Gender differences in the response of CD-1 mouse bone to parathyroid hormone: potential role of IGF-I

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Abstract

Parathyroid hormone (PTH) exerts both catabolic and anabolic actions on bone. Studies on the skeletal effects of PTH have seldom considered the effects of gender. Our study was designed to determine whether the response of mouse bone to PTH differed according to sex. As a first step, we analyzed gender differences with respect to bone mass and structural properties of 4 month old PTH treated (80 µg/kg per day for 2 weeks) male and female CD-1 mice. PTH significantly increased fat free weight/body weight, periosteal bone formation rate, mineral apposition rate, and endosteal single labeling surface, while significantly decreasing medullary area in male mice compared with vehicle treated controls, but induced no significant changes in female mice. We then analyzed the gender differences in bone marrow stromal cells (BMSC) isolated from 4 month old male and female CD-1 mice following treatment with PTH (80 µg/kg per day for 2 weeks). PTH significantly increased the osteogenic colony number and the alkaline phosphatase (ALP) activity (ALP/cell) by day 14 in cultures of BMSCs from male and female mice. PTH also increased the mRNA level of receptor activator of nuclear factor κB ligand in the bone tissue (marrow removed) of both females and males. However, PTH increased the mRNA levels of IGF-I and IGF-IR only in the bones of male mice. Our results indicate that on balance a 2-weeks course of PTH is anabolic on cortical bone in this mouse strain. These effects are more evident in the male mouse. These differences between male and female mice may reflect the greater response to PTH of IGF-I and IGF-IR gene expression in males enhancing the anabolic effect on cortical bone.


Introduction

Parathyroid hormone (PTH) is a major regulator of calcium and phosphate homeostasis. It has complex effects on bone. When given intermittently, PTH increases bone mass and strength (Alexander et al. 2001, Dempster et al. 2001) by increasing the number (Kostenuik et al. 1999) or function of osteoblasts (Rubin et al. 2002) and increasing osteoblast life span by inhibiting osteoblast apoptosis, an anabolic effect (Jilka et al. 1999). Continuous infusion of PTH decreases bone mass by stimulating a net increase in bone resorption, a catabolic effect (Lee & Lorenzo 1999, Ma et al. 2001). Recent studies indicate that the anabolic action of PTH on bone is site specific (Halloran et al. 1997, Iida-Klein et al. 2002). Some factors, such as insulin-like growth factor I (IGF-I), are required for the anabolic actions of PTH on bone (Bikle et al. 2002). The responses of bone to PTH are also dose- (Frolik et al. 1999) and time-dependent (Ishizuya et al. 1997, Schiller et al. 1999), and vary among species and strains (Hock 2000).

In some of our previous experiments, we observed a gender difference regarding the response of bone to PTH in bone mass in mice, but no detailed reports have been published on this observation to our knowledge. Because of the increasing use of genetically altered mice and the extrapolation of such studies to human physiology and pathophysiology, we undertook the current study.

Gender has been found to exert effects on skeletal development (Naganathan et al. 2002), peak bone mass (Orwell et al. 2001), and peak appendicular bone strength (Kim et al. 2003) in human, mice and rats. These effects have been attributed to sex steroid action (Turner 1999, Zhang et al. 1999) or genetic (Eisman 1999, Orwell et al. 2001) mechanisms. Recent clinical studies also indicate that gender affects the bone response to growth hormone (Johansson et al. 1999, Span et al. 2000), suggesting that gender may affect the bone response to other hormones.

To determine whether the response of mouse bone to PTH differed according to gender, we determined the bone response to PTH at the tissue level, cellular level and
molecular level by assessing fat free weight (FFW), structure by micro computed tomography (µCT), bone histomorphometry, colony forming units in bone marrow stromal cell (BMSC) cultures, and mRNA levels of resorption and formation markers using quantitative real-time PCR.

Materials and Methods

Animals

16 week old male and female CD-1 mice were treated with either PTH (PTH 1–34, rat, Bachem, CA, USA) 80 µg/kg body weight (BW) or vehicle everyday by s.c. injection for 2 weeks. To determine bone formation during the period of PTH administration, the mice were injected s.c. with calcein on day 0 of PTH administration followed in 12 days by demeclocycline (each 15 mg/kg) to label the mineralization fronts of the bone. Two days later, the mice were weighed and then sacrificed. The right tibias were obtained for fat-free weight and micro computed tomography (µCT); the left tibias were obtained for mineral appositional rate (MAR) and bone micro computed tomography (µCT); the left tibias were measured in each sample, covering a total of 1·15 mm of the metaphysis. To analyze only cancellous fractions of the bone were removed, and the bone marrow was flushed with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, USA), supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, USA), 100 U/ml penicillin/streptomycin (Mediatech), 0·25 µg/ml fungizone (Life Technologies). A single-cell suspension was obtained by repeated passage through an 18 gauge needle. A pool of BMSCs was made from the tibia and femur of each mouse. The cells were plated at 2 × 10^6 cells/well in 6-well plates. Non-adherent cells were removed by aspiration, and then the

Bone histomorphometry

MAR and BFR at the tibiofibular junction (TFJ) were measured as follows. Diaphyseal segments of the tibia were dehydrated, defatted in acetone followed by ether, and then embedded in bioplastic (Tap Plastics, Dublin, CA, USA). After polymerizing overnight, the blocks were sectioned at a thickness of 60 µm using a Leica SP 1600 circular bone saw (Leica Inc, Deerfield, IL, USA). The section containing the TFJ was digitized with a Hamamatsu video camera (Carl Zeiss Inc, Thornwood, NY, USA) coupled to a Leica DMR microscope, and periosteal MAR, BFR, single labeling surface of endosteal surface, cortical bone area and medullary area were determined using the National Institutes of Health (NIH) Image program.

BMSC culture

The left tibial and femoral bone marrow stromal cells were harvested using techniques previously described (Kostenuik et al. 1999). Briefly, the tibias and femurs were cleaned of adherent tissue, extracted in ethanol and diethyl ether using a soxhlet apparatus (Fisher Scientific, Pittsburgh, PA, USA), then dried at 100 °C overnight and weighed.
primary medium was replenished on day 5. On day 7, the cells were provided with secondary medium (the primary medium with 3 m\(\mu\)M \(\beta\)-glycerophosphate and 50 \(\mu\)g/ml ascorbic acid; Sigma). Subsequent medium changes were performed every 2 days for up to 28 days.

**Alkaline phosphatase (ALP) assay**

On day 14, cells were fixed with 10% phosphate-buffered formalin for 30 min for determining ALP activity and colony number. To determine ALP activity, the fixed cells were rinsed with distilled water, and then incubated for 30 min at 37\(^\circ\)C with a solution containing equal parts \(p\)-nitrophenol phosphate (Sigma) and alkaline buffer solution (Sigma). After the incubation, the reaction was stopped with equivolume 0.1 M NaOH. The absorbance (arbitrary units) was measured with a Uvikon spectrophotometer 930 (Research Instruments, San Diego, CA, USA) at 410 nm. To determine the number of colony forming units, the same cultures were rinsed with distilled water, and then stained with a 0.2% crystal violet (Sigma) in 2% ethanol for 1 h at room temperature. The staining solution was aspirated, and unbound stain was removed by rinsing the cultures 4 times with distilled water. The number of colonies over 1 mm in diameter was recorded. The crystal violet solution was eluted with 0.2% Triton X-100 (Sigma), and the absorbance was measured with a spectrophotometer at 590 nm for the determination of cell number as previously described (Kostenuik et al. 1999).

**mRNA levels in bone**

The right femurs and tibias were cleaned of adherent tissue, the marrow flushed out with PBS using a 26 gauge needle, and the bones frozen in liquid nitrogen and stored at \(-80^\circ\)C until processed. The bones from each animal were pooled, and bone pools were pulverized in a steel mortar and pestle cooled in liquid nitrogen. RNAs were isolated by a RNA Stat-60 kit (Tel-Test, Friendswood, TX, USA). For each pool 2000 ng of total RNA was reverse-transcribed in 100 \(\mu\)l of a reaction mixture that contained 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 7·5 mM MgCl\(_2\), 1 mM of each deoxynucleoside triphosphate, 5 \(\mu\)M of random primers (GIBCO BRL), 0·4 U/\(\mu\)l of RNAse inhibitor (Roche), and 2·5 U/\(\mu\)l of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) at 25\(^\circ\)C for 10 min, 48\(^\circ\)C for 40 min, 95\(^\circ\)C 5 min, and then stored at 4\(^\circ\)C. The sequences of the PCR primers and probes are listed in table 1. These primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Primers were synthesized by the Biomolecular Resource Center (University of California, San Francisco, CA, USA). Probes were synthesized by Integrated DNA Technologies, Inc (Coralville, IA, USA). The internal probe was labeled at the 5’ end (labeled as ‘a’ in table 1) with the reporter dye 6-carboxyfluorescein (FAM) and at the 3’ end (labeled as ‘b’ in table 1) with quencher dye 6-carboxy-tetramethylrhodamine. During PCR, the 5’–3’ nuclease activity of Taq DNA polymerase releases the reporter fluorescence (Heid et al. 1996). The fluorescence intensity is proportional to the accumulation of PCR product and was detected with an ABI Prism 7900HT sequence detection system (Applied Biosystems, Branchburg, NJ, USA). The sequence of the probe or primers for each target gene spans an exon/exon boundary to minimize the signal generated by genomic DNA that may contaminate the RNA sample, thereby eliminating the need to DNase treat the sample.

**Table 1** Primers and probes for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and probe</th>
<th>Sequence (5’–3’)</th>
<th>PCR products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward primer</td>
<td>TGCACCAACCACTGCTTAG</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GGTGAGGTTGATTTTGTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>aCAAGAAGCTGGGTGATGCCCTC</td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td>Forward primer</td>
<td>TTCCGTCTGTGTTGGACCG</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GCCTCGGAAACCACTCAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>aCTTATTTCAACAGGCTATGG</td>
<td></td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Forward primer</td>
<td>CAGCGAGAATGATGTC</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GAGCGAGAATCAGGAAATCGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>aTTCCTTCGCAAGCCCTGC</td>
<td></td>
</tr>
<tr>
<td>RANKL</td>
<td>Forward primer</td>
<td>GCCACAGCGCTTCCTTCAG</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GATGACTTATGGGAAACCGAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>aCAAGATATGAGGCGCTCATGGTGGG</td>
<td></td>
</tr>
<tr>
<td>OPG</td>
<td>Forward primer</td>
<td>ATCCGCTGGAAGCCGGAGT</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CCATCTGCGACATTTGTGCAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>aTGCAGCTGGAACCCAGACC</td>
<td></td>
</tr>
</tbody>
</table>

a, 5’ end labeled with the reporter dye FAM; b, 3’ end labeled with the quencher dye TAMRA.
PCR was carried out in triplicate with 20-µl reaction volumes of 1X TaqMan Universal PCR Master Mix, (Applied Biosystems), 500 nM of each primer, 200 nM of probe and 1µl of cDNA template. The PCR reaction was performed in an ABI Prism 7900HT sequence detection system using the following cycles: 95 °C for 12 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 minute. Analysis was carried out using the sequence detection software supplied with the ABI Prism 7900HT sequence detection system. The number of PCR cycles, threshold cycles (Ct), required for the FAM intensities to exceed a threshold above background was calculated for the test reactions (Ginzinger et al. 2000). The Ct values were determined for three test reactions in each sample and averaged. The ΔCt values were obtained by subtracting the GAPDH (as endogenous control) Ct values from the target gene Ct values of the same samples. The relative quantification of the target genes was given by 2-ΔCt.

**Statistical analysis**

Data are presented as mean ± s.d All data were analyzed using two-factor (gender and treatment) ANOVA followed by a post-hoc Fisher’s protected least significant difference test with a SuperANOVA program. Statistical significance was taken as P<0.05.

**Results**

**Bone mass**

The fat-free weights of tibias (normalized by body weight, FFW/BW) are shown in Fig. 1. By one-way ANOVA analysis, in control mice, FFW/BW of females (1.23 ± 0.12 mg/g) was significantly greater than FFW/BW of males (0.89 ± 0.15 mg/g; P<0.05). PTH significantly increased FFW/BW by 30% in male mice (1.18 ± 0.19 mg/g; P<0.05), but induced no significant changes in female mice (1.16 ± 0.21 mg/g). Two-way ANOVA revealed a significant PTH treatment-gender interaction (F=7.802; P=0.0099).

**Bone structure-proximal tibia**

µCT was used to evaluate the gender differences in bone structure. The results are shown in Table. 2. In control male mice, cortical bone thickness and mean trabecular separation were significantly less (87 and 71% of females, respectively), while TV, BV, BV/TV, connectivity density and mean trabecular number were significantly greater (138, 189, 133, and 237% of females). PTH significantly decreased BV (30%) in male mice compared with the control, but induced no significant changes in female mice although the trend was comparable. PTH had no significant effect on connectivity density or proximal cortical thickness in either gender.

**Bone formation: TFJ**

We determined gender differences in bone formation by bone histomorphometry at the TFJs. The results are shown in Fig. 2. PTH treatment significantly increased periosteal BFR (Fig. 2A), MAR (Fig. 2B) and endosteal sLS/BS (Fig. 2C) (60, 44, 40, respectively, P<0.05), decreased medullary area (Fig. 2D) (39%, P<0.05) at the TFJ in male mice, but induced no significant changes in female mice although the trends were comparable. Two-way ANOVA analysis showed significant interactions between PTH effects and gender on these measurements (P=0.05, P=0.05, P=0.04, and P=0.03, respectively). The increase in cortical bone area in male mice with PTH treatment (Fig. 2E, 13%) did not reach statistical significance.

**BMSC differentiation**

We analyzed bone marrow stromal cell differentiation by examining both colony number and ALP activity. On day 14 of cell culture, colony number and ALP activity tended to be greater in BMSC from male mice than from female mice (Fig 3), although these differences were not statistically significant. PTH given in vivo increased colony number
mRNA levels of bone markers

mRNA levels of bone markers were determined by quantitative real-time PCR. The results are shown in Fig 4 and 5. These values are expressed as a percentage of the female controls. All values have been normalized to GAPDH mRNA levels in the same samples. PTH significantly increased the mRNA levels of IGF-1 (Fig.4A) and IGF-1 receptor (IGF-1R, Fig. 4B) in male mice (605, 320% respectively), but not in female mice. The interactions between PTH effects and gender were significant (P=0.01, P=0.02, respectively, by two way ANOVA). Basal levels of IGF-1 mRNA were lower in male mice, however. PTH increased the mRNA levels of receptor activator of nuclear factor \( \kappa \)B ligand (RANKL) (Fig.5 A) in both male (14-fold) and female (16-fold) mice, but it did not alter osteoprotegerin (OPG) mRNA levels at least at this time point (1 h after last PTH treatment) (Fig. 5 B).

Discussion

In this mouse strain, females had greater tibial bone mass as a proportion of body weight as indicated by FFW/BW and cortical thickness of the proximal tibia. However, males had more trabecular bone as indicated by higher TV, BV, BV/TV, mean trabecular number and less mean trabecular separation in the proximal tibia. The thicker cortex in females may be the result of greater endocortical expansion. These results are consistent with Kim and coworkers’ observations in rats (Kim et al. 2003). These gender differences in bone mass and structure have been assumed to exist based on sex steroid action (Orwoll et al. 2001) but less studies have been carried out. Somjen et al. (1994) reported that the skeletal response to sex hormones is gender-specific. Cell proliferation in the rat diaphysis is stimulated only by estrogens in females and only by androgens in males. Androgen is growth promoting; it stimulates periosteal bone formation in males, while estrogen is growth limiting and accounts for the cessation of longitudinal growth and periosteal expansion in females (Zhang et al. 1999). Orwoll et al. (2001) also reported that the marked gender differences in peak bone size/mass were, in part, associated with quantitative trait loci, suggesting the effects of genetic influences on gender differences in bone.

In our study, gender also exerts a profound effect on the skeletal response to PTH on bone mass and structure. PTH significantly increased FFW/BW, which represents total bone mass (including cortical bone and trabecular bone), in male mice, but not in female mice, indicating that PTH has a greater overall anabolic effect on bone in males than in females. Furthermore, histomorphometric analysis showed that PTH increased cortical bone parameters, including periosteal BFR and MAR, endosteal sLS/BS, and decreased medullary area in male mice, but not in female mice, while reducing trabecular bone volume. Thus the overall increase in bone mass in male mice following PTH is primarily due to an increase in cortical bone, consistent with earlier studies (Parfitt 2002).

At the cellular level, we analyzed the effects of PTH given in vivo on bone marrow stromal cell (BMSC) proliferation and differentiation in vitro. We found that PTH increased colony number and ALP activity in both male and female mice. These results are consistent with previous observations that PTH promotes bone marrow stromal cell precursor differentiation (Nishida et al. 1994, Ishizuya et al. 1997, Locklin et al. 2003), increasing osteoblast number (Sabatini et al. 1996) or their functional activities (Schiller et al. 1999). The increase in colony number and ALP activity was greater in female mice (2.5-, 4.0-fold, respectively) than in male mice (1.5- and 2.5-fold, respectively). However, male mice in the absence of PTH tended to have more osteoprogenitor cells and higher ALP activities than female mice, and this difference persisted following PTH administration. In a previous study, Nasu et al.(2000) demonstrated that PTH stimulated osteoblast function such as ALP activity and collagen synthesis following pretreatment with estrogen, but it did not affect osteoblast function in the absence of estrogen.
pretreatment, suggesting that estrogen plays an important role in the anabolic effects of PTH on osteoblast differentiation. This suggests that the gender difference in the anabolic effects of PTH on BMSC is at least partly based on estrogen action. The gender differences in our studies are ones of degree, not significance. Conceivably, such differences could be exaggerated or eliminated in longer term studies.

In this study, we used quantitative real-time PCR to analyze gender differences in the bone response to PTH at

Figure 2 Gender differences in periosteal and endosteal bone formation at the tibiofibular junction as assessed by bone histomorphometry. Results are expressed as mean ± S.D. (A) Periosteal bone formation rate (BFR/BS). (B) Mineral apposition rate (MAR). (C) Endosteal single labeling surface (sLS/BS). (D) Medullary area. (E) Cortical bone area. There are nine females, five males in the vehicle group, and six females, five males in the PTH-treated group. a, P<0.05 PTH treatment vs control; b, P<0.05 female control vs male control.
the molecular level. In a pilot study (data not shown), we treated CD-1 mice with 80 µg PTH/kg per day or vehicle for 2 weeks, and sacrificed the mice at 0, 1, 3, 6, 12 and 24 h after the last PTH injection. This study showed that the greatest effects of PTH on RANKL (a stimulator of osteoclastogenesis) expression occurred 1 h after the last PTH injection, whereas OPG (a decoy receptor for RANKL) was stimulated maximally at 6 h. In this study, we collected our samples 1 h after the last PTH injection. Our results showed that PTH significantly stimulated gene expression of RANKL in male and female mice, consistent with other studies (Iida-Klein et al. 2002). The increases in male and female mice were comparable, indicating no gender differences in this response to PTH.

Previous studies indicated that PTH potently stimulates synthesis of IGF-I by osteoblasts, and thus newly synthesized IGF-I may directly increase osteoblast replication and bone formation (McCarthy & Centrella 2001). Our group (Bikle et al. 2002) has reported that IGF-1 was required for the anabolic effects of PTH on cortical bone. In this study, we found that PTH increased the mRNA levels of IGF-I and its receptor (IGF-IR) only in male mice. Although several reports suggested a stimulatory effect

**Figure 3** Gender differences in BMSC colony number and ALP activity (Day 14). Results are expressed as mean ± S.D. (A) Colony number and (B) ALP activity (ALP/cell). n=3 in each group. a, P<0.05 PTH treatment vs control.

**Figure 4** Gender differences in the response to PTH with respect to mRNA levels of bone formation markers. (A) IGF-1 and (B) IGF-IR. Results are expressed as percentage of female control, all values have been normalized to GAPDH mRNA levels in the same sample. n=3 in each group. a, P<0.05 PTH treatment vs control.
by estrogen on IGF-I synthesis by osteoblasts (Watson et al. 1995, Nasu et al. 2000), recent findings indicate that the influence of estrogen on IGF-I gene expression is complex and perhaps indirect (McCarthy et al. 1997). Estrogen is able to diminish IGF-I gene activation by PTH or PGE2 as well as by GH (McCarthy & Centrella 2001). In this way, it may limit the effects of these hormones on bone formation rates. Thus, the greater response of bone from male mice to PTH with respect to IGF-I and IGF-IR may account for the greater response of bone from male mice with respect to periosteal bone formation.

In summary, our results indicate that, on balance, in the CD-1 mouse strain, a 2-week course of PTH is anabolic on cortical bone. These effects are modified by gender with male mice showing greater sensitivity to PTH than female mice. These differences may reflect the greater response to PTH of IGF-I and IGF-I receptor gene expression in males, a response which we have previously shown are required for the anabolic effects of PTH in this mouse strain.

Acknowledgments

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