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ORIGINAL ARTICLE

Skeletal and mineral metabolic effects of risedronate in a rat model of high-turnover renal osteodystrophy

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Abstract

Introduction High-turnover bone disease is a major consequence of SHPT and may explain the high risk for fracture in patients with advanced chronic kidney disease (CKD). Bisphosphonates suppress bone turnover and improve bone strength, but their effects have not been fully characterized in advanced CKD with severe SHPT. Bisphosphonates also increase 1,25-dihydroxyvitamin D levels in normal and uremic rats, but the underlying mechanism remains to be determined.

Materials and methods We investigated the skeletal and mineral metabolic effects of RIS, a pyridinyl bisphosphonate, in rats with severe SHPT induced by 5/6 nephrectomy plus a high phosphate diet.

Results Nephrectomized rats developed severe SHPT, along with hyperphosphatemia, low 1,25-dihydroxyvitamin D, and markedly increased FGF23. Moreover, these rats exhibited characteristic features of high-turnover renal osteodystrophy, including increased indices of trabecular bone turnover, decreased cortical bone thickness, inferior cortical biomechanical properties, and a prominent increase in peritrabecular fibrosis. RIS treatment increased bone volume and partially attenuated trabecular bone remodeling, cortical bone loss, and mechanical properties, whereas it produced a marked improvement in peritrabecular fibrosis along with a corresponding decrease in osteogenic gene markers. RIS treatment also suppressed the elevation of FGF23, which was associated with increased 1,25-dihydroxyvitamin D.

Conclusions In a rat model of severe SHPT, treatment with RIS partially attenuated histological manifestations of high-turnover bone disease. RIS treatment also suppressed the elevation of FGF23, which may explain the increased 1,25-dihydroxyvitamin D production during the treatment.

Keywords Bisphosphonate · Chronic kidney disease · Fibroblast growth factor 23 · Secondary hyperparathyroidism · Vitamin D

Introduction

Patients with chronic kidney disease (CKD) have an increased risk of fracture. The risk of fracture increases as kidney function declines, with end-stage renal disease (ESRD) patients at particularly high risk for fracture [1–3]. Disturbances in mineral metabolism are common in CKD patients and result in a systemic disorder termed CKD-mineral and bone disorder (CKD-MBD) [4]. Renal osteodystrophy (ROD) is one component of CKD-MBD and may explain the high risk of fracture in this population. High-turnover bone disease caused by secondary hyperparathyroidism (SHPT) is the predominant form of ROD and likely contributes to bone fragility, as several, but not all, observational studies have shown significant associations of higher parathyroid hormone (PTH) levels with an elevated risk for

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fracture [5–7]. Notably, recent clinical studies suggested that treatment of SHPT with either calcimimetics or parathyroidectomy could improve high-turnover ROD and reduce the risk of fracture [8–11]. However, the fracture incidence in ESRD patients remains unacceptably high [12, 13], underscoring the need for additional therapeutic options.

Risedronate (RIS) is a potent pyridinyl bisphosphonate that reduced the risk of fracture in women with postmenopausal osteoporosis in randomized, controlled clinical trials [14, 15]. In a secondary analysis of these trials, RIS effectively preserved bone mineral density and reduced the incidence of vertebral fractures in osteoporotic women with mild CKD [16]. However, these trials excluded patients with elevated creatinine or PTH levels; hence, the efficacy and safety of RIS in patients with more advanced CKD and high-turnover ROD remain unknown. According to experimental studies, several types of bisphosphonates improve high-turnover ROD in animal models of CKD [17–23]. However, most of these studies used rats with less severe renal failure and relatively mild SHPT. Thus, the skeletal effect of bisphosphonate in more severe renal impairment and SHPT remains to be determined.

One of the key features of CKD-MBD is a progressive decline in 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) levels, which contributes to the development and progression of SHPT. The etiology of the progressive reduction in $1,25(OH)_2D$ levels in CKD is multifactorial, but recent evidence indicates that fibroblast growth factor 23 (FGF23), a bone-derived hormone, plays a central role [24–26]. Interestingly, previous experiments have shown that bisphosphate treatment increased $1,25(OH)_2D$ production in normal rats [27] and nephrectomized rats [19]. This effect was also observed in parathyroidectomized rats [28, 29], suggesting that an additional mechanism independent of PTH likely contributes.

Therefore, the purpose of this study was twofold: [1] to determine the effects of RIS on bone metabolism in rats with severe SHPT induced by 5/6 nephrectomy plus a high phosphate diet and [2] to investigate the underlying mechanism of the increased $1,25(OH)_2D$ production during bisphosphonate treatment.

Materials and methods

Animals and experimental design

Five-week-old male Sprague–Dawley rats were purchased from CLEA Japan (Tokyo, Japan). The rats were housed under standard environmental conditions (23 ± 2 °C, 55 ± 10% humidity, 12:12 h light–dark cycle) and had free access to water and standard rodent chow that contained 0.9% calcium and 0.8% phosphate (CLEA Japan), except when otherwise specified. All experiments were approved by the Institutional Review Board of Tokai University School of Medicine.

The experimental design is summarized in Fig. 1. At 6 weeks of age, rats were subjected to 5/6 nephrectomy or sham surgery. In the 5/6 nephrectomy, rats were anesthetized, and two-thirds of the right kidney (upper and lower poles) was resected and returned to the renal fossa. After 1 week of recovery, total nephrectomy of the left kidney was performed. To promote the severity of ROD, we switched nephrectomized rats to a high phosphate diet, which contained 0.9% calcium and 1.2% phosphate (CLEA Japan), 1 week after the second surgery. Sham-operated rats were kept on the standard diet.

After 6 weeks of disease progression, nephrectomized rats were randomly assigned to vehicle (0.9% saline) or 5 µg/kg body weight RIS (a gift from EA Pharma Co.,

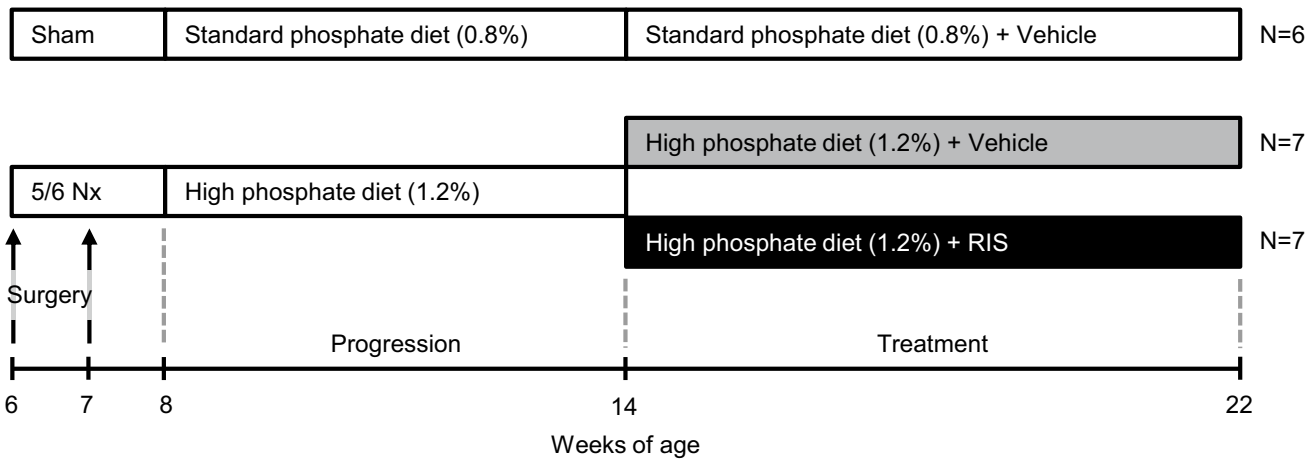

Fig. 1 Schematic of the study design. Vehicle or 5 µg/kg body weight risedronate (RIS) was administered subcutaneously twice weekly between 14–22 weeks of age. *Nx* nephrectomy

Ltd., Tokyo, Japan) administered subcutaneously twice weekly for 8 weeks. The drug dose was chosen on the basis of previous publications on RIS treatment in rats [30]. All sham-operated rats received twice-weekly injections of vehicle. After 8 weeks of treatment with RIS or vehicle, rats were placed in metabolic cages for 24-h urine collection and were killed for analyses.

Serum and urine biochemistries

Serum and urine calcium, phosphorus, creatinine, and blood urea nitrogen (BUN) levels were measured using standard methods. Serum PTH levels were measured using a rat intact PTH ELISA (Immutopics, San Clemente, CA). Serum FGF23 levels were measured using both an intact FGF23 ELISA, which exclusively measures the full-length protein (Kainos Laboratories, Tokyo, Japan), and a murine C-terminal FGF23 ELISA, which recognizes the intact protein and its C-terminal cleavage fragments (Immutopics, San Clemente, CA). Serum $1,25(OH)_2D$ levels were measured using a radioimmunoassay (Immunodiagnostics Systems, Fountain Hills, AZ). Serum levels of the N-terminal propeptide of type I procollagen (PINP) and C-terminal telopeptide fragments of type I collagen (CTX) were measured using enzyme immunoassays (Immunodiagnostic Systems).

Bone histology and histomorphometry

For dynamic histomorphometry, rats were injected intraperitoneally with fluorochromes tetracycline hydrochloride (25 mg/kg body weight; Nacalai Tesque, Kyoto, Japan) and calcein (8 mg/kg body weight; Nacalai Tesque) 9 and 2 days prior to sacrifice, respectively. At the time of sacrifice, the right tibia was harvested and fixed in 70% ethanol. The fixed bones were dehydrated in acetone and embedded in methyl methacrylate. Undecalcified bones were cut into 6-µm-thick sections by a motorized microtome (RM2255; Leica, Nussloch, Germany). The first consecutive section was left unstained to analyze fluorescent labeling for dynamic parameters. The second section was stained with Villanueva-Goldner for analysis of osteoclasts, osteoblasts, and osteoids. The bone sections were viewed using an Olympus BX51 microscope equipped with a Penguin 150CL digital camera (Pixera, Los Gatos, CA). Bone histomorphometric analysis was performed within the secondary spongiosa (0.8–1.2 mm away from the growth plate) under 20 × magnification using a semiautomatic image analysis system (Histometry RT Camera; System Supply, Nagano, Japan). Structural, dynamic, and cellular parameters are presented according to the standardized nomenclature [31].

Biomechanical testing

The mechanical strength of the left femur was determined with a three-point bending test using MZ-500D (Maruto, Tokyo, Japan). Bones were thawed, hydrated in saline, and then placed posterior side down on the bottom support (span = 13 mm). Load was applied vertically downwards to the midshaft with a constant rate of displacement of 2 mm/min until fracture. Based on the load-deformation curve, the maximum load (*N*), displacement (mm), stiffness (N/mm), and energy to failure (N mm) were determined.

Gene expression

Total RNA was isolated from the right femur and kidney using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) and an RNAqueous-4PCR kit (Thermo Fisher Scientific), respectively. For isolation from femurs, epiphyses were cut off, bone marrow was removed by centrifugation, and bone tissue was homogenized using a Shake Master NEO (Bio Medical Sciences, Tokyo, Japan) together with TRIzol reagent and 5-mm stainless beads. cDNA synthesis was performed using 0.5 µg total RNA and SuperScript IV VILO Master Mix with ezDNase Enzyme (Thermo Fisher Scientific). Quantitative real-time PCR was performed on the StepOnePlus System using the TaqMan One-Step RT-PCR Master Mix Reagents kit (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene to normalize expression.

Statistical analysis

Data are represented as mean ± SD. Differences were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Correlations were examined by Spearman's rank test. $P < 0.05$ was considered statistically significant. All analyses were performed using IBM SPSS Statistics 24 (IBM, Tokyo, Japan).

Results

Biochemistry of 5/6 nephrectomized rats treated with RIS and vehicle

Compared to sham-operated control rats, nephrectomized rats developed progressive uremia (Fig. 2a–c) and displayed reduced body weight (Fig. 2d) regardless of the treatment received. The kidney function of nephrectomized rats was approximately 15–20% of that of sham-operated rats as assessed by creatinine clearance, which corresponds to late stage 4 and early stage 5 CKD. These uremic rats showed low serum calcium (Fig. 2e), elevated serum phosphorus

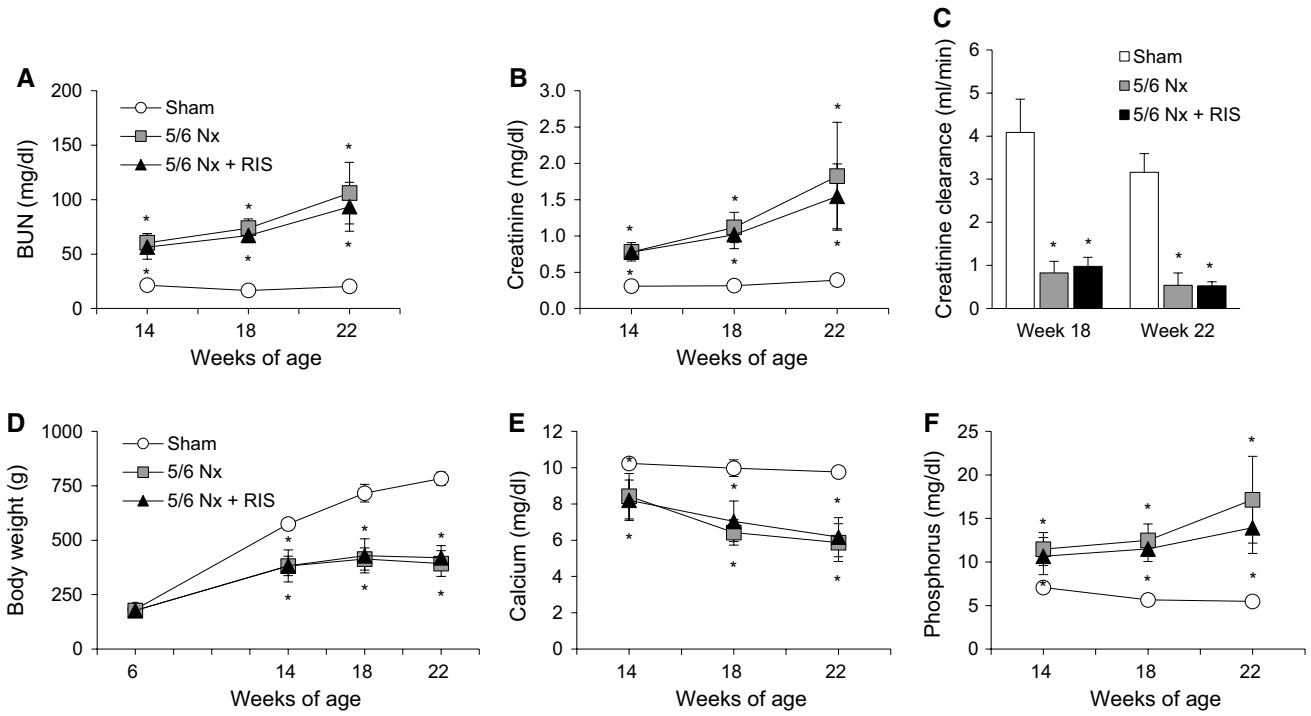

Fig. 2 **a** Blood urea nitrogen (BUN), **b** serum creatinine, **c** creatinine clearance, **d** body weight, **e** serum calcium, and **f** serum phosphorus in sham-operated rats receiving vehicle (sham), 5/6 nephrectomized rats receiving vehicle (5/6 Nx), and 5/6 nephrectomized rats receiving RIS (5/6 Nx + RIS). Data are shown as mean ± SD. $^*P < 0.05$ versus sham (one-way ANOVA followed by Tukey's post hoc test). $n = 6$–7 in each group

(Fig. 2f), and severe SHPT, but there was a nonsignificant trend towards lower PTH levels in those receiving RIS (Fig. 3a). Interestingly, nephrectomized rats receiving vehicle showed low serum 1,25$(OH)_2$D levels, but this decrease in serum 1,25$(OH)_2$D was not observed in RIS-treated uremic rats (Fig. 3b), despite the similar degree of uremia and SHPT. These results were consistent with those of a previous work [19] and led us to investigate whether FGF23 could be involved in the increased 1,25$(OH)_2$D levels. Compared to the sham-operated control rats, nephrectomized rats receiving vehicle showed markedly elevated intact FGF23 levels. However, these changes were attenuated and not significant in uremic rats treated with RIS (Fig. 3c, d). Similar findings were observed for C-terminal FGF23 (Fig. 3d). Furthermore, there was a modest correlation between 1,25$(OH)_2$D and FGF23 levels ($r = -0.545$, $P = 0.01$; Fig. 3e) after 8 weeks of treatment with RIS or vehicle (at 22 weeks of age). Quantitative real-time PCR analysis demonstrated a large but nonsignificant increase in *Cyp27b1* expression and a trend towards lower *Cyp24a1* expression in the kidney of RIS-treated uremic rats compared to those in rats receiving vehicle (Figs. 3f). These results suggest that RIS treatment for ROD led to decreased FGF23 production, which in turn resulted in stimulation of renal 1,25$(OH)_2$D synthesis. Consistent with the relatively low FGF23 in the RIS-treated uremic rats, there was a trend towards lower urinary phosphorus excretion (Fig. 3g) and higher renal expression of *Napi2a* (Fig. 3f) compared to those in uremic rats receiving vehicle, although the differences were nonsignificant.

Bone histomorphometry and biomechanics in uremic rats treated with RIS and vehicle

Next, we performed histomorphometric analysis of the tibia to evaluate the impact of RIS on ROD. Nephrectomy resulted in an increase in bone volume fraction and trabecular number and a decrease in trabecular separation (Table 1), but this finding is consistent with previous observations in uremic animals [20, 32–35]. Nephrectomized rats exhibited a significant increase in osteoblast surface and mineral apposition rate along with a trend towards increased mineralizing surface, bone formation rate, activation frequency (Table 1), and bone formation marker PINP (Fig. 4a). These rats also showed a trend towards an increase in osteoclasts (Table 1) and significantly elevated levels of bone resorption marker CTX (Fig. 4b). Moreover, we found a prominent increase in peritrabecular fibrosis, which was not detected in the sham-operated controls (Table 1 and Fig. 4c). We also found that the nephrectomized rats showed unequivocal loss of cortical bone (Fig. 4c). These histological changes were associated

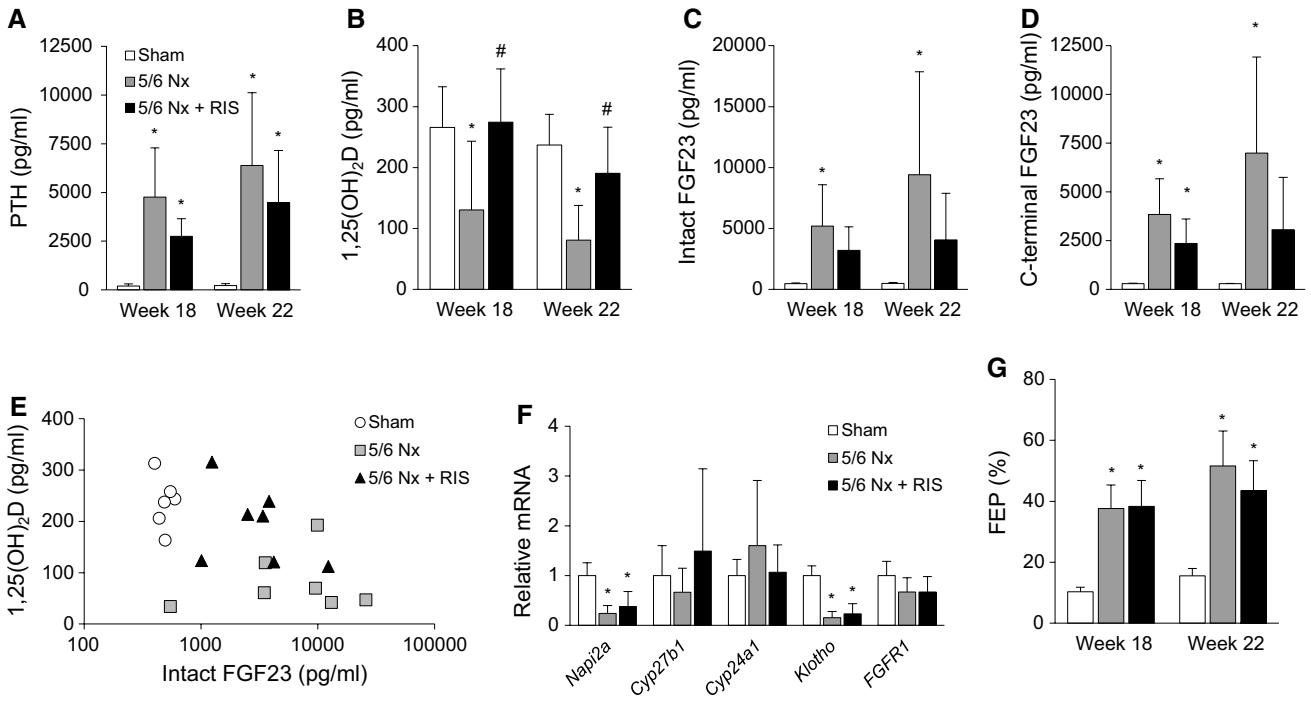

Fig. 3 **a** PTH, **b** 1,25(OH)$_2$D, **c** intact FGF23, and **d** C-terminal FGF23 in sham-operated rats receiving vehicle (sham), 5/6 nephrectomized rats receiving vehicle (5/6 Nx), and 5/6 nephrectomized rats receiving RIS (5/6 Nx + RIS) at 18 and 22 weeks of age. **e** Relationship between the 1,25(OH)$_2$D and intact FGF23 levels at 22 weeks of age ($r = -0.545$, $P = 0.01$ by Spearman's rank test). **f** Quantitative real-time PCR in the kidney at 22 weeks of age. **g** Fractional excretion of phosphate (FEP) at 18 and 22 weeks of age. Data are shown as mean ± SD. $^*P < 0.05$ versus sham; $^\#P < 0.05$ versus 5/6 Nx (one-way ANOVA followed by Tukey's post hoc test). $n = 6$–7 in each group

Table 1 Bone histomorphometry

	Sham	5/6 Nx	5/6 Nx + RIS
BV/TV (%)	12.4 ± 4.7	44.4 ± 9.5[a]	60.1 ± 7.8[a,b]
Tb.Th (μm)	80 ± 8	93 ± 16	145 ± 17[a,b]
Tb.N (/mm)	1.53 ± 0.44	4.87 ± 1.29[a]	4.16 ± 0.32[a]
Tb.Sp (μm)	622 ± 219	126 ± 53[a]	97 ± 26[a]
OS/BS (%)	12.9 ± 5.1	26.5 ± 16.4	29.2 ± 10.1
Ob.S/BS (%)	5.7 ± 3.6	19.4 ± 10.3[a]	20.4 ± 6.4[a]
ES/BS (%)	7.0 ± 2.5	11.3 ± 4.7	11.3 ± 4.2
Oc.S/BS (%)	3.48 ± 1.49	5.92 ± 2.98	4.92 ± 1.98
MAR (μm/d)	1.59 ± 0.30	2.42 ± 0.21[a]	2.18 ± 0.56[a]
MS/BS (%)	28.1 ± 7.2	25.8 ± 6.9	27.8 ± 6.2
BFR/BS (μm^3/μm^2/d)	0.46 ± 0.20	0.63 ± 0.22	0.61 ± 0.18
Ac.f (/y)	7.1 ± 3.2	10.3 ± 4.0	8.4 ± 2.8
Fb.V/TV (%)	0.0 ± 0.0	16.0 ± 5.4[a]	5.5 ± 2.0[a,b]

Data are shown as mean ± SD

Ac.f activation frequency, *BFR/BS* bone formation rate, *BV/TV* bone volume fraction, *ES/BS* eroded surface, *Fb.V/TV* fibrosis volume, *MAR* mineral apposition rate, *MS/BS* mineralizing surface, *Ob.S/BS* osteoblast surface, *Oc.S/BS* osteoclast surface, *OS/BS* osteoid surface, *RIS* risedronate, *Tb.N* trabecular number, *Tb.Sp* trabecular separation, *Tb.Th* trabecular thickness

[a] $P < 0.05$ versus sham

[b] $P < 0.05$ versus 5/6 Nx + vehicle (one-way ANOVA followed by Tukey's post hoc test). $n = 6$–7 in each group

with marked deterioration of the biomechanical properties of the femur (Table 2). Collectively, these findings indicate that our uremic rats fed for 14 weeks on a high phosphate diet demonstrated characteristic features of osteitis fibrosa, a high-turnover state driven by SHPT.

The RIS treatment of the uremic rats resulted in a further increase in the already elevated bone volume fraction and trabecular number (Table 1). There was no significant difference in the osteoblast surface, mineral apposition rate, mineralizing surface, bone formation rate, or activation frequency between nephrectomized rats receiving vehicle and RIS (Table 1), but bone formation marker PINP tended to decrease in uremic rats treated with RIS (Fig. 4a). The eroded surface did not change, but uremic rats treated with RIS showed a trend towards decreases in osteoclasts (Table 1) and bone resorption marker CTX (Fig. 4b). Moreover, RIS treatment led to a striking reduction in peritrabecular fibrosis (Table 1 and Fig. 4c). Furthermore, RIS treatment attenuated cortical bone loss in nephrectomized rats (Fig. 4c). These results indicate that the histological features of high-turnover bone disease could be ameliorated by RIS treatment. We also found a significant increase in the stiffness of femurs in uremic rats that received RIS, although the improvement in other biomechanical properties was small and nonsignificant (Table 2).

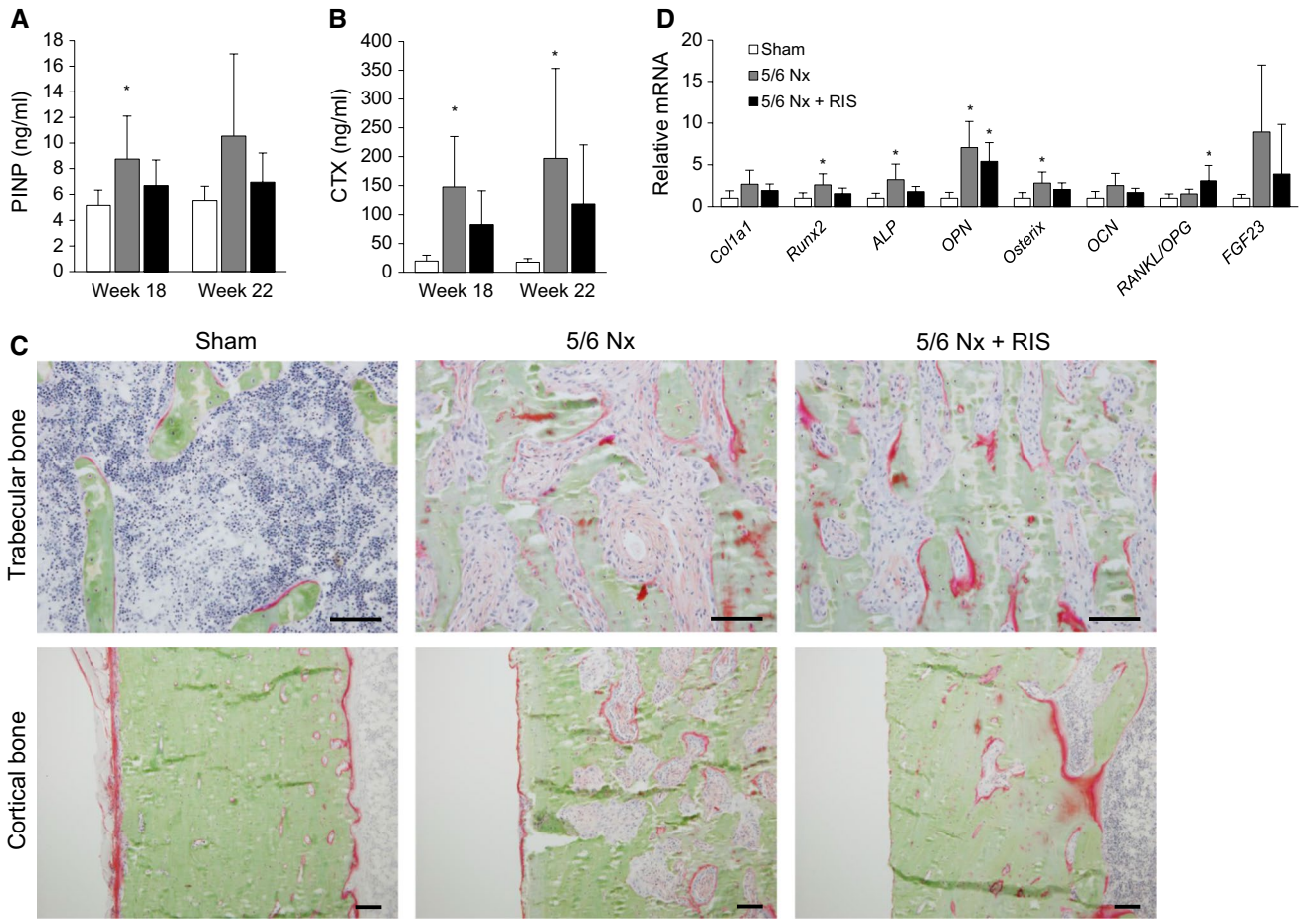

Fig. 4 **a** Serum N-terminal propeptide of type I procollagen (PINP) and **b** C-terminal telopeptide fragments of type I collagen (CTX) in sham-operated rats receiving vehicle (sham), 5/6 nephrectomized rats receiving vehicle (5/6 Nx), and 5/6 nephrectomized rats receiving RIS (5/6 Nx + RIS) at 18 and 22 weeks of age. **c** Villanueva-Goldner staining of trabecular and cortical bone in the proximal tibia at 22 weeks of age. **d** Quantitative real-time PCR in the femur at 22 weeks of age. Data are shown as mean ± SD. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus 5/6 Nx (one-way ANOVA followed by Tukey's post hoc test). $n = 6–7$ in each group

Table 2 Biomechanical properties of the femur

	Sham	5/6 Nx	5/6 Nx + RIS
Ultimate force (N)	239 ± 30	123 ± 25[a]	136 ± 15[a]
Displacement (mm)	1.39 ± 0.25	0.75 ± 0.14[a]	0.73 ± 0.12[a]
Stiffness (N/mm)	660 ± 54	338 ± 106[a]	470 ± 83[a,b]
Energy to failure (N mm)	243 ± 59	60 ± 24[a]	67 ± 14[a]

Data are shown as mean ± SD

RIS risedronate

[a] $P < 0.05$ versus sham

[b] $P < 0.05$ versus 5/6 Nx + vehicle (one-way ANOVA followed by Tukey's post hoc test). $n = 6–7$ in each group

Skeletal gene expression in nephrectomized rats treated with RIS and vehicle

Finally, we investigated the underlying mechanism by comparing gene expression profiles in femurs from these rats (Figs. 4D). Nephrectomy resulted in a significant upregulation of osteogenic gene markers, including *runt-domain transcription factor 2 (Runx2)*, *alkaline phosphatase (ALP)*, *osteopontin (OPN)*, and *osterix,* along with a trend towards increased *collagen type 1 (Col1a1)* and *osteocalcin (OCN)*, indicating increased osteoblastic activity. These changes were partially attenuated by RIS treatment. Nephrectomy also resulted in a trend towards a higher ratio of *receptor activator of NF-κB ligand (RANKL)* to *osteoprotegerin (OPG)*, which is a marker of osteoblast- and osteocyte-mediated osteoclastogenesis. In contrast to the inhibited bone resorption in the uremic rats administered RIS, these rats revealed a further increase in the *RANKL/OPG* ratio. These findings are in agreement with recent in vitro studies [36] and suggest that RIS treatment activated the osteoclastogenic action of osteoblast lineage cells. However, the direct pharmacological effect

on osteoclasts was even more potent and resulted in inhibition of bone resorption.

In accordance with the results of serum FGF23 levels, compared to sham-operated control rats, nephrectomized rats receiving vehicle showed a large increase in *FGF23* expression of borderline significance ($P = 0.07$), but this increase in *FGF23* expression was partially attenuated in uremic rats treated with RIS.

Discussion

High-turnover ROD is a major consequence of SHPT and could lead to increased bone fragility in CKD patients. In this study, we successfully established a rat model of hyperparathyroid bone disease induced by 5/6 nephrectomy and a high phosphate diet. These rats developed severe SHPT and demonstrated characteristic features of high-turnover ROD, including increased trabecular bone turnover, decreased cortical bone thickness, and inferior cortical biomechanical properties. Notably, these skeletal alterations were accompanied by a prominent increase in peritrabecular fibrosis, which was remarkably enhanced compared to that in previous studies [17–23]. These rats also showed an increase in trabecular bone volume, but this finding is consistent with previous rodent studies [20, 32–35] as well as the clinical observation that ESRD patients with high PTH levels have increased cancellous volume but decreased cortical thickness [37, 38].

Several previous studies have evaluated the skeletal effects of bisphosphonates, such as ibandronate, pamidronate, and zoledronate, in rats with CKD and relatively mild SHPT [17–23]. In this study, we examined the effects of RIS on bone metabolism in rats with more severe SHPT driven by 5/6 nephrectomy and a high phosphate diet. In accordance with previous animal studies [17–23], RIS treatment resulted in increased bone volume along with suppression of bone formation and resorption markers, supporting the improvement in high-turnover ROD. However, there was no significant improvement in the bone formation rate or eroded surface and only a partial recovery of mechanical properties. These results indicate a limited skeletal effect of RIS in the context of severe SHPT and may indicate the relative importance of lowering PTH levels over using bisphosphonate.

Interestingly, however, we found striking improvement in peritrabecular fibrosis in uremic rats treated with RIS. Peritrabecular fibrosis is a hallmark of osteitis fibrosa where fibroblast-like preosteoblasts produce a copious amount of poorly organized extracellular matrix in response to persistently elevated PTH [39, 40]. Because uremic rats treated with RIS showed elevated $1,25(OH)_2D$ levels, as discussed below, along with a trend towards lower PTH compared to those receiving vehicle, these metabolic changes might explain the improvement in peritrabecular fibrosis by RIS treatment. Another more likely mechanism for the improvement in peritrabecular fibrosis is the direct effect of RIS on bone-resident cells. We observed a consistent decrease in the expression of osteogenic gene markers in uremic rats treated with RIS, suggesting that RIS treatment suppressed the activity of preosteoblasts and thereby the synthesis of fibrotic extracellular matrix. In agreement with our findings, several animal studies have shown that bisphosphonate inhibits osteoblastic differentiation and activity [41–43]. Our in vivo findings are not consistent with prior in vitro studies [44, 45] but could be explained by assuming that in vivo bisphosphonate can inhibit osteoclast activity, which in turn leads to disruption of osteoclast-osteoblast coupling and subsequent depression of osteoblastic activity. However, we should note that in a previous study, pamidronate, another bisphosphonate, did not improve osteitis fibrosa in uremic rats with SHPT [19]. These different results may be explained by differences in the types of bisphosphonates; treatment duration; and the severity of uremia, SHPT, and high-turnover bone disease. Thus, further research is clearly needed to confirm the effect of bisphosphonate on osteitis fibrosa and identify factors that may modify the effect.

Peritrabecular fibrosis is a cause of renal anemia. Thus, the marked reduction in peritrabecular fibrosis by RIS treatment raise the hypothesis of improved anemia. Unfortunately, as we did not measure red blood cell counts or hemoglobin levels in the current study, this possibility remains to be confirmed in future experiments.

Another interesting finding of this study is that RIS treatment attenuated the decrease in $1,25(OH)_2D$ levels in uremic rats with SHPT. According to several previous studies, bisphosphonate administration increases $1,25(OH)_2D$ levels [19, 27], but the underlying mechanisms have not been identified. Bisphosphonate stimulates renal 1-hydroxylase activity in vivo but not in vitro [29], suggesting an indirect mechanism. Early studies suggested that bisphosphonate-induced inhibition of bone resorption causes increased systemic demand for calcium, which stimulates PTH secretion and thereby enhances renal $1,25(OH)_2D$ production [46, 47]. However, bisphosphonates also increase $1,25(OH)_2D$ levels even in parathyroidectomized rats [28, 29], suggesting a PTH-independent action. In this study, RIS treatment in uremic rats led to decreased FGF23 levels, which were associated with increased $1,25(OH)_2D$ levels. Although this finding does not establish a direct causal relationship, we suggest that the reduction in FGF23 by RIS treatment contributed to increased $1,25(OH)_2D$.

The molecular mechanism for decreased FGF23 upon RIS treatment is unknown but is consistent with the decrease in other osteogenic genes. Furthermore, a recent study demonstrated sustained suppression of osteoblast and osteocyte

activity after alendronate treatment [43]. Although we could not find clear evidence of increased apoptotic osteocytes or empty lacunae, it could be inferred that RIS-induced inhibition of the biological activity of osteoblast lineage cells underlies the decreased FGF23 synthesis. Importantly, recent experimental and epidemiological studies suggest but do not prove that FGF23 has a causal role in left ventricular hypertrophy, inflammation, and immunosuppression [48–50]. Whether bisphosphonates such as RIS can decrease FGF23 levels in CKD patients and if so, whether the reduction in FGF23 translates into improved clinical outcomes are intriguing questions and should be explored further.

Both PTH and FGF23 increase urinary phosphate excretion by inhibiting the renal sodium-phosphate co-transporters. However, there was no difference in FEP between RIS- and vehicle-treated uremic rats, despite the trend toward lower levels of PTH and FGF23 in those receiving RIS. We have no clear explanation for this observation, but it could be assumed that the PTH and FGF23 levels in RIS-treated uremic rats were still sufficient to maximize the tubular phosphate reabsorption. It is also noteworthy that in uremic rats treated with RIS, serum calcium levels did not increase despite increased $1,25(OH)_2D$ levels. Again, the underlying mechanism is unclear, but these findings might be explained by the increased influx of calcium into bone. Regulation of mineral metabolism is a complex process, and additional studies are required to confirm these possibilities.

In this study, we observed that nephrectomized rats exhibited increased trabecular bone volume despite severe high-turnover bone disease. This finding was unexpected but have been observed by other investigators [20, 32–35]. The exact mechanism for the increased bone volume in uremic animals has not been determined, but it is possible that the bone anabolic action of PTH overrode the activation of osteoclasts in response to elevated PTH. In this regard, it should be noted that rats have continuous longitudinal bone growth unlike humans, which might have favored the anabolic action of PTH and contributed to the increased bone volume after nephrectomy. Thus, our results should be interpreted with caution, and additional studies are required to determine whether the results of this animal study could be extrapolated to patients with advanced CKD.

Bisphosphonates are excreted primarily by the kidney. Thus, renal impairment leads to decreased clearance of bisphosphonates [51], but there is no evidence showing whether this issue actually leads to increased skeletal accumulation [52]. In this study, the dose of RIS was based on previous studies of rats with normal renal function [30]; hence, one may argue that the dose we used might be relatively high for uremic rats. However, the skeletal effect of RIS in our uremic rats was rather modest, and there were no signs of adynamic bone disease, suggesting that an even higher dose might be required in the setting of high-turnover bone disease associated with severe SHPT. Nonetheless, it should be noted that high concentrations of bisphosphonates are toxic to kidney cells. Furthermore, if oversuppression of bone formation occurs following bisphosphonate use, it might lead to serious adverse effects such as osteonecrosis of the jaw and atypical femoral fractures. Although not yet proven, this risk might be increased in ESRD given the decreased renal clearance. Thus, the pharmacokinetics of bisphosphonates in ESRD should be better defined, and further studies should determine if the optimal dose of bisphosphonates varies with the severity of SHPT or the degree of bone turnover.

There are several limitations to the present study. First, we did not confirm our histomorphometric findings on bone volume with other imaging methods such as dual-energy X-ray bone densitometry. Second, the sample size was small, which limited statistical power to detect relevant differences in some analyses. Third, the experimental design featured nephrectomy prior to achievement of adult size, which could have confounded the interpretation of the biomechanics.

In conclusion, in a rat model of severe SHPT and high-turnover ROD, treatment with RIS partially attenuated trabecular bone remodeling, cortical bone loss, and mechanical properties, whereas it produced a marked improvement in peritrabecular fibrosis along with a corresponding decrease in osteogenic gene markers. Additionally, RIS treatment suppressed the elevation of FGF23, which may explain the increased $1,25(OH)_2D$ production during the treatment. Although PTH-lowering treatment should be offered as the first-line therapy and use of bisphosphonates should be considered with caution in patients with advanced CKD, our data support the need for clinical trials to test the efficacy and safety of RIS or other bisphosphonates in this population with increased bone fragility and increased risk of clinical events and mortality associated with high FGF23.

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Compliance with ethical standards

Conflict of interest Dr. Komaba has received honoraria, consulting fees, and/or grant support from Bayer Yakuhin, Chugai Pharmaceutical, Japan Tobacco, Kyowa Kirin, Novartis, and Ono Pharmaceutical. Dr. Wada has received honoraria, consulting fees, and/or grant support from Chugai Pharmaceutical, Daiichi Sankyo, Kyowa Kirin, and Otsuka Pharmaceutical. Dr. Nakamura received grant support from Astellas Pharma and Novartis. Dr. Fukagawa has received honoraria, consulting fees, and/or grant support from Astellas Pharma, Bayer Yakuhin, EA Pharma Co., Ltd., Kissei Pharmaceutical, Kyowa Kirin, Ono Pharmaceutical, and Torii Pharmaceutical. The remaining authors declare no competing interests.

Ethical approval All experiments were approved by the Institutional Review Board of Tokai University School of Medicine.

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