Sulfate and thiosulfate inhibit oxalate transport via a dPrestin (Slc26a6)-dependent mechanism in an insect model of calcium oxalate nephrolithiasis


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Nephrolithiasis (kidney stones) is a major healthcare burden (>$10.3 billion/yr in US in 2012, i.e., one-third of National Institutes of Health budget) affecting ~12% of men and 6% of women during their lifetime in industrialized countries (2). However, given its prevalence, it is still poorly understood at both the physiological and genetic levels because of its significant complexity and varied etiology. The most common type of human kidney stone is largely composed of calcium oxalate (CaOx), which likely results from a combination of environmental and genetic factors. Mutations affecting oxalate metabolism, cell membrane transporters, ion receptors, and transmembrane ion channels have led to CaOx stone manifestations in diseases such as Dent’s disease, Barter’s syndrome, and hyperoxaluric metabolic disease (14).

Recently, our laboratory developed a useful genetic model in Drosophila melanogaster, where major renal pathophysiology (CaOx kidney stones), particularly cation/anion movement, can be studied rather precisely (7). Additionally, we identified a key transporter, Drosophila prestein (Slc26a5/6; dPrestin) that participates in this process (7). dPrestin has been shown to mediate electrogenic Cl-/oxalate2-, Cl-/sulfate2-, and electroneutral Cl-/formate exchange similar to what we and others have reported for other species orthologs of Slc26a6 (4, 6, 7, 9–12, 15, 24, 26). Interestingly, this transporter acts as a homolog to mouse Slc26a6 (mSlc26a6), whereby mice lacking this protein present with renal and intestinal transport defects in addition to CaOx urolithiasis (9, 24). Our model has shown that Drosophila can specifically mimic CaOx crystal formation, gut and renal tubule Cl-/ox2- exchange, and whole animal gut oxalate absorption to CaOx crystal formation compared with mammalian models (3, 5, 7). These data also provide additional evidence illustrating dPrestin as a homolog of mammalian Slc26a6 function in the pathophysiology of CaOx renal stone formation (3, 5, 7). Previous reports showed that dPrestin and mSlc26a6 exhibit sulfate-dependent currents (7, 11). However, unlike mSlc26a6, the steady-state kinetics of dPrestin-mediated sulfate transport has not been reported.

The sulfates, particularly thiosulfate, have a number of medical uses, such as an antidote to cyanide poisoning and concurrent administration with cisplatin to decrease nephrotoxicity (16). Interestingly, thiosulfate is reported as a treatment in nephrolithiasis. In 1985, Yatzidis (28) suggested that thiosulfate could reduce urinary ionized calcium levels, thereby decreasing rates of nephrolithiasis. In 1994, Agroyannis (1) reported that a patient receiving thiosulfate for treatment of renal tubular acidosis type I with nephrocalcinosis had no worsening of renal function and remained without clinical symptoms. Asplin and colleagues (2) reported that thiosulfate administration significantly reduced spontaneous calcium phosphate stone formation in genetic hypercalciuric stone (GHS)-forming rats (2). More recently, Rodgers and coworkers (18) reported that sulfate but not thiosulfate decreased calculated urinary, ionized Ca2+ and supersaturation in vitro and in silico. Given this information, it can be concluded that evidence for the use of sulfate and thiosulfate in humans...
Fig. 1. Sulfate and thiosulfate transport mediated by Drosophila prestin (dPrestin) and mouse (m) Slc26a6. Shown are representative current-voltage (I-V) relationships of oocytes expressing dPrestin (red), mSlc26a6 (blue), or control oocytes (black) in the presence of 1 mM sulfate or 0.1 mM thiosulfate and 20 mM Cl\(^{-}\). Values are tabulated from experiments performed using 3–6 oocytes from at least 2 donor frogs.

Since there are different systems and apparent data conflicts, our studies were designed to test the roles of these sulfate compounds on oxalate transport, rather than urinary calcium, and subsequent CaOx crystallization. Given that our Drosophila model can be used to easily and rapidly study genetic and physiological factors, the current study sought to investigate the effects of sulfate and thiosulfate on both dPrestin (dSlc26a5/a6) and mSlc26a6 function as potential inhibitors of transport, as well as CaOx crystallization in Drosophila Malpighian tubules (MT).

MATERIALS AND METHODS

Drosophila. Flies were kept on standard medium or dietary salt substitution in vials at 22°C, 12:12-h photoperiod, and 40% relative humidity. Wild-type (Oregon R) flies were used in ex vivo and whole fly feeding experiments, except where noted.

Ex vivo oxalate ± inhibitor birefringence experiments. Adult flies (7d) were allowed to feed on standard food. Malpighian tubules (MT) from female flies were dissected in Schneider’s medium and transferred immediately to poly-L-lysine-coated slides with insect PBS containing either 20 mmol/l sulfate or 20 mmol/l thiosulfate and allowed to preincubate for 20 min. An inhibitor containing PBS was

Fig. 2. Sulfate and thiosulfate kinetic curves. Kinetic curves were generated from voltage-clamp experiments in Xenopus oocytes expressing either mSlc26a6 or dPrestin (see MATERIALS AND METHODS) (A–E). Each line represents a different pulse voltage while the x-axis indicates changes in sulfate or thiosulfate in the bathing solution. Values are means ± SE; n = 3 oocytes.
Table 1. Steady-state kinetics of sulfate and thiosulfate transport

<table>
<thead>
<tr>
<th>$V_{\text{m}}$, mV</th>
<th>Sulfate</th>
<th>Thiosulfate</th>
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<tr>
<td></td>
<td>dPrestin $K_m$, mM</td>
<td>mSlc26a6 $K_m$, mM</td>
</tr>
<tr>
<td>−60</td>
<td>33.67 ± 24.58</td>
<td>9047 ± 1.72</td>
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<tr>
<td>−40</td>
<td>7.07 ± 0.78</td>
<td>4.97 ± 2.70</td>
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<tr>
<td>−20</td>
<td>5.06 ± 0.55</td>
<td>10.42 ± 4.38</td>
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<tr>
<td>0</td>
<td>4.53 ± 0.38</td>
<td>13.22 ± 7.27</td>
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<tr>
<td>20</td>
<td>3.70 ± 0.33</td>
<td>7.17 ± 3.49</td>
</tr>
<tr>
<td>40</td>
<td>3.06 ± 0.27</td>
<td>6.44 ± 2.91</td>
</tr>
<tr>
<td>60</td>
<td>2.59 ± 0.22</td>
<td>5.76 ± 3.58</td>
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Values are means ± SE; $n = 3$. dPrestin, Drosophila presstin. Michaelis-Menten constants ($K_m$) are shown. Sulfate- and thiosulfate-elicited currents were measured by addition of 0, 0.185, 0.556, 1.67, 5, and 15 mM or 0, 0.0206, 0.0617, 0.185, 0.556, 1.667, and 5 mM, respectively, in the presence of 20 mM Cl⁻.

then aspirated and replaced with PBS containing 5 mmol/l Na-oxalate and either 20 mmol/l sulfate or 20 mmol/l thiosulfate. These were allowed to co-incubate for 1 h with fresh solution replaced every 15 min. Crystallization was monitored for 60 min with a Zeiss Observer microscope using differential interference contrast (DIC) microscopy as previously (7).

**Cell type-specific knockdown of dPrestin.** This was performed as described previously (7). Briefly, the GAL4/UAS system allows for cell- and/or tissue-specific genetic manipulation in Drosophila, whereby a fly line which possesses a “GAL4-driver” can be crossed to a second transgenic fly line containing a construct of choice placed downstream of a UAS promoter. This allows the transgene to only be expressed in cells and/or tissues where GAL4 is also expressed. To specifically knock down dPrestin, we utilized the Uro-GAL4 driver, whereby the promoter of the tubule principal cell-specific gene urate oxidase (Uro) drives GAL4 expression, and crossed it to the BIGFly line 5843R3 (National Institute of Genetics) containing a hairpin dsRNA sequence directed against dPrestin.

**Feeding oxalate ≥ inhibitor birefringence experiments.** Adult flies were placed on food containing 5 mmol/l Na-oxalate and either 20 mmol/l sulfate or 20 mmol/l thiosulfate and were allowed to feed for 24 or 48 h. We chose 20 mM sulfate and thiosulfate based on our $V_{\text{m}}$ data (Fig. 2). We also wanted to make sure that we used the same molar additive to our fly food to minimize the possibility of ion concentration effects. At each respective time point, MT were dissected (above) and transferred to coated slides with insect PBS. Tubules were imaged immediately with a Zeiss Observer microscope using DIC microscopy as previously (7).

**CaOx crystal quantitation.** CaOx crystals were quantified using ImageJ software. Pictures were ×10 magnification, and the scale was set to reflect the full length of the image obtained as above. Six hundred micrometers were measured from the terminal end of the respective MT and quantified to achieve consistency among pictures. Once measured, brightness and contrast were adjusted, resulting in birefringent crystals in the designated 600 μm of tubule being visible, and the remaining tubule artifacts were blacked out. A threshold was then set, resulting in binary coloration (black and white) so that crystals could be counted.

Using the Analyze Particles feature in ImageJ, the minimum desired size of stone detection was specified at 2 μm², and parameters were set to overlay masks, display results, summarize, and to include holes. Data generated included an accurate and specific crystal count, total area of the 600 μm of tubule containing crystals, and the average size per individual crystal in said area. Data were then analyzed via one-way ANOVA with Dunnett’s post hoc test ($P < 0.05$) or Student’s t-test ($P < 0.05$) using GraphPad Prism.

**Animal health and welfare.** X. laevis were housed and cared for in accordance and approval of the Institutional Care and Use Committees of the Mayo Clinic College of Medicine.

**Drosophila Slc26a5 constructs.** The sequence of dPrestin (Slc26a5; CG5485) has been previously reported (25). As reported previously (7), we amplified this sequence by PCR, verified the sequence, and functionally tested the resulting clone. Our experiments indicate that dPrestin (CG5485) is the functional homolog of mammalian Slc26a6 (7) and is molecularly more closely aligned with Slc26a6b clones (8).

**Oocyte experiments.** dPrestin as well as mSlc26a6 were subcloned into the pGEMHE Xenopus expression vector, capped cRNA synthesized. Oocytes were injected with 50 nl cRNA (0.2 μg/μl, 10 ng/oocyte) or water as previously for other transporters (7, 11, 17, 19, 20) and incubated at 16°C in OR3 media. Oocytes were studied 3–10 days after injection.

**Electrophysiology.** Electrophysiology protocols were performed as we previously reported for Slc26a6 (8, 11, 12, 26). All solutions were either ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.5) or isoosmotic ion replacements (21). Kinetic experiments were done with the addition of either increasing sulfate (0, 0.185, 0.556, 1.67, 5 and 15 mM) or thiosulfate (0, 0.0206, 0.0617, 0.185, 0.556, 1.667, and 5 mM), Cl⁻ ND96 solutions (20 and 70 mM) were prepared as previously described (11).

**Two-electrode voltage clamp.** For these experiments, membrane currents were recorded with an OC-725C voltage clamp (Warner Instruments, Hamden, CT), filtered at 2–5 kHz, and digitized at 10 kHz. Current-voltage protocols consisted of 40-ms steps from $V_e$ (−60 mV) to −140 mV and +60 mV in 20-mV steps (11, 21).

**RESULTS**

dPrestin and mSlc26a6 transport sulfate and thiosulfate. To examine sulfate and thiosulfate transport function, dPrestin and mSlc26a6 proteins were expressed separately in Xenopus oocytes. Figure 1 shows two-electrode voltage-clamp experiments. Sulfate elicited slightly rectifying currents in both dPrestin- and mSlc26a6-expressing oocytes, whereas thiosulfate elicited outwardly rectifying current only in mSlc26a6-expressing oocytes. Control (water-injected) oocytes do not show these responses. The dPrestin-mediated sulfate activity (Fig. 1A) is similar to our previous report (7). Thiosulfate, however, was transported by both dPrestin and mSlc26a6 with similar current magnitudes (Fig. 1B). These data indicate that thiosulfate is another substrate for both transporters, but it is not merely a sulfate substitute.

Next, we determined the affinity of dPrestin and mSlc26a6 for sulfate and thiosulfate (Fig. 2). To do this, we used a voltage clamp and measured the current responses (kinetics) to

Fig. 3. Sulfate (48 h) and thiosulfate (24 and 48 h) decrease Malpighian tubule (MT) CaOx crystallization in vivo, but have no effect ex vivo. A: representative MT images from female flies fed oxalate (5 mM) or oxalate (5 mM) + sulfate (20 mM) or thiosulfate (20 mM) or pretreated with sulfate (20 mM) or thiosulfate (20 mM) and subsequently exposed to oxalate (5 mM) ± sulfate (20 mM) or thiosulfate (20 mM) for 1 h. Yellow arrows indicate CaOx crystals within the lumen of the MT. CaOx crystal number, total crystal area, and average area/crystal per 600 μm² of tubule were determined using ImageJ as described in MATERIALS AND METHODS. B: thiosulfate significantly decreases CaOx crystal number and total crystal area at both 24 and 48 h in vivo, while sulfate significantly decreases CaOx crystal number and total crystal area at 48 h only compared with oxalate alone. These effects are not seen ex vivo at 1 h. For feeding and ex vivo experiments, values are expressed as means ± SE. (A) 24 h feeding, n = 14 for oxalate controls and n = 6 for oxalate ± sulfate or thiosulfate; at 48 h feeding, n = 6 for all groups; for ex vivo experiments, n = 15 for all groups). *Significant difference from oxalate only controls as determined by 1-way ANOVA followed by Dunnett’s post hoc test, P < 0.05.
Flies fed oxalate (5 mM) + sulfate (20 mM) showed a significant decrease in CaOx crystal number and total crystal area at both time points (24 and 48 h) compared with oxalate alone (Fig. 4B, C, and D). These results indicate that both sulfate and thiosulfate have the ability to decrease CaOx crystallization by 24 h, with sulfate taking 48 h to show any significant effect. Interestingly, these results were only observed in flies when they were fed experimental compounds and not seen ex vivo, indicating an important role for transport/transport inhibition at the level of the gut.

dPrestin knockdown in MT significantly reverses sulfate-and thiosulfate-induced prevention of CaOx crystallization at 48 h. Knockdown of dPrestin (dsRNA directed against dPrestin) reduces MT-mRNA levels by ~70% (7). Since sulfate and thiosulfate both serve as competitive inhibitors of dPrestin-mediated oxalate transport and can prevent CaOx crystallization at 48 h, we examined the effect of these compounds on the formation of CaOx crystals in Drosophila MT both through feeding and ex vivo exposure (Fig. 3A). Flies fed oxalate (5 mM) + thiosulfate (20 mM) showed significant decreases in CaOx crystal number and total crystal area at both time points (24 and 48 h) compared with oxalate alone (Fig. 3B). Flies fed oxalate (5 mM) + sulfate (20 mM) showed a significant decrease in crystal number and total area only at the later time point (48 h) compared with oxalate alone. In contrast, when anterior MT were dissected and exposed to the same conditions ex vivo, there were no significant decreases in crystal number, total area, or size of individual crystals (Fig. 3B). These results indicate that both sulfate and thiosulfate have the effect of decreasing CaOx crystallization when fed to flies over a 24- to 48-h period. Thiosulfate appears to be more effective at decreasing CaOx crystallization by 24 h, with sulfate taking 48 h to show any significant effect.
were only significant with oxalate and thiosulfate addition (Fig. 4D). These results indicate that sulfate and thiosulfate competitively inhibit oxalate transport via luminal dPrestin in the MT and that with no dPrestin expression both compounds lose their abilities to effectively decrease CaOx crystallization.

**DISCUSSION**

*Drosophila melanogaster* has emerged as a useful model in exploring the pathophysiology of CaOx nephrolithiasis (3, 5, 7, 8). Our previous experiments illustrated that renal tubule oxalate export is mediated by the Slc26a6 ortholog dPrestin, which can also transport sulfate (7, 8). To better understand the factors determining CaOx crystallization and how sulfate-related substrates may interact with oxalate transport and subsequent CaOx crystallization, we investigated the effects of sulfate and thiosulfate on CaOx crystallization in *Drosophila* MT as well as solute transport by dPrestin and mSlc26a6. We measured the apparent affinity ($K_m$) of dPrestin and mSlc26a6 to transport these compounds as putative proteins significantly involved in the epithelial movement of oxalate and subsequent CaOx crystal formation.

Dose-dependent CaOx crystal formation is visible as early as 24–48 h in the MT of flies fed increasing concentrations of sodium oxalate, and 14 days in flies fed increasing concentrations of ethylene glycol, hydroxy-L-proline, or melamine (3, 7, 14). The dPrestin gene model of nephrolithiasis was developed by selective knockdown of Slc26a6 known to be a luminal oxalate transporter in MT (7, 14). Nine homologs of the 11 human Slc26 transporters are contained within the *Drosophila* genome, with the “prestin” gene encoding the functional ortholog of mammalian Slc26a6 (7, 14). These transporter homologs in *Drosophila* are found in various tissues, with the highest expression patterns located within the MT, midgut, and hindgut (flyatas.org, query “cg5485”). Interestingly, like the human and mouse Slc26a6, dPrestin mediates Cl⁻/oxalate exchange (8). Preliminary studies from our laboratory demonstrate that this model can be used to rapidly study factors which may influence CaOx crystallization within the MT (13, 22, 23). Additionally, given that the nucleation and subsequent growth of the crystals can be observed in real time and in an intact MT, this MT model provides a powerful tool for assessing enhancers or inhibitors of CaOx crystallization.

The current study was designed to evaluate sulfate and thiosulfate transport by dPrestin and mSlc26a6, as well as to determine whether these compounds affected CaOx crystallization in MT. dPrestin and mSlc26a6 have been shown to transport sulfate, with steady-state kinetics being reported for mSlc26a6 having an average $K_m$ of 12.7 ± 5.9 mM in the presence of 70 mM Cl⁻ (8). However, these kinetic parameters of sulfate transport via dPrestin have not been reported. Before this study, neither thiosulfate transport nor kinetics via dPrestin or mSlc26a6 have been reported. Our results indicate that dPrestin and mSlc26a6 transport both sulfate and thiosulfate, with a 7–8-fold greater affinity for thiosulfate. This transport is of particular interest as thiosulfate administration reduced the occurrence of calcium urolithiasis (27), and thiosulfate is used as a treatment for calcium phosphate nephrolithiasis (2) and nephrocalcinosis (1). Rodgers and coworkers (18) reported that sulfate, but not thiosulfate, had a modest effect of reducing urinary ionized calcium concentration, and concomitantly reducing urinary supersaturation of calcium salts, such as CaOx. Thiosulfate unfavorably increased CaOx supersaturation (18). This study also concluded that the unfavorable increase in CaOx supersaturation was due to decrements in urine pH following thiosulfate ingestion, rather than urinary thiosulfate concentrations. However, none of these studies were able to test mechanisms, other than ionized calcium and supersaturation, which may affect thiosulfate’s ability to decrease nephrolithiasis, particularly at concentrations which do not affect urinary pH.
Our results indicate that thiosulfate is more effective at preventing CaOx crystallization at 24 and 48 h with both compounds being as effective at 48 h. These data seem most consistent with oxalate transport inhibition in the gut, rather than effects on CaOx supersaturation and/or urinary ionized calcium concentrations, as thiosulfate treatment of MT did not prevent crystallization. Thiosulfate $K_m$ values for both mammalian and Drosophila transporters are significantly lower than those for sulfate transport, indicating an increased affinity for thiosulfate explaining its effects at the earlier time point. Such an affinity increase implies that thiosulfate could act as a competitive inhibitor of oxalate at concentrations which should have no effect on ionized calcium or supersaturation (in mM: dPrestin average $K_m^{\text{oxalate}} = 0.87 \pm 0.16$ and $K_m^{\text{thiosulfate}} = 0.22 \pm 0.03$; mSlc26a6 average $K_m^{\text{oxalate}} = 2.33 \pm 0.65$ and $K_m^{\text{thiosulfate}} = 1.27 \pm 0.22$). 

Comparatively, sulfate prevented CaOx crystallization, but only at the later time point (48 h). This delayed inhibition could be explained by sulfate’s relatively low transporter affinity compared with oxalate and even thiosulfate (in mM: dPrestin average $K_m^{\text{oxalate}} = 0.87 \pm 0.16$ and $K_m^{\text{thiosulfate}} = 8.7 \pm 3.9$; mSlc26a6 average $K_m^{\text{oxalate}} = 2.33 \pm 0.7$ and $K_m^{\text{thiosulfate}} = 7.99 \pm 4.1$). Interestingly, when dPrestin is knocked down in the MT, the beneficial effects of sulfate and thiosulfate are reversed to what is observed in wild-type flies fed oxalate alone, indicating the mechanism by which both sulfate and thiosulfate exert their biological effects. In flies fed sulfate or thiosulfate plus oxalate, we can assume that sulfate and thiosulfate are rendering the bulk of their competitive properties on gut dPrestin since MT-specific knockdown seems to have no effect on CaOx crystallization. Unfortunately, to our knowledge, there is no available, reliable gut cell-specific GAL4-driver; therefore, given these differences in CaOx crystallization observed in feeding vs. ex vivo, this conclusion is deductive rather than inclusive. Additionally, the Uro-GAL4 driver used to suppress the expression of luminally localized principal cell dPrestin is not significantly penetrant in the MT (~50–70%), especially in the terminal ends where the majority of calcium is stored, and hence where the majority of CaOx crystals are formed. This allows a portion of oxalate to still be secreted into the lumen by currently unknown mechanisms. In this case, CaOx crystallization still occurs, explaining why we still observe significant crystallization, especially when dPrestin knockdown is Uro-GAL4-driven. Given our MT-specific data, Fig. 5 postulates that sulfate and thiosulfate lessen CaOx crystallization via competitive interaction with oxalate at the level of the gut, thereby inhibiting transport out of the gut and/or into the lumen via a dPrestin- or mSlc26a6-dependent mechanism, subsequently and successfully preventing CaOx crystal formation and stone occurrence. Given our data, sulfate and thiosulfate are inhibiting oxalate transport at the luminal boundary of the MT since ex vivo exposure, which could allow oxalate to gain luminal access from ureter entry, had no effect. Hypothetically, by effectively competing with oxalate transport from the gut into the hemocoel and from the principal cell into the lumen, oxalate is prevented from accessing the calcium stores within the tubule lumen and is excreted uncomplexed via the gut pathway. However, the fact that CaOx crystals are still observed in tubules where dPrestin has been effectively knocked down indicates an alternative tubule oxalate secretion mechanism not further characterized in this study, but does indicate that the role of action of sulfate and thiosulfate is competitive inhibition of oxalate via a dPrestin (mSlc26a6)-dependent mechanism.

This current study has investigated, as a proof of principle, the mechanism of sulfate- and thiosulfate-mediated inhibition of oxalate transport via dPrestin and mSlc26a6, as well as these compounds’ effectiveness to limit CaOx crystallization within the MT via a dPrestin dependent-mechanism. Our findings thus far conclude that higher-affinity thiosulfate mimics or dPrestin/ mSlc26a6 oxalate mimics could be effective competitive inhibitors of CaOx crystallization and that this competition is most likely to involve luminal dPrestin in the gut, as well as luminal dPrestin in the MT, although other secretion mechanisms may exist. While blocking or limiting crystallization is an important step, it would be better yet to discover compounds capable of reversing CaOx crystallization.

ACKNOWLEDGMENTS

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DISCLOSURES

M. F. Romero is on the American Journal of Physiology-Renal Physiology Editorial Board and is an employee of the Mayo Clinic College of Medicine.

AUTHOR CONTRIBUTIONS


REFERENCES