Neonatal Mortality in an Aquaporin-2 Knock-in Mouse Model of Recessive Nephrogenic Diabetes Insipidus*

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Hereditary non-X-linked nephrogenic diabetes insipidus (NDI) is caused by mutations in the aquaporin-2 (AQP2) water channel. In transfected cells, the human disease-causing mutant AQP2-T126M is retained at the endoplasmic reticulum (ER) where it is functional and targetable to the plasma membrane with chemical chaperones. A mouse knock-in model of NDI was generated by targeted gene replacement using a Cre-loxP strategy. Along with T126M, mutations H122S, N124S, and A125T were introduced to preserve the consensus sequence for N-linked glycosylation found in human AQP2. Breeding of heterozygous mice yielded the expected Mendelian distribution with 26 homozygous mutant offspring of 99 live births. The mutant mice appeared normal at 2–3 days after birth but failed to thrive and generally died by day 6 if not given supplemental fluid. Urine/serum analysis showed a urinary concentrating defect with serum hyperosmolality and low urine osmolality that was not increased by a V2 vasopressin agonist. Northern blot analysis showed up-regulated AQP2-T126M transcripts of identical size to wild-type AQP2. Immunoblots showed complex glycosylation of wild-type AQP2 but mainly endoglycosidase H-sensitive core glycosylation of AQP2-T126M indicating ER-retention. Biochemical analysis revealed that the AQP2-T126M protein was resistant to detergent solubilization. Kidneys from mutant mice showed collecting duct dilatation, papillary atrophy, and unexpectedly, some plasma membrane AQP2 staining. The severe phenotype of the AQP2 mutant mice compared with that of mice lacking kidney water channels AQP1, AQP3, and AQP4 indicates a critical role for AQP2 in neonatal renal function in mice. Our results establish a mouse model of human autosomal NDI and provide the first in vivo biochemical data on a disease-causing AQP2 mutant.

The formation of concentrated urine by the kidney requires high osmotic water permeability across the collecting duct epithelium. Collecting duct epithelial cells express aquaporin water channels AQP2,1 AQP3, and AQP4 (1–4). AQP2 is the vasopressin (antidiuretic hormone)-regulated water channel (5, 6). Vasopressin induces the fusion of intracellular vesicles containing AQP2 with the apical plasma membrane resulting in increased water permeability (7–9). AQP2 is of considerable clinical importance in fluid and electrolyte balance. Mutations in human AQP2 cause hereditary non-X-linked nephrogenic diabetes insipidus (10–13). Down-regulation of AQP2 expression occurs in several forms of acquired NDI (14), and AQP2 up-regulation is important in the pathophysiology of fluid-retaining states such as congestive heart failure (15, 16). AQP3 and AQP4 are expressed constitutively at the basolateral membrane of collecting duct epithelial cells, with AQP3 mainly in cortical collecting duct and AQP4 in inner medullary collecting duct. Transgenic mice lacking AQP3 have low water permeability in cortical collecting duct and manifest NDI with marked polyuria and decreased urine osmolality (17). In contrast, mice lacking AQP4 show only a mild defect in urinary concentrating ability (18) despite a 4-fold reduction in transepithelial water permeability in inner medullary collecting duct (19). Although animal models exist of NDI caused by abnormalities in neurohypophysial vasopressin secretion, such as the Brattleboro rat (20, 21), there are no animal models of renal AQP2 deficiency/mutation.

The AQP2 point mutation T126M was identified as a cause of non-X-linked recessive NDI in humans (22). In transfected mammalian cells, AQP2-T126M protein is retained at the endoplasmic reticulum (ER) and degraded more rapidly than wild-type AQP2 (23). We also showed recently that ER-retained AQP2-T126M is mildly misfolded but functional (24). Treatment of transfected cells with chemical chaperones such as glycerol resulted in relocation of AQP2-T126M to the cell plasma membrane where it was able to function as a water channel and correct the defective cell phenotype (23, 24). Chemical chaperones or other agents that modify interactions with molecular chaperones were proposed as possible therapies for NDI. However therapy development requires demonstration that disease-causing AQP2 mutants are ER-retained in native kidney cells and that chaperone-modifying strategies are effective in vivo.

The purpose of this study was to generate and characterize a mouse model of recessive NDI caused by AQP2 mutation. We previously isolated and analyzed the mouse AQP2 cDNA and gene and showed that the mouse AQP2-T126M ortholog behaved like human AQP2-T126M in its ER localization, function, and correction by chemical chaperones (25). The homozygous AQP2-T126M knock-in mice created here had NDI and expressed the mutant AQP2 protein in collecting duct epithelial cells, as engineered, but the mice had an unexpectedly severe phenotype with neonatal mortality.

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1 The abbreviations used are: AQP, aquaporin; NDI, nephrogenic diabetes insipidus; ER, endoplasmic reticulum; kb, kilobases; tk, thymidine kinase; ES, embryonic stem cell; CHO, Chinese hamster ovary cell; RT-PCR, reverse transcription-polymerase chain reaction; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
MATERIALS AND METHODS

Generation of AQP2-T126M Knock-in Mutant Mice—Based on the reported mouse cDNA sequence and gene structure (25), a gene replacement targeting vector was constructed to knock-in the T126M mutation (see Fig. 1). The vector contained a 5-kb mouse genomic DNA fragment containing AQP2 exons 1–3. The T126M mutation was generated by site-directed mutagenesis. To retain the human consensus sequence for N-linked glycosylation, mutations H122S, N124S, and A125T were also introduced into the targeting vector. A silent FseI restriction site was engineered for mouse genotype analysis. A Pol2neobpA selection cassette flanked by loxP sites was inserted into intron 2 for positive selection, and a PGKk cassette was inserted at the 3′-end of the AQP2 targeting sequence for negative selection. The vector was linearized at a unique downstream NorI site and electroporated into CB1–4 embryonic stem (ES) cells. Transfected ES cells were selected with G418 and FIAU for 7 days, yielding 2 targeted clones of 246 doubly resistant colonies upon PCR screening using a sense primer specific for the neo cassette (5′-CAGTTCAAGGGTGAATGCTG-3′) and an antisense primer specific for the AQP2 gene (5′-CAAGAGCTCTGAGAAGCGC-3′) located downstream from the 3′-end of the construct. Homologous recombination was confirmed by Southern blot analysis using as probe a 3.2-kb genomic fragment as indicated in Fig. 1. ES cells were injected into PC 2.5-day 8-cell morula stage CD1 zygotes, cultured overnight to blastocysts, and transferred to pseudopregnant B6D2 females. Offspring were genotyped by PCR followed by Southern blot analysis as described above. Heterozygous founder mice were bred to produce homozygous AQP2 mutant mice. Heterozygous females were also mated with males (mouse strain FVB/N) homozygous for FseI-actin-cre (26) to remove the Pol2neobpA selection cassette flanked by loxP sites in intron 2 of the mutant AQP2 gene. After confirming absence of the Pol2neobpA sequence, the heterozygous mice were bred to produce homozygous mutant mice.

Northern Blot Analysis—RNA from kidney was isolated using TRIzol reagent (Life Technologies, Inc.). RNAs (10 μg/lane) were resolved on a 1.2% formaldehyde-agarose denaturing gel, transferred to a Nylon membrane (Amersham Pharmacia Biotech) and hybridized at high stringency with a 32P-labeled probe corresponding to the mouse AQP2 cDNA coding sequence.

Cell Transfection—Full-length cDNAs encoding wild-type and mutant AQP2 were PCR-amplified from kidney cDNA of wild-type and mutant mice, sequenced, and subcloned into pCDNA3 plasmid (Invitrogen) for transfection in CHO cells as described previously (25).

Immunocytochemistry—Immunofluorescence localization of AQP2 protein in fixed frozen kidney sections was done using a rabbit polyclonal antibody against the 30-amino acid C terminus of AQP2 as described previously (19, 25).

Endoglycosidase Digestion—100 μg of proteins from kidney homogenates were suspended in 9 μl of incubation buffer (20 mM sodium phosphate pH 7.5, 50 mM EDTA, 0.02% sodium azide, 0.1% SDS, 50 mM β-mercaptoethanol). Endoglycosidase F (0.5 units) was added, and mixtures were incubated at 37 °C for 2 h prior to immunoblot analysis.

Detergent Extraction—Membranes from kidney homogenates were incubated with phosphate-buffered saline containing 1% CHAPS at 4 °C for 30 min. Detergent-insoluble proteins were pelleted at 100,000 × g for 45 min and resuspended in the buffer containing 0.1% SDS, 1% deoxycholate, and 1% Triton X-100. Buffer was also added to the supernatant to give a final concentration of 0.1% SDS, 1% deoxycholate, and 1% Triton X-100. Supernatant and pelleted samples were then subjected to immunoblot analysis.

Immunoblot Analysis—Equal amounts of protein (5 μg/lane) were resolved on a 12% SDS-polyacrylamide gel and electroblotted to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in 10 mM Tris, 150 mM NaCl, pH 7.4 (TBS) for 1 h, followed by a 1-h incubation in anti-AQP2 polyclonal antibody (1:500). Membranes were washed in TBS containing 0.05% Tween and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Urine and Serum Osmolality—Urine and serum osmolalities were measured by freezing point depression osmometry (micro osmometer, Precision Systems, Inc.). In some experiments mice were injected with DDAVP (0.4 μg/kg) intraperitoneally at 60 min prior to obtaining the urine sample.

Xenopus Oocyte Expression—Full-length cDNAs encoding wild-type and mutant AQP2 were subcloned into an oocyte expression plasmid. Complementary RNA was transcribed in vitro using SP6 polymerase.

FIG. 1. Targeting strategy for AQP2 gene replacement. Homologous recombination results in replacement of the indicated segment (thick line) of the AQP2 gene by a 1.8-kb Pol2neobpA selection cassette flanked by loxP sites (arrowheads). Small arrows indicate the mutant sites. The probe used for Southern blot analysis is indicated probe, and the expected sizes of hybridized fragments are shown by dashed lines. Stage V and VI oocytes from Xenopus laevis were isolated, defolliculated with collagenase, and microinjected with 50- to 100-ng samples of cRNA (0–200 ng/μl). After incubation at 18 °C for 24 h, osmotic water permeability (P′) was measured from the time course of oocyte swelling at 10 °C in response to a 5-fold dilution of the extracellular Barth’s buffer with distilled water.

RESULTS

Targeted gene replacement in embryonic stem cells was done to replace wild-type AQP2 with AQP2-T126M. As shown in Fig. 1, a positive-negative selection strategy was used in which a loxP-flanked polII-neo selection cassette was introduced in intron 2. In addition to the T126M mutation, mutations H122S, N124S, and A125T were engineered in exon 2 of the coding sequence to replace wild-type AQP2 with AQP2-T126M.

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confirmed the four mutations for strain A and strain B mice (Fig. 2C).

Functional analysis and cellular localization of the RT-PCR amplified wild-type and mutant AQP2 sequences were done in heterologous expression systems. In Xenopus oocytes microinjected with transcribed cRNAs, osmotic water permeability was increased by expression of the mutant AQP2, though to a lesser extent than wild-type AQP2 (Fig. 3A), in agreement with previous results for human and mouse AQP2-T126M (23, 25).

Transient expression in CHO cells revealed an endosomal/plasma membrane expression pattern for the wild-type AQP2, but an endoplasmic reticulum (ER) expression pattern for the mutant AQP2 (Fig. 3B), in agreement with results for human and mouse AQP2-T126M (23, 25). Therefore the mutant AQP2 coding sequence expressed in mice has the expected functionality and ER-localization when expressed in heterologous systems.

Although the homozygous mutant pups appeared grossly normal at 1–2 days after birth, by 3–4 days they were much smaller than litter mates and failed to thrive (Fig. 4A). For the strain A mice, averaged body weights at 5 days (in grams, mean ± S.E.) were 3.8 ± 0.3 (wild-type), 3.9 ± 0.3 (heterozygous), and 1.7 ± 0.2 (mutant). Most strain A mutant mice died at 5–6 days after birth when no intervention was done to prolong survival. Survival of some pups was prolonged up to 8 days by separation of most wild-type and heterozygous pups from the homozygous pups (to minimize competition for milk) and by fluid supplementation by oral feeding (30–50 ml of milk, four times per day) or subcutaneous fluid injections (50–100 µl of half-normal saline, three times per day). Interestingly, many hybrid strain B mice were able to survive to day 8, although they were remarkably smaller than wild-type litter mates from day 3 onward.

Measurement of urine osmolality showed relatively hypertonic urine in the mutant mice that was not sensitive to DDAVP administration (Fig. 4B); however, the urine osmolality of 221 ± 9 mosmol probably underestimates the severity of the nephrogenic diabetes insipidus because of serum hyperosmolality and progressive renal failure. (It was not possible to obtain urine from very young mutant mice.) Serum osmolality was remarkably elevated in the dehydrated mutant mice (Fig. 4A, top). Blood urea nitrogen of the mutant pups was 7-fold greater than in wild-type pups (79 ± 15 mg/dl versus 11.6 ± 0.6 mg/dl). To determine whether the heterozygous mice manifest any abnormality in urinary concentrating ability, urine osmolalities were compared in adult (4–5 weeks of age) wild-type and heterozygous mice. Averaged urine osmolalities were 1544 ± 69 mosmol (heterozygous) and 1585 ± 187 mosmol (wild type), increasing after a 36-hour water deprivation to 3001 ± 126 mosmol (heterozygous) and 3081 ± 291 mosmol (wild type, differences not significant).

Immunoblot analysis of whole kidney homogenates showed that the majority of immunoreactive protein in wild-type and heterozygous mice migrated as a diffuse band of apparent molecular size 34–40 kDa (Fig. 5A), which represents fully processed AQP2 with complex glycosylation. A band of lower intensity at the predicted molecular size of nonglycosylated AQP2 was also seen. In contrast, the mutant AQP2 protein migrated mainly as a single band at ~31 kDa, which from previous data in oocytes and transfected cells probably represents an ER-retained form of AQP2 with core glycosylation. Sometimes bands at higher molecular weights were seen that probably represent AQP2 multimers.

To confirm the interpretation of the glycosylated bands, immunoblot analysis was done on endoglycosidase-treated samples. Endoglycosidase F treatment, which cleaves all sugars, gave a single band at 29 kDa for all genotypes (Fig. 5B). Endoglycosidase H treatment, which cleaves high-mannose core sugars of proteins that have not exited the ER, did not affect wild-type AQP2 but digested most of the 31 kDa form of the mutant AQP2. We found previously that AQP2-T126M protein was resistant to detergent extraction in a transfected CHO cell model (24), suggesting a different folding/aggregation state than wild-type AQP2. To determine whether this difference in detergent solubility occurs in native kidney, membrane homogenates were treated with CHAPS for 30 min (as done in Ref. 24) and centrifuged to separate supernatant (soluble AQP2) and pellet (insoluble AQP2). Fig. 5C shows remarkably greater detergent sensitivity of wild-type versus mutant AQP2. The in vivo biochemical data thus confirm several of the biochemical properties of the mutant AQP2 established in cell culture models.

Last, renal morphology and AQP2 immunolocalization were studied in kidneys of wild-type and mutant mice. Remarkably, kidneys from the mutant mice showed signs of obstructive uropathy including dilatation of collecting ducts and papillary atrophy (Fig. 6A, right). Many kidneys of the mutant mice contained a large fluid-filled cavity where the medulla should be. Immunofluorescence of kidneys from 5-day-old wild-type mice showed AQP2 localization in medullary collecting duct, but unlike adult mice, very little AQP2 was found in cortical collecting duct (Fig. 6B and C, left). AQP2 was found at the apical membrane, in agreement with previous results in rat and human kidney (27, 29, 30). At low magnification, AQP2 immunostaining was also found mainly in the medulla of kidneys from mutant mice, albeit at a substantially greater level than in wild-type mice (Fig. 6B, right). At higher magnification, AQP2 was seen both within cells, probably in the ER, as well as at the plasma membrane in some tubules (Fig. 6C, right). Insets in Fig. 6C show higher magnification.

DISCUSSION

The impaired neonatal survival of the mutant mice expressing AQP2-T126M indicates an important role for AQP2 in fluid and electrolyte balance early in life. The mutant mice had a urinary concentrating defect resulting in dehydration, failure to thrive, and death. Interestingly, mice lacking AQP1 (31), AQP3 (17), and AQP4 (18), which manifest urinary concentrat-
ing defects to differing extents, do not have reduced neonatal survival. The severe NDI in the AQP2 mutant mice supports the conclusion that transepithelial water permeability in collecting duct is rate-limited by apical membrane water permeability.

The neonatal mortality in the mutant mice was an unexpected finding, especially in view of the limited urinary concentrating ability of young normal mice. For several reasons, we believe that the early death is because of polyuria and its consequences, rather than to other causes related to expression of the mutated AQP2 protein. First, mice lacking an unrelated protein (the V2 receptor), which probably have comparable polyuria to the mutant AQP2 mice, also fail to thrive and die early (32). Other knockout mouse models (AQP3, NKCC2) having polyuria appear to develop kidney damage (33, 34). The mouse kidney is probably particularly sensitive to damage by polyuria compared with rat (e.g. Brattleboro rat) or human (e.g. subjects with NDI). We think it is unlikely that mutant AQP2 expression is itself damaging to the kidney, because very little AQP2 is expressed at the time of birth or before, and because the mutant mice appear grossly normal at 1–2 days after birth. Last, the low urine osmolality and high serum osmolality measured in the mutant mice indicates marked polyuria and probably underestimates the severity of the polyuria because the mice are in renal failure by the time they are large enough to permit collection of urine and serum samples.

Initial efforts to prolong the survival of the mutant mice by fluid supplementation were only partially successful. The improved survival of the strain B mice with the hybrid CD1 and FVB/N background is probably related strain differences, because the mutant AQP2 sequence is identical in both mouse strains, and the amount of expressed protein was comparable. More work involving fluid supplementation and transferring the mutant genotype to mice of different genetic backgrounds will be needed to determine whether survival of the mutant mice until adulthood is possible.

Northern blot analysis indicated increased mutant AQP2 expression in the homozygous mutant mice, which is probably related to dehydration and high serum vasopressin concentrations. The AQP2 promoter contains a cAMP response element that is responsible for up-regulation of AQP2 expression in states of high circulating vasopressin (35). Immunoblot analysis showed that the mutant AQP2 protein was core glycosylated as demonstrated by its size and sensitivity to endoglycosidase H. As found previously in transfected cell models, mutant AQP2 in kidney was also relatively resistant to detergent solubilization. However, immunofluorescence showed a significant fraction of the mutant AQP2 at the apical plasma membrane. It is possible that transfected cell cultures, where the mutant AQP2 is found exclusively at the ER, are not good...
models for the in vivo targeting of mutant AQP2. However, the grossly abnormal collecting duct morphology in mutant mice precludes such interpretations. The plasma membrane localization of some mutant AQP2 molecules might be a consequence of the many changes in epithelial cell physiology resulting from increased intraluminal pressure. Another interesting possibility is that the accumulation of osmolytes in renal medulla might facilitate the plasma membrane targeting of some mutant AQP2 molecules, as was found in cell culture models (23).

In summary, we have created and characterized the first mouse model of human NDI. The AQP2-T126M-expressing mutant mice permit in vivo studies of the biochemistry of a disease-causing AQP2 mutant. Adult AQP2 mutant mice, if and when available, should be useful in addressing fundamental issues about the pathophysiology of NDI caused by AQP2 mutations, and for testing the efficacy of established and novel therapies to improve urinary concentrating ability.

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