Loss of Cystic Fibrosis Transmembrane Regulator Impairs Intestinal Oxalate Secretion


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ABSTRACT

Patients with cystic fibrosis have an increased incidence of hyperoxaluria and calcium oxalate nephrolithiasis. Net intestinal absorption of dietary oxalate results from passive paracellular oxalate absorption as modified by oxalate back secretion mediated by the SLC26A6 oxalate transporter. We used mice deficient in the cystic fibrosis transmembrane conductance regulator gene (Cftr) to test the hypothesis that SLC26A6–mediated oxalate secretion is defective in cystic fibrosis. We mounted isolated intestinal tissue from C57BL/6 (wild-type) and Cftr−/− mice in Ussing chambers and measured transcellular secretion of [14C]oxalate. Intestinal tissue isolated from Cftr−/− mice exhibited significantly less transcellular oxalate secretion than intestinal tissue of wild-type mice. However, glucose absorption, another representative intestinal transport process, did not differ in Cftr−/− tissue. Compared with wild-type mice, Cftr−/− mice showed reduced expression of SLC26A6 in duodenum by immunofluorescence and Western blot analysis. Furthermore, coexpression of CFTR stimulated SLC26A6–mediated Cl−–oxalate exchange in Xenopus oocytes. In association with the profound defect in intestinal oxalate secretion, Cftr−/− mice had serum and urine oxalate levels 2.5-fold greater than those of wild-type mice. We conclude that defective intestinal oxalate secretion mediated by SLC26A6 may contribute to the hyperoxaluria observed in this mouse model of cystic fibrosis. Future studies are needed to address whether similar mechanisms contribute to the increased risk for calcium oxalate stone formation observed in patients with cystic fibrosis.


Cystic fibrosis (CF) is a life–shortening inherited disease common among many white populations. In recent years, improved treatment and management of respiratory and pancreatic disorders, including organ transplantation, have led to longer life expectancies for patients with CF.1 This increased longevity has increased the risk to patients with CF from additional conditions affecting the general adult population, including nephrolithiasis. Several studies have shown that incidence of nephrolithiasis is higher in patients with CF than in age-matched individuals.2–6 This difference corresponds to increased risk factors favoring kidney stone formation among patients with CF, including urinary abnormalities, such as low urine volume, hypercalciuria, metabolic acidosis, hypocitraturia, and hyperoxaluria.6–9

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Hyperoxaluria can result from increased endogenous production of oxalate or increased net absorption of dietary oxalate.\textsuperscript{10} We have recently reported that net intestinal absorption of dietary oxalate depends on the relative balance between oxalate absorption \textit{via} the paracellular leak pathway and transcellular back secretion dependent on SLC26A6 activity.\textsuperscript{11,12} Indeed, SLC26A6 gene–deficient mice have a phenotype of defective intestinal oxalate secretion, increased net intestinal oxalate absorption, and hyperoxaluria.\textsuperscript{11,13} Cystic fibrosis transmembrane conductance regulator (CFTR) has been shown to stimulate SLC26A6 transport activity as assayed by chloride-base exchange.\textsuperscript{14} Accordingly, the purpose of this study is to test the hypothesis that loss of CFTR reduces SLC26A6–mediated oxalate secretion in the intestine as a molecular mechanism that may contribute to hyperoxaluria.

RESULTS

Comparison of Oxalate and Mannitol Fluxes in Mouse Duodenum of Wild-Type and \textit{Cftr}–/– Mice

We have previously shown the presence in mouse duodenum of a secretory flux of oxalate dependent on expression of the apical transporter SLC26A6.\textsuperscript{11,12} To test the hypothesis that SLC26A6–mediated oxalate secretion is dependent on CFTR, we, therefore, focused on this segment of intestine. Using mouse duodenum mounted in an Ussing chamber, we performed measurements of the apparent permeability ($P_{app}$) for unidirectional secretion of [14C]oxalate simultaneously with flux of [3H]mannitol. Mannitol is an inert, water–soluble, nonmetabolized sugar frequently used as a marker for trans-epithelial transport \textit{via} the paracellular pathway.\textsuperscript{12} Figure 1 shows that the $P_{app}$ for unidirectional secretion of oxalate exceeded that for mannitol in wild-type mice as previously shown.\textsuperscript{12,15} The excess of oxalate secretion over that of mannitol reflects transcellular secretion mediated by SLC26A6, because it is abolished in \textit{Slc26a6}–/– mice.\textsuperscript{12,15} Importantly, in duodenum from \textit{Cftr}–/– mice, the higher secretory $P_{app}$ of oxalate compared with mannitol as seen in wild-type mice was very greatly reduced (Figure 1, inset). These findings indicate that, in the absence of CFTR, transcellular oxalate secretion is profoundly decreased.

Comparison of Glucose and Mannitol Fluxes in Mouse Duodenum of Wild-Type and \textit{Cftr}–/– Mice

To exclude the possibility that the reduced duodenal oxalate secretion in the absence of CFTR results from general downregulation of intestinal transport processes, we next measured the unidirectional absorption of [14C]α-methyl glucose simultaneously with flux of [3H]mannitol. Glucose is actively absorbed in the small intestine \textit{via} sodium-glucose cotransport.\textsuperscript{16} As shown in Figure 2, the $P_{app}$ for unidirectional absorption of glucose exceeded that of mannitol in duodenum from wild-type mice, indicating transcellular absorption of glucose. In the absence of CFTR, the excess of absorption of glucose compared with mannitol was not different from that observed in wild–type duodenal tissue (Figure 2, inset). These findings indicate that CFTR deletion does not reduce transcellular glucose absorption. Accordingly, the effect of CFTR deletion to reduce oxalate secretion as observed in Figure 1 is not the result of a general downregulation of intestinal transport processes.

It should be noted in Figures 1 and 2 that the $P_{app}$ for trans-epithelial flux of mannitol in either the absorptive or secretory direction was significantly reduced in duodenum from \textit{Cftr}–/– mice compared with wild-type tissue. Such a difference in passive permeability could reflect a difference in surface area, tight junction properties, and/or unstirred layers. Independent of this change in passive permeability, the results shown in Figures 1 and 2 taken together indicate that CFTR deletion causes a profound defect in transcellular oxalate secretion, with no effect on transcellular glucose absorption.

Effect of CFTR Channel Inhibitor on Oxalate Secretion

SLC26A6 functions as a Cl$^{-}$–oxalate exchanger.\textsuperscript{17} Therefore, cell to lumen secretion of oxalate by this transporter should be affected by the transmembrane Cl$^{-}$ gradient. One possible mechanism to explain the dependence of SLC26A6–mediated oxalate secretion on CFTR as observed in Figure 1 is that it is secondary to Cl$^{-}$ efflux \textit{via} CFTR, resulting in reduced intracellular Cl$^{-}$ and an increased lumen to cell Cl$^{-}$ gradient. To examine this possibility, we tested the effect of CFTR...
channel inhibitor CFTRinh-172 on oxalate secretion by wild-type duodenum. CFTRinh-172 at 10⁻⁵ M blocks CFTR channel activity in duodenum. As shown in Figure 3, we did not detect an effect of 20 μM CFTRinh-172 on oxalate secretion, suggesting that the defect in oxalate secretion in Cfr⁻/⁻ tissue observed in Figure 1 is not the result of loss of CFTR Cl⁻ channel activity.

**SLC26A6 Expression in Duodenum of Wild-Type and Cfr⁻/⁻ Mice**

Another possibility to explain the defect in transcellular oxalate secretion in duodenum of Cfr⁻/⁻ mice is that there is reduced expression of SLC26A6. This possibility was examined by two methods: immunocytochemistry and immunoblotting. As shown in Figure 4, immunofluorescence microscopy revealed greatly reduced SLC26A6 expression on the apical membrane of enterocytes in both the upper and lower portions of the villi of the duodenum of Cfr⁻/⁻ compared with wild-type mice.

To evaluate the expression of SLC26A6 by a more quantitative method, we performed immunoblotting of homogenates of epithelial cells from duodenum of wild-type and Cfr⁻/⁻ mice. As illustrated in Figure 5A, SLC26A6 polypeptide abundance was clearly lower in Cfr⁻/⁻ than in wild–type duodenal cell homogenates. The densitometric measurements in Figure 5B revealed a significant reduction in SLC26A6 expression in Cfr⁻/⁻ compared with wild-type tissue.

**Coexpression of SLC26A6 and CFTR in Xenopus Oocytes**

We also evaluated whether CFTR expression regulates SLC26A6 activity independent of changes in SLC26A6 expression. In fact, coexpression of CFTR was reported to stimulate SLC26A6 transport activity assayed as Cl⁻–base exchange. However, whether CFTR similarly regulates Cl⁻–oxalate exchange activity of SLC26A6 was not examined.

We, therefore, used Xenopus oocytes as a functional expression system to test whether CFTR regulates Cl⁻–oxalate exchange activity of SLC26A6 as illustrated in Figure 6. Figure 6A shows the time course of oxalate remaining in oocytes placed in different solutions. Thus, decline in oxalate content represents oxalate efflux. Efflux rate constants under the various conditions are summarized in Figure 6B. Oocytes expressing CFTR alone exhibited no oxalate efflux under any of the tested conditions, confirming both the lack of significant endogenous oxalate efflux pathways in the oocyte and the lack of ability of CFTR to mediate oxalate flux as previously reported. Oocytes expressing SLC26A6 alone exhibited oxalate efflux during their initial incubation in Cl⁻–containing ND96 medium followed by reversible abolition of efflux on transition to Cl⁻–free medium and then, block of efflux by the inhibitor 4,4'–disothioctyanostilbene–2,2'–disulfonic acid (DIDS). These findings are consistent with SLC26A6–mediated oxalate efflux by Cl⁻–oxalate exchange sensitive to DIDS inhibition as previously reported. Most importantly, coexpression of CFTR significantly stimulated SLC26A6–mediated oxalate efflux. Because CFTR conductance is reversibly increased by phosphorylation subsequent to elevations in intracellular cAMP, we also examined the effect of forskolin and 3-isobutyl-1-methylnanthine (IBMX) on oxalate efflux. Addition of forskolin and IBMX to ND96 buffer stimulated oxalate efflux compared with ND96 buffer alone regardless of whether CFTR was coexpressed with SLC26A6. Indeed, the increment in SLC26A6–mediated oxalate efflux induced by coexpression of CFTR was nearly identical in the presence and absence of
forskolin and IBMX. Taken together, the results in Figure 6, A and B indicate that CFTR stimulates activity of SLC26A6 as a Cl−-oxalate exchanger.

We next evaluated whether CFTR-mediated stimulation of SLC26A6 transport activity in Xenopus oocytes resulted at least in part from altered surface membrane expression of SLC26A6 as observed in vivo in mouse duodenum. Quantitative confocal immunofluorescence microscopy was performed on SLC26A6-expressing oocytes expressed with or without co-expression of CFTR. As shown in Figure 6, C and D, coexpression with CFTR did not significantly affect expression of SLC26A6 protein at or near the surface plasma membrane of Xenopus oocytes. Thus, the effect of CFTR to stimulate SLC26A6-mediated oxalate transport activity in oocytes is likely caused by direct modulation of its transport function.

URINE AND PLASMA OXALATE CONCENTRATION IN WILD-TYPE AND Cftr−/− MICE

The presence of a severe defect in intestinal oxalate secretion in Slc26a6−/− mice correlates with a phenotype of hyperoxalemia and hyperoxaluria.11,13 It was, therefore, of interest to evaluate whether the profound defect in transcellular oxalate secretion observed in Cftr−/− mice as shown in Figure 1 is associated with a similar in vivo phenotype. Accordingly, we measured urinary oxalate excretion and plasma oxalate concentration in wild-type and Cftr−/− mice. As shown in Figure 7, A and B, both urinary oxalate levels and plasma oxalate concentration were 2.5-fold higher in Cftr−/− compared with wild-type mice. Thus, the defect in intestinal oxalate secretion in Cftr−/− mice correlates with a phenotype of marked hyperoxalemia and hyperoxaluria. In contrast, as seen in

DISCUSSION

Patients with CF have an increased incidence of nephrolithiasis,6–8 for which one of the predisposing risk factors is hyperoxaluria.6–8 Because CFTR had been shown to interact with SLC26A6 and stimulate its transport activity as a Cl−-base exchanger14 and because SLC26A6–mediated oxalate secretion in the intestine is important for oxalate homeostasis,11,13 we investigated whether CF mice have a defect in SLC26A6–mediated oxalate secretion in the intestine as a molecular mechanism that may contribute to hyperoxaluria.

In fact, we show that genetic deletion of CFTR causes a profound defect in intestinal oxalate secretion. Moreover, the defect in intestinal oxalate secretion in Cftr−/− mice is associated with a phenotype of hyperoxalemia and hyperoxaluria, the same phenotype that results from a defect of intestinal oxalate secretion in Slc26a6−/− mice.11,13

We identified at least two mechanisms that may contribute to the greatly reduced level of intestinal oxalate secretion in CF mice. First, CFTR deletion results in a significant reduction of SLC26A6 expression in duodenal epithelial cells in vivo as revealed by immunofluorescence microscopy and immunoblotting. Second, shown by functional expression studies in Xenopus...
oocytes, CFTR stimulates activity of SLC26A6 as a Cl⁻-oxalate exchanger. Expression of SLC26A6 polypeptide at or near the surface of *Xenopus* oocytes was not directly affected by CFTR, suggesting that the reduction in membrane and total SLC26A6 expression in the intestine of CF mice is an indirect consequence of the loss of CFTR. For example, loss of CFTR is associated with inflammation in the intestine. Inflammation has been shown to be associated with reduced intestinal SLC26A6 expression and hyperoxaluria in a human patient with celiac disease. A direct effect of CFTR to activate Cl⁻-base exchange mediated by SLC26 family transporters was previously reported. Subsequent work attributed mutual stimulation of CFTR and SLC26 transporters to direct physical interaction between the R domain of CFTR and the STAS domain of SLC26 transporters, including SLC26A6.

It should be noted that we detected no significant difference in calcium excretion between wild-type and CF mice. It is, therefore, likely that the hypercalciuria sometimes observed in patients with CF is a secondary phenomenon. For example, aminoglycosides, which are commonly used in patients with CF, can cause hypercalciuria by activation of the calcium-sensing receptor and induction of a Barter-like syndrome with hypercalciuria.

It should be noted that urine calcium excretion in the CF mice was quite low compared with the level that was found in *Slc26a6*^2/2^ mice that developed urolithiasis (4.3 versus 21.3 mmol/g creatinine, respectively). This difference in calcium excretion, which is probably caused by use of different mouse strains and diets in the two studies, likely accounts for the absence of urolithiasis in the CF mice in this study.

It is important to note that hyperoxaluria and calcium oxalate nephrolithiasis are established complications of malabsorptive states as observed in the setting of pancreatic insufficiency. Calcium is presumed to bind preferentially to fatty acids in the intestinal lumen rather than to oxalate, facilitating increased absorption of soluble oxalate. We cannot exclude the possibility that malabsorption as observed in patients with CF may also contribute to an increase in soluble oxalate available for absorption.

Nevertheless, our model of hyperoxaluric *Cfr⁻/⁻* mice shows a profound defect of intestinal oxalate secretion that phenocopies the defect in intestinal oxalate secretion and hyperoxaluria that occurs in *Slc26a6*^2/2^ mice. Human SLC26A6 has been shown to mediate efflux of oxalate in exchange for...
Figure 7. Cfr<sup>+/−</sup> mice show hyperoxaluria and hyperoxalemia. (A) Urinary oxalate-to-creatinine ratio and (B) plasma oxalate are 2.5-fold higher in Cfr<sup>+/−</sup> compared with age- and sex-matched wild-type (WT) mice. (C) Urinary calcium-to-creatinine ratio was not significantly different between Cfr<sup>+/−</sup> and WT mice. Data are means±SEM from four mice in each group. **P<0.01 versus WT mice. n.s., P>0.05 versus WT mice.

extracellular Cl at a rate equivalent or greater than the mouse ortholog. The possible relevance of SLC26A6 expression to oxalate homeostasis in humans is evident from the report of a patient with subclinical celiac disease and absence of fat malabsorption, in whom hyperoxaluria correlated with markedly reduced expression of SLC26A6 in the small intestine. It is, therefore, possible that the defect in intestinal oxalate secretion that we describe in Cfr<sup>+/−</sup> mice may contribute to the hyperoxaluria and increased incidence of calcium oxalate nephrolithiasis observed in patients with CF.

In conclusion, we have shown that Cfr<sup>+/−</sup> mice have a phenotype of hyperoxalemia and hyperoxaluria associated with a profound defect in SLC26A6–mediated oxalate secretion in the intestine. Future studies are needed to address whether similar mechanisms contribute to the increased risk for calcium oxalate stone formation observed in patients with CF.

CONCISE METHODS

Mouse Studies and Diets
All measurements were performed on 8- to 12-week-old sex-matched mice. Transgenic CFTR knockout mice (Cfr<sup>+/−</sup>; B6.129P2-knockout Cfr<sup>tm1Unc</sup>/Cfr<sup>+/−</sup>) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the Yale University Animal Facility using pathogen-free, ventilated cages. Mice were backcrossed on a C57BL/6 background for a minimum of 10 generations. Wild-type mice were littermates. Experimental mice (wild type and Cfr<sup>+/−</sup>) were fed postweaning with a liquid diet (Peptamen; Nestle, Deerfield, IL) as previously described. All animal protocols were approved by the Yale University Institutional Animal Care and Use Committee.

Measurement of Oxalate, Mannitol, and Glucose Fluxes Across Mouse Intestine
Intestinal segments of wild-type and Cfr<sup>+/−</sup> mice were opened longitudinally along the mesenteric border and mounted as an intact sheet in a modified Ussing chamber as previously described. In brief, mucosal and serosal surfaces were bathed with 8 ml warmed (37°C), oxygenated bicarbonate Ringer solution (140 mM Na<sup>+</sup>, 119.8 mM Cl<sup>−</sup>, 5.2 mM K<sup>+</sup>, 1.2 mM Ca<sup>2+</sup>, 1.2 mM Mg<sup>2+</sup>, 25 mM HCO<sub>3</sub>−, 2.4 mM HPO<sub>4</sub>−<sub>2</sub>, 0.4 mM H<sub>2</sub>PO<sub>4</sub>−<sub>2</sub>, and 10 mM glucose at pH 7.4). We added 2 μM [14C]oxalate (specific activity, 117 mCi/mmol; Amersham Biosciences, Pittsburgh, PA), 0.08 μM [3H]mannitol (specific activity, 20 Ci/mmol; MP Biomedicals, Santa Ana, CA), or 0.6 μM [14C]α-methyl glucose (specific activity, 310 mCi/mmol; Amersham Biosciences) to either the mucosal or serosal bath and unlabeled 2 μM oxalate, 0.08 μM mannitol, or 0.6 μM α-methyl glucose, respectively, to the opposite bath. For [14C]α-methyl glucose absorption assay, Ringer solution was modified by replacing 10 mM glucose with 10 mM sodium acetate. After a 150-minute equilibration period to achieve steady-state flux rates, we collected samples before and after the 60-minute flux period to calculate values for unidirectional mucosa to serosa flux and serosa to mucosa flux. The P<sub>app</sub> values for oxalate, mannitol, and glucose were calculated according to the following equation: J=P<sub>app</sub>×A×[S], where J is the flux (mucosa to serosa or serosa to mucosa), A is the cross-sectional tissue area of the Ussing chamber, and [S] is the substrate concentration of [14C]oxalate, [3H]mannitol, or [14C]α-methyl glucose. We performed all flux studies under voltage clamp conditions using the multichannel voltage/current clamp model VCC MC6 (Physiologic Instruments, San Diego, CA).

Immunochemistry for Intestinal SLC26A6 Expression
Mice were euthanized by intraperitoneal injection of sodium pentobarbital using standard protocols. Duodena were removed, cleared with PBS, cut into 4-mm lengths, and then, immersion fixed in 2% paraformaldehyde, 750 mM lysine, and 10 mM sodium periodate in phosphate buffer, pH 7.4 for 4 hours at room temperature. Tissue was embedded in Epon 812 (Electron Microscopy Sciences, Hatfield, PA) and processed for immunocytochemistry as described previously. 1-μm sections were subjected to antigen retrieval and then, double labeled with a mouse mAb directed against SLC26A6 (7A7; 1:16,000 dilution) and a rabbit polyclonal antibody directed against villin (PA5–22072; 1:1000 dilution; Thermo Fisher Scientific, Vernon Hills, IL). Primary antibody labeling was visualized by incubation with fluorochrome–labeled secondary donkey anti–mouse Alexa-Fluor 488 and donkey anti–rabbit Alexa-Fluor 594, respectively (1:200 dilution) and a rabbit polyclonal antibody directed against villin (PA5–22072; 1:1000 dilution; Thermo Fisher Scientific, Vernon Hills, IL). Primary antibody labeling was visualized by incubation with fluorochrome–labeled secondary donkey anti–mouse Alexa-Fluor 488 and donkey anti–rabbit Alexa-Fluor 594, respectively (1:200 dilution).
dilution; Thermo Fisher Scientific). Specificity of 7A7-A2 mAb was verified by comparison of immunofluorescence in wild-type and Slc26a6−/− tissue (Supplemental Figure 1).

**Western Analyses of Slc26a6 Protein Expression in Duodenum**

Mice were euthanized by intraperitoneal injection of sodium pentobarbital as above. Duodena were removed and cleared with ice-cold PBS containing protease inhibitors (Roche Complete; Sigma-Aldrich, St. Louis, MO). Connective tissue was removed, and the duodena were everted and sealed by ligature. The mucosa was then scraped into cold PBS containing protease inhibitors, homogenized with a Thomas–style glass homogenizer and a serrated pestle, and stored at −80°C. The duodenal homogenates were solubilized directly in SDS sample buffer and then, subjected to SDS-PAGE and Western analysis as described previously.34 Western blots were probed with a rabbit anti-human Slc26a6 antibody (R29; 1:50,000 dilution) and a mouse anti–avian β-actin antibody (SC-47778; 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). HRP–conjugated anti–rabbit and anti–mouse secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used at dilutions of 1:20,000. Antibody labeling was visualized by enhanced chemiluminescence (Clarity; Bio–Rad, Hercules, CA) and captured on film. Quantitative assessments of Western blot results were performed by densitometry (Image Software; National Institutes of Health, Bethesda, MA), and all values were normalized to their respective sample β-actin levels before statistical analysis with an unpaired two–tailed t test. Specificity of R29 polyclonal antibody was verified by comparison of immunoblotting in wild-type and Slc26a6−/− tissue (Supplemental Figure 2).

**Heterologous Expression Studies of Human CFTR and Slc26a6 in Xenopus Oocytes**

Capped cRNA was transcribed with T7 RNA polymerase (Ambion, Austin, TX) from linearized cDNA template encoding human Slc26a6 (S-Q)30,35 and purified with an RNeasy Mini-Kit (Qiagen, Valencia, CA). cRNA concentration (A260) was measured by a Nanodrop Spectrometer (Thermo Fisher Scientific), and integrity was confirmed by formaldehyde agarose gel electrophoresis. Mature female *Xenopus laevis* (Department of Systems Biology, Harvard Medical School or NASCO, Madison, WI) were maintained and subjected to partial ovariectomy under hypothermic tricaine anesthesia following protocols approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center. Ovarian fragments were incubated overnight with 1.3 mg/ml collagenase B (Roche Diagnostics, Indianapolis, IN) in PBS containing 85 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, and 10 mM HEPES (pH adjusted to 7.40 with NaOH). Collagenase–treated oocytes were then rinsed for 20 minutes in Ca2+-free PBS, after which stages 5–6 oocytes were selected and manually defolliculated. Oocytes were injected on the same day with cRNA in a volume of 50 nl. Injected and un.injected oocytes were then maintained before use for 3–4 days at 19°C in PBS containing gentamicin.

[14C]oxalate efflux assays with tests of drug sensitivity were performed as previously described.36 Briefly, oocytes were injected with 50 nl 50 mM Na [14C]oxalate (6000–8000 cpm; with final estimated intracellular postequilibration concentration of 5 mM). After a recovery period of at least 20 minutes, efflux was measured in the indicated bath solutions. [14C]oxalate was from Amersham UK and GE Healthcare (Waukesha, WI). DIDS was from Calbiochem (La Jolla, CA). Forskolin was from Abcam, Inc. (Cambridge, MA). All other chemical reagents were from Sigma–Aldrich or Fluka (Milwaukee, WI) and were of reagent grade.

Confocal immunofluorescence microscopy was performed 2 days after cRNA injection. Individual oocytes were fixed for 30 minutes at room temperature in 1 ml 3% paraformaldehyde in PBS. Fixed oocytes (n=8–10 per group) were extensively rinsed with PBS supplemented with 0.002% sodium azide, epitope unmasked with 1% SDS for 1–5 minutes, and blocked in PBS with 1% BSA and 0.05% saponin (PBS–BSA) for 1 hour at 4°C. Oocytes were then incubated 4–16 hours at 4°C with affinity–purified rabbit polyclonal antibody specific for human Slc26a6 C–terminal peptide (1:200),35,37 washed several times in PBS–BSA, incubated for 1 hour with Cy3–conjugated secondary donkey anti–rabbit Ig (Jackson Immunoresearch Laboratories), and again, thoroughly washed in PBS–BSA. Oocytes were uniformly oriented along a Plexiglas groove and sequentially imaged through the 10× objective of a Zeiss LSM510 Laser Confocal Microscope (Carl Zeiss GmbH, Jena, Germany) at 512×512 resolution using the 543-nm laser line at 80% intensity at uniform settings of pinhole at 132 (1.68 Airy units), detector gain of 682, Amp gain of one, and zero offset. Polypeptide abundance at or near each oocyte surface was estimated by quantitation (Image, version 1.38; National Institutes of Health) of specific fluorescence intensity at the circumference of one quadrant of an equatorial focal plane.

**Measurement of Urine and Plasma Oxalate in Mice**

Urine and plasma samples were acidified, and oxalate was measured by ion chromatography using a Dionex ICS 2000 System (Dionex, Sunnyvale, CA) as previously described.31 Chromelon software (version 6.5; Dionex) was used to measure peak area and calculate the oxalate concentration.

**Statistical Analyses**

Results are given as the means±SEM for the indicated number of experiments. For analyses, we performed unpaired t tests (Prism 6.0 program; GraphPad Software, La Jolla, CA). A type 1 error of 0.05 (two tailed) was used as the level of statistical significance for all analyses.

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DISCLOSURES

None.

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