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Differential effects of intermittent PTH(1-34) and PTH(7-34) on bone microarchitecture and aortic calcification in experimental renal failure

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Abstract

PTH(1-84) and PTH(7-84) are elevated in chronic kidney disease (CKD). These peptides, as their shorter analogs PTH(1-34) and PTH(7-34) both promote PTH receptor (PTH1R) internalization but only PTH(1-34) and PTH(1-84) activate the receptor. Here, we examined the effects of intermittent administration of PTH(1-34) and PTH(7-34) on mineral ion metabolism, bone architecture, and vascular calcification in rats with experimental CKD. CKD with or without parathyroidectomy (PTX) was established by 5/6 nephrectomy (NPX) in rats. Animals were divided into 4 groups: Sham PTX + sham NPX (Sham); PTX + sham NPX (PTX); Sham PTX+NPX (NPX); PTX+NPX (PTX/NPX). Rats were treated with single daily doses of 40 µg/kg PTH(1-34), PTH(7-34), or vehicle. Creatinine was higher in NPX and Ca lower in PTX and PTX/NPX groups than in Sham or NPX rats. Plasma phosphate was higher in PTX, NPX and PTX/NPX than in Sham rats. PTH(1-34) was more hypercalcemic than PTH(7-34) in PTX rats. Fractional bone volume in rats treated with PTH(1-34) increased significantly in all groups compared to that of vehicle treatment. In addition, trabecular number, thickness and volumetric bone density increased in rats treated with PTH(1-34). In contrast, PTH(1-34) diminished vascular calcification. Bone and renal PTH1R mRNA expression was reduced as much or more in PTX/NPX rats as in NPX alone, whereas PTH(7-34) had no effect on PTH1R expression. Renal but not bone PTH1R mRNA increased in response to PTH(1-34). These findings suggest that PTH(1-34) exerts greater hypercalcemic and anabolic effects in parathyroidectomized and/or nephrectomized rats than does PTH(7-34). There was no evidence for significant bone or vascular actions of PTH(7-34). We conclude that PTH(1-34) protects against vascular calcification and bone demineralization in experimental renal failure.

Keywords

nephrectomy; parathyroidectomy; PTH(1-34); PTH(7-34); bone; microarchitecture; PTH receptor

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Introduction

Disturbances of bone and mineral metabolism are well recognized features of chronic kidney disease (CKD) [1,2]. Vascular calcification and ectopic calcification of other soft tissues are commonly observed. These actions on mineral metabolism in patients with CKD are accompanied by increased synthesis, secretion and circulating levels of parathyroid hormone (PTH) and PTH fragments, which lead to the development of secondary hyperparathyroidism (SHPT) [3,4]. The factors involved in the pathogenesis of SHPT in CKD, include phosphate retention, low 1,25(OH)₂D₃ and vitamin D receptors, decreased serum free calcium and parathyroid calcium-sensing receptors [5,6], downregulation of PTH receptors [7-10], and the accumulation of uremic toxins and acidosis [9,11] that contribute to skeletal resistance to the actions of PTH and a spectrum of bone diseases collectively called osteodystrophy. Although the mechanism of resistance is incompletely understood, uremic patients require PTH levels 2-3 times normal to maintain bone turnover [56]. Excessive suppression of PTH secretion, however, may lead to low bone turnover, so-called adynamic bone disease [12,13].

The type I PTH receptor (PTH1R) is the predominant form expressed in bone and kidney and is a member of class B of the superfamily of G protein-coupled receptors (GPCRs) [14,15]. It mediates the actions of PTH and of PTH fragments containing an intact amino terminus. In the kidney, the PTH1R mediates the regulation of PTH on the renal transport of phosphate and calcium [16]. In renal tubular epithelial cells, signal and expression of PTH1R appears to be modulated in a cell-specific manner. Mounting evidence supports the view that in renal failure serum PTH(7-84) accumulates to high levels [17,18] that may approach those of PTH(1-84) [18-20]. It has been suggested that the competitive inhibition of PTH1R binding by amino-truncated PTH fragments contributes to the PTH resistance of uremia [21]. However, as indicated above, PTH1R downregulation has been described in the same pathological setting [7,22], which is inconsistent with the view that PTH(7-34) acts exclusively as an antagonist.

Recent work shows that PTH(7-84) and its synthetic analog, PTH(7-34), internalize [23,24] and downregulate [25] the PTH1R without antecedent or concomitant receptor activation, whereas PTH(1-34) promotes synchronized PTH1R activation and internalization. This phenomenon occurs in a cell-specific manner that depends on the expression of the scaffolding protein Na/H exchange regulatory factor (NHERF1) [23,26], a cytoplasmic adaptor that interacts with the carboxy-terminus of the PTH1R and is implicated in protein targeting and in the assembly of protein complexes [26,27]. These findings provide a plausible alternative explanation for PTH1R downregulation and resistance in renal failure. According to this hypothesis, PTH(7-84) may contribute to hormone resistance by inducing PTH1R internalization and downregulation without accompanying activation. Reducing the number of plasma membrane-delimited PTH receptors would diminish the action of full-length or amino-terminal PTH fragments.

Other studies addressed competitive interactions between amino-truncated PTH fragments and PTH(1-34) or PTH(1-84) [28,29]. PTH(7-34) induces PTH1R internalization on its own and is only a competitive inhibitor at higher concentrations [23]. In the present study, we used exogenous PTH(1-34) and the amino-truncated peptide PTH(7-34) in animal models of CKD without secondary hyperparathyroidism to test the hypothesis that amino-truncated PTH fragments downregulate the PTH1R, thereby reducing bone demineralization and vascular calcification.

Material and methods

Animals and experimental protocol

All experiment protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC).

Nine-weeks old male Wistar rats weighing 250 - 300 grams, were purchased from Charles River Laboratories. Animals were fed standard diet (LabDiet, PMI Nutrition LLC, MO, US) containing 1.0% calcium, 0.75% phosphorus and 22.5% protein. The animals were allowed free access to food and water and housed in individual cages at constant room temperature with a 12-h light and dark cycle. Rats were randomly assigned to one of four experimental groups according to the type of surgery. Group 1: sham parathyroidectomy and sham nephrectomy (Sham), 23 rats; Group 2: parathyroidectomy and sham nephrectomy (PTX), 22 rats; Group 3: Sham parathyroidectomy and 5/6 Nephrectomy (NPX) (described below), 22 rats; Group 4: parathyroidectomy and 5/6 nephrectomy (PTX/NPX), 22 rats. The Animals were anesthetized by intraperitoneal injection of ketamine and xylazine before surgery and treated with subcutaneous buprenorphine for 3 days after the procedure to alleviate pain. During all surgical procedures, the vitals signs, electrolytes, and arterial blood gases were monitored and the animals were weighed twice per week.

Following an adaptation period of 48 h, the rats were parathyroidectomized under microscopic observation, dissecting and removing both parathyroid glands, while preserving the thyroid gland. The time course for the experimental protocol is shown in Fig. 1. Serum ionized calcium and serum PTH concentrations were measured before the surgical procedure, one week after surgery and at the end of the experiment. A decrease in serum calcium concentration <0.9 mM and undetectable serum PTH were taken as evidence of successful and complete parathyroidectomy. Ten days after PTX, the rats underwent right nephrectomy, and seven days later underwent 2/3 left nephrectomy using a suture placed and ligated around each pole of the kidney at 1/3 position. Each pole was then excised to produce total 5/6 nephrectomy. Sham operations (decapsulation) of the parathyroid glands and/or kidneys were performed at the same time intervals and served as controls. The day after nephrectomy (Day 21), animals from each surgical procedure were assigned to one of three treatment groups: vehicle (0.9% sodium chloride), PTH(1-34) (40 μ g/Kg/day) (Bachem California, Torrance, CA), or PTH(7-34) (40 μ g/Kg/day) (Molecular Medicine Institute, Peptide Synthesis Facility, University of Pittsburgh, PA). Vehicle or PTH was administered by subcutaneous injection once daily each morning for four weeks. At day 52 the rats were euthanized by intraperitoneal injection of pentobarbital. Blood samples were drawn and the femurs, tibia, kidneys and aorta were removed.

Biochemistry

On days 3, 13, 20 and 52 the animals were anesthetized and a blood sample was obtained from the tail vein and blood collected into a heparinized tube. The blood samples were taken 24 hrs after the last PTH injection and 3 hrs after the last food access. Plasma was separated from whole blood by centrifugation at 10000 rpm for 10 min and an aliquot of plasma was used for ionized calcium and pH determination (Rapidlab 348, Bayer, East Walpole, MA). Plasma was stored at -80°C until assays were performed. Creatinine was determined spectrophotometrically (Beckman DU 530) using Infinity Creatinine (Thermo Fisher Scientific, Inc., Waltham, MA). Phosphorus was determined using the Inorganic Phosphorus Reagent Set (UV) (Pointe Scientific, Inc., Canton, MI). Serum levels of intact PTH(1-84) were measured with a rat PTH (ELISA) kit (Rat Bio Intact ELISA Kit, Immutopics, San Clemente, CA).

Micro-computed tomography

Trabecular bone volume and skeletal architecture of the distal femur of each rat was measured by micro-computed tomography using a μ CT 40 (Scanco Medical AG, Bassersdorf, Switzerland) using the manufacturer's software. In brief, cross-sectional images were obtained with a voxel size of 16 μ m in all dimensions. Semi-automated contouring was used to select a region of interest (ROI) extending 3.2 mm proximal to the femoral growth plate, but excluding the cortex and subcortical bone, composed of 150 adjacent 16-micron slices. The three-dimensional volume and architecture of the distal femur was calculated by stacking the volume of each slice prior to applying an optimized Gaussian noise filter and grey scale threshold [30]. Fractional bone volume (bone volume per tissue volume, BV/TV), architectural parameters (trabecular thickness (Tb.Th), number (Tb.N) separation (Tb.Sp), and structural indices including connectivity density (Conn.D.) and structure model index (SMI) were calculated directly from the reconstructed trabecular structures as described elsewhere [31].

Vascular Calcification

At the time of euthanasia, the abdominal aorta was removed, flushed with phosphate-buffered saline through the intraluminal space and fixed in 10% buffered formalin. Four micrometer sections were stained for calcification by the Von Kossa method. Vascular calcification was analyzed histologically and examined by fluorescence microscopy (LEICA DMI600b, Center for Biological Imaging, University of Pittsburgh, PA). The extent of vascular calcification was determined by quantitative image analysis. Four independent samples for each experimental condition were digitized at 32-bit depth and uniformly thresholded and size analysis of particles 4-10² pixels was determined using Image J (<http://rsb.info.nih.gov/ij>).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) of bone and kidney

The dissected left tibia of each rat was flushed with phosphate-buffered saline (PBS) to remove the bone marrow and then frozen in liquid nitrogen. The proximal metaphysis and diaphysis were ground to a fine powder using a mortar and pestle under liquid nitrogen. The dissected kidney was stored in RNA stabilization solution (RNAlater, Ambion, Austin, TX, USA) until analysis.

RNA was extracted using Trizol (Invitrogen, CA, US) following the manufacturer's instructions. Briefly, total RNA from bone or kidney cortex was used as the template for cDNA synthesis in a 10- μ l volume using an RT-PCR kit (AccuScript High Fidelity Reverse Transcriptase, Stratagene, US) according to the manufacturer's instructions. The reaction mixture contained RNase H-deficient Moloney murine leukemia virus reverse transcriptase, 0.5 M Tris-HCl (pH 8.3), 0.75 M KCl, 0.03 M MgCl₂, 60 ng random primers, 12.5 mM dNTP, and 100 mM DTT (dithiothreitol). The mixture was incubated at 65 °C for 5 min, 42 °C for 30 min and stored at 4 °C. The PCR mixture (total 50 μ l) contained 10X PCR buffer, 1 μ l of cDNA, 1 μ l of PfuUltra HF DNA polymerase (Stratagene, US), 12.5 mM dNTP and 1 μ l (100 ng/ μ l) each of forward and reverse primers (Table 1). β -Actin was used as an internal control. The thermal cycling protocol was 95 °C for 120 sec in Segment 1; 95 °C for 30 sec, 51 °C for 30 sec and 72 °C for 60 sec in Segment 2 for 30 cycles; and 72 °C for 10 min in Segment 3 (PCR Sprint, Hybaid Limited, UK). The amplification reaction products (50 μ l) were analyzed using 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Semiquantitative analysis was performed using NIH Image 1.62.

Bone turnover markers

Osteocalcin (OC) and C-terminal fragments of type I collagen (CTX I) served as bone formation and turnover markers, respectively. OC and CTX I were measured in serum drawn

at the time of euthanasia (day 52) and stored at -80 C until the time of assay. Serum intact osteocalcin was measured using Rat-MID™ Osteocalcin ELISA (Nordic Bioscience Diagnostics, Chesapeake, VA). CTX I was measured with RatLaps ELISA (Nordic Bioscience Diagnostics, Chesapeake, VA)

Statistical analysis

Data are expressed as the mean $\pm$ SD or $\pm$ SEM as indicated. Statistical significance was determined by analysis of variance (ANOVA) and post hoc comparison using the Bonferroni test (Prism, GraphPad). *P* values < 0.05 were considered significant.

Results

All animals gained weight from the time of entry in the protocol. Rats undergoing PTX gained as much weight as Sham animals. However, rats subjected to NPX or PTX/NPX gained significantly less weight (83% and 73% compared to Sham; *P*<0.05, *P*<0.01, respectively).

Renal and parathyroid function

Serum creatinine (Cr), calcium (Ca), and inorganic phosphorus (Pi) were measured to determine the effectiveness of PTX and 5/6 NPX. Table 2 summarizes the plasma biochemistry results. Pre-treatment levels of Cr, Ca and Pi were comparable among groups. At the time of euthanasia, Cr, Ca and Pi in the Sham groups were similar to their respective baseline values. PTX did not affect serum Cr but was associated with the expected increases of Pi (*P*<0.01) and decreases of Ca (*P*<0.01). Intermittent PTH(1-34) administration to PTX rats blunted the reduction of serum Ca but did not fully normalize these values. PTH(7-34) treatment did not ameliorate either Pi or Ca. NPX caused profound elevations in serum Cr and Pi that were not accompanied by changes in serum Ca. Serum calcium was also unaffected by PTH in NPX animals. Finally, combined PTX/NPX increased serum Cr and Pi, and caused a reduction of serum Ca comparable to that observed with PTX alone. Thus, the changes seen individually in PTX and NPX rats were not additive in the PTX/NPX animals. Treatment with PTH(1-34) or PTH(7-34) did not affect serum biochemistries except for an increase of Pi in PTX/NPX (*P*<0.05) compared to PTX alone. The calcium phosphate product ($\text{Ca} \times \text{Pi}$, mg^2/dl^2) increased significantly following NPX but was unaffected by PTX alone or by the combination PTX/NPX. PTH(1-34) and PTH(7-34) increase the $\text{Ca} \times \text{Pi}$ product in the NPX rats (*P*<0.001).

Baseline plasma pH averaged 7.41-7.42 in Sham, NPX, and PTX/NPX groups but was significantly elevated (7.45) in PTX rats before treatment commencing on day 20. At the end of the 52-day protocol, pH had not changed in the Sham animals and had normalized in PTX rats. However, both NPX and PTX/NPX groups became significantly alkalotic (Fig. 2) (pH 7.48, 7.50, respectively).

PTH levels were determined before surgery and four weeks after surgery. PTH levels were undetectable in the PTX and PTX/NPX groups. At the time of euthanasia, serum PTH was unaltered in Sham groups but increased significantly in NPX rats from 69 ± 2 to 182 ± 24 pg/ml, *P*<0.01).

Bone Microarchitecture

The left femur of each rat was removed after euthanasia, preserved in 10% buffered formalin and analyzed by μCT . Representative cross-sectional images from Sham, PTX, NPX and PTX/NPX groups treated with vehicle (upper panel) or PTH(1-34) (lower panel) are shown in Fig. 3. The trabecular microarchitecture of animals treated with PTH(1-34) was denser than that of untreated animal in all samples.

Trabecular bone volume and architectural parameters of the distal femur were quantified by μ CT. These parameters are summarized in Fig. 4 and Table 3. Fractional bone volume (BV/TV) increased with daily injection of PTH(1-34) by 39%, 20%, 54%, and 86% in Sham, PTX, NPX and PTX/NPX rats ($P<0.05$), respectively compared to vehicle treatment (Fig. 4). The BV/TV ratio was the same for Sham and NPX. PTH(1-34) also increased the trabecular number (Tb.N) in NPX and PTX/NPX animals (Table 3) ($P<0.05$). The trabecular thickness (Tb.Th.) increased by 27% and 64% upon injection of PTH(1-34) in Sham and PTX/NPX groups, respectively ($P<0.05$). There were no statistically significant changes in the Conn.D. index in or between groups. PTH(1-34) decreased the trabecular spacing (Tb.Sp.) in the PTX/NPX group using by 50% when compared to vehicle ($P<0.05$).

PTH1R in Bone and Kidney

PTH1R mRNA expression was examined in samples extracted from the proximal metaphysis and diaphysis of the tibia. PTX alone had no significant effect on PTH1R mRNA expression in bone (Fig. 5, top panel). Treatment with PTH(1-34) or PTH(7-34) did not change PTH1R mRNA levels in the PTX group. NPX and PTX/NPX caused highly significant decreases in receptor mRNA expression when compared to Sham ($P<0.01$). The daily injection of PTH(1-34) significantly increased bone PTH1R mRNA expression in NPX rats ($P<0.01$), whereas PTH(7-34) administration had no demonstrable effect.

PTH predominantly exerts its effects through the PTH1R [32]. PTH resistance in CKD may arise from receptor downregulation. The effect of CKD with or without PTX on PTH1R mRNA extracted from the kidney cortex was examined using RT-PCR (Fig. 5, bottom panel). The renal expression of PTH1R mRNA decreased significantly in PTX/NPX animals treated with vehicle ($P<0.05$). The PTX and PTX/NPX animals expressed higher levels of PTH1R mRNA when compared to Sham ($P<0.05$ and $P<0.01$, respectively) after administration of PTH(1-34).

Bone markers

The C-terminal telopeptide of type I collagen, (CTX I) is a degradation product of type 1 collagen, a component of the peptide-bound cross-link fraction and is a marker of bone resorption [33]. The level of serum CTX I was measured at the end of the experiment. The levels of CTX I were comparable in Sham and PTX animals, independent of the treatment (Fig. 6, top panel). CTX I significantly increased in the NPX and PTX/NPX animals ($P<0.05$ and $P<0.01$, respectively) compared to Sham. Administration of PTH(1-34) did not affect CTX I. However, PTH(7-34) reduced CTX I in PTX/NPX rats compared to vehicle or PTH(1-34)-treated rats.

Osteocalcin (OC) is a hydroxyapatite-binding protein synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes that is expressed mainly during osteoid mineralization and serves as a marker of bone formation [34]. All animals exhibited high concentrations of serum OC after 4 weeks of treatment with PTH(1-34) (Fig. 6, bottom panel). NPX rats, with or without PTX, had higher OC concentrations compared to Sham animals. Consistent with the present results, Higher OC and CTX levels have been reported in chronic renal failure patients [60, 61]. However, OC also increased in Sham and PTX rats with PTH(1-34) treatment ($P<0.05$, $P<0.01$, respectively). The effect of PTH(1-34) also was associated with a significant increase of OC in NPX rats when compared to the effects of vehicle or PTH(7-34) treatment ($P<0.01$).

Vascular Calcification

Vascular calcification is a well recognized complication of CKD [35,36]. The abdominal aortas of NPX and PTX/NPX rats and their corresponding Sham controls were examined by the von Kossa staining procedure to determine the extent of calcification. Representative results are illustrated in Fig. 7a. Vehicle-treated rats are shown in the upper panel, and animals receiving

intermittent PTH(1-34) are displayed in the lower panel. There was conspicuous calcification in NPX rats receiving saline vehicle (arrows). PTH(1-34) decreased calcification in this group of rats. However, there was no difference in the vascular calcification after the administration of PTH(1-34) in PTX/ NPX rats. The results of quantitative analysis of calcification are displayed in Fig. 7b and bear out the visual results.

Discussion

The present studies were designed to analyze and compare the effects of intermittent PTH (1-34) or PTH(7-34) treatment on bone microarchitecture, vascular calcification, and PTH1R expression in an animal model of experimental renal failure. It has been reported that the administration of PTH prevents osteoporotic fractures in postmenopausal women, improves fracture healing and implant fixation, and osteogenesis in model animals [37-40]. We show here that in an animal model of experimental CKD, the intermittent administration of PTH (1-34) increases bone formation and expression of renal but not bone PTH1R mRNA, while decreasing vascular calcification.

To establish an experimental model of CKD without the effect of secondary hyperparathyroidism, we performed combined 5/6 nephrectomy and parathyroidectomy (PTX/ NPX). The higher values of Cr, Pi and weight loss 4 weeks after NPX verified the success of the renal surgery, and the combination of lower levels of plasma Ca with undetectable circulating serum PTH confirm complete PTX. We performed PTX to eliminate PTH and other amino-truncated PTH derivatives. Considerable evidence demonstrates that CKD is accompanied by substantial increases in serum levels of amino-truncated PTH fragments [19,41]. For instance PTH(7-84) [42], which has no effect on serum calcium or bone turnover in PTX/NPX rats, and blocked the action of PTH(1-84) on serum calcium and osteoblast activity [29]. Thus, in the present investigation, the effects of the administration of PTH peptides can be analyzed without the confounding influence of endogenous PTH1R ligands.

Under physiological conditions the kidneys excrete acid and regenerate bicarbonate to replenish the extracellular buffering capacity. In CKD, however, the ability to maintain acid-base balance is impaired because of the progressive reduction of functional renal mass with an increasing burden and inadequate capacity to eliminate the daily acid load [43]. Unexpectedly, the present findings show that plasma pH in both NPX and PTX/NPX rats became alkalotic (Fig.2). In PTX rats, plasma pH was significantly elevated (7.45) before treatment but had normalized by the time of euthanasia. Similar increases in the plasma pH of PTX rats have been noted [43]. In this case, the absence of PTH presumably increases phosphate absorption by proximal tubules. In PTX/NPX rats, PTH(1-34) and PTH(7-34) further decreased phosphate excretion resulting in greater increases of serum phosphate (Table 2). This finding suggests that the presence of amino-truncated PTH fragments may contribute to the hyperphosphatemia of CKD.

PTH was administered as single daily injections because intermittent PTH administration promotes bone formation, whereas continuous infusion causes bone resorption [44-46]. The mechanisms underlying these differences are not entirely clear. We elected to inject comparable amounts of PTH(1-34) and PTH(7-34). We used 40µg/Kg/day because the effect of PTH(1-34) treatment is dose-dependent, increasing mineral apposition rate and bone formation at this concentration [47], which in other studies was shown to be the lowest dose that elicited maximal effects when administered on an intermittent schedule [48]. It should be noted, however, that the K_a of the PTH1R for PTH(7-34) is an order of magnitude lower than that for PTH(1-34) [49,50]. An effect of C-type PTH receptors, CPTH1R, which would not be expected to bind or respond to PTH(7-34) but to PTH(7-84) cannot be excluded [51,52]. It should be noted that PTH(1-34) and PTH(7-34) have different affinities for the PTH1R. Thus, it is possible that

PTH(7-34) may exhibit effects when used at higher doses. The rapid clearance of PTH(1-34) leads to brief exposure (<3 h/d), which is clearly sufficient to increase bone mineral density [37]. The present findings show that several bone turnover and micro-architectural parameters were increased by intermittent treatment with PTH(1-34). In contrast, PTH(7-34) neither increased nor decreased bone micro-architecture.

In CKD, the changes in trabecular bone correlate with the degree of renal failure [53,54]. The increase of PTH levels [53] and the downregulation of PTH1R are major contributing causes of renal osteodystrophy [44,55]. In CKD rats, the use of pharmacological doses of intermittent PTH ameliorated the decreased bone formation in presence of both thyroparathyroidectomy and nephrectomy [63] by mechanisms not fully understood. Previous reports show decreased osteoblast activity and recruitment in PTX rats, decreased 1,25(OH)₂D levels and osteoporosis [39,56]. Measured standard bone microarchitectural parameters demonstrate that the anabolic effect of intermittent PTH(1-34) increased the fractional bone volume (BV/TV) and osteocalcin independent of NPX or PTX. OC and CTX I increase in CKD [60,61]. In PTX/NPX animals, the present findings show that intermittent administration of PTH(1-34) increased both trabecular number and thickness, and decreased trabecular separation. These findings suggest improvement in bone formation after PTH(1-34) but the results imply that different mechanisms are involved in PTX, NPX or PTX/NPX.

Here, we show that PTH1R mRNA expression in bone and kidney of untreated animals decreased after PTX, NPX, or PTX/NPX. Since PTH1R mRNA expression declined further in PTX/NPX animals compared with NPX rats, this finding confirms that the downregulation of PTH1R mRNA expression in CKD is not prevented by PTX [57]. These results suggest that the involvement of as yet undefined uremic factors may contribute to or mediate this effect [58]. PTH(1-34) blunted the decline of renal PTH1R mRNA expression in PTX and PTX/NPX rats, and increased PTH1R mRNA expression in bone in NPX animals when compared to vehicle. PTH(1-34) effects on PTX and PTX/NPX animals did not achieve statistical significance. Contrary to our prediction that amino-truncated PTH fragments would downregulate the PTH1R, PTH(7-34) had no effect on receptor expression. The failure of PTH(7-34) to promote PTH1R downregulation in the present setting may well be due to the relative short duration of treatment and the intermittent dosing protocol. With this caveat in mind, these data nonetheless predict that in bone, PTH1R mRNA reductions in NPX and PTX/NPX rats should result in PTH resistance in these animals if receptor protein is reduced proportionately, and if receptor abundance is rate-limiting to generating an anabolic response. Notably, intermittent daily PTH(1-34) administration effectively increased BV/TV under all conditions. This may be a consequence of the exquisite sensitivity of the rat skeleton to PTH or that the residual PTH1R expression was sufficient to mediate the anabolic actions of PTH(1-34).

Calcification of the intima and media of the aorta is common in CKD [59]. Vascular calcification is considered to be a regulated process that is influenced by tissue-specific cellular mechanisms and by discrete plasma components [60]. Treatment with vitamin D sterols may aggravate vascular calcification. The mechanism by which PTH causes vascular calcification remains uncertain. However, calcification of the rat aorta *in vitro* requires elevation of both Ca and Pi [61]. Although amino-truncated PTH fragments promote PTH1R downregulation in bone and kidney cells *in vitro*, no evidence was found that intermittent PTH(7-34) administration exerts biological actions that exacerbate or improve vascular calcification or skeletal deterioration *in vivo*. This may reflect a number of potential mechanistic considerations. We found that PTH(1-34) reduced vascular calcification in NPX rats. Rats with PTX/NPX showed similar decreased vascular calcification independent of the treatment. These observations suggest that the accumulation of antagonistic PTH fragments may contribute to the calcemic vasculopathy characteristic of end-stage renal disease. We suggest that PTH1R signaling plays important roles in promoting skeletal mineral accumulation, while

simultaneously decreasing vascular calcification. We propose that PTH(1-34) continues to inhibit proximal tubule phosphate transport [23], while the reduced effect of PTH on the distal nephron likely represents a protective action to avoid concomitant hypercalcemia in the face of frank hyperphosphatemia. These effects would limit the rise of the $\text{Ca} \times \text{Pi}$ product, which is an important risk for vascular calcification and mortality [62].

Based on these findings, we conclude that intermittent administration of PTH(1-34) exerts a protective action that reduces bone demineralization and vascular calcification in short-term experimental renal failure. PTH(7-34) exhibited no detectable effects of its own in the absence of endogenous PTH. The lack of an effect of PTH(7-34) may be ascribed to pharmacokinetic effects, or to an action at the level of the PTH1R or CPTHr, or downstream signaling.

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Fig. 1.

Experimental protocol for 5/6 nephrectomy and parathyroidectomy and time line for PTH administration. Sham = Sham operation; PTX = Parathyroidectomy; NPX 1st = First step of 5/6 nephrectomy; NPX 2nd = Second step of 5/6 nephrectomy.

Fig. 2.

Serum pH measurement. Serum pH was measured in the 4 experimental groups under baseline conditions (before surgery, day 3) and at euthanasia (day 52). Sham = Sham operation; PTX = parathyroidectomized; NPX = 5/6 nephrectomized; PTX/NPX = parathyroidectomized + 5/6 nephrectomized rats.

Fig. 3.

MicroCT renderings of the left femur of the rat. PTH(1-34) increased bone density in all animals compared to vehicle group. Sham = Sham operation; PTX = parathyroidectomized; NPX = 5/6 nephrectomized; PTX/NPX = parathyroidectomized + 5/6 nephrectomized rats.

Fig 4. PTH(1-34) increased BV/TV in all the groups. PTH(7-34) exerted no significant effects. Results are expressed as mean $\pm$ SEM. * $P < 0.05$ vs. respective vehicle. Abbreviations as in Fig. 2.

Fig. 5. Expression of PTH1R. PTH1R mRNA was measured by RT-PCR in the rat tibia (top) and kidney (bottom) and normalized to β -actin determined simultaneously. * $P < 0.05$, ** $P < 0.01$ vs. respective Sham, ^c. ^a $P < 0.01$ vs. NPX vehicle. Abbreviations as in Fig. 2.

Fig. 6. Bone turnover markers. C-terminal telopeptide of type I collagen, CTX I, (top) and, Osteocalcin, OC (bottom) were measured in serum collected at the time of euthanasia. Results are expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. respective vehicle. Abbreviations as in Fig. 2.

Fig. 7.

Reduction of vascular calcification by PTH(1-34). *A)* Histological sections of rat aorta. The arrows indicate calcification of the abdominal aorta staining by Von Kossa. PTH(1-34) decreased calcification in the aorta in NPX rats but there is not evident effect in the absence of parathyroid glands. * $P < 0.05$, ** $P < 0.01$ vs. respective Sham, ^c. ^a $P < 0.01$ vs. NPX vehicle. *B)* Summary of 4 independent representative samples. * $P < 0.05$, ** $P < 0.01$ vs. vehicle-treated NPX. Abbreviations as in Fig.2

Table 1

PCR primer sequences and their product sizes

Primer	Forward, 5'-3'	Reverse	Product size, bp
PTH1R	AGCGAGTGCCTCAAGTTCAT	ACAGCGTCCTTCACGAAGAT	236
β -actin	TCATGAAGTGTGACGTTGACATCCGT	CCTAGAAGCATTGCGGTGCACGATG	285

Table 2

Plasma biochemistry

Maneuver	Treatment	n	Cr (mg/dl)		P (mmol/L)		Ca (mmol/L)		Ca xP (mg ² /dl ²)	
			Baseline	Euthanasia	Before treatment	Euthanasia	Before treatment	Euthanasia	Before treatment	Euthanasia
Sham	Vehicle	10	0.54±0.08	0.56±0.11	1.96±0.05	1.85±0.21	1.26±0.03	1.25±0.04	30.75±1.31	30.27±1.85
	PTH(1-34)	7	0.54±0.09	0.57±0.13	2.15±0.10	2.02±0.11	1.22±0.04	1.23±0.06	33.19±2.46	31.89±3.12
	PTH(7-34)	6	0.48±0.16	0.48±0.07	2.00±0.08	2.02±0.15	1.27±0.07	1.31±0.07	32.04±2.47	33.18±2.87
	Vehicle	10	0.59±0.11	0.59±0.15	2.62±0.34	2.70±0.37	0.84±0.11	0.74±0.08 ^b	27.5±5.53	24.65±6.3
PTX	PTH(1-34)	6	0.52±0.07	0.61±0.08	2.70±0.14	2.65±0.17	0.84±0.13	0.89±0.11	26.99±5.51	27.22±1.59
	PTH(7-34)	6	0.53±0.08	0.53±0.08	2.67±0.45	2.55±0.37	0.83±0.05	0.77±0.17	27.82±4.61	22.07±4.56
NPX	Vehicle	10	0.49±0.08	1.20±0.12 ^a	2.02±0.09	2.82±0.67 ^b	1.24±0.13	1.27±0.05	32.15±3.03	38.74±6.14
	PTH(1-34)	6	0.52±0.09	1.27±0.18 ^a	2.1±0.10	2.84±0.20 ^c	1.24±0.04	1.28±0.07	32.39±1.23	46.23±3.77 ^c
	PTH(7-34)	6	0.50±0.12	1.25±0.11 ^a	2.06±0.11	2.90±0.24 ^c	1.20±0.03	1.25±0.04	31.2±1.39	45.13±5.35 ^c
	Vehicle	8	0.49±0.18	1.13±0.11 ^a	2.80±0.15	2.97±0.40	0.79±0.15	0.77±0.21	25.04±4.52	24.10±8.59
PTX/NPX	PTH (1-34)	8	0.55±0.11	1.28±0.18 ^a	2.65±0.37	3.31±0.61 ^d	0.90±0.05	0.89±0.09	29.90±3.94	30.96±3.08
	PTH (7-34)	6	0.53±0.08	1.18±0.07 ^a	2.42±0.42	3.15±0.82 ^d	0.83±0.12	0.84±0.14	24.21±8.02	26.55±6.40

Bone. Author manuscript; available in PMC 2008 December 1.

Sham = sham operation; PTX = parathyroidectomized; NPX = 5/6 nephrectomized; PTX/NPX = parathyroidectomized + 5/6 nephrectomized rats. Results are expressed as mean ± SD. Baseline values are those measured at Day 3 of the protocol (see Materials and Methods, Fig. 1); Before treatment are values determined at Day 20. Cr = Creatinine; Pi = Phosphorus; Ca = Calcium.

^a $P < 0.05$ compared to Sham and PTX of the same group of injection in Euthanasia;

^b $P < 0.05$ compared to Before treatment (Day 20) of the same group of injection;

^c $P < 0.001$ compared to Before treatment (Day 20) of the same group;

^d $P < 0.005$ vs pre-treatment of the same group.

Table 3

Micro CT parameters

	<i>n</i>	Trabecular Number 1/mm	Trabecular Thickness μm	Trabecular Spacing μm	Connectivity Density $1/\text{mm}^3$
Sham	Vehicle	6	2.92 $\pm$ 0.5	0.11 $\pm$ 0.002	0.45 $\pm$ 0.11
	PTH(1-34)	6	3.63 $\pm$ 0.2	0.14 $\pm$ 0.006 ^a	0.28 $\pm$ 0.02
	PTH(7-34)	6	3.21 $\pm$ 0.5	0.11 $\pm$ 0.003	0.39 $\pm$ 0.11
PTX	Vehicle	6	5.41 $\pm$ 0.6 ^b	0.14 $\pm$ 0.010 ^b	0.31 $\pm$ 0.09
	PTH(1-34)	6	5.76 $\pm$ 0.7	0.17 $\pm$ 0.015	0.14 $\pm$ 0.02
	PTH(7-34)	6	5.10 $\pm$ 0.9	0.15 $\pm$ 0.020	0.23 $\pm$ 0.06
NPX	Vehicle	6	2.67 $\pm$ 0.6	0.11 $\pm$ 0.008	0.40 $\pm$ 0.13
	PTH(1-34)	6	4.05 $\pm$ 0.2 ^a	0.13 $\pm$ 0.005	0.22 $\pm$ 0.02
	PTH(7-34)	6	2.30 $\pm$ 0.2	0.11 $\pm$ 0.004	0.51 $\pm$ 0.06
PTX/NPX	Vehicle	7	3.06 $\pm$ 0.5	0.11 $\pm$ 0.003	0.32 $\pm$ 0.06
	PTH(1-34)	8	5.41 $\pm$ 0.4 ^a	0.18 $\pm$ 0.010 ^a	0.16 $\pm$ 0.01 ^a
	PTH(7-34)	6	4.46 $\pm$ 0.9	0.13 $\pm$ 0.012	0.27 $\pm$ 0.01

^a $P < 0.05$ vs respective vehicle;

^b $P < 0.05$ vs respective Sham vehicle