Regulation of AQP6 mRNA and protein expression in rats in response to altered acid-base or water balance

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Promeneur, Dominique, Tae-Hwan Kwon, Masato Yasui, Gheun-Ho Kim, Jørgen Frøkjær, Mark A. Knepper, Peter Agre, and Søren Nielsen. Regulation of AQP6 mRNA and protein expression in rats in response to altered acid-base or water balance. Am J Physiol Renal Physiol 279:F1014–F1026, 2000.—In the rat, aquaporin-6 (AQP6) is mainly localized in intercalated cells (ICs) in collecting ducts, where it is exclusively associated with intracellular vesicles. In this study, we examined whether AQP6 protein and mRNA expression were regulated in the inner medulla or inner stripe of the outer medulla. Rats treated with dietary acid or acid load for 7 days with a fixed daily water intake revealed appropriate changes in urine pH but unchanged urine output. AQP6 protein and mRNA abundance were increased in alkali-loaded rats (187 ± 18 and 151 ± 17% of control, respectively), whereas no changes were observed in acid-loaded rats. Immunohistochemistry revealed increased IC AQP6 labeling in alkali-loaded rats but not in acid-loaded rats. In contrast, administration of NH₄Cl in the drinking water for 2 wk (free access to water) revealed a significant increase in AQP6 protein abundance (194 ± 9% of control), but this was associated with increased water intake. Combined, this suggests that AQP6 expression was not affected by acid loading per se but rather was in response to changes in water intake. Consistent with this, water loading for 48 h was associated with increased AQP6 protein abundance, compared with thirsted rats. Moreover, rats with lithium-induced nephrogenic diabetes insipidus had a threefold increase in both AQP6 protein and mRNA expression. Overall, these results suggest that AQP6 expression in collecting duct ICs is regulated by altered acid/alkali load or water balance. Thus AQP6 may contribute to maintenance of acid-base homeostasis and water balance.

aquaporin-6; intercalated cell; principal cell

THE AQUAPORINS (AQPs) are a family of membrane proteins that function as water channels (1). A series of studies has demonstrated that altered targeting/expression of AQPs in kidney plays a critical role in water balance disorders (19, 33, 43). Aquaporin-1 (AQP1) is highly abundant in the proximal tubule and descending thin limb (34, 35), and the critical role of AQP1 in urinary concentration was recently confirmed in transgenic knockout mice lacking AQP1 (25). At least four aquaporins (AQP2, AQP3, AQP4, and AQP6) are known to be expressed in kidney collecting ducts (33, 49). AQP2 (11) is the apical water channel of collecting duct principal cells (33). Water reabsorption in the collecting duct is regulated by both short-term regulation and long-term adaptational mechanisms, both of which have been shown to depend critically on AQP2. For instance, water restriction or chronic vasopressin treatment increases AQP2 expression as well as collecting duct water permeability, whereas water loading or treatment with vasopressin V₂-receptor antagonists decreases these parameters (33). Water transport across the basolateral plasma membrane of collecting duct principal cells is thought to be mediated by AQP3 (9) and AQP4 (40). Consistent with this, rats with lithium-induced nephrogenic diabetes insipidus (NDI) have dramatically reduced AQP2 and AQP3 expression levels along with a marked polyuria and urinary concentrating defect (20, 28). Moreover, transgenic mice lacking AQP3 (23) are severely polyuric, and inner medullary collecting ducts from AQP4-deficient mice have a significant reduction in vasopressin-stimulated water permeability (24).

In addition to AQP2, AQP3, and AQP4, we have recently demonstrated that AQP6 [previously referred to as rat WCH3 and human hKID (22, 26)] is also present in the collecting duct. Interestingly, we demonstrated that 1) AQP6 resides in intracellular vesicles in intercalated cells of cortical, outer medullary collecting duct (OMCD), and initial inner medullary collecting duct (IMCD) but not in the principal cells (49); 2)

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AQP6 exhibits low water permeability \( (P_f) \) when expressed in *Xenopus laevis* oocytes (48), which is consistent with a previous study (26); 3) with treatment with Hg\(^{2+} \), the \( P_f \) of AQP6 oocytes rapidly rises, unlike in the case of other AQP6s (48); 4) when exposed to low pH, the \( P_f \) of AQP6 oocytes is also rapidly activated, and this is accompanied by a selective chloride conductance (48); and 5) immunoelectron microscopy revealed that AQP6 co-localizes with \( \text{H}^+ \)-ATPase in the same intracellular vesicles of acid-secreting type A intercalated cells in kidney collecting duct (48). Therefore, it is speculated that AQP6 may be involved in acid-base homeostasis, or regulation of water balance; hence expression of AQP6 may be altered in the corresponding physiological or pathophysiological conditions.

In the present studies, we examined whether AQP6 protein abundance and mRNA expression in collecting duct intercalated cells is regulated by altered acid/alkali intake or by altered water intake. Using immunoblotting, Northern blotting, and immunohistochemistry, we examined 1) the changes in AQP6 protein abundance and mRNA expression in kidneys of rats with chronically altered acid/alkali intake; and 2) the changes in AQP6 protein abundance and mRNA expression in kidneys of rats with altered water balance (such as water loading, thirsting, or lithium-induced NDI).

**METHODS**

**Experimental Protocols for Altered Acid/Alkali Load in Food With Clamped Water and Food Intake**

Experiments were performed by using male Munich-Wistar rats (250–300 g, Møllegard Breeding Centre), which were maintained on a standard rodent diet (Altromin, Lage, Germany). Control and treated rats were chosen randomly and were provided a diet mixed with water to ensure a fixed diet at 7.2 mmol \( \text{Na}^+ \) per 24 g body wt \( \text{day}^{-1} \). All rats had free access to tap water and food, as described above.

**Protocol 1: NH\(_4\)Cl loading.** NH\(_4\)Cl was included in the diet at 7.2 mmol 220 g body wt \( \text{day}^{-1} \) and was administered for 7 days \( (n = 10) \). Control rats received the same diet but without NH\(_4\)Cl \( (n = 10) \). Rats received fixed amounts of food and water, as described above.

**Protocol 2: NaHCO\(_3\) loading.** NaHCO\(_3\) was included in the diet at 7.2 mmol 220 g body wt \( \text{day}^{-1} \) and was administered for 7 days \( (n = 13) \). Control rats received the same diet without NaHCO\(_3\) but with equimolar sodium in the form of NaCl \( (n = 12) \). This was done to determine whether the expression of AQP6 observed with NaHCO\(_3\) loading is attributable specifically to the effect of HCO\(_3^-\) administration.

Detailed characterization of the physiological responses of the rats or changes in thick ascending limb sodium transporter expression to NH\(_4\)Cl loading or HCO\(_3^-\) loading has been well reported previously (5, 16).

**Experimental Protocol for Altered Acid Load in Drinking Water**

**Protocol 3.** Experiments were performed by using male Munich-Wistar rats (250–300 g, Møllegard Breeding Centre). Rats were treated orally with 0.28 M NH\(_4\)Cl in their drinking water (tap water) for 2 wk ad libitum \( (n = 8) \). Control rats received tap water ad libitum without NH\(_4\)Cl \( (n = 6) \). All rats had free access to a standard rodent food (Altromin). This acid load averaged 12 \( \pm \) 1 mmol \( \text{day}^{-1} \cdot \text{rat}^{-1} \), which was very similar to the dietary acid load administered in protocol 1 \( (10 \pm \) 1 mmol \( \text{day}^{-1} \cdot \text{rat}^{-1} \)).

**Experimental Protocols for Altering Water Balance**

**Protocol 4: Thirsting or water loading.** Rats were deprived of water for 2 days (thirsting group, \( n = 12 \)) or were given a water-rich agar gel diet for 3 days (water-loading group, \( n = 11 \)). The increase in water intake was achieved by incorporating the daily food \( (18 \text{ mg/day}) \) in a water-rich agar gel, bringing the level to 60 ml of water and 11 mg agar/g of food to each rat daily, as described by Bouby et al. (4). The same amount of agar was given to the thirsted rats.

**Protocol 5: Lithium-induced NDI.** For lithium treatment, rats received food containing 40 mmol lithium/kg of dried food for the first 10 days and 60 mmol lithium/kg of dried food for the following 20 days \( (n = 8) \). All rats on lithium treatment had free access to a solid NaCl block for supplying adequate NaCl to prevent lithium intoxication and fatal outcome (42). Control rats were maintained on standard rat diet \( (n = 7) \). All rats had free access to water and food.

**Membrane Fractionation for Immunoblotting**

All rats were killed under light halothane anesthesia, and kidneys were rapidly removed. Whole kidney tissues or tissue samples from the dissected inner stripe of the outer medulla (ISOM) or inner medulla (IM; see RESULTS) were finely minced and homogenized in dissection buffer \( (0.3 \text{ M sucrose}, 25 \text{ mM imidazole}, 1 \text{ mM EDTA, pH 7.2}) \) containing 8.5 \( \mu \text{M leupeptin}, 1 \text{ mM phenylmethylsulfonyl fluoride}) \) by using an ultra-turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany). This homogenate was centrifuged in an Eppendorf centrifuge at 4,000 \( \text{g} \) for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was then centrifuged at 17,000 \( \text{g} \) for 1 h to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles. Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

**Primary Antibodies**

For semiquantitative immunoblotting and immunohistochemistry, we used previously characterized affinity-purified polyclonal antibodies against rat AQP6 (49). This antibody was raised against a peptide corresponding to amino acids 251–274 near the COOH terminal of rat AQP6 (GenBank accession no. AF083879) plus a cysteine added to the NH\(_2\)-terminal portion \( \text{NH}_2\text{CKV}EKVKVDLEPQKKESQTNSEDTEV}_{\text{COOH}} \).

**Electrophoresis and Immunoblotting**

Samples of membrane fractions were run on 12% SDS-polyacrylamide minigels (Bio-Rad Mini Protein II) for AQP6. For each gel, an identical gel was run in parallel and subjected to Coomassie staining to ensure identical loading (41). Proteins were transferred to nitrocellulose membranes by electroelution. After transfer by electroelution, blots were blocked with 5% milk in PBS-T \( (80 \text{ mM Na}_2\text{HPO}_4, 20 \text{ mM NaH}_2\text{PO}_4, 100 \text{ mM NaCl}, 0.1\% \text{Tween 20, pH 7.5}) \) for 1 h and incubated overnight at 4°C with anti-AQP6 affinity-purified antibodies. The labeling was visualized with horseradish peroxidase-conjugated secondary antibodies (P448, DAKO, Germany).
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Glostrup, Denmark, diluted 1:3,000) by using enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Preparation of RNA Samples and Northern Blotting

Total RNA was extracted from the ISOM or IM by the acid-phenol method using RNA-B (Quantum Bioprobe). Quantification of the message for AQP6 was performed by using a digoxigenin-labeled AQP6 RNA probe. The synthesis and digoxigenin labeling of the AQP6 RNA probe were performed by in vitro transcription by using a Maxiscript in vitro transcription kit (Ambion, Austin, TX).

RNA samples were separated by electrophoresis on agarose gel (1.2%) containing 0.6 M formaldehyde. Equal RNA loading was verified by visual inspection after coloration with ethidium bromide. RNA were transferred overnight from gel to nylon membranes (Hybond-N, Amersham Life Science, Buckinghamshire, UK), which were then baked in a vacuum oven (2 h at 80°C). Blots were placed in a glass hybridization tube containing 5× standard sodium citrate (SSC), 50% formamide, 0.1% sarcosyl, 0.02% SDS, and 2× blocking solution (blocking reagent in maleic acid, Boehringer Mannheim). Prehybridization was performed at 55°C for 30 min in a hybridization oven. The digoxigenin-labeled AQP6 RNA probe was then added to prehybridization medium, and membranes were incubated overnight at 55°C. The blots were washed twice for 15 min in maleic acid solution containing 0.3% Tween 20 and blocked for 30 min. After incubation with diaminobenzidine (21, 49), labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P448, 1:100, DAKO), followed by incubation with diamobenzidine (21, 49).

Quantitation of AQP6 Protein Abundance and mRNA Expression

Enhanced chemiluminescence films with bands within the linear range were scanned (30). For AQP6, both the 30- and the ~55-kDa band were scanned (49). The labeling density was corrected by densitometry of Coomassie-stained gels. For Northern blotting, the band of 2.5 kb, corresponding to AQP6 mRNA, was scanned. Values were corrected for potential differences in loading of total RNA by densitometry of 18S and 28S bands visualized by ethidium bromide on the same gel. Values were presented in the text as means ± SE. Labelings in the samples from experimental rats and control rats were calculated as a fraction of the mean control value for that gel. Comparisons between groups were made by unpaired t-test. P < 0.05 was considered significant.

Immunohistochemical Analyses

Kidneys from experimental rats and control rats were fixed by retrograde perfusion via the aorta with 4% paraformaldehyde in 0.1 M cacodylate buffer (21, 49). Tissue blocks prepared from cortex, outer stripe of inner medulla and ISOM, and IM were cryoprotected with 2.3 M sucrose containing 2% paraformaldehyde for 30 min, mounted on holders, and rapidly frozen in liquid nitrogen (35). For preparation of cryostat sections, tissue was cryoprotected in 25% sucrose. Cryostat sections (10 μm) and semithin sections (0.8–1 μm, Reichert Ultracut S Cryoultramicrotome) were incubated overnight at 4°C with anti-AQP6 antibodies, and labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P448, 1:100, DAKO), followed by incubation with diamobenzidine (21, 49).

RESULTS

Urine pH Levels and Output in Chronically Altered Dietary Acid/Alkali-Loaded Rats

Chronically acid- or alkali-loaded rats demonstrated persistent and marked changes in urine pH levels. In chronic acid-loaded rats (protocol 1), urine pH levels at the end of the experiment were significantly decreased to 5.93 ± 0.05, compared with in control rats (6.97 ± 0.04, P < 0.05, Table 1). In contrast, in chronic alkali-loaded rats (protocol 2), urine pH levels at the end of the experiment were significantly increased to 8.8 ± 0.04, compared with controls (7.0 ± 0.03, P < 0.05, Table 1). Urine output remained constant with clamped water and food intake (i.e., a fixed amount of water and food intake: 2 ml/day in alkali-loaded rats, compared with 1 ml/day in acid-loaded rats, compared with controls [24 ± 1.4 ml/day, not significant (NS)]) and 21 ± 1 ml/day in alkali-loaded rats, compared with controls (22 ± 0.6 ml/day, NS, Table 1).

Table 1. Functional data in rats with chronic acid/alkali loads (protocols 1 and 2)

<table>
<thead>
<tr>
<th></th>
<th>Acid</th>
<th>Control</th>
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<tr>
<td></td>
<td>Loaded (n = 10)</td>
<td>Control (n = 10)</td>
</tr>
<tr>
<td>Urine pH</td>
<td>5.93 ± 0.05*</td>
<td>6.97 ± 0.04</td>
</tr>
<tr>
<td>Urine output, ml/day</td>
<td>22 ± 12</td>
<td>24 ± 1.4</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kg H₂O</td>
<td>1,437 ± 57*</td>
<td>833 ± 35</td>
</tr>
<tr>
<td>(U/P)osm</td>
<td>4.6 ± 0.2*</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>T₃H₂O, ml/day</td>
<td>77.4 ± 2.3*</td>
<td>39 ± 1.2</td>
</tr>
<tr>
<td>FEN₆, %</td>
<td>0.14 ± 0.06</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>Uₒ₆V, μmol/min</td>
<td>1.4 ± 0.07</td>
<td>1.5 ± 0.08</td>
</tr>
<tr>
<td>Plasma Na</td>
<td>141 ± 0.6</td>
<td>140 ± 0.9</td>
</tr>
<tr>
<td>Plasma K</td>
<td>4.9 ± 0.1</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Plasma Cr</td>
<td>31.7 ± 1.6</td>
<td>32.2 ± 1.1</td>
</tr>
<tr>
<td>Cₒ₆, ml/min</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.1</td>
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Values are means ± SE; n, No. of rats; (U/P)osm, urine-to-plasma osmolality ratio; T₃H₂O, solute-free water reabsorption; FEN₆, fractional excretion of sodium; Uₒ₆V, urinary sodium excretion rate; Cₒ₆, creatinine clearance. *P < 0.05 when rats with altered acid or alkali loads were compared with control rats, respectively.
Changes in Urine Osmolality and Solute-Free Water Reabsorption in Chronically Altered Dietary Acid/Alkali-Loaded Rats

Oral loading with NH₄Cl for 7 days with clamped water and food intake in rats (protocol 1) caused a significant increase in urine osmolality and solute-free water reabsorption ($T_{cH2O}$) compared with control rats, despite unchanged urine output (Table 1). In contrast, oral loading with NaHCO₃ for 7 days with clamped water and food intake in rats (protocol 2) resulted in significantly decreased urine osmolality and $T_{cH2O}$ compared with control rats (Table 1). Urinary sodium excretion, plasma sodium and potassium concentration, plasma creatinine, and creatinine clearance were not changed by either NH₄Cl or NaHCO₃ treatment compared with controls (Table 1).

Intrarenal Distribution of AQP6 Protein and mRNA Expression

As demonstrated in Fig. 1, immunoblot and Northern blot demonstrated that AQP6 protein and mRNA are expressed in rat kidney, cortex, outer stripe of the outer medulla, ISOM, and IM. This is consistent with the immunolabeling pattern of AQP6 in the intercalated cells of the collecting ducts of cortex, outer medulla or IM, and other structures (49). In rat kidney membranes, the strongest signals were obtained in membranes from the ISOM (Fig. 1A), and this is consistent with the strongest AQP6 mRNA expression in the same zone (Fig. 1B). Because AQP6 is only known to be expressed in intercalated cells in the ISOM and IM, we prepared kidney membranes from whole kidney or from these two zones to examine the changes in the AQP6 protein abundance and AQP6 mRNA expression in intercalated cells in response to chronically altered acid/alkali-load or during altered water balance. The immunoblotting data were confirmed by immunohistochemistry.

Semiquantitative immunoblots of membrane fractions prepared from the ISOM of chronic alkali-loaded rats and control rats revealed that NaHCO₃ loading for 7 days with clamped water and food intake was associated with a marked increase in AQP6 protein abundance ($187 \pm 18$ compared with $100 \pm 16$% in control rats, $P < 0.05$, Fig. 2, A and B). Consistent with this, AQP6 mRNA expression in the ISOM was also significantly increased ($151 \pm 17$ compared with $100 \pm 14$% in control rats, $P < 0.05$, Fig. 3, A-C). Similar changes were observed in IM and whole kidney (not shown).

Immunohistochemistry using cryostat sections of rat kidneys revealed that intercalated cells in collecting segments of the collecting ducts of cortex, outer stripe of the outer medulla, ISOM, and IM. Digoxigenin-labeled rat AQP6 RNA probe labeled a band of ~2.5 kb, consistent with the predicted size of AQP6 mRNA. Strong expressions of AQP6 protein (A) and AQP6 mRNA (B) were obtained in the ISOM.

Fig. 1. Immunoblot and Northern blot analyses of aquaporin-6 (AQP6) in rat kidney. A: anti-AQP6 immunoblot of membranes from rat kidney cortex, outer and inner stripe of the outer medulla, and inner medulla. Anti-AQP6 specifically recognized both 30- and ~55-kDa bands. B: Northern blot of AQP6 mRNA in rat kidney cortex (Cor), outer (OSOM) and inner stripe of the outer medulla (ISOM), and inner medulla (IM). Digoxigenin-labeled rat AQP6 RNA probe labeled a band of ~2.5 kb, consistent with the predicted size of AQP6 mRNA. Strong expressions of AQP6 protein (A) and AQP6 mRNA (B) were obtained in the ISOM.

Fig. 2. A: immunoblot analysis of AQP6 in membrane fractions prepared from the ISOM of kidneys of rats with chronic NaHCO₃ loading and from control rats (protocol 2). B: densitometric analysis of AQP6 protein abundance from all experiments. Chronic dietary NaHCO₃ loading (with clamped water and food intake) was associated with a marked increase in AQP6 protein abundance ($187 \pm 18$ compared with $100 \pm 16$% in control rats, $P < 0.05$, Fig. 2, A and B). Consistent with this, AQP6 mRNA expression in the ISOM was also significantly increased ($151 \pm 17$ compared with $100 \pm 14$% in control rats, $P < 0.05$, Fig. 3, A-C). Similar changes were observed in IM and whole kidney (not shown).

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1 $T_{cH2O} = C_{osm} - V = V(U_{osm}/P_{osm}) - 1$, where $C_{osm}$ is osmolal clearance, $P_{osm}$ is plasma osmolality, $T_{cH2O}$ is solute-free water clearance, $U_{osm}$ is urine osmolality and $V$ is total daily urine output.
Load With Clamped Water and Food Intake was associated with significant increases in response to chronic NH4Cl loading with clamped water and food intake (109 ± 11 compared with 100 ± 9% in control rats, NS, Fig 6, A-C).

Consistent with immunoblotting and Northern blotting data, immunohistochemistry revealed that the AQP6 labeling in intercalated cells of both the ISOM (Fig. 7A) and IM (not shown) from chronic acid-loaded rats was unchanged, compared with control rats (Fig. 7B). AQP6 labeling was also primarily associated with intracellular domains in response to chronic dietary acid loading (Fig. 7A).

**Increased Whole Kidney AQP6 Abundance in Response to Chronic Acid Loading in Drinking Water With Free Access to Water and Food**

Rats treated with NH4Cl in the drinking water (protocol 3) had significantly reduced plasma HCO3− concentration (19 ± 1 compared with 31 ± 0.4 mmol/l in control rats, P < 0.05) or total CO2 levels (21 ± 1 compared with 33 ± 0.5 mmol/l in control rats, P < 0.05), due to chronic metabolic acidosis. Urine pH levels were also significantly decreased (5.2 ± 0.04 compared with 6.9 ± 0.1 in control rats, P < 0.05). In contrast to acid-loaded rats in protocol 1 (chronic acid loading in food with clamped water and food intake), the rats treated with NH4Cl in the drinking water had a significant increase in urine output at the end of the experiment (85 ± 6 compared with 48 ± 4 μl-min⁻¹-kg⁻¹ in control rats, P < 0.05). In parallel, increases in urine output were accompanied by a significant increase in water intake (138 ± 6 compared with 118 ± 3 μl-min⁻¹-kg⁻¹ in control rats, P < 0.05). However, urine osmolality was unchanged (1,697 ± 98 compared with 1,480 ± 56 mosmol/kgH2O in control rats, NS), despite high urine output and water intake. Moreover, NH4Cl treatment was associated with significantly increased TcH2O (353 ± 56 compared with 176 ± 7 μl-min⁻¹-kg⁻¹ in control rats, P < 0.05). When TcH2O was corrected for the osmolar clearance (Cosm), the value of TcH2O in rats treated with NH4Cl was not changed (279 ± 5 compared with 273 ± 3 μl-min⁻¹-kg⁻¹ in control rats, NS). This suggests that the increased solute load per nephron may be responsible for the increased urine output in rats with chronic NH4Cl treatment.

Chronic NH4Cl loading in drinking water for 2 wk was not associated with altered AQP6 mRNA expression in the ISOM: 105 ± 10% (n = 7) of control rats (100 ± 5%, n = 7, NS, Fig. 8). However, semiquantitative immunoblots of membrane prepared from the whole kidneys of rats treated with NH4Cl in drinking water ad libitum revealed a significant increase in whole kidney AQP6 abundance (194 ± 9 compared with 100 ± 8% in control rats, P < 0.05, Fig. 9, A and B). Consistent with the immunoblotting data, immunoperoxidase labeling of AQP6 using cryostat sections revealed that AQP6 labeling in the intercalated cells of both the ISOM (Fig. 10A) and IM (Fig. 10C) was increased compared with control rats (Fig. 10, B and D). This may suggest that AQP6 expression is also likely to be regulated by water intake.
Increased AQP6 Abundance in Water-Loaded Rats Compared With Thirsted Rats

Rats with a water-rich agar gel diet for 3 days (protocol 4) had a high urine output (45 ± 2 ml/day), whereas rats deprived of water for 2 days had a low urine output (3 ± 1 ml/day, P < 0.05). Semiquantitative immunoblots using membrane fractions prepared from whole kidneys of water-loaded and thirsted rats demonstrated that water loading in rats was significantly associated with increased whole kidney AQP6 abundance compared with thirsted rats (154 ± 11 compared with 100 ± 11% in thirsted rats, P < 0.05, not shown). Semiquantitative immunoblots of membrane fractions prepared from the IM of water-loaded and thirsted rats consistently revealed that water loading was associated with markedly increased AQP6 abundance in IM (339 ± 90% compared with 100 ± 16% in thirsted rats, P < 0.05, Fig. 11, A and B).

Increased AQP6 Abundance and mRNA Expression in Rats with Lithium-Induced NDI

Previously, we demonstrated that lithium-induced NDI in rats is associated with severe downregulation of inner medullary AQP2 or whole kidney AQP2 and AQP3 abundance, concomitant with the development of severe polyuria and impaired urinary concentration (20, 28). Rats fed with lithium-containing food for 4 wk had significantly increased urine output at the end of experiment (449 ± 53 compared with 28 ± 4 μl·min⁻¹·kg⁻¹ in control rats, P < 0.05). This was accom-
panied by a parallel increase in water intake (695 ± 60 compared with 99 ± 4 μl·min⁻¹·kg⁻¹ in control rats, P < 0.05). Moreover, lithium-treated rats had markedly lower urine osmolality (150 ± 8 compared with 1,385 ± 263 mosmol/kgH₂O in control rats, P < 0.05), indicating that chronic lithium treatment is associated with a severe impairment of urinary concentration (20, 28).

In contrast to the markedly reduced AQP2 expression in IM of rats with lithium-induced NDI (28), semiquantitative immunoblots of membrane fractions prepared from the IM revealed that rats with lithium-induced NDI (protocol 5) had a significantly increased AQP6 abundance in IM (307 ± 64 compared with 100 ± 28% in control rats, P < 0.05, Fig. 12, A and B). Consistent with this, AQP6 mRNA expression in the IM from rats with lithium-induced NDI was significantly increased (327 ± 56 compared with 100 ± 36% in control rats, P < 0.05, Fig. 12, C and D). Whole kidney AQP6 abundance was also significantly increased (257 ± 49 compared with 100 ± 20% in control rats, P < 0.05, not shown). Moreover, immunoperoxidase labeling of AQP6 in semithin sections revealed that AQP6 labeling in the intercalated cells of the ISOM from rats with lithium-induced NDI (Fig. 13A) was increased compared with control rats (Fig. 13B).

DISCUSSION

We have demonstrated that the expression of AQP6 mRNA and protein in collecting duct intercalated cells is subjected to significant regulation in response to chronic alkalosis and in conditions with altered water balance. Chronic NaHCO₃ loading of rats induced a significant increase in AQP6 mRNA and protein expression in the ISOM. In contrast, chronic NH₄Cl-loading of rats (with clamped daily water and food intake) did not have changes in AQP6 mRNA and protein expression. Chronically NH₄Cl-loaded rats with free access to water and food had a markedly increased AQP6 protein abundance associated with high urine output and water intake. An upregulation was only found in metabolic acidosis when rats had free access to water and, hence, had increased water intake. This suggests that AQP6 expression is also likely to be regulated in response to altered water intake. Consistent with this view, we also demonstrated that water loading of rats was associated with increased AQP6 abundance, compared with thirsted rats. Moreover, rats with lithium-induced NDI experienced a dramatic increase in water intake and urine output (20, 28) and had a marked increase in AQP6 mRNA and protein expression in opposition to the downregulation of AQP2 and AQP3 seen previously with these conditions. These results suggest that kidney AQP6 expression is upregulated in response to chronic metabolic alkalosis or increased water intake.

Increased AQP6 Expression in Response to Metabolic Alkalosis

AQP6 expression in intercalated cells was significantly increased in response to chronic NaHCO₃ loading with...
clamped water and food intake (protocol 2). However, despite a marked increase in AQP6 protein abundance, we also found no evidence of trafficking or redistribution of AQP6 from intracellular vesicles to plasma membrane domains in the intercalated cells in response to NaHCO₃ loading. Verlander and colleagues (44) demonstrated by immunoelectron microscopy that chloride-depleted metabolic alkalosis was associated with withdrawal of H⁺-ATPase from the apical plasma membrane to subapical cytoplasmic tubulovesicles in type A intercalated cells.

Fig. 7. Immunohistochemical analyses of AQP6 using immunoperoxidase labeling of cryostat sections of kidneys from rats with chronic NH₄Cl loading with clamped water and food intake (A) and from control rats (B). AQP6 immunolabeling was associated with intercalated cells in collecting ducts in ISOM (arrows in A and B). In chronic acid-loaded rats (A), AQP6 labeling in intercalated cells of the ISOM was similar to the labeling seen in control rats (B). Magnification, ×1,000.

Fig. 8. A: Northern blot of AQP6 mRNA in the ISOM from kidneys of rats treated with NH₄Cl in drinking water for 2 wk (ad libitum) and from control rats (protocol 3). B: ribosomal 18S and 28S rRNA bands visualized with ethidium bromide on the same gel. C: chronic NH₄Cl loading in drinking water for 2 wk was not associated with altered AQP6 mRNA expression: 105 ± 10% (n = 7) of control rats (100 ± 5%, n = 7, NS).

Fig. 9. A: immunoblot analysis of AQP6 in membrane fractions prepared from whole kidneys of rats treated with NH₄Cl in drinking water for 2 wk (ad libitum) and from control rats (CON; protocol 3). Anti-AQP6 recognized a major 30-kDa band, with 55- and 75-kDa bands in membrane fractions prepared from whole kidneys. B: rats treated with NH₄Cl in drinking water for 2 wk revealed a significant increase of whole kidney AQP6 abundance to 194 ± 9% (n = 8) of control levels (100 ± 8%, n = 6, *P < 0.05).
in cortical collecting duct or intercalated cells in the OMCD. Thus this is in opposition to the changes in H\textsuperscript{+}-ATPase trafficking observed with acidosis (27, 45). Moreover, type A intercalated cells had an increased number of numerous subapical tubulovesicles, with much reduced apical plasma membrane surface area (and only a few apical microprojections), suggesting that the apical plasma membrane is internalized during alkalosis (8).

During endocytosis, vesicles are acidified along the endocytic pathway and intravesicular acidification is known to be critical for several sorting events (31). Because protons are actively transported into vesicles by a vacuolar-type H\textsuperscript{+}-ATPase (12), the ClC-5 chloride channel was recently suggested to be important for apical endocytosis, probably by providing an electrical shunt for the H\textsuperscript{+}-ATPase (13). AQP6, which colocalizes with H\textsuperscript{+}-ATPase in the same vesicles in the intercalated cells, is also accompanied by selective chloride conductance when it is exposed to low pH (48). Thus upregulation of AQP6 expression in response to alkali loading may also be important for this endocytic process with acidification of vesicles and thus may provide intravesicular electroneutrality via chloride conductance (48). Additional studies will be needed to identify the mechanisms of the increase in AQP6 expression in response to alkalosis.

AQP6 Expression in Response to Metabolic Acidosis

In kidney, AQP6 colocalizes with H\textsuperscript{+}-ATPase in the same intracellular vesicles of acid-secreting type A

Fig. 10. Immunohistochemical analyses of AQP6 using immunoperoxidase labeling of cryostat sections of kidneys from rats with chronic NH\textsubscript{4}Cl loading with free access to water and food (A and C) and from control rats (B and D). AQP6 immunolabeling was associated with intercalated cells in collecting ducts in both ISOM (arrows in A and B) and in IM (arrows in C and D). In chronic acid-loaded rats with free access to water (A and C), AQP6 labeling in intercalated cells of the ISOM and IM was increased compared with the labeling seen in control rats (B and D), respectively. Magnification, ×650.
intercalated cells (48). When exposed to low pH, the $P_f$ of AQP6 is rapidly activated; thus in intracellular vesicles AQP6 may contribute to vesicle swelling and membrane fusion during exocytosis (15). Because intercalated cells in collecting duct are mainly associated with acid-base transport (46), these findings suggest that AQP6 is likely to be involved in acid-base metabolism, in the maintenance of intracellular vesicular electroneutrality with chloride conductance, and possibly in the tubular exocytosis within these cells.

Our studies demonstrate that AQP6 expression is not changed in response to chronic acid loading by using a protocol with fixed daily water and food intake (protocol 1). The absence of a change in expression has also been demonstrated for other intercalated cell transporters in acidosis. It was previously demonstrated that chronic oral acid loading induced no significant changes in $H^+\cdot$ATPase protein abundance (measured by dot immunobinding assay) or mRNA levels (determined by Northern blotting) in either cortex or medulla (3). This lack of a change in AQP6 or $H^+\cdot$ATPase expression is also consistent with the observation that, during acute respiratory acidosis and chronic metabolic acidosis, the number of the intercalated cells in the OMCD remains constant (14).

We did not find any evidence of AQP6 trafficking in response to acidosis. In contrast, acid loading was demonstrated to be associated with a shift in the distribution of $H^+\cdot$ATPase from cytoplasmic vesicles to the plasma membranes in the rat OMCD (3). Moreover, OMCD intercalated cells respond to chronic metabolic acidosis, exhibiting significant ultrastructural changes with an increase in the surface density ($S_v$) of the apical plasma membrane and a decrease in the subapical number of tubulovesicular structures to enhance urinary acidification (27). These findings suggest acidosis-induced redistribution of $H^+\cdot$ATPase within intercalated cells without changes in the $H^+\cdot$ATPase abundance. However, in contrast to the redistribution

![Fig. 11. A: immunoblot analysis of AQP6 in membrane fractions prepared from the IM of water-loaded rats and thirsted rats (protocol 4). B: densitometric analysis of AQP6 abundance from all experiments revealed that water loading in rats ($n = 12$) was associated with markedly increased AQP6 abundance compared with AQP6 levels in thirsted rats ($n = 11$): 339 ± 90% of thirsted rats (100 ± 16%, *$P < 0.05$).

![Fig. 12. A: immunoblot analysis of AQP6 in membrane fractions prepared from the IM of rats with lithium-induced nephrogenic diabetes insipidus (NDI) and from control rats (protocol 5). B: rats with lithium-induced NDI ($n = 8$) exhibited significantly increased AQP6 abundance (30-kDa band) in kidney IM: 307 ± 64% of control levels (100 ± 28%, *$P < 0.05$). C: Northern blot of AQP6 mRNA in the IM. D: AQP6 mRNA expression in the IM from rats with lithium-induced NDI ($n = 8$) was significantly increased to 327 ± 56% of control levels (100 ± 36%, *$P < 0.05$).]
of H\(^+\)-ATPase in acidosis, AQP6 remained predominantly associated with intracellular domains in response to acid loading, and there was no evidence of trafficking or redistribution to the plasma membrane domains.

Chronic NH\(_4\)Cl-loaded rats excreted more chloride into the urine than controls due to increased chloride loading (5). Previous studies by Kim et al. (17) have demonstrated that increased delivery of chloride to cortical collecting duct, which was induced by chronic infusion of bumetanide and a high salt intake, was associated with significant hypertrophy of type B intercalated cells (with increase in both cell profile area and in the boundary length), whereas type A intercalated cells were small compared with those in controls and exhibited a flat luminal surface with short and sparse microprojections. Because AQP6 labeling is mainly associated with type A intercalated cells in cortical collecting ducts and type A-like intercalated cells in OMCD and IMCD, this adaptation to chloride may contribute to the unchanged AQP6 expression in chronic NH\(_4\)Cl loading, although the cellular and molecular mechanisms are still not established.

Interestingly, AQP6 expression was upregulated in response to chronic acid loading in animals with free access to water and food, which was associated with an increase in urine output and water intake (protocol 3). This is consistent with a recent study showing an increase in AQP6 mRNA expression in the same condition (36). However, AQP6 expression remained mainly associated with intracellular domains, despite the increased AQP6 abundance. Thus our data taken together suggest that AQP6 expression is not affected by acid loading per se but rather seems to be regulated by water intake. It should be emphasized that the daily amount of acid loading in the two protocols was very similar. The marked difference between these two acidosis models was the water intake and urine output. In protocol 3, using chronic acid loading with free access to water and food, rats had increased water intake and increased urine output, which was possibly due to osmotic diuresis with NH\(_4\)Cl, in conjunction with a significant increase in whole kidney AQP6 abundance. This suggests that AQP6 expression in intercalated cells is also likely to be regulated by altered water metabolism.

**AQP6 Expression in Response to Altered Water Balance**

\(P_f\) in the renal collecting ducts is known to be regulated by vasopressin (18, 33). In collecting duct principal cells, apically expressed AQP2 is the chief target for vasopressin in regulating collecting duct \(P_f\) (29, 33). Acute short-term regulation involves vasopressin-regulated trafficking of AQP2 between intracellular vesicles and the apical plasma membrane (6, 32, 38, 47). AQP2 is also involved in chronic adaptational regulation of body water balance achieved through regulation of AQP2 abundance (29). Importantly, multiple studies have now emphasized a critical role of AQP2 in several inherited (7) and acquired water balance disorders (33). AQP3 and AQP4 are also present in the collecting duct principal cells and are abundant in the basolateral plasma membranes, representing exit pathways.

In contrast to AQP2, AQP3, and AQP4, AQP6 is not present in the principal cells but resides exclusively in intracellular vesicles in intercalated cells of collecting ducts (49). Generally, intercalated cells can be viewed as the chief site of the acid-base and potassium trans-

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Fig. 13. High-resolution immunocytochemical analyses using immunoperoxidase labeling of AQP6 in semithin sections of kidneys from rats with lithium-induced NDI (A) and from control rats (B). AQP6 immunolabeling was associated with intercalated cells in collecting ducts in ISOM (arrows in A and B). In rats with lithium-induced NDI (A), AQP6 labeling in intercalated cells of the ISOM was increased compared with the labeling seen in control rats (B). In rats with lithium-induced NDI, principal cells were hypertrophied and enlarged (arrowheads in A). Magnification, \(\times 1,000\).
port in the distal nephron and collecting duct (46). In contrast, principal cells are regarded as the main site in the regulation of water and sodium absorption and potassium secretion in these segments (18). Moreover, AQP6 exhibits low basal $P_f$ like AQP0 (48). Therefore, AQP6 is not likely to function as a simple conduit for transepithelial water reabsorption or secretion in nephron.

Our studies demonstrate that water loading of rats, a condition where AQP2 abundance in kidney is significantly reduced (33), was associated with a marked increase in AQP6 abundance compared with thirsted rats (37). This is consistent with a recent study demonstrating reduced AQP6 mRNA expression in dehydrated rats (36). To further explore this, we used rats with lithium-induced severe NDI, where total kidney AQP2 and AQP3 abundance are dramatically reduced, playing a crucial role for the development of lithium-induced polyuria and impaired urinary concentration (20, 28). The results demonstrated that lithium-treated polyuric rats had a marked increase in AQP6 expression. The reciprocal changes in AQP6 expression in intercalated cells in response to altered water balance compared with AQP2 and/or AQP3 expression in principal cells is unexplained thus far. The changes in intracellular pH levels or intracellular vesicle pH levels in type A intercalated cells in response to altered water metabolism may be important in this process. However, it has been demonstrated that arginine vasopressin increases cAMP levels in both principal cells and intercalated cells from rabbit cortical collecting ducts, although maximal stimulation was significantly greater with principal cells (10). Moreover, both principal cells and intercalated cells from rabbit cortical collecting ducts responded to antidiuretic hormone by increasing apical cell membrane $P_f$ (39). Thus it might be possible that intercalated cells are also involved in water metabolism and that AQP6 may be somehow involved.

**Summary**

In summary, our results demonstrated that the AQP6 abundance and mRNA expression in kidney are significantly regulated in response to chronically altered acid/alkali load, and to changes in water balance. AQP6 expression is significantly upregulated in chronic alkali-loaded rats, water-loaded rats, and in rats with lithium-induced NDI, suggesting that AQP6 is involved in the acid-base metabolism and water balance. Immunocytochemistry studies exhibited no evidence for trafficking and redistribution of AQP6 from intracellular vesicles to plasma membranes, despite an increase in AQP6 expression. Further studies will be needed to identify the mechanistic basis and the exact role of altered AQP6 expression in the intercalated cells in diverse pathophysiological conditions.

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