31); 5) AQP8 at apical membrane of epithelia in rat small intestine and colon crypt cells (8); and 6) AQP9 in goblet cells in duodenum, jejunum, ileum, and colon (35). Studies on AQP4-null mice showed that AQP4 facilitated transepithelial osmotic water permeability in the colon but had little or no effect on colonic fluid secretion or fecal dehydration (50). Here, we found AQP8 protein expression at the luminal membrane crypt epithelial cells in the ascending colon; however, no differences in cholera toxin- or agonist-stimulated maximal fluid secretion were found. Although specific AQP8 staining could not be detected elsewhere in colon and small intestine, we also measured osmotically driven water transport in the jejenum and active fluid absorption in jejunum and descending colon. No differences were found, nor was there a difference in stool water content in wild-type vs. AQP8-null mice.

The colon carries out constitutive fluid absorption to dehydrate feces, and under some conditions, such as in cholera, the colon is capable of rapid fluid secretion. Recent data suggest that colonic fluid absorption primarily occurs across crypt epithelium, where absorptive convection (47) and pericryptic hyperosmolality (46) have been demonstrated. Here, we found no significant impairment of colonic fluid absorption or fecal dehydration in colon in AQP8-deficient mice. Estimating a crypt surface area of $0.96 \text{cm}^2$ per cm of colon (47), the data here indicate a fluid absorptive rate of $2.4 \mu\text{L}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$. This value is substantially lower than that of $>20 \mu\text{L}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ in kidney proximal tubule and salivary gland, where aquaporins facilitate isosmolar fluid transport (23, 40), but higher than that of $0.016 \mu\text{L}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ in lung alveolus, where aquaporin deletion was found not to impair fluid transport (2, 21). In the ascending colon, maximally stimulated fluid secretion was $\sim 0.7 \mu\text{L}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ in the wild-type and AQP8-null mice. Thus the lack of effect of AQP8 deletion on colonic fluid absorption and secretion is consistent with the relatively low absolute rate of fluid absorption per surface area of crypt epithelium. The physiological role(s) of AQP8 expression in intestine thus remain unclear.

Weak expression of transcript encoding AQP8 was reported in mouse kidney (27), with immunocytochemical localization of AQP8 protein in intracellular structures of proximal tubules and collecting ducts in rat (8). We were unable to detect specific AQP8 immunostaining using an NH2 terminus anti-AQP8 antibody that was able to detect AQP8 in other tissues. Functional studies showed no impairment of urinary concentrating ability in AQP8-null mice. Double-knockout mice lacking AQP8 and AQP1 together were generated, reasoning that a subtle defect in renal function might be seen in the absence of AQP1. However, urinary concentrating function under basal conditions and after water deprivation was not impaired in the AQP8/AQP1 double-knockout mice compared with mice lacking AQP1 alone. These results provide evidence against a significant role of AQP8 in the urinary concentrating function in mice.

In summary, we found few and only mild phenotype differences between wild-type and AQP8-deficient mice. This was an unexpected finding, given the wide and strong AQP8 pattern particularly in gastrointestinal organs and our prior results showing multiple phenotype abnormalities in mice lacking functional AQP1–5. However, a negative study cannot be definitive in that all possible organ functions and physiological/pathological stresses were not tested. Also, although we think it is unlikely based on prior studies in AQP knockout mice, the possibility cannot be ruled out that compensatory changes in the expression of other water or solute transporters in the AQP8-null mice might account for their unremarkable phenotype.

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