The Journal of Experimental Biology 209, 1630-1638
Published by The Company of Biologists 2006
doi:10.1242/jeb.02185

Parathyroid hormone may maintain bone formation in hibernating black bears (*Ursus americanus*) to prevent disuse osteoporosis

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Accepted 22 February 2006

Summary

Mechanical unloading of bone causes an imbalance in bone formation and resorption leading to bone loss and increased fracture risk. Black bears (*Ursus americanus*) are inactive for up to six months during hibernation, yet bone mineral content and strength do not decrease with disuse or aging. To test whether hibernating bears have biological mechanisms to prevent disuse osteoporosis, we measured the serum concentrations of hormones and growth factors involved in bone metabolism and correlated them with the serum concentration of a bone formation marker (osteocalcin). Serum was obtained from black bears over a 7-month duration that included periods of activity and inactivity. Both resorption and formation markers increased during hibernation, suggesting high bone turnover occurred during inactivity. However, bone formation appeared to be balanced with bone resorption. The serum concentration of parathyroid hormone (PTH) was higher in the hibernation (P=0.35) and post-hibernation (P=0.006) seasons relative to pre-hibernation levels. Serum leptin was lower (P<0.004) post-hibernation relative to pre-hibernation and hibernation periods. Insulin-like growth factor I (IGF-I) decreased (P<0.0001) during hibernation relative to pre-hibernation and reached its highest value during remobilization. There was no difference (P=0.64) in 25-OH vitamin D between the three seasons. Serum osteocalcin (bone formation marker) was significantly correlated with PTH, but not with leptin, IGF-I or 25-OH vitamin D. Osteocalcin and PTH were positively correlated when samples from all seasons were pooled and when only hibernation samples were considered, raising the possibility that the anabolic actions of PTH help maintain bone formation to prevent disuse osteoporosis. Prostaglandin E_2 (PGE_2) release from MC3T3 osteoblastic cells was significantly affected by treatment with bear serum from different seasons (i.e. hibernation versus active periods). The seasonal changes in PGE_2 release showed trends similar to the seasonal changes in serum IGF-I. Since both PGE_2 and IGF-I are associated with collagenous bone formation, it is possible that seasonal changes in a circulating factor influence IGF-I levels *in vivo* in bears and PGE_2 release in osteoblastic cells in vitro. The significant decrease in serum leptin following arousal from hibernation may promote bone formation during remobilization, assuming there is a similar decrease in intracerebroventricular leptin. These findings support the idea that seasonal changes in the concentration of circulating molecules help regulate bone formation activity and may be important for preventing disuse osteoporosis in bears.

Key words: black bear, *Ursus americanus*, bone remodeling, disuse osteoporosis, hibernation, leptin, IGF-I, PTH.

Introduction

Many clinical and experimental conditions that mechanically unload bone cause disuse osteoporosis and increase fracture risk. For example, disuse due to spinal cord lesion significantly decreases bone mineral density (BMD) in the tibia and femur (Dauty et al., 2000), and significantly reduces the cross-sectional moment of inertia of the femoral diaphysis (Modlesky et al., 2005). Thus, bone bending strength is reduced by spinal cord injury and fracture risk is increased (Wang et al., 2001). These disuse-induced structural changes to bone are due to unbalanced bone remodeling, which typically increases bone resorption and decreases bone formation (Rantakokko et al., 1999; Weinreb et al., 1989).

Inactivity-induced changes in bone remodeling are reflected in serum and urinary markers of bone turnover. Human bedrest increases bone resorption, leading to increased urinary calcium excretion and decreased BMD (Arnaud et al., 1992; Inoue et al., 2000; LeBlanc et al., 1995; Watanabe et al., 2004; Zerwekh

et al., 1998). Serum markers of bone resorption consistently increase during bedrest, whereas serum markers of bone formation have been shown to increase, decrease or remain unchanged during bedrest (Inoue et al., 2000; Pedersen et al., 1995; Watanabe et al., 2004; Zerwekh et al., 1998). Bone biopsies have shown that bedrest decreases bone formation rate, decreases osteoblast surface and increases osteoclast surface (Arnaud et al., 1992; Zerwekh et al., 1998). Bone lost during rat hindlimb suspension is primarily due to decreased bone formation (Sakata et al., 2004). Immobilization of canine forelimbs increases both resorption and formation, but substantial bone loss occurs because of increased remodeling space, unbalanced relative increases in resorption and formation, and possibly because of an abnormally long lag time between resorption and formation (Li et al., 2005; Yang Li et al., 2005). Taken together, these studies suggest that bone is lost during disuse as a result of unbalanced remodeling (i.e. more resorption than formation).

Disuse also alters circulating hormone concentrations. Serum parathyroid hormone (PTH) concentration is unchanged or decreased during bedrest (Arnaud et al., 1992; Inoue et al., 2000; Shackelford et al., 2004; Watanabe et al., 2004). Decreased PTH is expected since serum calcium concentration increases during bedrest. A decrease in PTH probably contributes to decreased 1,25 $(OH)_2$ vitamin D (25-OH D) during bedrest, which reduces intestinal calcium absorption leading to increased calcium excretion (LeBlanc et al., 1995; Shackelford et al., 2004; Zerwekh et al., 1998). Insulin-like growth factor I (IGF-I) has been shown to increase in a human bedrest study that also showed a decrease in the bone formation marker PICP (carboxy-terminal propeptide of type 1 procollagen) (Inoue et al., 2000). The negative correlation between IGF-I and PICP is surprising since IGF-I has anabolic effects on bone. However, rat hindlimb suspension did not affect serum levels of IGF-I and prevented the ability of exogenous IGF-I to stimulate bone formation (Sakata et al., 2004). This suggests that disuse prevents the anabolic effects of IGF-I. Two weeks of rat hindlimb suspension decreased serum leptin, bone formation rate, and trabecular bone volume (Martin et al., 2005). However, low dose intraperitoneal injection of leptin was able to restore serum leptin levels and prevent bone loss by restoring bone formation rate.

Hibernation is a mechanism for surviving prolonged periods of food shortages. Bears do not eat, drink, urinate or defecate during hibernation (Nelson, 1987). During hibernation, bears have a remarkable ability to recycle catabolic waste products (e.g. urea) through neuroendocrine control (Nelson, 1978; Nelson et al., 1983). Bone catabolic products also appear to be recycled in bears during hibernation, probably to keep blood calcium concentration within a homeostatic range. Hibernation causes bone loss in ground squirrels, golden hamsters and little brown bats (Haller and Zimny, 1977; Kwiecinski et al., 1987; Steinberg et al., 1979; Steinberg et al., 1981; Steinberg et al., 1986). However, serum markers of bone formation, trabecular bone volume and BMD do not decrease in black bears during hibernation (Donahue et al., 2003a; Floyd et al., 1990; Pardy et al., 2004). Additionally, cortical bone strength and ash fraction increase with age, and porosity does not change with age in black bears, despite annual periods of disuse (Harvey and Donahue, 2004). Taken together, these findings suggest that bears have evolved biological mechanisms to prevent disuse osteoporosis (Donahue et al., 2005). Systemic hormones and growth factors may be involved in maintaining bone formation in hibernating bears to maintain bone mass in the absence of mechanical loading. We hypothesized that the serum concentrations of PTH, 25-OH D, leptin and IGF-I are correlated with the serum concentration of the bone formation marker osteocalcin. Since histomorphometric indices of bone resorption and formation increase during hibernation (Floyd et al., 1990), we hypothesized that serum markers of bone resorption and formation also increase. We also hypothesized that serum collected in different seasons would cause different levels of prostaglandin E_2 (PGE_2) to be released from MC-3T3 osteoblastic cells, because exogenous PGE_2 prevents disuse-induced bone loss (Akamine et al., 1992; Bakker et al., 2003) possibly by stimulating IGF-I production (McCarthy et al., 1991) to increase collagen production (Canalis et al., 1989). Elucidating the biological mechanisms that regulate bone formation in hibernating bears may help us design anabolic therapies to mitigate bone loss in human osteoporoses. This may be accomplished by targeting genes and circulating hormones (e.g. PTH), which are differentially expressed in bears and humans during disuse.

Materials and methods

Serum samples

Blood samples were collected from 16 different female black bears (*Ursus americanus* Pallas) held in the Virginia

Table 1. *The number of bears and the assays that were performed in each hibernation season*

Season	Bears (Cubs)*	Ages (years)	Assays	Data location
2001–2002	5 (2)	2–19	PICP, ICTP	Fig. 1
2002–2003	3 (3)	6–19	PGE_2 in MC3T3 culture media	Fig. 3
2003–2004	5 (5)	3–12	Osteocalcin, PTH, 25-OH D, leptin, IGF-I	Figs 1,2; Table 2
2004–2005	3 (1)	3–10	Ionized calcium	Table 2

*The number of bears that gave birth to cubs is in parentheses.

PICP, carboxy-terminal propeptide of type 1 procollagen; ICTP, carboxy-terminal cross-linked telopeptide of type 1 procollagen; PGE_2, prostaglandin E_2; PTH, parathyroid hormone; 25-OH D, 1,25 $(OH)_2$ vitamin D; IGF-I, insulin-like growth factor I.

Tech Center for Bear Research between 2001 and 2005 (Table 1). The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069-F&WS). The bears were anesthetized with a 2:1 mixture of ketamine (100 mg ml^{-1}):xylazine (100 mg ml^{-1}); the dosage was 1 cc of the mixture per 45.5 kg of body mass. Body temperatures were 4°C to 6°C cooler during winter collection, confirming that the bears were in a state of hibernation. No urine or scat was present in the hibernation dens. Behavior indicating stress was not observed during any of the handling procedures. Blood samples were drawn from the femoral vein while the bears were anesthetized, and the samples were transported to the laboratory in an ice-packed cooler. Immediately on return to the laboratory, the blood was spun to isolate the serum, which was frozen at –20°C. Blood samples were collected from each bear every 10 days from the beginning of October until the end of May. Hibernation began in early January and ended in early April. Thus, the collection dates encompassed an active pre-hibernation period, a disuse hibernation period, and an active post-hibernation remobilization period.

Biochemical assays

The serum collected from five female bears in the 2003–2004 hibernation season was assayed for PTH, 25-OH D, leptin, IGF-I and osteocalcin (a bone formation marker) using radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). The bears were between 2- and 12-years old. Each bear gave birth to 2–4 cubs between January 17 and February 16. PTH was assayed with an ELISA kit from Immutopics International (San Clemente, CA, USA); the intra-assay coefficient of variation was 4.7%. 25-OH D was assayed with an ELISA kit from ALPCO Diagnostics (Windham, NH, USA); the intra-assay coefficient of variation was 5%. Leptin was measured by radioimmunoassay (Linco, St Charles, MO, USA); the intra-assay coefficient of variation was 3.4%. IGF-I was measured by acid ethanol extraction radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA); the intra-assay coefficient of variation was 4.3%. Serum osteocalcin was measured by radioimmunoassay (the methods are explained in detail in the section below). Ionized calcium concentration was measured with an ion-selective electrode (Bayer Rapidlab 865, Leverkusen, Germany) in serum samples collected from three bears during the 2004–2005 hibernation season. For all the serum metabolites, the mean values (for all bears and all time points within a given season) were calculated for each season (pre-hibernation, hibernation, and post-hibernation) and compared by analysis of variance (ANOVA). ANOVAs were followed up with Fisher's PLSD tests for multiple mean comparisons. Natural log transformations were used to correct non-constancy of variance for osteocalcin, PTH, 25-OH D and IGF-I to validate the ANOVAs. Linear regressions were used to assess the correlations between osteocalcin and the hormones. The volume of some serum samples was insufficient to run all assays; sample sizes for each assay are indicated with the results.

Black bear osteocalcin purification and radioimmunoassay procedures

To validate the rat osteocalcin assay (Patterson-Allen et al., 1982) for use with bear samples, osteocalcin was purified from black bear cortical bone by modification of a previously described method (Colombo et al., 1993). The bear bone was broken into small fragments, defatted with a mixture of three parts hexane and two parts isopropanol, and lyophilized. The dried bone was ground to a fine powder under liquid nitrogen, and the osteocalcin was solubilized in 0.5 mol l^{-1} EDTA as described by Hauschka et al. (Hauschka et al., 1989). Osteocalcin was purified from the resulting EDTA extract by a modification of the method of Colombo et al. (Colombo et al., 1993). Briefly, the crude EDTA solution was diluted twofold and passed over a bulk column containing 10 g Sepralyte C18 particles (Analytichem International, Harbor City, CA, USA) previously activated with methanol and equilibrated with 0.1% trifluoroacetic acid in water (0.1% TFA). An extensive wash with 0.1% TFA was followed by 30% methanol/0.1% TFA until UV absorbance dropped to baseline. Osteocalcin was eluted with 80% methanol/0.1% TFA. Methanol was evaporated under a stream of air and the remaining solution was lyophilized. The resulting dried protein was suspended in 0.05 mol l^{-1} Tris buffer, pH 8.0 and applied to a 5 ml Bio-Rad Econo-Q column previously equilibrated with the same buffer. The column was developed with a gradient from 0.1 to 0.6 mol l^{-1} NaCl in 0.5 mol l^{-1} Tris, pH 8.0. Osteocalcin eluted in a symmetric peak, the last to elute from the column. Identity of this peak as osteocalcin was qualitatively verified by reacting fraction aliquots with diazobenzene sulfonic acid yielding a pink color in those fractions containing carboxyglutamic acid, characteristic of osteocalcin, with intensity corresponding to peak height (Nishimoto, 1990). Both the C18 and the Econo-Q column were new and never exposed to protein from other species. Previous experience with other species suggests the final osteocalcin peak is greater than 99% pure. Concentration of bear osteocalcin in the final elute was determined with BCA reagents from Pierce Chemical (Rockford, IL, USA).

Highly purified bear osteocalcin and bear serum were assayed by radioimmunoassay (Patterson-Allen et al., 1982). The antibody was guinea-pig anti-rat osteocalcin and tracer was ^{125}I-labeled rat osteocalcin. Dose dilutions of both rat osteocalcin standard (Biomedical Technologies, Inc, Stoughton, MA, USA) and purified bear osteocalcin were completely parallel in the assay. Aliquots of 10 μl bear serum were assayed in duplicate. Intra-sample measurements varied by less than 5%.

Bone resorption and formation markers during hibernation

To observe changes in bone formation during disuse (hibernation), the mean values of serum osteocalcin concentration for five bears (serum collected in 2003–2004) were calculated for each time point during the hibernation period. These values were normalized by the maximum osteocalcin value during the hibernation period. Similar

calculations were done for PICP (bone formation marker) and ICTP (C-terminal cross-linked telopeptide of type 1 procollagen; a bone resorption marker) data that we measured in five different bears during the 2001–2002 hibernation season (Donahue et al., 2003a). The concentrations of these markers were also normalized by their maximum hibernation values because each marker has a different unit of concentration. This enabled the normalized values of the resorption and formation markers to be plotted on the same graph for the evaluation of the temporal and relative magnitude of changes in bone resorption and formation during disuse.

Effects of bear serum on prostaglandin E_2 release from MC-3T3 osteoblastic cells

To assess the effects of seasonal variations in bear serum on osteoblast metabolism, MC-3T3 cells were treated with bear serum, and prostaglandin E_2 (PGE_2) release was quantified. MC-3T3 cells were grown in alpha minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin–streptomycin solution at 37°C in 5% CO_2 for 24 h. The medium was aspirated and replaced with 10 ml of fresh medium containing 10% bear serum collected prior to hibernation, during hibernation, or after hibernation. This bear serum was collected from three bears in the 2002–2003 hibernation season; the volume was insufficient to assay the bone remodeling markers and hormones as described in the previous sections. The cells were allowed to grow for an additional 24 h, and then the medium was collected and frozen at –20°C for PGE_2 analysis. The cells were removed from the culture dishes using 0.25% trypsin in EDTA, pelleted by centrifugation, and, after staining with Trypan Blue were quantified using a hemocytometer.

The PGE_2 levels were determined using the Biotrak^{TM} PGE_2 competitive enzyme immunoassay (Amersham Biosciences, Piscataway, NJ, USA). The assay was performed in duplicate using 50 μl samples from all experimental media samples. The reaction was halted prior to endpoint determination using 1 mol l^{-1} sulfuric acid and measurements were made at 450 nm using a microplate reader (VERSAmax, Molecular Devices Corporation, Sunnyvale, CA, USA). The duplicate optical density values were corrected for nonspecific binding, averaged, and compared to a calibration curve to determine the amount of PGE_2 in each well. These values were corrected for total medium volume and normalized by the number of cells in the sample. ANOVA was used to compare the normalized PGE_2 among the three serum groups.

Results

Both bone resorption and formation markers increase during hibernation and appear to remain coupled and balanced

The bone resorption marker (ICTP) began to increase immediately after the onset of hibernation (Fig. 1). After 10–20 days, the bone formation markers (osteocalcin and PICP) also increased. This is consistent with the 1–2 week histological 'reversal' period between resorption and formation (Jee, 2001). These remodeling markers showed trends of increased resorption and formation throughout the hibernation period, and formation appeared to remain coupled and balanced with resorption. Mean osteocalcin levels were higher (P<0.0001) during and after hibernation compared to pre-hibernation (Table 2).

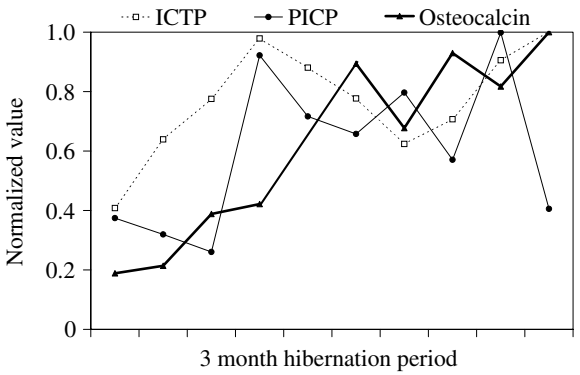

Fig. 1. Normalized serum resorption (ICTP) and formation (PICP and osteocalcin) marker concentrations during the 3-month hibernation period. Each data point is the mean value from five bears. Serum was collected every 10 days; the first sample was taken December 31, the last was March 31. The resorption marker increased immediately after the onset of hibernation. After 10–20 days, the formation markers increased and appeared to remain coupled to the increased resorption for the duration of hibernation. The PICP and ICTP concentration values for the entire study period (i.e. pre-hibernation, hibernation and post-hibernation seasons) have previously been published (Donahue et al., 2003a).

Serum ionized calcium significantly increases during hibernation

The concentration of ionized calcium during hibernation was significantly (P=0.0062) higher than the pre-hibernation concentration (Table 2). During remobilization following arousal from hibernation, ionized calcium concentration did not significantly (P=0.37) increase relative to hibernation, but remained higher (P=0.015) than the pre-hibernation concentration.

PTH was significantly correlated with osteocalcin, and PTH showed significant seasonal variations but vitamin D did not

Osteocalcin was positively correlated with PTH (Fig. 2), but not with 25-OH D, leptin, or IGF-I. PTH was significantly higher in the post-hibernation season than in the pre-hibernation (P=0.006) and hibernation (P=0.014) seasons. The increase in PTH during hibernation relative to pre-hibernation was not significant (P=0.35), possibly due to the low sample size (power=0.39). 25-OH D did not show seasonal variations (P=0.64).

Serum leptin and IGF-1concentrations showed seasonal variations

Serum leptin did not change during hibernation relative to

Table 2. *Mean serum metabolite concentrations for the three study periods*

Metabolite	Pre-hibernation	Hibernation	Post-hibernation
Osteocalcin (ng ml^{-1})	16.9±7.4^{a} (15)	70.4±43.1^{b} (22)	49.3±26.8^{b} (19)
Ionized calcium (nmol l^{-1})	0.709±0.150^{a} (12)	0.871±0.093^{b} (10)	0.960±0.014^{b} (2)
PTH (pg ml^{-1})	14.6±4.5^{a} (6)	25.3±27.3^{a} (14)	41.4±20.2^{b} (9)
25-OH D (nmol ml^{-1})	16.6±16.3 (9)	11.2±6.2 (9)	16.0±13.4 (9)
Leptin (ng ml^{-1})	4.0±0.7^{a} (15)	3.8±0.7^{a} (22)	3.1±0.7^{b} (19)
IGF-I (ng ml^{-1})	387±88^{a} (15)	209±52^{b} (22)	594±207^{c} (19)

PTH, parathyroid hormone; 25-OH D, 1,25 $(OH)_2$ vitamin D; IGF-I, insulin-like growth factor I.

Values are means ± s.d.; sample sizes in parentheses. For a given metabolite, values with the same superscript letter are not significantly (P<0.05) different. 25-OH D did not show significant seasonal differences. All of the metabolites were measured in the same serum samples, with the exception of ionized calcium.

pre-hibernation, but was significantly (P<0.004) lower during post-hibernation remobilization (Table 2). IGF-I significantly (P<0.0001) decreased during hibernation relative to pre-hibernation and reached its highest value during remobilization (Table 2).

PGE_2 release by MC-3T3 osteoblasts is affected by seasonal variations in bear serum

The amount of PGE_2 released by osteoblastic cells treated with bear serum *in vitro* was higher for treatment with post-hibernation serum compared to pre-hibernation serum (P=0.058) and hibernation serum (P=0.014), (Fig. 3). The PGE_2 release for cells treated with the hibernation serum was not significantly (P=0.48) different compared to the pre-hibernation serum. The seasonal changes in PGE_2 release showed trends similar to the seasonal changes in serum IGF-I.

Discussion

For many animals, mechanical unloading of bone decreases bone formation and/or increases bone resorption, resulting in net bone loss. However, both resorption and formation increase during canine forelimb immobilization, but significant bone loss occurs because of unbalanced increases in resorption over formation (Li et al., 2005; Yang Li et al., 2005). Likewise, high bone turnover occurs in patients with spinal cord injury, which leads to bone loss and increased fracture incidence (Modlesky et al., 2005; Pitsillides et al., 1995). Our previous analyses of serum remodeling markers suggested that bears may lose some bone during hibernation because of increased resorption and unchanged formation, but that they may be able to gain it back more quickly than other animals and thus prevent bone loss with aging despite long annual periods of inactivity (Donahue et al., 2003a; Donahue et al., 2003b). However, these conclusions were based on the mean seasonal values of the remodeling markers, not the relative, temporal changes in formation and resorption markers during hibernation. Our current analyses on serum markers of bone metabolism suggest that both resorption and formation increase during disuse in bears, with a normal lag time (i.e. reversal period) between resorption and formation. The increase in formation appears to remain coupled and balanced with the increase in resorption, which probably prevents bone loss during hibernation. This is corroborated by histological data from black bear iliac crest biopsies, which also showed increased resorption and formation activity during hibernation, and by the

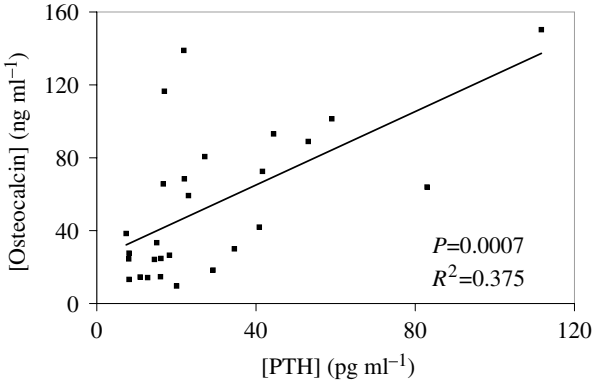

Fig. 2. Serum osteocalcin levels were significantly correlated with serum PTH levels for pre-hibernation, hibernation and post-hibernation. Samples were pooled (N=27).

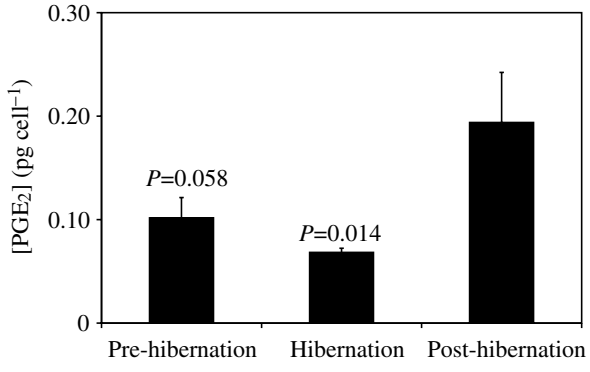

Fig. 3. The amount of PGE_2 released by osteoblastic cells was greatest when the cells were treated with serum collected in the post-hibernation period (N=9). P-values are for comparisons with the post-hibernation value. Pre-hibernation (N=8) and hibernation (N=7) values were not different from each other (P=0.48). Values are means ± s.e.m.

fact that bears do not lose bone volume or BMD during hibernation (Floyd et al., 1990; Pardy et al., 2004).

Inactivity during hibernation causes bone loss in ground squirrels, golden hamsters and little brown bats by increasing bone resorption and decreasing formation (Haller and Zimny, 1977; Kwiecinski et al., 1987; Steinberg et al., 1979; Steinberg et al., 1981; Steinberg et al., 1986). However, aestivation does not decrease bone strength or cross-sectional area in the long bones of green-striped burrowing frogs (*Cyclorna alboguttata*) (Hudson et al., 2004). After 9 months of aestivation there was no evidence of osteoclast activity; however, there was also no intracortical remodeling in non-aestivating frogs. Unfortunately, little is known about the endocrinology of aestivating frogs (Hudson et al., 2004), and thus the mechanisms that preserve bone during aestivation are unknown.

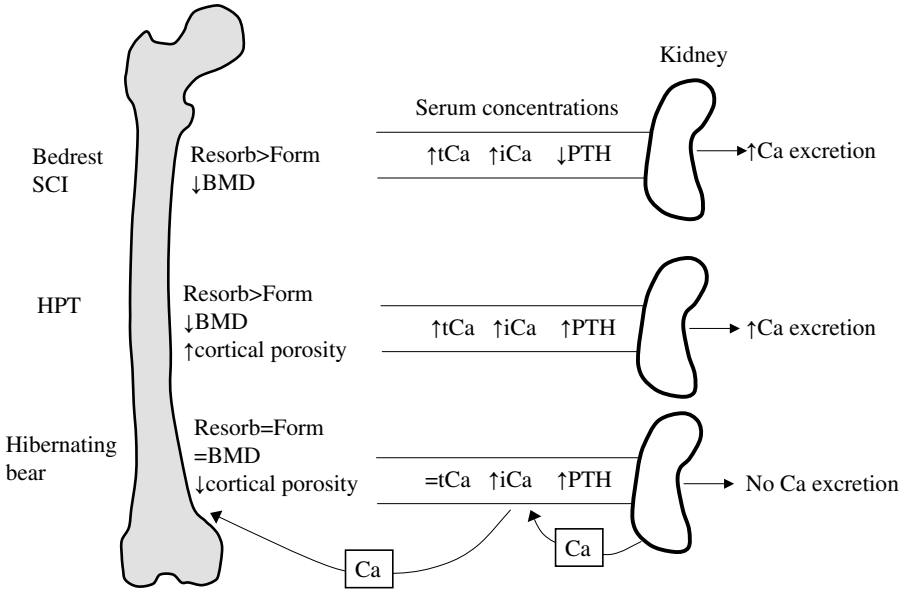

Fig. 4. Proposed differences in bone remodeling and calcium metabolism between human bedrest (Shackelford et al., 2004), spinal cord injury (SCI) (Maimoun et al., 2005; Modlesky et al., 2005), hyperparathyroidism (HPT) (Brockstedt et al., 1995; Monchik and Gorgun, 2004) and hibernating bears. There is increased remodeling activity in all four conditions; however, bone resorption (Resorb) and formation (Form) appear to be balanced in hibernating bears whereas they are unbalanced in the other three conditions. This imbalance leads to increased cortical porosity and decreased bone mineral density (BMD) and cortical thickness in humans. However, cortical porosity decreases and cortical cross-sectional area and mineral content are unchanged in hibernating bears (McGee et al., 2006). As in human inactivity, resorption increases and formation decreases in hibernating bats and hamsters, leading to increased cortical porosity and decreased cortical thickness (Kwiecinski et al., 1987; Steinberg et al., 1979; Steinberg et al., 1981; Steinberg et al., 1986). In the human conditions, calcium liberated by bone resorption is excreted in urine and increases total (tCa) and ionized (iCa) serum calcium concentration. Bears, however, do not urinate during hibernation and thus it is likely that the calcium liberated by bone resorption is recycled back into bone, which is possibly facilitated by PTH increasing renal reabsorption of calcium.

Bears have evolved many unique biological mechanisms to survive long periods of immobilization without food (Nelson, 1987). These mechanisms appear to include the recycling of calcium and other products of bone catabolism, since bears increase bone turnover but do not excrete waste during hibernation. In humans, bedrest-induced disuse osteoporosis is caused primarily by increased resorption without a corresponding increase in formation (Shackelford et al., 2004; Zerwekh et al., 1998). This results in hypercalcemia and a negative calcium balance brought about by increased urinary and fecal calcium. Since bears do not urinate or defecate during hibernation, it is probable that most of the calcium released from bone by resorption is recycled back into bone *via* osteoblastic bone formation. Similar extensive bone turnover and recycling was documented during prolonged fasting in northern elephant seals (*Mirounga angustirostris*) that remain active, but consume neither food nor water for up to 3 months (Patterson-Buckendahl et al., 1994). Ionized calcium did increase in bears by 23% during hibernation, possibly because of the lag time between resorption and formation. Paradoxically, PTH was highest when ionized calcium was highest (Table 2).

Hibernation is a mechanism to survive famine. Bears do not eat, drink, urinate, or defecate during hibernation (Nelson, 1973). Metabolic energy and water is derived from fat catabolism, and water is conserved by not urinating (Nelson, 1973). Urea, which is produced by protein catabolism and normally excreted in urine, does not increase in the blood of bears during hibernation despite the fact that they do not urinate (Nelson, 1978). During hibernation, bears recycle urea nitrogen by increasing protein synthesis (Nelson et al., 1983). Similar recycling mechanisms may help maintain collagenous bone formation; ICTP and PICP are collagen peptide fragments which were found to be increased, indicating an increased, but balanced bone turnover during hibernation (Fig. 1). Bone calcium is also likely to be recycled during hibernation (Fig. 4). The effect of PTH on the kidneys is to increase calcium reabsorption and decrease phosphate. This mechanism seems to be at work in hibernating bears since we found serum ionized calcium concentration to increase and serum phosphorous concentration has been shown to decrease during hibernation (Hellgren et al., 1993). PTH may also facilitate calcium recycling (*via* increasing bone formation) by acting directly on osteoblasts to increase differentiation and prevent apoptosis (Hock et al., 2001).

Numerous substances influence bone cell metabolism in concert with mechanical stimulation. Further complicating the regulation of bone cell metabolism is the fact that many

molecules (e.g. PTH, PGE_2, leptin) can exert both anabolic and catabolic influences. Cortisol, which inhibits osteoblast activity, increases in bears during hibernation (Donahue et al., 2003a). Here we found that IGF-I (which typically promotes bone formation) decreases during hibernation. Thus, it appears that changes in cortisol and IGF-I that occur during hibernation, which would be expected to reduce bone formation, are dominated by the anabolic influence of other molecules, since bone formation markers increase during hibernation. In the hierarchy of factors that regulate bone metabolism, those that regulate calcium concentration probably dominate, followed by the sex steroids and mechanical stimulation (Harada and Rodan, 2003). Since PTH is the main regulator of blood calcium concentration and can have potent anabolic effects on bone (Neer et al., 2001; Zanchetta et al., 2003), it is possible that PTH helps maintain bone formation in hibernating bears. The anabolic actions of PTH require IGF-I in normally loaded bone (Bikle et al., 2002; Kostenuik et al., 1999). However, even though PTH reduces bone loss during disuse (Halloran et al., 1997; Turner et al., 1998), unloaded bone does not respond to the anabolic actions of IGF-I (Bikle et al., 1994), raising the possibility that the anabolic actions of PTH occur via a mechanism independent of IGF-I during disuse. This is consistent with our findings that serum concentration of the bone formation marker osteocalcin increased during hibernation despite decreased IGF-I (Table 2), and that serum PTH was positively correlated with osteocalcin (Fig. 2). These findings support the idea that PTH may have a dominant role in preserving bone formation in hibernating bears, possibly by stimulating osteoblast differentiation and inhibiting osteoblast apoptosis (Hock et al., 2001).

The anabolic effects of PTH may be enhanced when physical activity is resumed following arousal from hibernation. Mechanical loading and PTH act synergistically to increase bone formation *in vivo* and biochemical signaling *in vitro* (Ma et al., 1999; Ryder and Duncan, 2000). During remobilization in the spring, bone formation, as indicated by serum osteocalcin, remains higher than during pre-hibernation periods. Serum levels of PTH and IGF-I are also highest in the post-hibernation period, and serum from this season induced the greatest PGE_2 release from osteoblasts in vitro. These findings are consistent with the idea of PTH inducing bone formation *via* mechanisms involving IGF-I and PGE_2 (Bakker et al., 2003; Bikle et al., 2002; McCarthy et al., 1991). PTH stimulates PGE_2 production in osteoblasts and exogenous PGE_2 prevents disuse-induced bone loss by activating more formation than resorption (Akamine et al., 1992; Bakker et al., 2003). PGE_2 stimulates IGF-I production (McCarthy et al., 1991), which can increase collagen production (Canalis et al., 1989). This raises the possibility that the anabolic actions of PTH occur via different mechanisms during periods of normal mechanical loading compared to periods of unloading, since IGF-I was lowest during hibernation when osteocalcin was highest.

Sympathetic tone also has potent effects on bone metabolism and may contribute to the regulation of bone remodeling in bears. During hibernation, resting heart rate drops to approximately one-fourth to one-half of non-hibernating rates, indicating low sympathetic tone during hibernation (Hellgren, 1998; Nelson et al., 2003). Low intracerebroventricular leptin decreases sympathetic tone, which increases bone formation and decreases resorption (Elefteriou et al., 2005; Takeda et al., 2002). We found that serum leptin did not change between pre-hibernation and hibernation; however, it was significantly lower during remobilization following hibernation. Similar seasonal changes have been reported in brown bears (Hissa et al., 1998). This finding suggests that leptin is not involved in the increased bone turnover that occurs during hibernation. However, decreased serum leptin during remobilization following arousal from hibernation may promote bone formation and decrease resorption, assuming there is a corresponding decrease in intracerebroventricular leptin.

Although our findings on seasonal changes in serum metabolites and their effects on in vitro osteoblast metabolism are provocative, they are somewhat limited because a complete complement of measurements was not made on all serum samples in a given year because of volume limitations. However, for the bears studied, hibernation starts and stops within a few days of the same date each year because hibernation is regulated by the availability of food supplies in the captive facility. Furthermore, some molecules (e.g. ICTP and leptin) have been measured over a number of years in different groups of bears and have shown strikingly similar seasonal variations from year to year. Another limitation is that we have focused our discussion on how inactivity may mediate changes in bone metabolism. However, the bears are also anorectic during hibernation and significant bone loss occurs in humans with anorexia nervosa (Legroux-Gerot et al., 2005). In these patients, serum markers of formation are markedly decreased and resorption markers are increased, explaining why up to 40% of women with anorexia nervosa become osteoporotic. Thus, it is even more remarkable that bears can maintain bone mass during hibernation (Pardy et al., 2004) since they are presented with nutritional challenges as well as challenges resulting from mechanical unloading.

Since disuse-induced bone loss is largely caused by increased bone resorption, anti-resorption drugs (i.e. bisphosphonates) are a logical prophylactic treatment. However, bisphosphonates do not prevent disuse induced loss of BMD following spinal cord injuries (Nance et al., 1999). Furthermore, treating dogs with bisphosphonates had little effect on mitigating disuse-induced bone loss (Yang Li et al., 2005). An alternative approach to treating disuse osteoporosis is to increase bone formation to preserve balance between bone resorption and formation. Hibernating bears may do just that during hibernation, possibly in part through the anabolic actions of PTH. Indeed, recombinant PTH reduces fracture risk by increasing bone mineral density in postmenopausal women (Neer et al., 2001). A more thorough understanding of the mechanisms that maintain osteoblastic bone formation in

hibernating bears may provide insight for treating disuse osteoporosis.

List of abbreviations

25-OH D	1,25 $(OH)_2$ vitamin D
BMD	bone mineral density
ELISA	enzyme-linked immunosorbent assay
ICPT	C-terminal cross-linked telopeptide of type 1 procollagen
IGF-I	insulin-like growth factor I
	prostaglandin E_2 (PGE_2)
PTH	parathyroid hormone
PICP	C-terminal propeptide of type 1 procollagen
TFA	trifluoroacetic acid

This work was supported by a grant from NIH (NIAMS AR050420) and a gift from Timothy Floyd, M.D. to S.W.D., and grants from NSF (IBN-0343515) and NIH (NIA R21 AA 14399-01A2) to P.P.-B. Leptin, IGF-I, PICP, ICTP and ionized calcium levels were determined in the Core Endocrine Laboratory at the Penn State University Hershey Medical Center.

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