Proteasomal Degradation of Runx2 Shortens Parathyroid Hormone-induced Anti-apoptotic Signaling in Osteoblasts

A PUTATIVE EXPLANATION FOR WHY INTERMITTENT ADMINISTRATION IS NEEDED FOR BONE ANABOLISM

It is unknown why sustained elevation of parathyroid hormone (PTH) stimulates bone resorption, whereas intermittent administration stimulates bone formation. We show in mice that daily injections of PTH attenuate osteoblast apoptosis, thereby increasing osteoblast number, bone formation rate, and bone mass, but do not affect osteoclast number. In contrast, sustained elevation of PTH, achieved either by infusion or by raising endogenous hormone secretion with a calcium-deficient diet, does not affect osteoblast apoptosis but increases osteoclast number. Attenuation of apoptosis by PTH in cultured osteoblastic cells requires protein kinase A-mediated phosphorylation and inactivation of the pro-apoptotic protein Bad as well as transcription of survival genes, like Bcl-2, mediated by CREB (cAMP response element-binding protein) and Runx2. But, PTH also increases proteasomal proteolysis of Runx2. Moreover, the anti-apoptotic effect of PTH is prolonged by inhibition of proteasomal activity, by overexpressing a dominant negative form of the E3 ligase (ubiquitin-protein isopeptide ligase) that targets Runx2 for degradation (Smurf1), or by overexpressing Runx2 itself. The duration of the anti-apoptotic effect of PTH, thus, depends on the level of Runx2, which in turn is decreased by PTH via Smurf1-mediated proteasomal proteolysis. The self-limiting nature of PTH-induced survival signaling might explain why intermittent administration of the hormone is required for bone anabolism.

Cyclic activation of cell surface receptors often leads to a different biologic response than sustained activation. A classic example is the stimulation of luteinizing hormone and follicle stimulating hormone production by pituitary gonadotroph cells in response to pulsatile gonadotropin releasing hormone (GnRH) versus the inhibition of hormone production by continuous GnRH treatment (1). The differential response of the skeleton to intermittent versus continuous elevation of parathyroid hormone (PTH) is another long known example with important clinical and therapeutic implications. Indeed, chronic elevation of the hormone as in primary or secondary hyperparathyroidism stimulates bone resorption leading to bone loss (2, 3). In contrast, intermittent administration of PTH stimulates new bone formation by increasing osteoblast number (4). Heretofore, a mechanistic explanation for the dependence of this effect on repeated transient increases in PTH has remained unknown. Albeit, intermittent PTH administration by daily injections was recently approved by the United States Food and Drug Administration as the first form of osteoporosis therapy that increases bone mass de novo, reverses the bone deficit, and dramatically reduces the incidence of fractures (5).

We had previously shown that daily injections of PTH to mice for 28 days reduces the prevalence of osteoblast apoptosis and increases the number of osteoblasts (6), providing an explanation for the increase in bone formation rate and bone mineral density (BMD) seen with intermittent PTH administration. In the studies reported herein we show that in contrast to daily injections, sustained elevation of PTH in mice does not reduce the prevalence of osteoblast apoptosis but, as expected, stimulates osteoclastogenesis. Furthermore, we show that the anti-apoptotic effect of PTH requires runt related transcription factor 2 (Runx2)-mediated transcription of survival genes like Bcl-2 and is short-lived because PTH itself also stimulates the proteasomal degradation of Runx2 (7), as known as Cbfa1, Osf2, Aml3, and PEBP2αA). The self-limiting nature of PTH-induced survival signaling suggests a mechanistic explanation for the necessity of intermittent administration to elicit the bone anabolic properties of this hormone.

EXPERIMENTAL PROCEDURES

Animal Studies—Female Swiss-Webster mice (4–6 months old, Harlan, Indianapolis, IN) were maintained and used in accordance with National Institutes of Health guidelines. The Institutional Animal Care and Use Committees of the University of Arkansas School for Medical Sciences and the Central Arkansas Veterans Healthcare System approved the animal protocols. Mice were fed standard rodent diet (Agway

□ The abbreviations used are: PTH, parathyroid hormone; BMD, bone mineral density; Runx2, runt related transcription factor 2; dn, dominant negative; nGFP, enhanced green fluorescent protein containing a nuclear localization sequence; DBA, dibutyryl-cAMP; IGF-1, insulin-like growth factor 1; wt, wild type; CREB, CAMP response element-binding protein; ANOVA, analysis of variance; RANKL, receptor activator of NF-κB ligand; PKA, protein kinase A; Smurf, Smad ubiquitin regulatory factor; IL, interleukin; LIF, leukemia inhibitory factor; MEEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; Z-E(Or-Bu)AL-pNA, benzoyloxy carbonyl-Glu(O-t-butyl)-Ala-Leu-p-nitroanilide.
RMH 3000; Arlington Heights, IL) containing 22% protein, 0.97% calcium, and 0.01% phosphorous. The age of the animals used in each experiment varied by 2 weeks or less, and mice with a body weight 10% higher or lower than the mean of the group were excluded. Mice were given daily subcutaneous injections of human PTH-(1-34), human PTH-(1-84) (Bachem California, Inc., Torrance, CA) or vehicle (0.9% saline, 0.01 mM β-mercaptoethanol, 0.1% acetic acid) as described (6). Alternatively, mice were infused with PTH-(1-84) using 3- or 5-day micro-osmotic pumps delivering hormone at 1 or 0.5 μl/h, respectively (Durect Corp., Cupertino, CA). Pumps were implanted into an interscapular subcutaneous pocket under Metaphane anesthesia as previously described (7). There was no effect of PTH administration whether by injection or infusion on body weight (not shown). In the experiments shown in Fig. 1, treatment mice were euthanized 60% as determined by the number of cells labeled with nGFP. 60% as determined by the number of cells labeled with nGFP. 60% as determined by the number of cells labeled with nGFP. 60% as determined by the number of cells labeled with nGFP.

Cell Cultures and in Vitro Quantification of Apoptosis—HeLa cells, osteoblastic OB-6 cells, and osteoblastic cells from neonatal murine calvaria were prepared and used as described (12–14). Calvaria cells from fos-deficient mice (15) were provided by L. McCauley, University of Texas, TX; and wild-type siblings were prepared from neonatal mice from our breeding colony of B6;129S2-Bcl-x<sup>tm1Sjk</sup> heterozygotes (The Jackson Laboratory, Bar Harbor, ME). The genotype of each mouse was established by the presence of neo and Bcl-2 sequences by PCR of tail genomic DNA and confirmed by Western blotting of calvaria cell lysates. OB-6 cells harboring the rTA/TET-OPF) protein were infected with a tetra-cycline-regulated retroviral construct to conditionally express dominant negative (dn) Runx2 (16).

Cells were seeded at 1.5 × 10<sup>4</sup>/cm<sup>2</sup> and maintained in the presence of α-minimal essential medium supplemented with 10% fetal bovine serum (calvaria) or 2% fetal bovine serum (OB-6 cells) for 18–24 h before the addition of PTH, PTHrP, or pro-apoptotic stimuli. The effector stimulus, for example, TNFα, was present for 40–60% as determined by the number of cells labeled with nGFP. Because apoptosis is only determined in the transfected cells, this approach has the advantage of being independent of differences in the efficiency of transfection among experiments and eliminates the contribution of untransfected cells that would otherwise mask the effect of the concentration of PTH. At least 250 transfected cells were evaluated, and the identity of the samples was unknown to the person scoring the live and dead cells. Experiments were repeated at least once and assessed by a different blinded reader. Critical findings were confirmed by in situ nick-end labeling, measurement of caspase-3 activity, or immuno-
Chicago, IL). Data were analyzed by t test or one-way analysis of variance (ANOVA) after establishing normal distribution of data and homogeneity of variances. Significance values were adjusted with Bonferroni’s correction. Pearson Product moment analysis was used to detect relationships between osteoblast apoptosis and indices of bone formation. For longitudinal analysis of the effect of PTH on BMD, a mixed-effects longitudinal ANOVA model was used with SAS software (SAS Institute Inc., Cary, NC) to allow specification of the covariance structure.

RESULTS

The Prevalence of Osteoblast Apoptosis Is Inversely Related to Bone Formation in Mice Receiving Intermittent PTH—To firmly link suppression of osteoblast apoptosis and the stimulation of bone formation during intermittent PTH administration, we sought evidence for the correlation of the two phenomena in dose-response- and time-course studies in mice. In these studies we used 3–300 ng PTH-(1–34)/g to determine the minimum effective dose requirement for the anabolic effect of PTH in mice and also to eliminate the possibility that the anti-apoptotic effect of PTH was a phenomenon only associated with the 400 ng/g dose we had used in our earlier work (6). Daily injection of as little as 30 ng of PTH-(1–34)/g for 28 days increased hindlimb and spine BMD. These changes were associated with a reduction in osteoblast apoptosis at both sites as well as an increase in the level of serum osteocalcin, a biochemical index of osteoblast number (Fig. 1a). At the same dose that PTH inhibited osteoblast apoptosis, it also increased osteoblast number, bone formation rate, and the amount of cancellous bone in the distal femur, but the number of osteoclasts was not affected at any dose examined (not shown). The prevalence of osteoblast apoptosis exhibited a strong inverse correlation with three independent measures of bone formation, serum osteocalcin, bone formation rate, and osteoblast number (Fig. 1a). Temporal correlation between osteoblast apoptosis and bone formation was demonstrated in a separate experiment in which mice were given daily injections of 200 ng of PTH-(1–34)/g for 7, 14, or 28 days (Fig. 1b). Spine and hindlimb (not shown) BMD increased time-dependently in the PTH-treated mice, and this increase became statistically significant at days 14 and 28. A decrease in osteoblast apoptosis and an increase in osteoblast number in vertebral bone along with an increase in serum osteocalcin were evident as early as 7 days in mice receiving PTH, as compared with the vehicle controls. These changes persisted throughout the course of the experiment.

Sustained PTH Elevation Does Not Affect Osteoblast Apoptosis—We next compared the effects of sustained versus intermittent PTH elevation on the prevalence of osteoblast apoptosis and on the number of osteoblasts and osteoclasts at 2, 4, and 6 days (Fig. 1c). In this experiment we used human PTH-(1–84), the full-length PTH molecule that has identical biological effects as the 1–34 peptide but, unlike 1–34, can be quantified using a commercially available immunoassay. Infusion of 140 ng of PTH-(1–84)/h raised the serum level of the hormone from 13 ± 7 pg/ml to 160 ± 54, 164 ± 56, and 201 ± 66 pg/ml as determined at 2, 4, or 6 days after initiation of the infusion. Sustained elevation of PTH caused an increase in the number of osteoclasts at day 2 and was followed by an increase in osteoblast number at day 4, consistent with the expected increase in bone remodeling by continuous PTH elevation (Fig. 1c and Supplemental Fig. 2). The level of serum pyridinoline cross-links, a biochemical marker of bone resorption, was also elevated by day 6 (Fig. 1c). Consistent with previous findings in the rat (31), 6 days of PTH infusion at this dose did not affect vertebral cancellous bone area (not shown). Strikingly, sustained elevation of PTH had no effect on the prevalence of osteoblast apoptosis at any time during the experiment (Fig. 1c), whereas daily injections of 230 ng PTH-(1–84)/g reduced osteoblast apoptosis and increased osteoblast number beginning on day 2. Osteoclast number and pyridinoline cross-links were unchanged by intermittent PTH. Importantly, the increase in osteoblast number seen with daily injections was significantly greater and occurred earlier than that caused by sustained infusion of PTH. Consistent with these differences and the association of increased osteoblast number with reduced apoptosis only in the case of the daily injections, 6 days of intermittent PTH increased the amount of bone made by each osteoblast team (wall width), but infused PTH did not (Fig. 1c). Peritrabecular fibrosis was not observed in this experiment (not shown).

Confirmation of the inability of sustained PTH elevation to affect osteoblast apoptosis was provided in an additional experiment whereby infusion of 500 ng of PTH-(1–84)/h resulted in higher circulating levels (453 ± 120 pg/ml). Yet there was no effect on osteoblast apoptosis after 6, 18, or 48 h (Fig. 1d). By contrast, sustained elevation of PTH induced a progressive increase in the mRNA level of RANKL, the cytokine that mediates the pro-osteoclastogenic effect of PTH (16). As previously reported in the rat (31), infusion of PTH at this high level caused hypercalcemia and peritrabecular fibrosis (not shown) and led to mortality beginning on day 4. Elevation of endogenous PTH-(1–84) from 18 ± 4 to 54 ± 8 pg/ml by feeding mice a calcium-deficient diet for 7 days (a model of secondary hyperparathyroidism) also failed to affect the prevalence of apoptotic osteoclasts in vertebral bone (normal diet: 7.4 ± 2.4%; calcium-deficient diet: 7.2 ± 2.0%, n = 5 per group) even though it increased bone remodeling and caused bone loss (not shown).

PTH Suppression of Osteoblast Apoptosis Is Short-lived—The demonstration of the anti-apoptotic effect of PTH with repeated injections, but not sustained elevation, led us to suspect that sustained elevation may have self-limited the effect of the hormone, as for example occurs with the effect of PTH on CAMP generation resulting from receptor desensitization. Therefore, we proceeded to determine the duration of the anti-apoptotic effect of PTH in osteoblastic cells and its determinants in vitro. Treatment of calvaria-derived cells with 50 nM PTH-(1–34) starting 1 h before exposure of the cells to the pro-apoptotic agent etoposide protected cells from apoptosis when the phenomenon was measured at 6 or 12 h after the addition of this agent. However, the anti-apoptotic effect of the hormone was no longer evident at 24 h, even though PTH was present during the entire period of the experiment (Fig. 2d). In a separate experiment, PTH protected against apoptosis if added to the calvaria cells 1–3 h before the addition of etoposide, but this protective effect was lost if PTH was added 5–24 h before etoposide, clearly demonstrating that the anti-apoptotic action of the hormone was short-lived (Fig. 2c). In contrast to the short-lived survival signaling, the PTH-induced increase in RANKL mRNA was maintained for 48 h (Fig. 2c).

The short duration of PTH-induced anti-apoptosis was reproduced in a murine bone marrow-derived osteoblast-like cell line, OB-6 (13) (Fig. 2, d and e). Using this cell line, we also demonstrated that 1 h of exposure to PTH was sufficient to trigger survival signals, as evidenced by retention of the anti-apoptotic effect when the hormone was removed from the cultures just before the addition of etoposide (Fig. 2e) or cell detachment (Supplemental Fig. 3c) or when further PTH receptor signaling was blocked with the competitive antagonist PTH-(3–34) (not shown). PTH, however, did not protect against apoptosis if added simultaneously with, or 3 h after etoposide. As in the case of PTH, the stable CAMP analog DBA produced a short-lived anti-apoptotic effect (Fig. 2, d and e), indicating that PTH receptor desensitization or intracellular CAMP degradation did not shorten survival signal-
FIG. 1. Effect of daily injection or sustained elevation of PTH on bone formation, osteoblast apoptosis, and bone turnover. Swiss-Webster female mice (4–8 per group) were injected daily for 28 days with vehicle (V) or 3–300 ng/g/day hPTH-(1–34) (a), with vehicle or 200 ng/g/day hPTH-(1–34) for 7, 14, or 28 days (b), left untreated (basal) or given hPTH-(1–84) by either continuous infusion using micro-osmotic pumps (140 ng/h, continuous) or by daily injections (230 ng/g/day, intermittent) for 2, 4, or 6 days (c), or infused with 500 ng PTH(1–84)/h for 6, 18, or 48 h (d). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *, p < 0.05 versus vehicle by mixed effects longitudinal ANOVA (a) or by AVOVA (b–d). In panel a, bone formation rate, osteoblast (Ob) number, and bone area per tissue area were determined in femoral sections. In the regression plots of this panel, each point corresponds to the value of serum osteocalcin, bone formation rate, or osteoblast number for each animal plotted as a function of the prevalence of osteoblast apoptosis in a femoral bone section in that animal. Linear regression lines (95% confidence intervals) are shown. Pearson Product moment analysis was used to determine the strength of the correlation (r) and its significance level (p < 0.0001). Vertebral sections were used for histologic measurements shown in panels b–d (Oc, osteoclast). Representative photomicrographs depicting the changes in osteoclast and osteoblast number as well as osteoblast apoptosis are shown in Supplemental Fig. 2. The table in panel c lists values for wall width and the level of serum pyridinoline cross-links (pyr) in animals that were untreated or given PTH for 6 days. In panel d, RANKL mRNA in tibial bone was quantified by RNase protection.
ing. Similar results were obtained when cell detachment (anoikis) or dexamethasone were used to stimulate apoptosis (Supplemental Fig. 3, c, e, and f). Medium from cells incubated for 20 h with PTH or DBA was fully capable of preventing etoposide-induced apoptosis when added to another set of osteoblastic cells (Supplemental Fig. 3g). Thus, the short duration of PTH-induced attenuation of apoptosis was not due to loss of PTH or DBA bioactivity.

**The Anti-apoptotic Effect of PTH Requires Protein Kinase A (PKA)-mediated Non-transcriptional and Transcriptional Events and the Participation of Runx2.** Secreted factors did not contribute to the anti-apoptotic effect of PTH on cultured
FIG. 3. The anti-apoptotic effect of PTH is not due to a secreted factor. a, conditioned medium from OB-6 cells cultured for 4 h with either vehicle (veh) or with 50 nM PTH-(1–34) (PTH) was added to OB-6 cells alone or with 10 µM PTH-(3–34) to block the effect of the PTH remaining in the conditioned medium and allow detection of the effect of a putative secreted anti-apoptotic factor(s). One hour later apoptosis was induced by the addition of etoposide, and apoptosis was quantified by trypan blue staining after 6 h. b, OB-6 cells transfected with the indicated constructs (along with nGFP) were exposed to PTH for 1 h followed by a 6-h treatment with etoposide. Apoptosis was evaluated by nuclear morphology of fluorescent cells. c, osteoblastic cells derived from calvaria were exposed to PTH for 1 h in the absence or presence of 20 µg/ml blocking antibodies to IL-6, IL-11, or LIF or 0.4 µg/ml IGF-binding protein 4. These amounts block the activity of ~1 nM cytokine or IGF-1. Apoptosis was induced by the addition of 100 nM dexamethasone and quantified by trypan blue uptake. d, OB-6 cells were pretreated with the IGF-1 signaling inhibitor
osteoblastic cells. Thus, conditioned medium from cells treated with PTH-(1–34) failed to prevent etoposide-induced apoptosis when the actions of the hormone remaining in the conditioned medium were blocked with PTH-(3–34) (Fig. 3a). Moreover, inhibition of IL-6 type cytokines or IGF-1 signaling did not interfere with the anti-apoptotic effect of PTH (Fig. 3, b–e). Therefore, we proceeded to elucidate the PTH-stimulated intracellular signaling pathways responsible for promoting the survival of osteoblastic OB-6 cells. In view of evidence that DBA mimics the time course and potency of the inhibitory effect of PTH on apoptosis (Fig. 2, d and e), we focused on the role of cAMP-activated kinases. The anti-apoptotic effect of PTH was blocked by the PKA inhibitors H89 and RpCAMP (Fig. 4a). Even though PTH stimulated phosphorylation of extracellular signal regulated kinases, neither de MEK (the extracellular signal regulated kinase kinase) nor the MEK inhibitor PD98059 blocked the anti-apoptotic effect of PTH (Fig. 4b). Furthermore, sphingosine phosphate did not mimic the anti-apoptotic effect of PTH, indicating that sphingosine kinase was also not involved in this action (not shown). Thus, of the known kinases stimulated by PTH-induced cAMP, only PKA is evidently required for the anti-apoptotic effect of the hormone.

PKA phosphorylates Bad and thereby inactivates its pro-apoptotic function by facilitating interaction with 14-3-3, an important protein that sequesters proteins containing phosphoserine (19, 32). As shown in Fig. 4, c and d, PTH-treated OB-6 cells exhibited a transient increase in Ser112-phospho-Bad that was blocked by H89 or RpCAMP. Furthermore, Bad mutants containing an alanine for serine substitution at positions 136, 155, or both prevented the ability of PTH to block apoptosis stimulated by etoposide (Fig. 4e) or by anokins (Supplemental Fig. 4e). Alanine substitution at position 112Ser in Bad on the other hand did not abrogate the effect of PTH, consistent with the fact that 112Ser phosphorylation does not directly promote interaction of Bad with 14-3-3 (19, 32). None of the Bad mutants affected viability of cells cultured under basal conditions (not shown).

Besides Bad phosphorylation, the anti-apoptotic effect of PTH required RNA synthesis, as indicated by the abrogation of the effect of actinomycin D in OB-6 cells (Fig. 5c). Based on this we proceeded to examine the role of several transcription factors. PTH induced a rapid and transient phosphorylation of CREB (Fig. 5b), and dn CREB blocked the anti-apoptotic effect of PTH (Fig. 5c), indicating a role of this transcription factor in the anti-apoptotic effect. Other transcription factors known to be activated by PTH, including Fos, Jun, CCAAT/enhancer-binding protein β, and Elk-1 (33), were not required. The dispensability of Fos was confirmed using osteoblastic cells derived from Fos-deficient mice. Strikingly, a dn mutant of the osteoblast-specific transcription factor Runx2 also blocked the anti-apoptotic effect of PTH (Fig. 5c). dn CREB and dn Runx2 also abolished the anti-apoptotic effect of DBA, excluding the possibility that the effect of the mutants resulted from a reduced number of PTH receptors or interference with cAMP production (Fig. 5c). Thus, besides its essential role in the differentiation of osteoblasts (21), Runx2 may also play a role in their survival. The unexpected requirement for Runx2 was confirmed in HeLa cells that lack this transcription factor. As shown in Fig. 5d, PTH inhibited apoptosis only in HeLa cells co-transfected with Runx2 and the PTH receptor, and DBA inhibited apoptosis in cells transfected only with Runx2. The ability of PTH to inhibit apoptosis showed the same transcription factor dependence (Supplemental Fig. 4).

Activation of the PTH receptor in growth plate chondrocytes stimulates the synthesis of the anti-apoptotic protein Bcl-2 (34). We, therefore, examined whether PTH-induced synthesis of Bcl-2 is involved in suppression of osteoblast apoptosis. PTH caused a 40–60% increase in the level of Bcl-2 in OB-6 cells, reaching a peak level 2–4 h after the addition of PTH and declining to basal levels thereafter (Fig. 6b). A similar response was obtained with osteoblastic calvaria cells (not shown). The anti-apoptotic effect of PTH as well as the stimulatory effect of the hormone on both Bcl-2 mRNA and protein was dependent on Runx2 because conditional expression (TET-OFF) of dn Runx2 in OB-6 cells prevented these responses (Fig. 6, b–d). It is unknown whether the recently reported PTH-induced increase in Runx2 transcriptional activity is involved in up-regulation of Bcl-2 synthesis or inhibition of osteoblast apoptosis (35).

We next examined the role of Bcl-2 in the anti-apoptotic effect of PTH using osteoblastic cells from calvaria of mice genetically deficient in this protein. PTH failed to prevent either etoposide- or dexamethasone-induced apoptosis in osteoblastic cells from Bcl-2 null mice (Fig. 6e). Thus, Bcl-2 is critical for the anti-apoptotic effect of the hormone in osteoblasts.

The Duration of the Anti-apoptotic Effect of PTH Is Limited by PTH-induced Proteasomal Degradation of Runx2—Modulation of proteasomal proteolysis is known to regulate the duration of intracellular signaling after receptor activation (36, 37). Moreover, it is known that PTH stimulates proteasomal degradation of Runx2 (38) and that cAMP promotes CREB degradation (39). We, therefore, examined the role of proteasomal proteolysis in the PTH-induced suppression of apoptosis. Blockade of proteasomal degradation with lactacystin (40) prolonged the anti-apoptotic effect of PTH to 24 h, whereas the proteasome activator Z-E(Ot-Bu)AL-pNA (41) completely prevented PTH suppression of apoptosis (Fig. 7a). Notably, neither inhibition nor stimulation of proteasomal activity affected the basal viability of OB-6 osteoblastic cells. Thus, the level of key mediators of PTH-induced survival, the survival proteins themselves, or both must be modulated by the proteasome. dn Runx2, but not dn CREB or dn Bad, blocked the anti-apoptotic effect of PTH in the presence of lactacystin at 24 h (Fig. 7b), suggesting that the prolongation of survival signaling is due to prevention of Runx2 degradation. In agreement with this, 3 h of PTH treatment caused a decline in the amount of Runx2 and a corresponding increase in ubiquitinated Runx2 marked for degradation (Fig. 7c). At 6 h, both total and ubiquitinated Runx2 were decreased below basal levels, indicating that the ubiquitinated Runx2 had been degraded. Lactacystin increased both total and ubiquitinated Runx2 and prevented the PTH-induced decline in both forms of Runx2.

The E3 ligase (ubiquitin-protein isopeptide ligase) responsible for targeting Runx2 for proteasomal degradation is Smurf1, which binds to a PY motif in the C-terminal domain of Runx2 (23, 42). Overexpression of Smurf1, but not the related ligase Smurfl, blocked the pro-survival effect of PTH at 6 h, and this action was reversed by lactacystin. Conversely, a dn Smurf1 lacking ligase activity extended the anti-apoptotic effect of PTH to 24 h (Fig. 7d). As was the case with lactacystin, the prolong-
The anti-apoptotic effect of PTH requires PKA-dependent phosphorylation of Bad. OB-6 cells transfected with the indicated constructs (along with nGFP) or treated with the indicated inhibitors for 30 min were exposed to 50 nM PTH for 1 h followed by 6 h of treatment with etoposide. For the evaluation of extracellular signal regulated kinase phosphorylation (b), cells maintained in the absence of serum for 5 h were treated for 30 min with 50 μM PD98059 followed by 15 min of treatment with PTH-(1–34). Ser155-phosphorylated Bad ([155S]pBAD), and total Bad were determined by Western blot analysis of lysates of OB-6 cells treated with PTH-(1–34) for the indicated times (c) or with 10 μM H-89 or 50 μM RpcAMP for 1 h followed by 45 min with PTH-(1–34) (d). Phosphorylated Bad/Bad levels are expressed as the fold increase induced by PTH over the respective vehicle control without (−) or with H-89 or RpcAMP. Apoptosis was determined by trypan blue staining (a and the left panel of b) or by nuclear morphology (b, right panel, and e). Data shown represent the mean (±S.D.) of triplicate determinations. *, p < 0.05 versus cells maintained in the absence of etoposide, determined by ANOVA.

Runx2-(1–443), which lacks the PY motif for targeting Runx2 for degradation but retains transcriptional activity (42), also prolonged the anti-apoptotic effect of PTH (Fig. 7g). More important, whereas co-transfection of Smurf1 blocked the prolonging effect of Runx2 on PTH-induced suppression of apoptosis, it did not affect the ability of Runx2-(1–443) to extend the anti-apoptotic
FIG. 5. PTH suppression of apoptosis requires the transcriptional activity of CREB and Runx2.  

*a*, OB-6 cells treated with vehicle or 0.2 μM actinomycin D for 30 min were exposed to 50 nM PTH[1-34] for 1 h followed by the addition of 50 μM etoposide for 6 h. Actinomycin D treatment inhibited ~90% of RNA synthesis, as indicated by [3H]uridine incorporation (vehicle, 118,300 ± 18,600 cpm/mg of protein; actinomycin D: 12,600 ± 6,700 cpm/mg of protein).  

*b*, Ser133-phospho-CREB (pCREB) and total CREB were determined by Western blot analysis. OB-6 or HeLa cells transfected with the indicated constructs (along with nGFP) or calvaria cells from wt or fos<sup>−/−</sup> mice (c) were treated with vehicle, 50 nM PTH, or 100 μM DBA for 1 h followed by 6 h of treatment with etoposide. Apoptosis was evaluated by trypan blue staining (a and the right panel of c) nuclear morphology or by nuclear morphology (c, left panel, and d). Data shown represent the mean ± S.D. of triplicate determinations.  

*, p < 0.05 versus cells maintained in the absence of etoposide, determined by ANOVA. PTHR, PTH receptor.
Fig. 6. PTH-stimulated increase in Bcl-2 is required for the anti-apoptotic effect of PTH. a, effect of 50 nM PTH on the level of Bcl-2 protein in OB-6 cells, determined by Western blotting. The bar graph shows the mean (±S.D.) -fold change in Bcl-2 relative to baseline in 3 experiments. b–d, OB-6 cells conditionally expressing dn Runx2 were cultured in the presence (+) or absence (dn Runx2) of 100 ng/ml doxycycline.
effect of the hormone. The prolongation of the anti-apoptotic effect of PTH by lactacystin or by overexpression of dn Smurf1, Runx2, or Runx2 (1–433) was confirmed by active caspase-3 immunostaining of cells co-transfected with nGFP (Supplemental Fig. 5).

Taken together with earlier evidence that Smurf1 targets Runx2 to the proteasome (42), the above findings demonstrate that PTH-stimulated Smurf1-mediated Runx2 degradation limits the duration of the anti-apoptotic signaling of the hormone. Moreover, increasing the level of Runx2 by inhibition of its proteasomal degradation or by overexpression it is sufficient for producing a long-lived suppression of osteoblast apoptosis in response to PTH.

**DISCUSSION**

**Attenuation of Osteoblast Apoptosis Plays a Major Role in the Anabolic Effect of PTH**—We previously estimated that 60–80% of osteoblasts in the trabecular bone of rodents and humans die by apoptosis and that the average life span of osteoblasts during murine bone remodeling is 6–8 days (14, 43). Moreover, we had provided evidence that attenuation of osteoblast apoptosis by daily injections of PTH in mice accounts, at least in part, for the increased number of osteoblasts and, thereby, the increased bone formation produced by this regimen (6). In support of this earlier evidence we demonstrate here that the anti-apoptotic effect of intermittent PTH administration occurs with daily doses of as little as 10 ng/g and that the magnitude of the hormonal effect on apoptosis in vivo is indeed dependent on the dose of the daily injection. More important, we document that the prevalence of osteoblast apoptosis is a critical determinant of the number of osteoblasts, the level of serum osteocalcin, and the rate of bone formation because it quantitatively and temporally correlates with these key indices of new bone formation.

An impact of PTH on apoptosis in the present studies was observed as early as after only two daily injections. After 4 daily injections there was a 50% reduction in the prevalence of apoptosis, and at that time the number of osteoblasts had increased by 2-fold. These changes could have only occurred if a large portion of the existing osteoblasts were destined to die by apoptosis during this relatively short period of time. Consistent with this, ~10% of osteoblasts in the untreated control mice exhibited features of apoptosis, as measured by in situ nick-end labeling, a highly sensitive technique that detects as few as 1000 DNA strand breaks, which can develop 24–48 h before the nuclear and cellular degenerative signs of apoptosis are fully manifest (44). Our in vitro experiments (Fig. 2, b and e) indicate that PTH cannot prevent apoptosis of osteoblasts that have received a prior death signal. Thus, even if PTH had exerted its anti-apoptotic effect in vivo as soon as after the first injection, the reduction in the prevalence of apoptosis could not have been detected until the existing dying osteoblasts had fully disintegrated and disappeared.

The increase in osteoblasts caused by intermittent PTH could also be due to increased proliferation and differentiation of progenitors (45). Nonetheless, daily PTH injections stimulated bone formation in rodents without affecting preosteoblast replication (46) or increasing the number of early mesenchymal progenitors (6), and the anabolic effect of the hormone was not hindered in a mouse strain with reduced osteoblastogenesis (6). Intermittent PTH may also increase osteoblast number by transformation of quiescent lining cells into osteoblasts (46, 47). This mechanism may be important in periosteal bone (48), where the rate of bone formation is very low compared with that in cancellous bone, reducing the potential contribution of reduced osteoblast apoptosis to the anabolic effect (49). It is difficult to ascertain if lining cell activation occurred in the cancellous bone we studied, but we noted that increased osteoblast number was always associated with decreased apoptosis in animals receiving intermittent PTH. If some of the new osteoblasts arose from lining cells, intermittent PTH must have also delayed their apoptosis. Attenuation of osteoblast apoptosis is evidently a powerful mechanism for increasing bone mass because it may also account at least in part for the anabolic effect of a new class of synthetic ligands for the androgen or estrogen receptor in gonadectomized mice (50) as well as the high bone mass of humans and rodents caused by mutation of the low density lipoprotein receptor-related protein (51). This contention is further supported by our recent finding that implants of marrow-derived osteoblast progenitors over-expressing Bcl-2 exhibit decreased osteoblast apoptosis and increased bone formation (52).

**Self-limited Anti-apoptotic Signaling Explains Why Intermittent PTH Is Required for Bone Anabolism**—In contrast to daily injections, continuous PTH administration did not affect the prevalence of osteoblast apoptosis. Inadequate PTH levels could not explain this lack of an effect because the infused hormone increased RANKL synthesis and osteoclastogenesis, which require PKA activation (16, 53). Inevitably, therefore, continuous PTH administration should have initiated the anti-apoptotic signals that, as we show here, also originate from PKA activation. Why then did continuous PTH administration, in contrast to intermittent administration, not affect the prevalence of osteoblast apoptosis?

The mechanistic studies presented herein provide an explanation for this dichotomy. Moreover, they offer for the first time an explanation for the dependence of the anabolic properties of PTH on intermittent administration of the hormone. Specifically, as summarized in the model provided in Fig. 8. PTH acting through cAMP and PKA prevents osteoblast apoptosis by inactivating the proapoptotic protein Bad and by stimulating the transcription of the anti-apoptotic gene Bcl-2. These effects combine to increase the ratio of the amount of anti- to pro-apoptotic members of the Bcl-2 family, which is known to determine the ability of the cell to resist death signals (54). Nevertheless, other survival genes also could be involved. The increased transcription of these genes in response to PTH requires at least two transcription factors, CREB and the osteoblast-specific transcription factor Runx2. However, PTH also decreases the level of Runx2 by promoting its proteasomal degradation in a process involving the ligase Smurf1. In turn, reduction of Runx2 levels below a critical threshold terminates the ability of PTH to maintain its anti-apoptotic signal by removing this critical factor on which the transcription of the pro-survival genes by PTH depends. This provides for a negative feedback loop that shortens the duration of the anti-apoptotic effect of the hormone. In other words, the longevity of the anti-apoptotic signal of PTH is limited by a separate action of PTH on intermittent administration of the hormone on the rate of proteasomal proteolysis of Runx2.

*Runx2 Degradation Plays a Pivotal Role in Determining the Duration of PTH-induced Survival Signaling*—The PTH-induced decrease of Runx2 in our in vitro studies was transient, for 48 h. Cells were then cultured for 1 h with 50 nM PTH before the addition of etoposide (etop), and apoptosis was evaluated by trypan blue staining 6 h later (b). Bcl-2 mRNA was determined by real-time PCR (c), and Bcl-2 protein was determined by Western blotting (d) at the indicated times after the addition of PTH. e, apoptosis induced by etoposide (etop) or dexamethasone (dex) in calvaria cell cultures obtained from neonatal wt (+/+) or Bcl-2 null (−/−) littermates was determined as in b. The basal level of apoptosis was the same for both genotypes, and identical results were obtained in a second experiment. Data shown represent the mean (± S.D.) of triplicate determinations. *, p < 0.05 versus vehicle (veh)-treated cells by ANOVA.
FIG. 7. The duration of the anti-apoptotic effect of PTH is limited by PTH-induced proteasomal degradation of Runx2. a, OB-6 cells were treated with vehicle, lactacystin (10 μM), or the proteasome activator Z-E(Ot-Bu)Al-pNA (100 μM) for 30 min followed by the addition of 50 nM PTH. After 1 h, etoposide was added, and apoptosis was evaluated by trypan blue staining 6 or 24 h later. b and d–g, OB-6 cells transfected with the indicated constructs (along with nGFP) were treated with PTH followed by the addition of etoposide. Nuclear morphology of fluorescent cells was determined 6 or 24 h later. Basal apoptosis was increased to 20% in cells transfected with dn Bad and treated with lactacystin and in cells cotransfected with dn Bad and dn Smurf1, as compared with 5% in cells transfected with vector or the other constructs. An additional 15% of the cells were killed by etoposide in all conditions. Data shown represent the mean (±S.D.) of triplicate determinations. *, p < 0.05 versus cells maintained in the absence of PTH by ANOVA.

c, upper panel, OB-6 cell lysates were analyzed by Western blotting using antibodies to Runx2 or β actin. Values shown represent the mean (±S.D.) fold change in 3 replicate experiments (*, p < 0.05 versus untreated cells at the same time point). c, lower panel, cell lysates were immunoprecipitated (IP) with anti-Runx2 antibody and analyzed by Western blotting (WB) with anti-ubiquitin antibody.
with levels of the protein reaching a nadir between 3–6 h after exposure of the cells to the hormone and returning toward basal line by 24 h. Nevertheless, restoration of Runx2 to base line was insufficient for re-establishment of increased synthesis of anti-apoptotic proteins like Bcl-2, because following a peak level at 2–4 h after PTH exposure, Bcl-2 declined to basal levels by 6 h. Thus, PTH-induced increase in Bcl-2 synthesis depends on signals generated during the initial exposure to the hormone, but these signals are not present after prolonged exposure. PTH receptor desensitization was excluded as a mechanism responsible for the refractoriness to the anti-apoptotic signals of the hormone. Instead, a decline in phospho-CREB seems likely, as this transcription factor is known to activate Bcl-2 synthesis (55). Interestingly, the ligase responsive to targeting Smad1 for proteolysis is also Smurf1. However, the involvement of Smad1 or other targets of Smurf1 in determining the duration of PTH-induced survival signaling seems unlikely because overexpression of Runx2 alone is sufficient to extend the anti-apoptotic effect of PTH. In any case our findings call for studies aiming to establish the in vivo relevance of the level of Runx2 for the ability of PTH to attenuate osteoblast apoptosis.

In conclusion, we propose that the necessity of repeated episodes of PTH elevation to reduce osteoblast apoptosis in vivo is a reflection of two distinct effects of PTH, namely Runx2-dependent stimulation of survival factor synthesis and self-induced refractoriness to this effect by acceleration of Runx2 proteasomal degradation (Fig. 8). Because osteoblasts are inherently susceptible to endogenous death signals when pro-survival factors decrease, short bursts of PTH elevation provide temporary stays of execution. The cumulative effect of repeated delays of apoptosis causes a reduction in the prevalence of dying osteoblasts and a corresponding increase in osteoblast number. Conversely, the inability of sustained PTH elevation to affect osteoblast apoptosis in vivo could be due to transient elevation of phospho-Bad and phospho-CREB, and refractoriness resulting from suppression of Runx2 levels below the critical threshold needed to maintain survival signaling by the hormone.

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