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Lithium treatment inhibits renal GSK-3 activity and promotes cyclooxygenase 2-dependent polyuria

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Lithium, a metallic monovalent cation, has been used therapeutically for more than 150 years and remains an important pharmacotherapeutic agent for treatment of bipolar disorder and Alzheimer’s disease (10, 24, 34). It is estimated that ~1 of every 1,000 individuals in the population is on lithium treatment (33). The use of lithium is frequently complicated by impaired renal water reabsorption, resulting in nephrogenic diabetes insipidus (NDI) (3). The mechanism by which lithium induces diabetes insipidus is incompletely understood. Sugawara et al. (31) reported that after LiCl treatment, rats exhibit increased urinary PGE2 excretion. They also found the nonselective cyclooxygenase-inhibiting NSAID indomethacin reduced lithium-induced PGE2 excretion and polyuria, suggesting that a diuretic cyclooxygenase product may be involved in lithium-associated diabetes insipidus. COX2, an inducible isoform of cyclooxygenase, is expressed in the kidney together with COX1, a constitutively expressed isoform. Furthermore, recent studies demonstrate that lithium can increase COX2 in cultured cells via inhibition of glycogen synthase kinase-3 (GSK-3) (27). The present studies were designed to test the possibility that COX2 activity rather than COX1 contributes to lithium-induced polyuria and determine whether inhibition of GSK-3β is involved.

METHODS

Experimental animals and treatment. Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and individually housed in metabolic cages specially designed for mice. Mice were allowed free access to water and standard rodent chow placed in containers outside the cage to avoid urine contamination. To minimize stress related to the new environment, mice were housed in metabolic cages for at least 3 days before initiation of the studies.

Eighteen mice were divided into three experimental groups consisting of six mice each. Two groups were administered LiCl (4 mmol/kg body wt ip) daily for 16 days. On day 5 of LiCl injection, one of the groups was provided with a COX2-specific inhibitor SC-58236, 6 μg/ml (22) in the drinking water. Another group was provided with vehicle. The third group was injected with saline and used as a control. Daily water intake and urine output were monitored.

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and COX1 was analyzed by immunoblotting. Distribution of COX2 within the kidney was examined by immunohistochemistry. The COX1 homozygous null mice (COX1−/−) were provided by Dr. R. Langenbach and maintained as described (22). A total of seven COX1−/− mice were housed individually in metabolic cages and treated with LiCl for 5 days as described before for C57BL/6J mice. Four of these mice were concurrently treated with MF-tricyclic (another COX2 inhibitor) (21), beginning on day 1 of a 5-day LiCl treatment. The remaining three mice were treated with vehicle. Urine volume, urinary osmolality, and urine PGE$_2$ were determined. All protocols were approved through the institutional animal care and use committee of Vanderbilt University.

**Isolation of microsomes.** Since COX2 is mainly localized to the endoplasmic reticulum-associated plasma membrane (30), microsomes were isolated to determine COX2 protein expression as previously reported (36). Briefly, kidneys were homogenized in buffer (30 mM Tris, pH 8, 1 mM EDTA, and 2 mM EGTA) using a homogenizer (PowerGen 700, Fisher Scientific) and then centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was spun again at 150,000 g for 60 min. The microsomal pellet was resuspended in RIPA buffer [1% Triton X-100, 0.5% deoxycholate, 1% SDS, 150 mM NaCl, 50 mM Tris·HCl (pH 8), 2 mM EDTA, and 1 mM sodium orthovanadate], and protein levels were measured with subsequent analysis by immunoblotting for COX2 and COX1 protein expression.

**Cell culture.** Primary cultures of medullary interstitial cells from C57BL/6J mice were established as reported (12). Cells were cultured in DMEM supplemented with 10% (vol/vol) FBS at 37°C in 95% air-5% CO$_2$. Subconfluent cultures in passages 10–15 were used for the study.

**Immunoblotting.** Cultured renal medullary interstitial cells (RMICs) were washed with PBS and lysed in RIPA buffer followed by sonication for 12 s. Protein concentration of kidney microsomes and whole cell lysate was determined using a biocinchonic acid protein assay (Sigma, St. Louis, MO). Thirty micrograms of protein extract in SDS Laemmli buffer were loaded in each lane of a 10% SDS-PAGE minigel and separated at 120 V. Proteins were transferred to a nitrocellulose membrane at 22 V overnight at 4°C. The membrane was washed three times with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and then incubated in blocking buffer (150 mM NaCl, 50 mM Tris, 0.05% Tween 20, and 5% Carnation nonfat dry milk, pH 7.5) for 1 h at room temperature. The membrane was then incubated with anti-GSK-3 (1:2500, BD Transduction Laboratories), anti-pGSK (ser9) (1:1000, Cell Signaling), anti-COX1 (1:300, Santa Cruz Biotechnology), or anti-COX2 (1:1000, Cayman Chemicals, Ann Arbor, MI) antibodies in blocking buffer overnight at 4°C. After being washed three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000 for anti-mouse and 1:10,000 for anti-rabbit) for 1 h at room temperature, followed by three 15-min washings. Antibody labeling was visualized by addition of chemiluminescence reagent (Renaissance, DuPont-New England Nuclear, Boston, MA), and the membrane was exposed to Kodak XAR-5 film and developed.

**Immunohistochemistry.** In general, at the termination of an experiment, one kidney was removed from each rat for Western blot analysis, and the other was perfused with fixative for histological analysis. Under deep anesthesia with Nembutal (70 mg/kg ip), rats were exsanguinated with 50 ml/100 g heparinized saline (0.9% NaCl, 2 units heparin, 0.02% sodium nitrite) through a transthoracic aortotomy cannula and fixed with 3.7% formaldehyde in an acidic solution (pH 4.5) containing phosphate, periodate, acetate, and sodium chloride as described (25). The acidified aldehyde fixatives were crucial for reliable preservation of inner medulla/papillary structure and COX2 antigenicity. The fixed kidney was dehydrated through a graded series of ethanol, embedded in paraffin, sectioned (4 μm), and mounted on glass slides. Internal controls and comparisons were facilitated by creating compound blocks with multiple specimens that were sectioned and stained together. COX2 was immunolocalized with affin-
3.22 ± 0.24 (lithium alone) to 1.85 ± 0.52 ml (lithium+SC-58236-treated mice). Thus, while SC-58236 blunted lithium-induced polyuria, it did not completely restore urine volume to control values (P < 0.001, SC-58236 vs. vehicle-treated control group) (Fig. 1A).

Urinary osmolality also decreased following LiCl treatment (P < 0.001 vs. vehicle control) (Fig. 1B) from 4,562 ± 230 mosmol/kgH2O before LiCl to 3,341 ± 737 and 1,476 ± 135 mosmol/kgH2O by days 4 and 8 of LiCl injection, respectively. Coadministration of SC-58236 with LiCl increased urine osmolality compared with mice treated with LiCl alone (P < 0.001). For instance, on day 10 of lithium treatment, urine osmolality was 1,378 ± 402 mosmol/kgH2O in LiCl-treated mice vs. 2,359 ± 492 mosmol/kgH2O in mice treated with SC-58236+LiCl. Despite this increase, urine osmolality in COX2 inhibitor-treated mice remained significantly lower compared with vehicle-treated mice (P < 0.001).

Lithium increases COX2-derived urine PGE2 excretion. Since PGE2 is an endogenous diuretic autacoid derived from COX1 or COX2, urinary PGE2 excretion following LiCl treatment was measured in the presence or absence of a COX2 inhibitor. LiCl treatment increased urinary PGE2 excretion from 2.96 ± 0.08 (day 0) to 26.7 ± 0.2 pg/24 h by day 4 (P < 0.0001). Urine PGE2 was also significantly higher in lithium-treated mice than in saline-injected mice (2.98 ± 0.7 pg/24 h, P < 0.0001) (Fig. 2). The increase in urinary PGE2 associated
with LiCl treatment temporally correlated with increased COX2 protein expression (see below). Furthermore, this lithium-associated increase in PGE2 excretion was halved when mice were treated with the COX2 inhibitor SC-58236 ($P < 0.0001$ by repeated-measures ANOVA). On day 10, the urine PGE2 excretion rate was 39.57 ± 2.1 (LiCl alone) vs. 19.97 ± 3.4 pg/24 h (LiCl+SC-58236).

Effect of lithium treatment on COX1-deficient mice. To more definitively exclude a role of COX1 in lithium-induced polyuria, the effect of LiCl on mice deficient in COX1 was examined. LiCl increased urinary PGE2 excretion by sixfold above basal levels in COX1-deficient mice (14 ± 6 on day 0 to 88 ± 15 pg/24 h on day 5, $P < 0.0001$). Importantly, when COX1−/− mice were treated with a COX2 inhibitor (MFtricyclic), lithium failed to increase urinary PGE2 levels (13 ± 4 pg/24 h) (Fig. 3C). Furthermore, while LiCl treatment also increased urine output in COX1-deficient mice from 0.75 ± 0.1 ml on day 0 to 3.2 ± 0.4 ml by day 5 ($P < 0.01$), coadministration of LiCl with the COX2 inhibitor dramatically suppressed lithium-induced diuresis. Following 5 days of SC-58236 treatment, urine output was only 1 ± 0.1 vs. 3.2 ± 0.04 ml with LiCl alone ($P < 0.01$) (Fig. 3A). Similarly, while lithium decreased the urine osmolality significantly in the COX1-deficient mice (4,287 ± 414 on day 0 vs. 1,231 ± 176 mosmol/kgH2O on day 5, $P < 0.001$), urinary osmolality of the COX2 inhibitor-treated COX1−/− mice remained unchanged from control values and were significantly greater than with lithium alone (3,569 ± 213 mosmol/kgH2O at day 5 with lithium+COX2 inhibitor vs. 1,231 ± 176 mosmol/kgH2O with LiCl alone) ($P < 0.0005$) (Fig. 3B).

Lithium increases COX2 expression in RMICs. LiCl also increased COX2 protein expression on immunoblots of kidney microsomes isolated from mice killed on days 3, 4, 5, or 16 of LiCl treatment. Renal COX2 expression increased beginning on day 4 (the same day that urine volume increased) and remained elevated throughout the period of LiCl-associated polyuria (day 16) (Fig. 4A). COX1 levels remained unchanged. To further examine whether LiCl directly induced COX2 expression, mouse RMICs were cultured. As previously observed in rabbit RMICs, LiCl treatment (30 mM) markedly induced COX2 protein expression in cultured mouse RMICs (Fig. 4B), consistent with a direct effect of LiCl on interstitial cell COX2 expression.

It has been reported that lithium can elevate circulating antidiuretic hormone (ADH) levels, which can lead to increased urinary PGE2 excretion (31). To further determine whether increased medullary COX2 expression is caused by a direct cellular effect of LiCl rather than an effect secondary to
altered ADH levels, rats with a hereditary ADH deficiency (Brattleboro rats) were studied. LiCl treatment significantly increased renal medullary COX2 protein expression compared with vehicle treatment in Brattleboro rats (Fig. 5). Because the Brattleboro rats are already in a state of maximum polyuria, no further increase in urine volume was observed in these rats during lithium treatment. From this we infer that lithium can stimulate COX2 expression, independently of changes in circulating ADH or as a consequence of altered urine volume. Immunohistochemical staining of kidneys from LiCl-injected rats showed perinuclear COX2 staining in medullary interstitial cells. COX2 immunoreactivity was not detected in the collecting ducts (Fig. 6). These studies are consistent with lithium-associated upregulation of COX2 observed on immunoblots (Fig. 4) being restricted to RMICs.

**Lithium inhibits renal GSK-3β activity.** Recent studies indicate that lithium acts as an ATP-noncompetitive inhibitor of GSK-3β (28). GSK-3β is a constitutively active kinase whose activity can be inhibited by phosphorylation of the Ser9 residue. Constitutive GSK-3β activity has recently been shown to tonically suppress COX2 expression in cultured renal interstitial cells (27). To test whether LiCl-induced COX2 expression is associated with reduced renal GSK-3 activity in vivo, the effect of LiCl treatment on renal GSK-3β activity (32P incorporation) was determined using primed glycogen synthase as a substrate. LiCl treatment significantly decreased renal GSK-3β kinase activity over the same time frame of increase in COX2 expression (3-fold by day 5 and 10-fold by day 7 of lithium treatment) (Fig. 7). Total GSK-3β protein level remained unaltered. Serum Li levels were 2.14 ± 0.13 mmol/l in these mice.

Similarly, treatment of cultured mouse RMICs with LiCl for 8 h decreased GSK-3β activity by 45% (Fig. 8A). Increased phosph- GSK-3β Ser9, an inactive form of GSK-3β, was also observed in these cells, further supporting the inhibition of GSK-3 in RMICs by LiCl. Total GSK-3β remained unaltered (Fig. 8B).

**DISCUSSION**

Lithium is commonly prescribed for treatment of bipolar illness and Alzheimer’s disease (10, 24, 34). Its use is routinely associated with polyuria due to NDI. Several studies have addressed the potential mechanisms underlying lithium-induced NDI. Previous studies have focused on direct effects of lithium on the collecting duct, demonstrating impaired V2-receptor responsiveness of the collecting duct associated with decreased vasopressin-stimulated cAMP generation (6, 7). Lithium-treated animals also show reduced expression of the water channels aquaporin-2 and -3, which are critical mediators of epithelial water permeability in the collecting ducts (5, 20, 23). Other studies have demonstrated reduced protein abundance of collecting duct urea transporters A1 and B and reduced vasopressin-stimulated phosphorylation of urea trans-
porter A1 following LiCl treatment (18). The preceding findings demonstrate that lithium treatment alters collecting duct function in vivo.

Collecting duct function may be significantly affected by paracrine factors elaborated by adjacent RMICs, which are a major site of COX2 expression in the medulla (12, 13). The present studies show that lithium increases both total renal medullary COX2 expression and medullary interstitial cell COX2 both in vivo and in vitro. Importantly, COX2 inhibition significantly reduced polyuria following lithium treatment, consistent with a role for interstitial cell COX2-derived prostaglandins in the pathogenesis of lithium-induced NDI.

The potential for a paracrine effect of interstitial cell-derived PGE2 is supported by several previous studies. Cultured interstitial cells produce abundant PGE2 via COX2 (12). Furthermore, basolateral PGE2 potently suppresses ADH-stimulated hydraulic conductivity in isolated, perfused renal collecting ducts (16), mainly through increased intracellular Ca2+ and reduced cAMP levels (16, 17). Previous studies in rats indicate that urinary PGE2 is increased by lithium treatment and that indomethacin, a nonselective COX inhibitor, reduced both urine PGE2 production and urine volume by 80% in lithium-induced NDI (31). These findings are consistent with a role for increased renal PGE2 production in promoting lithium-induced diuresis.

Both COX1 and COX2 prostaglandin synthases are present in the kidney and contribute to renal PGE2 production (26, 29, 32). Of the two COX isoforms identified, COX1 is regulated by a housekeeping-like promoter (19). It is constitutively expressed in most tissues and is particularly abundant in the renal collecting duct (11, 14). In the kidney, the inducible COX2 isoform is also highly expressed but is restricted to the macula densa and RMICs (11, 15, 35). The present studies demonstrate that LiCl treatment markedly increased renal medullary interstitial COX2 expression but did not alter COX1 expression.

Furthermore, the present studies of COX1−/− mice definitively exclude a role for COX1 in lithium-induced polyuria and increased urinary PGE2 excretion. While COX1 deficiency failed to prevent LiCl-induced polyuria, COX2-selective inhibition prevented polyuria induced by LiCl. LiCl also increased urinary PGE2 excretion in COX1-deficient mice, and this was abolished by a COX2 inhibitor. These studies suggest that increased RMIC COX2 expression is a major source of lithium-induced urinary PGE2.

LiCl administration is reported to stimulate ADH release from the central nervous system and thus increase plasma ADH levels (31). In turn, ADH may directly stimulate renal prosta-
directly inhibit ADH action in isolated, perfused collecting ducts, failed to completely restore urine volume and urine osmolality in polyuric rabbit RMICs. Since delayed treatment with the COX2 inhibitor SC-58236 resulted in increased renal excretion of water and sodium, these findings were consistent with a model whereby increased renal interstitial cell GSK-3β activity both in the mouse kidney and in cultured RMICs. Suppression of renal GSK-3β activity temporally correlated with increased renal COX2 expression. Thus constitutive GSK-3β activity may tonically suppress renal COX2 expression in vivo as well as in vitro. Since LiCl did not change renal COX1 expression and COX1 gene disruption did not prevent lithium-induced polyuria, COX2 appears to be a primary cause of polyuria and source of increased urinary PGE2 production following LiCl treatment.

It is well documented that prostaglandins exert biological effects via cell-surface G protein-coupled receptors (9). PGE2 has four cell-surface receptors, including EP1, EP2, EP3, and EP4. Importantly, both basolateral EP1 and EP3 receptors have been detected in renal collecting ducts, where they suppress ADH-stimulated water absorption (4). These findings are consistent with a model whereby increased renal interstitial cell COX2 produces increased PGE2, activating adjacent basolateral COX2 receptors, thus reducing water reabsorption by the collecting duct. This action may contribute to inhibition of collecting duct cAMP generation and decreased aquaporin-2 expression (12, 14), resulting in polyuria (Fig. 9).

LiCl likely has additional effects on collecting duct function, since delayed treatment with the COX2 inhibitor SC-58236 failed to completely restore urine volume and urine osmolality to control levels. Lithium has been previously shown to directly inhibit ADH action in isolated, perfused collecting duct tubules (8). The mechanism of action could involve collecting duct GSK-3β, since this enzyme is also expressed in cultured cells from this segment (unpublished observations).

In summary, LiCl-induced polyuria in mice is accompanied by increased renal COX2 expression and increased urine PGE2 excretion. Renal GSK activity progressively decreased in these animals over a time course matching increased COX2 expression (Fig. 8). Since COX2 selective inhibition significantly reduced this polyuria, these findings support an important paracrine role for interstitial cell-associated COX2 activity in lithium-associated polyuria. We further conclude that COX2 inhibition could provide a therapeutic option in the treatment of LiCl-induced diabetes insipidus in clinical practice.

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