Sildenafil reduces polyuria in rats with lithium-induced NDI

Talita Rojas Sanches, Rildo Aparecido Volpini, Maria H. Massola Shimizu, Ana Carolina de Bragança, Fabiola Oshiro-Monreal, Antonio Carlos Seguro, and Lúcia Andrade

Nephrology Department, University of São Paulo School of Medicine, São Paulo, Brazil

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Sildenafil reduces polyuria in rats with lithium-induced NDI. Lithium (Li)-treated patients often develop urinary concentrating defect and polyuria, a condition known as nephrogenic diabetes insipidus (NDI). In a rat model of Li-induced NDI, we studied the effect that sildenafil (Sil), a phosphodiesterase 5 (PDE5) inhibitor, has on renal expression of aquaporin-2 (AQP2), urea transporter UT-A1, Na+/K+2Cl− cotransporter (NKCC2), epithelial Na channel (ENaC; α-, β-, and γ-subunits), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase. We also evaluated cGMP levels in medullary collecting duct cells in suspension. For 4 wk, Wistar rats received Li (40 mmol/kg food) or Li and Sil (Li+Sil). In Li+Sil rats, urine output and free water clearance were markedly lower, whereas urinary osmolality was higher, than in Li rats. The cGMP levels in the suspensions of medullary collecting duct cells were markedly higher in the Li+Sil and Sil groups than in the control and Li groups. Semiquantitative immunoblotting revealed the following: in Li+Sil rats, AQP2 expression was partially normalized, whereas that of UT-A1, γ-ENaC, and eNOS was completely normalized; and expression of NKCC2 and NHE3 was significantly higher in Li rats than in controls. Inulin clearance was normal in all groups. Mean arterial pressure and plasma arginine vasopressin did not differ among the groups. Sil completely reversed the Li-induced increase in renal vascular resistance. We conclude that, in experimental Li-induced NDI, Sil reduces polyuria, increases urinary osmolality, and decreases free water clearance via upregulation of renal AQP2 and UT-A1.

LITHIUM (Li) has been widely used as a pharmacological agent in psychiatric therapy and is the established drug of choice for treating bipolar affective disorders (20, 21, 29, 31). It is eliminated primarily through renal excretion and competes with the Na-K-2Cl cotransporter (NKCC2) and the epithelial sodium channel (ENaC) (12, 13, 39). In addition, Li is transported by Na-K-ATPase (12, 13, 39). Approximately 60% of the filtered load of Li is reabsorbed in the proximal tubule, whereas 20% is reabsorbed in the thick ascending limb, connecting tubule, and cortical collecting duct (12, 13). Nephrogenic diabetes insipidus (NDI), increased urinary excretion of sodium (UVNa), and distal renal tubular acidosis have all been associated with Li treatment (16, 17, 22, 31). In particular, Li-induced NDI is associated with dysregulation of aquaporin-2 (AQP2) and ENaC in the collecting duct (20, 22, 23, 26, 41). Furthermore, reduced urine concentrating ability has been shown to accompany decreased expression of the inner medullary urea transporter UT-A1 in Li-treated rats (2, 18).

The most widely understood pathway leading to AQP2 membrane accumulation is arginine vasopressin (AVP) type 2 receptor stimulation of adenyl cyclase, cAMP-mediated activation of PKA, and AQP2 phosphorylation, the last being necessary for AVP-stimulated membrane accumulation of AQP2 (6, 7, 15, 28). Bouley et al. (4) showed that acute elevation of intracellular cGMP levels induced by nitric oxide (NO), l-arginine, or atrial natriuretic peptide also leads to elevation of intracellular cAMP levels and is therefore independent of stimulation of the AVP type 2 receptor signaling pathway. The authors demonstrated elevated intracellular cGMP levels and greater plasma membrane accumulation of AQP2 in LLC-AQP2 cells exposed to sildenafil citrate (Sil; Viagra), a phosphodiesterase 5 (PDE5) inhibitor (5). These data suggest that cGMP-specific PDE inhibitors induce activation of a parallel cGMP-mediated signal transduction pathway, thereby allowing AQP2 membrane insertion to occur without activation of the AVP type 2 receptor (4, 5). This indicates that pharmacological activation of AVP-independent cGMP signaling pathways can aid in the treatment of some forms of NDI.

We hypothesized that Sil increases AQP2 expression and would thus be useful in treating Li-induced NDI. Therefore, we investigated the effects of Sil on renal expression of UT-A1, AQP2, Na+/K+/H+ exchanger 3 (NHE3), NKCC2, and ENaC (α-, β-, and γ-subunits) in rats with Li-induced NDI.

METHODS

Animals and Experimental Protocol

Male Wistar rats, weighing 150–250 g, were obtained from the animal facility of the University of São Paulo School of Medicine. Experimental NDI was induced by administration of Li, and the rats were then treated or not with Sil. The experimental protocol was approved by the Ethics Committee of the University of São Paulo School of Medicine. During the 4-wk study period, all animals were fed standard rat chow, with a fixed concentration of sodium chloride (0.5%), and were given ad libitum access to tap water. The rats were divided into four groups: control (n = 5), fed standard rat chow for 4 wk; Sil (n = 5), fed standard rat chow for 4 wk plus Sil (200 mg/kg food) for the last 3 wk of that period; Li (n = 9), fed standard rat chow plus Li (40 mmol/kg food) for 4 wk; and Li+Sil (n = 8), fed standard rat chow plus Li (40 mmol/kg food) for 4 wk plus Sil (200 mg/kg food) for the last 3 wk of that period.

We performed a second set of experiments to further measure mean arterial pressure (MAP) and renal vesicle resistance (RVR; via renal blood flow [RBF]), as well as to determine plasma AVP levels.

To determine whether, in Li-treated animals, a higher dose of Sil would have different effects than did the standard dose (200 mg/kg food) for the last 3 wk of that period.

Address for reprint requests and other correspondence: L. Andrade, Nephrology Dept., Univ. of São Paulo School of Medicine, Av. Dr. Arnaldo, 455, 3º andar, sala 3310, CEP 01246–903, São Paulo, Brazil (e-mail: luciacan@usp.br).
food), we also evaluated a group of rats receiving Li and a higher dose of Sil, the Li + Sil-hi group (n = 5), which comprised rats fed standard rat chow plus Li (40 mmol/kg food) for 4 wk and receiving Sil (400 mg/kg food) for the last 3 wk of that period. Initial tests (urine output, urinary osmolality, and water intake) showed no significant differences between the Li + Sil and Li + Sil-hi groups. Therefore, the remaining parameters were not determined in the Li + Sil-hi group.

Metabolic cage studies. Rats were housed one per cage, maintained on a 12:12-h light-dark cycle, and acclimated to the housing conditions for 2 days before the experimental procedures. They were then placed in metabolic cages to collect 24-h urine samples, at baseline and at the end of weeks 1, 2, 3, and 4. At the end of week 4, rats were anesthetized with thiopental sodium (50 mg/kg body wt). To measure MAP, the right or left carotid artery was catheterized with a PE-50 catheter. A median incision was made to measure RBF; the left renal pedicle was carefully dissected, and the renal artery was isolated with care to avoid disturbing the renal nerves. An electromagnetic flow probe was placed around the exposed renal artery, and RBF was measured with an electromagnetic flowmeter (T 106 XM; Transonic Systems, Bethesda, MD). Whole blood was then collected from the carotid artery, and the kidneys were immediately removed. The rats were then euthanized with an overdose of anesthesia. Kidneys were dissected to obtain cortex and medulla samples. Cortex and medulla tissue samples were frozen in liquid nitrogen and stored at −80°C. To calculate RVR, we divided MAP by RBF (results expressed as mmHg·ml⁻¹·min⁻¹).

Clearance studies. To study renal function, inulin clearance studies were conducted, additional groups of rats being submitted to the 4-wk study protocol described above: control (n = 5); Sil (n = 4); Li (n = 4); and Li + Sil (n = 5).

Before the performance of the clearance studies, each animal was anesthetized intraperitoneally with 50 mg/kg body wt of thiopental sodium. The trachea was cannulated with a PE-240 catheter, and spontaneous breathing was maintained. To control MAP and allow blood sampling, a PE-60 catheter was inserted into the right carotid artery. To collect urine samples, a suprapubic incision was made, and the urinary bladder was cannulated with a PE-240 catheter. Following the surgical procedure, a loading dose of inulin (100 mg/kg body wt) was administered through the jugular vein. We started and maintained constant infusion of inulin (10 mg/kg body wt at 0.04 ml/min) throughout the experiment. A total of three urine samples were collected at 30-min intervals. Blood samples were obtained at the beginning and end of the experiment. After the clearance study, the kidneys were flushed with saline and fixed in methacarn solution for immunohistochemical analysis. We determined inulin, in plasma and urine, using the anthrone method.

Analysis of blood and urine. The volume of each 24-h urine sample was measured gravimetrically. Urine samples were centrifuged in aliquots to remove suspended material, and the supernatants were analyzed. We measured urinary and plasma osmolality using a freezing-point osmometer (3D3; Advanced Instruments, Norwood, MA). We used flame photometry to measure serum and urinary levels of sodium and potassium, whereas we used kinetic techniques to measure serum levels of creatinine and urinary levels of urea. We measured serum Li levels using an ion-selective electrode.

Osmolar clearance was calculated by the following formula

\[ C_{\text{osm}} = \frac{U_{\text{osmolality}} \times U_{\text{volume}} / 1440 \text{ min}}{P_{\text{osmolality}}} \]

where \( C_{\text{osm}} \) is osmolar clearance, \( U_{\text{osmolality}} \) is urinary osmolality, \( U_{\text{volume}} / 1440 \text{ min} \) is urine output in microliters per day (1,440 min), and \( P_{\text{osmolality}} \) is plasma osmolality.

Free water clearance was also calculated

\[ C_{\text{H2O}} = U_{\text{volume}} / 1440 \text{ min} - C_{\text{osm}} \]

where \( C_{\text{H2O}} \) is free water clearance.

Plasma AVP. We determined plasma AVP concentrations using enzyme immunoassay kits (Assay Designs, Ann Arbor, MI). All assays were run in duplicate. The kits include recombinant AVP ([Arg⁸]-vasopressin), with which we established a standard curve for calculating AVP concentrations.

**AQP2, urea-, sodium transporter-, PDE5-, eNOS-, and iNOS-specific antibodies.** The peptide-derived polyclonal antibodies specific to NKC2, α-ENaC, and NHE3 were kindly supplied by Dr. Mark Knepper (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). The peptide-derived polyclonal antibody specific to UT-A1 was kindly supplied by Dr. Jeff Sands (Emory University, Atlanta, GA). We obtained the peptide-derived polyclonal antibodies specific to AQP2, PDE5, and actin from Santa Cruz Biotechnology (Santa Cruz, CA); the peptide-derived polyclonal antibody specific to eNOS and iNOS from Transduction Laboratories (Lexington, KY); and the peptide-derived polyclonal antibodies specific to β-ENaC and γ-ENaC from Millipore (Billerica, MA).

Preparation of membrane fractions. Using a Teflon pestle glass homogenizer (Schmidt and Co., Frankfurt am Main, Germany), we homogenized cortex and medulla samples in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, and 41 mM KOH, pH 7.5) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). The tissue samples were frozen in liquid nitrogen and stored at −80°C.

To determine AVP concentrations, serum and urine samples were aliquoted to remove suspended material, and the supernatants were analyzed. We measured serum AVP concentrations using a specific antibody specific to AVP from Millipore (Billerica, MA). The peptide-derived polyclonal antibody specific to AVP was kindly supplied by Dr. Michael Hendriksen (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The kits include recombinant AVP ([Arg⁸]-vasopressin), with which we established a standard curve for calculating AVP concentrations.

**Fig. 1.** Time course of changes in urine output and free water clearance. A: urine output. Rats developed nephrogenic diabetes insipidus (NDI) within 1 wk after the introduction of lithium (Li). Among the rats with Li-induced NDI, those also receiving sildenafil (Sil) presented significantly lower urine output than did those receiving lithium alone. Values are means ± SE. *P < 0.01 vs. control. **P < 0.001 vs. Sil. *P < 0.01 vs. control. *P < 0.01 vs. Li. *P < 0.05 vs. control. *P < 0.01 vs. Sil. *P < 0.001 vs. Li. *P < 0.05 vs. Sil. B: free water clearance at week 4.
Table 1. Physiological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 8)</th>
<th>Sil (n = 10)</th>
<th>Li (n = 13)</th>
<th>Li+Sil (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>355 ± 9.0</td>
<td>339 ± 15.2</td>
<td>284 ± 11.9</td>
<td>315 ± 7.0</td>
</tr>
<tr>
<td>Urine output, ml/day</td>
<td>94 ± 1.8</td>
<td>104 ± 1.7</td>
<td>60 ± 5.7</td>
<td>28.3 ± 3.4</td>
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<tr>
<td>Water intake, ml/day</td>
<td>12 ± 1.2</td>
<td>13.4 ± 1.9</td>
<td>59.6 ± 3.3</td>
<td>29.2 ± 2.6</td>
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<tr>
<td>Food intake, g/day</td>
<td>28 ± 1.5</td>
<td>31.4 ± 2.3</td>
<td>29.3 ± 1.7</td>
<td>29 ± 1.7</td>
</tr>
<tr>
<td>Uosm, mosmol/kg H2O</td>
<td>1098 ± 212</td>
<td>893 ± 107.5</td>
<td>212 ± 13.4</td>
<td>301 ± 9.5</td>
</tr>
<tr>
<td>C18:2, µL/min</td>
<td>−15 ± 1.4</td>
<td>−14.6 ± 2.9</td>
<td>9.1 ± 3.1</td>
<td>−0.7 ± 0.7</td>
</tr>
<tr>
<td>UVNa, meq/day</td>
<td>0.37 ± 0.04</td>
<td>0.9 ± 0.2</td>
<td>0.75 ± 0.15</td>
<td>0.38 ± 0.09</td>
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<tr>
<td>UVR, meq/day</td>
<td>1.14 ± 0.13</td>
<td>1.0 ± 0.17</td>
<td>0.71 ± 0.11</td>
<td>0.6 ± 0.13</td>
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<tr>
<td>UUrea, mg/day</td>
<td>221 ± 47</td>
<td>250 ± 37</td>
<td>368 ± 41.4</td>
<td>196 ± 35</td>
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<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.29 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Serum lithium, meq/l</td>
<td>0.3 ± 0.05</td>
<td>0.26 ± 0.04</td>
<td>0.26 ± 0.04</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Serum sodium, meq/l</td>
<td>144 ± 2.0</td>
<td>142 ± 2.8</td>
<td>149 ± 2.7</td>
<td>146 ± 1.0</td>
</tr>
<tr>
<td>Serum potassium, meq/l</td>
<td>3.6 ± 0.11</td>
<td>3.7 ± 0.1</td>
<td>3.4 ± 0.1</td>
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</tr>
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</table>

Values are means ± SE. *Sil, sildenafil; Li, lithium; Uosm, urine osmolality; C18:2, free water clearance; UVNa, 24-h urinary excretion of sodium; UVR, 24-h urinary excretion of potassium; UVUrea, 24-h urinary excretion of urea; UVNa, 24-h urinary excretion of sodium; UVK, 24-h urinary excretion of potassium; UVurea, 24-h urinary excretion of urea.*
P < 0.05 vs. control. *P < 0.01 vs. control. *P < 0.001 vs. control. +P < 0.05 vs. Sil. +P < 0.01 vs. Sil. +P < 0.001 vs. Sil. +P < 0.001 vs. control.

Table 2. Hemodynamic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 8)</th>
<th>Sil (n = 10)</th>
<th>Li (n = 13)</th>
<th>Li+Sil (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>129 ± 5.3</td>
<td>128 ± 5.4</td>
<td>131 ± 6.0</td>
<td>129 ± 3.4</td>
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<tr>
<td>RVR, mmHg·ml⁻¹·min⁻¹</td>
<td>19.3 ± 0.75</td>
<td>20.8 ± 1.1</td>
<td>23.6 ± 0.7</td>
<td>20.2 ± 1.0</td>
</tr>
<tr>
<td>RBF, ml/min</td>
<td>6.3 ± 0.09</td>
<td>6.1 ± 0.13</td>
<td>5.5 ± 0.12</td>
<td>5.8 ± 0.09</td>
</tr>
<tr>
<td>AVP, pg/ml</td>
<td>23 ± 2.2</td>
<td>23.3 ± 2.7</td>
<td>27.5 ± 4.5</td>
<td>26.7 ± 3.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. *MAP, mean arterial pressure; RVR, renal vascular resistance; RBF, renal blood flow; AVP, arginine vasopressin. *P < 0.05 vs. control. *P < 0.01 vs. Sil. *P < 0.001 vs. Li+Sil. +P < 0.01 vs. Sil.
The suspensions were treated or not as follows: those from rats fed NaH$_2$PO$_4$, and 5.0 mM d-glucose, and finely minced. Subsequently, analysis of samples from control, Li, Li/H11001, and Sil rats markedly decreases urine output, free water clearance, and water intake in Li-treated rats. The Li rats gained less weight than did the control and Sil rats. However, this condition was ameliorated in Li+Sil rats (Table 1). Urine output was significantly higher in Li rats than in Li+Sil rats (Fig. 1A; Table 1). Although urine output was 50% lower in Li+Sil rats than in Li rats, it was significantly higher in Li+Sil rats than in control and Sil rats. As expected, polyuria and water intake were significantly greater in Li rats than in control and Sil rats. In Li+Sil rats, free water clearance was significantly lower than it was in Li rats (Table 1; Fig. 1B), although it remained significantly higher than that seen in the control and Sil rats, both of which presented free water reabsorption (Table 1; Fig. 1B). The marked increase in urine output in Li rats was accompanied by decreased urinary osmolality, which was significantly lower in Li rats than in control and Sil rats. Li+Sil rats, urinary osmolality increased from week 1 to week 4 (220 ± 25 vs. 301 ± 9.5 mosmol/kgH$_2$O; $P = 0.013$, paired $t$-test) and was significantly higher than that observed in Li rats ($P = 0.0002$, Mann-Whitney test). However, the ANOVA revealed no statistical differences among groups in terms of urinary osmolality or food intake (Table 1). Although urinary osmolality was higher in the Li+Sil-hi rats than in the Li+Sil rats (404 ± 54.3 vs. 301 ± 9.5 mosmol/kgH$_2$O), the difference was not statistically significant. 

**RESULTS**

**Li induces NDI.** During the 4-wk study period, all animals received standard rat chow with a fixed concentration of sodium chloride and were given ad libitum access to tap water. Animals receiving Li developed NDI within 1 wk. At the end of week 1, urine output was significantly higher in Li and Li+Sil rats than in control and Sil rats (Fig. 1A). In Li rats, NDI persisted throughout the study period (Fig. 1A; Table 1).

Intracellular cGMP accumulation assays in triplicate.

**Statistical analysis.** Quantitative data are expressed as means ± SE. Differences among the means of multiple parameters were analyzed by ANOVA followed by the Student-Newman-Keuls test. Differences between parameters were analyzed by an unpaired $t$-test or by nonparametric methods (Mann-Whitney test). Values of $P < 0.05$ were considered statistically significant.

**Table 3. Quantitation of immunoblotting of membrane fractions prepared from kidney medulla samples**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control (n = 3)</th>
<th>Sil (n = 4)</th>
<th>Li (n = 4)</th>
<th>Li+Sil (n = 4)</th>
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</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>105 ± 3.5</td>
<td>120.3 ± 2.0$^{a,b,d}$</td>
<td>31.2 ± 6.6$^{c,d}$</td>
<td>60.7 ± 2.2$^{c}$</td>
</tr>
<tr>
<td>Intracellular vesicle</td>
<td>103 ± 3.5</td>
<td>109 ± 5.1</td>
<td>81.5 ± 14</td>
<td>94.4 ± 2.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. Shown is a densitometric analysis of all samples from control, Li, Li+Sil, and Sil rats. *$P < 0.05$ vs. control. $^{a}P < 0.001$ vs. control. $^{b}P < 0.001$ vs. Li. $^{c}P < 0.001$ vs. Sil. $^{d}P < 0.001$ vs. Li+Sil.
was there any significant difference between the Li+Sil-hi and Li+Sil rats in terms of water intake (28.6 ± 5.4 vs. 29.2 ± 2.6 ml/day) or urine output (21.8 ± 5.6 vs. 28.3 ± 3.4 ml/day).

Sil has no effect on renal Li clearance. To rule out any influence of Sil treatment on renal Li clearance, we measured serum Li levels, and we found no significant difference between Li rats and Li+Sil rats (Table 1).

Sil has no effect on renal function. To address the question of whether the decrease in urine output observed in Li+Sil rats was due to a decrease in the glomerular filtration rate (GFR), we measured inulin clearance (the gold standard for determining GFR). We found no significant difference among the groups (control: 0.86 ± 0.05; Sil: 0.69 ± 0.03; Li: 0.94 ± 0.04; and Li+Sil: 0.83 ± 0.06 ml·min⁻¹·100 g body wt⁻¹). Serum creatinine levels were comparable among the groups.

Sil does not alter blood pressure or AVP levels in Li-treated rats. To assess whether the marked decreases in urine output and free water clearance, as well as the increase in urinary osmolality, observed in Li+Sil rats could be a result of Sil-induced systemic vasodilatation, we measured MAP, RVR, RBF, and plasma AVP. As can be seen in Table 2, there were no differences in terms of MAP. Although mean RVR was higher in the Li group than in any of the other groups, it normalized completely after Sil treatment. Mean RBF was

Fig. 4. Immunostaining for AQP2 in kidney medulla samples. Samples are from control (A), Li (B), Li+Sil (C), and Sil (D) rats (magnification ×40). Note that the AQP2 staining is less intense in the Li group sample than in the control and Sil group samples, as well as being more intense in the Li+Sil group sample than in the Li group sample. Also shown is immunostaining for AQP2 in kidney medulla samples from control (E), Li (F), Li+Sil (G), and Sil (H) rats (magnification ×100). Note that plasma membrane staining for AQP2 is less intense in the Li group sample than in the control and Sil group samples, as well as being more intense in the Li+Sil group sample than in the Li group sample.
In Li rats presented decreased expression of UT-A1. Levels of UT-A1 were comparable to that observed in controls (99.7 ± 0.3%; NS), although different from that observed in Sil rats (108.3 ± 4.7%; P < 0.05). There was no significant difference between Sil and control rats in terms of UT-A1 expression (Fig. 6).

Sil does not reverse Li-induced changes in renal cortex abundance of NHE3. Semiquantitative immunoblotting revealed that renal cortex expression of NHE3 was significantly lower in Li rats than in control, Li+Sil, and Sil rats. Treatment with Sil partially restored RBF in Li rats. Mean plasma AVP levels did not differ among the groups.

Sil treatment does not alter serum or urinary levels of sodium and potassium but decreases urinary excretion of urea. No significant differences were observed among the groups in terms of serum sodium, serum potassium, UVNa, or UVK. Although UVurea was higher in Li rats than in controls, it was significantly lower in Li+Sil rats than in those from Li rats (163 ± 3.3 and 151 ± 4.3 vs. 163 ± 3.3%; NS). Expression of NKCC2 was significantly higher in Li and Li+Sil rats than in control and Sil rats (163 ± 3.3 and 151 ± 4.3 vs. 100 ± 0.88 and 93 ± 3.3%, respectively; P < 0.001 for both). In Sil rats, NKCC2 expression did not differ significantly from that observed for the controls.

Sil reverses Li-induced downregulation of UT-A1. In Li+Sil rats, UT-A1 expression was higher than that seen in Li rats (92 ± 0.4 vs. 50.2 ± 3.3%; P < 0.001), and UT-A1 expression was comparable to that observed in controls (99.7 ± 0.3%; NS), although different from that observed in Sil rats (108.3 ± 4.7%; P < 0.05). There was no significant difference between Sil and control rats in terms of UT-A1 expression (Fig. 6).

Sil does not reverse Li-induced changes in renal cortex abundance of NHE3. Semiquantitative immunoblotting revealed that renal cortex expression of NHE3 was significantly lower in Li rats than in control, Li+Sil, and Sil rats. Treatment with Sil partially restored RBF in Li rats. Mean plasma AVP levels did not differ among the groups.

Sil treatment does not alter serum or urinary levels of sodium and potassium but decreases urinary excretion of urea. No significant differences were observed among the groups in terms of serum sodium, serum potassium, UVNa, or UVK. Although UVurea was higher in Li rats than in controls, it was significantly lower in Li+Sil rats than in Li rats (163 ± 3.3 and 151 ± 4.3 vs. 60 ± 0.88 and 93 ± 3.3%, respectively; P < 0.001 for both). In Sil rats, NKCC2 expression did not differ significantly from that observed for the controls.
higher in Li-treated rats than in controls (134 ± 5.5 vs. 99.3 ± 0.7%; P < 0.001). In addition, NHE3 expression was higher in Li rats than in Sil rats (134 ± 5.5 vs. 98 ± 4.2%; P < 0.001), although there was no significant difference between Li+Sil rats and Li rats (138 ± 1.4 vs. 134 ± 5.5%; NS). However, NHE3 expression was significantly higher in Li+Sil rats than in the control and Sil group samples (P < 0.001), although there was no significant difference between Sil and control rats (98 ± 4.2 vs. 99.3 ± 0.7%; NS).

Sil reverses Li-induced downregulation of γ-ENaC. Neither Li alone nor the Li-Sil combination has any effect on renal cortex abundance of α-ENaC or β-ENaC. In Li rats, α-ENaC expression in the renal cortex remained stable (105 ± 2.7%) and was comparable to that observed for control, Sil, and Li+Sil rats (99.3 ± 0.67, 102.3 ± 3.8, and 105 ± 3.2%, respectively; NS, Fig. 8A). We also evaluated β-ENaC and γ-ENaC protein expression in the renal cortex. Abundance of β-ENaC was unaffected across the groups (control: 99.3 ± 0.8, Sil: 98 ± 1.4, Li: 95.7 ± 2.2, and Li+Sil: 97.0 ± 1.3; NS, Fig. 8B). Expression of γ-ENaC was significantly lower in Li rats than in control, Sil, and Li+Sil rats (34.3 ± 9.2 vs. 99.2 ± 0.54, 99.6 ± 1.0, and 97.7 ± 1.2%, respectively; P < 0.001.

Fig. 7. Immunostaining for UT-A1 in kidney medulla samples. Shown are samples from control (A), Li (B), Li+Sil (C), and Sil (D) rats (magnification ×40). Also shown is immunostaining for UT-A1 in kidney medulla samples from control (E), Li (F), Li+Sil (G), and Sil (H) rats (magnification ×100). Note that plasma membrane staining for UT-A1 is less intense in the Li group sample than in the control and Sil group samples, as well as being more intense in the Li+Sil group sample than in the Li group sample.
for all). There were no significant differences among the control, Sil, and Li+Sil groups in terms of γ-ENaC expression (Fig. 8C).

**PDE5 is expressed in the renal medulla.** Expression of PDE5 in the renal medulla was comparable among the groups (control: 100 ± 2.2, Sil: 105 ± 8.5, Li: 91.4 ± 5.2, and Li+Sil: 96 ± 1.3%; NS, Fig. 9). Each of the two subunits of PDE5 possesses a catalytic domain and a regulatory domain, the former being the target of PDE5 inhibitors. It should be borne in mind that we measured only the protein abundance of PDE5 (i.e., PDE5 activity was not measured).

**Sil increases cGMP levels in medullary collecting duct cell suspensions.** Investigating the effect that incubation with Sil had on intracellular cGMP levels in medullary collecting duct cell suspensions, we found that the mean cGMP level (expressed in fmol/g of papilla) was higher in the Li+Sil-susp group than in the control-susp group and Li-susp group (6,402 ± 1,162 vs. 1,790 ± 262 and 1,890 ± 302, respectively; P < 0.001 for both). Although not significantly different from the Li+Sil-susp group value, the mean cGMP level observed for the Sil-susp group (5,012 ± 699) differed significantly from the control-susp group value (P < 0.01). In addition, the mean cGMP level was significantly higher in the Sil-susp group than in the Li-susp group (P < 0.01, Fig. 10).

**DISCUSSION**

Chronic Li treatment is known to be a major cause of NDI (20, 23, 31), and downregulation of AQP2 has been associated with the development of severe polyuria (20, 23, 31). Here, we have demonstrated that, in rats with Li-induced NDI, chronic Sil treatment upregulates expression of AQP2 and UT-A1.

In the basolateral membrane of collecting duct principal cells, AVP binds to the AVP type 2 receptors (6, 19, 27, 28). Acting through the GTP-binding protein Gs, the interaction between AVP and the AVP type 2 receptor activates adenylyl cyclase, accelerating the production of cAMP by ATP (6, 19, 28, 38). Subsequently, cAMP binds to the regulatory subunit of PKA, resulting in dissociation of the regulatory subunit from the catalytic subunit. This activates the catalytic subunit, phosphoraying AQP2 (9, 27, 28), which is then translocated from intracellular vesicles to the plasma membrane (6, 9, 27, 28). It has been shown that Li inhibits AVP-induced adenylyl cyclase activity and cAMP levels in the medullary collecting duct (8), and this inhibition is the likely cause of the reduced expression of AVP-regulated AQP2 (8). However, it has also been shown that Li inhibits such activity by reducing AQP2 mRNA (likely through decreased gene transcription), and that Li-induced AQP2 degradation and NDI occur independently of principal cell adenylyl cyclase activity (3, 22). In addition, Li alters neither AVP-stimulated cAMP production nor PKA-dependent phosphorylation of AQP2 and cAMP-responsive element-binding protein (22).

In 2000, Bouley et al. (4) identified another pathway of AQP2 plasma membrane insertion. The authors described a cAMP-independent, cGMP-dependent pathway for AQP2 membrane insertion in renal epithelial cells. This suggests that AQP2 phosphorylation at S256 is required for cGMP-stimulated AQP2 trafficking, although it remains unknown whether the final phosphorylation step is through PKA or through cGMP-activated PKG (4, 5, 10). In our study, we were unable to measure S256-phosphorylated AQP2. Although we demonstrated that plasma membrane AQP2 expression was higher in Li+Sil rats than in Li rats, we were also unable to determine whether the final phosphorylation step is through PKA or PKG.

It has also been shown that AQP2 accumulates in the plasma membrane of collecting duct principal cells and LLC-AQP2 cells upon exposure to the selective PDE5 inhibitor Sil, which also inhibits cGMP degradation (5). In cultured cells, this occurs with no detectable increase in cAMP (4). It is unknown whether the Sil effect is due to increased AQP2 exocytosis, decreased AQP2 endocytosis, or both (4). In our study, Sil administration in Li-treated rats decreased urine output and increased urinary osmolality, as well as increasing AQP2 expression. Therefore, Sil treatment might represent a novel therapeutic approach for patients with Li-induced NDI. The exact mechanism by which Sil increases AQP abundance and trafficking in Li treated rats remains unknown.

We also investigated the question of whether UT-A1 expression is affected by Sil treatment in Li-induced NDI. Chronic Li administration is a model of NDI in which UT-A1 expression decreases (2, 18), as confirmed in the present study. Acting through PKA, AVP increases the UT-A1 phosphorylation in inner medullary collecting duct cell suspensions (1, 35, 36, 37, 42). It has been demonstrated that UT-A1 sensitivity to AVP-induced phosphorylation is lower in Li-treated rats (18). In the present study, Sil treatment significantly increased UT-A1 expression in the medulla, despite ongoing Li therapy. The increased collecting duct water reabsorption after Sil treatment would increase urea
significant, there was a 50% decrease in UVNa in the Li
ence among the groups in terms of UVNa. However, although not
model, as expected, there was no statistically significant differ-
sion, which was completely normalized by Sil treatment. In our
transporter in the thick ascending limb. Expression of
increased, as was expression of NKCC2, the major apical sodium
which could be prevented by the NO precursor L-arginine (34).
expression in the inner medullary collecting ducts, an effect that
is mediated by cGMP (25, 30). Further studies are needed to
identify the exact mechanism by which Sil increases UT-A1
expression in Li-treated rats.

Chronic Li treatment has been associated with a significant
increase in UVNa in rats (20, 23), especially in a model of
high-dose Li treatment (60 mmol/kg food) (20). Even in rat
models of low-dose Li treatment (40 mmol/kg food), UVNa has
been shown to increase, albeit to a lesser degree than in the
high-dose model (20). In Li and Li + Sil rats, expression of NHE3,
the major apical sodium transporter in the proximal tubule, was
increased, as was expression of NKCC2, the major apical sodium
transporter in the thick ascending limb. Expression of α- and
β-ENaC was not affected by Li treatment, with or without Sil. In
Li-treated rats, there was a decrease in γ-ENaC protein expres-
sion, which was completely normalized by Sil treatment. In our
model, as expected, there was no statistically significant differ-
ence among the groups in terms of UVNa. However, although not
significant, there was a 50% decrease in UVNa in the Li + Sil
group. Further studies, in a high-dose model with a fixed sodium
intake, are needed to elucidate the role that Sil plays in sodium
channel dysregulation.

The levels of cGMP were increased in the Sil and Li + Sil
groups. However, in the Sil group rats, urine output, UT-A1
expression, and expression of sodium transporters did not differ
from the values obtained for the rats in the control group (in which
there was no increase in cGMP levels). One interpretation of these
results is that the lack of any apparent Sil-induced effect on urine
output, UT-A1 expression, or sodium transporter expression in the
Sil group is related not to PDE5 inhibition in the collecting ducts
but rather to systemic PDE5 inhibition that affects a circulating
factor and thus modifies protein expression in the collecting duct,
independently of cGMP levels. However, it should be borne in
mind that the animals were studied after having received Sil for 3
wk, which translates to chronic treatment. In addition, when Sil is
given to normal rats, the mechanisms at work might be quite
different from those activated when Sil is given to animals under
treatment with Li. Furthermore, the increased cGMP levels in the
medullary collecting duct tubules after Sil administration was an
in vitro finding and should therefore be interpreted with caution.

Body weight, which is a measure of extracellular volume expan-
sion, was not significantly higher in the Sil group than in the other
groups; nor did the Sil group rats show any decrease in urine
output or increase in urinary osmolality, although AQP2 expres-
sion was higher in this group. We hypothesized that these differ-
ces (no increase in body weight; no increase in urine output, no
increase in urinary osmolality) were due to the fact that our
animals received Sil for 3 wk, with the following consequences:
extracellular volume expansion and altered sodium balance (al-
though not significant, there was a tendency toward higher UVNas
and lower serum levels of sodium compared with the control
group). In response to the extracellular volume expansion, sodium
is excreted in urine (the mechanism of sodium balance). Hence,
the characteristic findings in a steady state are a discrete increase
in UVNa and a discrete decrease in serum levels of sodium. It is
likely that (after 3 wk of treatment with Sil) our animals had
reached a steady state in terms of sodium and water balance.

In Li-treated rats, RVR was increased but was completely
normalized by Sil treatment. We also demonstrated decreased
eNOS expression in Li-treated rats. We hypothesized that this
decrease was responsible for the increase in RVR in those rats.
The phenotypes of mice lacking all three NO synthases are
polyuria, polydipsia, and renal unresponsiveness to AVP, char-
acteristics consistent with NDI (40). In our study, Sil treatment also
completely restored eNOS expression in Li-treated rats.

To date, there have been no studies demonstrating decreased
eNOS expression in this model of Li-induced NDI. However,
some studies have reported that the erectile dysfunction seen in
patients receiving Li can be attributed to Li blockade of the NO
pathway, as demonstrated in endothelium-mediated relaxation of
rat corpus cavernosum (34). Chronic Li treatment might impair
the NO-mediated neurogenic relaxation of rat corpus cavernosum,
which could be prevented by the NO precursor L-arginine (34).
Although Sil has been used in the treatment of erectile dysfunction
(24), it is currently also used for the management of pulmonary
hypertension (11, 14). Rostaing et al. (33) showed that, in kidney
transplant recipients, GFR (inulin clearance) increases within 120
min after Sil administration. Rodriguez-Iturbe et al. (32) demon-
strated that early treatment with Sil slows the progression of renal
damage.

In conclusion, using Sil to treat Li-induced NDI reduces
polyuria, increases urinary osmolality, and decreases free water
clearance via upregulation of renal AQP2 and UT-A1, as well as
normalizing RVR and eNOS expression. A therapeutic
approach that includes Sil could have significant clinical im-
plications in patients suffering from Li-induced NDI.

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Fig. 10. Determination of cGMP levels in suspensions of medullary collecting
duct tubules. Differences among the means were analyzed by ANOVA
followed by the Student-Newman-Keuls test.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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