

Review Article

Npt2a as a target for treating hyperphosphatemia

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Hyperphosphatemia results from an imbalance in phosphate (P_i) homeostasis. In patients with and without reduced kidney function, hyperphosphatemia is associated with cardiovascular complications. The current mainstays in the management of hyperphosphatemia are oral P_i binder and dietary P_i restriction. Although these options are employed in patients with chronic kidney disease (CKD), they seem inadequate to correct elevated plasma P_i levels. In addition, a paradoxical increase in expression of intestinal P_i transporter and uptake may occur. Recently, studies in rodents targeting the renal Na^+/P_i cotransporter 2a (Npt2a), responsible for $\sim 70\%$ of P_i reabsorption, have been proposed as a potential treatment option. Two compounds (PF-06869206 and BAY-767) have been developed which are selective for Npt2a. These Npt2a inhibitors significantly increased urinary P_i excretion consequently lowering plasma P_i and PTH levels. Additionally, increases in urinary excretions of Na^+ , Cl^- and Ca^{2+} have been observed. Some of these results are also seen in models of reduced kidney function. Responses of FGF23, a phosphaturic hormone that has been linked to the development of left ventricular hypertrophy in CKD, are ambiguous. In this review, we discuss the recent advances on the role of Npt2a inhibition on P_i homeostasis as well as other pleiotropic effects observed with Npt2a inhibition.

Introduction

Plasma phosphate (P_i) is tightly controlled in the range from 0.9 to 1.45 mmol L⁻¹ in healthy adults [1], and requires a concerted interplay between intestinal uptake, storage/release from bone, and renal excretion. The kidney plays an important role in fine-tuning total body P_i via modulating its reabsorption. Hyperphosphatemia is a common consequence of deranged P_i homeostasis and is classified as plasma P_i levels >1.45 mmol L⁻¹ [1,2]. Hyperphosphatemia can further be subclassified as mild (1.44–1.76 mmol L⁻¹), moderate (1.76–2.08 mmol L⁻¹), or severe (>2.08 mmol L⁻¹) [3].

In the kidney, P_i transport occurs mainly in the proximal tubule where the sodium–phosphate cotransporters Npt2a (SLC34A1), Npt2c (SLC34A3), Pit1 (SLC20A1), and Pit2 (SLC20A2) are expressed (for a review see [4–6]). The overall contribution of some of these transporters in P_i reabsorption has been elucidated by using genetically modified mice. The renal contribution of Npt2a has been estimated to be $\sim 70\%$, based on studies in brush border membrane vesicles of Npt2a^{-/-} mice [7], and is associated with increased urinary P_i excretion and reduced plasma P_i levels [8]. The contribution of Npt2c to P_i reabsorption is less clear. Kidney-specific Npt2c knockout does not affect P_i homeostasis [9] and Npt2a/c double knockout mice show similar urinary P_i /creatinine ratios compared with Npt2a^{-/-} mice [10]; however, a compensatory up-regulation (~ 2.5 -fold) of Npt2c protein in brush border membrane vesicles of Npt2a^{-/-} mice was observed [11]. The contribution of Pit1 to renal P_i transport seems small and no differences in plasma P_i between Pit1^{-/-} and wild-type mice were found [12]. Knockout of Pit2 does not affect urinary P_i /creatinine or plasma P_i when fed a control diet but high dietary P_i causes elevated plasma P_i compared with wild-type mice [13]. Of note, a recent study discovered that Npt2b, a transporter described to be primarily expressed in the intestine and lungs, was localized to the thick ascending limb; however, Npt2b was not regulated by dietary P_i [14].

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Received: 13 November 2021

Revised: 8 December 2021

Accepted: 13 December 2021

Version of Record published:
7 January 2022

The primary regulators of renal P_i excretion are PTH and FGF23, both mediating the retrieval of Npt2a/c from the luminal membrane (for a review see [15]). PTH is released from the parathyroid glands in response to low plasma Ca^{2+} levels as well as high plasma P_i levels [16]. The mechanism how FGF23 is released in response to elevated plasma P_i is less clear; however, it has been postulated that activation of FGF receptor 1 in response to elevated plasma P_i can regulate FGF23 production as well as via production of GALNT3, an O-glycosylation enzyme that protects FGF23 from proteolytic cleavage [17]. Even though elevated plasma P_i is considered a driver for secondary hyperparathyroidism and elevated plasma FGF23 levels, hyperphosphatemia does not occur until later stages in CKD. Plasma FGF23 starts to increase in the early stages of CKD which is followed by an increase in PTH. These changes are sufficient to maintain P_i levels in the ‘physiological’ range until stage 4–5 CKD. This hypothesis is supported by studies showing that administration of a FGF23 neutralizing antibody increased plasma P_i in a rat model of reduced kidney function (5/6 Nx) [18], implying that elevated FGF23 levels are important for reducing plasma P_i levels in CKD. In addition, mice with drastically reduced FGF23 production in bone also showed increased plasma P_i levels in a mouse model of adenine-induced CKD [19]. Although elevated FGF23 levels are associated with a poor outcome in CKD (causing left ventricular hypertrophy [20]), FGF23 also protects the body from the detrimental cardio-renal consequences of elevated P_i . Hyperphosphatemia alone is associated with cardiovascular morbidity and is also a substantial risk factor for the development of vascular calcification in CKD [21].

Consequences of CKD on renal P_i transporters

How does CKD affect Npt2a expression? A continuous decline in nephron number in conjunction with elevated PTH and FGF23 levels potentially reduces Npt2a (and possibly Npt2c) expression. Consistent with this, in mouse and rat CKD models induced by adenine feeding, reduced expression levels of Npt2a protein and mRNA were observed [22–24]. In a different model of reduced kidney function in rats and mice (5/6 Nx), both species showed reduced Npt2a mRNA expression compared with sham animals [23,25]. Of note, renal Npt2b became highly expressed in the thick ascending limb in a model of oxalate-induced kidney failure [14]. Similarly, in adenine-induced CKD, renal Npt2b mRNA levels were ~20-fold greater compared with control mice [23]. The significance of these findings remains to be determined but raises the question if increased renal Npt2b expression in CKD paradoxically increases P_i reabsorption. Another feature of CKD is low urine pH, and the activity of Npt2a increases with higher pH [26]. Some of these observations could impact the therapeutic efficacy when targeting renal Na^+/P_i cotransporters.

Therapeutic approaches to reduce P_i burden in CKD

Treating hyperphosphatemia in CKD remains a significant challenge. Currently, the treatment options for hyperphosphatemia are limited to dietary P_i restriction, oral P_i binders, and niacin/nicotinamide; however, all have been proven to have limitations [27–29]. The efficacy of dietary P_i restriction and P_i binders is further limited due to increased maladaptive P_i uptake in the gastrointestinal tract [30,31]. With the vision of inhibiting P_i uptake from the intestine, a non-absorbable Npt2b inhibitor (in the intestine Npt2b is responsible for >90% of active P_i uptake [32]) was developed [33]. Unfortunately, clinical trials with this compound were discontinued due to ineffectiveness in reducing plasma P_i in healthy volunteers and patients on hemodialysis [33]. A novel pan-phosphate transporter inhibitor (EOS789: Npt2a, Pit1/2) was able to maintain lower levels of plasma P_i in parallel with lower FGF23 and PTH in a long-term study [34] and was found to be safe in a phase 1b clinical trial in patients on hemodialysis [35]. Further studies are needed to prove its efficacy. Tenapanor, a non-absorbable intestinal-specific Na^+/H^+ exchanger isoform 3 inhibitor, was found to be an effective drug to reduce plasma P_i and FGF23 levels in patients on hemodialysis [36,37]. However, the United States Food and Drug Administration denied approval because of an effect that was ‘...small and of unclear clinical significance’ [38], and requested additional studies before approval will be granted.

Development of Npt2a inhibitors – *in vitro* studies

Until recently, no selective inhibitors were developed that target renal Npt2a. Pfizer developed PF-06869206 [39], which has a 3-chloro-2-methylazaindole core. Cell culture experiments in HEK cells, transfected with human, rat or mouse P_i transporters (Npt2a, Npt2c, Pit1 or Pit2), showed selectivity for Npt2a over all other tested transporters, with an IC_{50} ~500 nmol L^{-1} [39]. An i.v. dose of 1 mg kg^{-1} in rats showed a half-life of 4.8 h, while a dose of 5 mg kg^{-1} in mice showed a half-life of 0.75 h. At higher oral doses, supra-proportional increases in plasma concentrations were observed, possibly indicative of saturation of clearance or enterohepatic

recycling resulting in longer apparent half-lives. Bayer developed BAY-767 (structure not publicly available) with an IC_{50} of ~ 4 nmol L^{-1} for rat/human Npt2a and >100 -fold selectivity over Npt2b, Npt2c or Pit-1 in stably transfected CHO cells [40]. No data regarding half-life are available for this compound.

The inhibitory kinetics of PF-06869206 were also studied by our group in OK cells which endogenously express Npt2a, Npt2c, Pit1 and Pit2 and are a commonly used model to study electrolyte and P_i transport in the proximal tubule [41–43]. In OK cells, PF-06869206 caused a dose-dependent inhibition of Na^+ -dependent $^{32}P_i$ uptake ($IC_{50} \sim 1$ $\mu\text{mol } L^{-1}$), reaching a maximum inhibitory effect of $\sim 70\%$ at 100 $\mu\text{mol } L^{-1}$. The non-selective Npt2 inhibitor PFA inhibited $\sim 90\%$ of Na^+ -dependent P_i uptake, indicating that, in addition to Npt2a, Npt2c is inhibited [8]. Comparable results were found in acutely isolated rat proximal tubule cells: PF-06869206 inhibited $\sim 55\%$ of ^{32}P uptake at the maximum tested concentration of 30 $\mu\text{mol } L^{-1}$ and PFA inhibited 70% of Na^+ -dependent P_i uptake [44]. Michaelis–Menten kinetics in OK cells identified no significant difference in apparent maximum velocity of reaction; however, showed a ~ 2.4 -fold higher substrate concentration for P_i in response to PF-06869206, which is consistent with a competitive mode of inhibition [8].

In vivo effects of Npt2a inhibition – urinary P_i excretion

PF-06869206 shows good bioavailability in rats and mice. In our studies, acute oral gavage of PF-06869206 caused a dose-dependent increase in urinary P_i excretion in C57Bl/6J mice. The maximum dose of 100 mg kg^{-1} caused a ~ 6 -fold increase (3 h period) in urinary P_i excretion compared with vehicle ($ED_{50} \sim 21$ mg kg^{-1}) [45]. Studies looking at fractional excretion index (FEI) showed that FEI increased with higher doses of the inhibitor. At the highest dose tested (500 mg kg^{-1}), a ~ 17 -fold increase in the FEI of P_i was observed (4 h period). BAY-767 caused a ~ 1.7 -fold increase in fractional urinary P_i excretion over a 16 h period at the highest dose tested (10 mg kg^{-1}) [40]. Studies by 2 different groups confirmed the specificity of PF-06869206 by using Npt2a $^{-/-}$ mice which completely lack a change in urinary P_i excretion [8,44]. In terms of the increase in urinary P_i excretion, Npt2c $^{-/-}$ mice were indistinguishable from wild-type mice. Other models in which the phosphaturic effect of PF-06869206 has been tested are FGF23 $^{-/-}$ mice (characterized by growth retardation, abnormal bone phenotype, hyperphosphatemia and short life span [46]) and GALNT3 $^{-/-}$ mice (characterized by increased FGF23 proteolysis consequently lowering intact FGF23 levels and leading to hyperphosphatemic familial tumoral calcinosis [47]). In both models, FEI of P_i increased in response to PF-06869206 ~ 9 -fold and ~ 2 -fold, respectively, at a dose of 300 mg kg^{-1} compared with vehicle [44].

Effects of Npt2a inhibition on plasma P_i , PTH and FGF23

Do the above-described effects of Npt2a inhibition on urinary P_i excretion reduce plasma P_i levels and subsequently affect PTH and FGF23 levels? Our studies in C57Bl/6J mice show that PF-06869206 at a dose of 30 mg kg^{-1} reduced plasma P_i starting after 1 h and reaching a maximum (-35%) 2 h after administration, with a full recovery after 24 h [8,45]. Somewhat unexplained is the 10-fold higher dose (300 mg kg^{-1}) required to demonstrate a significant decrease in plasma P_i in C57Bl/6 mice in the studies performed by Clerin et al. [44]. Two to four hours after administration of PF-06869206, plasma P_i significantly decreased in Npt2c $^{-/-}$ (-33%), FGF23 $^{-/-}$ (-20%) and GALNT3 $^{-/-}$ mice (-20%) [44], the latter two providing evidence for efficacy of PF-06869206 under hyperphosphatemic conditions. Our unpublished observations also showed that PF-06869206 reduced plasma P_i levels in mice fed a high P_i diet. However, both studies [40,41] independently confirmed the specificity of these effects by using Npt2a $^{-/-}$ mice. Three-day treatment of rats with BAY-767 resulted in $\sim 20\%$ lower plasma P_i levels at 10 mg kg^{-1} [40].

The membrane abundance of Npt2a can be directly regulated by PTH and FGF23. Our studies in C57Bl/6J mice showed a $\sim 50\%$ reduction in PTH 3 h after administration of PF-06869206 at a dose of 30 mg kg^{-1} . Clerin et al. [44] reported that in mice, PTH levels were $\sim 65\%$ lower in response to 300 mg kg^{-1} PF-06869206 compared with vehicle 2 and 4 h after administration. Both studies indicated that 24 h after administration PTH levels returned to baseline. Rats treated with BAY-767 also showed $\sim 50\%$ lower PTH after 3 days of treatment with 10 mg kg^{-1} compared with vehicle [40]. Of note, the Ca^{2+} -sensing receptor in parathyroid glands also functions as a P_i sensing receptor explaining the stimulatory effect of P_i on PTH release [16]. In contrast with PTH, FGF23 levels were not significantly affected by PF-06869206 treatment in mice [44,45]. Of note, in rats treated with BAY-767, a $\sim 25\%$ reduction in FGF23 was observed [40]. The reason for these discrepancies in FGF23 responses remains to be determined.

Npt2a inhibition in CKD/reduced kidney function

As a model of reduced kidney function, our group employed 5/6 Nx in mice. However, in our studies, this model lacked some key features of CKD, such as hyperphosphatemia and secondary hyperparathyroidism. Eight weeks after surgery, acute administration of PF-06869206 showed a dose dependence for urinary P_i excretion (Figure 1A); however, the maximum effect (at 100 mg kg^{-1}) was smaller in 5/6 Nx mice compared with sham mice (~ 2 -fold versus ~ 10 -fold, respectively). A dose of 100 mg kg^{-1} resulted in a significant decrease in plasma P_i ($\sim 35\%$ versus $\sim 50\%$, respectively; 1–3 h after administration, Figure 1C) and PTH ($\sim 60\%$ versus $\sim 65\%$, respectively, 3 h after administration, Figure 1D). Clerin et al. [44] studied 5/6 Nx rats that were treated with PF-06869206 (300 mg kg^{-1} , q.d. via oral gavage) for 8 weeks. This long-term administration showed that FEI of P_i was ~ 2.5 -fold higher compared with vehicle treated 5/6 Nx rats. Plasma P_i was $\sim 15\%$ lower in PF-06869206-treated compared with vehicle treated 5/6 Nx rats. In rats with 5/6 Nx, there was also no hyperphosphatemia, but secondary hyperparathyroidism and elevated FGF23 levels were observed compared with

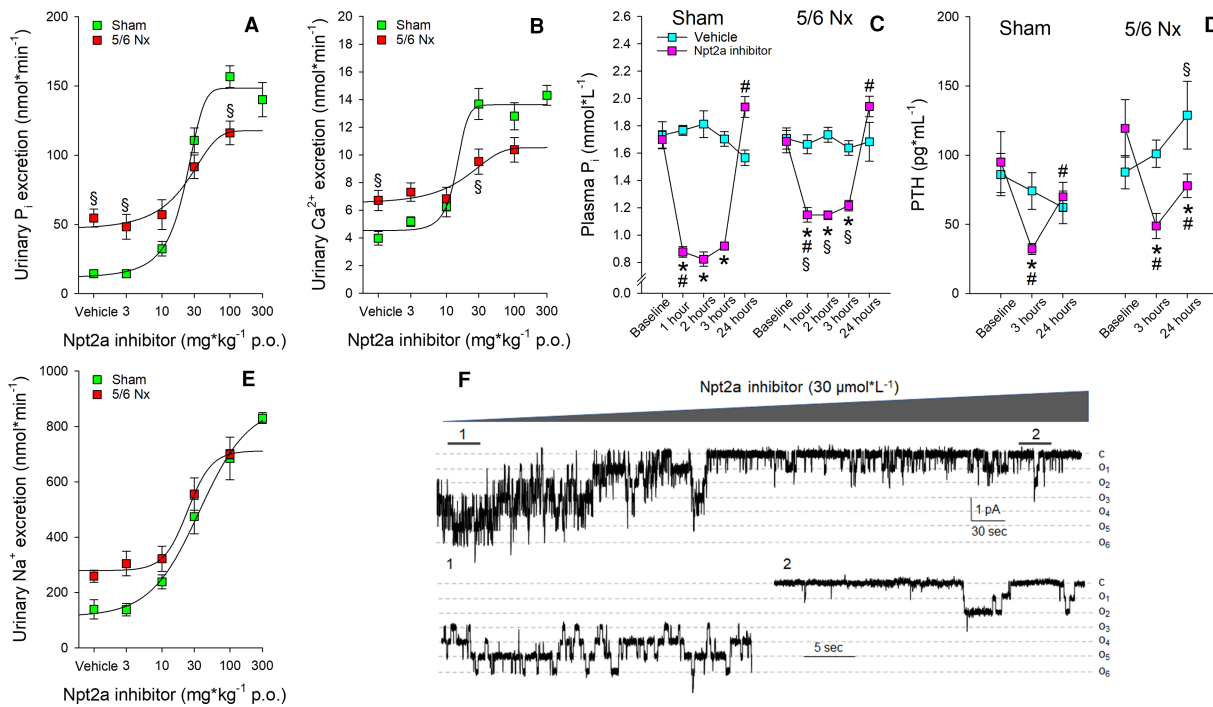


Figure 1. Pharmacological effects of Npt2a inhibition with PF-06869206 on renal and plasma parameters in mice with normal kidney function and 5/6 nephrectomy.

Eight weeks after subtotal nephrectomy (5/6 Nx) or sham surgery, inhibition of Npt2a by PF-06869206 (given via oral gavage, p.o.) caused a dose-dependent increase in urinary (A) P_i and (B) Ca^{2+} excretion (3 h metabolic cage experiments). This was associated with reductions in (C) plasma P_i and (D) PTH levels in sham and 5/6 Nx mice, while plasma Ca^{2+} remained unaffected (not shown). At a dose of 100 mg kg^{-1} , effects on plasma P_i were smaller in magnitude in 5/6 Nx compared with sham mice; however, the effect on plasma PTH was not different between groups. In addition to the effect of PF-06869206 on urinary P_i and Ca^{2+} excretion, a dose-dependent increase in urinary Na^+ excretion (E) was found in both groups, that was not significantly different from each other. Due to a lack of effect on urinary K^+ excretion and unaffected natriuresis in Npt2a $^{-/-}$ mice (both not shown), we hypothesized that the natriuresis is a result of inhibition of Na^+ transport in the connecting tubule/collecting duct, rather than in the proximal tubule, where the epithelial Na^+ channel ENaC is expressed. In electrophysiological studies in acutely split-open cortical collecting ducts of C57BL/6 mice, ENaC open probability was measured in cell-attached patches formed on the apical membrane of principal cells. The pipette was backfilled with Npt2a inhibitor ($30 \mu\text{mol L}^{-1}$). A continuous current trace is shown in (F). The areas under the bars over the continuous traces are shown below at expanded timescales. Dashed lines indicate the respective current levels, with c denoting the closed state and o denoting the open state. Open probability was acutely inhibited ($\sim 85\%$) by PF-06869206, providing evidence that the natriuresis might be the consequence of off-target effects on ENaC. Data taken from [8,45]. * $P < 0.05$ versus vehicle, § $P < 0.05$ versus sham, # $P < 0.05$ versus previous time point.

sham rats; however, treatment with PF-06869206 did not reduce PTH or FGF23 levels. So far, BAY-767 has not been tested in CKD models.

Pleiotropic effects of Npt2a inhibition

Are other minerals and electrolytes affected by Npt2a inhibition? In addition to effects on urinary P_i excretion, PF-06869206 increased urinary Na^+ , Cl^- and Ca^{2+} excretion (~5-fold, ~5-fold and ~3-fold greater at 300 mg kg^{-1} compared with vehicle, respectively) in our studies without affecting their respective plasma levels [45]. Effects on urinary Ca^{2+} excretion (~5-fold greater at 300 mg kg^{-1} compared with vehicle) have been confirmed by Clerin et al. [44], other parameters were not determined and are also not reported for BAY-767. The mechanism for the increased Ca^{2+} excretion remains elusive but could be caused by either inhibition of Ca^{2+} reabsorption in the proximal tubule or in the distal convoluted tubule where TRPV5 is expressed. The latter could be inhibited by decreased PTH in response to PF-06869206, leading to increased Ca^{2+} excretion. Of note, we did not observe effects on urinary K^+ , glucose or amino acid excretion, and urinary pH remained unaffected. Together this implies that there is no generalized effect on the proximal tubule as found, for example, in Fanconi syndrome. Similarly, in mice with 5/6 Nx, PF-06869206 caused dose-dependent responses

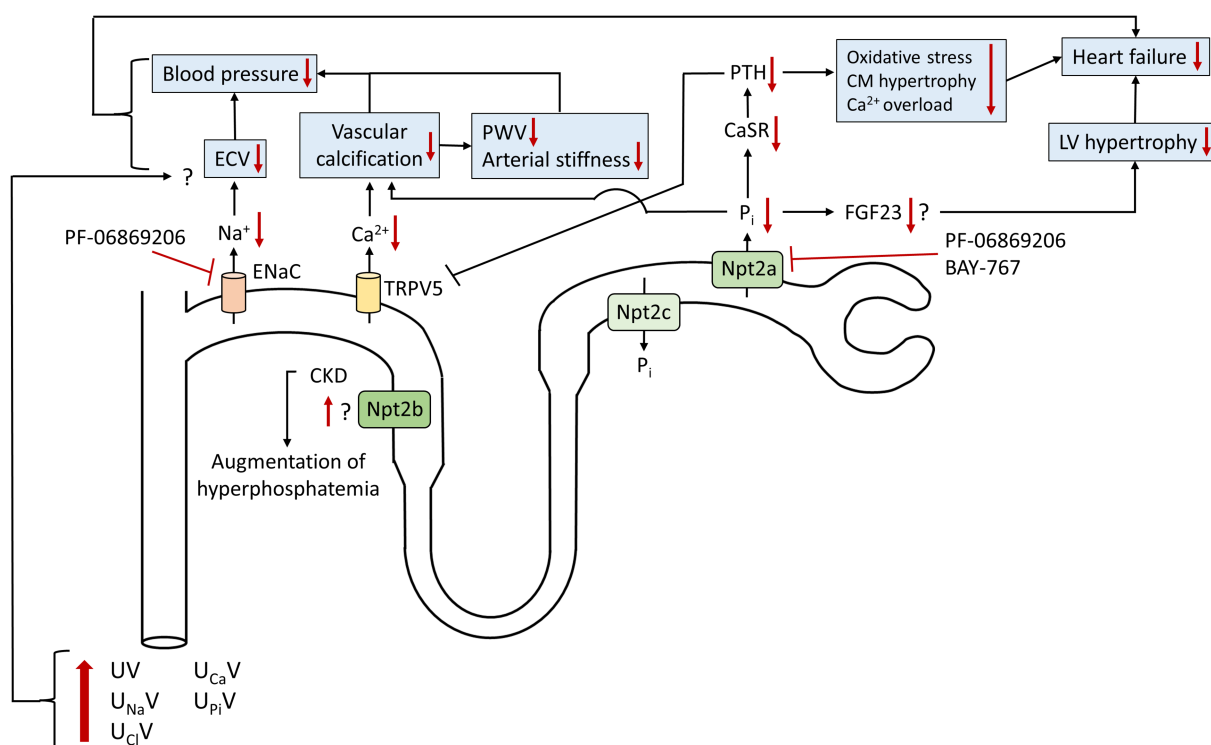


Figure 2. Proposed effects of Npt2a inhibition on renal electrolyte/mineral excretion and the potential role in cardiovascular protection.

Npt2a blockade with either PF-06869206 or BAY-767 increases urinary P_i excretion and consequently, plasma P_i and PTH are reduced. The reduction in PTH, which most likely occurs via reduced activation of the calcium-sensing receptor (CaSR) on the parathyroid glands, may be protective against development of heart failure due to reduced PTH-induced cardiomyocyte (CM) hypertrophy, cardiac Ca^{2+} overload, and increased oxidative stress. Elevated FGF23 causes left ventricular (LV) hypertrophy in chronic kidney disease (CKD). Npt2a inhibition with BAY-767, but not PF-06869206, resulted in a decrease in plasma FGF23. Lowering FGF23 could reduce LV hypertrophy and possibly the development of heart failure. The diuretic and natriuretic effects of Npt2a blockade, the latter via reduced open probability of the epithelial sodium channel (ENaC), should reduce the effective circulating volume (ECV) and blood pressure. Either via a direct effect of Npt2a blockade in the proximal tubule or indirectly via a reduction in PTH and transient receptor potential cation channel 5 (TRPV5)-mediated Ca^{2+} reabsorption, urinary Ca^{2+} excretion is increased. The calciuretic effect, in combination with the phosphaturic effect, should reduce vascular calcification, pulse wave velocity (PWV), and arterial stiffness. This is expected to reduce blood pressure and further slowdown the progression of heart failure. New data have provided evidence of increased Npt2b expression in CKD; however, further studies are needed to determine its (patho)physiological relevance.

in urinary Na^+ (Figure 1E), Cl^- and Ca^{2+} (Figure 1B) excretion without affecting excretion of urinary K^+ , glucose, or pH [45].

We expected that the increase in urinary Na^+ excretion would be blunted in $\text{Npt2a}^{-/-}$ mice; however, to our surprise, the PF-06869206-induced natriuresis was still present in $\text{Npt2a}^{-/-}$ mice [8]. In conjunction with the lack of effect on urinary K^+ excretion, we hypothesized that this might be an off-target effect in the aldosterone-sensitive distal nephron via inhibition of the ENaC. Subsequent studies in acutely isolated, split-open cortical collecting ducts (Figure 1F) showed that ENaC open probability was reduced by ~85% in response to PF-06869206 [8], giving a possible explanation why $\text{Npt2a}^{-/-}$ mice still showed a natriuresis. Total body Na^+ and blood pressure are interdependent variables; however, despite the acute natriuresis and diuresis observed in 5/6 Nx mice in response to PF-06869206 [45], long-term treatment with PF-06869206 in 5/6 Nx rats did not affect systolic blood pressure [44]. The reason(s) for these differences remain to be determined.

Effect of *Npt2a* inhibition on vascular calcification

Another pleiotropic effect that needs special consideration relates to the increase in Ca^{2+} (and P_i) excretion in response to PF-06869206 and BAY-767. Hormonal, mineral and other changes observed in CKD provide the perfect storm for accelerated vascular calcification. The latter goes along with reduced arterial elasticity, hypertension and augmented pulse-wave velocity (Figure 2). Together with elevated FGF23 levels this can result in left ventricular hypertrophy and ultimately heart failure (Figure 2), which is most exaggerated in patients on hemodialysis [48] and contributes substantially to cardiovascular mortality in CKD. BAY-767 has been studied in this regard [40]. Vascular calcification in rats was induced via administration of a pan-FGF receptor inhibitor for 10 days, which increased plasma P_i levels ~2-fold. Concomitant oral treatment with BAY-767 (10 mg kg^{-1} , q.d.), resulted in a reduced (~1.4-fold) increase in plasma P_i . Calcium content in the aorta was reduced by ~75% in response to BAY-767 treatment. Of note, 2.2% lanthanum carbonate (administered via diet), an oral phosphate binder, did not affect aortic Ca^{2+} content [49].

Perspectives

- Current treatment options for hyperphosphatemia in patients with or without reduced kidney function are insufficient. Inhibitors of renal *Npt2a* offer a novel treatment avenue to correct this condition and its detrimental consequences (vascular calcification, left ventricular hypertrophy, heart failure).
- Recent research has shown the feasibility of this approach in animal models of normal and reduced kidney function. Elevated PTH and FGF23 levels suppress renal *Npt2a* in CKD; however, PF-06869206 was still effective in reducing plasma P_i and PTH in CKD models.
- Further studies in different CKD models are needed to investigate the role of *Npt2a* inhibition on the course of CKD as well as possible reductions in cardiovascular complications.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases grant 1R01DK110621 (to Dr. Rieg), VA Merit Review Award IBX004968A (to Dr. Rieg) and an American Heart Association Transformational Research Award 19TPA34850116 (to Dr. Rieg). Financial support for this work was also provided by the NIDDK Diabetic Complications Consortium (RRID:SCR_001415, www.diacomp.org), grants DK076169 and DK115255 (to Dr. Rieg). Dr. Thomas was supported by an American Heart Association postdoctoral fellowship (19POST34400026 and 828731).

Author Contributions

All authors wrote and reviewed the article.

Abbreviations

CHO, Chinese hamster ovary; CKD, chronic kidney disease; ED₅₀, effective dose 50; ENaC, epithelial sodium channel; FEI, fractional excretion index; FGF23, fibroblast growth factor 23; GALNT3, Polypeptide N-Acetylgalactosaminyltransferase 3; HEK, human embryonic kidney; OK, opossum kidney; PFA, phosphonoformic acid; PTH, parathyroid hormone; q.d., quaque die; TRPV5, transient receptor potential vanilloid 5; 5/6 Nx, 5/6 nephrectomy.

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