Lithium treatment induces a marked proliferation of primarily principal cells in rat kidney inner medullary collecting duct

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Lithium treatment induces a marked proliferation of primarily principal cells in rat kidney inner medullary collecting duct. Am J Physiol Renal Physiol 291: F39–F48, 2006. First published January 24, 2006; doi:10.1152/ajprenal.00383.2005.—Lithium (Li) treatment for 4 wk has previously been shown to increase the fraction of intercalated cells in parallel with a decrease in the fraction of principal cells in the kidney collecting duct (Christensen BM, Marples D, Kim YH, Wang W, Frøkiær J, and Nielsen S. Am J Physiol Cell Physiol 286: C952–C964, 2004; Kim YH, Kwon TH, Christensen BM, Nielsen J, Wall SM, Madsen KM, Frøkiær J, and Nielsen S. Am J Physiol Renal Physiol 285: F1244–F1257, 2003). To study how early this fractional change starts, the origin of the cells and the possible mechanism behind the changes, we did time course studies in rats subjected to different durations of Li treatment (i.e., for 4, 10, and 15 days). Increased urine output was already observed at day 4 of Li treatment with decreased AQP2 levels although not statistically significant. At days 10 and 15, both a significant polyuria and down-regulation in AQP2 expression were observed. At day 10, the density of H+/HCO3-ATPase-positive cells was increased in the IMCD of Li-treated rats and this was further pronounced at day 15. Some of the H+/HCO3-ATPase-positive cells did not contain Cl-/HCO3- exchange AE1, indicating that they were not fully differentiated to type A IC. By double labeling for either H+/HCO3-ATPase and proliferating-cell nuclear antigen (PCNA) or for AQP4 and PCNA, we found that proliferation mainly occurred in proximal IMCD cells at day 4 and it increased toward the middle part of the IMCD in response to prolonged Li treatment. Most cells expressing PCNA were stained with AQP4 but not with H+/ATPase. Triple-labeling for H+/ATPase, AQP4, and PCNA showed a subset of cells negative for all three proteins or only positive for PCNA. In contrast, a 4-wk recovery period after 4 wk of Li treatment reversed the enhanced proliferative rate to the control levels. In conclusion, the Li-induced increase in the density of intercalated cells is associated with a high proliferative rate of principal cells in the IM-1 and IM-2 rather than a selective proliferation of intercalated cells as expected. This is likely to contribute to the remodeling of the collecting duct after Li treatment.

Lithium (Li)-induced nephrogenic diabetes insipidus (NDI) in rats has been shown to be associated with a dramatic decrease in aquaporin-3 (AQP3) expression in the kidney collecting duct (18, 22). Moreover, we recently demonstrated that 4 wk of Li administration in rats also lead to marked alterations in the cellular composition of the inner medullary collecting duct (IMCD) and cortical collecting duct (CCD), with a major increase in the fraction of intercalated cells (IC) and a decrease in the fraction of principal cells (PC) (6, 14). These remarkable structural and cellular changes as well as the downregulation of AQP2 and the polyuria were completely reversed 4 wk after removal of Li from the diet in rats, suggesting that the Li-induced urinary concentrating defect could be a consequence of 1) the downregulation of collecting water channels AQP2 and AQP3 (18, 22) and 2) the change in the cellular composition in the collecting duct (6, 14).

The time course in the development of Li-induced polyuria including the time course of the changes in AQP2 and AQP3 expression as well as the structural changes in the collecting duct are unknown. Moreover, the mechanisms behind the changes in cellular composition are also unknown. It may be hypothesized that the increase in the fraction of intercalated cells and the decrease in the fraction of principal cells potentially are caused by one or several factors, including 1) selective proliferation of existing intercalated cells, 2) differentiation of principal cells to intercalated cells, and/or 3) selective loss of principal cells by the process of cell death (e.g., by apoptosis). To provide further insights into this, a series of protocols was performed aimed at investigating the time course of the changes in AQP2 expression and especially the changes in the cellular composition of kidney collecting duct cells in response to chronic Li treatment. This was achieved by immunoblotting and by single, double, and triple immunocytochemical labeling of kidney sections from control rats and rats treated with Li for 4, 10, 15, or 28 days. Specifically, immunoblotting was performed to examine the time course of the changes in AQP2 expression and to correlate this with functional changes in urine output. Immunocytochemical analyses were performed to examine the time course of the changes in cellular composition by use of single labeling of AQP4 (a marker for collecting duct principal cells) and H+/ATPase (a marker for intercalated cells) as well as double labeling using the specific markers for collecting duct principal cells (AQP4) and intercalated cells (H+/ATPase and the anionic exchanger AE1). Immunocytochemical analysis was also performed to examine whether there was proliferating-cell nuclear antigen (PCNA), an indicator of cell proliferation-positive collecting duct cells, and if this was the case then the cell type was identified by double or triple labeling with AQP4 and H+/ATPase. Finally, immunocytochemical analysis was performed to investigate whether there were any apoptosis-inducing factor (AIF)-labeled collecting duct cells so as to examine whether apoptosis is involved in the changes in cellular composition in Li-induced NDI.
MATERIALS AND METHODS

Experimental Animals and Protocols

The animal protocols were approved by the board of the Institute of Anatomy, University of Aarhus, according to the licenses for use of experimental animals issued by the Danish Ministry of Justice. Wistar rats were obtained from M&B (Ejby, Denmark). Li-containing food was prepared as previously described (6, 22). Rats were subjected to the following protocols. In protocol 1, rats were given 40 mmol Li/kg of dry food for 4 days ($n = 3$), and in protocol 2 rats were given 40 mmol Li/kg of dry food for 7 days, followed by 60 mmol Li/kg of dry food for 3 days ($n = 3$). In protocol 3, rats were given 40 mmol Li/kg of dry food for 7 days, followed by 60 mmol Li/kg of dry food for 8 days ($n = 3$). In all three protocols, corresponding control rats received normal food ($n = 3/protocol$). Rats on Li treatment had access to a solid NaCl block for supplying an adequate NaCl, preventing Li intoxication and a fatal outcome. In protocol 4, rats received food containing 40 mmol Li/kg of dry food for the first 7 days, followed by 60 mmol Li/kg of dry food the next 3 wk ($n = 4$). In protocol 5, rats were given 40 mmol Li/kg of dry food for 7 days, followed by 60 mmol Li/kg of dry food for 3 wk, and control rats received normal food ($n = 10$). After the 4-wk treatment, one-half of the Li-treated rats and one-half of the control rats were killed ($n = 5$) and the remaining rats in both groups were fed a Li-free normal diet for an additional 4 wk ($n = 5$). All rats (Li-treated and controls) had free access to water intake. During the last 2–3 days of the experiments, the animals were housed in metabolic cages to measure daily urine output.

Primary Antibodies

AQP4 (LL182AP). An affinity-purified rabbit polyclonal antibody raised to a COOH-terminal peptide of rat AQP4 has been characterized previously (33).

AQP2 (LL127 AP). An affinity-purified rabbit polyclonal antibody raised to a COOH-terminal peptide of rat AQP2 has been characterized previously (24).

PCNA. A monoclonal antibody recognizing PNA was purchased from Sigma.

AIF. A goat polyclonal antibody recognizing AIF was purchased from Santa Cruz Biotechnology.

H+-ATPase (H7659AP). An affinity-purified rabbit polyclonal antibody raised to a COOH-terminal peptide of the bovine intercalated cell-specific 56-kDa B1 subunit of vacuolar H+-ATPase (amino acids 499–513, SRGQAQQPDASDTAL) has been characterized previously (23). Characterization of this antibody confirmed the specificity, and both immunoblotting and immunocytochemistry reveal identical patterns compared with previously developed antibodies to the same peptide (14, 35). The antibody recognizes an ~62-kDa band on immunoblotting of rat kidney membrane preparations, and this band disappears on peptide preabsorption (not shown). Immunohistochemistry showed specific labeling of intercalated cells in the kidney collecting duct of both rat and mouse (not shown).

AE1 (rkAE1–1–759). An affinity-purified rabbit polyclonal antibody raised to a NH2-terminal peptide of rat band 3-like Cl–/HCO3– exchanger (amino acids 1–15 of the kidney isofrom, MDQRNQELQWVSEAHA) has been characterized previously (16). Characterization of this antibody confirmed the specificity and reveal identical patterns compared with previously developed antibodies against the erythrocyte isofrom of AE1 (1, 34). The antibody selectively recognizes type A intercalated cells in rat and mouse kidney as determined by immunohistochemistry (not shown). On immunoblot of kidney membrane preparations the antibody recognizes both a 90-kDa band corresponding to the kidney isofrom of AE1 and a 100-kDa band corresponding to the erythrocyte isofrom of AE1 (4). Both bands disappear on preabsorption with the immunizing peptide.

Immunocytochemistry

Fixation. In protocols 1–3, right kidneys were removed for homogenization and immunoblotting before the fixation procedure. Left kidneys were then retrograde perfusion-fixed in 3% paraformaldehyde in 0.1 M cacodylate, pH 7.4, via the abdominal aorta for 3 min and were subjected to postfixation for 30 min in 0.1 M sodium cacodylate, pH 7.4, and paraffin embedding. The fixation of kidneys from protocols 4 and 5 is described previously (6). In addition, control tissues (jejunum and ovary from normal adult rats and kidneys from 4-day-old rats) were immersion-fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, and were subjected to paraffin embedding.

Preparation of tissue for light and laser confocal microscopy. Before paraffin embedding, tissue blocks from whole kidneys were dehydrated in a graded series of ethanol (2 h in 70, 96, and 99%, respectively) and xylene (overnight). Paraffin sections (2 μm) were cut on a Leica RM 2126 microtome and dried overnight at 37°C.

Single Labeling. Sections were incubated with one of the following affinity-purified antibodies: 1) H+-ATPase antibodies (1:200 dilution), 2) PCNA (1:2,000 dilution), or 3) AIF (1:100 dilution). Labeling was visualized by use of a peroxidase-conjugated secondary antibody and 3,3’-diaminobenzidine (DAB).

Double Labeling. For immunofluorescence microscopy, sections were incubated with anti-H+-ATPase antibodies (1:100 dilution) and anti-PCNA antibodies (1:1,000 dilution). Labeling was visualized with Alexa 488- and Alexa 546-conjugated secondary antibodies (Alexa 488-anti-rabbit and Alexa 546 anti-mouse, respectively; Molecular Probes, Eugene, OR). Double labeling was also performed as follows: 1) anti-AQP4 antibodies (1:200 dilution) and anti-H+-ATPase antibodies (1:500 dilution); 2) anti-PCNA antibodies (1:4,000 dilution) and anti-AQP4 antibodies (1:20 dilution); 3) anti-H+-ATPase antibodies (1:1,000 dilution) and anti-AE1 antibodies (1:10 dilution); and 4) anti-PCNA antibodies (1:4,000 dilution) and anti-H+-ATPase antibodies (1:1,000 dilution). Sections were incubated overnight at 4°C with either AQP4, PCNA, or AE1, followed by incubation with horseradish peroxidase-conjugated secondary antibody, and labeling was visualized with DAB (brown).

Sections were then incubated in 3.5% H2O2 in methanol to remove remaining peroxidase from the first staining. Sections were incubated overnight at 4°C with the second primary antibody (H+-ATPase or AQP4) followed by incubation with horseradish peroxidase-conjugated secondary antibody. For detection of either H+-ATPase or AQP4, Vector SG (Vector Laboratories) was used as the chromogen to produce a blue gray label.

Triple Labeling. Triple labeling was performed as follows. Sections were incubated with both monoclonal anti-PCNA antibodies (1:12,000 dilution) and polyclonal anti-H+-ATPase antibodies (1:4,000 dilution) the first day of the procedure. After incubation overnight, sections were incubated with peroxidase secondary antibodies and labeling was visualized with DAB (PCNA in the nucleus and H+-ATPase in the apical plasma membrane). This was followed by a second incubation with anti-AQP4 antibodies (1:10 dilution) and visualization of AQP4 by Vector SG (AQP4 in the basolateral membrane).

Quantification of PCNA-Positive Intercalated Cells Using Anti-PCNA and Anti-H+-ATPase Antibodies

Cell counting was performed in kidney sections from the Li-treated rats that were double labeled with antibodies against PCNA and H+-ATPase. Counting was performed directly under the microscope. H+-ATPase-positive cells labeled with or without PCNA were counted. The counting was performed in the inner medulla, and the total number of cells counted was 198 in protocol 1, 123 in protocol 2, and 118 in protocol 3.

Immunoblotting

Whole kidney was homogenized in dissecting buffer as previously described (6). This homogenate was centrifuged at 4,000 g for 15 min
at 4°C. Laemmli sample buffer was added to the supernatant, and samples were run in duplicate. One gel was Coomassie stained to ensure that loading in the lanes was consistent, whereas the other was subjected to immunoblotting. After transfer by electroelution to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated with anti-AQP2 antibody (LL127, Amersham Pharmacia Biotech, Little Chalfont, UK). Films were scanned using an AGFA scanner (Arcus II), and labeling density was quantitated using specially written software (22).

Statistical Analyses

Values are presented as means ± SE. Statistical comparisons were accomplished by an unpaired t-test (with equal or unequal variances as determined by an F-test). P values <0.05 were considered statistically significant.

RESULTS

Effects of 4, 10, and 15 Days of Li Treatment on Urine Output and Whole Kidney AQP2 Expression

Chronic Li treatment (4 wk) has previously been shown to cause severe polyuria (6, 14, 18, 22). Consistent with this, we here demonstrate that urine output increased to 70 ± 5 vs. 27 ± 2 μl·min⁻¹·kg⁻¹ in controls (P < 0.05, n = 3, Table 1) at day 4 of Li treatment in parallel with a decrease in urine osmolality (763 ± 32 vs. 2,167 ± 425 mosmol/kgH2O, not significant, n = 3). Moreover, the urine output progressively increased to 216 ± 40 vs. 36 ± 2 μl·min⁻¹·kg⁻¹ (P < 0.05, n = 3) at day 10 and 478 ± 68 vs. 28 ± 5 μl·min⁻¹·kg⁻¹ (P < 0.05, n = 3) at day 15 of Li treatment. Consistent with this, urine osmolality was significantly decreased (246 ± 7 vs. 1,366 ± 79 mosmol/kgH2O at day 10 and 167 ± 18 vs. 1,872 ± 2 mosmol/kgH2O at day 15; P < 0.05, n = 3). Plasma Li concentration was 0.65 ± 0.04 mM at 4 days (protocol 1), 0.81 ± 0.07 mM at 10 days (protocol 2), and 1.01 ± 0.22 mM at 15 days (protocol 3) after Li treatment.¹

We previously reported that AQP2 expression was markedly decreased in response to 4 wk of Li treatment (6, 18, 22). To establish the time course of changes in AQP2 expression, immunoblotting was performed to evaluate the expression at days 4, 10, and 15 of Li treatment. The AQP2 protein level in total kidney was decreased at day 4 although not statistically significantly (58 ± 2 vs. 100 ± 21%, not significant, n = 3, Fig. 1A), whereas a significant downregulation was observed at day 10 (19 ± 2 vs. 100 ± 15%, P < 0.05, n = 3, Fig. 1B) and day 15 (7 ± 4 vs. 100 ± 22%, P < 0.05, n = 3, Fig. 1C) of Li treatment.

Li Treatment for 10 and 15 Days Increased Density of H⁺-ATPase-Labeled Cells in the Inner Medulla

We have previously shown that Li treatment for 4 wk increased the density of apical H⁺-ATPase-positive cells in the IMCD and that these cells all had basolateral AE1 immunolabeling, indicating that they correspond to type A-intercalated cells (6, 14). Immunoperoxidase microscopy of paraffin-embedded kidney sections revealed unchanged density of H⁺-ATPase-labeled cells in the IMCD of rats treated with Li for 4 days (Fig. 2D) compared with control rats (Fig. 2A). In contrast, a markedly increased density of H⁺-ATPase-labeled cells in the IMCD was observed after 10 or 15 days of Li treatment (Fig. 2, E and F) compared with controls (Fig. 2, B and C).

Fig. 1. Immunoblot and corresponding densitometric analysis of aquaporin-2 (AQP2) in total kidney of control rats and rats treated with lithium (Li) for 4 (A), 10 (B), and 15 days (C), respectively. Anti-AQP2 antibody recognizes 29- and 35- to 50-kDa bands that correspond to nonglycosylated and glycosylated forms of AQP2. AQP2 protein levels were decreased at day 4 although not statistically significantly (0.58 ± 0.02 vs. 1.00 ± 0.21 in controls, not significant, n = 3; A). After 10 (0.19 ± 0.02 vs. 1.00 ± 0.15 in controls, P < 0.05, n = 3; B) and 15 days of treatment (0.07 ± 0.04 vs. 1.00 ± 0.22 in controls, P < 0.05, n = 3; C), a significant downregulation was observed.

1 The plasma lithium concentration observed at days 10 and 15 corresponds to therapeutic levels (0.8–1.3 mM).

Table 1. Functional data

<table>
<thead>
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<th>Control</th>
<th>Lithium</th>
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<tr>
<td>BW, g</td>
<td>307 ± 34</td>
<td>286 ± 29</td>
</tr>
<tr>
<td>Urine output, μl·min⁻¹·kg⁻¹</td>
<td>27 ± 2</td>
<td>70 ± 5*</td>
</tr>
<tr>
<td>Uosm mosmol/kgH₂O</td>
<td>2,167 ± 425</td>
<td>763 ± 32</td>
</tr>
<tr>
<td>Plasma lithium levels, mM</td>
<td>ND</td>
<td>0.65 ± 0.04</td>
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Values are means ± SE; n = 3 rats/protocol. BW, body weight; Uosm, urine osmolality; ND, not detectable. *P < 0.05.
region of the labeled cells. Double labeling with $\text{H}^+\text{-ATPase}$ and AE1 revealed that many $\text{H}^+\text{-ATPase}$-labeled cells were also labeled with basolateral AE1, indicating that these cells were type A-like intercalated cells (Fig. 3, D–I). However, some cells exhibiting apical $\text{H}^+\text{-ATPase}$ labeling exhibited no AE1 immunolabeling in the basolateral plasma membrane (Fig. 3, H and I).

Double Labeling with Antibodies Against AQP4 and $\text{H}^+\text{-ATPase}$

Next, we performed double immunolabeling with antibodies against AQP4, a marker for principal cells, and $\text{H}^+\text{-ATPase}$, a marker for intercalated cells. AQP4 is localized to the basolateral plasma membrane in IMCD cells, as previously demonstrated (33). The double labeling revealed that all the cells in the IMCD were labeled with either AQP4 or $\text{H}^+\text{-ATPase}$ in sections from kidneys of rats treated with Li for 4 days and in nontreated control rats (Fig. 4, A and D). In contrast, after 10 and 15 days of lithium treatment, there were some cells in the proximal part of inner medulla (IM-1) that were negative for both AQP4 and $\text{H}^+\text{-ATPase}$ (B and C, arrowheads), and after 15 days there were also some cells in the middle part of inner medulla (IM-2) that were negative for both proteins (F, arrowheads). Magnification, ×225.

Fig. 2. Immunocytochemistry using kidney sections (inner medulla; IM) from control rats (A–C) and rats treated with Li for 4 (D), 10 (E), and 15 days (F). Sections were incubated with anti-$\text{H}^+\text{-ATPase}$ and peroxidase-conjugated secondary antibody. The density of $\text{H}^+\text{-ATPase}$-positive cells was increased in rats treated with Li for 10 and 15 days (E and F) compared with the controls (B and C). Magnification, ×450.

Fig. 3. Double labeling with peroxidase-conjugated secondary antibody of kidney sections (IM) from control (A–C) and Li-treated rats (4 days, D and G; 10 days, E and H; 15 days, F and I). Labeling was performed with primary antibodies that recognize anion exchanger isoform 1 (AE1; brown) and $\text{H}^+\text{-ATPase}$ (blue gray). In the control rats and rats treated with Li for 4 days, basolateral AE1 staining was observed in all cells that contained $\text{H}^+\text{-ATPase}$ (always apical, A–D and G, arrows). After 10 and 15 days of Li treatment, most cells containing $\text{H}^+\text{-ATPase}$ were also positive for AE1 (E and F, arrows). However, there were also $\text{H}^+\text{-ATPase}$-positive cells that had no or very little basolateral AE1 staining (H and I, arrowheads). Magnification, ×450.

Fig. 4. Double labeling with peroxidase-conjugated secondary antibody of kidney sections (IM-1 and IM-2) from rats treated with Li for 4 (A and D), 10 (B and E), and 15 days (C and F). Labeling was performed with primary antibodies that recognize AQP4 (brown) and $\text{H}^+\text{-ATPase}$ (blue gray). After 4 days of Li treatment, all cells in the IM were either labeled for basolateral AQP4 or apical $\text{H}^+\text{-ATPase}$ (A and D). After 10 and 15 days of lithium treatment, there were some cells in the proximal part of inner medulla (IM-1) that were negative for both AQP4 and $\text{H}^+\text{-ATPase}$ (B and C, arrowheads), and after 15 days there were also some cells in the middle part of inner medulla (IM-2) that were negative for both proteins (F, arrowheads). Magnification, ×225.
IM-3). Compared with controls, there were also cells that exhibited considerably weaker AQP4 labeling, albeit still detectable.

**Li Treatment was Associated with an Increased Number of PCNA-Positive Cells That were Mainly H\(^+\)-ATPase Negative**

To address whether Li has an effect on proliferation of principal and/or intercalated cells in the IMCD, we performed immunocytochemistry using an antibody against PCNA. PCNA is an auxiliary protein of DNA polymerase \(\delta\), which is essential for DNA replication during the S phase of the cell cycle (9, 21, 30, 31). The protein begins to accumulate during the G1 phase and is most abundant during the S phase, followed by a decline during the G2/M phase of the cell cycle. To test the specificity of the antibody, we used rat jejunum as a positive control. Cells containing positive nuclei were primarily seen in the crypts (not shown) as previously described (9).

Double immunofluorescent labeling with antibodies against PCNA and H\(^+\)-ATPase showed that there were no or very few PCNA-positive cells in the inner medulla of the control rats in different protocols of Li treatment (Fig. 5, A–D). In contrast to the controls, 4-day Li treatment was associated with a very dramatic increase in PCNA labeling. At day 4 of Li treatment, this increase was particularly prominent in the initial part of the inner medulla (IM-1; Fig. 5E), and there were only few cells labeled with PCNA in the middle part of the inner medulla (IM-2; Fig. 5F). Moreover, there was a remarkable increase in the number of PCNA-positive cells in both IM-1 (Fig. 5, F and G) and IM-2 (Fig. 5, J and K) at days 10 and 15. In IM-1, most of the PCNA-positive cells were H\(^+\)-ATPase negative (Fig. 5,

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**Fig. 5.** Fluorescent double labeling of kidney sections (IM-1 and IM-2) from control rats (A–D) and rats treated with Li for 4 (E and I), 10 (F and J), and 15 days (G and K) or 4 wk (H and L). Sections were labeled with polyclonal anti-H\(^+\)-ATPase antibody (green) and monoclonal anti-proliferating-cell nuclear antigen (PCNA) antibody (red), and labeling was visualized by Alexa 488- and Alexa 546-conjugated secondary antibodies. In the IM of control rats, no or very few PCNA-positive cells were observed (A–D). After 4 days of Li treatment, cell nuclei staining positive for PCNA were seen almost exclusively in H\(^+\)-ATPase-negative cells in IM-1 (E). Multiple PCNA-positive nuclei were also seen in IM-1 after 10 and 15 days, and the labeling was mainly confined to H\(^+\)-ATPase-negative cells (F and G). Some PCNA-positive nuclei were also observed in cells with apical H\(^+\)-ATPase staining in IM-1 (F and G, arrows). In IM-2, PCNA-positive nuclei were observed after 10 days, and the density increased after 15 days of treatment (J and K). After 4 wk, the density of PCNA-positive nuclei was decreased in the IM (H and L). PCNA-positive nuclei were almost exclusively observed in H\(^+\)-ATPase-negative cells in IM-1 (H). In IM-2, there were also a few H\(^+\)-ATPase-positive cells with PCNA-staining nuclei (L). Magnification, \(\times\)250.
F and G), although a subset of the PCNA-positive cells had apical H\(^+\)-ATPase labeling, representing type A-like intercalated cells that underwent proliferation. Cell counting in kidney sections from the Li-treated rats labeled with H\(^+\)-ATPase and PCNA showed that 11 ± 0.2% of the H\(^+\)-ATPase-positive cells were positive for PCNA at day 4 of Li treatment, and the number increased up to 38 ± 9% at day 10 and then again decreased to 10 ± 6% at day 15 after Li treatment. At 4 wk after Li treatment, the density of PCNA-positive cells was markedly decreased in the inner medulla (Fig. 5, H and L) compared with 10 or 15 days of Li treatment.

Li Treatment was Associated with an Increased Number of PCNA-Positive Cells That were Mainly AQP4 Positive

To identify whether the cells that were PCNA positive but H\(^+\)-ATPase negative were principal cells, we performed double immunolabeling with antibodies against PCNA and AQP4 as a marker for principal cells (Fig. 6). At day 4 of Li treatment, virtually all PCNA-positive cells exhibited AQP4 labeling (Fig. 6A). After 10 and 15 days of Li treatment, AQP4 expression was reduced especially in IM-1, and PCNA-positive cells exhibited either distinct AQP4 expression or weak AQP4 expression (Fig. 6, B and C). Moreover, there were cells that were positive for PCNA but negative for AQP4 in the IM-1 region at days 10 and 15 after Li treatment (Fig. 6, B and C).

Next, we performed triple labeling with antibodies recognizing PCNA, AQP4, and H\(^+\)-ATPase to confirm whether the PCNA-positive/AQP4-negative cells were all PCNA positive/H\(^+\)-ATPase positive. The result revealed that some PCNA-positive/AQP4-negative cells did not exhibit H\(^+\)-ATPase labeling at days 10 and 15 of Li treatment (Fig. 7, E and F). Thus there were PCNA-positive cells in the inner medulla that were negative for both AQP4 and H\(^+\)-ATPase (Fig. 7, E and F). This indicates that the cells that were PCNA positive but AQP4 negative were not type A-like intercalated cells. In addition, there were also cells that were negative for all three proteins after 10 and 15 days of Li treatment (Fig. 7, E and F).

No PCNA-Positive Cells Were in the Inner Medulla After a 4 wk-Recovery Period in Rats Treated with Li

We have previously shown that a 4-wk recovery period following Li treatment for 4 wk reversed the decreased AQP2 expression and the altered cellular profile back to control levels (6). To investigate whether the same was the case for the PCNA labeling, kidney sections from the rats treated with Li for 4 wk followed by a 4-wk Li-free diet (protocol 5) were labeled with anti-PCNA antibody (Fig. 8). The PCNA-positive cells were observed in the IMCD of rats treated with Li for 4 wk (Fig. 8D) as described above, but after the 4-wk recovery period no PCNA-positive cells were observed in the kidney inner medulla (Fig. 8D).

Indications of Apoptosis in the IMCD After 10 or 15 Days of Li Treatment

To investigate whether Li treatment for 4, 10, and 15 days induced apoptosis of cells in the IMCD, we performed immunocytochemistry using an antibody recognizing AIF. AIF is a protein that normally resides in the mitochondria. When apoptosis is induced, AIF is undergoing translocation to the nucleus,
where it causes chromatin condensation and DNA fragmentation (29, 32). Ovaries from adult rat and kidneys from 4-day-old rats were used as control tissues (Fig. 9, A–C). In the ovary, anti-AIF antibody labeled the nuclei of cells in the corpus luteum (Fig. 9, A and B) (2). Nuclear AIF staining was also observed in the kidney inner medulla of 4-day-old rats (Fig. 9C), consistent with previous studies showing apoptosis in the developing kidney medulla, e.g., in the transformation of the thick ascending limb to the ascending thin limb as well in the removal of intercalated cells from the IMCD (12, 13).

In the present study, a few AIF-positive nuclei were observed in the IMCD after 10 and 15 days of Li treatment (Fig. 9, E and F). In contrast, AIF-positive nuclei were not observed in the inner medulla of control rats (Fig. 9D), suggesting that apoptosis in the IMCD may be involved, at least in part, in the changes in cellular composition in Li-induced NDI.

Rats Already Developed Polyuria After 4 Days of Li Treatment

Rats already developed polyuria at day 4 of Li treatment, and the urine output progressively increased at days 10 and 15. The polyuria was associated with a significant decrease in AQP2 protein expression at days 10 and 15, consistent with previously studies demonstrating a downregulation of both AQP2 protein and mRNA levels after 10 days of Li treatment (19, 22).

DISCUSSION

We demonstrated polyuria, downregulation of AQP2, and changes in the cellular profile in the IMCD of rats with Li-induced NDI, which are consistent with previous studies (6, 14, 18, 19, 22). Particularly in this study, we examined the time course of changes in these findings and investigated the cell proliferation in the IMCD as well as the identity of the cells presenting increased mitosis. Consistent with a recent study (6), we confirmed a marked decrease in the fraction of principal cells that exhibited AQP4 labeling and a significant increase in the fraction of intercalated cells that displayed apical H⁺-ATPase labeling in the IMCD. A number of cells in the IMCD, particularly in the proximal part of the inner medulla, exhibited increased mitosis evidenced by positive PCNA staining even in an early phase of Li treatment (day 4). The increased mitosis was also seen in the middle part of the inner medulla in response to prolonged Li treatment (days 10 and 15). Surprisingly, most of the PCNA-positive cells exhibited AQP4 labeling but not H⁺-ATPase labeling. This suggests that Li treatment causes a selective increase in the proliferative rate of the principal cells in the IMCD despite the fact that the fraction of principal cells in the IMCD was markedly decreased compared with the fraction of intercalated cells. Thus these data do not support the hypothesis of a selective proliferation of existing intercalated cells to explain the changes in the cellular composition in response to Li treatment. In contrast, a 4-wk recovery period after 4 wk of Li treatment reversed the enhanced mitotic rate to the control levels in parallel with the previously shown reversal of both the altered cellular profile and the downregulation of AQP2 (6).
Cell Proliferation in IMCD in Response to Li Treatment

We demonstrated that there were no or very few PCNA-positive cells in the inner medulla of control rats under normal conditions, consistent with a previous study revealing that collecting duct cells in the mature kidney divide at a very slow rate (36). In contrast, we demonstrated that Li treatment was associated with an increased PCNA labeling in the initial part of the inner medulla even in an early phase (day 4) and that the number of PCNA-positive cells was markedly increased in the initial and middle part of the inner medulla in response to prolonged Li treatment (10 or 15 days). This finding was consistent with a previous study showing that Li treatment of rats resulted in an increase in the mitotic rate in the collecting duct cells, as shown by [3H]thymidine incorporation (10). They demonstrated that the marked increase in DNA synthesis in the collecting ducts seen after Li treatment was pronounced at the border region between the outer and inner medulla. Thus their finding was compatible with our study revealing that the most marked increase in the number of PCNA-positive cells was observed in the initial and middle parts of the IMCD. Moreover, we previously demonstrated that Li treatment in rats caused a marked increase in the number of intercalated cells in the initial and middle part of the IMCD (6).

Using cDNA microarray analysis of the inner medulla from Li-treated rats, we also recently demonstrated that long-term Li treatment induces a decrease in cyclin-dependent kinase inhibitor p27Kip1 mRNA and protein levels (28). Importantly, immunohistochemistry revealed that decreased p27Kip1 labeling intensity and an increased number of p27Kip1-negative IMCD cells were most pronounced in the initial part and middle part of the inner medulla. Because the cyclin-dependent kinase inhibitor p27Kip1 controls cell proliferation in response to normal mitogenic stimuli, we speculated that a decreased p27Kip1 expression level could contribute to the cell proliferation or cell hypertrophy in response to Li treatment, particularly in the initial and middle part of the inner medulla. This view was further supported by a previous study demonstrating that cell proliferation and apoptosis of tubular epithelial cells were increased in p27 knockout mice following ureteral obstruction (26).

Origin of Increased Density and Cellular Organization of Intercalated Cells in Li-Treated Rats

Prolonged Li treatment is associated with a marked change in the cellular composition and structural organization of the collecting duct. In the proximal part of the inner medulla, there is a dramatic increase in intercalated cells and many intercalated cells are seen adjacent to each other. This was never seen in control rats. There are several possible explanations for the increase in intercalated cells, including 1) increased cell death of principal cells, leaving intercalated cells; 2) increased proliferation of intercalated cells; 3) conversion of principal cells to intercalated cells; 4) detachment of principal cells (rather than cell death), leaving intercalated cells in greater density; 5) intercalated cells deriving from residing or extra renal stem cells.

Regarding the increased death of principal cells, we observed that some collecting duct cells were labeled positive for an AIF after 10 and 15 days of Li treatment. These cells were likely principal cells because double labeling with AIF and H+-ATPase showed no coexpression with these two proteins (not shown). However, it cannot be ruled out that AIF-positive cells were also negative for principal cell markers. However, the results indicate that Li treatment accelerated apoptosis of cells that likely are principal cells. However, this finding probably does not completely explain the decreased fraction of principal cells because several previous studies did not reveal any cellular damage in the inner medulla after prolonged Li treatment. Jakobsen et al. (10) demonstrated no signs of cellular damage in Li-treated rats. Laursen et al. (19) also investigated the ultrastructure of the IMCD cells in the middle part of the inner medulla in rats treated with Li for 4 wk, and they also observed no signs of cellular damage. This study, however, investigated neither cell damage in the proximal part of the inner medulla (IM-1) nor the changes in response to short-term treatment of Li. The AIF antibody recognizes a protein involved in apoptosis and does not detect cells dying from necrosis. Therefore, we could suggest that apoptosis (but not necrosis) in the IMCD may be involved, at least in part, in the changes in cellular composition in Li-induced NDI.

Regarding the increased proliferation of intercalated cells, the increased fraction of intercalated cells cannot completely be explained by the disappearance of principal cells only. There were actually more intercalated cells in Li-treated rats compared with the controls. Moreover, in contrast to the findings seen after 4 wk of Li treatment (6) we also observed that some of the intercalated cells did not contain AE1 in the short-term protocols. This suggests that they were not fully differentiated intercalated cells. It is therefore possible that the increased fraction of intercalated cells in Li-treated rats may also arise from the proliferation of intercalated cells. Although the majority of PCNA-labeled nuclei were observed in principal cells, it might be possible that the proliferation seen in intercalated cells would give rise to the increased fraction of intercalated cells observed after 10 days, 15 days, and 4 wk of Li treatment. However, in Li-treated rats there was an appearance of intercalated cells in the middle part of the inner medulla, but there are no intercalated cells in this area of the inner medulla under normal conditions. Therefore, these intercalated cells cannot have arisen from existing intercalated cells. These findings lead to the third possibility.

Regarding the conversion of principal cells to intercalated cells, intercalated cells may have arisen directly from the differentiation of principal cells to intercalated cells. This would also be consistent with the decreased fraction of principal cells despite the increased proliferation of principal cells. If this is the case, it would be expected that one would see some cells with both AQP4 and H+-ATPase when cell conversion had occurred between principal and intercalated cells. However, the double-labeling results showed that only very few cells from Li-treated rats labeled positive for both AQP4 and H+-ATPase even at an earlier stage during treatment. This finding was consistent with a previous study showing that only very few cells were stained positive for both AQP2 and H+-ATPase after 4 wk of Li treatment (6). Intercconversion could also explain that the situation reverses after cessation of Li; i.e., the intercalated cells convert back to principal cells. It is also possible the principal cells differentiate to intercalated cells after they have undergone mitosis; i.e., they no longer express the principal cell markers but start expressing intercalated cell markers.
The fourth possibility is that principal cells could disappear by detachment from the basement membrane, leaving intercalated cells in greater density. A similar mechanism has been observed in the developing kidney where intercalated cells are removed by extrusion from the epithelium (12). Previous studies have shown that the basolateral extracellular spaces between the principal cell and the basement membrane are consistently more dilated in collecting ducts of Li-treated animals compared with controls (18, 19). There are fewer junctions between the cell and the basement membrane. One could imagine that eventually the contacts between the cell and the basement membrane will disappear and the cell will be extruded to the lumen. In the present study, we did see cells that were poorly attached to the basal membrane, but it is unclear whether this is caused by the fixation.

A fifth possibility is that intercalated cells may arise from stem/progenitor cells. After 10 and 15 days of Li treatment, there were some cells in the collecting duct in the initial part of the inner medulla that were all negative for both PCNA, AQP4, and H+−ATPase; i.e., they were neither proliferating cells nor fully differentiated principal or intercalated cells. These cells were not observed in the control rats and might be a kind of progenitor cells that have entered the collecting duct on Li treatment and possibly differentiated to intercalated cells. There have been several recent studies suggesting that stem cells are residing in the adult kidney. Recently, possible renal progenitor cells that lack the expression of hematopoietic markers but expressed PAX-2, an embryonic renal marker, have been isolated from adult human kidney (5). In another study, 5′-bromo-2′-deoxyuridine administration to rat and mouse pups followed by a 2-mo chase period resulted in the appearance of 5′-bromo-2′-deoxyuridine-retaining cells with stem cell characteristics in papilla of the adult kidney, suggesting that adult kidney stem cells are located in the renal papilla (25). Moreover, Duffield et al. (7) demonstrated that intrinsic tubular cell proliferation could contribute to the restoration of epithelial integrity after renal ischemia in mice and this was not by bone marrow-derived tubular cells. In contrast, several studies also revealed that some bone marrow-derived cells do appear to incorporate into the injured tubule as epithelial cells (8, 11, 20, 27). It could be possible that bone marrow-derived cells may secrete a factor that promotes proliferation of endogenous renal cells as well as promoting the migration of potential renal stem cells from the interstitium into the kidney tubule rather than the bone marrow-derived cells themselves enter the epithelium (15). It has also recently been demonstrated from a mouse knockout model that intercalated and principal cells arise from a common progenitor cell (3). In these mice lacking the transcription factor Foxi1, principal and intercalated cells in the distal nephron were replaced by a single cell type that was positive for both AQP2 (marker for principal cells) and carbonic anhydrase II (marker for intercalated cells). This study also showed that the Foxi1 knockout mice had altered morphology of the cortical collecting duct epithelium, which was possible due to the lack or altered structure of the intercalated cells. These mice lacked cells that were positive for the intercalated cell markers pendrin, AE1, H+−ATPase, and AE4. Furthermore, because Foxi1 was only expressed in intercalated cells and not in principal cells, it suggests that activation of Foxi1 is required for differentiation of precursor cells into intercalated cells. It has also recently been shown that the Foxi1 directly activates the AE4 promoter (17).

However, it should be emphasized that additional investigations are needed to understand the potential differentiation of stem/progenitor cells to the intercalated cells and/or principal cells in response to prolonged Li treatment.

**No Proliferation in Rats Recovered From Lithium-Induced NDI**

There were no proliferating cells in kidneys of rats treated with Li for 4 wk followed by a 4-wk recovery period. We have previously shown that urine output, AQP2 protein levels, as well as the cellular composition are reversed in these animals (6). The level and organization of intercalated cells were the same as in control rats. Although we did not see any proliferating cells in the recovered animals after 4 wk of Li treatment, it is possible that cells proliferated at an earlier time point of the recovery period. A potential proliferation of intercalated cells at an earlier time point may lead to an increased turnover of intercalated cells and increased cell death. There may also be continuous proliferation of principal cells during the recovery period, resulting in an increased number of these cells. Intercalated cells may also disappear by interconverting to principal cells, which would also lead to an increased number of principal cells. Finally, intercalated cells may also disappear by extrusion from the epithelium.

However, the mechanisms behind the removal of intercalated cells in kidneys of rats treated for 4 wk with Li followed by normal diet for 4 wk are still unknown.

**Summary**

We demonstrated the time course of changes in polyuria, downregulation of AQP2, and the cellular profile in the IMCD of rats with Li-induced NDI. We demonstrated that a number of cells in the IMCD (IM-1 and IM-2) exhibited a high proliferative rate in response to Li treatment. Most cells expressing PCNA, which are reflective of a high proliferative rate, were stained with AQP4, a marker for principal cells, but not with the intercalated cell marker H+−ATPase. In contrast, a 4-wk recovery period after 4 wk of Li treatment reversed the enhanced mitotic rate to the control levels in parallel with the previously shown reversal of both the altered cellular profile and the downregulation of AQP2. In conclusion, this study demonstrated that Li treatment progressively induces an increased proliferation of primarily principal cells in the IMCD. This may lead to an increased turnover of principal cells. The increased fraction of intercalated cells may arise from proliferation of the existing intercalated cells as well as differentiation of principal cells to intercalated cells or differentiation of potential stem cells residing in the kidney.

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