The Role of Extracellular Phosphate Levels on Kidney Disease Progression in a Podocyte Injury Mouse Model

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Abstract

Background: Hyperphosphatemia is a major accelerator of complications in chronic kidney disease and dialysis, and phosphate (Pi) binders have been shown to regulate extracellular Pi levels. Research on hyperphosphatemia in mouse models is scarce, and few models display hyperphosphatemia induced by glomerular injury, despite its relevance to human glomerular disease conditions. In this study, we investigated the involvement of hyperphosphatemia in kidney disease progression using a mouse model in which hyperphosphatemia is induced by focal segmental glomerulosclerosis (FSGS). **Methods:** We established the NEP25 mouse model in which FSGS-hyperphosphatemia is induced by podocyte injury and evaluated the effect of a Pi binder, sevelamer. **Results:** After disease induction, we confirmed a gradual increase in serum Pi accompanied by reduced renal function and observed increases in serum FGF23 and PTH. Treatment with sevelamer significantly reduced serum Pi and urinary Pi fractional excretion and suppressed increases in serum FGF23 and PTH. A high dose improved serum creatinine and tubular injury markers, and pathological analysis confirmed amelioration of glomerular and tubular damage. Gene expression and marker analysis suggested protective effects on tubular epithelial cells in the diseased kidney. Compared to disease control, NEP25 mice treated with sevelamer retained their mRNA expression of Klotho, a known FGF23 co-receptor and renoprotective factor. **Conclusions:** Hyperphosphatemia caused by renal function decline was observed in a FSGS-induced NEP25 mouse model. Studies using this model showed that Pi regulation had a positive impact on kidney disease progression, and notably on tubular epithelial cell injury, which indicates the importance of Pi regulation in the treatment of kidney disease progression.

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Introduction

Inorganic phosphate (Pi) is an important nutrient for maintaining energy balance and bone mineral metabolism. Blood Pi levels are regulated with calcium (Ca), which in turn is tightly regulated by a complex endocrine system that includes PTH, FGF23, klotho, vitamin D, and so on [1]. Blood Pi and Ca levels are higher in outside cells

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than inside, which facilitates rapid availability when needed within cells but also increases the risk of undesired calcification in other tissues [1–4]. Usually, calcification is tightly regulated by proteins in the endocrine system, such as Fetuin-A, and so on [5, 6], but in kidney disease or other conditions requiring dialysis, reduced expression of Fetuin-A levels in blood compromises Pi regulation [5]. The dysfunction of the Pi regulation system leads to secondary hyperparathyroidism, bone disease, ectopic calcification, and high mortality. Lowering Pi levels with Pi binders that inhibit Pi absorption in the gut improves clinical outcomes in such cases [7].

The amelioration of hyperphosphatemia and other complications by regulating Pi with Pi binders was demonstrated in several preclinical studies with rodent kidney disease models, such as the adenine model [8–10]. Moreover, studies in anti-GBM nephritis [11] or chronic anti-Thy1 nephritis rat models [12] show that lowering serum Pi with Pi binders or restricted Pi intake improves not only abnormal mineral metabolism but also the progression of kidney disease itself. Although these studies showed the renoprotective effects of Pi regulation in glomerular injury models, little has been reported on the effect of Pi regulation in cases of direct tubular injury. The adenine model commonly used for rat and mouse studies primarily uses tubular injury to induce hyperphosphatemia [8–10], but this method is too artificial for evaluating the renoprotective effects of Pi regulation. Because there are only a few studies on hyperphosphatemia induced by glomerular injury, the renoprotective effect of Pi regulation in mice remains unexplored [13]. In this study, we used NEP25 mice, which are genetically engineered transgenic mice expressing human CD25 in only the podocytes, thus creating a podocyte-specific injury model without any direct effect on other cell types [14]. When these mice are injected with an immunotoxin, LMB2, they show strong proteinuria, reduced renal function accompanied by collapsing focal segmental glomerulosclerosis, and secondary tubule-interstitial fibrosis [15]. Since little is known about Pi metabolism in this NEP25 mouse model, this study evaluated the role of extracellular Pi in kidney disease progression using the Pi binder sevelamer.

Materials and Methods

Animals

NEP25 mice, heterozygous podocyte-specific hCD25 transgenic mice, were bred by Chugai Pharmaceutical Co. Ltd. and also kindly provided by Matsusaka et al. [14] in Tokai University. All mice were kept in well-controlled animal housing with free access to water and pellet or powder food. Animal procedures and protocols were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical Co. Ltd. and approved by the Institutional Animal Care and Use Committee.

Experimental Design

Study 1: Nine-week-old male NEP25 mice were fed a normal pellet diet (CE-2, Japan CLEA) containing 1.1% of Pi and 1.18% of Ca. To induce disease, NEP25 mice, other than in the control group, were injected with 0.8 μg/kg of immunotoxin LMB2 (provided from National Institutes of Health, MD, USA) via the tail vein. Blood and urine samples were collected on day 7, 14 or 21. Blood samples were collected from the abdominal portion of the vena cava under isoflurane anesthesia, transferred into MiniCollect tubes, and centrifuged (3,000 g, 15 min, 4°C) to prepare serum samples.

Study 2: Nine-week-old male NEP25 mice were fed a normal powder diet (CE-2, Japan CLEA) or diet containing 1% or 3% of sevelamer-H_2CO_3 (Sanofi-Genzyme) starting the day before disease induction. In the captopril group, captopril (TCI Chemicals, Japan; 0.15 mg/mL in drinking water, approximately 30 mg/kg/day) was first given the day before LMB2 injection and continued until sacrifice. Blood samples were collected from the jugular vein on day 14 or the abdominal portion of the vena cava under isoflurane anesthesia on day 21 and serum samples were prepared as in Study 1. Urine samples were collected on day 14 or 21. After sacrifice on day 21 under isoflurane anesthesia, serum samples and kidney tissues were collected. The collected kidney tissues were dissected for pathology (formalin-fixed and methyl carnoy-fixed) and mRNA expression analysis (for mRNA, only the cortex region was collected and frozen in liquid nitrogen).

Biochemical Analysis

Serum and urine samples were measured (cystatin-C, urea nitrogen, creatinine [urine], total protein, Pi, Ca) using a Hitachi 7,100 Autoanalyzer. Serum creatinine levels were measured using the LC-MS method. Creatinine was measured using hydrophilic interaction ultra-high-performance liquid chromatography coupled with API4000 (HILIC-UPLC-MS/MS) and stable isotopically labeled creatinine-d3 was used as an internal standard to measure endogenous creatinine. Urine neutrophil gelatinase-associated lipocalin (NGAL) was measured using ELISA (BioPORTO, Denmark). Serum PTH and FGF23 were measured using mouse PTH (1–84) ELISA (Immutopics, Quidel, San Diego, CA, USA) or an FGF23 ELISA kit (Kainos, Japan). Serum T50 was measured with a nephelometer assay, adjusted to the method described by Pasch et al. [16].

Pathological Analysis

Kidney tissues were fixed with 10% neutral buffered formalin solution (for hematoxyline-eosin [HE] staining) or methyl carnoy's fixative (for periodic acid-Schiff [PAS] staining) and then embedded in paraffin. The paraffin blocks were cut in 4 μm and stained for HE or PAS. As in previous studies, PAS-positive areas in each glomerulus were graded using a score of 0–4 [14, 15]. Over 50 randomly selected glomeruli from each mouse were evaluated for glomerulosclerosis. A score of 0 represents no lesion, and 1, 2, 3, and 4 represent mesangial matrix expansion, hyalinosis, or sclerosis involving ≤25%, 25–≤50%, 50–≤75%, and >75% of the glomerular tuft area respectively. Tubulointerstitial damage on HE-stained sections from each mouse was graded using a score of 0–4.
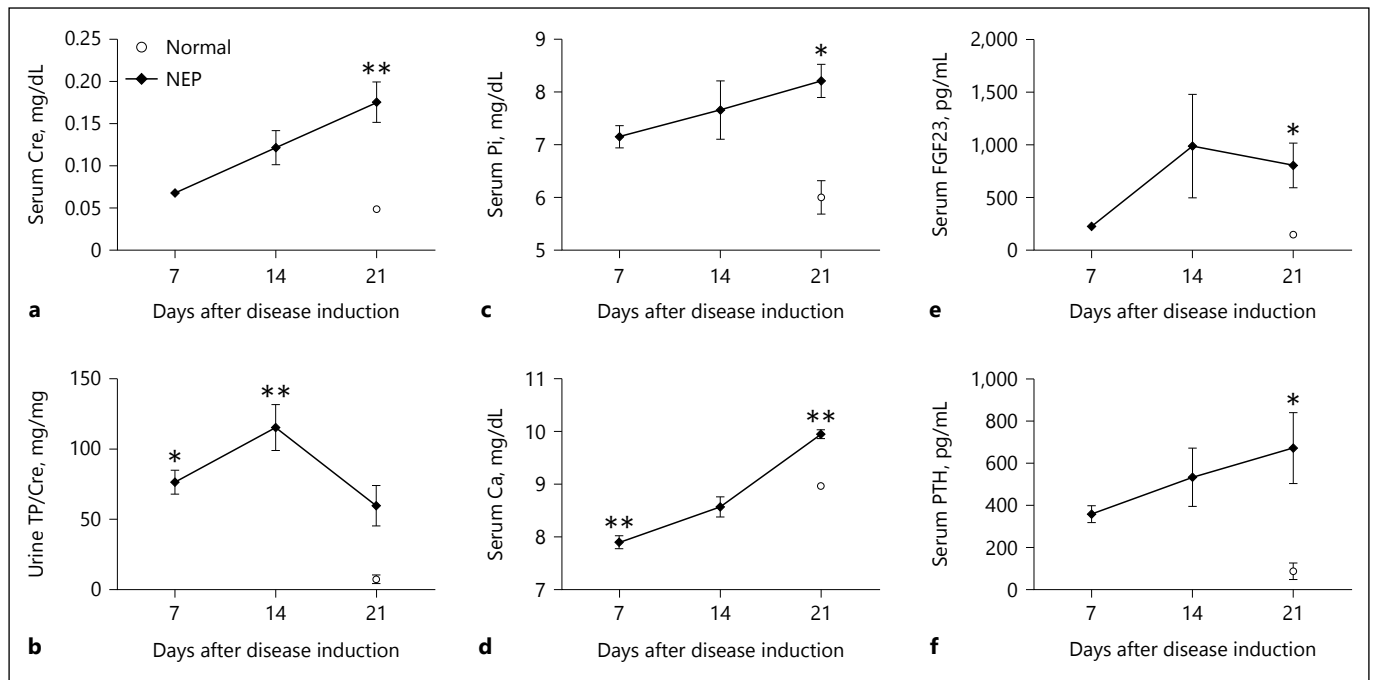
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Fig. 1. Serum creatinine, proteinuria, serum Pi, Ca, FGF23, and PTH in NEP25 mice. **a** Serum creatinine increased gradually in NEP25 mice after LMB2 injection. **b** Proteinuria was observed from day 7, increased further on day 14, and then dropped on day 21. **c** Serum Pi increased from day 7 gradually. **d** Serum Ca was below normal levels on days 7 and 14 and then increased above levels in the normal control. **e**, **f** Serum FGF23 and PTH increased gradually from day 7. Mean ± SE. Normal control: $n = 3$, disease: day 7, $n = 10$; day 14, $n = 10$; day 21, $n = 8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Dunnett's test or Kruskal-Wallis test vs. NC. Pi, phosphate; Ca, calcium; Cre, creatinine.

Score 0 represents no lesion, and 1, 2, 3, and 4 represent lesions of tubulointerstitial fibrosis/inflammation, involving ≤25%, 25–≤50%, 50–≤75%, and >75% of the cortex tubulointerstitial area respectively [15]. The pathological index was scored unblinded.

Immunohistochemistry
p57 was stained by p57 Kip2 rabbit polyclonal antibody (PA5–16,539, Invitrogen ThermoFisher) in a methyl carnoy-fixed paraffin block. Antigen retrieval was performed by microwave (98 °C, 20 min). Podocyte numbers were counted in over 50 randomly selected glomeruli from each animal.

Gene Expression
Kidney mRNA samples were extracted from frozen kidney cortex samples using an RNeasy Mini kit (Qiagen). cDNA samples were synthesized from RNA samples using a reverse transcription kit (Roche Applied Science). Gene expression levels were measured by real-time PCR, using Taqman probe/primer sets (Applied Biosystems) for each gene.

Statistical Analysis
Statistical analysis except for the pathological analysis was performed by Student t test or Dunnett's test using a Prism GraphPad7 (GraphPad Software, La Jolla, CA, USA). A one-way ANOVA was performed before Dunnett's test. Pathological analysis was performed by a Mann-Whitney test or Kruskal-Wallis test versus DC.

Results

Evaluation of NEP25 Mouse Model as a Hyperphosphatemia Model

As in the previous study by Moll et al. [15], after injecting mice with LMB2 to induce kidney disease, serum creatinine levels gradually increased (Fig. 1a). Proteinuria, which was present on day 7 and further elevated on day 14, decreased on day 21, but was still at abnormal levels (Fig. 1b). Serum Pi increased gradually (Fig. 1c). Serum Ca remained below normal levels on day 7 and 14, but had exceeded them by day 21 (Fig. 1d). Corresponding to serum Pi, serum FGF23 and intact PTH were also elevated (Fig. 1e, f). These results confirm that hyperphosphatemia occurred in the NEP25 mice as a result of primary glomerular injury-induced chronic renal dysfunction.

The Effects of Sevelamer on Pi and Ca Levels

We evaluated the effects of sevelamer, a Pi binder, on Pi in the NEP25 model. On days 14 and 21, serum Pi increased significantly in the disease control. Sevelamer 3% treatment significantly suppressed the serum Pi in-
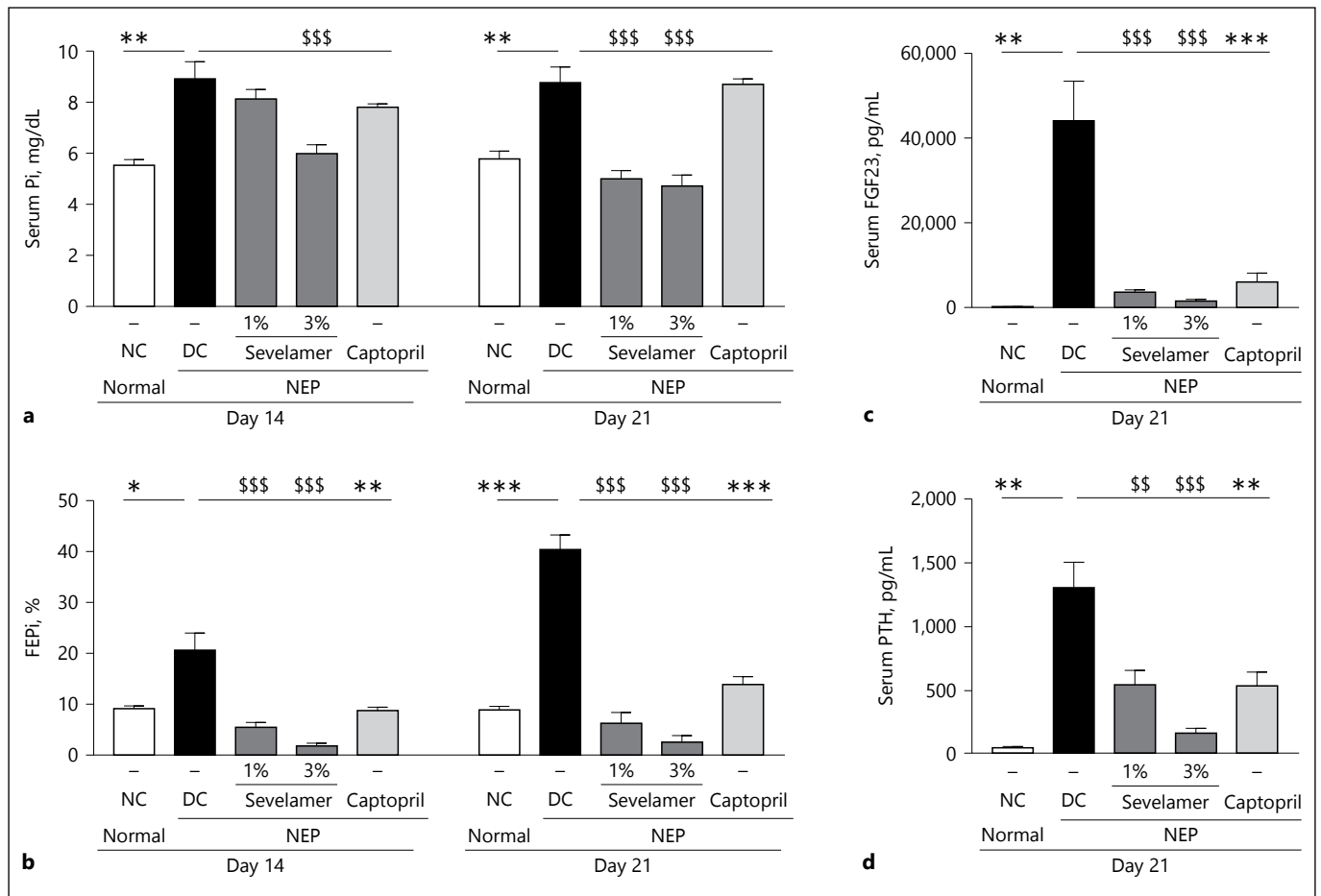
Fig. 2. Effects of sevelamer and captopril on serum Pi, FEPi, serum FGF23, and PTH in NEP25 mice. **a** Serum Pi levels in NEP25 mice were higher than in the normal control on days 14 and 21. Sevelamer 3% ameliorated the increases on days 14 and 21, sevelamer 1% only on day 21. Captopril did not significantly alter the serum Pi. **b** FEPi was increased on days 14 and 21 in NEP25 mice. Sevelamer ameliorated the increases to below normal control levels in a dose-dependent manner on days 14 and 21. Captopril also brought levels down to those in the normal control. **c, d** Serum FGF23 and PTH levels in NEP25 mice increased on day 21. Sevelamer reduced the increase in both proteins. Captopril also reduced these increases. The actual values of FGF23 for mean ± SE were NC: 210 ± 16 pg/mL, DC: 44,050 ± 9,325 pg/mL. Mean ± SE, normal control: $n = 3$, disease control: $n = 7$, sevelamer 1%, 3%, and captopril: $n = 8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student t test vs. DC. $^\$ p < 0.05$, $^{\$\$} p < 0.01$, $^{\$\$\$} p < 0.001$, Dunnett's test vs. DC. Pi, phosphate; FEPi, fractional excretion of Pi.

creases on days 14 and 21, but sevelamer 1% treatment significantly suppressed serum Pi only on day 21. We set the captopril treatment group as a control to compare renoprotective effects. The Captopril treatment failed to suppress the serum Pi increase (Fig. 2a). On days 14 and 21, urinary fractional excretion of Pi % (FEPi%) increased significantly in the disease control. Sevelamer treatment drastically ameliorated the FEPi% to below normal levels in a dose-dependent-manner on days 14 and 21. Captopril treatment brought FEPi% close to normal levels (Fig. 2b). Of note, kidney Slc34a1 and Slc34a3 mRNA were downregulated, possibly due to proximal tubular injury. Sevelamer and captopril significantly ameliorated the reduction in Slc34a3 expression but not the reduction in Slc34a1 expression (online suppl. Fig. S1a, b; for all online suppl. material, see www.karger.com/doi/10.1159/000497118). Serum FGF23 and PTH increased significantly in the disease control. Both sevelamer and captopril treatments significantly suppressed increases in serum FGF23 and PTH. Sevelamer 3% showed the strongest suppression in serum FGF23 and PTH (Fig. 2c, d). Sevelamer mitigated hyperphosphatemia, FGF23 increases, and secondary hyperparathyroidism in NEP25 mice, but a high dose was needed
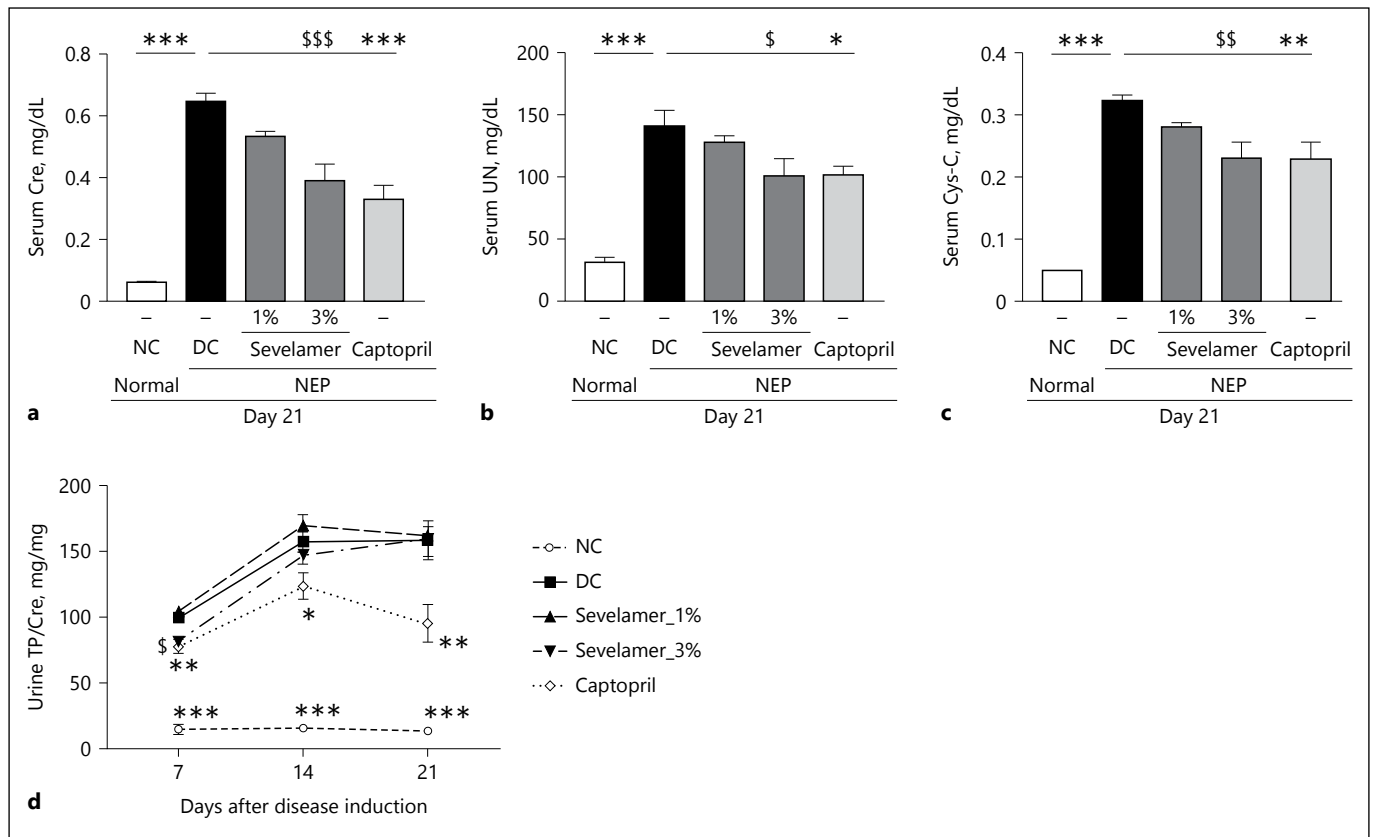
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Fig. 3. Effects of sevelamer and captopril on renal function markers, serum creatinine and proteinuria in NEP25 mice. **a–c** All renal function markers increased in NEP25 mice on day 21. Sevelamer ameliorated all the increases in a dose-dependent manner. Captopril also ameliorated all the increases. **d** Only captopril amelio-rated the proteinuria. Mean ± SE, normal control: $n = 3$, disease control: $n = 7$, sevelamer 1%, 3%, and captopril: $n = 8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student t test vs. DC. $ $p < 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$, Dunnett's test vs. DC. UN, urea nitrogen; Cys-C, cystatin-C; Cre, creatinine.

to keep Pi to the normal range. Regarding Ca, sevelamer and captopril treatment did not alter elevations in serum Ca in the disease control (online suppl. Fig. S2a, b). The Ca increases in the disease control might be related to the upregulation of kidney Cyp27b1 and the downregulation of Cyp24a1, but the results varied between treatment groups (online suppl. Fig. S1c, d). Serum Ca × Pi product increased significantly in the disease control, but this was significantly suppressed by sevelamer 3% on days 14 and 21 (online suppl. Fig. S2c, d). Sevelamer 1% suppressed the increase in Ca × Pi product only on day 14, and captopril did not alter it at all. Additionally, to determine the propensity for calcification, we measured serum T50, which indicates the precipitation time of se-rum Ca Pi, using an in vitro assay system [16, 17]. In this study, serum T50 in the normal control was longer than that in disease control and sevelamer treatment extend-ed it even further (online suppl. Fig. S2e).

The Effects of Sevelamer on Renal Function and Proteinuria

NEP25 mice showed significant increases in serum cre-atinine, indicating renal dysfunction. The measurements for urea nitrogen and cystatin-C also confirmed changes in renal function in the mice. The positive control, captopril, significantly ameliorated these serum markers. Sevelamer also ameliorated these serum markers in a dose-dependent manner (Fig. 3a–c). The proteinuria observed in the dis-ease control was significantly mitigated by captopril treat-ment, but unaffected by sevelamer (Fig. 3d). Sevelamer ex-hibited different renoprotective effects than captopril.

The Effects of Sevelamer on Podocyte Injury

In NEP25 mice, injection of the immunotoxin LMB2 induced podocyte injury and podocyte detachment or death. As in previous studies, immunostaining with the podocyte-specific marker p57 was used to confirm the

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remaining podocyte numbers (Fig. 4a–d) [18, 19]. In the normal control, about 7 p57-positive cells, in other words podocytes, were observed in each glomerulus. In the disease control, the number of p57-positive cells plunged to less than one. Captopril treatment ameliorated this reduction, but sevelamer did not show a clear improvement (Fig. 4e). To support the results, podocyte-specific gene expression was evaluated. The expression of Nphs1 and Nphs2 (nephrin and podocin) mRNA decreased dramatically, but captopril treatment ameliorated this (Fig. 4f, g). The Wt1 mRNA expression is unrelated to the results because it is expressed in activated parietal epithelial cells as well as in podocytes, so it may not be a specific marker for podocytes in focal segmental glomerulosclerosis (Fig. 4h) [20]. These data suggested that sevelamer treatment did not affect podocyte injury directly.

Pathological Analysis in Glomerular and Tubular Lesions

Histopathological and semi-quantitative analyses of glomerular and tubular lesions were performed (Fig. 5). The disease control revealed glomerulosclerosis (Fig. 5a, b, e). The glomerular PAS score showed that sevelamer 3% and captopril slightly ameliorated this (Fig. 5c–e). The disease control revealed severe tubulointerstitial damage (Fig. 5f, g, j). Sevelamer and captopril treatments ameliorated the tubulointerstitial damage score in HE sections. Even sevelamer 1% led to some improvement (Fig. 5j). In NEP25 mice, sevelamer seemed to have a more distinct effect on tubules than on glomeruli. Of note in the disease control, HE staining showed no clear calcification in kidneys.

Effects of Sevelamer and Captopril on Tubular Injury Markers

To support the pathological analysis of tubular injury, we measured urinary cystatin-C and NGAL [21]. Under normal conditions, the small peptide cystatin-C in blood is filtered in glomeruli and then reabsorbed in tubules, but when tubules are injured, it is excreted into urine without being reabsorbed. NGAL is also upregulated in urine and kidneys when tubules are injured. In the disease control, both urinary cystatin-C and NGAL levels increased. Sevelamer 3% and captopril ameliorated these increases (Fig. 6a, b). Increases in kidney Lcn2 mRNA, Lcn2 gene-coded NGAL, were also seen in the disease control. Sevelamer 3% and captopril suppressed these increases (Fig. 6c). These results confirmed that sevelamer ameliorated tubular injury.

Effects of Sevelamer and Captopril on Klotho mRNA Expression

It is known that αKlotho, expressed in distal tubular cells, is an important Pi regulator that also coregulates FGF23 and plays a role in tubulointerstitial fibrosis. In NEP25 mice, Klotho mRNA expression was drastically decreased. Sevelamer 3% and captopril ameliorated this reduction (Fig. 7a). Reports show that the Edn1 gene is down-stream of the Klotho signaling pathway [22]. Edn1 mRNA expression increased in the disease control. Sevelamer 3% and captopril ameliorated this as well (Fig. 7b).

Discussion/Conclusion

The decline in renal function seen in patients with chronic kidney disease leads to elevated extracellular Pi levels due to low glomerular filtration of small molecules. NEP25 mice have already been reported to show proteinuria, glomerulosclerosis, tubular injury, and a decline renal function induced by podocyte-specific injury [14, 15]. In the current study, NEP25 mice also showed chronic hyperphosphatemia and a corresponding elevation in serum FGF23 and PTH, which confirms that the NEP25 mice showed signs of abnormal Pi metabolism, and the study also showed an accompanying loss of kidney function due to glomerular injury, as seen in most human kidney diseases. Moreover, a clinical Pi binder, sevelamer, alleviated hyperphosphatemia in the mice. Thus, this study supports the suitability of the NEP25 mouse model as a relevant glomerular injury-induced model of hyperphosphatemia.

In this study, sevelamer suppressed hyperphosphatemia and significantly improved renal function. It ameliorated tubular injury, as evidenced by histopathological analysis and measurements in tubular injury markers. This effect on tubular injury may elucidate a mechanism by which Pi regulation protects renal function. In the NEP25 model, glomerular injury led to secondary tubular injury and, finally, renal dysfunction. Many hypotheses posit that tubular injury and renal dysfunction are caused by downstream mechanisms of glomerular injury, such as proteinuria, hypoxia due to low blood flow, oxidative stress, inflammation, and fibrosis and so on [23]. High serum Pi levels induced by glomerular damage may somehow be involved in the disease progression scheme from glomerular to tubular injury, but it is difficult to evaluate the renoprotective effects of Pi regulation if tubular injury is too direct, as in the adenine model [24]. A previous publication explored hyperphosphatemia in Alport mice; these mice, lacking the gene Col4a3, show abnormality in
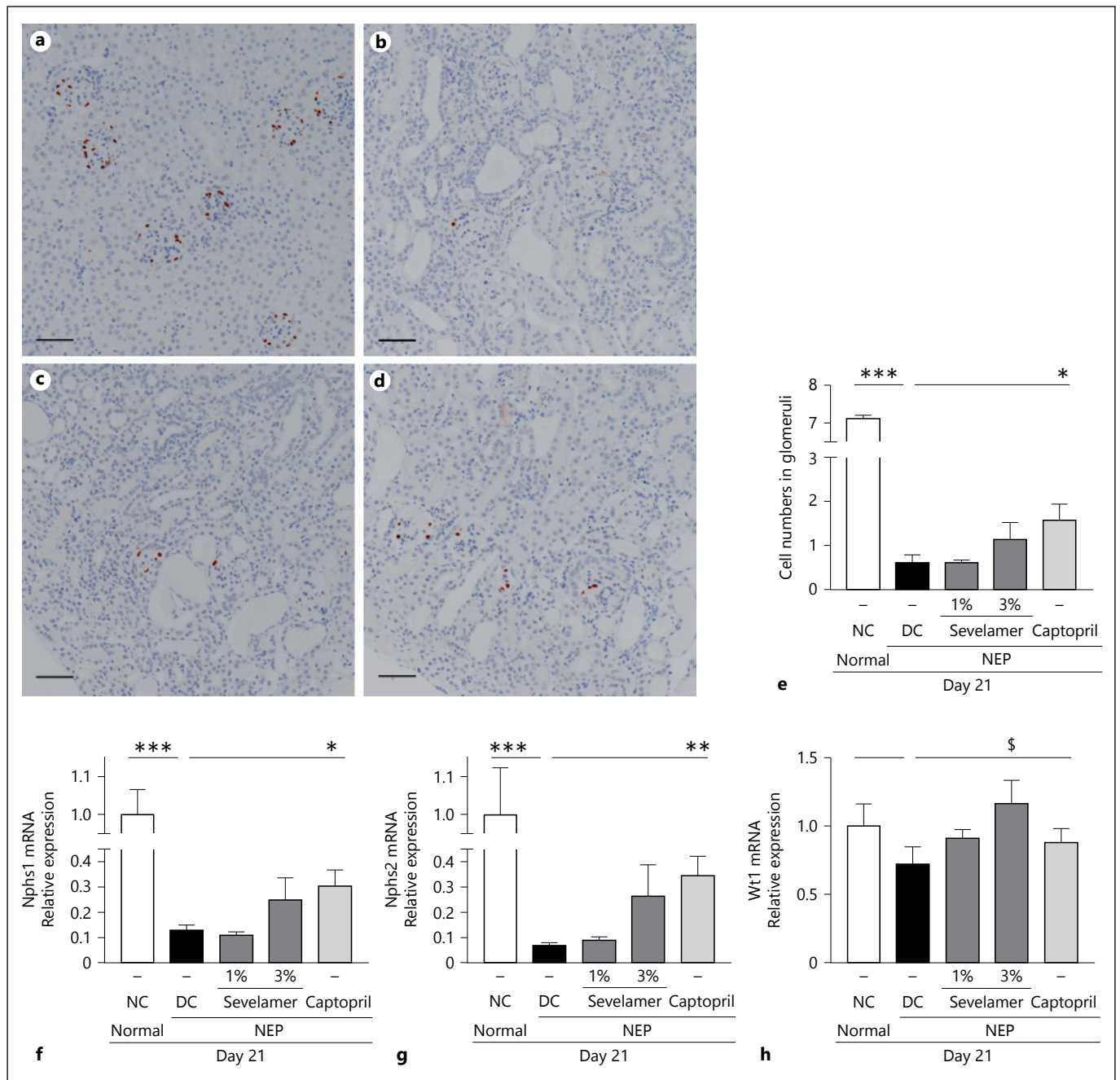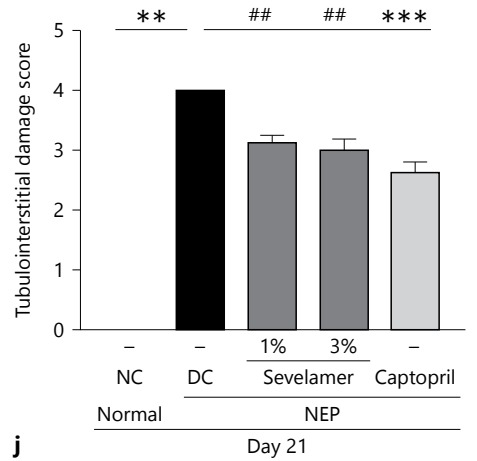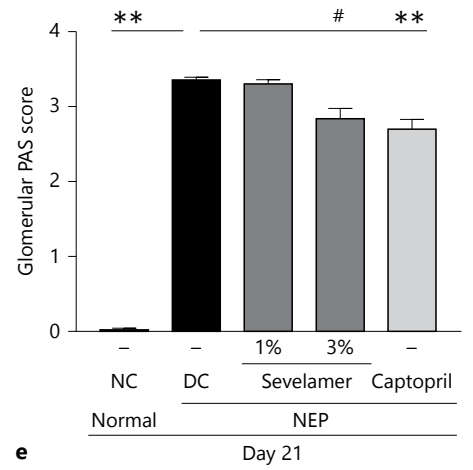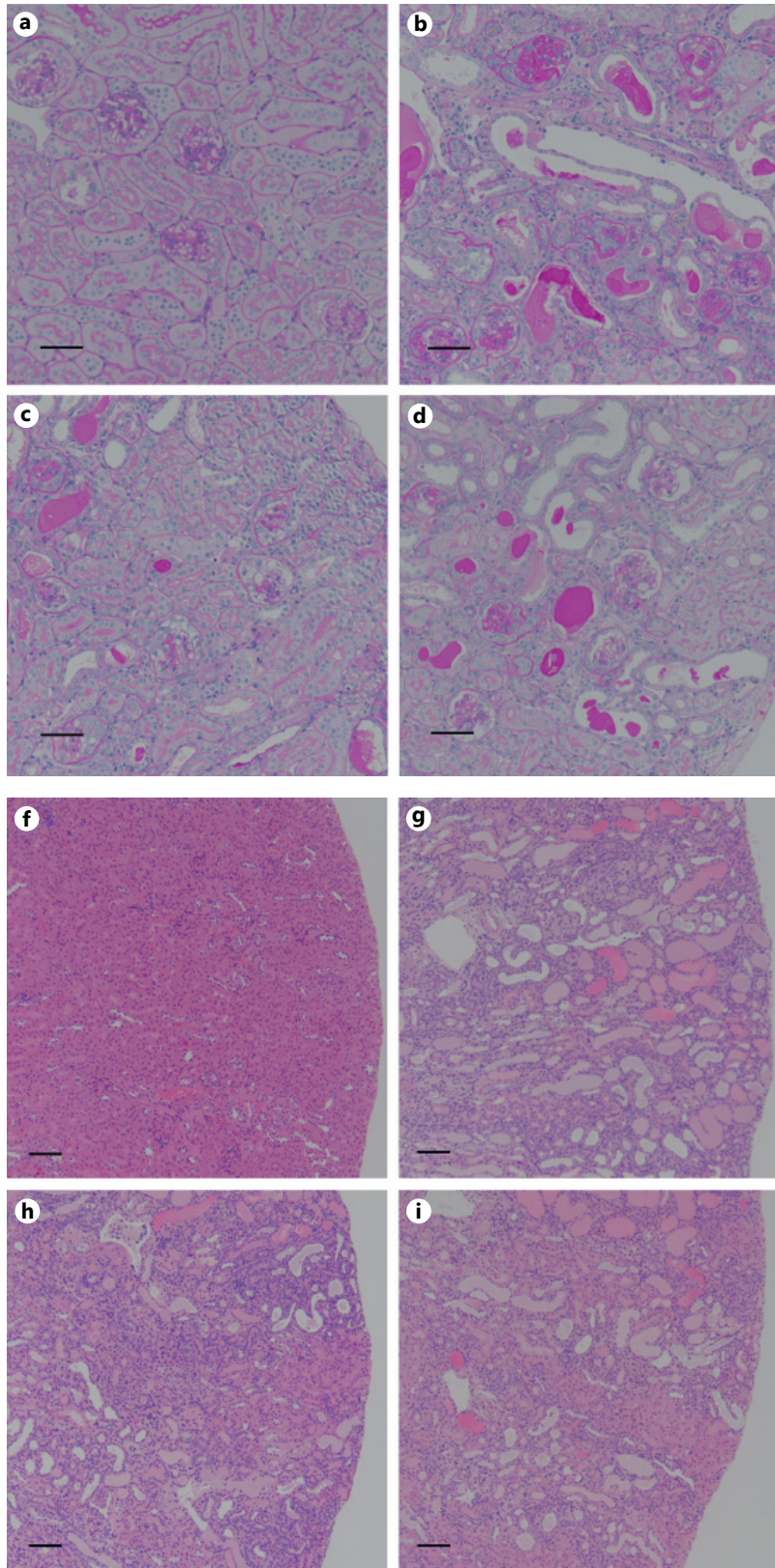
Fig. 4. Effects of sevelamer and captopril on podocyte injury: number of p57-positive cells, kidney mRNA expressions, Wt1, Nphs1, and Nphs2, in NEP25 mice. **a–d** Representative images of immunostaining for p57 in kidney slices from tissue that is (**a**) normal, (**b**) diseased, (**c**) treated with sevelamer 3%, and (**d**) treated with captopril. Scalebar: 50 μm. **e** The number of p57-positive cells in glomeruli. Average number of p57-positive cells in >50 glomeruli of each mouse was calculated. Disease control showed a drastic decrease in podocyte numbers. Captopril treatment ameliorated the decrease. **f–h** Expression levels of mRNA specific to kidney podocytes (**f**) Nphs1, (**g**) Nephs2, (**h**) Wt1. Disease control showed a drastic decrease in Nphs1 and Nphs2 mRNA expression. Captopril treatment ameliorated the decrease. Mean ± SE, normal control: $n = 3$, disease control: $n = 7$, sevelamer 1%, 3%, and captopril: $n = 8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student t test vs. DC. $ $p < 0.05$, Dunnett's test vs. DC.

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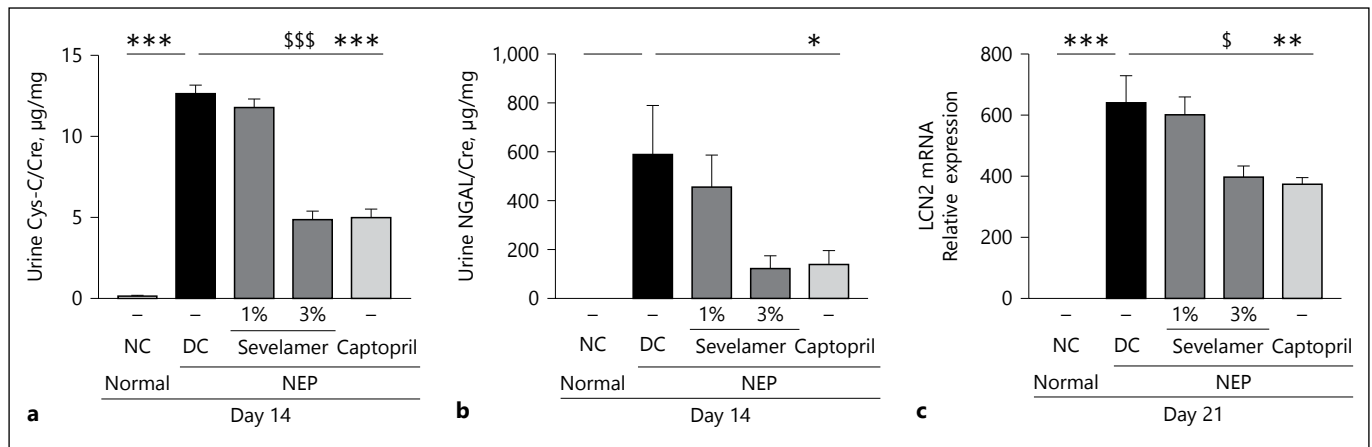
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Fig. 6. Effects of sevelamer and captopril on tubular injury markers in NEP25 mice. **a** Urinary Cystatin-C excretion. **b** Urinary NGAL excretion. **c** Kidney Lcn2 mRNA expression. Disease control showed clear increases in the tubular injury markers. Sevelamer 3% and captopril ameliorated the increases. The exact values of NGAL for mean ± SE were NC: 0.051 ± 0.005 μg/mg-Cre, DC: 587 ± 202 μg/mg-Cre. Mean ± SE, normal control: $n = 3$, disease control: $n = 7$, sevelamer 1%, 3%, and captopril: $n = 8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student t test vs. DC. $ $p < 0.05$, $$$ $p < 0.001$, Dunnett's test vs. DC. Cre, creatinine.

the glomerular basement membrane and renal dysfunction resembling that of Alport syndrome patients [13]. In the Alport study, restricted Pi intake did not suppress serum Pi sufficiently to exhibit renoprotective effects, but in the current study, sevelamer treatment was able to reduce serum Pi to normal or lower levels. The degree to which serum Pi was suppressed may be an important aspect of the renoprotective effect of Pi regulation.

A Pi binder may also mitigate kidney disease progression by regulating Klotho expression. In this study, Klotho expression that had been down-regulated under disease conditions recovered when Pi was regulated by treatment with sevelamer. Reduced Klotho expression in 5/6NX uremic rats was associated with increased FEPi% [25], and the authors showed that Pi stimulation decreases Klotho expression via wnt signaling in cultured cells. In this study, the drastic reduction in urinary FEPi% after sevelamer treatment may have recovered the reduced Klotho expression by the mechanism they proposed. Klotho was also reported to downregulate endothelin-1 expression [22], which may be a factor of progressive disease in podocytes, endothelial cells, or tubular cells [26].

In this study, sevelamer treatment suppressed kidney Edn1 mRNA. Additionally, the shed form of Klotho has been reported to exert anti-fibrosis effects on various cell types via TGFβ1, Wnt, or FGF2 pathways. In NEP25 mice, the recovered Klotho expression is a possible renoprotective mechanism in Pi regulation.

According to our analysis of podocyte injury, captopril treatment ameliorated podocyte injury and glomerulosclerosis, possibly because captopril suppressed blood pressure in glomeruli. On the other hand, though sevelamer ameliorated glomerulosclerosis, it had no significant impact on podocyte injury. High Pi levels in cultured mesangial cells have been reported to directly enhance the production of collagen IV induced by TGFβ1 by affecting the Smad pathway [27]. This mechanism might explain how the reduction in serum Pi by sevelamer treatment seen in the NEP25 mice would suppress extracellular matrix in injured glomerul. Taking these findings together, Pi restriction would have beneficial effects on injured glomeruli.

In a previous study using the anti-GBM nephritis rat model, sevelamer treatment ameliorated renal dysfunc-

Fig. 5. Effects of sevelamer and captopril on kidney histology, glomerulosclerosis, and tubulointerstitial damage in NEP25 mice. **a–d** Representative images of histopathology by PAS staining. Scalebar: 50 μm (**a**) normal control, (**b**) disease control, (**c**) sevelamer 3%, (**d**) captopril. **e** Glomerular PAS score, a score for glomerular sclerosis. Disease control showed severe glomerulosclerosis, while sevelamer 3% and captopril ameliorated the sclerosis. **f–i** Representative images of HE staining of kidney slices. Bar: 100 μm (**f**): normal control, (**g**) disease control, (**h**) sevelamer 3%, (**i**) captopril. **j** Tubular HE score, a score for tubular injury. Disease control showed severe tubular injury, while sevelamer 3% and captopril ameliorated the injury. Mean ± SE, normal control: $n = 3$, disease control: $n = 7$, sevelamer 1%, 3%, and captopril: $n = 8$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney test vs. DC. # $p < 0.05$, ## $p < 0.01$, Kruskal-Wallis test vs. DC.
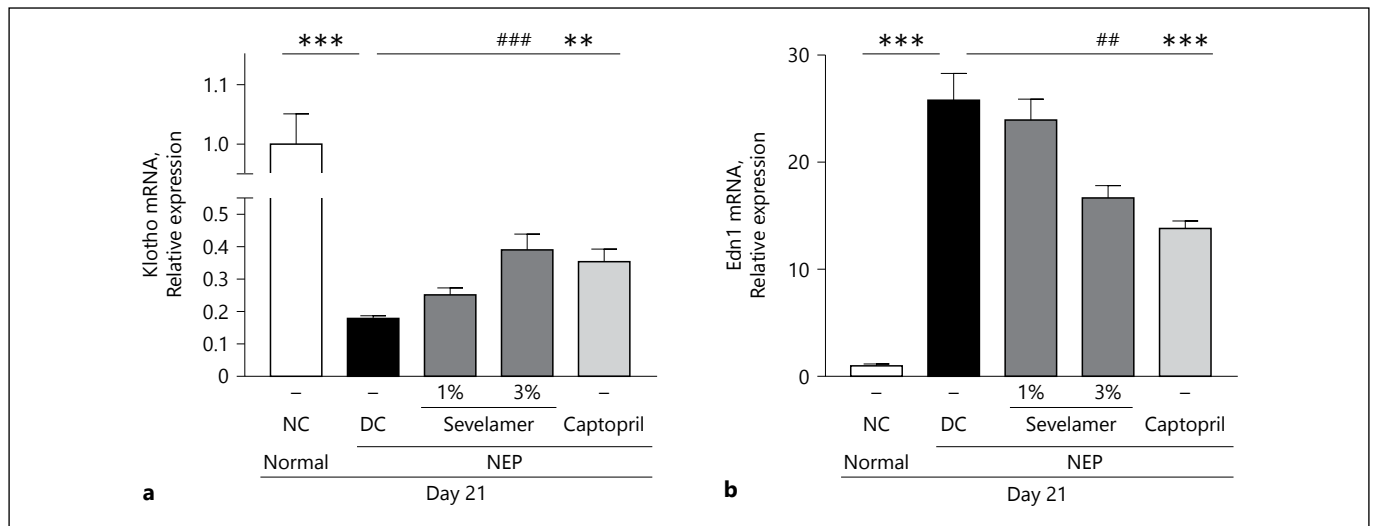
Fig. 7. Effects of sevelamer and captopril on kidney Klotho and Edn1 mRNA expression in NEP25 mice. **a** Kidney Klotho mRNA. Disease control showed a decrease in Klotho mRNA levels. Sevelamer 3% and captopril ameliorated the decrease. **b** Kidney Edn1 mRNA. Disease control showed clear increases in Edn1 mRNA levels. Sevelamer 3% and captopril ameliorated the increases. Mean ± SE, normal control: $n = 3$, disease control: $n = 7$; sevelamer 1%, 3%, and captopril: $n = 8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student t test vs. DC. ## $p < 0.01$, ### $p < 0.001$, Dunnett's test vs. DC.

tion and associated pathological changes, such as glomerular and tubulointerstitial lesions [11]. Although this study confirmed a similar renoprotective effect of sevelamer in NEP25 mice, the anti-GBM rat study was conducted for 5 months, whereas the NEP25 mouse study lasted for only 3 weeks. HE staining of sections from the NEP25 disease control group did not reveal the renal calcification observed in the anti-GBM rats, which suggests that entopic calcification may not be involved in kidney disease progression in the NEP25 model. However, the significant increase in serum Ca × Pi product seen in NEP25 mice was significantly suppressed by sevelamer 3% on days 14 and 21 (online suppl. Fig. S2c, d) as a result of serum Pi regulation, not serum Ca (online suppl. Fig. S2a, b; Note that serum Ca was elevated in the disease control, and kidney Cyp27b1 was upregulated and Cyp24a1 downregulated [online suppl. Fig. S1c, d]). On the other hand, sevelamer 1%, exerting a weaker renoprotective effect than 3%, suppressed the increase only on day 21, not on day 14. Suppression of the serum Ca × Pi product might be related to the renoprotective effect of Pi regulation.

In CKD-MBD, calciprotein particles (CPPs) have been reported as a new biological marker associated with tissue and organ impairments, such as cardiovascular damage and inflammation [28, 29]. CCPs, which comprise Ca, Pi, and other proteins, such as Fetuin-A, have primary and secondary forms. Primary CPPs form small, soluble colloidal protein-mineral nanoparticles that prevent supersaturated Ca and Pi from precipitating. Secondary CPPs are more elongated than primary CPPs and form bigger, insoluble crystals. Low values of serum T50 predict cardiovascular events and all causes of mortality in dialysis patients and pre-dialysis kidney disease patients [17, 30, 31]. In this study, low serum T50 values reflected shorter precipitation times in the disease control than in normal control, and sevelamer treatment significantly extended the shortened serum T50 (online suppl. Fig. S2e). This data suggested that sevelamer reduced the risk of the formation of secondary CPPs in NEP25 mice. Secondary CPPs are reported to induce cell necrosis, mineralization, and TNF-α mRNA and protein in experiments in primary human aortic vascular smooth muscle cells [32]. Although there is currently no direct evidence that secondary CPPs damage kidney cells, they may play a similar role in kidney cells in NEP25 mice as they do in vascular smooth muscle cells.

In conclusion, regulation of extracellular Pi by sevelamer showed a protective effect on kidney disease progression in a hyperphosphatemia NEP25 mouse model in which glomerulosclerosis was induced by podocyte injury. Pi regulation mitigated tubular injury in the mice, possibly by controlling Klotho expression and/or suppressing secondary CPP formation. Future studies should be conducted to determine other potential mechanisms. The NEP25 mouse model set-

ting can be utilized with various transgenic mice (Klotho tgm, Npt2b KO, etc.) to further and more precisely assess the effects of Pi regulation on kidney disease progression.

Ethics Statement

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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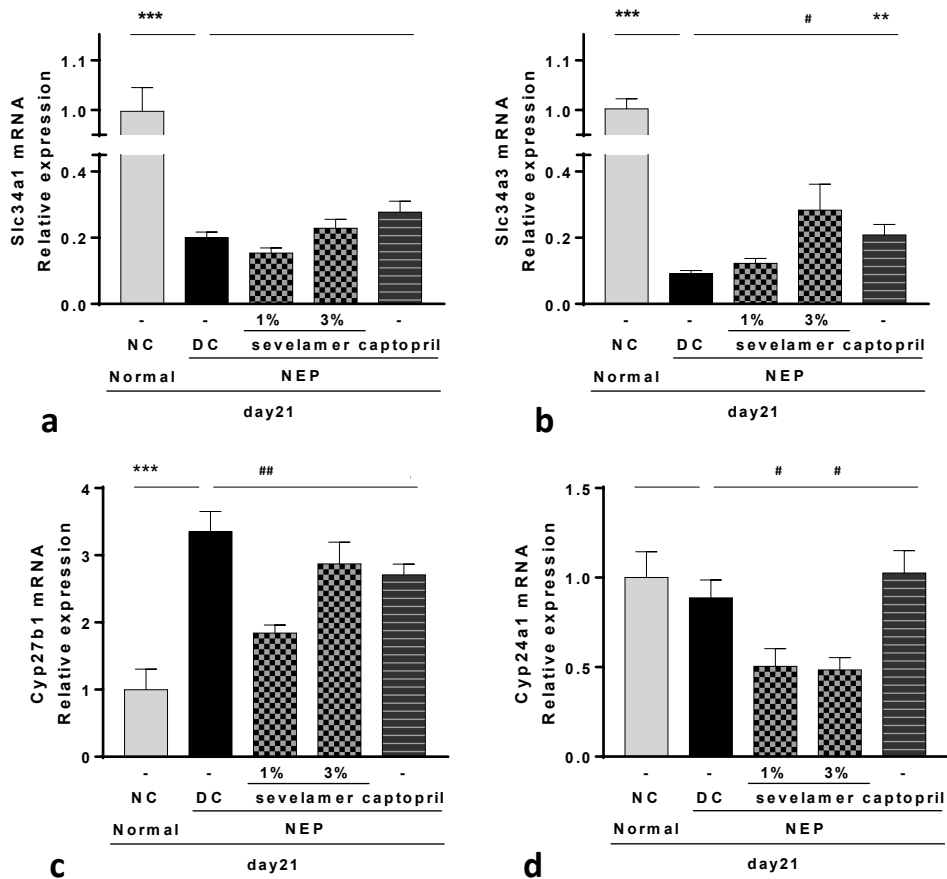
Fig.S1

Fig.S1. Effects of sevelamer and captopril on kidney mRNA expressions, Slc34a1, Slc34a3, Cyp27b1, and Cyp24a1 in NEP25 mice. **a** Kidney Slc34a1 mRNA expressions. Slc34a1 mRNA levels were drastically decreased in NEP25 mice. The decrease was not ameliorated by any treatment. **b** Kidney Slc34a3 mRNA expressions. Slc34a3 mRNA levels were drastically decreased in NEP25 mice. The decrease was not ameliorated by sevelamer 3% and captopril treatments. **c** Kidney Cyp27b1 mRNA expressions. Cyp27b1 mRNA was increased in NEP25 mice. Only sevelamer 1% suppressed the increase. **d** Kidney Cyp24a1 mRNA expressions. Cyp24a1 mRNA was not changed in NEP25 mice. Only sevelamer suppressed Cyp24a1 mRNA levels. Mean + SE, *p<0.05, **p<0.01, ***p<0.001, Student's t-test vs. DC. $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001, Dunnett's test vs. DC
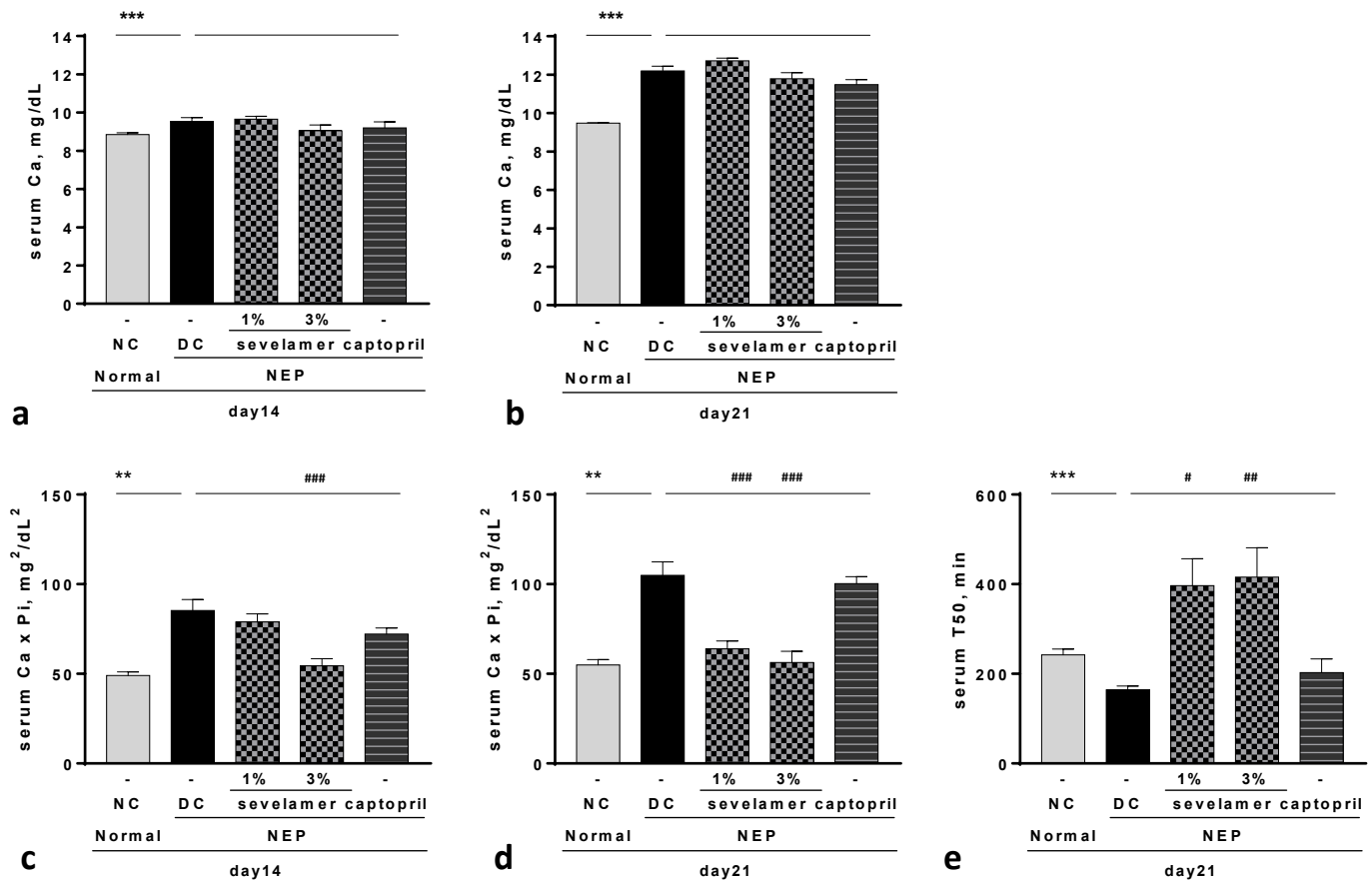
Fig.S2

Fig.S2. Effects of sevelamer and captopril on serum Ca, Ca x Pi product, T50 in NEP25 mice. **a,b** Serum Ca levels increased on day 14 and 21 in NEP25 mice. There were no differences in serum Ca levels in the sevelamer, captopril from those in disease control. **c,d** Serum Ca x Pi product was increased on day 14 and 21. Sevelamer treatment ameliorated the increase on both day 14 and 21. Dose-dependent amelioration was observed only on day 14. Captopril treatment did not change the increase. **e** Serum T50 time as calcification indicator on day 21. The T time was decreased in NEP25mice. Serum T50 time in the sevelamer group was greater than in normal control. Captopril did not show a clear effect on serum T50. Mean + SE, *p<0.05, **p<0.01, ***p<0.001, Student's t-test vs. DC. $p<0.05, $$ p<0.01, $$$ p<0.001, Dunnett's test vs. DC