Small intestine resection increases oxalate and citrate transporter expression and calcium oxalate crystal formation in rat hyperoxaluric kidneys

Short bowel induces hypocitraturia via NaDC-1

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Abstract

Short bowel (SB) increases the risk of kidney stones. However, the underlying mechanism is unclear. Here, we examined how SB affected renal oxalate and citrate handleings for in vivo hyperoxaluric rats and in vitro tubular cells. SB was induced by small intestine resection in male Wistar rats. Sham-operated controls had no resection. After 7 days of recovery, the rats were divided into control, SB (both fed with distilled water), EG, and SB+EG (both fed with 0.75% ethylene glycol (EG) for hyperoxaluric induction) groups for 28 days. We collected the plasma, 24 h of urine, kidney, and intestine tissues for analysis. Hypocitraturia were found and persisted to 28 days for the SB group. Hypocalcemia and high plasma parathyroid hormone (PTH) levels were found in the 28-day SB rats. SB aggravated EG-mediated oxalate nephropathy by fostering hyperoxaluria and hypocitraturia, and increasing the degree of supersaturation and calcium oxalate (CaOx) crystal deposition. These effects were associated with renal upregulations of the oxalate transporter solute carrier family 26 (Slc26)a6 and citrate transporter sodium-dependent dicarboxylate cotransporter-1 (NaDC-1) but not Slc26a2. The effects of PTH on the SB kidneys were then examined in NRK-52E tubular cells. Recombinant PTH attenuated oxalate-mediated cell injury and upregulated NaDC-1 via protein kinase A activation. PTH, however, showed no additive effects on oxalate-induced Slc26a6 and NaDC-1 upregulation. Together,
these results demonstrated that renal NaDC-1 upregulation-induced hypocitraturia weakened the defense against Slc26a6-mediated hyperoxaluria in SB kidneys for excess CaOx crystal formation. Increased tubular NaDC-1 expression caused by SB relied on PTH.

**Keywords**: short bowel, hyperoxaluria, hypocitraturia, calcium oxalate crystal, parathyroid hormone

**Clinical Perspectives**

SB increased the risk of kidney stones.

This study showed that SB mediated Slc26a6 and NaDC-1 upregulation, which aggravated oxalate nephropathy in rats by fostering hyperoxaluria and hypocitraturia, increasing the degree of supersaturation and CaOx crystal deposition via the parathyroid hormone (PTH)/protein kinase A (PKA) pathway.

Our results provide novel insights into the intrarenal mechanism of SB patients associated with kidney stones and evidence regarding how PTH affects the citrate transporter.
INTRODUCTION

Patients who have undergone bowel resection often have kidney stones. The short bowel (SB) patients had a largely increased incidence of calcium oxalate (CaOx) kidney stones 8–10 years after small intestine resection (1, 2). Low urine volume and pH, hyperoxaluria, and hypocitraturia are known to be the risk factors for patients with SB surgery who develop kidney stone formation (2). These factors were attributed as a result of intestinal dysfunction after resection. It however is unclear whether there is a renal mechanism involved in these changes.

Abnormal oxalate handling usually leads to CaOx stones, which is the most common type of kidney stone. Oxalate can be eliminated by intestinal and renal clearances after dietary intake or endogenous synthesis from liver. Oxalate is secreted actively by the anion transporter solute carrier family 26 (Slc26) protein in intestinal epithelium and renal tubular cells. Two members of this family, Slc26a2 and Slc26a6, are present in both intestine and kidney and contribute to different degrees of oxalate exchange activity with other anions (3, 4). In the Slc26a6 null mice, the most striking phenotype was hyperoxaluria-mediated CaOx urolithiasis, which was caused by defective intestinal secretion of ingested oxalate (3). In SB syndrome, unabsorbed fats bind to Ca\(^{2+}\) and leave the increased oxalate to be absorbed from the colon as a result of hyperoxaluria and CaOx stone formation (5). Nephrolithiasis in SB is known to be
prevented by maintaining the patient on a low oxalate diet (6). Moreover, oxalate is toxic to renal tubular cells (7). The cell debris of injured tubular cells favors CaOx crystal formation as a seed in crystal growth and aggregation other than hyperoxaluria.

Apart from hyperoxaluria, SB also decreased the intestinal absorption and therefore diminished the urinary excretion of citrate as a result of hypocitraturia in patients (2). Citrate normally acted as an inhibitor of CaOx crystallization, which had a higher affinity for Ca\(^{2+}\) than oxalate and reduced free Ca\(^{2+}\) in the urine to prevent supersaturation in stone formation (8, 9). In addition, citrate also adhered to the formed crystal surface to reduce crystal growth and attach to the epithelia (10). The sodium-dependent dicarboxylate cotransporter-1 (NaDC-1) is the primary citrate transporter, most of which are distributed in the intestine and the proximal tubule of the kidneys (11). We previously showed enhanced renal NaDC-1 expression in the hyperoxaluric rats and this contributed to hypocitraturia with a loss of the anticrystallization defense against CaOx crystal formation (12). A previous study showed that NaDC-1 reciprocally regulated Slc26a6 activity as Slc26a6 inhibited NaDC-1, and NaDC-1 activated Slc26a6 for citrate reabsorption and oxalate secretion, respectively (13). The destruction of this function likely leads to CaOx formation.

Researchers developed an experimental platform in the rat model of SB that
mimics human kidney stone formation after SB resection or bypass (14). In that study, resected rats fed with high oxalate and lipids presented higher hyperoxaluria than rats without resection (14). They found that crystal formation was not related to urinary supersaturation and suggested that oxalate transporters induced local saturation inside the kidney, which may be responsible for crystal deposition (14). This raises an interesting question regarding whether enhanced oxalate secretion via its transporter boosts CaOx crystal formation as a renal mechanism in SB rats when challenged with hyperoxaluria. This issue however was not yet tested. We, therefore, hypothesized that inappropriate renal handling of oxalate and citrate may contribute to CaOx crystal formation in SB kidneys. The present study used ethylene glycol (EG) as a precursor of oxalate to induce hyperoxaluria and CaOx crystal formation in male Wistar rats and to assess the in vivo effects of short bowel (SB). Changes in oxalate and citrate transporter expressions in the intestines and the kidneys were examined to see how SB affects oxalate and citrate handling. In order to gain insight of the SB effects, we examined the cellular mechanism of changes in oxalate and citrate transporter expressions in renal tubular cells in vitro.
MATERIALS AND METHODS

Experimental animals and study designs

Male Wistar rats (BioLASCO, the authorized distributor for Charles River Laboratories in Taipei, Taiwan) weighing approximately 200–250 g were used in this study as experimental animals. A total of four groups included six rats in each group, and the experimental period was 28 days. The control group received the sham operation as described below. Distilled water was given as drinking water for the control (C) group. The ethylene glycol (EG) group received a sham operation and were fed with 0.75% of EG in the drinking water. The short bowel (SB) group received an 80% small intestine resection as described below and were fed with distilled water. The SB+EG group received an 80% resection and were fed with 0.75% of EG. All rats had free access to food and consumed standard chow. Drinking water was limited to 40 mL per day for each rat. The rats were housed in metabolic cages on days 1 and 28 for a 24 h collection of urine, feces, and monitoring their food intake.

After 28 days, the rats were anesthetized using sodium pentobarbital (65 mg/kg, intraperitoneal) for the collection of arterial blood and sacrificed to harvest the kidneys and colons after transcardiac perfusion (12). The kidneys were sliced and kept in 10% formalin for CaOx crystal analysis. The renal tissues and colons were
kept at -80°C for further biochemical examination. All animal experiments were
performed at the Far Eastern Memorial Hospital in compliance with the Guide for the
Care and Use of Laboratory Animals (published by National Academy Press,
Washington DC, 2011) and were reviewed and approved by the Institutional Animal
Care and Use Committee of Far Eastern Memorial Hospital (IACUC-2018-FEMH-08)
in Taiwan.

**Surgical procedure of small intestine resection**

The rats were anesthetized using sodium pentobarbital sodium (65 mg/kg,
intraperitoneal). The rats were then placed on a servo-null heating plate to maintain
their body temperature around 37°C via a rectal thermometer. As shown in Figure 1,
the abdomen was opened under disinfection. The duodenum, small intestine, and
cecum were all exposed to the abdominal cavity. To avoid resected tissue bleeding,
the branches of the superior mesenteric artery were ligated as indicated in Figure 1.
Approximately 80% of the small intestine was removed between the duodenum and
cecum. Electrocautery was used to stop the bleeding during the resection with
minimal blood loss. The residual intestine and cecum were anastomosed with a 6-0
prolene suture, and the abdominal wall was sutured. A sham operation was performed
with abdominal opening and exposure of the gastrointestinal tract but without
resection. After surgery, the rats were placed in individual cages and warmed by a heat lamp to avoid body temperature loss. Distilled water containing 5% glucose was supplied for one day after surgery and standard chow was given 24 h after surgery. The mortality rate of SB rats is 20% (3/15). Two of them died because of intra-abdominal bleeding, the third rat was sacrificed because of malnutrition by loss of more than 20% of body weight after 4 days of surgery.

**Plasma chemistry and urinalysis**

The blood and 24 h urine samples were then centrifuged and the supernatant was sampled for the following biochemical analyses. The urinary pH was measured using a standard pH meter. The concentration of Ca\(^{2+}\) in the plasma and urine were measured using an electrolyte analyzer (Dri-Che 3500i, Fuji, Tokyo, Japan) as described previously (12). The plasma PTH levels were determined using a commercial kit (RayBiotech, Norcross, GA) following the manufacturer’s instruction. The oxalate and creatinine levels in the plasma and urine were determined using commercial kits (Trinity Biotech Plc, Bray, Co. Wicklow, Ireland; BioQuant, Reinach, Switzerland, respectively) as described previously (12). Creatinine clearance (Ccr) was then calculated accordingly. The urinary magnesium and citrate levels were determined using commercial kits (BioAssay Systems, Hayward, CA, USA;
r-Biopharm, Woodfield Boulevard, Caringbah, Australia, respectively) as described previously (12). Urinary lactate dehydrogenase (LDH, a marker of tubular damage) was measured using a commercial kit (Roche Applied Science, IN, USA) as described previously (18). The degree of urine supersaturation was calculated according to the formula of the AP(CaOx) index

\[ (4076 \times \text{calcium}^{0.9} \times \text{oxalate}^{0.96})/((\text{citrate}+0.015)^{0.60} \times \text{magnesium}^{0.55} \times \text{urine volume}^{0.99}) \]

as described previously (15).

Detection of CaOx crystals in rat kidneys

The kidney tissue was sectioned into 5 μm of thickness and Pizzolato's staining was applied to recognize the presence of CaOx crystals as described previously (16). In brief, a mixed solution of equal quantities of 5% silver nitrate and 30% hydrogen peroxide was applied to slides to cover the tissue sections. The slides were exposed to light from a 60-W incandescent lamp at a distance of 15 cm for 30 min. The slides were washed with distilled water, counterstained with nuclear first red solution for 5 min, and dehydrated in 95% alcohol. The black regions were identified as CaOx crystals. The renal section was magnified at 200X under an optical microscope and five images with an area of 100 μm² in one section were taken to count and average the crystal numbers.
Tubular cell culture and drug treatment

To examine the effects of PTH on NaDC-1, NRK-52E cells were used because the PTH receptor is known to be present in this cell line as previously reported (17).

The cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). This cell line was originally derived from the American Type Culture Collection lines, CRL-1571. All culture medium and supplements were purchased from Thermo Scientific HyClone (South Logan, UT, USA). The cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in 5% CO2 at 37 °C. The cells were maintained and subcultured every 3 days when they reached confluence. The cells were seeded in a 6-well plate for 2 days. On the day of experiment, 100 μL of culture medium of each well was sampled and mixed with chemicals to achieve the final concentration as below. The cells were treated with phosphate-buffered saline (as control, pH 7.4), oxalate (Oxa, 0.5 mM, Sigma-Aldrich, St. Louis, MO, USA), recombinant PTH (1-34) (0.1 μM, Sigma-Aldrich), selective protein kinase A (PKA) inhibitor H89 (0.5 μM, Sigma-Aldrich), or KT5720 (1 μM, Sigma-Aldrich) alone or in combination for 48 h. The doses for PKA blockers were chosen based on their IC50 values.
**Cell viability and assays**

We used a commercial kit to measure the levels of lactate dehydrogenase (LDH) (Roche Applied Science, IN, USA) in the cell culture medium to evaluate cell damage as described previously (18). An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) assay was used to measure the cell viability. Briefly, the medium was removed and the cells were washed three times with phosphate buffered saline (PBS, pH 7.4) after 48 h. MTT (0.5 mg/mL in PBS) was then added to cells and incubated for 4 h at 37°C. MTT formazan crystals were then dissolved in dimethyl sulfoxide and we measured the optical density (O.D.) with an enzyme-linked immunosorbent assay plate reader (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) at 570 nm. The cell viability (%) was calculated using the following formula: (O.D. of treated sample/O.D. of control sample) × 100.

**Western blot analysis for protein expression**

We examined the expressions of Slc26a2, Slc26a6, NaDC-1, and β-actin by immunoblot analysis in the cultured cells and kidney tissue, as described previously (12). Primary antibodies against the above proteins were obtained from Santa Cruz.
Biotechnology (Santa Cruz, CA, USA) for Slc26a6, NaDC-1, and β-actin, and from abcam (Cambridge, UK) for Slc26a2. Briefly, equal amounts of total protein were separated in denaturing sodium dodecyl sulfate polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham-Pharmacia Biotech, Little Chalfont, UK). The membranes were then incubated with the appropriate primary antibody (1:1000 for Slc26a2 and Slc26a6; 1:500 for NaDC-1; and 1:2000 for β-actin) overnight at 4°C. After washing, the membranes were incubated for 1 h at room temperature with the corresponding horseradish-peroxidase-conjugated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA). The bound antibodies were visualized using an enhanced chemiluminescence kit (Amersham-Pharmacia Biotech) and Kodak film. The band density was measured semi-quantitatively using an image analysis system (Diagnostic Instruments, Sterling Heights, MI, USA). The amount of each protein was expressed relative to the amount of actin.

**Statistics**

Numerical data are presented as the mean ± S.E.M (the standard error of the mean). We conducted our statistical analysis using Prism 3.0 for Windows software (GraphPad Software Inc, San Diego, CA, USA). The differences between subgroups
were analyzed using an unpaired *t*-test or one-way ANOVA. Differences were regarded as significant at P < 0.05.
RESULTS

Basic health parameters before hyperoxaluric induction

Before hyperoxaluric induction, SB rats (n=12) showed similar body weight, daily food intake, urine output and pH, and feces when compared to those in the Sham rats (n=12) after 7 days of recovery from surgery (Table 1). SB significantly decreased the daily urine excretion of Ca\(^{2+}\) and citrate when compared to the Sham rats. A power analysis showed that the sample size of 12 in Table 1 has an 80% power to detect an effect size as a Cohen’s $d$ greater than 0.8 unit assuming a 5% significance level.

Effects of SB and EG on basic health parameters for 28 days of treatment

After 28 days of hyperoxaluric induction, the gain of body weight was similar among four subgroups (n=6 in each, Figure 2A). There was no statistical difference in the amount of daily food intake, urine output, and feces after 28 days (Figures 2B–2D). The ratio of the kidney weight to body weight (KW/BW) increased significantly in the SB+EG group (1.3±0.1%) when compared to the SB (1.1±0.1%) or EG (1.1±0.1%) group (Figure 2E).
Changes in plasma levels of Ca\(^{2+}\), citrate, oxalate, and PTH

The plasma Ca\(^{2+}\) levels were lowered in the SB (8.6±0.2 mM) and SB+EG (8.6±0.1 mM) groups when compared with their corresponding control groups (9.6±0.2 and 9.3±0.2 mM in the C and EG groups, respectively) with a similar degree of hypocalcemia (Figure 3A). There was a slight increase in the plasma oxalate in the EG group; however, this was insignificant when compared with the control group (Figure 3B). The SB or SB+EG groups showed no effect on the plasma oxalate level. The plasma citrate levels were similar among groups although insignificant low levels were found in the SB group (Figure 3C). The plasma PTH levels were significantly increased in both the SB (36.6±5.1 pg/mL) and SB+EG (42.7±5.5 pg/mL) groups when compared to their corresponding control groups (22.1±2.1 and 26.4±2.6 pg/mL in the C and EG groups, respectively) (Figure 3D).

Effects of SB and EG on urinary chemistry

The urinary pH 7.1±0.1 was lowered in the EG group; a similar low urinary pH 7.0±0.3 was also found in the SB+EG group when compared to 8.0±0.1 in the control group and 7.7±0.3 in the EG group, respectively (Figure 4A). The urinary Ca\(^{2+}\) excretion was similar among groups (Figure 4B). Hyperoxaluria was successfully induced in both EG groups (Figure 4C). SB alone did not affect the oxalate excretion.
but did significantly augment the severity of hyperoxaluria in the SB+EG group (39.4±3.9 mg/day) when compared to the EG group (18.6±1.5 mg/day). EG lowered the urinary citrate level (Figure 4D); this was similar to our previous finding (12). SB also induced hypocitraturia and augmented EG-induced hypocitraturia in the SB+EG group. Creatinine clearance (Ccr) was similar among groups, indicating these treatments did not affect renal function after 28 days (Figure 4E). Because tubular damage aggregates CaOx crystals, we next tested whether SB may affect tubular damage in the EG kidney. Using a reliable marker of tubular damage, our results showed that urinary excretion of lactate dehydrogenase (LDH) in the EG group (6.4±0.8 units/day) increased significantly compared with that in the control group (1.3±0.5 units/day) (Figure 4F). SB itself had no effect on LDH excretion (2.1±0.7 units/day), but markedly aggravated LDH excretion in the SB+EG group (10.6±1.5 units/day). In calculating the AP(CaOx)index to estimate the degree of supersaturation, both EG groups had higher AP(CaOx)index than their corresponding control groups (Figure 4G). The increased AP(CaOx)index, however, was more prominent in the SB+EG group (125±48 unit) when compared to the SB (6±3 unit) or EG (50±11 unit) group.

Renal CaOx crystal deposition
Using Pizzolato’s staining, we found that there was no Ca\(^{2+}\) crystal deposition in the kidneys of the control and SB groups (the left two pictures in Figure 5A). Ca\(^{2+}\) crystals were found in the hyperoxaluric kidneys of the EG group and this deposition caused tubular lumen dilation (the right upper picture in Figure 5A). There was more CaOx crystal deposition in the SB+EG kidney and more tubular dilation, possibly due to severe obstruction (the right lower picture in Figure 5A). Moreover, a mild interstitial fibrosis surrounding the dilated tubule and tubular atrophy were found in the SB+EG kidney. The number of crystals per area was significantly increased in the EG group (582±21), and this was aggravated in the SB+EG group (948±124) (Figure 5B).

Changes in oxalate and citrate transporter expression in the kidneys and intestines

The severe hyperoxaluria and hypocitraturia seen in the SB+EG rats were possibly a result of impaired oxalate and citrate handling in the kidneys and intestines. We, therefore, examined the changes in oxalate and citrate transporter expression in both tissues. The renal expressions of Slc26a6, but not Slc26a2, in the EG and SB+EG groups were significantly increased when compared with those in their corresponding control groups (Figures 6A and 6B). SB alone showed no effect on the
renal expression of Slc26a6 and Slc26a2. Compared to the control, EG increased NaDC-1 expression (Figure 6C); this was consistent with our previous finding (12).

NaDC-1 expression was markedly upregulated in the SB group, this prominent change in the NaDC-1 expression was also found in the SB+EG group. These changes were correlated to hypocitraturia. The intestinal expression of Slc26a6 was significantly increased in the SB group but not in the EG and SB+EG groups when compared to the control group (Figure 6D). The intestinal expressions of Slc26a2 and NaDC-1 were similar among groups (Figures 6E and 6F).

**PTH directly upregulates NaDC-1 via PKA signaling**

As a citricemic hormone, we further tested the effect of PTH in the tubular cells. An increase in LDH release (28.9±3.1 units/L) and a reduction in cell viability (59±5%) as tubular cell damage were found in the oxalate (Oxa) group when compared to the control group (7.9±0.8 units/L in LDH and 100±6% in cell viability, n=6 in each group, Figures 7A and 7B), indicating tubular cell damage caused by oxalate. These were attenuated in the PTH+Oxa group (18.9±1.8 units/L in LDH and 77±3% in cell viability). No effect on the cell viability, however, was found in the PTH group. The Slc26a6 and NaDC-1 expressions were increased in the Oxa group (Figures 7C and 7D). The treatment of PTH alone showed no effect on Slc26a6
expression but significantly upregulated NaDC-1 in the PTH group. The increased
Slc26a6 and NaDC-1 expressions in the PTH+Oxa group were similar to those seen in
the Oxa and PTH groups, respectively.

The main signaling pathway underlying the PTH receptor is PKA activation (19).
We, thus, examined whether the PKA pathway was involved in changes in Slc26a6 or
NaDC-1 expression. Again, Slc26a6 expression was not affected in the PTH group.

Two specific blockers of PKA, H89 and KT5720, did not affect Slc26a6 expression in
the PTH-treated groups (Figure 7E). The inhibition of PKA activity by the selective
blockers H89 and KT5720 significantly attenuated the NaDC-1 upregulation in the
PTH-treated groups (Figure 7F).
DISCUSSION

This study demonstrated more CaOx crystal deposition in the SB-treated hyperoxaluric kidneys and confirmed previous findings in the rat model and in the SB patients. As in the schematic diagram shown in Figure 8, secondary hyperparathyroidism to enhance PTH release, as a result of hypocalcemia in the SB rats, directly upregulated the citrate transporter NaDC-1 via the PKA pathway in tubular cells. This possibly contributed to hypocitraturia in the SB rats and would weaken the intrarenal defense mechanisms against CaOx crystal formation. Not only hypocitraturia but also severe hyperoxaluria caused by oxalate transporter Slc26a6 upregulation after oxalogenic treatment aggravated urinary supersaturation for CaOx crystal formation and deposition in the SB kidneys.

Our results showed that 7-day SB rats demonstrated hypocalciuria (Table 1). While we did not measure plasma Ca\(^{2+}\) level at this induction time, it was significantly lowered after 28 days (Figure 3A). Previous studies demonstrated that SB induced steatorrhoea with less intestinal Ca\(^{2+}\) absorption and insufficient nutrients for the impaired formation of albumin, and these, in turn, lowered the plasma Ca\(^{2+}\) concentration (20). In this study, hypocalciuria was possibly due to intestinal malabsorption, but this was no longer seen after 28 days of SB induction even when there was hypocalcemia (Figures 3A and 4B). Hypocalciuria was associated with
normal urine Ca\(^{2+}\) excretion and clearly indicated that a deficiency of the Ca\(^{2+}\) handling in the SB kidneys was present. We suggest that the hypocalcemia seen in our SB rats was not only dependent on intestinal malabsorption but also relied on impaired renal Ca\(^{2+}\) handling.

Hypocalcemia is known as a strong stimulus to the parathyroid gland for PTH release (21). PTH is not only related to the maintenance of Ca\(^{2+}\) homeostasis; a previous study revealed that PTH also acts as a citricemic hormone to maintain the bodily citrate balance (22). In this study, severe hypocitraturia was found in the SB rats from day 7 to 28 (Table 1 and Figure 4D). This was consistent with clinical observations in SB patients with persistent hypocitraturia (23). In addition to exogenous resources from the diet, citrate can be synthesized endogenously in mitochondria and enters the tricarboxylic acid cycle for energy production (24).

Energy consideration is important to SB patients who are thought to require a long-term parenteral supplement (25). SB surgery significantly decreased the available tissues of the intestines for citrate absorption from the diet and left a short passage time for citrate in the remaining intestinal tissues. Plasma citrate is known to be maintained by bone turnover, intestinal absorption, and renal clearance (22). NaDC-1 is the main transporter responsible for citrate reabsorption with cotransport of Na\(^+\) in the intestines and kidneys (13). We partially ruled out the bone effects
because there was a similar weight gain between the SB and control groups (Table 1 and Figure 2A). Our results showed that intestinal NaDC-1 expression in SB rats was similar to other groups (Figure 6F), indicating there was no compensation for enhancing citrate absorption in the remaining intestinal tissues.

Our results further demonstrated that NaDC-1 expression was upregulated in the SB kidneys (Figure 6C); this likely enhanced the citrate reabsorption by the reduction of renal excretion. Enhanced citrate reabsorption via NaDC-1 upregulation in SB rats possibly maintained plasma citrate as a result of normocitrinemia (Figure 3C). A reduction of renal citrate excretion might benefit SB rats by restoring the plasma level of citrate for energy utilization. However, this might induce hypocitraturia (Figure 4D) and weaken the intrarenal defenses against CaOx crystal formation, especially when facing hyperoxaluria.

To consider the underlying mechanism for NaDC-1 upregulation-induced hypocitraturia, our results showed a parallel increase in plasma PTH and renal NaDC-1 expression in the SB rats (Figures 3D and 6C). The reduction of renal citrate clearance by PTH was postulated to increase tubular reabsorption via the effect of citrate transporters (22). In this study, we also confirmed a direct effect of PTH on the NaDC-1 expression in the PTH receptor-expressed tubular cells, NRK-52E (17), as that recombinant PTH significantly increased the NaDC-1 expression (Figure 7D).
The underlying signal pathway was dependent on PKA activation as PKA inhibition abrogated the PTH-mediated NaDC-1 upregulation (Figure 7F). According to our knowledge, this was not known before. We concluded that hypocalcemia in our SB rats stimulated citricemic hormone PTH secretion, which increased the plasma levels of Ca\(^2+\) and citrate, but only citrate returned to the normocitrericemic level.

PTH affected the NaDC-1 expression, and we also found that hyperoxaluria in rats or a direct oxalate treatment in tubular cells also increased the NaDC-1 expression (Figures 6C and 7D). A previous study showed that chronic metabolic acidosis led to an increase in rat renal cortical NaDC-1 mRNA and protein expression in the apical membrane of proximal tubules (26). Acidosis directly increased NaDC-1 activity and induced hypocitraturia (27). As low urinary pH was found in the EG-treated rats (Figure 4A), we speculated that the metabolic acidosis seen in hyperoxaluric kidneys may account for NaDC-1 upregulation. Oxalate might also induce intracellular acidosis in tubular cells and this likely enhanced the NaDC-1 expression. Considering the effect of PTH together with oxalate (or hyperoxaluria), our results showed that there was no additive effect of acidosis and PTH on NaDC-1 upregulation. We speculated that in vivo hyperoxaluria or in vitro oxalate possibly shares similar signal pathways, such as PKA activation after PTH treatment.

EG is known to be oxidized to oxalate by the liver and eliminated from
circulation by intestinal secretions and urinary excretions (28). SB might decrease the intestinal permeability of EG and further reduce its efficacy for hepatic oxalogenesis. However, the hyperoxaluria seen in the SB+EG group was more severe than that in the EG group (Figure 4C); this indicated that a shorter length of intestines in SB rats did not affect the EG absorption in the remaining intestinal tissues (duodenum and colon) for hyperoxaluric induction. For intestinal oxalate elimination, our results showed that the oxalate secretion protein Slc26a6, but not Slc26a2, was upregulated in SB intestines (Figures 6D and 6E). This likely enhanced the intestinal elimination of oxalate via Slc26a6 in SB rats and prevented hyperoxalemia caused by EG absorption. A previous study showed that SB rats fed with a normal diet for 8 weeks demonstrated more urinary oxalate excretion (14). Contrary, our SB rats without EG treatment showed no hyperoxaluria. The shorter induction time (4 weeks) likely limited spontaneous hyperoxaluric formation in our SB rats. Intestinal Slc26a6 and Slc26a2 expressions in the SB+EG group were similar to those in the control group (Figures 6D and 6E). This largely limited the intestinal oxalate elimination of SB rats in response to EG-induced oxalogenesis. The unchanged intestinal Slc26a6 expression in the SB+EG rats was consistent with the previous findings. They showed that Slc26a6 expression in the intestinal or colon segments was not changed in the mini-gastric bypass rats fed with a high fat and high oxalate diet to induce
hyperoxaluria (29).

These results clearly indicated that there was a renal effect for the compensatory elimination of plasma oxalate if there was a deficiency in the intestinal oxalate secretions. Increased renal Slc26a6 expression contributes to hyperoxaluria (30). Our results showed consistent results for a significant increase in Slc26a6 expression not only in the EG kidneys but also in the SB+EG kidneys (Figures 6A). A slight elevation in the Slc26a2 expression in the SB+EG kidneys when compared with the control kidneys might also contribute to hyperoxaluria (Figure 6B). Together with hypocitraturia, severe hyperoxaluria increased the degree of supersaturation in SB+EG group for CaOx formation and deposition (Figures 4E and 5). A previous study in the SB patients revealed urinary CaOx supersaturation and CaOx plugs present in the renal inner medullary collecting ducts (31). Compared to the present study, the rat animal model showed a dispersed distribution of CaOx crystals in the SB+EG kidneys with an increase in the number, which led to tubular obstruction and dilation (Figure 5). Therefore, post-obstruction in the hyperoxaluric SB kidneys might impair renal function and lead to end-stage kidney disease (32).

In conclusion, SB aggravated hyperoxaluria-induced renal tubular damage and CaOx crystal formation through hypocitraturia. The underlying mechanism of hypocitraturia was dependent on the enhanced effects of PTH. Increased PTH levels
from SB directly increased citrate transporter NaDC-1 expression via the PKA pathway in tubular cells. Together with the effect of hyperoxaluria or oxalate on the increased Slc26a6 expression, hypocitraturia worsened CaOx crystal formation in SB through poor anticrystallization against hyperoxaluria.

LIMITATION

The present study induces SB for 35 days only, and this induction time was shorter than clinical presentation in the resected patients who developed oxalate nephropathy. Severe hyperoxaluria in our SB rats was induced by feeding the rats with an oxalogenic diet; this was not a spontaneous formation in terms of the disease progression in SB patients. However, our animal model shared some important features similar to SB patients, such as hypocalcemia, hypocalciuria, hypocitraturia, and hyperoxaluria (33). This study only focused on changes related to secondary parathyroidism in our experimental models. Other hormones related to the maintenance of plasma Ca$^{2+}$ levels, such as calcitriol and calcitonin might have an influence on CaOx crystal formation caused by SB. Further study is required to examine the mechanisms in more detail regarding the hormonal effects on the SB kidneys.
GRANTS

This study was funded by grants from Far Eastern Memorial Hospital (FEMH-2018-C-046 and 105-FEMH-FJU-04) to Tseng YS and Ma MC and the Ministry of Science and Technology (108-2314-B-030-004-MY3) to Ma MC, Taiwan.

AUTHOR CONTRIBUTIONS

Y.-S.T. and M.-C.M. conceived and designed the research plan. Y.-S.T., W.-B.W., Y.C., F.L.Y., and M.-C.M. performed the experiments and analyzed the data. Y.-S.T., W.-B.W., F.L.Y., and M.-C.M. interpreted the results. Y.-S.T. and M.-C.M. drafted the manuscript, with editing and revisions from W.-B.W. and Y.C. All authors approved the final version of the manuscript.

ABBREVIATIONS

SB, short bowel; EG, ethylene glycol; Slc26a2/6, Solute Carrier Family 26 Member 2 or 6; NaDC-1, sodium-dependent dicarboxylate cotransporter-1; AP(CaOx) index, the ion activity product of calcium oxalate; Oxa, oxalate; PTH, parathyroid hormone; PKA, protein kinase A.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.
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Table 1. Basic health parameters of rats before hyperoxaluric induction. The data were obtained from a metabolic cage study collected over a 24 h period after the rats treated with a sham operation (Sham) or short bowel resection (SB) for 7 days. Hypocalciuria and hypocitraturia were noticed in SB rats when compared with the Sham rats. N = 12 in each treatment. *P<0.05, SB vs. Sham rats.

| Index/groups          | Sham    | SB      |
|-----------------------|---------|---------|
| Body weight (g)       | 233.6±3.4 | 229.1±5.3 |
| Food intake (g)       | 27.1±1.1  | 23.9±3.1  |
| Urine amount (ml/day) | 27.7±2.3  | 24.7±4.2  |
| Feces (g/day)         | 12.6±0.9  | 13.0±1.5  |
| Urine pH              | 8.1±0.2   | 8.1±0.2   |
| Urine Ca^{2+} (mg/day)| 0.40±0.05 | 0.23±0.02* |
| Urine oxalate (mg/day)| 3.0±0.9   | 2.4±0.8   |
| Urine citrate (mg/day)| 3.1±0.2   | 1.2±0.1*  |
Figure 1. Anatomic diagram of the small intestine resection in SB rats. A: The duodenum, small intestine, and cecum were exposed from the abdominal cavity under aseptic conditions after the abdomen was opened. B: Four star signs (red) indicate the ligation of the vascular branches of the superior mesenteric artery, which will be cut distal during the resection. Two scissor signs (blue) indicate the resection sites of the small intestine for further anastomosis.

Figure 2. Basic health parameters after 28 days of treatment. The data were obtained from a metabolic cage study collected over a 24 h period after 28 days of induction of body weight (BW) gain (A), daily food intake (B), feces amount (C), urine output (D), and kidney weight (KW) to BW ratio (E). A significant increase in the KW/BW ratio was found in the SB+EG group when compared to the EG or SB group. N = 6 in each group. C, control; EG, ethylene glycol (hyperoxaluria); SB, small intestine resection; SB+EG, small intestine resection co-treated with ethylene glycol. #P<0.05, SB+EG vs. EG group; @P<0.05, SB+EG vs. SB group.

Figure 3. Changes in the plasma biochemistry and PTH levels. The blood was collected from the inferior vena cava after 28 days of treatment for determining the plasma levels of Ca^{2+} (A), oxalate (B), citrate (C), and PTH (D). The plasma levels of
oxalate and citrate were similar among groups. Hypocalcemia and increases in the plasma PTH were found in both SB-treated groups. N = 6 in each group. The group abbreviations are the same as in Figure 2. *P<0.05, SB vs. C group; #P<0.05, SB+EG vs. EG group.

Figure 4. Urinalysis and CaOx supersaturation. Urine was collected from metabolic cages over a 24 h of period after 28 days of induction and analyzed for changes in the urinary pH (A), Ca^{2+} (B), oxalate (C), and citrate (D) excretion, and creatinine clearance (Ccr, E), and urinary lactate dehydrogenase (LDH) levels (F), and the degree of supersaturation as expressed as the AP(CaOx) index (G). Low urinary pH was found in both EG-treated rats. Urinary Ca^{2+} excretion and Ccr was similar among groups; these were associated with hyperoxaluria, hypocitraturia, enzymuria, and supersaturation in the SB or EG group with a more prominent change in the SB+EG group. U, units. N = 6 in each group. The group abbreviations are the same as in Figure 2. *P<0.05, EG or SB vs. C group; #P<0.05, SB+EG vs. EG group; @P<0.05, SB+EG vs. SB group.

Figure 5. The effects of SB on renal CaOx crystal deposition. Representative micrographs of light microscopy after Pizzolato’s staining and counterstaining with
eosin Y in rat kidneys with 28 days of treatment showed CaOx crystal deposition (A). CaOx crystal deposition (indicated by open arrows) in the sections. CaOx crystals in the SB+EG kidney led to renal tubular obstruction and dilation (asterisk). A mild interstitial fibrosis (star signs) surrounding the dilated tubule and tubular atrophy (arrow head) were found in the SB+EG kidney. The amount of CaOx crystals in renal sections was counted under high power field (HPF) (B). No crystals were found in the kidneys of the control and SB groups. The group abbreviations are the same as in Figure 2. *P<0.05, EG vs. C group; #P<0.05, SB+EG vs. EG group; @P<0.05, SB+EG vs. SB group.

**Figure 6. Changes in oxalate and citrate transporter expression.** The protein expressions of Slc26a6, Slc26a2, and NaDC-1 in whole kidneys (A-C) and intestinal (D-F) tissue extracts were examined by immunoblotting. Representative blots from three rats showed typical protein expression of Slc26a6, Slc26a2, and NaDC-1 (20 μg of total protein per lane) at appropriate molecular weights. The lower bar graph (N = 6 in each group) showed the ratio of the band density of the interested protein to β-actin. Slc26a6, but not Slc26a2, was upregulated in both EG-treated groups; this was associated with increases in the NaDC-1 expression in all experimental groups. The Slc26a6 expression was also increased in the intestines of SB group. The group
abbreviations are the same as in Figure 2. *P<0.05, SB or EG vs. C group; #P<0.05, SB+EG vs. EG group; @P<0.05, SB+EG vs. SB group.

**Figure 7. The effects of PTH on oxalate and citrate transporter expression.** The responses to oxalate (Oxa), parathyroid hormone (PTH), and the protein kinase A (PKA) blockers, H89 and KT5720, were evaluated in NRK-52E cells. (A) Lactate dehydrogenase (LDH) was released under the treatment of oxalate (Oxa) or PTH alone or in combination. (B) The cell viability was examined using an MTT assay. (C, D) Slc26a6 and NaDC-1 expression in cells treated with oxalate or PTH. (E, F) Slc26a6 and NaDC-1 expression in cells treated with PTH and PKA blockers, H89 or KT5720. The lower bar graphs (N = 6 in each group, C-F) shows the ratio of the band density of the interested protein to β-actin. *P<0.05, Oxa or PTH vs. C group; #P<0.05, PTH+Oxa vs. Oxa group; @P<0.05, PTH+Oxa vs. PTH group.

**Figure 8. Schematic diagram showing how short bowel (SB) induced hypocitraturia in hyperoxaluric kidneys.** SB induced hypocalcemia and PTH release. PTH acted on renal tubules and increased citrate transporter NaDC-1 expression via the PKA pathway, which induced hypocitraturia and lowered the anticrystallization ability of SB kidneys. The oxalogenic treatment of EG increased
the Slc26a6 expression in the kidney but not in the intestine of SB rats, aggravating hyperoxaluria for CaOx crystal formation.
