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Reduced active transcellular intestinal oxalate secretion contributes to the pathogenesis of obesity-associated hyperoxaluria

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Abstract

Most kidney stones are composed of calcium oxalate, and minor changes in urine oxalate affect the stone risk. Obesity is a risk factor for kidney stones and a positive correlation of unknown etiology between increased body size and elevated urinary oxalate excretion has been reported. Here, we used obese *ob/ob* (*ob*) mice to elucidate the pathogenesis of obesity-associated hyperoxaluria. These *ob* mice have significant hyperoxaluria (3.3-fold) compared with control mice, which is not due to overeating as shown by pair-feeding studies. Dietary oxalate removal greatly ameliorated this hyperoxaluria, confirming that it is largely enteric in origin. Transporter SLC26A6 (A6) plays an essential role in active transcellular intestinal oxalate secretion, and *ob* mice have significantly reduced jejunal A6 mRNA (– 80%) and total protein (– 62%) expression. While net oxalate secretion was observed in control jejunal tissues mounted in Ussing chambers, net absorption was seen in *ob* tissues, due to significantly reduced secretion. We hypothesized that the obesity-associated increase in intestinal and systemic inflammation, as reflected by elevated proinflammatory cytokines, suppresses A6-mediated intestinal oxalate secretion and contributes to obesity-associated hyperoxaluria. Indeed, proinflammatory cytokines (elevated in *ob* mice) significantly decreased intestinal oxalate transport *in vitro* by reducing A6 mRNA and total protein expression. Proinflammatory cytokines also significantly reduced active mouse jejunal oxalate secretion, converting oxalate transport from net secretion in vehicle-treated tissues to net absorption in proinflammatory cytokines-treated tissues. Thus, reduced active intestinal oxalate secretion, likely secondary to local and systemic inflammation, contributes to the pathogenesis of

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Disclosures

John Asplin and Ignacio Granja are employed by LabCorp.

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obesity-associated hyperoxaluria. Hence, proinflammatory cytokines represent potential therapeutic targets.

Keywords

Obesity; intestinal oxalate secretion; hyperoxaluria; inflammation; SLC26A6

INTRODUCTION

Kidney stone disease (KSD) is the second most prevalent kidney disease in USA after hypertension, with a rising prevalence and complications including CKD and ESRD.¹ It remains a major source of patient discomfort and disability, lost working days, and health-care expenditure (~\$10 billion annually). Hyperoxaluria is a major risk factor for KS, and 70–80% of KS are composed of calcium oxalate (CO).² Urinary oxalate is an important determinant of the level of supersaturation than calcium, and the risk for stone formation is affected by small increases in urine oxalate.³

Obesity is a leading preventable cause of death worldwide, with increasing prevalence in adults and children, and reaching an epidemic level in USA. Obesity is a critical causative factor in the development of metabolic syndrome (MS).⁴ Obesity and MS are associated with higher rates of KS in several studies.^{4–6} Although a greater proportion of KS in obese patients are uric acid stones, COKS are also common in these patients, as reported by Li et al who found that 53.1% of uric acid stone formers were obese compared with 42.7% COKS formers.⁷ Mild to moderate hyperoxaluria is frequently seen in obese stone formers and a positive correlation between increased body size and elevated urinary oxalate excretion has been reported in population based studies.^{8–10} Urinary oxalate is significantly higher in COKS formers with MS compared to those without MS ($\mu\text{mol/day}$: no MS = 353 ± 141 ; MS = 454 ± 206), and it significantly rises with increasing features of the MS (356 ± 141 to 504 ± 203 ; zero to four features).¹¹ In addition, analysis of individuals with and without KS in the NHSI, NHSII, and HPFS, revealed that urinary oxalate excretion is directly related to BMI in women.¹² Moreover, overweight children have significantly higher urine oxalate compared with normal weight children.¹³ A reduced man-woman ratio for KS from 1.7:1 in 1997 to 1.3:1 in 2002 was reported, with the greater increase of overweight and obese women compared with men a possible explanation for the changing trend.¹⁴

The mechanism(s) underlying the positive correlation between body size and urinary oxalate excretion and hence increased incidence of KSD, beyond dietary indiscretion, remain(s) unknown. With the rising prevalence of obesity and MS, it is expected that the incidence of KSD will continue to increase at a significant rate, leading to a substantial increase in care costs. Better understanding of the mechanisms underlying the obesity-associated hyperoxaluria (OAH) is crucial for identification of potential targets for therapeutic intervention. To this end, we initiated studies in the obese *ob/ob* (*ob*; an extensively used animal model of obesity in which the obesity is primarily due to leptin deficiency¹⁵) mice and observed that these mice have significant hyperoxaluria compared with their lean controls. We find reduced active transcellular intestinal oxalate secretion, which is likely

mediated by proinflammatory cytokines (PCs), contributes to OAH. We also find that several PCs significantly reduce SLC26A6 (A6)-mediated oxalate transport, both *in vitro* and *in vivo*. For the convenience of oxalate measurements, oxalate uptake and not efflux (secretion), has been determined in human colonic Caco2-BBE (C2) cells since A6 operates in both directions.¹⁶

RESULTS

We used the *ob* mice to elucidate the molecular mechanisms underlying the positive correlation between body size and urinary oxalate excretion.^{8, 9} To establish the *ob* mice as a useful model for the OAH, we assessed whether the *ob* mice have increased urinary oxalate excretion compared to their lean controls. Shown in Figure 1A, *ob* mice have significantly higher (3.3-fold) urine oxalate (adjusted for creatinine = Cr) compared to controls using urine samples collected directly from the bladders at the time of euthanasia or collected over a 1 h period as described in Methods. Importantly, we also observed significantly higher (3.2-fold) urine oxalate in the *ob* mice compared to controls using 24 h urine samples collected by placing the mice in metabolic cages (Figure 1B). These results show that the *ob* mice have similar levels of hyperoxaluria when urinary oxalate is adjusted for Cr, which corrects for any variations in urinary water excretion, or expressed as an excretion rate over 24 h. Collectively, these results indicate that the *ob* mice have significant hyperoxaluria, and therefore establish them as a useful model to elucidate the mechanisms of OAH.

db/db (*db*) mice are deficient in leptin receptor and are another commonly used animal model of obesity.¹⁷ We similarly assessed urinary oxalate levels in the *db* mice and their controls to ensure that the observed hyperoxaluria is not specific to the *ob* mice. Interestingly, the *db* mice have significantly higher (2.3-fold) urine oxalate compared to their lean controls (Figure 2), indicating the hyperoxaluria observed in the *ob* mice is not specific to these mice.

ob and *db* mice are hyperphagic, and therefore it is highly possible that the observed hyperoxaluria is largely coming from diet. To control for this possibility, *ob* mice and their controls were housed individually (one mouse/cage) and their daily food consumption was assessed over a period of 4 days, during which they have free access to food. The *ob* mice consumed significantly higher (1.8-fold) food per day compared to controls (Figure 3A). To rule out the possibility that the observed hyperoxaluria is due to overeating, the *ob* mice and their controls were pair-fed (4 g/mouse/day) for up to 4 days, with the pair-fed mice in the two groups consuming all the food. Importantly, the *ob* mice were still found to have significantly higher (2.6-fold) urine oxalate compared with controls (Figure 3B). Taken together, these results strongly support the hypothesis that the observed hyperoxaluria is largely due to specific defect(s) in the *ob* mice and it is clearly not solely due to overeating.

Anion exchanger SLC26A6 (A6) is apically expressed in enterocytes and plays an essential role in small intestinal active transcellular oxalate secretion, thereby preventing hyperoxaluria and related KS (A6 null mice develop hyperoxalemia, hyperoxaluria, and KS).^{18, 19} SLC26A2 (A2) is also apically expressed in enterocytes and it transports oxalate when expressed in heterologous systems;^{20–22} however, its role in intestinal oxalate

transport is unknown. In addition, SLC26A3 (A3) is also apically expressed in enterocytes and plays a critical role in mouse intestinal active transcellular oxalate absorption.²³ Active transcellular intestinal oxalate secretion requires oxalate influx into the enterocyte from the blood side, where SLC26A1 (A1) is likely involved, and then its efflux from the luminal side by A6 ($\pm$ other transporters, potentially including A2). Therefore, we evaluated whether there is altered mRNA expression of one or more of these transporters in intestinal tissues from the *ob* mice using qPCR. Interestingly, Jejunal A6 mRNA expression is significantly reduced (80%) in *ob* mice compared with controls, without a change in A1, A2, and A3 mRNA expression levels (Figure 4). These results indicate that the observed decrease in A6 mRNA is specific and is not due to global changes in mRNA expression in the *ob* mice.

To evaluate whether the observed reduction in A6 mRNA expression is translated into decreased A6 protein expression, A6 total protein expression was assessed by immunoblotting. Indeed, the *ob* mice have significantly reduced (62%) jejunal A6 total protein expression compared with controls (Figure 5A & B). Due to lack of good antibodies, we did not assess A1, 2, & 3 total protein expression. Of note is that we did not observe a significant change in the expression of the apical membrane protein villin (data not shown).

Since A6 plays a crucial role in small intestinal oxalate secretion,^{18, 19} it is possible that the reduced jejunal A6 mRNA and total protein expression could lead to decreased active intestinal oxalate secretion and therefore potentially contribute to the observed hyperoxaluria. To test this possibility, jejunal tissues were isolated from the *ob* mice and their controls and mounted in Ussing chambers. Of note is that the jejunum was chosen because jejunal wall inflammation was reported in the *db* mice²⁴, and which we confirmed in our *ob* mice (Fig. 8B), as well as the fact that A6 is highly expressed in mouse jejunum (at an equivalent level to the duodenum).²⁵ Interestingly, while a net oxalate secretory flux (-14 ± 11) was observed in control tissues, a large net oxalate absorptive flux (16 ± 8) was seen in the *ob* tissues (Figure 6). This was mainly due to a significant reduction (31%) in jejunal oxalate secretion (J_{SM}). In addition, we observed a non-significant increase (19%) in the absorptive flux (J_{MS}), which also contributed to the reversal of oxalate transport from net secretion in control tissues to net absorption in *ob* tissues. Collectively, these findings indicate that reduced active transcellular intestinal oxalate secretion and potentially increased transcellular intestinal oxalate absorption contributes to the hyperoxaluria observed in the *ob* mice, and hence to the pathogenesis of OAH.

Reduced active intestinal oxalate secretion contributes to the pathogenesis of OAH by increasing the amount of net oxalate absorbed by the intestine, and indicates that the OAH is mainly enteric in origin. To support the hypothesis that the OAH is enteric in origin, the *ob* mice and their controls were placed on an oxalate-free diet to evaluate the effect of dietary oxalate removal on the observed hyperoxaluria. Shown in Figure 7, an oxalate-free diet greatly ameliorated the observed hyperoxaluria, confirming that it is largely enteric in origin.

Although the oxalate-free diet greatly ameliorated the observed hyperoxaluria, urine oxalate still remains significantly higher (~ 1.3 -fold) in the *ob* mice compared to urine oxalate in the control mice on regular diet, suggesting that a small component of the observed hyperoxaluria might be due to increased endogenous hepatic oxalate synthesis. To evaluate

whether increased hepatic oxalate synthesis contributes to the observed hyperoxaluria, urinary glycolate (an indicator of hepatic oxalate synthesis²⁶) levels in the *ob* mice and their controls were measured. There is no significant difference in the glycolate levels in the *ob* and their controls ($\mu\text{g}/\text{mg Cr}$: Control = 86.5 ± 8 ; *ob* = 82.8 ± 6.9 ; $n = 6$), suggesting that the *ob* mice have no evidence of enhanced hepatic oxalate synthesis through a glycolate-dependant pathway.

Obesity is characterized by chronic systemic inflammation²⁷ and both *ob* and *db* mice have higher plasma levels of several PCs (e.g. TNF- α [TNF], IFN- γ [IFN], IL-1 β , & IL-6).²⁸ Obesity is also characterized by increased small intestinal inflammation.^{24, 29} To evaluate whether our *ob* mice have increased systemic and/or small intestinal wall inflammation under our housing conditions, plasma and jejunal TNF, IFN, and IL-6 levels were measured by ELISA and qPCR, respectively. The *ob* mice were found to have significantly higher plasma TNF, IFN, & IL-6 (Fig. 8A) and jejunal TNF & IFN (Fig. 8B) levels, confirming systemic and jejunal wall inflammation in these mice. The *ob* mice also have a non-significant increase in jejunal wall IL-6 levels.

A6-mediated intestinal oxalate secretion plays a critical role in limiting net intestinal absorption of ingested oxalate, thereby preventing hyperoxaluria and KS.^{18, 19} It is possible that the obesity milieu is associated with factors that could cause defective A6 regulation, leading to reduced active intestinal oxalate secretion. We hypothesized that the high circulating and intestinal PCs levels observed in obesity, which inhibit several intestinal transporters,^{30, 31} suppress A6-mediated intestinal oxalate secretion and thus contribute to OAH. To test this hypothesis, we examined the effects of several PCs on apical oxalate uptake (49% of which is mediated by A6^{32, 33}) by human intestinal Caco2-BBE (C2) cells (human epithelial colorectal adenocarcinoma cells). C2 cells were treated basolaterally with TNF (25 ng/ml $\times$ 48 h), IFN, IL-6, or IL- β (50 ng/ml $\times$ 48 h), before assessing ¹⁴C-oxalate uptake. TNF, IFN, and IL-6 significantly inhibited (30, 33, and 32%, respectively) apical ¹⁴C-oxalate uptake by C2 cells, while IL-1 β had no effect (which reflects the specific inhibitory effects of TNF, IFN, and IL-6) (Figure 9). On the other hand, the PCs IL-2 and IL-8 (50 ng/ml $\times$ 48 h), which are elevated in obese human subjects,³⁴ had no effect (data not shown). The PCs concentrations were selected based on previous work^{30, 35, 36} and on our findings in pilot studies (where several concentrations were tested) that the described doses gave the best inhibition. Taken together, these results show that active DIDS-sensitive (100 μM DIDS inhibits it by 91% as we previously reported³⁷) apical ¹⁴C-oxalate uptake by C2 cells, is suppressed by several PCs.

Since A6 mRNA and total protein expression levels are significantly reduced in the *ob* mice, we examined whether TNF, IFN, and/or IL-6 inhibit(s) apical ¹⁴C-oxalate transport by C2 cells by reducing A6 mRNA and/or total protein expression level(s). Using qPCR, TNF and IFN significantly reduced (38 and 36%, respectively) A6 mRNA expression, whereas IL-6 had no significant effect (Figure 10). These results show that TNF and IFN inhibit oxalate transport by C2 cells by reducing A6 mRNA expression.

To confirm that the observed PC-induced reduction in A6 mRNA expression in C2 cells is translated into decreased A6 protein expression, A6 total protein expression was assessed by

immunoblotting. TNF and IFN also significantly reduced A6 total protein expression in C2 cells (37 and 42%, respectively) (Figure 11). However, IL-6 had no significant effect on A6 total protein expression (data not shown). These results indicate that TNF and IFN decrease apical oxalate transport by C2 cells by reducing A6 total protein expression.

To ensure that PCs similarly regulate anion exchange-mediated active intestinal oxalate secretion *in vivo* as observed *in vitro* in C2 cells, age- and gender-matched BALB/c mice were given TNF [2 µg; IP × 1³⁵] or vehicle and then jejunal tissues were isolated and mounted in Ussing chambers 48 h later. Importantly, TNF caused significant inhibition (46%) of mouse jejunal active transcellular oxalate secretion, converting oxalate transport from net secretion in vehicle-treated tissues (−22±23) to net absorption in TNF-treated tissues (13±15) (Figure 12). TNF had no significant effect on the oxalate absorptive flux (J_{ms}). These results confirm that PCs similarly inhibit active oxalate secretion *in vivo* as observed *in vitro*. Taken together, the above described findings clearly show that PCs can directly inhibit A6-mediated active intestinal oxalate secretion, and thus strongly support our hypothesis that PCs play an important role in the pathogenesis of OAH.

DISCUSSION

In the present study we used the *ob* mouse model of obesity to define the pathogenesis of OAH. The *ob* mice have significant hyperoxaluria compared with lean controls, which is not due to overeating as shown by pair-feeding experiments. Significant hyperoxaluria is also observed in another mouse model of obesity (*db*). Removal of dietary oxalate greatly ameliorated the hyperoxaluria observed in the *ob* mice, confirming that it is largely enteric in origin (i.e. dietary oxalate is the source of most of the excess oxalate in the urine of the *ob* mice). We also shown that the *ob* mice have remarkably reduced jejunal A6 mRNA and total protein expression. Assessment of unidirectional oxalate fluxes in jejunal tissues mounted in Ussing chambers showed net oxalate secretion in control tissues but net oxalate absorption in *ob* tissues, which is largely due to a significant reduction in active oxalate secretion. In addition, systemic and jejunal wall inflammation is demonstrated in the *ob* mice, and that several PCs significantly reduced A6-mediated oxalate transport, both *in vitro* and *in vivo*. Taken together, we have demonstrated that reduced active transcellular intestinal oxalate secretion, which is likely mediated by PCs, contributes to the pathogenesis of OAH.

Although dietary oxalate removal greatly ameliorated the observed hyperoxaluria and confirms that it is mainly enteric in origin, the finding that urine oxalate still remains significantly higher (~1.3-fold) in the *ob* mice compared to urine oxalate in the lean control mice on regular diet, suggests that a small component of the observed hyperoxaluria might be due to increased endogenous hepatic oxalate production. However, our finding that the *ob* and control mice have similar urine glycolate (a marker of hepatic oxalate synthesis²⁶) levels does not support this notion. It should be noted that although urine glycolate is high in primary hyperoxaluria (PH) type 1, it is normal in PH types 2 & 3.^{38, 39} Therefore, the lack of an increase in urine glycolate does not totally rule out relative hepatic oxalate overproduction in the *ob* mice, and future studies will further explore this possibility and its contribution to the OAH.

We hypothesized that PCs are key mediators of the observed reduction in active transcellular intestinal oxalate secretion based on the following observations: 1. Obesity is characterized by chronic low-grade systemic inflammation in humans and animals,^{27, 40, 41} and both *ob* and *db* mice have high PCs circulating levels.²⁸ 2. In addition to chronic systemic inflammation, increased small intestinal inflammation is also observed in the *db* mice and obese human subjects with insulin resistance.^{24, 29} 3. We demonstrated systemic and jejunal wall inflammation in our *ob* mice. 4. PCs are known to negatively regulate several intestinal transporters,^{30, 31} and we hypothesized that they inhibit A6-mediated active intestinal oxalate secretion. Indeed, we have shown that PCs can directly inhibit apical oxalate transport by C2 cells, through mechanisms including reduced A6 mRNA and total protein expression. The physiological relevance of the C2 findings is underscored by the fact that TNF significantly decreased (46%) mouse jejunal active oxalate secretion, converting oxalate transport from net secretion in vehicle-treated tissues to net absorption in TNF-treated tissues. Collectively, these findings strongly support our hypothesis that PCs play an important role in the pathogenesis of OAH by reducing active transcellular intestinal oxalate secretion. Of interest in this regard is that IFN was shown to reduce A6 mRNA expression, promoter activity, and transport function (assayed as DIDS-sensitive Cl/OH exchange activity) in C2 cells.⁴² In addition, TNF and IL-6, which are elevated in obese patients, contribute to the pathogenesis of obesity-associated insulin resistance.⁴³ It will be of significant therapeutic interest to evaluate in future studies whether neutralizing antibodies against TNF, IFN, and IL-6 will normalize and/or ameliorate the hyperoxaluria observed in the *ob* mice. Future studies will also elucidate the mechanism(s) by which IL-6, which did not affect A6 mRNA and total protein expression, inhibits oxalate transport in C2 cells, which might involve modulation of the intrinsic activity of the preexisting A6 membrane transporters.

KSD remains a major public health problem in USA, with a rising prevalence and complications including CKD and ESRD.¹ Importantly, the risk of stone formation begins to rise in both men and women even at urinary oxalate levels traditionally considered to be within the normal range (25–30 mg/day).³ Despite being a major risk factor for KSD, hyperoxaluria has no specific therapy. Lack of in-depth understanding of the pathophysiologic mechanisms leading to secondary hyperoxaluria, including OAH, is one reason behind delayed progress in new treatments for hyperoxaluria. An interesting and clinically relevant finding of this study is that reduced active transcellular intestinal oxalate secretion, which is likely mediated by the obesity-associated increased systemic and intestinal inflammation, plays an important role in the pathogenesis of OAH. Importantly, our findings should provide the basis for further mechanistic and interventional studies for the prevention and/or treatment of OAH, including evaluating the *in vivo* therapeutic potential of neutralizing antibodies against the involved PCs as described above.

Our findings could also have potential pathophysiologic relevance for other conditions characterized by chronic intestinal inflammation, including IBD and celiac disease.^{44, 45} IBD patients have a significantly increased risk of KS, often due to enteric hyperoxaluria.^{46, 47} This enteric hyperoxaluria has been largely attributed to fat malabsorption,⁴⁸ where malabsorbed fat complexes with calcium and as a result oxalate becomes free and easily absorbed. Malabsorption occurs in crohn's disease (CD) but not in ulcerative colitis (UC);

however, hyperoxaluria is also seen in UC.⁴⁹ Therefore, malabsorption might not be the only important risk factor predisposing to the IBD-associated hyperoxaluria, and it is possible that other mechanism(s) must be operative. In view of our findings that systemic and intestinal inflammation contribute to OAH, by likely mediating the observed reduction in active intestinal oxalate secretion, we hypothesize that the IBD-associated chronic intestinal inflammation also contribute to the enteric hyperoxaluria seen in IBD. Future studies will be directed at testing this hypothesis using IBD animal models. In addition, a case of subclinical celiac disease with significant hyperoxaluria leading to CKD was reported.⁵⁰ Of remarkable interest is that this patient had significantly reduced small intestinal A6 apical expression compared with a controlled subject, which had likely contributed to reduced A6-mediated active small intestinal oxalate secretion and therefore to the reported hyperoxaluria.⁵⁰ Moreover, diabetic patients [who also have increased systemic inflammation⁵¹] excrete 2 mg/day more urinary oxalate than those without diabetes.⁸ Furthermore, diabetic KS formers excrete significantly higher urine oxalate (>6 mg/day) than nondiabetic stone formers.⁵² Our findings could also potentially explain the association between DM and KSD with regards to increased urinary oxalate excretion.

The obesity-associated altered enteric microbiome might be another potential mediator of the reduced active intestinal oxalate secretion. Compared with lean individuals, an altered gut microbiota was reported in obese humans and animals.^{53, 54} LPS (endotoxin) is a structural part of the cell walls of gram-negative bacteria,⁵⁵ and endotoxemia induces systemic inflammation through cytokine release.^{28, 56–58} Microbiota-mediated cytokine (e.g. TNF) secretion also contributes to intestinal mucosal inflammation.⁵⁹ Significantly reduced gut colonization with *oxalobacter formigenes* (*Of*) is observed in obese subjects (16%) compared to 50–70% in the general population.⁶⁰ In addition to degrading intraluminal dietary oxalate, *Of* also interacts with colonic epithelium by inducing distal colonic oxalate secretion, leading to reduced urinary excretion.⁶¹ *Of* colonization is associated with reduced stone risk,^{62–65} and the obesity-associated decreased *Of* colonization might potentially contribute to hyperoxaluria in obese subjects. However, it is unlikely contributing to the hyperoxaluria observed in the *ob* mice since laboratory mice are usually found not to be colonized with *Of*.⁶⁶ Of interest in this regard is that we have recently shown that *Of*-derived bioactive factors remarkably stimulate oxalate transport by C2 cells (through mechanisms including increased intrinsic activity of A6 membrane transporters), as well as significantly reduced urinary oxalate excretion in hyperoxaluric mice.³³ Besides *Of*, several other gut microbes also degrades intraluminal dietary oxalate, including *Lactobacillus Acidophilus*, *Bifidobacterium lactis*, *Eubacterium Lentum*, *Enterococcus faecalis*, and *Providencia Rettgeri*.⁶⁷ However, whether one or more of these bacteria is/are also capable of interacting with intestinal cells and modulate(s) intestinal oxalate transport as reported with *Of*⁶¹ remains unknown. By degrading dietary oxalate, these bacteria will reduce net intestinal oxalate absorption and lower urinary oxalate excretion. Based on these observations, it is possible that the altered gut microbiota also play an important role in the pathogenesis of OAH, by contributing to the observed reduced active intestinal oxalate secretion. Therefore, future studies will explore the role of gut microbiome in OAH, especially if neutralization and/or reduction of the associated inflammation is/are found to have no effect.

Our study has several limitations and they are summarized as follows: we concentrated on the jejunum for the reasons described above; however, we did not examine whether the *ob* mice also have reduced A6 mRNA/total protein expression and reduced active oxalate secretion in the duodenum and ileum. In addition, we did not determine whether the *db* mice have similar reduction in small intestinal A6 expression and active oxalate secretion. Moreover, due to lack of good antibodies, the potential roles of A1, 2, & 3 in the pathogenesis of the OAH could not be assessed at a protein level.

In summary, we have shown that obese mice have significant hyperoxaluria, which is largely enteric in origin, and that reduced active transcellular intestinal oxalate secretion contributes to the pathogenesis of OAH. The observed reduction in active intestinal oxalate secretion is likely mediated by the obesity-associated increased systemic and intestinal inflammation, and that PCs represent potential therapeutic targets for the prevention and/or treatment of OAH and related KSD.

METHODS

Animals

Mice were purchased and maintained as detailed in Supplementary Methods.

Urine collection for oxalate and glycolate measurements

Urine collection and oxalate & glycolate measurements were performed as detailed in Supplementary Methods. To evaluate the effects of dietary oxalate removal on the observed hyperoxaluria, the *ob* and control mice were placed on an oxalate-free diet, which has no detectable oxalate ($<0.2 \mu\text{mol/g}$), for 4 weeks.

Measurement of fluxes across jejunal tissues

Unidirectional oxalate fluxes (mucosa to serosa, J_{MS} , and serosa to mucosa, J_{SM}) were assessed in jejunal tissues mounted in Ussing chambers as we recently reported³³ (see Supplementary Methods for details). Following a 15–30-min equilibration period, ^{14}C -oxalate fluxes were measured for 75 min at 15-min intervals or for 150–180 min at 30 min intervals and the measurements were averaged.

Cell culture

C2 cells were grown and maintained as previously reported^{37, 68} and described in Supplementary Methods.

Radioactive flux studies

Apical ^{14}C -oxalate flux studies in C2 cells were performed following our published methods,^{37, 68} and detailed in Supplementary Methods.

Real-time PCR

qPCR was performed as we recently reported³³ and detailed in Supplementary Methods.

SDS/PAGE and Western Blotting

Total A6 protein expression is assessed by immunoblotting in jejunal and C2 cell lysates as previously reported^{33, 35} and detailed in Supplementary Methods.

Statistical Analysis

Experimental data are presented as means $\pm$ SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, or by Student's *t* test for unpaired samples when comparing two groups. *P* values < 0.05 were considered statistically significant

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Urinary oxalate levels in the *ob/ob* (*ob*) mice and their lean controls. **A:** Urine samples were collected either directly from the bladders of the *ob* mice and their controls at the time of euthanasia or collected over a 1 h period as described in Methods. The *ob* mice have significantly higher (μmol Oxalate/mg creatinine) urinary oxalate levels (* $P < 9.9 \times 10^{-20}$ for *ob* compared with Controls, by unpaired t-test, $n = 47-49$). **B:** The *ob* mice and their controls were placed individually in metabolic cages and 24 h urine samples were collected. The *ob* mice have significantly higher (μmol/24 h) urinary oxalate levels (* $P < 0.0002$ for *ob* compared with Controls, by unpaired t-test, $n = 7-8$).

Fig. 2.

Urinary oxalate levels in the *db/db* (*db*) mice and their lean controls. Urine samples were collected directly from the bladders of the *db* mice and their controls at the time of euthanasia. The *db* mice have significantly higher (μmol Oxalate/mg creatinine) urinary oxalate levels (* $P < 0.03$ for *db* compared with Controls, by unpaired t-test, $n = 3$).

Fig. 3.

Effect of pair-feeding on urinary oxalate levels in the *ob/ob* (*ob*) mice and their lean controls. **A:** Average daily food consumption by the *ob* mice and their controls. The *ob* mice and their controls were housed individually and their daily food consumption was assessed over a period of 4 days, during which they have free access to food. The *ob* mice consumed significantly higher food per day (* $P < 0.006$ for *ob* compared with Controls, by unpaired t-test, $n = 4-5$). **B:** Urinary oxalate levels in pair-fed (4g/mouse/day) *ob* mice and their controls. Urine samples were collected either directly from the bladders at the time of euthanasia or collected over a 1 h period as described in Methods. The *ob* mice have significantly higher (μmol Oxalate/mg creatinine) urinary oxalate levels (* $P < 0.007$ for *ob* compared with Controls, by unpaired t-test, $n = 4$).

Fig. 4.

SLC26A1 (A1), SLC26A2 (A2), SLC26A3 (A3), and SLC26A6 (A6) mRNA expression in the *ob/ob* (*ob*) mice and their lean controls. Total RNA was isolated from the *ob* mice and their controls for real-time PCR analysis. Values are means $\pm$ SD of 4–5 independent experiments each of which was done in duplicate or triplicate. Relative A1, A2, A3, and A6 mRNA expression levels were expressed as a percentage of Control normalized to GAPDH. The *ob* mice have significantly reduced A6 mRNA expression level (* $P < 0.002$ for *ob* compared with Controls, by unpaired t-test).

Fig. 5. SLC26A6 (A6) total protein expression in the *ob/ob* (*ob*) mice and their lean controls. **A:** A representative Western blot analysis of total A6 protein expression. A6 protein expression was evaluated in jejunal mucosal scrapings (15 µg protein/lane) isolated from the *ob* mice and their controls (CON). The lower part of the same blot was probed with an anti-β-actin (Actin) antibody to normalize loading of protein in each lane (lower panel). **B:** Densitometry of immunoblot results. Western blot band density was quantified using ImageJ software. Values are means ± SD for 4 independent experiments (blots shown in Supplementary Fig. S1) of relative total A6 abundance to β-actin and are presented as a percentage of the Control value. The *ob* mice have significantly reduced A6 protein expression (* $P < 0.004$ for *ob* compared with Controls, by unpaired t-test).

Fig. 6.

Unidirectional [mucosa to serosa = J_{MS} (absorptive flux), and serosa to mucosa = J_{SM} (secretory flux)] and net (J_{net}) transepithelial oxalate fluxes across jejunal tissues ($n = 19$ tissue pairs) isolated from the *ob/ob* (*ob*) mice and their lean controls and mounted *ex vivo* in modified Ussing chambers. While a net baseline oxalate secretory flux is observed in control tissues, a large net baseline oxalate absorptive flux is seen in *ob* tissues, which is mainly due to a significant reduction in J_{SM} (* $P < 0.007$ and $< 1.6E^{-10}$ for *ob* mice compared with Controls, with regards to J_{SM} and J_{net} , respectively, by unpaired t-test).

Fig. 7.

Effect of dietary oxalate removal on urinary oxalate levels in the *ob/ob* (*ob*) mice and their lean controls. Urinary oxalate levels were assessed as described in Methods in *ob* and control mice consuming oxalate-containing diet (Regular Diet) or an oxalate-free diet. The oxalate-free diet significantly reduced urinary oxalate levels in the *ob* mice (* $P < 8.2E^{-12}$ for the *ob* mice on the oxalate-free diet compared with the regular diet, by unpaired t-test, $n = 7-10$).

Fig. 8.

Plasma and jejunal cytokines levels in the *ob/ob* (*ob*) mice and their lean controls. **A:** Plasma TNF- α (TNF), IFN- γ (IFN), and IL-6 levels were measured as described in the Methods. Values are means $\pm$ SD of 10–19 independent measurements each of which was done in duplicate. The *ob* mice have significantly higher plasma TNF, IFN, and IL-6 levels (* $P < 0.002$, $3.2E^{-06}$, and $1.4E^{-09}$ for *ob* compared with Controls, with regards to TNF, IFN, IL-6, respectively, by unpaired t-test). **B:** Jejunal total RNA was isolated from the *ob* mice and their controls for real-time PCR analysis. Values are means $\pm$ SD of 7–11 independent experiments each of which was done in triplicate. Relative TNF, IFN, and IL-6 mRNA expression levels were expressed as a percentage of Control normalized to GAPDH. The *ob* mice have significantly higher jejunal wall TNF and IFN mRNA expression levels (* $P < 0.002$ and 0.008 for *ob* compared with Controls, with regards to TNF and IFN, respectively, by unpaired t-test).

Fig. 9.

Effect of TNF- α , IFN- γ , IL- β , and IL-6 on apical ¹⁴C-oxalate uptake by Caco2-BBE (C2) cells. C2 cells were treated basolaterally with TNF- α (25 ng/ml $\times$ 48 h), IFN- γ , IL- β , or IL-6 (50 ng/ml $\times$ 48 h), or vehicle (Control) and then ¹⁴C-oxalate uptake was measured as described in Methods. Values are means $\pm$ SD of 7 independent experiments each of which was done in triplicate and was normalized to the respective Control value. TNF- α , IFN- γ , and IL-6 significantly inhibited ¹⁴C-oxalate uptake (* P < 0.001 for TNF- α , IFN- γ , and IL-6 compared with Control or IL- β , by ANOVA).

Fig. 10.

Effect of TNF- α (TNF), IFN- γ (IFN), and IL-6 on SLC26A6 (A6) mRNA expression in Caco2-BBE (C2) cells. C2 cells were treated basolaterally with TNF (25 ng/ml $\times$ 48 h), IFN or IL-6 (50 ng/ml $\times$ 48 h), or vehicle (Control), and then total RNA was isolated for real-time PCR analysis. Values are means $\pm$ SD of 5–6 independent experiments each of which was done in triplicate. Relative A6 mRNA expression level was expressed as a percentage of Control normalized to GAPDH. TNF and IFN significantly reduced A6 mRNA expression level (* $P < 0.05$ for TNF and IFN compared with Control, by ANOVA).

Fig. 11.

Effect of TNF- α (TNF) and IFN- γ (IFN) on SLC26A6 (A6) total protein expression in Caco2-BBE (C2) cells. **A:** A representative Western blot analysis of total A6 protein expression. A6 protein expression was evaluated in C2 cell lysate (10 μ g protein/lane: CON, TNF, and IFN = C2 cells treated with vehicle, TNF, and IFN, respectively). The lower part of the same blot was probed with an anti-GAPDH antibody to normalize loading of protein in each lane (lower panel). **B:** Densitometry of immunoblot results. Western blot band density was quantified using ImageJ software. Values are means $\pm$ SD for 4 independent experiments (blots shown in Supplementary Fig. S2) of relative total A6 abundance to GAPDH and are presented as a percentage of the Control value. TNF and IFN significantly reduced A6 protein expression (* $P < 0.01$ for TNF and IFN compared with Control, by ANOVA).

Fig. 12.

Unidirectional [mucosa to serosa = J_{MS} (absorptive flux), and serosa to mucosa = J_{SM} (secretory flux)] and net (J_{net}) transepithelial oxalate fluxes across jejunal tissues ($n = 11$ tissue pairs) isolated from vehicle - and TNF- α -treated BALB/c mice and mounted *ex vivo* in modified Ussing chambers. TNF- α caused significant inhibition of jejunal active transcellular oxalate secretion (J_{SM}), converting oxalate transport from net secretion in vehicle-treated tissues to net absorption in TNF- α -treated tissues (* $P < 0.011$ and < 0.0008 for TNF- α -treated tissues compared with vehicle-treated tissues, with regards to J_{SM} and J_{net} , respectively, by unpaired t-test).