HHS Public Access

Author manuscript

Pflugers Arch. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Pflugers Arch. 2021 January ; 473(1): 95–106. doi:10.1007/s00424-020-02495-x.

The anion exchanger PAT-1 (Slc26a6) does not participate in oxalate or chloride transport by mouse large intestine

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Abstract

The membrane-bound transport proteins responsible for oxalate secretion across the large intestine remain unidentified. The apical chloride/bicarbonate (Cl^-/HCO_3^-) exchanger encoded by Slc26a6, known as PAT-1 (Putative Anion Transporter 1), is a potential candidate. In the small intestine, PAT-1 makes a major contribution to oxalate secretion but whether this role extends into the large intestine has not been directly tested. Using the PAT-1 knockout (KO) mouse, we compared the unidirectional absorptive $\left(J_{ms}^{ion}\right)$ and secretory $\left(J_{sm}^{ion}\right)$ flux of oxalate and Cl^- across cecum, proximal colon and distal colon from wild-type (WT) and KO mice *in vitro*. We also utilized the non-specific inhibitor DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid) to confirm a role for PAT-1 in WT large intestine and (in KO tissues) highlight any other apical anion exchangers involved. Under symmetrical, short-circuit conditions the cecum and proximal colon did not transport oxalate on a net basis, whereas the distal colon supported net secretion. We found no evidence for the participation of PAT-1, or indeed any other DIDS-sensitive transport mechanism, in oxalate or Cl^- by the large intestine. Most unexpectedly, mucosal DIDS concurrently stimulated J_{ms}^{Ox} and J_{sm}^{Ox} by 25–68 % across each segment without impacting net transport. For the colon, these changes were directly proportional to increased transepithelial conductance suggesting this response was the result of bidirectional paracellular flux. In conclusion, PAT-1 does not contribute to oxalate or Cl^- transport by the large intestine, and urge caution when using DIDS with mouse colonic epithelium.

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Author contributions

J.W. and M.H. conceived and designed the study; J.W. performed the research; J.W. and M.H. analyzed the data; J.W. wrote the paper.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics approval

All animal experimentation was approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Keywords

Ussing chamber; radiotracer; dicarboxylic acid; solute carrier

Introduction

Oxalate is part of the diet and a non-functional end-product of metabolism. It is normally cleared without complication by the kidneys, although elevated oxalate in the urine (hyperoxaluria) is a risk factor for the formation of insoluble calcium oxalate, a primary constituent of the vast majority of kidney stones [6,21,64]. The gastrointestinal (GI) tract also has a vital role where absorption of ingested oxalate makes an important contribution to its appearance in the urine [90,44,12]. Additionally, the intestine is also a valuable extra-renal pathway for oxalate elimination, displaying a substantial adaptive capacity for secretion of this waste metabolite, capable of reducing urinary output [34,35]. Identifying and characterizing the intestinal transport mechanisms responsible, and how they are regulated, is necessary to help understand oxalate homeostasis and whether these pathways might serve as potential therapeutic targets for hyperoxaluria [63,87].

Oxalate transport across the intestinal epithelium takes place in response to prevailing electrochemical gradients through a combination of transcellular and paracellular routes. The former pathway has been characterized as a secondary active process involving several different anion exchange proteins located within the apical and basolateral membranes of the enterocytes [34]. So far, two members of the Slc26 (Solute carrier) gene family, Slc26a3, known as DRA (Down-Regulated in Adenoma), and Slc26a6 (referred to as PAT-1; Putative Anion Transporter 1) have prominent roles in oxalate handling. DRA and PAT-1 are expressed along the length of the intestine to varying degrees and both are localized to the apical membrane [81,82,45,65], where they primarily function as chloride/bicarbonate (Cl^-/HCO_3^-) exchangers contributing to electroneutral sodium chloride and fluid absorption, HCO_3^- secretion, and intracellular pH (pH_i) regulation [67,80,71,91,66].

Using knockout (KO) mice, deletion of either DRA or PAT-1 brought about major changes to intestinal oxalate transport which, in turn, had significant, corresponding impacts on its overall homeostasis. Two independently developed PAT-1 KO mouse models revealed a key role for PAT-1 in oxalate secretion by the distal ileum [24] and duodenum [47], with its absence causing a distinct hyperoxaluria [24,47], as well as hyperoxalemia and urolithiasis [47]. A long standing, unanswered question is whether this secretory function of PAT-1 also extends into the large intestine. Subsequent characterization of the DRA-KO model found ~ 40 % of oxalate absorption by the cecum and distal colon involved DRA [26], but the apical and basolateral transporters responsible for secretion remain unidentified [89]. PAT-1 is therefore an obvious candidate, even though relative expression levels in the large intestine are very low [81,3,48], or in some cases undetectable [62]. Numerous studies have nevertheless shown PAT-1 is present in mouse [57,41,54,16,74,60] and human [53,58] large intestine, and we previously demonstrated its functional contribution to sulfate (SO_4^{2-}) secretion by mouse cecum [83], but a role in oxalate transport has yet to be reported.

The aim of this study was to determine whether the apical Cl^-/HCO_3^- exchanger, PAT-1 is involved in oxalate transport by the large intestine. We simultaneously measured oxalate and Cl^- flux across cecum, proximal colon and distal colon from PAT-1 KO mice, compared to their wild-type (WT) counterparts, under symmetrical, short-circuit conditions *in vitro*. In addition, with PAT-1 characterized as sensitive to non-specific anion transport inhibitor, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) [51,46,61,17,20,25], and taking advantage of the relative insensitivity of DRA to DIDS [59,18,56,8,75,83], we used this chemical probe as part of a paired experimental design to confirm a role for PAT-1 in WT large intestine and (when applied to KO tissues), highlight any other DIDS-sensitive oxalate transporters at the apical membrane.

Materials and Methods

Experimental animals.

PAT-1 KO (Slc26a6 −/−) mice were obtained from a colony of breeding pairs maintained on a C57BL background. Information on the targeting vector construction and subsequent generation of this model have been described elsewhere [82]. Genotype analysis of all offspring was performed by PCR of DNA isolated from tail snips, as detailed previously [82]. Mice were housed at the University of Florida, where they were given free access to standard chow (diet 7912; Harlan Teklad, Indianapolis, IN) and sterile drinking water. A total of 22 mice of both sexes with a mean body mass of 24.6 ± 1.0 g, n = 12 (PAT1-KO), and 26.5 ± 1.2 g, n = 10 (WT) were used in the following experiments. All animal experimentation was approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were euthanized by inhalation of 100 % CO_2 followed by exsanguination *via* cardiac puncture or cervical dislocation after which the lower portion of the intestinal tract (distal ileum to distal colon) was removed to ice-cold buffer (Figure 1).

Transepithelial flux experiments.

In addition to oxalate, for a number of experiments we also simultaneously measured Cl^- transport since this is one of the main substrates of PAT-1 [81,67], which operates as a DIDS-sensitive Cl^-/oxalate exchanger in mouse distal ileum [24]. Tracing the accompanying flux of Cl^- may therefore assist our interpretation of changes to oxalate, as well as providing information on the contribution of PAT-1 to Cl^- transport in the large intestine. To measure unidirectional oxalate and Cl^- flux, intact pairs of tissues were taken from cecum, proximal colon and distal colon (Figure 1), and mounted in modified Ussing chambers (Physiologic Instruments, San Diego, CA) under short-circuit conditions, as detailed previously [84]. Briefly, each tissue was mounted flat on a slider (P2304, Physiologic Instruments) exposing a gross surface area of 0.3 cm^2 and secured between two halves of a modified Ussing chamber (P2300, Physiologic Instruments), bathed on both sides by 4 mL of buffered saline (pH 7.4), and maintained at 37 °C while being simultaneously gassed and stirred with a humidified 95 % O_2/5 % CO_2 gas mixture. These preparations were continuously voltage-clamped, the mucosal bath serving as ground, and approximately 10 min after mounting 0.27 μCi ^{14}C-oxalate (Specific activity = 115 mCi/mmol) and 0.09 μCi $^{36}Cl^-$ (Specific

activity = 571 μCi/mmol) were added to either the mucosal (M) or serosal (S) half-chamber which was then designated as the 'hot side'. Sodium oxalate (1 mmol/L) was used to achieve the desired final concentration of 1.5 μmol/L oxalate in each half-chamber. To calculate the specific activity (dpm/mmol) of each isotope, 50 μL samples were collected from the 'hot side' at the beginning and end of each experiment. After 15 min, and for every subsequent 15 min interval for 105 mins, the appearance of ^{14}C-oxalate and $^{36}Cl^-$ were detected in 1 mL samples from the opposing 'cold side', which were immediately replaced with 1 mL of appropriate, warmed buffer. Transepithelial potential difference (mV) and short-circuit current, I_{sc} (μA) were also recorded at each 15 min sampling interval. Experiments were divided into two time periods, consisting of an initial 'control' period (Period I, 0–45 min), followed by a second 'experimental' period (Period II) extending from 60–105 min. For this study, DIDS (200 μmol/L) was added to the mucosal half-chamber at the end of Period I (45 min). The activity of ^{14}C-oxalate and $^{36}Cl^-$ in each sample was determined by liquid scintillation spectrophotometry (Beckman LS6500, Beckman-Coulter Inc., Fullerton, CA) with quench correction following the addition of 5 mL scintillation cocktail (Ecoscint A, National Diagnostics, Atlanta, GA). A series of external standards established the validity of counting dual-labeled samples, thus allowing the individual activities of ^{14}C-oxalate and $^{36}Cl^-$ to be calculated based on their relative counting efficiencies after minimizing and accounting for overlap of their energy spectra (Supplementary Figure 1).

Buffer solutions and reagents.

A standard bicarbonate buffer was used in these experiments and contained the following (in mmol/L): 139.4 Na^+, 122.2 Cl^-, 21 HCO_3^-, 5.4 K^+, 2.4 HPO_4^{2-}, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 0.6 $H_2PO_4^-$, 0.5 SO_4^{2-}, and adjusted to pH 7.4 (after equilibrating with 95 % O_2/5 % CO_2), with 10 D-glucose included in the serosal buffer and 10 D-mannitol added to the mucosal. To inhibit spontaneous prostanoid production all buffers contained 5 μmol/L indomethacin (Sigma, St. Louis, MO). The radioisotope ^{14}C-oxalate was a custom preparation from ViTrax Radiochemicals (Placentia, CA) and $^{36}Cl^-$ was purchased as $H^{36}Cl$ from Amersham Biosciences (Piscataway, NJ). Stock solutions of DIDS (Molecular Probes, Eugene, OR) in DMSO were made fresh on the day of each experiment and added to the mucosal half-chamber for a final concentration of 200 μmol/L. The total amount of DMSO presented to the tissues never exceeded 0.1 %.

Calculations and statistical analyses.

The unidirectional flux of oxalate and Cl^- in the absorptive, mucosal-to-serosal $\left(J_{ms}^{ion}\right)$ direction, and secretory, serosal-to-mucosal $\left(J_{sm}^{ion}\right)$ direction across each tissue were calculated from the change in activity of ^{14}C-oxalate and $^{36}Cl^-$ detected on the 'cold side' of the chamber at each 15 min sampling point, having corrected for dilution by replacement buffer between samples. The flux of each anion was expressed per cm^2 of tissue per hour. The recordings of short-circuit current (I_{sc}, μA/cm^2) and potential difference (mV) were used to calculate transepithelial conductance (G_T, mS/cm^2) using Ohm's Law. Net flux of each ion was calculated as: $J_{net}^{ion} = J_{ms}^{ion} - J_{sm}^{ion}$ for pairs of tissues matched on the basis of G_T (no greater than a ± 25 % difference in G_T between pairs). The following data are presented as mean ± SE. Significant differences in oxalate and Cl^- flux, G_T and I_{sc} between WT and

PAT1-KO epithelia were determined by independent t-test based on the mean values obtained during Period I. The effect of mucosal DIDS (200 μmol/L) was evaluated by paired t-test comparing the mean value for Period II to the prior 'control' period (Period I). Where data failed to meet the assumptions of approximate normality and equality of variance, equivalent non-parametric tests were performed. The results of all tests were accepted as significant at $P \leq 0.05$. Statistical analysis was performed with SigmaPlot v14.0 (Systat Software Inc. San Jose, CA).

Results

The transport of oxalate and Cl^- was examined under symmetrical, short-circuit conditions. In most tissues, the flux of these two anions were measured simultaneously, for some experiments only oxalate flux was determined. The accompanying electrophysiological characteristics, I_{sc} and G_T, were combined for all tissues and are presented as a single value for each segment.

Oxalate and chloride transport by the cecum.

Table 1 shows the WT cecum undertakes no overall net transport of oxalate while robustly absorbing Cl^-. To probe the possible role of apical Cl^-/HCO_3^- exchange, 200 μmol/L DIDS was applied to the mucosal bath. Contrary to the anticipated DIDS-sensitivity of oxalate transport, J_{ms}^{Ox} and J_{sm}^{Ox} increased significantly by 43 % and 25 %, respectively. There was, however, no impact on net oxalate flux by WT cecum and these increases in unidirectional flux were not associated with any overt changes to transepithelial permeability, as indexed by G_T. The presence of mucosal DIDS did not affect Cl^- flux in the same way although it significantly reduced the size of the I_{sc} from -0.49 ± 0.21 to -0.28 ± 0.18 μeq/cm^2·h. The net transport of oxalate and Cl^- by the PAT-1 KO cecum were remarkably consistent with WT tissues. The absence of PAT-1 did not produce any significant alterations to the unidirectional flux of either anion. Additionally, G_T was not different between WT and KO ceca, and I_{sc} was unchanged in PAT-1 KO tissues. The PAT-1 KO cecum also exhibited the same response to mucosal DIDS, rather than diminishing oxalate transport, rates were significantly increased by 50 % $\left(J_{ms}^{Ox}\right)$ and 34 % $\left(J_{sm}^{Ox}\right)$ relative to the preceding control period, with no overall change in net flux. Mucosal DIDS also did not produce any corresponding changes to G_T in the PAT-1 KO cecum, while the magnitude of I_{sc} decreased to -0.83 ± 0.26 μeq/cm^2·h from -1.24 ± 0.38 μeq/cm^2·h.

Oxalate and chloride transport by the proximal colon.

The WT proximal colon did not transport of oxalate or Cl^- on a net basis (Table 2). The application of mucosal DIDS produced the same unexpected increases to both J_{ms}^{Ox} (28 %) and J_{sm}^{Ox} (68 %). Of note, these elevations in unidirectional flux were accompanied by enhanced ionic conductance (G_T), and reduced I_{sc} from -1.23 ± 0.23 to -0.92 ± 0.22 μeq/cm^2·h. Mucosal DIDS also significantly increased J_{sm}^{Cl} by 19 %, but did not impact J_{ms}^{Cl} and was insufficient to produce any change to net Cl^- transport. In the PAT-1 KO proximal colon, there was mean net oxalate secretion but this rate was highly variable and not quite different from zero ($P = 0.053$). The PAT-1 KO proximal colon also did not absorb Cl^- on a

net basis, similar to its WT counterpart. The addition of DIDS to the mucosal bath in the absence of PAT-1 failed to diminish oxalate transport but raised J_{ms}^{Ox} and J_{sm}^{Ox} by 55 % and 44 %, respectively, as well as J_{ms}^{Cl} (18 %) and J_{sm}^{Cl} (19 %). Again, these increases occurred in conjunction with a large rise in G_T. Unlike the response of I_{sc} across the WT proximal colon following treatment with DIDS, there was a very modest, but significant reduction in magnitude from −1.78 ± 0.26 to −1.54 ± 0.23 μeq/cm^2·h in the KO epithelium.

Oxalate and chloride transport by the distal colon

Contrary to preceding portions of the large intestine, the WT distal colon exhibited a clear, robust net secretion of oxalate (Table 3). Treatment with mucosal DIDS, however, failed to reduce this net transport. In common with proximal colon and cecum, there were increases of 57 % and 34 % to J_{ms}^{Ox} and J_{sm}^{Ox}, respectively. Unidirectional Cl^- flux was also modestly higher in the presence of DIDS, J_{sm}^{Cl} significantly so, but these changes did not alter net transport of either anion. Once again, these unexpected responses to DIDS coincided with increases to G_T and smaller I_{sc}. The distal colon from WT and PAT-1 KO mice were only significantly different from each other in terms of I_{sc} which was almost 2-fold higher in the latter. Mucosal DIDS produced the same surprising impact on oxalate fluxes, G_T and I_{sc} as seen in the proximal colon. The transport of Cl^- by the PAT-1 KO distal colon and its response to DIDS was also comparable to WT tissues with overall net absorption remaining unchanged.

Discussion

In mouse small intestine, the apical Cl^-/HCO_3^- exchanger, PAT-1 (Slc26a6) makes an important contribution to the secretion of oxalate, helping restrict its net absorption and limit urinary output. Using the PAT-1 KO mouse model, in conjunction with the classical anion transport inhibitor DIDS, we wanted to determine if this role for PAT-1 also extends into the large intestine, where the transporters involved in oxalate secretion have yet to be identified. Under symmetrical short-circuit conditions *in vitro*, we found the cecum and proximal colon did not transport oxalate on a net basis, whereas the distal colon consistently supported basal net secretion. We subsequently found no evidence of a role for PAT-1 or the participation of any DIDS-sensitive apical transport process in oxalate or Cl^- flux across any of these segments. Surprisingly, mucosal DIDS concurrently stimulated J_{ms}^{Ox} and J_{sm}^{Ox} along the entire length of the large intestine. For the colon, these increases to unidirectional oxalate flux were associated with enhanced epithelial ion permeability, as indexed by G_T, leading us to suggest this unexpected response to DIDS was the result of passive, paracellular movement of oxalate. We conclude PAT-1 does not contribute to secondary active oxalate or Cl^- transport by the large intestine, and caution that DIDS affects the permeability of the mouse colonic epithelium and may not be suitable for probing the mechanisms of anion transport.

Oxalate and chloride transport by the cecum

For cecum, J_{ms}^{Ox} and J_{sm}^{Ox} were some of the lowest measured (on average < 20 pmol/cm^2·h) and did not result in any net transport of oxalate (P = 0.116), in agreement with prior studies

[36,50,88,89]. Oxalate flux was unchanged by deletion of PAT-1 (Table 1), thus offering no evidence of a basal role in the cecum. In contrast to oxalate, the cecum supported high rates of Cl^- transport and these were similarly unaffected by the loss of PAT-1 (Table 1), confirming earlier findings that PAT-1 does not participate in net Cl^- absorption, which is deemed exclusively DRA-mediated [1,83]. Overall, this is consistent with mutations of human DRA causing congenital Cl^- diarrhea [43], a phenotype reproduced by the DRA-KO [65], but not PAT-1 KO mouse [82]. In addition to being the dominant apical Cl^-/HCO_3^- exchanger in this segment, DRA also contributes nearly 60 % of J_{ms}^{Ox} [26], although there was no evidence of any opposing synergy with PAT-1 in terms of oxalate transport. For example, the absence of PAT-1 failed to unmask or enhance (DRA-mediated) J_{ms}^{Ox} by the cecum (Table 1), as it does in distal ileum [24]. Similarly, in DRA-KO cecum, J_{sm}^{Ox} (which one might have assumed was PAT-1 mediated) also did not change [26].

In the cecum of germ-free mice, PAT-1 protein expression increased almost 2-fold following a fecal transplant [74]. This study did not examine the impacts on epithelial ion transport but implied this may be associated with PAT-1 mediated oxalate secretion into the cecum *in vivo*, since there was a corresponding 24 % reduction to urine oxalate: creatinine excretion [74]. However, based on our data presented here this may be an unlikely scenario. So far, only colonization with the oxalate-degrading gut bacterium *Oxalobacter formigenes* has been shown to stimulate J_{sm}^{Ox} and overall net secretion by mouse cecum [41,36,50]. While Stern *et al*. [74] do not determine whether this bacterium was present in the administered fecal transplant, subsequent work has revealed *O. formigenes* does not specifically require PAT-1 to induce oxalate secretion by the cecum [30]. An alternative explanation for seeing enhanced PAT-1 expression might therefore be the import of HCO_3^- to maintain pH_i, as documented in proximal small intestine [69,70,79,91]. This would help defend the epithelial cells from acidification due to the intense microbial activity taking place within the cecal pouch. In addition, having demonstrated its involvement in SO_4^{2-} secretion [83], another reason for increasing PAT-1 expression in the cecum may be to supply inorganic sulfur, an essential element for sustaining the gut microbiota [27,13,9], in these formerly germ-free mice.

A defining characteristic of PAT-1 is its sensitivity to disulfonic stilbene derivatives, such as DIDS, when presented at concentrations ≤ 100 μmol/L for various cell models *in vitro* [51,46,61,17,20,25]. Any contribution of PAT-1 to oxalate flux by the native cecal epithelium would therefore be inhibited by 200 μmol/L DIDS, yet we saw the opposite response, a stimulation of J_{ms}^{Ox} and J_{sm}^{Ox} in both WT and KO tissues (Table 1), further refuting a role for PAT-1. In contrast, the relative insensitivity of DRA to DIDS [59,18,56,8,75,83], could explain why there was no diminution of oxalate and Cl^- absorption (Table 1). Furthermore, this stimulation of J_{ms}^{Ox} in particular does not appear to be DRA-mediated, as we would have anticipated a corresponding increase to J_{ms}^{Cl}. Additionally, there was no indication mucosal DIDS had initiated net electrogenic HCO_3^- secretion, based on I_{sc} (Table 1). In mouse distal ileum, DIDS was successfully used to help show PAT-1 contributes to the secretion of oxalate [24] and SO_4^{2-} [86], but these two anions appear to

move across the cecum by completely separate apical pathways. For instance, mucosal DIDS blocks cecal SO_4^{2-} transport, including PAT-1 mediated secretion [83], but here it unexpectedly stimulated J_{sm}^{Ox} (Table 1). The concurrent increases to J_{ms}^{Ox} and J_{sm}^{Ox} suggest enhanced bidirectional paracellular permeability to oxalate. Transepithelial conductance (G_T), an indicator of ionic permeability, especially along the paracellular pathway, was unchanged by mucosal DIDS (Table 1), as reported for distal ileum [24,86], implying the tight junctions and overall epithelial integrity had not been compromised. This resilience of the cecum, in terms of G_T, is consistent with our own prior observations [83], along with those of others [49], following exposure to even higher DIDS concentrations (500 μmol/L). However, just because we have been unable to measure any increase in G_T, does not exclude the possibility of a smaller undetectable change having taken place with consequences for oxalate flux. For example, in WT cecum, the ΔJ^{ox} brought about by DIDS was a mere 5 pmol/cm^2·h (equivalent to a current of 0.08 nA), and with a mean transepithelial potential of 0.4 mV, this represents a conductance of 0.67 μS/cm^2, compared with overall G_T (18 mS/cm^2). Thus, an increase in epithelial ionic permeability of < 1 μS/cm^2 could conceivably account for the observed increases to J_{ms}^{Ox} and J_{sm}^{Ox}.

Oxalate and chloride transport by the proximal colon

The WT proximal colon did not transport oxalate on a net basis (Table 2), contrary to earlier studies of this segment from rabbit [38], rat [32,33], and mouse [41] which all sustained net oxalate secretion, although not all previous studies of the latter model detected net transport [52,60]. In PAT-1 KO proximal colon there were no significant changes to either J_{ms}^{Ox} or J_{sm}^{Ox} relative to WT (Table 2), and even though overall net flux was secretory (−11.29 ± 6.21 pmol/cm^2·h), it was not quite different from zero (P = 0.053). While PAT-1 is indeed expressed in mouse proximal colon [81,41,16], we have not revealed any evidence of a role in either oxalate or Cl^- transport (Table 2). Interestingly, PAT-1 mRNA and protein are strongly upregulated in the proximal colon of a mouse model of chronic kidney disease, associated with enhanced fecal oxalate excretion in WT, but not PAT1 KO, mice *in vivo* [60]. Of note, however, this extra-renal clearance did not result in stimulation of oxalate secretion by the proximal colon when examined *in vitro* [60]. Whether DRA is contributing to J_{ms}^{Ox} and J_{ms}^{Cl}, as it does elsewhere in mouse large intestine, is unknown since the proximal colon was not part of our prior investigations [26]. Furthermore, it is unclear whether DRA is even expressed in this segment. Some investigators have detected DRA mRNA [18,60] and protein [16] in mouse proximal colon, while others report this key apical Cl^-/HCO_3^- exchanger as conspicuously absent [77]. The lack of net Cl^- absorption shown here (Table 2), and in prior functional studies *in vitro* [19,14,15,55,7], would be consistent with no DRA being present. This would also explain the characteristically low rates of luminal alkalinization and why Cl^--dependent HCO_3^- secretion (a hallmark of Cl^-/HCO_3^- exchange) is undetectable [49,92].

Application of 200 μmol/L mucosal DIDS failed to inhibit either J_{ms}^{Ox} or J_{sm}^{Ox}, but unexpectedly provoked a simultaneous increase in each flux, alongside J^{Cl}, for WT and PAT-1 KO tissues, accompanied by a rise in G_T (Table 2), possibly representing oxalate and Cl^- moving through a ‘leakier’ paracellular pathway. In support of this interpretation, further

analysis revealed this change to transepithelial conductance in response to DIDS (ΔG_T) was positively correlated with the associated changes to oxalate (ΔJ^{ox}) and Cl^- (ΔJ^{cl}) flux between Period I and Period II (Figure 2). Of note, the predicted y-axis intercept at $\Delta G_T = 0$ for each of these relationships was either positive or zero, indicating no obvious inhibition by DIDS had taken place. This diverges from earlier work where 100 μmol/L DIDS significantly reduced oxalate and Cl^- flux across rabbit proximal colon, without any impact on G_T, thus offering evidence of a role for anion exchange [38]. However, since DIDS was applied to the mucosal and serosal baths simultaneously in this previous study [38], it is not possible to ascertain whether its actions were specific to the apical or basolateral membrane. Subsequent work using apical membrane vesicles from human proximal colon have identified a DIDS-sensitive SO_4^{2-}/OH^- exchanger, distinct from Cl^-/HCO_3^- exchange, and possessing a high affinity for oxalate [78]. However, the presence of any such mechanism in rabbit would likely have been inactive due to the use of SO_4^{2-}-free buffers [38]. Our present findings for mouse proximal colon offer no evidence of a role for PAT-1, or another DIDS-sensitive pathway for oxalate transport (despite the presence of SO_4^{2-} in the buffer), thereby highlighting the existence of important species differences in this segment of large intestine.

Oxalate and chloride transport by the distal colon

The distal colon displayed robust net secretion of oxalate under symmetrical, short-circuited conditions (Table 3), in common with the vast majority of reports so far for this animal model [41,36,50,29,88,89]. While PAT-1 protein is present in mouse distal colon [41,74], we found no evidence to suggest it was involved in oxalate transport. Neither J_{sm}^{Ox} nor net secretion by PAT-1 KO tissues were significantly reduced (Table 3), and comparable to the sustained net secretion of oxalate already shown for PAT-1 KO distal colon [30]. The mechanism responsible for J_{sm}^{Ox} across WT mouse distal colon is dependent on intracellular carbonic anhydrase (CA), extracellular HCO_3^-, and specifically stimulated by PCO_2 [84], but our observations suggest PAT-1 is an unlikely part of this, regardless of its ability to form a functional unit with CA [2] and respond to systemic acid-base disturbances [72]. Until very recently, PAT-1 was linked with *O. formigenes*-stimulated oxalate secretion by mouse large intestine [41,5,4]. However, colonization of PAT-1 KO distal colon by *O. formigenes* was still able to enhance J_{sm}^{Ox} and net secretion, thus ruling out PAT-1 as an essential target for this bacterium [30]. Furthermore, if there were a role for PAT-1, or a similar anion exchange transport mechanism, then it too would also have been inhibited by mucosal DIDS, but this was instead complicated by the unexpected stimulation of J_{ms}^{Ox} and J_{sm}^{Ox} (Table 3). Based on I_{SC} and Cl^- flux, there was no indication of net anion secretion, but G_T correspondingly increased 20 %. Similar to proximal colon, the change to oxalate flux caused by DIDS (ΔJ^{ox}) was positively associated with G_T, at least for WT (Figure 3A), and ΔJ^{cl}, for WT and KO tissues (Figures 3C and 3D, respectively), likely representing increased paracellular oxalate and Cl^- movement. Again, the predicted y-intercepts at $\Delta G_T = 0$ were either positive or zero, suggesting no underlying DIDS inhibition. Alongside PAT-1, the inability of DIDS to block J_{sm}^{Ox} would rule against the involvement of Slc26a2 (Diastrophic Dysplasia Sulfate Transporter) or Slc4a1 (Anion Exchanger 1), which have been also

suggested as alternative apical anion exchangers potentially involved in oxalate transport by the large intestine [84,87].

In contrast to net oxalate secretion and Cl^- absorption shown for mouse distal colon, both anions are routinely absorbed by this segment of rabbit [37,42,38,40] and rat [23,39,32,33,31,85] large intestine under similar experimental conditions. When the related stilbene inhibitor, SITS was added to the mucosal bath (100 μmol/L), net oxalate and Cl^- absorption by rabbit distal colon were abolished *via* J_{ms}^{Ox} and J_{ms}^{Cl}, respectively, without any change in G_T [37], thus contributing to the idea of oxalate and Cl^- sharing a common transcellular pathway, such as DRA [26]. However, Cl^- transport by rabbit DRA (like other mammalian homologs), is relatively insensitive to the disulfonic stilbenes, possessing an EC_{50} of 560 μmol/L for inhibition by DIDS [45]. This raises uncertainty about whether 100 μmol/L SITS would have been sufficient to reduce J_{ms}^{Ox} and J_{ms}^{Cl} by rabbit distal colon if DRA-mediated. Finally, the ability of mucosal DIDS to modify the ionic permeability of the colonic epithelium has not previously been documented and seems to be a peculiarity of the mouse model since we are not aware of similar impacts on native intestine from other species. Indeed, when mucosal DIDS or SITS have been applied to either rabbit or rat distal colon, even at concentrations as high as 1 mmol/L, G_T was unaffected [73,10,28,22,40,68]. Although our stock solutions were prepared fresh on the day of each experiment and stored in the dark prior to use, the quality of DIDS from commercial suppliers is inconsistent [11]. We therefore cannot exclude potential off-target effects any impurities may have exerted separately from DIDS [76], and were potentially responsible for the impacts on epithelial permeability.

Summary

The mechanism(s) and identity of the transporters responsible for intestinal oxalate secretion have yet to be fully elucidated [87]. In this study, we set out to determine whether the apical Cl^-/HCO_3^- exchanger, PAT-1 contributes to oxalate (and Cl^-) flux across the large intestine, using the PAT-1 KO mouse model, in conjunction with the anion transport inhibitor DIDS. Under symmetrical short-circuit conditions *in vitro*, WT cecum and proximal colon did not perform any significant net oxalate transport, while distal colon supported secretion on a net basis. Comparisons with PAT-1 KO tissues, revealed no evidence for the participation of PAT-1 (or indeed any other DIDS-sensitive apical transport process) in basal unidirectional oxalate or Cl^- flux across any segment. This is in keeping with prior observations where oxalate flux did not consistently correlate with PAT-1 protein expression in mouse large intestine [41], and recent demonstration that *O. formigenes* has no absolute requirement for PAT-1 to stimulate oxalate secretion by cecum or distal colon [30]. Our attempts to distinguish an alternative apical anion exchange mechanism using mucosal DIDS were complicated by unexpected increases to oxalate flux across all segments of the large intestine. These surprising responses likely represent enhanced bidirectional movement of oxalate along the paracellular pathway, since they could be linked to measureable increases in G_T, at least in the colon. Despite their lack of specificity and diverse off-target effects, the disulfonic stilbene derivatives (such as DIDS and SITS) have long been employed over a wide range of concentrations to probe the mechanisms of anion transport by native intestinal

epithelia from various model species without any of the issues encountered here. We therefore urge investigators to exercise caution when attempting to use DIDS with mouse large intestine. Having found no evidence of a role for PAT-1, continued investigations are necessary to resolve the mechanism(s) and transporters responsible for transcellular oxalate secretion by the mammalian large intestine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors wish to thank Carolyn Avila-Duran, Maureen Mohan and Morgan Parker for animal husbandry.

Funding

This work was supported by National Institutes of Health grants DK-108755 and DK-088892 to M. Hatch. J. Whittamore was also supported by a research grant from the Oxalosis & Hyperoxaluria Foundation.

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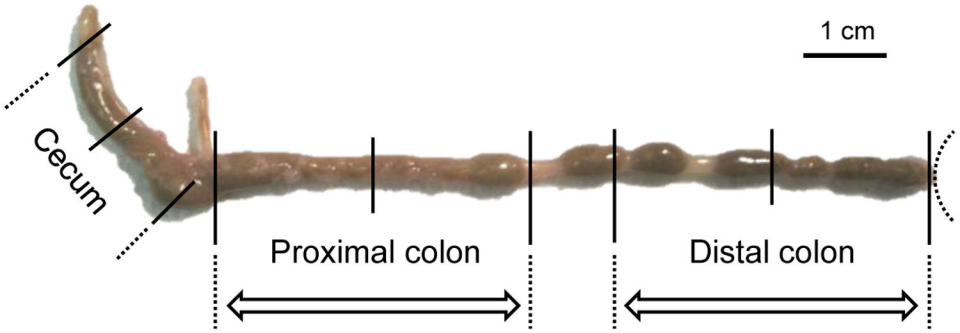

Fig. 1.

A scaled representation of mouse large intestine showing the designation of each segment which was divided into pairs for flux experiments

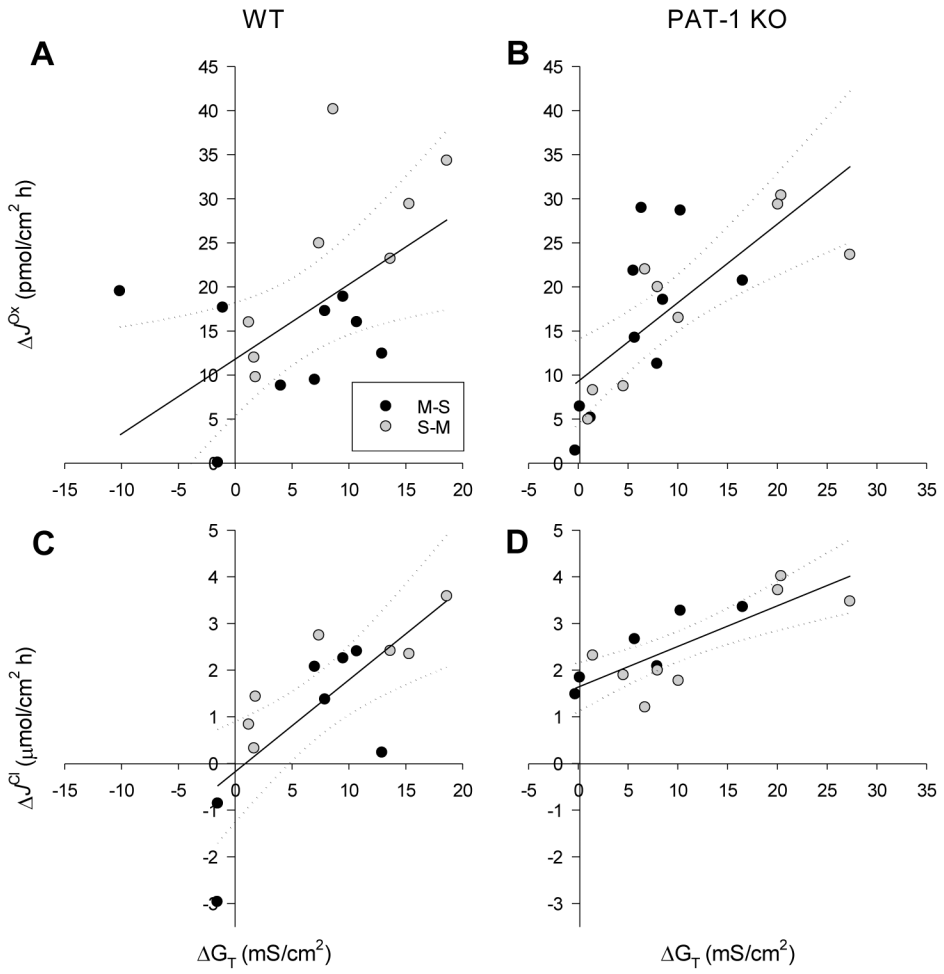

Fig. 2.

Changes to the unidirectional flux of oxalate ΔJ^{ox} (pmol/cm^2·h) and chloride, ΔJ^{cl} (μmol/cm^2·h), following application of 200 μmol/L mucosal DIDS, when plotted as a function of the corresponding change to transepithelial conductance (ΔG_T), for WT and PAT-1 KO mouse **proximal colon**. The presence of a significant correlation, described by linear regression analysis (solid line) with 95 % confidence intervals (dotted lines) for the M–S and S–M data points combined, suggests mucosal DIDS increased the bidirectional paracellular movement of oxalate and chloride. For **WT** proximal colon, oxalate (Panel A): $\Delta J^{ox} = 11.81 + 0.85\ \Delta G_T$ ($F_{1,16} = 6.606$, P = 0.021; $r^2 = 0.292$) and chloride (Panel C): $\Delta J^{cl} = -0.18 + 0.20\ \Delta G_T$ ($F_{1,12} = 14.865$, P = 0.002; $r^2 = 0.553$). For **PAT-1 KO** proximal colon, oxalate (Panel B): $\Delta J^{ox} = 9.29 + 0.89\ \Delta G_T$ ($F_{1,17} = 19.644$, P = <0.001; $r^2 = 0.536$) and chloride (Panel D): $\Delta J^{cl} = 1.64 + 0.09\ \Delta G_T$ ($F_{1,12} = 21.340$, P = <0.001; $r^2 = 0.640$). ΔJ^{ion} was calculated by: J^{ion} Period II (Mucosal DIDS) − J^{ion} Period I (Control), and ΔG_T was calculated by: G_T Period II (Mucosal DIDS) − G_T Period I (Control)

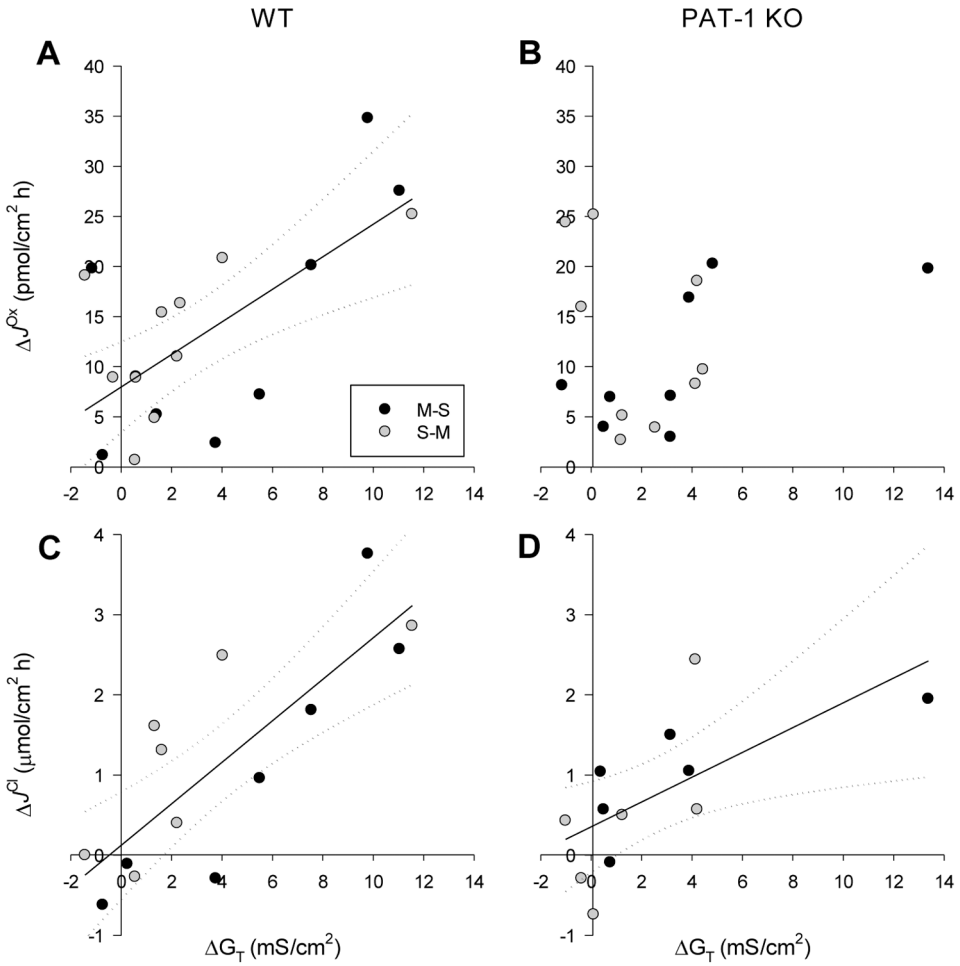

Fig. 3.

Changes to the unidirectional flux of oxalate ΔJ^{ox} (pmol/cm^2·h) and chloride, ΔJ^{cl} (μmol/cm^2·h), following application of 200 μmol/L mucosal DIDS, when plotted as a function of the corresponding change to transepithelial conductance (ΔG_T), for WT and PAT-1 KO mouse **distal colon**. The presence of a significant correlation, described by linear regression analysis (solid line) with 95 % confidence intervals (dotted lines) of M–S and S–M data points combined, suggests mucosal DIDS increased the bidirectional paracellular movement of oxalate and chloride. For **WT** distal colon, oxalate (Panel A): $\Delta J^{ox} = 7.97 + 1.62\ \Delta G_T$ ($F_{1,18} = 13.984$, P = 0.002; $r^2 = 0.437$) and chloride (Panel C): $\Delta J^{cl} = 0.12 + 0.26\ \Delta G_T$ ($F_{1,12} = 24.232$, P = <0.001; $r^2 = 0.669$). For **PAT-1 KO** distal colon, oxalate (Panel B): Pearson correlation, r = 0.277, P = 0.251, n = 19 and chloride (Panel D): $\Delta J^{cl} = 0.35 + 0.16\ \Delta G_T$ ($F_{1,10} = 7.477$, P = 0.021; $r^2 = 0.428$). ΔJ^{ion} was calculated by: J^{ion} Period II (Mucosal DIDS) − J^{ion} Period I (Control), and ΔG_T was calculated by: G_T Period II (Mucosal DIDS) − G_T Period I (Control)

Table 1

A comparison of mean ± SE transepithelial oxalate and chloride flux across the mouse **cecum** and their responses to mucosal DIDS (200 μmol/L) in the presence and absence of PAT-1 (Slc26a6) under symmetrical, short-circuit conditions *in vitro*. Significant differences between WT and PAT-1 KO (Effect of genotype) were determined by independent t-test based on the values obtained during Period I

Genotype	Treatment	J^{Ox} (pmol/cm^2/h)			J^{Cl} (μmol/cm^2/h)			I_{sc} (μeq/cm^2/h)	G_T (mS/cm^2)
		ms	*sm*	*net*	*ms*	*sm*	*net*		
WT	Control (Period I)	15.30 ± 1.65 (9)	19.40 ± 3.23 (9)	−4.10 ± 3.17 (9)	28.22 ± 0.97 (7)	21.10 ± 0.87 (7)	7.13 ± 1.14 (7)	−0.49 ± 0.21 (18)	18.40 ± 0.91 (18)
	+ DIDS (Period II)	21.82 ± 3.75 * (9)	24.21 ± 4.06 * (9)	−2.39 ± 5.53 (9)	27.99 ± 0.90 (7)	21.29 ± 0.88 (7)	6.70 ± 1.34 (7)	−0.28 ± 0.18 (18)	18.52 ± 1.02 (18)
(Effect of DIDS) P-value		0.008	0.006	0.587	0.644	0.750	0.518	0.017	0.870
PAT-1 KO	Control (Period I)	18.43 ± 2.97 (9)	19.71 ± 3.51 (9)	−1.27 ± 5.34 (9)	29.12 ± 1.76 (6)	21.88 ± 0.83 (6)	7.25 ± 1.73 (6)	−1.24 ± 0.38 (18)	20.10 ± 1.19 (18)
	+ DIDS (Period II)	27.59 ± 5.94 * (9)	26.34 ± 5.58 (9)	1.25 ± 9.95 (9)	27.79 ± 1.36 (6)	21.90 ± 0.73 (6)	5.89 ± 1.10 (6)	−0.83 ± 0.26 * (18)	22.78 ± 1.92 (18)
(Effect of DIDS) P-value		0.008	0.048	0.665	0.109	0.970	0.160	0.044	0.101
(Effect of genotype) P-value		0.370	0.930	0.656	0.650	0.532	0.954	0.275	0.262

*Significant difference between Control (Period I) and treatment with DIDS (Period II) by paired t-test.

Table 2

A comparison of mean ± SE transepithelial oxalate and chloride flux across the mouse **proximal colon** and their responses to mucosal DIDS (200 μmol/L) in the presence and absence of PAT-1 (Slc26a6) under symmetrical, short-circuit conditions *in vitro*. Significant differences between WT and PAT-1 KO (Effect of genotype) were determined by independent t-test based on the values obtained during Period I

Genotype	Treatment	J^{Ox} (pmol/cm^2/h)			J^{Cl} (μmol/cm^2/h)			I_{sc} (μeq/cm^2/h)	G_T (mS/cm^2)
		ms	*sm*	*net*	*ms*	*sm*	*net*		
WT	Control (Period I)	38.18 ± 4.27 (8)	34.78 ± 4.71 (8)	3.40 ± 3.02 (8)	12.73 ± 1.24 (6)	11.62 ± 1.15 (6)	1.11 ± 2.17 (6)	−1.23 ± 0.25 (15)	24.71 ± 2.10 (16)
	+ DIDS (Period II)	48.76 ± 5.35 * (8)	58.47 ± 7.70 * (8)	−9.71 ± 5.16 * (8)	13.11 ± 1.61 (6)	13.84 ± 1.48 * (6)	−0.73 ± 2.79 (6)	−0.92 ± 0.22 * (15)	30.31 ± 3.35 * (16)
(Effect of DIDS) P-value		0.016	<0.001	0.017	0.668	0.003	0.094	0.002	0.010
PAT-1 KO	Control (Period I)	30.45 ± 4.18 (9)	41.74 ± 6.74 (9)	−11.29 ± 6.21 (9)	14.21 ± 1.79 (5)	11.98 ± 1.42 (5)	2.23 ± 1.46 (5)	−1.78 ± 0.26 (18)	23.49 ± 1.43 (18)
	+ DIDS (Period II)	47.33 ± 7.09 * (9)	59.91 ± 9.25 * (9)	−12.58 ± 8.20 (9)	16.78 ± 1.86 * (5)	14.20 ± 1.61 * (5)	2.58 ± 1.39 (5)	−1.54 ± 0.23 * (18)	32.40 ± 3.12 * (18)
(Effect of DIDS) P-value		<0.001	<0.001	0.689	0.002	0.006	0.460	0.013	<0.001
(Effect of genotype) P-value		0.217	0.422	0.059	0.502	0.847	0.694	0.080	0.629

* Significant difference between Control (Period I) and treatment with DIDS (Period II) by paired t-test.

Table 3

A comparison of mean ± SE transepithelial oxalate and chloride flux across the mouse **distal colon** and their response to mucosal DIDS (200 μmol/L) in the presence and absence of PAT-1 (Slc26a6) under symmetrical, short-circuit conditions *in vitro*. Significant differences between WT and PAT-1 KO (Effect of genotype) were determined by independent t-test based on the values obtained during Period I

Genotype	Treatment	J^{Ox} (pmol/cm^2/h)			J^{Cl} (μmol/cm^2/h)			I_{sc} (μeq/cm^2/h)	G_T (mS/cm^2)
		ms	*sm*	*net*	*ms*	*sm*	*net*		
WT	Control (Period I)	22.11 ± 2.64 (10)	38.79 ± 3.21 (10)	−16.68 ± 3.13 (10)	15.99 ± 1.07 (7)	11.85 ± 0.27 (7)	4.14 ± 1.27 (7)	−0.62 ± 0.15 (20)	15.18 ± 0.94 (20)
	+ DIDS (Period II)	34.75 ± 6.04 * (10)	51.91 ± 4.01 * (10)	−17.16 ± 4.18 (10)	17.15 ± 0.74 (7)	13.05 ± 0.60 * (7)	4.10 ± 1.09 (7)	−0.17 ± 0.15 * (20)	18.21 ± 1.69 * (20)
(Effect of DIDS) P-value		0.010	<0.001	0.896	0.112	0.040	0.863	<0.001	0.002
PAT-1 KO	Control (Period I)	19.99 ± 3.37 (8)	29.72 ± 3.51 (8)	−9.73 ± 3.71 (8)	16.68 ± 0.65 (4)	11.29 ± 0.19 (4)	5.39 ± 0.76 (4)	−1.17 ± 0.19 † (16)	13.97 ± 1.05 (16)
	+ DIDS (Period II)	28.58 ± 4.93 * (8)	41.84 ± 5.25 * (8)	−13.25 ± 4.39 (8)	17.43 ± 0.60 (4)	11.98 ± 0.50 (4)	5.48 ± 0.96 (4)	−0.78 ± 0.22 * (16)	16.63 ± 1.74 * (16)
(Effect of DIDS) P-value		0.015	0.014	0.478	0.113	0.368	0.951	0.004	<0.001
(Effect of genotype) P-value		0.625	0.075	0.169	0.599	0.186	0.508	0.024	0.399

* Significant difference between Control (Period I) and treatment with DIDS (Period II) by paired t-test.

† Significant difference between WT Control and PAT-1 KO Control by independent t-test.