Low-dose PTH increases osteoblast activity via decreased \textit{Mef2c}/\textit{Sost} in senescent osteopenic mice

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Abstract

Intermittent administration of parathyroid hormone (PTH) 1–34 at a standard dose has been shown to induce anabolic effects in bone. However, whether low-dose PTH promotes bone formation during senescence is unknown. To address this issue, we determined the effects of low-dose PTH and analysed the underlying mechanisms in prematurely senescent mice that display osteopenia. Treatment of 9-week-old \textit{Samp6} mice for 6 weeks with PTH at a standard dose (100 \textmu g/kg per day) increased vertebral and femoral bone mass and improved bone microarchitecture as a result of increased bone-forming surfaces and mineral apposition rate (MAR). At a tenfold lower dose (10 \textmu g/kg per day), PTH increased axial bone volume and trabecular thickness, as detected by bone histomorphometry but not by micro-computed tomography analysis. This anabolic effect resulted from increased osteoblast activity, as reflected by increased serum N-terminal propeptide of type 1 procollagen (P1NP) levels and MAR, with unchanged bone-forming surface or osteoblast surface. Mechanistically, low-dose PTH increased the expression of osteoblast markers in bone marrow stromal cells and mature osteoblasts, which was associated with increased expression of the Wnt effector \textit{Wisp1}. Moreover, low-dose PTH decreased the expression of the \textit{Mef2c} transcription factor, resulting in decreased \textit{Sost} expression in osteoblasts/osteocytes. These results indicate that PTH at a low dose is effective at promoting bone formation and increased bone volume in senescent osteopenic mice through increased osteoblast activity and modulation of specific Wnt effectors, which raises the potential therapeutic use of intermittent PTH at low dose to increase bone forming activity and bone mass in skeletal senescence.

Key Words

\textbullet{} low-dose PTH
\textbullet{} senescent mice
\textbullet{} osteoblast activity
\textbullet{} Wnt signalling

Introduction

Bone loss associated with ageing is characterised by decreased bone formation relative to bone resorption, resulting in altered bone microarchitecture, osteoporosis and increased risk of fractures (Khosla \textit{et al.} 2011). The age-related decline in bone formation results from multiple intrinsic and extrinsic mechanisms that lead to decreased differentiation of bone marrow stromal cells (BMSCs) into osteoblasts and decreased osteoblast number and activity (Manolagas & Parfitt 2010, Kassem & Marie 2011). One important challenge is therefore to improve the number and functioning of osteoblasts in order to prevent the decrease in bone formation and bone mass in skeletal senescence. Intermittent parathyroid hormone (PTH) administration is the only proved strategy for inducing an anabolic effect that is
available in clinics for the treatment of severe osteoporosis. This treatment has been shown to increase bone formation to a greater extent than bone resorption in animals and humans, resulting in improved bone mass and microarchitecture (Compston 2007). In adult mice, the anabolic effect of PTH on bone is mediated by increased numbers and activity of osteoblasts (Canalis et al. 2007, Jilka 2007). The PTH-induced increase in osteoblast number in adult mice is achieved by increased osteoblast differentiation, by converting quiescent lining cells to active osteoblasts and by reducing osteoblast apoptosis (Jilka 2007, Kim et al. 2012).

The anabolic effect of PTH is mediated through direct and indirect mechanisms. PTH signals through G-protein-coupled type 1 PTH/PTH-related peptide receptor and subsequent activation of protein kinase A, protein kinase C coupled type 1 PTH/PTH-related peptide receptor and specific Wnt effectors.

To date, only a few studies have analysed the anabolic effect of PTH in the ageing skeleton (Knopp et al. 2005, Brennan et al. 2009, Jilka et al. 2010). Moreover, the effect of PTH 1–34 in osteopenic mice has been tested at standard doses ranging from 80 to 100 µg/kg per day, and it is not known whether PTH at a much lower dose (i.e. a tenfold lower dose) may be effective at promoting bone formation in senescent mice. To address this issue, we determined the skeletal response to low-dose PTH in prematurely senescent osteopenic mice and analysed the cellular and molecular mechanisms involved in this response. We show herein that intermittent PTH at a low dose is effective at increasing axial bone mass in prematurely senescent osteopenic mice by promoting osteoblast activity, through modulation of specific Wnt effectors.

Materials and methods

Animals and treatment

We used Samps6 mice, a murine model of ageing in the P6 strain of senescence-accelerated mice which is characterised by decreased bone formation and low bone mass (Jilka et al. 1996, Clement-Lacroix et al. 2005). Seven-week-old Samps6 mice (Harlan Laboratories, Derby, UK) were fed with mouse standard diet (A04 rat/mouse diet, SAFE, Augy, France) containing calcium (0.84%), phosphorus (0.57%) and vitamin D (1000 IU/kg), and were weighed once a week. Nine-week-old mice (eight mice per group) were treated with s.c. human PTH 1–34 at a high standard dose (100 µg/kg BW) or a low dose (10 µg/kg BW) (Sigma) or the vehicle, 5 days/week for 6 weeks. The rationale for choosing the low dose is that it is tenfold lower than the dose usually used in osteopenic mice (Knopp et al. 2005, Jilka et al. 2010, Hanyu et al. 2012, Kim et al. 2012). To label bone mineralisation fronts, control and treated mice were given tetracycline (20 mg/kg, Sigma) and calcinein (10 mg/kg) by s.c. injection, respectively, at days 8 and 3 before being killed by injection of ketamine/xylazine. The protocol was conducted according to the guidelines of the Local Ethical Committee (ref. no. CEEALV/2011.11.01).

P1NP analysis

The animals were killed and blood was collected and spun in order to recuperate the serum. Serum aliquots were frozen before analysis of N-terminal propeptide of type 1 procollagen (P1NP) levels, an established marker of bone formation, by ELISA (R&D Systems, Lille, France).
Bone microarchitecture and histomorphometry

The animals were killed and, lumbar vertebrae and right femurs (distal metaphysis) were obtained for analysis of micro- and macro-structures. The bones were scanned using a high-resolution micro-computed tomography (microCT) system (Skyscan 1172, MicroPhotonics, Allen-town, PA, USA) and analysed using a 3D morphometry evaluation program (NRecon reconstruction program). For histomorphometric analysis, the bones were embedded in methylmethacrylate and 5 μm sections were stained with aniline blue to analyse structural parameters (osteoblast surface, bone volume, trabecular number and thickness), as described previously (Hay et al. 2009). TRAP staining was carried out to evaluate the number of active osteoclasts (Hay et al. 2009). Unstained sections (8 μm thick) were used to assess dynamic parameters (mineral apposition rate (MAR), double labelled surface and bone formation rate (BFR); (Parfitt et al. 1987).

Quantitative PCR analysis

The animals were killed and the bone marrow was flushed from the tibia, yielding BMSCs and remaining bone containing mature osteoblasts and osteocytes. Total RNA was extracted from the two bone cell pools, and 1 μg of total RNA from each sample was reverse-transcribed using the Applied Biosystems Kit (High-Capacity cDNA RT Kit). The relative mRNA levels of osteoblast differentiation markers and Wnt effectors were evaluated by quantitative PCR analysis (LightCycler; Roche Applied Science) using a SYBR Green PCR Kit (ABGen, Courtabœuf, France) and specific primers (Hay et al. 2009, Andrews et al. 2012, Saidak et al. 2012). The signals were normalised to hypoxanthine phosphoribosyltransferase (HRPT) as an internal control.

Statistical analysis

Values are presented as the mean ± S.E.M. of six to eight animal groups. Data were analysed with the unpaired two-tailed Student’s t-test. A P value <0.05 was considered statistically significant.

Results

Low-dose PTH increases bone volume in senescent mice

We first compared the effect of the two doses of PTH on trabecular bone mass and microarchitecture as evaluated by microCT analysis. As it might be expected, intermittent PTH at the standard dose increased vertebral bone mass and volume in Samp6 mice (Fig. 1A and B). This effect resulted from increased trabecular thickness and number and decreased trabecular separation (Fig. 1C, D and E). Interestingly, the low dose of PTH was sufficient to increase trabecular bone thickness as evaluated by microCT analysis (Fig. 1C). To further investigate the anabolic effect of PTH at low doses, we performed a histomorphometric analysis of vertebral bone. We found that PTH at a low dose increased trabecular bone volume

Figure 1

Distinct effects of standard and low-dose intermittent PTH on bone mass and microarchitecture in prematurely senescent osteopenic Samp6 mice. Representative microCT images of lumbar vertebrae (A) showing that both doses of PTH increased bone mass in Samp6 mice. (B, C, D and E) Analysis of bone microarchitecture parameters showing the distinct effects of PTH at the standard and low doses on bone volume (BV, expressed relative to trabecular volume (TV)), trabecular thickness (Tb. Th), trabecular number (Tb. No) and trabecular separation (Tb. Sep). Mean ± S.E.M. of six to eight mice. *Statistically significant (P < 0.05).
in vertebrae (Fig. 2A and B) as a result of increased trabecular bone thickness (Fig. 2C), with no significant change in trabecular number or separation (Fig. 2D and E). The positive effect on bone volume was restricted to the axial bone because the standard dose, but not the low dose, increased bone volume and trabecular thickness and number and decreased trabecular separation in the femur (Supplementary Fig. 1A, B, C and D, see section on supplementary data given at the end of this article). The increased axial bone volume induced by low-dose PTH was not related to changes in bone resorption, because the trabecular separation was not affected (Figs 1 and 2) and the number of TRAP$^+$ osteoclasts was unchanged (Supplementary Fig. 2A). These results indicate that PTH at a low dose is effective at increasing trabecular bone thickness and bone volume in the axial skeleton in senescent osteopenic Samp6 mice.

Low-dose PTH increases osteoblast function but not number in senescent mice

To determine whether the low-dose PTH increased bone mass by increasing bone formation, we analysed the levels of serum P1NP, an established marker of bone-forming activity. Both the standard-dose and the low-dose PTH increased serum P1NP levels, indicating an anabolic response at both doses (Fig. 3). To confirm that the low dose of PTH was efficient at promoting trabecular bone formation, histomorphometric analysis was performed in axial bone. PTH at low dose had no effect on the extent of double-labelled surface (Fig. 4A). Consistently, the osteoblast surface, which reflects osteoblast number, was unchanged (Supplementary Fig. 2B). In contrast, the low-dose PTH increased the bone MAR, which reflects the bone-forming activity (Fig. 4B) without change in the BFR (Fig. 4C). These effects were not restricted to axial bone because similar results were obtained in the femur (Supplementary Fig. 3, see section on supplementary data given at the end of this article). In contrast, the standard high-dose PTH increased bone MAR, double-labelled surface and BFR by twofold (Supplementary Fig. 3). These results indicate that PTH at a low dose is effective at increasing bone formation in senescent osteopenic mice by increasing the activity but not the number of active osteoblasts.
To determine the mechanisms by which the low-dose PTH increased osteoblast activity, we performed a molecular analysis of genes that characterise osteoblast function in osteoprogenitor cells present in the bone marrow stroma. At a low dose, PTH was effective at increasing the expression of alkaline phosphatase (Alp) and type 1 collagen (Col1a1) and tended to increase the expression of osteocalcin (Oc), a marker of mature osteoblasts (Fig. 5A–C). In the bone-marrow-free tibia that contains mature osteoblasts and osteocytes, Alp and Col1a1 levels were not affected by low-dose PTH, whereas Oc was markedly increased (Fig. 5A–C). These results indicate that PTH at low dose acted by promoting osteoblast function in Samp6 mice.

**Low-dose PTH modulates specific Wnt effectors in senescent mice**

We next determined the molecular mechanisms underlying the positive effect of the low-dose PTH on osteoblast activity in Samp6 mice by determining the changes in direct Wnt effectors (Clevers 2006, Gordon & Nusse 2006). We first analysed the effect of low-dose PTH on

![Figure 4](http://joe.endocrinology-journals.org/C209/2014/Society%20for%20Endocrinology/D0:10.1530/JOE-14-0249 Printed%20in%20Great%20Britain)

**Figure 4**

Intermittent low-dose PTH increased osteoblast activity but not bone forming surface in the vertebrae of prematurely senescent osteopenic Samp6 mice. Low-dose PTH had no effect double-labelled surface (DLS) (A) or bone formation rate (BFR) (C) but increased the mineral apposition rate (MAR) (B). Mean ± s.d. of six to eight mice. *Statistically significant (P < 0.05).

**Low-dose PTH increases functional osteoblast markers in senescent mice**

To determine the mechanisms by which the low-dose PTH increased osteoblast activity, we performed a molecular analysis of genes that characterise osteoblast function in osteoprogenitor cells present in the bone marrow stroma. At a low dose, PTH was effective at increasing the expression of alkaline phosphatase (Alp) and type 1 collagen (Col1a1) and tended to increase the expression of osteocalcin (Oc), a marker of mature osteoblasts (Fig. 5A–C). In the bone-marrow-free tibia that contains mature osteoblasts and osteocytes, Alp and Col1a1 levels were not affected by low-dose PTH, whereas Oc was markedly increased (Fig. 5A–C). These results indicate that PTH at low dose acted by promoting osteoblast function in Samp6 mice.

![Figure 5](http://joe.endocrinology-journals.org/C209/2014/Society%20for%20Endocrinology/D0:10.1530/JOE-14-0249 Printed%20in%20Great%20Britain)

**Figure 5**

Intermittent low-dose PTH increased expression of the osteoblast differentiation markers Alp (A), Col1a1 (B) and Oc (C) in prematurely senescent osteopenic Samp6 mice. Quantitative RT-PCR analysis of osteoblast gene expression was carried out in the bone marrow stromal cells and osteoblasts/osteocytes extracted from long bones in control Samp6 mice or mice treated with low-dose PTH for 6 weeks. The transcript levels were normalized to the values for HPRT. Mean ± s.d. of six to eight mice. *Statistically significant (P < 0.05).
Wnt-induced secreted protein 1 (Wisp1) because it is a direct target of canonical Wnt signalling (Ono et al. 2011). The low-dose PTH increased Wisp1 mRNA levels in both BMSCs and osteoblasts/osteocytes, indicating that Wnt signalling was activated in both osteoblast precursor cells and more mature osteoblasts/osteocytes (Fig. 6A).

Wnt signalling is negatively regulated by inhibitors such as DKK1 and sFRP1 (Bodine et al. 2004). To investigate whether low-dose PTH acts by downregulating these Wnt inhibitors, we analysed the changes in Dkk1 and sFrp1 gene expression in bones of Samp6 mice. We found that low-dose PTH increased the expression of Dkk1 in both BMSCs and osteoblasts/osteocytes (Supplementary Fig. 4A, see section on supplementary data given at the end of this article). In contrast, sFrp1 mRNA levels remained unchanged (Supplementary Fig. 4B). This indicates that these Wnt antagonists were distinctly affected by low-dose PTH in Samp6 mice. Importantly, we found that Sost mRNA levels tended to be reduced in BMSCs and were greatly reduced by PTH at a low dose in osteoblasts/osteocytes (Fig. 6B). To determine how the low-dose PTH may affect Sost expression in Samp6 mice, we analysed the expression of Mef2c which upregulates Sost (Leupin et al. 2007, Kramer et al. 2010) and is regulated by PTH in vitro (Bonnet et al. 2012). We found that Mef2c in bones was decreased by low-dose PTH, which provides a mechanism by which low-dose PTH acts on Sost expression and promotes osteoblast activity in osteopenic senescent Samp6 mice (Fig. 6C).

Overall, these results provide the first evidence that low-dose PTH treatment in Samp6 mice is effective for increasing bone mass in the axial skeleton by increasing osteoblast activity, but not cell number, via upregulation of Wisp1 and downregulation of Mef2c/Sost in bone (Fig. 7).

**Discussion**

Intermittent PTH at a standard dose is known to produce an anabolic effect on bone in adult animals. However, it is not known whether a low dose (i.e. tenfold lower than the...
usual dose used) is effective in osteopenic ageing mice. In this study, we showed that low-dose PTH is efficient at increasing bone formation and axial bone volume in senescent osteopenic mice, and that this anabolic effect results from increased osteoblast activity and modulation of specific Wnt effectors. First, we showed that PTH treatment at a low dose increased trabecular bone thickness and volume in axial bone in \textit{Samp6} mice, demonstrating that a low dose of PTH is sufficient to augment bone mass in senescent osteopenic mice. This effect was evidenced by histomorphometric but not microCT analysis. This discrepancy is probably due to the distinct resolution between the two techniques. Second, the results indicate that the increased bone volume induced by low-dose PTH results from increased osteoblast activity, as shown by the increased PINP levels and MAR. In contrast, the osteoblast surface and double-labelled surfaces were unchanged, indicating that low-dose PTH acted by increasing osteoblast activity, but not the number of active osteoblasts, in \textit{Samp6} mice. This is in contrast with the effect produced by PTH at a standard dose, which increased the double-labelled bone forming surface in \textit{Samp6} mice (this study), as has been found in WT ageing mice (Jilka 2007). These results support the notion that PTH at a low dose is effective at increasing bone volume in senescent osteopenic mice through enhanced osteoblast activity.

In WT mice, the anabolic effect of PTH at a standard dose results from activation of multiple signals (Canalis \textit{et al.} 2007, Jilka 2007). In prematurely senescent osteopenic mice low-dose PTH increased the expression of \textit{Alp} and \textit{Col1a1}, which are targets of Wnt signalling (Gaut \textit{et al.} 2005, Bodine \& Komm 2006). Consistent with this finding, low-dose PTH increased the expression of \textit{Wisp1}, a direct target of Wnt signalling, as has been also previously reported for WT mice treated with a standard dose of PTH (Jilka \textit{et al.} 2010). \textit{WISP1} is a member of the CCN family that is upregulated by β-catenin (Xu \textit{et al.} 2000) and that positively regulates bone formation (French \textit{et al.} 2004, Ono \textit{et al.} 2011). Our findings that low-dose PTH increased \textit{Wisp1} expression in both BMSCs and osteoblasts/osteocytes therefore reveals one mechanism for the anabolic effect of low-dose PTH in senescent mice. We also found that low-dose PTH increased the expression of \textit{Dkk1} in BMSCs and osteoblasts/osteocytes. This is surprising because \textit{Dkk1} antagonises canonical Wnt signalling \textit{in vivo} by binding to Wnt ligands, leading to the attenuation of Wnt/receptor activation and bone formation (Bovolenta \textit{et al.} 2008). The increased expression of \textit{Dkk1} by low-dose PTH is likely to result secondarily from the activation of Wnt signalling which is known to increase \textit{Dkk1} expression (Chamorro \textit{et al.} 2005). This is consistent with a negative-feedback response restricting the exposure of bone cells to prolonged activation of Wnt signalling. The induction of \textit{Dkk1} expression is unlikely to reduce the overall response to low-dose PTH in senescent \textit{Samp6} mice because, although the suppression of Wnt signalling by \textit{Dkk1} was found to attenuate PTH-mediated stromal cell response and new bone formation (Guo \textit{et al.} 2010), targeted overexpression of \textit{Dkk1} in osteoblasts does not impair the anabolic response to PTH in mice (Yao \textit{et al.} 2011). In contrast to \textit{Dkk1}, the level of \textit{sFrp1}, a negative regulator of bone formation (Yao \textit{et al.} 2010), was unchanged by low-dose PTH in \textit{Samp6} mice. Results from previous studies have indicated that the bone anabolic effect of PTH is attenuated by either overexpression or deletion of \textit{sFrp1} in mice (Bodine \textit{et al.} 2007). The lack of change in \textit{sFrp1} expression in PTH-treated \textit{Samp6} senescent mice is thus unlikely to affect the anabolic effect of low-dose PTH in these mice.

Our finding that PTH at a low dose markedly decreased \textit{Sost} levels in osteoblasts/osteocytes provides another mechanism for the anabolic effect of low-dose PTH in senescent \textit{Samp6} mice. The decreased \textit{Sost} level with a low-dose of PTH in \textit{Samp6} mice is consistent with the effect of PTH at a standard dose in WT mice (Bellido \textit{et al.} 2005, Keller \& Kneissel 2005). \textit{Sost} expression in osteocytes has been shown to be positively regulated by the transcription factor MEF2C (Leupin \textit{et al.} 2007). Recent results have indicated that \textit{Mef2c} invalidation results in reduced \textit{Sost} expression and increased bone mass in mice (Kramer \textit{et al.} 2010, 2012, Collette \textit{et al.} 2012). In this study, we found that PTH at a low dose decreased \textit{Mef2c} levels which was associated with decreased \textit{Sost} levels, increased osteoblast activity and bone volume in senescent osteopenic \textit{Samp6} mice. Our data thus support the notion that the anabolic response to low-dose PTH involves downregulation of \textit{Mef2c} and subsequent reduction of \textit{Sost}, leading to increased Wnt/β-catenin signalling, osteoblast activity and bone mass in senescent osteopenic mice (Fig. 7). The finding that PTH at low dose is able to modulate specific Wnt signalling effectors in \textit{Samp6} mice is particularly important in the context of skeletal senescence because results from current studies indicate that bone ageing is associated with alterations in the local expression of Wnt ligands (Rauner \textit{et al.} 2008) among other mechanisms (Manolagas \& Parfitt 2010, Marie 2014). We previously reported that the anti-osteoporotic agent strontium ranelate promotes Wnt signalling in osteoblasts and
thereby increases bone formation and bone mass in senescent osteopenic Samp6 mice (Saidak et al. 2012). The results of the present study support the interest in targeting Wnt signalling for improving osteoblast activity and bone mass in skeletal ageing.

In summary, our results reveal that PTH at a low dose is effective at increasing bone volume in senescent osteopenic mice through activation of osteoblast activity and modulation of specific Wnt effectors, which supports the potential therapeutic interest in using intermittent PTH at a low dose for improving bone formation and bone mass in skeletal senescence.