Note

The Optimal Duration of PTH(1–34) Infusion Is One Hour per Day to Increase Bone Mass in Rats

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Parathyroid hormone (PTH) is a potential medicine for osteoporosis, and subcutaneous (s.c.) PTH treatment enhances bone mass; however, continuous infusion of PTH elicits bone resorption and induces bone loss. To clarify this contradictory phenomenon, we examined bone markers and bone mass in rats to assess the optimal duration of PTH(1–34) infusion. Continuous infusion of PTH at 1 µg/kg/h (C_{ss} , steady-state concentration *ca.* 300 pg/mL) for 1–4 h clearly stimulated the expression both of bone formation-related genes (*c-fos*, *Wnt4*, *EphrinB2*) and of bone resorption-related genes (*tnfsf11*, *tnfsf11b*, encoding receptor activator of nuclear factor-kappaB ligand (RANKL), osteoprotegerin (OPG)), but s.c. treatment stimulated these genes only 1-h after the injection. Rats were treated with 1-, 2-, or 4-h infusions of PTH daily using a totally implanted catheter system, and the femoral bone mineral density (BMD) was measured at 4 weeks. The 1-h infusion of PTH significantly stimulated serum bone formation markers (procollagen I N-terminal propeptide (PINP) and osteocalcin) on day 14 and femoral BMD at 2 and 4 weeks, but the 4-h infusion of PTH did not enhance BMD. Since the 4-h infusion increased the levels of both the bone formation markers and a bone resorption marker (urinary C-terminal telopeptide of type 1 collagen (CTX)), the increased bone resorption may predominate over bone formation. The intermittent elevation of plasma PTH to 300 pg/mL for 1-h each day is optimal for increasing bone mass in rats. In osteoporosis therapy in human, using the optimal duration for the clinical dose of PTH may selectively stimulate bone formation.

Key words parathyroid hormone; bone mineral density; infusion; bone formation; bone marker; rat

Intermittent administration of parathyroid hormone (PTH) is known to enhance bone mass in rats, while continuous infusion of PTH decreases bone mineral density (BMD) by increasing bone resorption.¹⁾ The contradictory phenomenon of PTH(1–34) action is caused by the dual effects of PTH on bone resorption and bone formation. To analyze the PTH action in bone, previous studies used an alzet infusion pump for programmed administrations or various dosing regimens of PTH(1–34) in rats,^{2,3)} but with this system it was difficult to identify the optimal duration of PTH treatment for anabolic and catabolic effects on bone.

In previous studies, dosing rats with PTH(1–34) at 80 µg/kg elevated the serum level of PTH to 3800–18000 pg/mL, which is higher than the serum level of PTH (C_{max} 160–360 pg/mL) induced by therapeutic doses of PTH (0.3–0.4 µg/kg) in human.^{2–5)} Furthermore, the pharmacokinetic profiles after intermittent subcutaneous (s.c.) administrations of PTH(1–34) in rats were shorter than those in human (T_{max} and $T_{1/2}$ in rats: 15 min and 30 min; T_{max} and $T_{1/2}$ in human: 30 min and 1 h).^{4–6)} These difficulties in establishing a relevant study mean that the optimal duration of PTH(1–34) to selectively exhibit anabolic effects on bone tissue has not been fully understood in either animals or humans.

To investigate the effects of different durations of continuous treatment of compounds *in vivo* in rats, a catheter-implanted system is useful.^{7,8)} In this system, the catheter is directly inserted into the femoral vein, so the compound can be infused continuously at constant plasma levels for desig-

nated periods without restraining the rats. Using this system, we examined the effect of PTH infusion for 1-, 2-, and 4-h on bone markers and BMD in rats. For bone markers related to anabolic action in bone, we focused on AP-1 family genes, such as *c-fos*, *ATF4*, *fra-1*, and bone formation related genes, *Jagged-1*, *Runx2*, *Wnt4*, *SOST*, and *EphrinB2*, and well known bone resorption related genes, receptor activator of nuclear factor-kappaB ligand (RANKL)-osteoprotegerin (OPG) system, encoded from *tnfsf11* (RANKL) and *tnfsf11b* (OPG).^{9–19)}

In the present study, we examined the optimal duration of PTH infusion for bone markers and bone mass, and found that the intermittent elevation of plasma PTH to 300 pg/mL for 1-h in each day is optimal for the increased bone mass in rats by selectively up-regulating bone formation.

MATERIALS AND METHODS

Reagents and Animals PTH(1–34) was purchased from Peptide Institute Co., Ltd. (Japan), and diluted in phosphate-citrate buffer with 0.05% Tween 80. All animal studies were approved by the institutional animal care and use committee of Chugai Pharmaceutical, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. In the *in vivo* study, implanting the catheter to establish catheter-implanted unrestrained rats was conducted as in previous reports.^{7,8)} PTH(1–34) was infused using an infusion pump (TE-361, Terumo, Japan) for 1-, 2-, and 4-h. For the pharmacokinetic

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(PK) study of PTH(1–34) infusion, each rat was placed in a Ballman cage, and plasma was prepared from the femoral vein. For the PK study of s.c. PTH(1–34) in rats, plasma was prepared from jugular vein.

Measurement of BMD BMD of the rat right femur was measured by dual-energy X-ray absorptiometry (DXA) using a DCS-600EX (Aloka, Japan). The length of the femur was divided into 10 parts numbered F1 to F10 from the proximal to distal ends, and BMD of the proximal (F1–F3), middle (F4–F7), distal (F8–F10) and whole (F1–F10) femur was calculated.

Measurement of PTH and Bone Markers by Enzyme-Linked Immunosorbent Assay (ELISA) Plasma level of PTH(1–34) was assessed by ELISA (Immutopics Inc., U.S.A.). Urinary C-terminal telopeptide of type 1 collagen (uCTX) was calculated by RatLaps ELISA (Immunodiagnostic System Ltd., U.K.). Serum levels of procollagen I N-terminal propeptide (PINP) and osteocalcin were measured by Rat/Mouse PINP EIA (Immunodiagnostic System Ltd.) and by rat osteocalcin ELISA (GE Healthcare, U.K.).

Gene Expression Analysis by Real-Time Polymerase Chain Reaction (PCR) Primary spongiosa of femurs were collected from each rat, and rapidly frozen in liquid nitrogen. Frozen samples were powdered, and total RNA was extracted using TRizol (Life Technologies, U.S.A.), and then purified by RNeasy-Mini-column (Qiagen, Germany). A real-time PCR was performed to analyze mRNA expression of respective genes.

Statistical Analysis Data are represented as the mean $\pm$ standard error (S.E.) and statistical significance was determined using SAS (Ver.5.00, SAS Institute Japan). The Dunnett test was performed to detect the significant differences in the PTH(1–34) infusion groups compared with the vehicle, and a Student's *t*-test of two samples was performed to detect the significant differences of s.c. PTH(1–34) compared with the vehicle.

RESULTS

Plasma Levels of PTH(1–34) by Continuous Infusion in Rats Plasma levels of PTH(1–34) achieved by continuous infusion in rats at 1.28, 7.69, or 23.2 $\mu\text{g/kg/h}$ were examined at various time points up to 4-h, when infusion was stopped, and then for an additional hour. Continuous PTH infusions of 1.28, 7.69, and 23.2 $\mu\text{g/kg/h}$ achieved plasma PTH of 327, 2048, and 9837 pg/mL, respectively (Fig. 1A), and after infusion, all plasma PTH levels decreased rapidly (Fig. 1A). In human therapeutic treatment for osteoporosis, the serum PTH level is about 300 pg/mL, so we decided to use an infusion of 1 $\mu\text{g/kg/h}$ PTH (C_{ss} , steady-state concentration *ca.* 300 pg/mL) for the present study. Literature shows that intermittent s.c. administration of PTH(1–34) at 5–25 $\mu\text{g/kg}$ elevated femoral BMD in ovariectomized (OVX) rats, in which plasma levels were 170–530 pg/mL; therefore, the positive control, 8.6 $\mu\text{g/kg}$ (2 nmol/kg) of PTH(1–34) was used for the s.c. administration. In the present study, the plasma levels of PTH(1–34) were elevated by the s.c. administration (8.6 $\mu\text{g/kg}$) of PTH in rats, and reached to 428 pg/mL at 5 min and 124 pg/mL at 30 min, and returned to the baseline levels at 60 min (Fig. 1B).

Gene Expression Regulated by PTH(1–34) Infusion and s.c. Administration Rats were treated with 1 $\mu\text{g/kg/h}$ PTH

Fig. 1. Plasma Levels of PTH(1–34) from Continuous Infusion of PTH(1–34) in Rats

(A) PTH(1–34) was infused intravenously with an inserted catheter for 4-h at three different infusion rates (1.28, 7.69, 23.2 $\mu\text{g/kg/h}$). Blood was collected at 0, 15, 30 min, 1-, 2-, and 4-h during infusion. After infusion was stopped at 4-h, blood was collected at 5, 15, 30, and 60 min. (B) PTH(1–34) was administered subcutaneously (8.6 $\mu\text{g/kg}$), and blood was collected at 0, 5, 15, 30 min, 1-, 2-, and 4-h. The plasma PTH(1–34) concentration was determined by ELISA. Data are the mean $\pm$ S.E.M.; 7.69 and 23.2 $\mu\text{g/kg/h}$ ($n=3$), 1.28 $\mu\text{g/kg/h}$ ($n=2$, error bar not shown), s.c. ($n=4$).

by continuous infusion, and femurs were collected at 1-, 2-, and 4-h to examine the expression of various genes in the primary spongiosa of femurs in rats. The s.c. administration of 8.6 $\mu\text{g/kg}$ of PTH(1–34) was also performed and femurs were collected at 0.5, 1, 2, and 4-h. The continuous PTH infusion elevated the mRNA expression of the transcription factors *c-fos* (40 fold at 1 h) in bone at all time points up to 4-h (Fig. 2A, left panel). PTH infusion also elevated the expression of the bone formation-related genes *EphrinB2* (25 fold at 1 h) and, *Wnt4* (23 fold at 2 h), in bone from 1- to 4-h, and suppressed the mRNA expression of *SOST* (0.3 fold) at 4-h (Fig. 2B left panel). In addition, PTH infusion elevated the mRNA expression of RANKL (5.3 fold at 4 h) and suppressed that of OPG (0.4 fold at 2 h), so that the ratio of RANKL/OPG (12.6 fold at 4 h) was clearly elevated from 1- to 4-h (Fig. 2C, left panel). The s.c. administration of PTH(1–34) transiently elevated the expression of *c-fos* (13.5 fold at 0.5 h), *Wnt4* (4.8 fold at 1 h), *EphrinB2* (18.6 fold at 1 h), and RANKL (3.8 fold at 0.5 h) at 0.5 h and 1-h, and the expression returned to the baseline levels at 2–4-h (Figs. 2A–C, right panel).

Effects of PTH(1–34) Infusion on Femoral BMD in Rats To examine the optimal duration of PTH(1–34) infu-

Fig. 2. Changes in the mRNA Expression in Rat Bone of Genes Related to Bone Formation and Bone Resorption after 4-h Infusion and s.c. Administration of PTH(1–34)

(A) AP-1-related genes (*c-fos*, *Fra-1*, *ATF4*). (B) Bone formation-related genes (*Jagged-1*, *Runx2*, *Wnt4*, *SOST*, *EphrinB2*). (C) Bone resorption-related genes (*RANKL*, *OPG*, *RANKL/OPG*) in bone. In A–C, data from PTH infusion are on the left and data on s.c. administration of PTH on the right. Distal femurs at each time point were collected, and total RNAs were isolated for real-time PCR. Data are given as the mean $\pm$ S.E.M. ($n=3$).

sion for increasing bone mass, rats were treated with $1\mu\text{g/kg/h}$ PTH(1–34) for 1-, 2-, and 4-h infusions daily using the catheter system (Fig. 3), and the femoral BMD was measured at 4 weeks. The daily 1-h infusion of PTH(1–34) significantly elevated the BMD of the whole, proximal, middle, and distal areas of the femur (Table 1), suggesting that PTH elevates bone mass in both trabecular bone and cortical bone. However, both the 2- and 4-h infusions of PTH did not enhance femoral BMD (Table 1). The s.c. administration of PTH significantly elevated femoral BMD in all areas at 4 weeks (Table 1). Therefore, the transient increase in plasma PTH for 1-h may be critical for the anabolic function of PTH in bone. The increase in femoral BMD by 1-h infusion of PTH(1–34) was dramatically shown in the proximal femur in rats. On the other hand, the s.c. administration of PTH markedly elevated femoral BMD in the distal area of the femur.

Effects of PTH(1–34) Infusion on Bone Markers in Rats

To clarify the difference in PTH action on bone mass between the 1-h infusion and the 4-h infusion, bone metabolic markers were measured for 14d, and femoral BMD were measured on day 14. Levels of serum PINP and osteocalcin (bone for-

Fig. 3. Experimental Protocol of Repeated PTH(1–34) Infusion in Rats

mation markers) were markedly elevated by the 1- and 4-h infusions of PTH (Figs. 4A, B), but uCTX (a bone resorption marker) was elevated by the 4-h infusion, and not by the 1-h infusion (Fig. 4C). The 1-h infusion of PTH(1–34) significantly elevated the femoral BMD only at the proximal area on day 14 (Table 2). The bone formation markers were elevated by both 1-h infusion and 4-h infusion, but only 4-h infusion stimulated bone resorption, indicating that 1-h infusion of PTH is optimal for increasing bone mass in rats.

Table 1. Effects of PTH(1–34) Infusion and s.c. Administration of PTH(1–34) on Femoral BMD for 4 Weeks in Rats

BMD	Whole femur (mg/cm ²)	Proximal femur (mg/cm ²)	Middle femur (mg/cm ²)	Distal femur (mg/cm ²)
Vehicle	134.4±2.2	135.3±4.2	121.9±1.4	148.1±3.1
1-h	151.0±2**	160.2±2.9**	131.9±1.4*	164.8±3.8*
2-h	143.2±5.2	142.5±6.6	129.0±4.0	160.7±6.1
4-h	140.6±0.9	144.0±2.8	127.5±2.1	152.7±1.9
s.c. PTH(1–34)	150.8±2.4 ^{sss}	149.0±3.3 ^s	131.8±1.9 ^{ss}	175.7±5.2 ^{ss}

Rats were treated with 1 µg/kg/h PTH(1–34) for 1-, 2-, and 4-h infusions daily using the catheter system, and the femoral BMD was measured at 4 weeks. BMD of the right femur was measured by DXA, and analyzed for the whole area and in three areas: whole, proximal, middle, and distal femur. Data are given as the mean±S.E.M. (*n*=6) **p*<0.05, ***p*<0.01 for PTH(1–34) infusion vs. vehicle, ^s*p*<0.05, ^{ss}*p*<0.01, ^{sss}*p*<0.005 for s.c. administration vs. vehicle.

Table 2. Effects of PTH(1–34) Infusion on Femoral BMD for 14d in Rats

BMD	Whole femur (mg/cm ²)	Proximal femur (mg/cm ²)	Middle femur (mg/cm ²)	Distal femur (mg/cm ²)
Vehicle	137.8±1.1	141.3±1.3	121.4±1.2	153.2±2.5
1-h	142.4±1.4	150.6±1.7*	121.5±1.4	158.4±2.5
4-h	135.7±2.1	139.1±2.7	121.5±1.6	148.4±3.1

Rats were treated with 1 µg/kg/h PTH(1–34) by 1-h or 4-h infusions daily, and the femoral BMD were measured at day 14. BMD of the right femur was measured by DXA, and analyzed for the whole area and in three areas: whole, proximal, middle, and distal femur. Data are given as the mean±S.E.M., Vehicle (*n*=5), PTH(1–34) for 1-h infusion (*n*=7), 4-h infusions (*n*=6), **p*<0.05 for PTH(1–34) infusion vs. vehicle.

Fig. 4. Effects of PTH(1–34) Infusion on Bone Metabolic Markers in Rats

Rats were treated with 1 µg/kg/h PTH(1–34) by 1-h or 4-h infusions daily, and bone metabolic markers were measured for 14d. Levels of bone formation markers, serum PINP (A) and osteocalcin (B), were measured on days 0, 3, 6, 10, and 14. Levels of uCTx (C), a bone resorption marker, were measured on day 14. Data are given as the mean±S.E.M., Vehicle (*n*=5), PTH(1–34) for 1-h infusion (*n*=7), 4-h infusions (*n*=6), **p*<0.05, ***p*<0.01, ****p*<0.005 for PTH(1–34) infusion vs. vehicle.

DISCUSSION

Since PTH stimulates both bone formation and bone resorption, the selective action of PTH is critical for achieving an increase in bone mass. In the present study, we found that the 1-h infusion of PTH enhances bone formation without any effects on bone resorption, while the 4-h infusion of PTH could induce both bone formation and bone resorption. In the s.c. administration of PTH, the expression of the bone formation-related genes *EphrinB2* and *Wnt4* was elevated at 0.5–1-h, and still higher than baseline level at 2-h (Fig. 2B).

However, the expression of RANKL completely returned to baseline level at 2-h (Fig. 2C), suggesting that when PTH is stopped, RANKL expression may quickly return to baseline levels. As demonstrated by the 1-h continuous infusion and the s.c. administration, an intermittent PTH elevation for 1-h may enhance BMD in rats. Indeed, the 4-h infusion stimulated the bone resorption marker uCTx, but the 1-h infusion did not (Fig. 4C).

Dobnig and Turner²⁾ used an alzet osmotic pump for programmed administrations of 80 µg/kg of PTH(1–34) in rats for 6d, and reported that the repeated 1-h infusions enhanced

the bone area and bone formation parameters, but the effects on BMD was not demonstrated probably because the treatment period was insufficient to demonstrate the effects on BMD in rats. Frolick *et al.*³⁾ examined the optimal duration of PTH(1–34) with 6 s.c. injections of 80 µg/kg of PTH(1–34) within 1-h or 6-h in rats, and reported that 6 injections within 1-h enhanced tibia BMD, while 6 injections over 6-h did not enhance BMD. The plasma level of PTH(1–34) in their study was more than 3800 pg/mL, much higher than that of the human therapeutic dose. In the present study, we clearly showed that the constant level of PTH at human therapeutic dose (C_{ss} ca. 300 pg/mL) for 1-h each day is the optimal duration of PTH for increasing bone mass with elevated bone formation without bone resorption in rats. The 1-h infusion of PTH showed the anabolic effects on all areas of femur at 4 weeks, but the proximal area was most sensitive and the increase in BMD was detected only in the proximal area at 2 weeks (Tables 1, 2). On the other hands, the s.c. administration of PTH dramatically elevated femoral BMD at distal area (Table 1). The difference of pharmacokinetic profile between the infusion and the s.c. administration may influence the phenomena. It is possible that the continuous elevation of PTH for 1 h is effective for cortical bone formation of femoral neck in the proximal area, and that the transient elevation of PTH for 15 min is effective for bone formation in distal trabecular bone. It is known that the femoral neck in proximal area is sensitive to mechanical stress, and PTH is involved in the mechanical signaling in bone tissues. Further studies are needed to define the difference of cellular response in osteoblasts between the infusion and the s.c. treatment of PTH.

Osteoblasts are thought to be the principal target cells for PTH. However, recent studies have shown that osteocytes are also cellular targets of PTH in bone tissues. On PTH treatment, osteocytes, which are the most abundant cells in adult bone, express RANKL *via* the PTH/parathyroid hormone-related protein (PTHrP) receptor to induce osteoclastic bone resorption.^{20,21)} Osteocytes produce sclerostin, a negative regulator for bone formation, and PTH signaling suppresses the sclerostin production by inhibiting *SOST* gene expression in osteocytes.^{11,12)} Indeed, the mRNA expression of *SOST* was suppressed by PTH infusion at 4-h (Fig. 2B, left). Therefore, osteocytes may be the target cells of PTH, and PTH may regulate sclerostin-regulated bone formation and RANKL-dependent bone resorption in bone. Further studies are needed to define the roles of osteocytes in the anabolic and catabolic function of PTH at optimal duration in animals and human.

In summary, we have shown here that the intermittent elevation of plasma PTH to 300 pg/mL by 1-h infusions each day enhances bone mass by increasing bone formation without increasing bone resorption.

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Conflict of Interest MS, HN, EJ, CN, KA, AH, MK, MN, TT are employees of Chugai Pharmaceutical Co., Ltd. MI and CM have no conflict of interest.

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