Starvation-induced increase in the parathyroid hormone/PTH-related protein receptor mRNA of bone and kidney in sham-operated and thyroparathyroidectomized rats

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Abstract
Parathyroid hormone (PTH) acts on bone and kidneys by binding to PTH/PTH-related protein (PTHRP) receptors and regulating calcium (Ca) and phosphorus (P) homeostasis. PTH/PTHRP receptor mRNA was expressed at high levels in PTH target tissues such as the kidneys and bone including the calvaria, femur, and tibia. Because short-term starvation influences Ca and P ion homeostasis, we measured changes in PTH/PTHRP receptor mRNA expression in the bone and kidneys. Food deprivation for 3 days decreased the serum Ca and P concentrations, and reinstitution of feeding for 2 days normalized the serum Ca level and significantly increased the serum P level. Concomitantly, rat immunoreactive PTH (riPTH) was increased during starvation and returned to the control level after 2 days of subsequent feeding. Serum 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3) concentrations did not significantly change during starvation and subsequent feeding. Starvation up-regulated PTH/PTHRP receptor mRNA expression in both bone and kidney. The effects of food deprivation on the receptor transcript abundance were greater in bone (threefold increase compared with control) than in the kidney (1.8-fold increase), whereas the mRNA level increase by food deprivation was more rapid in the kidneys than in bone. The PTH-induced adenyl cyclase activity of renal membranes increased in starvation. Feeding after starvation normalized the mRNA levels in both tissues. Serum PTH depression, initiated by thyroparathyroidectomy, did not affect PTH/PTHRP receptor mRNA levels in bone and kidney in rats that were fed or starved for 3 days. The abundance of receptor mRNA in bone and kidney was significantly lower in fed rats given either corticosterone or vehicle than in starved rats. These data indicate that starvation induces PTH/PTHRP receptor mRNA expression in bone and kidney, independently of serum PTH and corticosterone concentrations. The factors leading to up-regulated receptor mRNA induced by starvation remain unknown.

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Introduction
Parathyroid hormone (PTH) is an 84 amino acid polypeptide that acts mainly in the bone and kidneys by regulating calcium (Ca) and phosphorus (P) metabolism (1). PTH binding to the PTH/PTH-related protein (PTHRP) receptor increases the levels of several intracellular second messengers, including cAMP, ionized calcium and diacylglycerol (2–4). PTHrP is associated with the humoral hypercalcaemia of malignancy (5, 6) and is expressed in the various tissues (7), having effects similar to those of PTH through endocrine and paracrine mechanisms. Moreover, PTHrP binds to the same receptor as PTH and activates the effectors (1, 8). The number of PTH/PTHRP receptors and the increase of cAMP by PTH or PTHrP in the target cell lines established from bone and kidney varies and is controlled rigorously by bioactive substances such as PTH/PTHRP (9–11), 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3) (12) and retinoic acid (13).

Cloning of the PTH/PTHRP receptor cDNA allowed the receptor mRNA to be quantified (14). Regulation of PTH/PTHRP receptor gene expression has been studied in vivo. The PTH/PTHRP receptor mRNA in the kidneys is reduced in rats with secondary hyperparathyroidism caused by chronic renal failure, whereas thyroparathyroidectomy (TPTX) does not affect transcription in the rat kidney (15). Kilav et al. (16) have reported that hypoparathyroidism induced by surgical parathyroidectomy up-regulated the PTH/PTHRP receptor transcript in the kidney, whereas induced hyperparathyroidism in rats fed low calcium diets had no effect on renal receptor mRNA levels. Turner et al. (17) suggested that vitamin D status, not secondary hyperparathyroidism, is implicated in the regulation of the receptor mRNA expression in the
kidney and long bones, including the epiphysis and metaphysis. Thus findings concerning the regulatory effects of receptor mRNA expression by PTH and 1,25(OH)2D3 have not been consistent.

Starvation not only caused significant changes in the serum levels of thyroxine, corticosterone and gonadotropins and their production in endocrine tissues (18, 19), but also affects mineral ion homeostasis by the regulation of production and degradation of the calcium-regulating hormones such as PTH, insulin-like growth factor (IGF)-I and 1,25(OH)2D3, which is mainly inactivated by renal 24-hydroxylase (20, 21). In addition, short-term fasting markedly decreases both formation and resorption of bone and IGF-I administration significantly increases bone formation, but not resorption, in women (22). Thus nutritional states of humans and animals alter bone turnover and serum levels of the calcitropic hormones including PTH. As no other investigators have studied the effects of starvation on expression of the PTH/PTHrP receptor mRNA in PTH target tissues, we investigated the effect of food deprivation on the gene expression of bone and kidney. We also examined whether hormonal factors such as PTH, 1,25(OH)2D3 and corticosterone influence the gene expression of the PTH/PTHrP receptor in fed control rats.

Materials and methods

Materials

Megaprime DNA labeling kits, nylon membranes (Hybond-N+), Hyper-film and the rat 1,25(OH)2D3/corticosterone assay system were purchased from Amersham International (Buckinghamshire, UK). GeneAmp RNA PCR kits (Perkin–Elmer PCR reagents) were obtained from Roche Molecular System Inc. (Branchburg, NJ, USA) and pT7BlueT-vector kits were purchased from Novagen from Roche Molecular System Inc. (Branchburg, NJ, USA). PCR kits (Perkin–Elmer PCR reagents) were obtained from Oriental Yeast (Tokyo, Japan). All other reagents and chemicals were of analytical grade.

Preparation of PTH/PTHrP receptor cDNA probe

To prepare the probe for Northern blot analysis, we isolated total RNA from normal rat kidneys. Reverse transcription (RT)–polymerase chain reactions (PCR) were performed using a GeneAmp RNA PCR kit. Oligonucleotide-primed single-stranded cDNA was generated by reverse transcriptase. Briefly, 1 μg total RNA and 15 pmol reverse primer (5'-3'; nucleotide (nt) 1327–1346) (14) was reverse-transcribed with 50 U Moloney murine leukemia virus reverse transcriptase. The RT reaction was performed in 20 μl (total volume) RT buffer (50 mmol/l KCl, 5 mmol/l MgCl2 and 10 mmol/l Tris–HCl, pH 8.3) containing 1 mmol/l each deoxy-NTP and 20 U RNasin. The mixture was incubated for 30 min at 42°C, heated to 99°C for 5 min, and quickly chilled to 4°C. The single-stranded cDNA mixture was PCR amplified. The reaction was performed using 8 μl mixture in PCR buffer (50 mmol/l KCl, 2 mmol/l MgCl2 and 10 mmol/l Tris–HCl, pH 8.3), 200 mmol/l deoxy-NTPs, 15 pmol forward primer (5'-3'; nt 654–673) (14) and 2.5 U Taq DNA polymerase in 100 μl total volume. The amplification profile was 1 min of denaturation at 93°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C. Thirty cycles of PCR proceeded in a GeneAmp PCR system 2000 (24). The PCR products were separated in a low melting temperature agarose gel and cloned into the

Animals

Five-week-old male (150–170 g) Sprague–Dawley rats (SLC, Hamamatsu, Japan), were maintained on rodent chow (Ca 1.42%, P 1.16% and vitamin D3 1.6 U/g diet) for 1 week. Three to four rats per cage were housed in a temperature-regulated environment (22 ± 2°C), with a 14 h light : 10 h darkness cycle and free access to water. In the starvation experiments, control rats had free access to food, whereas the starved rats were denied food for 1, 2 or 3 days, then fed for 2 days after the starvation period. In the TPTX experiments, rats were deprived of food for 1 day before surgery. The rats were thyroparathyroidectomized (TPTX) or sham-operated under anesthesia with sodium pentobarbital (50 mg/kg body weight), maintained for 2 days and killed at indicated times by exsanguination from the aorta under ether anesthesia. Some fed rats received daily s.c. injections of either corticosterone (25 mg/kg body weight) or vehicle (isotonic saline) for 4 days.

The total serum Ca and P levels were measured colorimetrically. Serum rat immunoreactive PTH (riPTH) was determined using the IRMA kit. Serum 1,25(OH)2D3 was measured by a radioreceptor assay with a calf thymus receptor and C18/OH cartridge purification (23). Corticosterone levels in serum were determined by the rat 1,25(OH)2D3/corticosterone assay system. For total RNA isolation, all tissues, including bone, kidney, liver, lung, heart, skin and duodenum, were quickly frozen in liquid N2. The study was approved by the Institutional Animal Care and Use Committee of Ohu University and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals.
pT7Blue-T vector. The sequences of the products were determined by dideoxynucleotide chain termination (25). A clone with 100% homology with the confirmed sequence was used to prepare the probe (14).

**Determination of PTH/PTHrP receptor mRNA**

Total RNA from four or five animals per group was extracted from various tissues including bone and kidney, using guanidine thiocyanate. To isolate total RNA from the calvaria, tibia and femur, frozen bone tissues were powdered in a stainless steel chamber chilled with dry ice. The powders were homogenized in 4 mol/l guanidine thiocyanate, 25 mmol/l sodium citrate, pH 7.0, 0.5% laurylsarcosine, and 0.1 mol/l 2-mercaptoethanol. Total homogenate RNA was extracted with phenol saturated with 0.1 mol/l citrate buffer (pH 4.3). The aqueous phase was layered onto CsCl (0.96 g/ml in 0.1 mol/l EDTA, pH 7.8) cushions and centrifuged (400 000 g at 20°C) for 18 h (26). The RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water and precipitated with 0.1 vol 3 mol/l sodium acetate, pH 5.2, and 2.5 vol ice-cold 99.5% (v/v) ethanol. The RNA precipitates were washed with 75% ethanol (v/v) and resuspended in DEPC water. For Northern blots, total RNA was fractionated in a 1.2% agarose gel containing formaldehyde and transferred to a Hybond-N+ membrane. Blots were hybridized with the cDNA probe, then labeled with \[^{32P}\]dCTP using the Megaprime DNA labeling system (27). Northern blots of total RNA were used to assess the integrity of the isolated RNA assessed and to quantify PTH/PTHrP receptor mRNA. Hybridization was performed in 50% formamide, 5×Denhardt’s solution, 0.5% lauryl sulfate and 5×SSPE (80 mmol/l sodium chloride, 10 mmol/l sodium phosphate, and 1 mmol/l EDTA) at 42°C for 2 days then washed in 0.1×SSPE at 65°C for 15 min (28). After hybridization, the membranes were exposed to Hyper-film at −80°C with intensifying screens. Cyclophilin mRNA, which is constitutively gene-expressed regardless of physiological status, was used to verify the amount of mRNA on the Northern blots. The intensities of the 2.5 kb mRNA expressions were quantified by densitometric tracings of the autoradiographs. The PTH/PTHrP receptor mRNA concentration was normalized to the amount of cyclophilin mRNA expression.

**Adenylyl cyclase assay in renal membranes**

The kidney cortex from four rats per group was dissected and renal membranes were prepared according to the procedure described by Turner et al. (17). Adenylyl cyclase activity was assessed by measuring the amounts of cAMP generated after stimulation of the membrane preparations. Renal membrane preparations containing 50 μg protein were added to an incubation mixture con-taining 50 mmol/l Tris (pH 7.4), 1.2 mmol/l ATP, 1.7 mmol/l MgCl₂, 0.1% BSA, 1.0 mmol/l IBMX, 7.0 mmol/l phosphocreatine, 1.0 U creatine phosphokinase/ tube and distilled water in a final volume of 100 μl. The mixture was incubated for 15 min at 37°C with vehicle, 10⁻⁵ mol/l hPTH(1–84), 1.0 mmol/l forskolin or 10 mmol/l NaF. The reaction was stopped by the addition of 100 μl of 1.2 mol/l perchloric acid and the amount of cAMP generated was measured using a cAMP RIA kit.

**Statistical analysis**

The data are described as means ± S.E.M. Statistical analysis was performed using the one-way analysis of variance
followed by Fisher’s protected least significant difference (Statview 4.02, Abacus Concepts Inc., Berkeley, CA, USA). P < 0.05 was considered statistically significant.

**Results**

**Effects of starvation and feeding on PTH/PTHrP receptor mRNA expression and serum biochemistry in sham-operated rats**

Food deprivation for 3 days decreased the serum Ca and P levels whereas subsequent feeding for 2 days normalized the serum Ca. The P concentration increased upon feeding, to levels higher than those recorded immediately after 3 days of starvation. In contrast, concentrations of riPTH were increased during starvation and returned to normal after 2 days of feeding. Serum 1,25(OH)2D3 concentrations did not alter during starvation and feeding (Fig. 1). The effects of starvation and subsequent feeding on the amount of PTH/PTHrP receptor mRNA level in bone (calvaria) were analyzed by Northern blotting (Fig. 2). Two PTH/PTHrP receptor transcripts were detected at 2.4–2.5 kb with high abundance and at 1.8–2.0 kb with low abundance. The receptor transcripts in bone were markedly increased in starved rats and normalized after 2 days of feeding. Bone mRNA levels increased 1.4-, 2.5- and 3.0-fold after 1, 2 and 3 days of starvation respectively. Changes in the PTH/PTHrP receptor mRNA expression in the kidney during starvation and feeding are shown in
The renal mRNA concentrations were significantly increased 1 day after food deprivation and these levels were maintained until 3 days of starvation. Feeding tended to decrease the receptor mRNA level in the kidney, whereas the amounts of the receptor transcripts were not significantly different between animals immediately after 3 days of starvation and those fed for 2 days thereafter. The PTH/PTHrP receptor mRNA abundance during starvation was increased more in bone than in kidney (Fig. 4). Basal and maximal adenylyl cyclase activities in crude renal membrane preparations were similar in fed and fasted rats (Fig. 5A). PTH-stimulated adenylyl cyclase activities were increased during food deprivation. The PTH-induced activity was significantly increased in kidneys of rats after 1–3 days of starvation (Fig. 5B).

**Effect of serum PTH deprivation on PTH/PTHrP receptor transcripts and serum biochemistry in starved rats**

The effects of TPTX on serum biochemistry in rats 3 days after starvation are shown in Table 1. Serum Ca levels were decreased in TPTX animals compared with sham-operated rats under food deprivation and markedly declined in sham-operated, fasting rats compared with the fed control rats. Serum P concentrations significantly decreased during food deprivation. Serum rIPTH levels were increased during 3 days of starvation in sham-operated rats. TPTX rats had rIPTH concentrations that were significantly lower than those of the sham-operated rats. Serum 1,25(OH)2D3 levels were unchanged in the TPTX and sham-operated rats after 3 days of starvation (Table 1). Figure 6 depicts the PTH/PTHrP receptor mRNA expression in the bone and kidney of sham-operated and TPTX animals. TPTX did not affect the receptor mRNA abundance in the bone and kidney of rats fed or starved for 3 days.

**Effect of corticosterone on PTH/PTHrP receptor mRNA expression**

The results of this experiment are summarized in Table 2. Serum corticosterone concentrations increased markedly...
with starvation (P<0.001), and the daily administration of corticosterone (25 mg/kg body weight) in fed rats increased the serum corticosterone levels to those of starved rats. However, the abundance of PTH/PTHrP receptor mRNA in bone and kidney did not change significantly after 4 days of injections with corticosterone or saline in fed rats, whereas the abundance in these tissues was increased with starvation.

Discussion

Gene expression of the PTH/PTHrP receptor mRNAs is also developmentally and hormonally regulated by PTH in classical target tissues such as bones and kidneys (1, 29). The most prominent receptor transcript of 2.4–2.5 kb was found in the kidneys and bones including the calvaria, tibia and femur, as revealed by Northern blots. Smaller mRNA transcripts (1.8–2.0 kb) were also present and these findings are consistent with those previously reported (30). Our findings indicate that starvation causes an increase in the receptor transcripts and that subsequent feeding rapidly decreases receptor gene expression in target tissues. Furthermore, renal PTH/PTHrP receptor clearly increased in food deprivation when PTH-stimulated adenyl cyclase activities were assessed in crude renal membranes of fed and fasted rats.

During short-term starvation, intact rats experience hypocalcemia associated with mild hyperparathyroidism. Generally, the responsiveness of target tissue during continued exposure to hormones can be modulated by homologous down-regulation of its receptor expression and alterations in receptor-effector coupling (31). Excess serum PTH or exposure to PTH reduces the PTH/PTHrP receptor as measured by the radioreceptor assay, and down-regulates PTH-stimulated adenyl cyclase activities in rats with normal renal function (32) and chronic renal failure (15). Moreover, in vitro studies indicate that prolonged exposure to PTH desensitizes cells to further stimulation by the hormone (10, 11) and decreases the abundance of PTH/PTHrP receptor mRNA in bone cells (4, 33, 34). The PTH/PTHrP receptor transcripts in PTH target tissues were not down-regulated but up-regulated in starved rats, with increased levels of plasma riPTH. Starvation increased the abundance of the receptor mRNA in sham-operated and TPTX rats, suggesting that parathyroid function is not relevant to the gene expression of bone and kidney PTH/PTHrP receptors in in vivo studies. These findings are consistent with those of others (11, 15, 33, 35), suggesting that the in vivo desensitization mechanism to PTH in bone and kidney is due to diminished numbers of PTH/PTHrP receptors and to post-receptor events including receptor down-regulation in the target tissues.

Table 1 Effects of TPTX on serum biochemistry in rats fed or starved for 3 days. Five-week-old rats underwent sham-operation (Sham) or TPTX and were maintained for 2 days. One day before surgery, the animals were deprived of food. Blood was withdrawn from rats 2 days after surgery and biochemical serum determinations were performed. Sham-operated (fed) rats served as control. Results are expressed as means ± S.E.M. of four rats.

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<th>Fed</th>
<th>Fasted</th>
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<tr>
<td></td>
<td>Sham (control)</td>
<td>Sham</td>
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<tr>
<td>Total Ca (mg/dl)</td>
<td>9.6 ± 0.1 ***</td>
<td>7.1 ± 0.1</td>
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<tr>
<td>P (mg/dl)</td>
<td>9.1 ± 0.1 ***</td>
<td>6.0 ± 0.2</td>
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<tr>
<td>riPTH (pg/ml)</td>
<td>16.4 ± 1.0 **</td>
<td>31.5 ± 0.5</td>
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<td>1,25(OH)2D3 (pg/ml)</td>
<td>108 ± 13</td>
<td>85 ± 12</td>
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** P < 0.01, *** P < 0.001 compared with sham-operated (fasted) rats.

Table 2 Relationship between serum corticosterone concentrations and PTH/PTHrP receptor mRNA abundance of bone and kidney in fed and 3-day fasted rats. Either corticosterone (25 mg/kg body weight) or vehicle (isotonic saline) was injected s.c. into fed rats for four successive days. Last injection was 24 h before sacrifice. The fasted group was starved for 3 days. Values represent means ± S.E.M. of four rats.

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<th>Fed</th>
<th>Fasted</th>
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<td></td>
<td>Vehicle (control)</td>
<td>Corticosterone</td>
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<tr>
<td>Serum corticosterone (nmol/l)</td>
<td>440 ± 40</td>
<td>1580 ± 40 ***</td>
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<tr>
<td>PTH/PTHrP receptor mRNAs (fold of control)</td>
<td>Bone 1.00 ± 0.27</td>
<td>1.10 ± 0.21</td>
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<td></td>
<td>Kidney 1.00 ± 0.05</td>
<td>1.14 ± 0.09</td>
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** P < 0.01, *** P < 0.001 compared with fed control.
internalization, sequestration, degradation, phosphorylation or uncoupling from transducing G protein but not because of the transcriptional control of the receptor gene. In contrast, in vitro studies have shown reduced gene expression of PTH/PTHrP receptor mRNA expression, although a recent in vivo study (17) suggested that 1,25(OH)2D3 inhibits expression of the receptor gene. Our study indicates that amounts of PTH/PTHrP receptor mRNA in both bone and kidney of rats starved for 3 days increased, but that their serum 1,25(OH)2D3 levels were not affected. In addition, the gene expression of the receptor in starved TPTX rats was enhanced, although serum 1,25(OH)2D3 levels were not significantly altered. The increased expression of PTH/PTHrP receptor mRNA in young growing rats fed with a vitamin D-deficient diet (17) may be due to increased responsiveness of the target tissues to PTH when deficient in mineral ions and vitamers. In the present study, TPTX did not decrease the level of plasma 1,25(OH)2D3 in starved rats. The plasma 1,25(OH)2D3 levels may be increased in TPTX animals with severe hypocalcemia, through calcium-dependent and PTH-independent mechanisms (36).

The mechanism of regulating the gene expression of the PTH/PTHrP receptor in bone and kidney during fasting is not understood, but may involve a number of hormones that are dramatically changed by food deprivation. Food deprivation is a stressful situation, leading to an enhanced adrenal corticosteroid release (20). Chronic administration in vivo of glucocorticoids causes bone resorption and osteopenia (37). The present study was undertaken to define the involvement of glucocorticoids in the PTH/PTHrP receptor gene expression in PTH target tissues of fed rats. Our results showed that the increased abundance of PTH/PTHrP receptor mRNA in PTH target tissues of starved rats is not due to increased concentrations of serum corticosterone. Nutritional states have a role in IGF-I gene expression in the liver (18). Serum IGF-I levels decrease during food deprivation, and skeletal tissues are a target organ of IGF-I. As PTH/PTHrP receptor mRNA is expressed abundantly and is dramatically altered during endochondral bone formation in young rats (29), IGF-I may influence the gene expression of the receptor in bone.

In conclusion, starvation induces up-regulation of PTH/PTHrP receptor mRNA expression inrat bone and kidney independently of changes in serum PTH, 1,25(OH)2D3 and glucocorticoids.

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