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Crystal deposition triggers tubule dilation that accelerates cystogenesis in polycystic kidney disease

Jacob A. Torres*†, Mina Rezaei*†, Caroline Broderick†, Louis Lin†, Xiaofang Wang‡, Bernd Hoppe†, Benjamin D. Cowley, Jr.§, Vincenzo Savica¶, Vicente E. Torres‡, Saeed Khan¶, Ross P. Holmes¶, Michal Mrug¶,§, Thomas Weimbs**†

†University of California Santa Barbara, Department of Molecular, Cellular, and Developmental Biology, and Neuroscience Research Institute, Santa Barbara, CA 93106-9625, USA;
‡Mayo Clinic College of Medicine, Division of Nephrology and Hypertension, 200 First Street SW Rochester, MN 55905, USA;
¶University Children’s Hospital Bonn, Division of Pediatric Nephrology, Sigmund-Freud-Straße 25, 53127 Bonn, Germany;
§University of Oklahoma Health Sciences Center, Department of Medicine, Section of Nephrology, 1100 N Lindsay Ave, Oklahoma City, OK 73104, USA;
¶University of Messina, Department of Clinical and Experimental Medicine, Piazza Pugliatti, 1, 98122 Messina ME, Italy;
¶University of Florida, Department of Pathology, Gainesville, FL 32611, USA;
¶University of California, Department of Molecular, Cellular & Developmental Biology
University Children’s Hospital Bonn, Division of Pediatric Nephrology, Sigmund-Freud-Straße 25, 53127 Bonn, Germany;
¶Department of Urology, University of Alabama at Birmingham, 720 2nd Ave S, Birmingham, AL 35294, USA;
¶Department of Medicine, The University of Alabama at Birmingham, 720 2nd Ave S, Birmingham, AL 35294, USA;
§Department of Veterans Affairs Medical Center, 700 19th St S, Birmingham, AL 35233, USA.

*These authors contributed equally to this work.
**Corresponding author
Thomas Weimbs (weimbs@ucsb.edu)
Department of Molecular, Cellular & Developmental Biology
University of California Santa Barbara
Santa Barbara, California 93106-9625
Office Phone: (805) 893-4144

Abstract

The rate of disease progression in autosomal-dominant (AD) polycystic kidney disease (PKD) exhibits high intrafamilial variability suggesting that environmental factors may play a role. We hypothesized that a prevalent form of renal insult may accelerate cystic progression and investigated tubular crystal deposition. We report that calcium oxalate (CaOx) crystal deposition led to rapid tubule dilation, activation of PKD-associated signaling pathways, and hypertrophy in tubule segments along the affected nephrons. Blocking mTOR signaling blunted this response and inhibited efficient excetration of lodged crystals. This mechanism of “flushing out” crystals by purposefully dilating renal tubules has not previously been recognized. Challenging PKD rat models with CaOx crystal deposition, or inducing calcium phosphate deposition by increasing dietary phosphorous intake, led to increased cystogenesis and disease progression. In a cohort of ADPKD patients, lower levels of urinary excretion of citrate, an endogenous inhibitor of calcium crystal formation, correlated with increased disease severity. These results suggest that PKD progression may be accelerated by commonly occurring renal crystal deposition which could be therapeutically controlled by relatively simple measures.

Keywords: Polycystic kidney disease, mTOR, oxalate, PKD, nephrolithiasis, ADPKD

Introduction

ADPKD is a common, life-threatening genetic disease that causes immense human suffering and places a high economic burden on the health care system (1). Progressive renal cyst growth leads to deterioration of renal parenchyma, and ~50% of patients require dialysis or kidney transplantation in adulthood (2). Recently, a vasopressin receptor antagonist has been approved as the first treatment for ADPKD but the utility of this drug may be limited by aquareasis, potential liver toxicity and high cost (3). An urgent need remains for effective and safe treatment options. ADPKD is caused by mutations in either the PKD1 or PKD2 gene. Numerous functions for the respective gene products, polycystin-1 (PC1) and polycystin-2 (PC2), have been identified but the purpose of the polycystins in the kidney remains to be determined (4). The rate of disease progression in ADPKD exhibits high inter- and intrafamilial variability suggesting involvement of modifier genes and/or environmental factors, yet none have been clearly
identified (5). If an environmental factor influences disease progression it would be of utmost importance to identify it so that it can be controlled. Support for a role of environmental factors as determinants of disease progression in ADPKD has come from genetic mouse experiments that culminated in the widely accepted “third hit” model of cystogenesis (6). In this model, three events must occur to trigger the formation of individual cysts in ADPKD: the “first hit” is a germline mutation in the PKD1 or PKD2 gene; the “second hit” is a random somatic mutation in a single tubule cell that affects the other PKD1/PKD2 allele; the “third hit” is an insult to the affected kidney such as ischemic or toxic damage that triggers a repair response. Indeed, elimination of both PKD1 alleles in mature mouse kidneys alone does not lead to cyst growth for many months (7). However, subsequent renal injury in such mice – or similarly pre-conditioned mice - will trigger rapid cyst growth (6, 8, 9). In human ADPKD, new cysts are thought to arise throughout life (10). Since most “second hits” (somatic mutations) in patients presumably occur long after kidney maturation, it is likely that subsequent “third hits” (damage/repair) are also required for cystogenesis in humans. However, ischemic or toxic kidney damage are rare events in humans and unlikely explain the relatively constant progression of ADPKD although it is possible that subclinical levels of such injuries may play a role. We hypothesized that a much more prevalent, and clinically relevant, mechanism may drive disease progression as a “third hit” trigger, and considered tubule occlusion or injury by sporadically lodged microcrystals.

Kidneys are constantly challenged by super-saturated solutes – such as calcium oxalate (CaOx), calcium phosphate (CaP), uric acid and others – present in the urinary filtrate that may precipitate while passing through the tubular system. While millions of microscopic crystals may form within kidneys daily, most are excreted safely with the urine (11, 12). Diets and numerous pathologies greatly influence the range of crystal burden that the kidneys experience. Up to a quarter of fresh urines from normal subjects exhibit overt crystalluria (13) without immediate detriment to normal kidneys. Without innate mechanisms preventing excessive growth and retention of crystals within the luminal space, tubules would rapidly occlude and renal function would seize. Mechanisms include regulators of crystal nucleation and growth such as osteopontin, nephrocalcin and Tamm-Horsfall Protein (THP) (12). If crystals do become lodged in renal tubules, e.g. due to fast growth or aggregation, it can lead to nephrolithiasis. The fate of such lodged microcrystals is poorly understood but the main proposed mechanism for their removal is to cross the epithelium into the interstitial space leading to resorption or kidney stone formation (14). 70-80% of all human kidney stones are composed of CaOx indicating that oxalate constitutes a particular challenge.

Several correlative observations support a link between ADPKD progression and renal crystal burden. Symptomatic nephrolithiasis is very common (up to 20-28%) among ADPKD patients (15–17) and patients with nephrolithiasis exhibit more severe PKD than those without (17–19). Hyperoxaluria occurs in 18% of ADPKD patients with nephrolithiasis suggesting the presence of CaOx crystals in some patients (18). ADPKD patients also have high incidences of clinical gout (24%) and hyperuricemia (>60%)(18, 19), conditions that are associated with uric acid crystal formation in the kidneys. Hyperuricemia correlates with faster disease progression in ADPKD (20). In the general population, the incidence of nephrolithiasis is greater in males than females (21). Similarly, ADPKD progresses more rapidly in males than females (22). Likewise, male rats are naturally more prone to CaOx nephrolithiasis than female rats (23). In the Han:SPRD rat model of ADPKD, male animals are affected more severely than females (24). This dual gender bias in humans and rats may be consistent with a role of crystal deposition in accelerating ADPKD progression.

We have investigated the possible role of renal crystal deposition in affecting the rate of progression of PKD. In the process, we uncovered a previously unrecognized mechanism that facilitates the passage of microcrystals by tubule dilation. We report here that acute or chronic induction of CaOx crystal deposition in normal rats and mice leads to rapid activation of mTOR and Src/STAT3 signaling pathways accompanied by rapid dilation of tubule diameters along the entire tubular and collecting duct system. The same signaling pathways are known to be activated in ADPKD and contribute to renal cyst growth. ~7 days after acute oxalate treatment, CaOx crystals are cleared via the luminal space, these signaling pathways are inactivated and tubule diameters return to normal. Inhibition of mTOR blocks tubule dilation and leads to renal accumulation of large CaOx crystal aggregates. These results suggest that tubule dilation is a purposeful mechanism in response to lodged microcrystals with the apparent goal of “flushing out” such crystals. While tubule dilation in response to renal crystal burden has previously been observed in numerous studies it has not previously been recognized as an active protective mechanism. Furthermore, we show that chronic oxalate exposure leads to tubular CaOx crystal deposition in male, but not female, animals of the Han:SPRD rat model of PKD. This leads to increased cystogenesis and worsening of disease progression in male, but not female, animals. Similarly, a high phosphorous diet causes tubular CaP crystal deposition in the Pkd1^−/−;Pck (PCK) rat model of PKD leading to accelerated disease progression. We show that mTOR/STAT3 are similarly activated in dilated renal tubules in primary hyperoxaluria type 1 (PH1) patients, a genetic disease that leads to CaOx deposition and renal failure. Finally, we show that urinary citrate levels inversely correlate with more severe disease in ADPKD patients. Urinary citrate is known to antagonize the precipitation of CaOx crystals in tubule lumens. Altogether, these results suggest a model in which tubular crystal deposition normally triggers a protective mechanism leading to temporary tubule dilation. In PKD this mechanism acts as a “third hit” trigger that causes such dilated tubules to “overshoot” to form cysts. If this mechanism affects the rate of disease progression in human ADPKD then it may consequently be possible to treat patients with relatively simple and well-established interventions known to
reduce renal crystal formation in nephrolithiasis patients such as dietary changes, increased water intake and treatment with citrate as a calcium chelator. Intriguingly, increased water intake (25, 26) and citrate treatment (27–29), respectively, have already been shown to be effective in rat models of PKD but the underlying mechanisms have remained elusive and it has not previously been considered that an effective reduction of renal crystal burden may be involved.

Results

**Chronic CaOx crystal deposition leads to tubule dilation and activation of PKD-associated signaling pathways.**
To examine whether CaOx crystal deposition may lead to activation of PKD-associated signaling pathways, a well-established mouse model of chronic CaOx nephrolithiasis was utilized. Animals deficient in the renal sodium-phosphate cotransporter (NPT2a) are calcric and form renal CaOx crystal deposits when treated with either hydroxy-proline or glyoxylate as metabolic precursors of oxalate (30). Treated animals exhibit numerous luminal CaOx crystal deposits visible by polarized light microscopy after 28 days and tubule diameter dilation is apparent throughout the parenchyma (Figure 1A). Analysis with segment-specific markers revealed that proximal tubules (lotus tetragonolobus lectin-FITC; LTL), connecting tubules (CALB1), collecting ducts (dolichos biflorus agglutinin–Rhodamine; DBA) and the thick ascending limb (TAL;THP) all exhibit dilation (Figure 1B, C). Quantification of these segments show that all segments analyzed were significantly dilated (Figure 1C) accompanied by a significant decrease in cell height (Figure 1D). Strikingly, dilated tubules are strongly positive for phosphorylated S6 (pS6) a downstream effector of the mTOR signaling pathway indicating that mTOR is activated in these cells (Figure 1D). In contrast, adjacent non-dilated tubules do not exhibit mTOR activation (Figure 1D). Similarly, dilated tubules are also strongly positive for active, nuclear, tyrosine-phosphorylated STAT3 (Figure 1D). Both mTOR and STAT3 are activated in cyst-lining cells in human ADPKD and various rodent models, and are drivers of renal cyst growth (31–34). For comparison, kidneys from the Bicflm (Bpk) mouse model of PKD were analyzed side-by-side and showed a similar degree of mTOR and STAT3 activation (Figure 1D). Dilated tubules also frequently contained Ki67-positive cells (Figure 1E, F) indicating that chronic nephrolithiasis causes cell cycle entry.

**Rapid activation of PKD-associated signaling pathways and tubule dilation in response to acute CaOx crystal deposition.**
To test whether tubule dilation and activation of PKD-associated signaling pathways are slow or fast responses to CaOx crystal deposition wild-type mice were treated acutely by a single intraperitoneal injection of sodium oxalate and analyzed at different time points thereafter.

For unknown reasons, mice are relatively resistant to the formation of renal CaOx deposits (30, 35). A high dose of oxalate (0.7 mg/kg) was initially used to induce acute, severe nephrolithiasis which led to rapid, abundant crystal deposition appearing to be exclusively luminal (Figure 2A, B). This coincided with rapid activation of mTOR and STAT3 at 3 hours and peaking at 1 day following treatment (Figure 2C). High-dose oxalate treatment led to significant renal impairment and frequent death due to renal failure after several days.

To induce moderate, reversible crystal deposition, we next administered a low dose of oxalate (0.3 mg/kg) which resulted in CaOx crystal deposition as early as 6 hours after injection, peaking at 24 hours, with most crystals excreted by 3 days (Figure 2D). Abundant CaOx crystals are detectable in urine from 6 hours until 3 days after injection (data not shown). Tubule dilation can be seen as early as 1 day and peaking at 3 days (Figure 2D). By seven days post-injection all CaOx crystals are eliminated and tubule diameters return back to normal (Figure 2D). At all the time points that CaOx crystals are visible they are found nearly exclusively in tubule lumens, suggesting that the bulk of crystals are excreted with the urine via the luminal space (Figure 2D). Dilated tubules exhibit activation of mTOR and STAT3 as early as 1 day after oxalate administration indicating that this is an early event coinciding with the process of tubule dilation (Figure 2E). Mice survived this low-dose oxalate treatment and appeared to completely recover. However, some animals failed to form renal CaOx crystals consistent with the known resistance of mice to CaOx nephrolithiasis.

To be able to quantify responses more accurately we next investigated the response to moderate, reversible CaOx crystal deposition in a more robust experimental system. Rats are commonly used to experimentally induce CaOx nephrolithiasis because they are much more prone than mice to forming CaOx crystals following oxalate administration, are more resilient after crystal deposition and recover more consistently (36, 37). A single injection of sodium oxalate in rats again led to rapid renal CaOx crystal deposition within 6 hours accompanied by fast tubule dilation (Figure 3A). After seven days, all crystals were cleared. Analysis using segment-specific markers revealed that all investigated nephron segments undergo dilation, namely proximal tubules, the thick ascending limb, connecting tubules and collecting ducts (Figure 3B). Quantification of outer tubule and lumen diameters revealed that both parameters increased, peaking at day 3 and largely returned to normal by day 7 (Figure 3C-F). The tubule diameter increase is accompanied by a decrease in cell height (Figure 3G).
mTOR and Src/STAT3 signaling are activated in dilated tubules at six hours following injection, reaching a peak at three days (Fig 3H, I). Ki67-staining revealed that affected tubule cells enter the cell cycle as early as 6 hours following oxalate administration and peaking at 3 days (Figure J, K). However, very few cells stain positive for the G2/M marker phospho-histone H3 Ser10 (38) indicating that most cells that enter the cell cycle do not proliferate but appear to be arrested in G1 (Figure 3K) which is the hallmark of hypertrophy (39). A non-significant increase in the number of apoptotic tubule cells was observed (Figure 3L) indicating that this level of crystal burden does not cause extensive cell death.

We next investigated whether macrophages may be associated with tubule dilation. Macrophages are known to infiltrate cystic kidneys in a process involving monocyte chemoattractant protein-1 (Mcp1), reside in the pericystic interstitium and promote cyst growth (40(p1), 41). Macrophages are also known to be attracted to sites of crystal deposition, which may also involve Mcp1(12), and to promote fibrosis in CaOx-nephrolithiasis (42). The appearance of CD68-positive macrophages coincided with tubule dilatation (Figure 3M). Beginning at 6 hours post-oxalate treatment, increasing numbers of macrophages can be seen in the cortex and surrounding dilated tubules. After 7 days, as crystals have been excreted and most tubule diameters have returned to normal, the presence of macrophages decreases but remains elevated. These results suggest that macrophages are rapidly recruited after CaOx challenge and may play a role in promoting tubule dilation.

Altogether, these results indicate that both mice and rats respond to renal CaOx crystal deposition by rapidly recruiting macrophages, activating mTOR and STAT3 signaling in tubule cells, dilating tubule diameters throughout the nephron via hypertrophy, and clearing crystals via the luminal space for urinary excretion.

**mTOR Inhibition blunts tubule dilation and impairs crystal excretion.**

To investigate whether mTOR activation is necessary for tubule dilation in response to lodged CaOx crystals, rats were treated with rapamycin to inhibit mTOR, followed by acute oxalate challenge. As anticipated, rapamycin treatment prevented mTOR activation (Figure 4A, B). mTOR inhibition did not prevent the activation of Src and STAT3 (Figure 4A, B) indicating that these pathways are not downstream of mTOR. Rapamycin treatment greatly diminished, but not fully suppressed, cell cycle entry as measured by Ki67 (Figure 4C, Supplemental Figure 1). Remarkably, mTOR inhibition blunted the extent of tubule dilation after oxalate challenge (Figure 4D). Eventually, all investigated tubule segments were able to dilate in rapamycin-treated animals but to a diminished degree (Figure 4E) suggesting that mTOR activity is at least partially required for tubule dilation.

mTOR inhibition led to the striking accumulation of large aggregates of CaOx crystals in tubules located in a distinct band at the corticomedullary boundary (Figure 4F-H). Immuno-staining with segment specific markers demonstrated that these clusters of CaOx crystals primarily accumulate in the lumens of the thin descending limb of Henle and the preceding segment of the proximal tubule (Figure 4H). Crystal aggregates are still prominently observed at this border in rapamycin-treated animals at 3 days after treatment (Figure 4F, I). During this course, crystals are markedly deplet ed in the renal cortex in rapamycin-treated animals vs. control animals (Figure 4F, I). These results suggest that mTOR inhibition leads to inhibition of tubule dilation creating a “bottleneck” at the point of the nephron that naturally exhibits the smallest lumen diameter, the thin descending limb of Henle. This leads to accumulation of CaOx crystals at this location which, in turn, prevents the efficient transport of crystals back towards the distal convoluted tubule and the collecting ducts in the renal cortex. Altogether, these findings suggest that tubule dilation is a purposeful response to lodged renal crystals, that PKD-related signaling pathways are involved in this process, and that inhibition of this system leads to inefficient clearance of crystals. To our knowledge, this mechanism of crystal clearance has not previously been recognized.

**CaOx crystal burden leads to increased disease severity in the Han:SPRD rat model of PKD.**

We hypothesized that renal crystal deposition may accelerate PKD progression. To investigate this possibility we chose the well-characterized Han:SPRD (Anks6Cy or Cy+) rat model of PKD (24) because: (a) as a rat model these animals are more susceptible to CaOx nephrolithiasis than mice; (b) this is a slowly progressive model which may allow for the possibility that disease progression could be accelerated; and (c) male, heterozygous (Cy+/) animals in this model exhibit more severe disease progression than female Cy+/+ animals which would be consistent with the known propensity of male rats to be more susceptible to renal CaOx crystal deposition than female rats. We chose to investigate the early stage of PKD progression in this model (postnatal weeks 3-8) which represents the main period of cystogenesis during which renal function is not yet significantly affected(24).

Animals were treated from 3-8 weeks of age with 0.75% ethylene glycol in the drinking water (Figure 5A) for exposure to a chronic burden of CaOx crystals. Ethylene glycol is metabolized to oxalate leading to hyperoxaluria, and this treatment has previously been shown to lead to CaOx nephrolithiasis in male but not female rats (14, 43, 44). The renal effects of ethylene glycol treatment have been shown to be due to the accumulation of CaOx crystals, whereas other metabolic intermediates are not toxic to kidney cells at relevant concentrations (45). Numerous CaOx crystals are readily detectable in lumens of cysts and dilated tubules in male, but not female, Cy+/+ rats (Figure 5B). Remarkably, treated male Cy+/+ rats exhibit more severe renal cystic disease as compared to untreated animals (Figure 5B). In contrast, disease progression is unaffected by ethylene glycol treatment in female Cy+/+ rats. The two-
kidney/body weight ratio (Figure 5C) and renal cystic area (Figure 5D) are both significantly increased in ethylene glycol-treated male Cy/+ rats vs. controls. A significant increase in cyst number (Figure 5E), but not average cyst size (Figure 5F), is also observed suggesting that the increased disease severity is largely due to an increase in cystogenesis.

Cysts are known to arise from the proximal tubule in Cy/+ rats (24). Immuno-staining with segment-specific markers revealed that essentially all cysts are of proximal tubule origin in both ethylene glycol-treated and untreated male Cy/+ rats (Figure 5G). This indicates that CaOx crystal deposition leads to increased cystogenesis only in the tubule segment that is predisposed to cyst formation in this model.

Since female Cy/+ rats are exposed to the same ethylene glycol regimen as male Cy/+ rats but are resistant to renal CaOx crystal deposition and exhibit no altered cystic disease progression we conclude that the effect of ethylene glycol treatment in male Cy/+ rats is not due to any chemical action of ethylene glycol or oxalate per se but is due to the physical interaction of CaOx crystals with the renal epithelium. Altogether, these results suggest that renal crystal burden can exacerbate cystogenesis leading to overall acceleration of PKD progression.

**Citrate treatment reduces CaOx crystal burden in the Han:SPRD rat model of PKD.**

Previously, Tanner et al. demonstrated that treatment of Cy/+ rats with citrate leads to significant inhibition of disease progression (27–29). A mechanism for this beneficial effect could not be identified at the time and this work never led to clinical trials. Due to its ability to chelate Ca$^{2+}$, citrate is a major inhibitor of CaOx precipitation in renal tubules, and is clinically used to prevent recurring nephrolithiasis. We hypothesized that the previously observed beneficial effect of citrate therapy in the Cy/+ rat model may involve the prevention of normally occurring tubular Ca-crystals. To test this, Cy/+ rats were treated with citrate-supplemented drinking water as previously described (29) for 7 days and compared to untreated animals. Very few Ca-crystals are detectable in wild-type rats but male Cy/+ rats exhibit a significantly elevated crystal burden (Figure 5H). Female Cy/+ rats only show a marginal increase in Ca-crystals compared to wild-type. Importantly, citrate treatment led to a significant reduction of Ca-crystal burden in male Cy/+ rats down to the baseline levels observed in wild-type animals (Figure 5H, I). These results suggest that the previously observed amelioration of PKD progression by citrate therapy may be due to a reduction in renal crystal burden and consequent suppression of cystogenesis.

**High phosphate diet leads to calcium phosphate crystal deposition and increased disease progression in the PCK rat model.**

Next, we investigated whether the observed effect of renal crystal deposition on PKD progression is specific to CaOx crystals or may also be caused by crystals of a different chemical composition, and perhaps at a different location within the tubular/collection duct system. We investigated the PCK rat model, an orthologous model to autosomal-recessive PKD due to a mutation in the *Pkhd1* gene(46). In addition to liver cysts, PCK rats develop renal cysts of predominantly collecting duct origin which exhibit increased activity of mTOR (47, 48), Src(49) and STAT3 (50).

In contrast to CaOx nephrolithiasis that affects male humans and rats more severely than females, the opposite is true for CaP nephrolithiasis (23, 51). Intake of phosphate varies widely among individuals and may affect the risk of CaP nephrolithiasis (52, 53). To determine if increased dietary phosphate may affect PKD disease progression, male and female PCK rats were fed identical artificial diets except for the phosphorous content (control 0.6% P; low phosphate diet, LPD 0.2% P; high phosphate diet, HPD 1.2% P) between 3 and 10 weeks of age. LPD caused growth retardation compared to the control diet, but did not change cyst area or fibrosis (data not shown). HPD led to a marked increase in CaP crystal deposits in tubule lumens along the corticomedullary junction, to a larger extent in female than in male animals (Figure 6A, E). The location of CaP crystal deposition at the corticomedullary junction and spreading to involve the medulla is consistent with previous findings (23). Immunostaining for segment-specific markers revealed that CaP deposits occurred primarily in AQP1-positive tubules at the transition between proximal tubules and the thin descending loop of Henle (Figure 6C).

Strikingly, HPD led to a worse progression of PKD, to a larger extent in female than male animals. Two-kidney/body weight ratios (Figure 6F) and cysts scores (Figure 6G) were significantly increased in both male and female rats on HPD compared to controls (Supplemental Table 1). Increased serum BUN in female PCK rats in the HPD group indicates impairment of renal function (Figure 6H). In addition to an increase in AQP2-positive, collecting duct-derived cysts, HPD also led to the emergence of AQP1-positive, proximal tubule-derived cysts (Figure 6B). There was no effect on the liver cystic disease (Supplemental Table 1) suggesting that the effect of HPD is kidney specific. Altogether, these results show that a dietary-induced increase in tubular CaP precipitates leads to increased cystogenesis and PKD disease progression in this model.

**Tubule dilation and activation of PKD-associated signaling pathways in human primary hyperoxaluria.**

To validate our findings in human disease, we investigated renal tissues from primary hyperoxaluria type 1 (PH1) patients. In PH1, a genetic defect leads to defective glyoxylate metabolism, causing endogenous oxalate overproduction and thus hyperoxaluria, CaOx crystal deposition and eventually renal failure (54). Renal biopsy specimens from PH1 patients exhibit CaOx crystal deposition and tubule dilation (Figure 7A). Both mTOR and STAT3
pathways are strongly activated in PH1 to a degree comparable to cysts in human ADPKD (Figure 7B). This result suggests that tubular crystal deposition leads to activation of PKD-associated signaling pathways and tubule dilation in both rodents and humans.

**Urinary citrate inversely correlates with disease severity in ADPKD.**

Since urinary citrate is a major inhibitor of CaOx and CaP crystal formation, and low urine citrate levels are a risk factor for kidney stone formation (55), we tested whether urinary citrate levels may correlate with disease severity in ADPKD. 24-hour urines from a cohort of 26 ADPKD patients with varying degrees of disease progression (total kidney volume (TKV) range: 381-2787 ml; median: 1121 ml) but without renal functional impairment or only mild renal insufficiency based on eGFR and serum creatinine levels were analyzed. Oxalate excretion varied widely (range: 17-226 mg/d; median: 31 mg/d), presumably due to fluctuating differences in dietary oxalate intake, and did not correlate with disease progression. However, the urinary citrate levels correlated inversely with more advanced renal cystic disease progression as reflected by the log transformed TKV (Figure 7C) an FDA-approved enrichment biomarker for high-risk ADPKD patients The urinary citrate level correlated better with TKV than the urinary albumin excretion that was previously described as an independent predictor of TKV and renal function in ADPKD patients (Figure 7D) (56). It has previously been shown that renal clearance and urine excretion of citrate are not significantly decreased in mild renal insufficiency due to other, non-PKD reasons. Consistent with this, urine citrate excretion in our ADPKD patient cohort does not significantly correlate with eGFR (Figure 7E). These results are consistent with the hypothesis that urinary citrate may be protective in ADPKD patients by antagonizing renal calcium crystal formation.

These data complement outcomes of our animal studies. Together these findings are consistent with the conclusion that renal crystal deposition triggers accelerated progression of polycystic kidney disease by activation of an inherent, renoprotective mechanism that has the purpose of facilitating the excretion of tubular crystals.

**Discussion**

The results presented here point to a previously unrecognized renoprotective mechanism that involves purposeful dilation of renal tubules in response to lodged microcrystals with the apparent purpose of facilitating the “flushing out” of such crystals along the luminal space for excretion with the urine. Furthermore, our results suggest that this renoprotective mechanism acts as a “third-hit” trigger leading to accelerated disease progression in PKD.

Large numbers of microscopic crystals of various compositions (incl. CaOx, CaP, struvite, uric acid) may form in human kidneys daily, are transported through the tubules, and excreted safely with the urine (11, 57). Protective mechanisms have evolved to regulate crystal nucleation and growth and prevent functional deterioration of the kidney (12). However, the occurrence of nephrocalcinosis and nephrolithiasis indicates that these mechanisms are not always completely effective, and that crystals can sporadically lodge in tubule lumens due to aberrantly fast growth or aggregation. It is poorly understood how sporadically lodged micro-crystals are cleared from kidneys. One observed mechanism is that some crystals can somehow cross the epithelial barrier into the interstitial space (14) but their subsequent fate is uncertain and this may be a slow process occurring in the time-frame of weeks (58). It is difficult to see how such a cumbersome and disruptive interstitial mechanism could accomplish effective clearance of more than a small amount of lodged crystals at a time. Eventually, renal crystal deposition can lead to pathological nephrocalcinosis or nephrolithiasis including tubule occlusion and the formation of macroscopic kidney stones which are most commonly found in the urinary space attached to the renal papilla (59, 60).

Even though tubule dilation is universally observed in many forms of nephrocalcinosis and nephrolithiasis in humans and animal models (61) this phenomenon has not been thought of anything more than a secondary consequence to cell injury. Our results suggest that CaOx crystal deposition leads to rapid activation of mTOR and Src/STAT3 signaling in tubule epithelial cells accompanied by equally rapid dilation of tubule diameters. While occasional cell damage can be observed – particularly when cells are in direct contact with a lodged crystal - the rapid dilation response occurs in most tubule segments up- or downstream of any lodged crystals without any apparent evidence of cell damage or death. Our results suggest that segments all along an affected nephron dilate in response to lodged CaOx crystals, whether a crystal is detectable in the particular segment under observation or not.

Our chosen experimental conditions of oxalate challenge lead to a moderate degree of initial deposition of CaOx crystals, which can be completely resolved by the kidneys. This mild level of crystal burden does not lead to widespread tissue destruction or renal failure, and was intended to mimic the situation that kidneys may frequently be exposed to under non-pathological conditions. We find that CaOx crystals under these conditions are located almost exclusively in tubule lumens from the moment they first occur until they are cleared. Interstitial crystals were very rarely observed suggesting that the vast majority of crystals are cleared via the luminal space and excreted with the urine. The distribution of acutely induced CaOx crystals indicates that crystals initially form in the proximal tubule and are then passed along the tubular system. This is in agreement with previous studies in rats (61) and primary hyperoxaluria patients (62). It is intuitive that dilation along the entire length of the nephron would facilitate this mechanism of “flushing out” crystals and counter their accumulation. Indeed, we find that mTOR inhibition with
rapamycin blunts the ability of tubules to dilate in response to CaOx crystals and leads to accumulation of crystal aggregates, primarily at the transition from the proximal tubule to the descending loop of Henle. These transitions are located in the corticomedullary boundary and appear to represent a bottleneck for the passage of microcrystals, presumably due to the abrupt narrowing of tubule diameters. This is consistent with the previous finding of CaOx crystal aggregates in the same location in primary hyperoxaluria patients (62).

We propose that tubule dilation is a purposeful mechanism that involves activation of mTOR and STAT3 signaling pathways. Several possible scenarios can be envisioned for how a CaOx crystal may trigger a dilation response. In a possible short-range mechanism, the direct physical contact of a crystal with the apical surface of epithelial cells may be required while the crystal passes through a tubule. This model, however, would not explain how a tubule segment distal to a lodged crystal would be able to detect its presence and dilate in response. Alternatively, in a possible long-range mechanism, a lodged crystal may activate a response both up- and downstream of its location by affecting fluid flow in the given nephron due to partial or complete occlusion. Another possible long-range mechanism may involve diffusible factors – such as growth factors, cytokines or other signaling molecules – that may be secreted by injured epithelial cells in direct contact with a crystal. Primary cilia may play a role in both short- and long-range mechanisms by sensing crystals, signaling factors and/or fluid flow. Our observation that macrophages associate rapidly with dilating tubules may suggest that these infiltrating cells may also play a role in promoting tubule dilation. A role of macrophages in both nephrocalcinosis (42, 63) and renal cystic disease (40, 41) is well established. Future work is required to investigate these possibilities.

Our results suggest that tubule dilation involves - and at least partially requires - activation of mTOR signaling. mTOR functions in two complexes, mTORC1 and mTORC2, which regulate numerous cellular functions including cell size, proliferation and the actin cytoskeleton (64, 65). It is possible that mTOR-dependent cell size increase may play a role in tubule dilation. Our finding that dilation of tubule diameters comes at the expense of cell height indicates that a change in cell shape is important, raising the possibility that mTOR-mediated changes in the actin cytoskeleton play a role in this process. Interestingly, Pkd1–/– cyst-lining epithelial cells also exhibit a change in shape from cuboidal to flat (66) which suggests that PC1 may play a role in cell shape regulation. It is interesting to note that PC1 can regulate mTOR activity (32). It was also recently shown that osteoblastic cells react to stretch by PC1-dependent activation of STAT3 (67). Whether PC1 plays a direct role in the regulation of mTOR and/or STAT3 signaling during the dilation-response to crystal deposition remains to be investigated.

In addition to a relatively rapid increase of tubule diameters, peaking at 24 hours (Figure 3C-F), we also observe a slower induction of cell cycle entry, peaking at 3 days after acute oxalate challenge (Figure 3K). Our results indicate that cell cycle entry leads primarily to hypertrophy but not proliferation (Figure 3K-M). We suggest that affected cells transition from G0 to G1, and arrest in G1 as they increase their cell mass. After successful completion of crystal clearance, cells then exit the cell cycle again, presumably by reverting back to G0 (Figure 3K). Therefore, ultimately the cell number would remain relatively stationary which is consistent with our finding that the response to acute CaOx crystal deposition leads only to very minor increases in mitosis and apoptosis. It has previously been reported that acute CaOx crystal deposition in mice leads to necroptosis of tubule epithelial cells and an increase in TUNEL-positive cells (68). However, these studies were carried out by injection with an extremely high, lethal dose of sodium oxalate that was >300-fold higher than in our study. We suggest that the reason why we did not observe significant cell death in our study is due to our use of a more physiologically relevant dose of oxalate that allows complete crystal excretion and recovery.

mTOR activation appears to be intimately involved in the hypertrophic response because rapamycin treatment strongly inhibits cell cycle entry of tubule cells in response to CaOx crystal deposition (Figure 4C). This is consistent with the well-established role of mTOR activation in hypertrophy including the previously observed role in renal hypertrophy after unilateral nephrectomy (69). Our results, however, suggest that mTOR and STAT3 activation are independent of each other because STAT3 is still activated in the presence of rapamycin (Figure 4A, B).

In the natural progression of PKD, tubule dilation is the first step towards cystogenesis. Evidence from several laboratories clearly indicates that genetic inactivation of the Pkd1 gene, or ablation of primary cilia, in mice with mature kidneys, is insufficient to trigger cystogenesis. However, subsequent renal insults can trigger rapid cystogenesis in such models, which has led to the now widely accepted view that a “third-hit” mechanism determines the rate of cystogenesis and progression in PKD (6). Experimentally, ischemia-reperfusion injury, nephrotoxic injury and compensatory hypertrophy after unilateral nephrectomy have been shown to act as third-hit triggers in preconditioned mice. Given that such types of renal insults are rare in humans, they appear unlikely to explain the relatively steady rate of progression in human ADPKD. Our results suggest that activation of the renoprotective mechanism in response to crystal deposition described here can act as a third-hit trigger and accelerate the rate of PKD progression. Given the constant exposure of human kidneys to precipitating solutes we consider it likely that this mechanism represents a more clinically relevant trigger that contributes to the rate of progression in ADPKD.

In two rat models of PKD we showed that conditions that lead to renal deposition of different types of crystals (CaOx vs. CaP) both lead to increased cystogenesis and accelerated disease progression. These rat models are genetically independent of each other and exhibit different disease phenotypes. The gene coding for samcystin, a protein expressed in proximal tubule cells, is affected in the Han:SPRD (Cy+/) rat model leading to cysts of proximal...
Animals and treatment regimens

Methods

Established methods for the treatment of recurring nephrolithiasis and the associated disease progression in ADPKD may favorably affect the same therapeutic interventions that are well established for the treatment of ADPKD. 

Urine excretion of citrate is one of the major inhibitors of calcium crystal precipitation. It is likely that most or all of the lithogenic-associated proteins converge on the same molecular pathways that lead to renal cyst growth. PCK and Cy/+ rat models may be regarded as models for subsets of cysts that arise from distinct tubule segments. Nevertheless, despite this genetic and phenotypic distinctness between these two models, and despite the fact that we induced deposition of crystals of different chemical compositions, we observed an increase in cystogenesis in both cases. These results suggest that renal crystal deposition can lead to cystogenesis in a variety of tubule segments and in a variety of genetically predisposed models.

Our results indicate that the observed increase in cystogenesis was dependent on the physical presence of tubular crystals as opposed to any chemical effects of oxalate or phosphate per se. Treatment of male and female Cy/+ rats with the same dose of ethylene glycol resulted only in crystal deposition and increased cystogenesis in males but not females. Similarly, the effect of a high phosphate diet on CaP precipitation correlated with increased cystogenesis preferentially in female rather than male PCK rats. This suggests that increased cystogenesis is due to mechanical effects of crystals, such as disruption of fluid flow or physical cell damage.

Based on these results, we propose that renal crystal deposition may affect disease progression in human ADPKD. Although there is no definitive study, a link between ADPKD progression and renal crystal burden has been found in correlative studies (15–17). Generally, in these studies it is speculated that increased abnormalities in tissue architecture and/or metabolic abnormalities during ADPKD progression may increase risk for nephrolithiasis. However, the opposite may be the case, i.e. that increased crystal burden leads to accelerated ADPKD progression. It is also possible that ADPKD progression and nephrolithiasis positively reinforce each other leading to a vicious cycle.

If this conclusion is correct, then treatments that reduce renal crystal formation and deposition may slow progression in ADPKD. Such treatments typically are dietary changes to avoid foods rich in oxalate, phosphate, and uric acid precursors, increased water intake, and supplementation with citrate as a chelator of calcium to reduce CaOx and CaP precipitation.

Remarkably, increased water intake has already been shown to be effective and slows PKD progression in the PCK rat (25, 26). The mechanism of the protective effect is unknown but was hypothesized to involve a decrease in vasopressin signaling. We hypothesize that the protective effect may instead (or in addition) be due to increased urine output and dilution of solutes, which decreases the risk of tubular crystal deposition. Increased water intake is commonly prescribed for the treatment of recurring nephrolithiasis.

Even more remarkable, citrate treatment has been shown to be highly effective in reducing cyst growth, preserving renal function and extending life span in the Cy/+ rat model (27–29). The mechanism of this effect remained unexplained and these investigators speculated that the effect may be due to urine alkalization. In contrast, our results suggest that the observed beneficial effect of citrate was due to citrate’s action as a chelator and inhibitor of calcium crystal formation because citrate treatment very effectively reduced the calcium crystal burden in Cy/+ rats (Figure 5H, I). Citrate was found to be ineffective in the Nphp3(−/−) mouse model of PKD (78, 79). However, since mice are naturally highly resistant to CaOx crystal precipitation it is unlikely that tubular crystals play any role in the cystogenesis of this, or any other mouse model of PKD. Therefore, citrate therapy would not be expected to be effective in such models. Furthermore, cystogenesis in most genetic mouse models of PKD is pre-determined by the genetic abnormality and does not depend on any third-hit trigger. Consistent with this, no evidence for lithogenic risk factors were found in a Pkd1 mouse model (80).

In our retrospective analysis of urinary citrate excretion in ADPKD patients we found that lower citrate levels significantly correlate with more advanced disease progression as reflected by the TKV (Figure 7C). Given that urinary citrate is one of the major inhibitors of calcium crystal precipitation it is plausible that patients with high citrate excretion rates are relatively protected and exhibit a slower rate of cystic disease progression.

Altogether, our results suggest that renal tubules utilize a previously unrecognized mechanism, tubule dilation, to aid in the excretion of lodged luminal crystals. Activation of this mechanism also appears to inadvertently act as a third-hit trigger leading to cystogenesis in polycystic kidney disease. These findings suggest the intriguing possibility that disease progression in ADPKD may be favorably affected by the same therapeutic interventions that are well established for the treatment of recurring nephrolithiasis.

Methods

Animals and treatment regimens
Animals were allowed free access to water and standard chow, were weaned at 3 weeks of age and separated by sex. All animals were monitored for signs of distress during the course of the study. Individual animals were treated as the experimental unit for the purposes of this study. The researchers performing the experiments were not blinded to the treatment of animals. Sample size was determined using prior knowledge from similar experiments performed in our laboratory using PKD rodent models.

Wild-type mice: 8-10 week old male and female C57BL/6 mice weighing between 20-30g (Charles Rivers laboratories) were challenged with a single intraperitoneal injection of 0.22M sodium oxalate in 0.9% saline, sterile filtered and administered at 0.3mg/kg (low dose) and harvested at 6 hours (n=4), 1 day (n=12), 3 day (n=8) and 7 days (n=10); or 0.7mg/kg (high dose) and harvested at 3 hours (n=4) and 1 day (n=9) to induce acute CaOx crystal deposition. Control animals included (n=6) males and (n=5) females treated with saline. Low-dose trials were conducted in 5 separate experiments and high-dose trials were conducted in 2 separate experiments. Animals that did not exhibit CaOx crystal deposition were not included in analyses of crystal effects.

NPT2a-/- mice from a colony at the University of Florida were previously described as a model of chronic CaOx nephrolithiasis (36). 4-6-month-old NPT2a-/- mice weighing 18-30g were given 1.5% glyoxylate (n=3) or 5% hydroxy-proline (n=2) mixed with chow for 28 days to induce hyperoxaluria. By day 7 of the treatment NPT2a-/- mice become hyperoxaluric, and remained so throughout the experimental period(35).

Wild-type rats: 8-week-old Sprague Dawley rats weighing between 200-300g (Charles Rivers laboratories) were challenged with a single intraperitoneal injection of 0.22M sodium oxalate in 0.9% saline, sterile filtered and administered at 0.7mg/kg and analyzed after 6 hours (n=5), 1 day (n=5), 3 days (n=5), or 7 days (n=3). 6 hour, 1 day and 3 day treatments were conducted over 3 experiments and once for 7 days. For mTOR inhibition, rats were treated with 2mg/kg rapamycin (LC Laboratories) dissolved in DMSO 18 hours prior to, then 24 and 72 hours after oxalate challenge. Controls received DMSO only. Rapamycin treated animals were treated with oxalate as described above and euthanized 6 hours (n=5), 1 day (n=5), 3 days (n=5), or 7 days (n=2) later. 6 hour, 1 day and 3 day treatments were conducted over 3 experiments and once for 7 days. 5 age-matched untreated controls were used for this experiment. Animals that did not exhibit crystal deposition were not included in histological analyses.

Han:SPRD rats were obtained from the University of Oklahoma Health and Sciences Center and a colony established at the University of California Santa Barbara. Animals were genotyped as previously described (70). Ethylene glycol was administered to male and female Han:SPRD, Cy/+ and control rats to induce chronic CaOx crystal deposition as previously described(81). 0.75% ethylene glycol (Sigma-Aldrich) in purified water was administered ad libitum from 3 to 8 weeks of age. Animals treated for this experiment include; 9 wild-type male rats (264.0g±22.9), 9 Cy/+ male rats (251.1g±21.6), 6 wild-type female rats (202.8g±17.4), 7 Cy/+ female rats (188.9g±14.3) over 4 experiments. Untreated animals include; 4 wild-type male rats (274.3g±16.6), 5 Cy/+ male rats (267.3g±16.6) and 5 wild-type female rats (192g±12.1), 5 Cy/+ female rats (193.8g±13.4).

Citrate treatment of Han:SPRD rats began at week 7 until week 8 of age. As previously described (29), rats were treated ad libitum with drinking water supplemented with 55 mM tripotassium citrate/67 mM citric acid. Kidneys were cut into sequential, 5µm sections and subjected to Pizzolato staining prior to manual quantification. Calcium deposits were identified as black aggregates within lumen. The analyst was blinded to the treatment of the animals. Animals treated for this experiment include; 3 wild-type male rats (244.5g±45.23), 5 Cy/+ male rats (249.8.1g±21.6), 9 wild-type female rats (198.3g±16.8), 3 Cy/+ female rats (210.4g±11.5) over 3 experiments.

PKC-δ rat experiments were conducted at the Mayo Clinic College of Medicine (Supplemental Table 1) (46). 10 male and female wild-type and cystic rats were fed identical diets except for phosphorus content, 0.17% (TestDiet 5857), 0.057% phosphorous (TestDiet 5755) or 1.21% phosphorous (TestDiet 585Y), from 3 to 10 weeks of age.

Histological and Immunohistological analyses
Birefringent CaOx crystals were detected in H&E-stained paraffin sections by polarized light microscopy or Pizzolato’s stain. CaP deposits were detected by von Kossa staining
For immuno-fluorescence analysis, paraffin embedded renal sections were rehydrated, through xylene and decreasing ethanol series, washed in Tris-buffered saline and boiled in 10mM sodium citrate buffer pH 6 for 5 minutes in a pressure cooker. Cooled, then washed twice in TBST and blocked (1% BSA, 0.1% fish skin gelatin, 0.05% sodium azide in TBST) at 37°C for 30 minutes. Sections were incubated with antibodies diluted in blocking buffer overnight at 4°C (anti-pSG323/238, Cell Signaling #4858; anti-pSTAT3 Y705, Cell Signaling #9145; anti-aquaporin-1, Millipore AB2219; anti-aquaporin-2, Santa Cruz sc-9880; anti-Calbindin 1, Sigma C9848; anti-Tamm-Horsfall protein, Santa Cruz sc020631; anti-Ki-67, BD Pharmingen #550609). Sections were incubated with 1% Sudan Black diluted in 70% ethanol for 20 minutes, then two washes in TBST for 5 minutes. Sections were then incubated fluorescent-labeled antibodies diluted in blocking buffer at 37°C for one hour (anti-Rabbit-DyLight 488 and 594, #35552 and #35560, ThermoFischer; anti-mouse-DyLight 488, ThermoFisher #35502) and/or fluorescent-labeled lectins lotus tetragonolobus lectin-FITC (LT), Vector FL-1321; dolichos biflorus agglutinin–Rhodamine (DBA), Vector RL-1032. After washing, sections were post-fixed in 10% neutral-buffered formalin for 10 minutes, washed in TBS, and mounted with ProLong Gold antifade mounting medium with DAPI (Thermo Fisher).
For immuno-histochemical analysis, paraffin sections were rehydrated as described above followed by proteinase K (0.6U/ml in TE buffer pH 8) digestion, treatment with 3% H2O2, and blocking in 10% donkey serum. After successive incubations with primary antibody (anti-CD68/SR-D10 (ED1), Novus Biologicals #NB600-985), secondary antibody (anti-mouse-Biotin, Jackson ImmunoResearch 715-065-151), and HRP conjugated streptavidin (Streptavidin-HRP, BD Pharmingen #554066) slides were developed using DAB peroxidase substrate (Vector #SK-4100), followed by counterstaining with hematoxylin.

Cystic index was determined using H&E-stained whole kidney sections and analysis using ImageJ software to determine the percent of cystic area relative to total kidney area. Manual determination of inclusive areas was used to exclude artifacts from section.

To measure the intra-renal location and size distribution of CaOx crystals and aggregates, ImageJ software was used on polarized light microscopy images of H&E-stained whole kidney sections. 7-10 fields from 200x images were analyzed from both cortical and medullary areas.

For the analysis of cell heights, tubule diameters and lumen diameters, ImageJ software was used of H&E-stained whole kidney sections. Tubule diameters were measured from the basal membrane across the narrowest segment of a tubule to the basal membrane of the opposite side. Lumen diameters were determined by measuring the distance between the apical membranes across the narrowest portion of a tubule. Cell heights were determined by measuring three cells per tubule, measuring cell height from the basal to apical membrane across a cell nucleus. The researcher performing the measurements was blinded to the treatments.

**Immunoblotting**

Kidneys were bisected and placed into a mortar cooled with liquid nitrogen pulverized using a pestle and stored at -80°C for future analysis. Tissue samples were lysed in SDS sample buffer containing phosphatase and protease inhibitors (Sigma-Aldrich). Following standard SDS-PAGE and transfer, samples were analyzed using the following antibodies: Actin, Amersham Bioscience N350; pS6K1(S235/236), Cell Signaling #4858; S6, Cell Signaling #2217; Src, Cell Signaling #2109; psrcY416 #6943; pSTAT3Y705, Cell Signaling #9145; STAT3, Cell Signaling #9139.

**ADPKD patients and urine sample analyses**

Urine samples from patients in the Emory ADPKD Cohort Study were collected and analyzed according to protocols approved by Institutional Review Boards at Emory University and University of Alabama at Birmingham (82). Oxalate levels were determined by IC/MS (83) and citrate concentrations by LC-MS.

**Statistics**

Data sets were tested for normality prior to a nonparametric, one-tailed, Mann-Whitney analysis with GraphPad Prism software after determining a non-normal distribution and χ² analysis. PCK rats were analyzed with two-way ANOVA and two-tailed, unpaired t-test. Box whisker plots represent 90% of values with median displayed as a line in between the 2nd and 3rd quartiles and the mean displayed with “+”. A P value less than 0.05 was considered significant. Variances between statistically compared groups differed. Correlations between ADPKD patient data (urine citrate/creatinine/albumin vs. TKV or eGFP) were analyzed using a linear regression analysis and the sample Pearson correlation coefficient.

**Study Approval**

All animal experiments were conducted with approval of the Institutional Animal Care and Use Committees of the University of California Santa Barbara, University of Florida and the Mayo Clinic, respectively.
Author Contributions

JAT and MR share first authorship. This work was part of the PhD thesis of JAT who made the initial significant discoveries and was assigned first in the author series. JAT contributed to the design of experiments, animal procedures, immunofluorescence, western blotting, histology, statistical analysis, preparation of figures and writing of the manuscript. MR contributed histological, immunohistological and statistical analyses, and preparation of figures. LL contributed immunohistological analysis. CB contributed, animal procedures, immunoblotting and tubule measurements. BH contributed biopsy specimens of patients with PH1 and provided clinical support. BC contributed Han:SPRD rats for experiments and experimental advice. VT, VS and XW designed and conducted PCK rat experiments. SK contributed NPT2α−/− animals for analysis and contributed to experimental design. RH contributed urine analyses and nephrolithiasis risk interpretation. MM designed and supervised the ADPKD patient data analyses. TW acted as the principal investigator, contributed to the design and supervision of most experiments, and to the preparation of figures and writing of the manuscript.

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References


Figure 1: Chronic CaOx crystal deposition leads to tubule dilation and activation of PKD-associated signaling pathways

**A.** H&E-stained sections of kidneys from glyoxylate treated Npt2a−/− mice imaged by polarizing light microscopy. CaOx crystals appear as bright spots. **B.** Kidney sections from glyoxylate- or hydroxyproline-treated Npt2a−/− mice stained with LTL, DBA, calbindin 1 (CALB1) and Tamm-Horsfall Protein (THP). **C.** Quantification of tubule and lumen diameters from animals in B (CD=Collecting Duct). **D.** Quantification of cell heights from animals in B. **E.** Immunofluorescence microscopy for mTOR activity (phospho-S6 S235/236) and phospho-STAT3 Y705. Bpk mouse used as positive control. **F.** Immunostaining for cell cycle marker Ki67. **G.** Quantification of Ki67-positive cells as the percent of the total number of DAPI stained nuclei per field analyzed. Control n=3, n=3 for glyoxylate and n=2 for OH-Pro; *P<0.05; **P<0.01; ***P<0.001; ψP<0.0001; Error bars represent SD. Scale bar=50μm. Mann-Whitney used for statistical analysis.
Figure 2: Acute CaOx crystal deposition leads to rapid tubule dilation and activation of PKD-associated signaling pathways in wild-type C57/BL6 mice.

A.) H&E-stained kidney sections 1 day after administration of 0.7mg/kg NaOx visualized by normal and polarized light microscopy. Scale bar=1mm

B.) High magnification from A. using polarized light. CMB, corticomedullary boundary. Scale bar=100µm

C.) Immunoblot of total kidney lysates for phospho-S6\textsuperscript{S235/236}, phospho-STAT3\textsuperscript{Y705} or total proteins at 3 hours (n=4) and 1 day (n=9) post 0.7mg/kg NaOx treatment. Representative of 2 experiments.

D.) Polarized light micrographs of kidney sections from 0.3mg/kg NaOX treated mice at 6 hours (n=4), 1 day (n=12), 3 days (n=8) and 7 days (n=13) post treatment. bar=100µm

E.) Immunofluorescence staining of phospho-S6\textsuperscript{S235/236} and phospho-STAT3\textsuperscript{Y705} in 0.3mg/100g treated mice. Images of 0.3mg/kg treated animals representative of 5 experiments. Scale bar=50µm
Figure 3: Acute CaOx crystal deposition leads to rapid tubule dilation, activation of PKD-associated signaling pathways, and hypertrophy in wild-type rats.

A.) Polarized microscopy of H&E-stained renal sections of 0.7mg/kg NaOx-treated rats after 6 (n=5), 24 hours (n=5), 3 days (n=5) and 7 days (n=3), respectively. B.) Segment-specific immunostaining; collecting ducts (AQP2), proximal tubules (AQP1), connecting tubules (Calbindin 1, CALB1), and thick ascending loop (TAL) of Henle (Tamm-Horsfall Protein, THP) from treated rats. Bottom panel show separate images in the same panel when necessary. C-F.) Lumen and tubule diameters of the indicated tubule segments (CD=Collecting Duct). G.) Cell heights in the indicated tubule segments at 6 hours after NaOx challenge. H.) Immunoblot of PKD-associated signaling pathways in kidney lysates. I.) Immunofluorescence microscopy for phospho-S6\textsuperscript{235/236} and phospho-STAT3\textsuperscript{Y705} at indicated time points after NaOx challenge. J.) Ki67 immunostaining. K.) Kidney sections co-stained with Ki67 and phospho-histone H3\textsuperscript{S10} and quantification of positive cells as a percent of total cells. L.) Quantification of TUNEL-positive cells as a percent of total cells. M.) IHC for the macrophage marker CD68 in wild-type, untreated rats (control) or wild-type rats treated with NaOx at the indicated times post-NaOx administration. Cy\textsuperscript{+} rat kidney was stained for comparison. **P<0.01; ***P<0.001; ****P<0.0001; ψ P<0.0001. Scale bar=50 μm. Box whisker plots represent 90% of values with median displayed as a line in between the 2\textsuperscript{nd} and 3\textsuperscript{rd} quartiles and the mean displayed with “+”.

All panels representative of 3 experiments. Error bars represent SD. Mann-Whitney used for statistical analysis.
Figure 4: mTOR inhibition blunts tubule dilation and hypertrophy after NaOx challenge, and disrupts efficient CaOx crystal excretion.

A.) Immunoblotting of kidney lysates. B.) Immunostaining 6 hours after treatment with NaOx, with or without rapamycin pretreatment. C.) Quantification of Ki67-positive cells as a percent of total cells. D.) Immunostaining for segment-specific markers AQP1 and AQP2. E.) Quantification of tubular and lumen diameters with rapamycin (+R). F.) Inverted polarized light micrographs of whole kidneys after 6 hours or 3 days following NaOx treatment. G.) High magnification polarized light micrographs showing oxalate crystals in renal cortex and the corticomedullary boundary (CMB) at 6 hours following NaOx treatment with and without rapamycin pretreatment. H.) Pizzolato staining together with segment specific markers in rapamycin treated rats. Arrow heads denote crystals. I.) Quantification of intra-renal location of deposited CaOx crystals at 6 hours and 3 days after oxalate treatment with and without rapamycin. J.) Quantification of size distribution of CaOx crystals/aggregates in oxalate treated rats with and without rapamycin. Animals treated with oxalate: 6 hours (n=5), 1 day (n=5), 3 days (n=5), or 7 days (n=3). For oxalate and rapamycin treated animals; 6 hours (n=5), 1 day (n=5), 3 days (n=5), or 7 days (n=2) *P<0.05; ***P<0.001; **P<0.0001. Error bars represent SD. All panels representative of 3 experiments for oxalate treated animals. Scale bar=50μm. Box whisker plots represent 90% of values with median displayed as a line in between the 2nd and 3rd quartiles and the mean displayed with “+”. Mann-Whitney used for statistical analysis.
Figure 5: Chronic CaOx crystal deposition leads to increased cystogenesis and disease progression in the Han:SPRD rat model.

A.) Timeline of treatment. Han:SPRD rats were given 0.75% ethylene glycol in their drinking water starting at week 3 until 8 weeks of age. B.) Bright-field and polarized light microscopy of H&E-stained kidney sections. Scale bar=2mm C.) 2-Kidney/Body weight. D.) Renal cystic index. E.) Cyst numbers per animal kidney section. F.) Cyst sizes measured by surface area in H&E-stained sections. G.) Immunostaining for segment specific markers AQP1 and AQP2. Scale bar=50µm and 100µm. H.) Pizzolato staining from wild-type or Han:SPRD rats, either treated with or without citrate. I.) Pizzolato stained sections from untreated and citrate-treated male, Cy/+ Han:SPRD rats. Animals treated for this experiment; n=9 wild-type and n=9 Cy/+ males, 6 wild-type female, n=7 Cy/+ females. Untreated animals include; n=4 wild-type males, n=5 Cy/+ male rats and n=5 wild-type female, n=5 Cy/+ female. Scale bar=100µm and 50µm. *P<0.05; ***P<0.001. Images of ethylene glycol treated rats representative of 4 experiments and 3 experiments for citrate treated rats. Error bars represent SD. Mann-Whitney used for statistical analysis.
Figure 6: High phosphate diet induces renal tubular CaP deposition and accelerates disease progression in the PCK rat model.

Male and female PCK rats were given a control diet containing 0.6% P or a high phosphate diet (HPD) containing 1.2% P between 3 and 10 weeks of age. A.) Von Kossa staining showing CaP deposition (black). B.) Immunofluorescence microscopy for AQP1 and AQP2. C.) Overlay of von Kossa stain (black) with AQP1 and AQP2 immuno-staining CaP deposits. Primarily near the transition between proximal tubules and the descending loop of Henle (arrowheads). D.) Immunofluorescence staining of phospho-S60235/236PCK rats on HPD. E.) Quantification of CaP deposition. F.) 2-Kidney/Body weight ratios. G.) Kidney cyst scores. H.) BUN levels in female PCK rats on HPD. N=10 male, female, WT and cystic rats used for these experiments. Error bars represent SD. Data for E-H are transcribed from Supplemental Table 1. Scale bars 100μm (B), or 50μm (C,D). *, P<0.05, **,P<0.01, ***, P<0.001, ****, P<0.0001, unpaired two-tailed t-test used for statistical analysis.
Figure 7: mTOR and STAT3 activation in kidneys from primary hyperoxaluria (PH1), and citrate excretion inversely correlates with total kidney volume in ADPKD patients. 
A.) H&E-stained renal sections from a normal human kidney (NHK) or PH1 patient visualized by bright-field and polarized light microscopy (n=3). B.) Immunostaining for P-S6\textsuperscript{235/236} and P-STAT3\textsuperscript{Y705} in PH1 kidneys vs. NHK. Human ADPKD kidney was stained side-by-side for comparison. Scale bars=50 μm. C.) 24-hour urinary citrate excretion normalized to creatinine excretion correlated inversely with the log transformed total renal volumes of ADPKD patients. D.) The correlation between 24-hour urine albumin excretion normalized to creatinine excretion and the log transformed total renal volumes of ADPKD patients did not reach statistical significance. E.) 24-hour urinary citrate excretion normalized to creatinine and plotted against eGFR. The sample Pearson correlation coefficients are displayed for each graph. P values of C, D and E performed using linear regression analysis. Hashed lines represent 95% confidence intervals.