Increased expression of $H^+$-ATPase in inner medullary collecting duct of aquaporin-1-deficient mice

Young-Hee Kim, Jin Kim, A. S. Verkman, and Kirsten M. Madsen

Department of Medicine, University of Florida, Gainesville, Florida 32610-0215; Department of Anatomy, Catholic University Medical College, Seoul 137-701, Korea; and Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0521

Submitted 23 January 2003; accepted in final form 7 May 2003

AQUAPORIN-1 (AQP1), a water-transporting protein, is expressed in the apical and basolateral plasma membranes of the proximal tubule, the descending thin limb of Henle’s loop, and the microvascular endothelium of the descending vasa recta (12, 19, 20). Recent studies in AQP1 null mice generated by targeted gene disruption have established the role of AQP1 as a major water channel in the kidney (16, 25, 32). Phenotype analysis has demonstrated that AQP1 null mice are polyuric and manifest a urinary concentrating defect because of an inability to create a hypertonic medullary interstitium (9, 16, 23, 32) as well as defective isosmolar fluid absorption by the proximal tubule (25, 31). Further analysis of urine composition, reported in this study, revealed a significantly decreased urinary pH in AQP1 null mice; however, the mechanism behind the increased acidity of the urine is unclear.

The collecting duct plays an important role in acid-base transport in the kidney and is the main site of urine acidification (3). Acid secretion occurs along the entire collecting duct, whereas bicarbonate secretion has been demonstrated only in the cortical collecting duct (CCD). There is convincing evidence that intercalated cells are responsible for acid-base transport in the collecting duct. At least two types of intercalated cells, type A and type B, are present in the collecting duct (2, 13, 26, 29, 35). Type A intercalated cells secrete protons and absorb bicarbonate. Proton secretion is mediated, at least in part, by a vacuolar-type $H^+$-ATPase that is located in the apical plasma membrane and apical tubulovesicles (7, 8, 13, 34). Bicarbonate absorption occurs via AE1, a truncated form of the erythrocyte $Cl^-/HCO_3^-$ exchanger (13, 26, 29, 33). Type B intercalated cells are responsible for bicarbonate secretion in the CCD (39). There is also evidence that acid secretion in the collecting duct can occur via an $H^+-K^+$-ATPase that has been demonstrated in both type A and type B intercalated cells (36, 40, 41).

Intercalated cells gradually disappear in the inner medullary collecting duct (IMCD) and are not observed in the terminal one-half to two-thirds of the IMCD. However, there is evidence that acid secretion occurs along the entire IMCD, indicating that IMCD cells may also be capable of acid secretion (1). Studies using micropuncture and micropuncture techniques have demonstrated a decrease in urinary pH along the IMCD (10, 24, 30). Both acute and chronic metabolic acidosis is associated with increased urine acidification along the IMCD (6, 10), whereas acidification is abolished during acute and chronic alkalosis (5). Subse-

Address for reprint requests and other correspondence: K. M. Madsen, Dept. of Medicine, PO Box 100215, Univ. of Florida, 1600 SW Archer Rd., Gainesville, FL 32610-0215 (E-mail: MADSEKM @medicine.ufl.edu).
quent studies demonstrated net acid secretion in isolated IMCD segments from normal rats and reported that acid secretion was significantly increased in IMCD segments from ammonium chloride-loaded rats (37). The cellular mechanism of acid secretion in the IMCD is not known with certainty. However, there is evidence from studies in cultured IMCD cells that acid secretion may be mediated by both the bafilomycin-sensitive vacuolar-type H^+-ATPase (27) and the SCH-28080-sensitive H^+-K^+-ATPase (22). Studies in the isolated perfused IMCD from rats have also reported that acid secretion is mediated by an H^+-K^+-ATPase in the terminal IMCD (38). Although in vitro studies have provided evidence that acid secretion in IMCD cells can be mediated by H^+-ATPase as well as H^+-K^+-ATPase, immunohistochemical studies have failed to demonstrate expression of either protein in IMCD cells in vivo under any experimental conditions.

To explore the mechanism of the decrease in urinary pH observed in mice deficient in AQP1, we examined the expression of the vacuolar H^+-ATPase in kidneys of AQP1 null and wild-type mice by light and electron microscopic immunocytochemistry and Western blot analysis. Our results demonstrate a significant increase in the expression of H^+-ATPase in the inner medulla with induction of H^+-ATPase in the apical plasma membrane of IMCD cells and increased prevalence of type A intercalated cells in the IMCD of AQP1-deficient mice compared with wild-type mice. Based on these observations, we propose that the inability to create a hypertonic medullary interstitium may play a role in the expression of H^+-ATPase in IMCD cells and interfere with the deletion of intercalated cells from the IMCD that normally occurs during postnatal kidney development.

METHODS

Animals and Tissue Preservation

The transgenic AQP1-deficient mice that were generated by targeted gene disruption have been characterized in detail in previous studies (16). The pH of freshly spontaneously voided urine specimens from six AQP1 null and six wild-type mice, 4–6 wk of age, was measured with pH electrodes. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996) and were approved by the University of California, San Francisco, and University of Florida Institutional Animal Care and Use Committees.

A total of eight mice, four AQP1 (+/+) and four AQP1 (−/−), were used for studies of H^+-ATPase expression by immunohistochemistry and Western blot analysis. Additional animals were used for double-immunolabeling experiments. The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). The kidneys were perfused briefly with PBS to rinse away all blood. From animals used for studies of H^+-ATPase expression, the right kidney was excised and processed for immunoblot analysis. This was followed by perfusion of the left kidney with a periodate-lysine-2% paraformaldehyde (PLP) solution for 10 min. In animals used only for immunohistochemical studies, both kidneys were preserved by perfusion with PLP. The kidneys were removed and cut into 1- to 2-mm-thick slices that were fixed additionally by immersion in the same fixative for 2 h at room temperature and then overnight at 4°C. Sections of tissue were cut transversely through the entire kidney on a vibratome (Pelco 101, Sectioning series 1000, Ted Pella, Redding, CA) at a thickness of 50 μm and processed for immunohistochemical studies using a horseradish peroxidase preembedding technique.

Antibodies

Rabbit polyclonal antibodies were used in all experiments. H^+-ATPase immunoreactivity was detected by immunohistochemical and immunoblot analysis using an antibody against the 70-kDa subunit of the vacuolar H^+-ATPase (courtesy of Dr. Dennis Stone, Univ. of Texas Southwestern, resin. From IMCD cells, 50-μm-thick vibratome sections cut to AQFP (courtesy of Dr. Mark A. Knepper, National Institutes of Health, Bethesda, MD). Type A intercalated cells were identified with an antibody against AE1 (courtesy of Dr. Philip S. Low, Purdue Univ., West Lafayette, IN). Lack of AQFP immunoreactivity in AQFP null mice was confirmed using an antibody against AQFP (provided by Dr. M. A. Knepper).

Immunohistochemistry

Preembedding method. Sections of PLP-fixed tissue were cut transversely through the kidney on a vibratome at a thickness of 50 μm and processed for immunohistochemistry using an indirect immunoperoxidase method. All sections were washed three times with 50 mM NH₄Cl in PBS for 15 min. Before incubation with the primary antibodies, the sections were incubated for 3 h with PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin (solution A). The tissue sections were then incubated overnight at 4°C in a solution of polyclonal antibodies against H^+-ATPase (1:2,000), AQFP (1:500), AQFP (1:300), or AE1 (1:2,000) in PBS containing 1% BSA (solution B). After several washes with PBS containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin (solution C), the tissue sections were incubated for 2 h in peroxidase-conjugated donkey anti-rabbit IgG, Fab fragment (Jackson Immunoresearch Labs) diluted 1:100 in solution B. The sections were then rinsed, first in solution C and subsequently in 0.05 M Tris buffer, pH 7.6. For the detection of horseradish peroxidase, the sections were incubated in 0.1% 3,3′-diaminobenzidine (brown stain) in 0.05 M Tris buffer for 5 min, after which H₂O₂ was added to a final concentration of 0.01% and the incubation was continued for 10 min. After being washed three times with 0.05 M Tris buffer, the sections were dehydrated in a graded series of ethanol and embedded in TAAB resin. From all animals, 50-μm-thick vibratome sections cut through the entire kidney were mounted in TAAB between polyethylene vinyl sheets. For the observation at high magnification, sections from the inner medulla were excised and glued onto empty blocks of TAAB, and 1.5-μm semithin sections were cut for light microscopy. The sections were photographed on an Olympus Photomicroscope equipped with differential-interference contrast optics.

Double immunolabeling for AQFP and H^+-ATPase. To identify the cells labeled with H^+-ATPase in the IMCD, double labeling was performed with antibodies against H^+-ATPase and AQFP, a marker of principal cells and IMCD cells. From the flat-embedded 50-μm-thick sections processed for immunolabeling of AQFP, sections from the inner medulla were excised and glued onto empty blocks of TAAB 812, and consecutive 1.5-μm sections were cut for postembedding immunolabeling. The sections were treated for 15 min with a saturated solution of sodium hydroxide in absolute ethanol to remove the resin. After three brief rinses in
absolute ethanol, the sections were hydrated with graded ethanol and rinsed in tap water. The sections were rinsed with PBS, incubated in normal donkey serum for 30 min, and subsequently incubated overnight with antibody against H^+\text{-ATPase} (1:1,000) at 4°C. The sections were rinsed with PBS, incubated for 2 h in peroxidase-conjugated donkey anti-rabbit IgG, and washed again with PBS. For detection of H^+\text{-ATPase}, Vector SG (Vector Laboratories) was used as the chromogen to produce a blue gray label, which is easily distinguishable from the brown label produced by 3,3’-diaminobenzidine used in the preembedding procedure for detection of AQP4. The sections were washed with distilled water, dehydrated with graded ethanol and xylene, mounted in Canada balsam, and examined by light microscopy.

**Transmission Electron Microscopy**

Tissue slices immunostained for H^+\text{-ATPase} were fixed in 2% glutaraldehyde in 0.1 M Tyrode buffer for 1 h at 4°C and washed twice for 10 min, first with 0.1 M Tyrode buffer and then with 0.1 M sodium cacodylate buffer. This was followed by postfixation in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4°C. After being rinsed in 0.1 M sodium cacodylate buffer, the tissue was dehydrated in a graded series of alcohol and propylene oxide and embedded in TAAB resin. Ultrathin sections were stained with lead citrate and photographed with a Zeiss 10 A transmission electron microscope.

**Immunoblot Analysis**

Kidneys from four AQP1 (+/+), and four AQP1 (−/−) mice were excised and separated into cortex, outer medulla, and inner medulla. The tissue was homogenized in a solution containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS, and freshly added leupeptin (5 mg/ml) and 100 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 12,000 g for 15 min at 4°C, and protein concentrations were determined on supernatants using the BCA protein assay reagent (Pierce, Rockford, IL). The proteins were resolved by SDS-PAGE, electrophoretically transferred onto nitrocellulose membranes, and then probed with antibodies to H^+\text{-ATPase}.

**Statistical Analysis**

To quantify the level of expression of H^+\text{-ATPase}, autoradiographs were scanned on a Hewlett-Packard Scanjet 4C using Deskscan II software, and densitometry was performed using National Institutes of Health IMAGE 1.60 software. The results are presented as means ± SE. The statistical significance of the difference in H^+\text{-ATPase} expression between AQP1 (−/−) and AQP1 (+/+) mice was assessed using Student’s unpaired t-test. P values <0.025 were accepted as indicating significant differences between mean values.

**RESULTS**

Determination of pH in the spontaneously voided urine samples demonstrated a significantly lower pH in urine from AQP1-deficient mice than in samples from wild-type mice (5.63 ± 0.07 vs. 6.15 ± 0.1; P < 0.001) (Fig. 1).

**Immunohistochemistry**

As illustrated in Fig. 2, there was no AQP1 immunoreactivity in kidneys of AQP1 knockout mice, whereas AQP1 was expressed in the proximal tubule,

![Fig. 1. Diagram showing the pH of freshly voided urine samples from aquaporin-1 (AQP1) null (AQP1 −/−; ●) and wild-type (AQP1 +/+; ○) mice. There was significantly decreased urinary pH in association with AQP1 deletion. Values are means ± SE; n = 6/group. *P < 0.001.](http://ajprenal.physiology.org/)

![Fig. 2. Light micrographs of 50-μm-thick sections from AQP1 (+/+ and AQP1 (−/−) mouse kidney (A and B, respectively) illustrating immunostaining for AQP1. AQP1 was strongly expressed in the proximal tubule, descending thin limb of Henle’s loop, and descending vasa recta in kidney from AQP1 (+/+ mouse, but there was no labeling in the kidney of AQP1 (−/−) mouse. Co, cortex; OS, outer stripe of outer medulla; IS, inner stripe of outer medulla. Magnification: ×12 (A and B).](http://ajprenal.physiology.org/)
the AQP1 (positive type A intercalated cells in the middle part of the IMCD from In addition, there was an increase in the prevalence of H increased from the middle of the IMCD toward the tip of the papilla. However, in the AQP1 (mouse, there was strong H immunostaining of the basolateral plasma membrane (Fig. 4). There was a gradual increase in the intensity of H ATPase immunoreactivity from the middle part of the IMCD to the tip of the renal papilla (Fig. 4, B and C). However, there was no labeling of principal cells in the initial IMCD (Fig. 4A).

The expression of H ATPase in the apical plasma membrane was confirmed by transmission electron microscopy, which also revealed striking changes in the configuration of IMCD cells in AQP1 null mice (Fig. 5). The junctional complex appeared to be transposed toward the basement membrane, leading to a decrease in the height of the intercellular space and the lateral plasma membrane and a corresponding increase in the apical membrane. H ATPase immunostaining was observed on the entire apical plasma membrane of the IMCD cells, but there was no labeling of the basolateral membrane.

To confirm that the labeled cells were IMCD cells, we performed double labeling with antibodies against AQP4, which is expressed in the basolateral plasma membrane of IMCD cells, and H ATPase. The results demonstrated that in the AQP1 null mice, H ATPase immunoreactivity was located in the apical plasma membrane of AQP4-positive cells in the terminal half

thin descending limb, and vasa recta of wild-type mice, as reported in previous studies (12, 19, 20).

Immunostaining for H ATPase was observed in proximal tubule cells and in intercalated cells in the connecting tubule and collecting duct in both wild-type and AQP1 knockout mice (Fig. 3). As the collecting duct descended toward the renal papilla, H ATPase-positive cells decreased in number in wild-type mice, and there was no H ATPase immunolabeling from the middle part of the IMCD (Fig. 3A). However, in AQP1 knockout mice, strong H ATPase immunoreactivity was observed in IMCD cells, and the intensity of labeling increased in the terminal papilla (Fig. 3B). Furthermore, intercalated cells were also observed in the middle part of the IMCD (Fig. 3B), and the number of H ATPase-positive intercalated cells appeared to be increased in the initial part of the IMCD in AQP1 null mice compared with wild-type mice. Higher magnification light microscopy revealed that H ATPase immunostaining of IMCD cells in AQP1 null mice was located in the apical plasma membrane, and there was no H ATPase immunolabeling of the basolateral plasma membrane (Fig. 4). There was a gradual increase in the intensity of H ATPase immunoreactivity from the middle part of the IMCD to the tip of the renal papilla (Fig. 4, B and C). However, there was no labeling of principal cells in the initial IMCD (Fig. 4A).

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of the IMCD, thus identifying them as IMCD cells (Fig. 6A, and B). There was no H^+ -ATPase immunoreactivity in AQP4-positive cells in wild-type mice (not shown). Intercalated cells with strong apical H^+ -ATPase immunostaining, but no AQP4 immunolabeling, were also present in the middle portion of the IMCD in AQP1 null mice (Fig. 6A). Labeling with antibodies against AE1, the basolateral anion exchanger, identified the intercalated cells in the IMCD as type A intercalated cells (Fig. 7).

**Immunoblot Analysis**

To quantify the changes in H^+ -ATPase expression in AQP1 null mice, we carried out immunoblot analysis with kidney homogenates in which the cortex, outer medulla, and inner medulla were separated and probed with antibodies to H^+ -ATPase. There was a statistically significant increase in H^+ -ATPase expression in the inner medulla of AQP1 knockout mice (299 ± 19) compared with wild-type animals (153 ± 94; P < 0.025, n = 4). In contrast, there were no changes in H^+ -ATPase expression in the cortex or outer medulla of AQP1 knockout mice compared with wild-type animals (Fig. 8).

**DISCUSSION**

In this study, we demonstrated that deletion of AQP1 is associated with a significant decrease in urinary pH. Because acid secretion in the collecting duct is the main determinant of urinary pH, and is to a large degree mediated by a vacuolar-type H^+ -ATPase, we determined the expression of H^+ -ATPase in the kidney of AQP1 (−/−) and AQP1 (+/+ ) mice. Our results demonstrate a significant increase in the expression of H^+ -ATPase in the inner medulla of AQP1 (−/−) mice compared with AQP1 (+/+ ) animals. The increase in H^+ -ATPase expression was due to an induction of H^+ -ATPase in the apical plasma membrane of IMCD cells and an increase in the prevalence of type A intercalated cells in the IMCD. There were no
changes in H^+-ATPase expression in either cortex or outer medulla of AQP1 null mice. These results represent the first demonstration of apical H^+-ATPase immunoreactivity in IMCD cells in vivo and suggest that the decrease in urinary pH observed in AQP1 null mice is due to upregulation of H^+-ATPase in the IMCD.

Although numerous immunohistochemical studies have examined the expression and distribution of H^+-ATPase in the collecting duct, H^+-ATPase immunoreactivity has not been observed in IMCD cells in vivo in either normal or acidotic conditions. However, it is well established from microcatheterization and micropuncture studies of the papillary collecting duct that acid secretion occurs along the entire IMCD (10, 24, 30) and is increased in both acute and chronic metabolic acidosis (6, 10). In vitro studies in cultured IMCD cells have demonstrated the presence of bafilomycin- or SCH-28080-sensitive acid secretion in these cells, indicating that H^+-ATPase as well as H^+-K^+-ATPase can be expressed in IMCD cells in vitro (22, 27). Interestingly, most studies have reported only one of the two transporters in cultured IMCD cells, suggesting that the expression of both H^+-ATPase and H^+-K^+-ATPase may be dependent on the in vitro culture conditions or on the site of origin of the IMCD cells in the renal papilla. Thus discrepancies existed between the results of immunohistochemical and functional studies. However, the present demonstration of H^+-ATPase immunoreactivity in IMCD cells indicates that under certain conditions, acid secretion in the terminal IMCD may be mediated by a vacuolar-type H^+-ATPase located in the apical plasma membrane of IMCD cells. Previous studies by Stuart-Tilley et al. (28) have demonstrated expression of the anion exchanger AE2 in the basolateral plasma membrane of IMCD cells in normal mouse kidney, suggesting that bicarbonate absorption in these cells is mediated by AE2.

The mechanism of the increased expression of H^+-ATPase in the inner medulla and the induction of H^+-ATPase in the apical plasma membrane of the IMCD cells is not known. However, it is well estab-
lished that AQP1-deficient mice are unable to generate a hypertonic medullary interstitium (16, 32). The low interstitial osmolality is believed to be due to a disruption of the countercurrent multiplication mechanism because of decreased water permeability of the descending limb of Henle’s loop and the descending vasa recta (9, 23). Thus it is tempting to speculate that the inappropriately low tonicity in the renal medulla may play a role in the induction of H⁺-ATPase in the IMCD cells.

A previous study by Amlal and co-workers (4) has provided evidence that H⁺-ATPase activity is regulated by hypotonicity. In vitro studies in cultured IMCD cells demonstrated that the rate of sodium-independent pH recovery in response to an acid load was significantly greater in a hypotonic solution than in an isotonic solution. The pH recovery was independent of the presence of potassium and was inhibited by N-ethylmaleimide, indicating that it was mediated by vacuolar H⁺-ATPase. When IMCD cells were incubated and assayed in a hypertonic solution, the rate of pH recovery was decreased, indicating that H⁺-ATPase activity is decreased by hypotonicity (4). Whether a change in interstitial tonicity has a direct effect on H⁺-ATPase protein expression remains to be established. If a hypertonic environment inhibited not only H⁺-ATPase activity but also protein expression, it might prevent the expression of H⁺-ATPase in IMCD cells under normal in vivo conditions and thus explain the discrepancy between the results of in vivo immunohistochemical studies and studies of IMCD cells in vitro.

Interestingly, although the deletion of AQP1 was associated with induction of H⁺-ATPase and the appearance of strong immunoreactivity in the apical plasma membrane of IMCD cells, there were no changes in the level of H⁺-ATPase in the inner medulla, increased prevalence of intercalated cells in the IMCD, and induction of H⁺-ATPase in the apical plasma membrane of IMCD cells in the mouse kidney. The challenge will be to identify the molecular signal(s) responsible for the induction and regulation of H⁺-ATPase expression in the IMCD cells.

In summary, the present study demonstrates that AQP1 gene deletion is associated with a significant increase in the expression of H⁺-ATPase in the inner medulla, increased prevalence of intercalated cells in the IMCD, and induction of H⁺-ATPase in the apical plasma membrane of IMCD cells in the mouse kidney. The challenge will be to identify the molecular signal(s) responsible for the induction and regulation of H⁺-ATPase expression in the IMCD cells.

The authors thank Dr. Soren Nielsen, Aarhus University, Aarhus, Denmark, and Dr. C. Craig Tisher, University of Florida, Gainesville, FL, for advice and support during this project.

Present address of Y.-H. Kim: The Water and Salt Research Center, Univ. of Aarhus, DK-8000 Aarhus C, Denmark.

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