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CREM deficiency in mice alters the response of bone to intermittent parathyroid hormone treatment

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Abstract

CREM belongs to the ATF/CREB family of basic leucine zipper transcription factors. We previously showed that PTH induces ICER (inducible cAMP early repressor) in osteoblasts. ICER proteins, which are transcribed from the P2 promoter of the *Crem* gene, act as transcriptional attenuators. The objective of this study was to determine whether the *Crem* gene plays a role in the response of bone to intermittent PTH. Adult *Crem* knockout (KO) and wild type (WT) male mice were given daily subcutaneous injections of vehicle or hPTH(1–34) (160 μg/kg) for 10 days. Bone mineral content and density (BMC and BMD, respectively) were measured in femur and tibia by dual energy X-ray absorptiometry (DEXA). Bone morphometry was analyzed by X-ray computed microtomography (microCT) and histomorphometry. Serum bone turnover markers were measured. In vitro osteoclast formation assays were performed in bone marrow cultures treated with PTH or the combination of RANKL and M-CSF. KO mice had slightly higher basal bone mass than wild type mice. PTH treatment increased tibial BMC and BMD to a greater extent in WT mice compared to KO mice. PTH increased both cortical area and trabecular bone area in WT but not in KO femurs. PTH increased the bone formation rate and percent osteoblast surface to the same extent in femurs of WT and KO mice but increased osteoclast parameters and calvarial porosity to a greater extent in KO mice. PTH increased serum osteocalcin levels to the same extent in WT and KO mice. PTH-induced osteoclast formation was 2-fold greater in bone marrow cultures from KO mice. Collectively, our data suggest that the CREM deficiency in mice alters the response of bone to intermittent PTH treatment such that osteoclastogenesis is increased. *Crem* gene may specify the anabolic response to intermittent PTH treatment by restraining PTH-induced osteoclastogenesis.

Keywords: Osteoblast; Osteoclast; Bone resorption; Bone mass acquisition; PTH; CREM; ICER

Introduction

CREM belongs to the CREB/CREM/ATF family of basic leucine zipper transcription factors that bind to cAMP response elements (CREs) in the regulatory regions of cAMP-responsive genes [1]. CREM transcription factors play an important role in a variety of physiological systems including cardiac function [2,3], circadian rhythms [4] and locomotion [5]. However, CREM is most notable for its role in fertility as male CREM deficient mice have a block in the maturation of sperm [6,7].

The structure and regulation of the *Crem* gene are highly complex. *Crem* contains four known promoters encoding a variety of alternatively spliced transcripts that are expressed in a tissue-specific pattern during development and postnatal life [1,8]. CREM factors can function as either activators or inhibitors of transcription depending on whether or not they contain specific domains for transactivation and serine phosphorylation [1]. The most upstream of *Crem*'s four promoters (P1) is thought to be constitutively active [9]. By contrast, the intronic P2 promoter located near the 3′ end of the *Crem* gene directs the transcription of four inducible products collectively called ICER [4]. Recently, two newly-identified

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promoters, P3 and P4, have been shown to direct expression of *Crem* isoforms in testes [8].

The well-described anabolic effect of intermittent PTH on bone mass acquisition has been documented in rats [10], mice [11], monkeys [12] and humans [13]. These observations have been pivotal to the development of PTH as an anabolic therapy for treating osteoporosis [14]. However, the cellular and molecular mechanisms underlying the anabolic effect of PTH on bone are still unresolved. Cells of the osteoblast lineage contain PTH receptors and serve as the primary target cells of PTH signaling in bone [15]. Although PTH activates both the cAMP-protein kinase A (PKA) and PKC pathways in osteoblasts, cAMP-PKA signaling has been shown to be critical for the anabolic effect of PTH on bone mass acquisition [16,17]. It is shown that the time course of RANKL and OPG expression is different between acute and sustained PTH treatment in mice [18]. This suggests that the anabolic effect of intermittent PTH on bone may be related to the kinetics of gene expression in response to PTH.

We previously showed that PTH induces ICER expression in osteoblasts in vitro and in vivo [19]. The induction is rapid and transient and highly restricted to agents that signal through the cAMP-PKA pathway [20]. ICER proteins contain a DNA binding domain but they are devoid of transcriptional activation and phosphorylation domains [4]. Thus, ICER proteins function as powerful transcriptional repressors by binding to CREs and blocking their access to transcriptional activators. The availability of *Crem* KO mice provided a model to test the hypothesis that CREM/ICER factors play a role in regulating PTH responses in bone.

Materials and methods

Animals

Crem KO mice were previously developed in a mixed genetic background (129Sv/C57BL/6) and kindly provided by Dr. Gunter Schutz [7]. This model should have a disruption of all functional CREM isoforms. All animal care protocols were reviewed and approved by the University of Connecticut Health Center Animal Care Committee. We established heterozygous KO breeding units to generate WT and KO littermates. Because the litter sizes were small, we also established homozygous KO-heterozygous KO and WT–WT breeding units to generate additional experimental animals. In both cases, the results were similar and data from all breeding units were pooled. WT and KO male mice were given daily subcutaneous injections of vehicle (1 mg/ml BSA in acidified with 0.01 M acetic acid) or 160 μg/kg body weight of hPTH(1–34) (Bachem, Torrance, CA) dissolved in the same vehicle. There were 20–24 mice per group for the measurements of bone BMD and bone morphometry by microCT, 13–16 per group for static histomorphometry and 8–9 per group for dynamic histomorphometry. For dynamic histomorphometry, mice were given intraperitoneal injections of calcein (10 mg/kg) and xylenol orange (90 mg/kg) 6 days and 2 days, respectively, prior to sacrifice.

Bone density and morphometry

Bone mineral content (BMC) and bone mineral density (BMD) of whole dissected femurs and tibiae were measured by DEXA using a PIXImus densitometer (GE-Lunar, Madison, WI). Morphometry of calvaria and the trabecular and cortical compartments of femurs was quantified using conebeam X-ray computed microtomography (μCT40, Scanco Medical AG, Bassersdorf, Switzerland). Serial tomographic images were acquired at 55 kV and 145 μA, collecting 1000 projections per rotation at 300 ms integration time. Three-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering, and rendered within a 12.3 mm field of view at a discrete density of 578,704 voxels/mm^3 (isometric 12-μm voxels). Segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained Gaussian filter to reduce noise, applying density thresholds of 350 mg/cm^3 and 260 mg/cm^3 for the cortical and trabecular compartments of the femur, respectively, and 330 mg/cm^3 for calvaria. Volumetric regions for trabecular analysis were selected within the endosteal borders of the distal femoral metaphysis to include the secondary spongiosa located 960 μm (~6% of length) from the growth plate and extending 1 mm proximally. Trabecular morphometry was characterized by measuring the bone volume fraction (BV/TV), trabecular thickness (TbTh), trabecular number (TbN), and trabecular spacing (TbSp). Cortical morphometry was quantified and averaged for 50 serial cross-sections (600 μm) extending distally from the diaphyseal mid-point between proximal and distal growth plates. Cortical measurements included average cortical thickness, cross-sectional area of cortical bone, sub-periosteal cross-sectional area, and marrow area. Calvarial morphometry was quantified and averaged for 10 serial cross-sections (120 μm) within the central coronal plane of the parietal bones, transverse to the sagittal suture. Calvarial thickness and porosity (marrow space) were measured within the central third (~2100 μm length) of each half of the parietal arch between the sagittal and squamous sutures (Fig. 6A).

Histomorphometry

Static and dynamic histomorphometry were performed as described previously [21]. All static and dynamic parameters were measured according to the Report of the American Society of Bone and Mineral Research Histomorphometry Nomenclature Committee [22].

Serum bone turnover markers

Serum osteocalcin was measured by radioimmunoassay using a goat anti-mouse osteocalcin antibody as previously described [23]. The C-terminal telopeptide of α1(I) collagen was measured by an enzyme-linked immunosorbent assay (ELISA) using a RatLaps kit (Nordic Bioscience Diagnostics, A/S).

Osteoclast formation in bone marrow cultures

Femurs and tibiae from 10- to 11-week-old mice were dissected from surrounding tissues. The epiphyseal growth plates were removed and marrow collected by flushing with serum free α-modified essential medium (α-MEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin with a 25-gauge needle. Cells were cultured in α-MEM containing 10% heat inactivated fetal bovine serum (HI-FBS). For PTH treatment (100 ng/ml), cells were plated in 48-well dishes at 1×10^6 cells/well. For M-CSF and RANKL (each at 30 ng/ml) treatment, cells were plated in 48-well dishes at 2.5×10^5 cells/well. The medium was changed on day 3. Osteoclast formation was measured at each time point as the number of tartrate resistant acid phosphatase (TRAP) positive cells containing 3 or more nuclei as previously described [24].

CFU-GM assay

Bone marrow cells were plated at 50,000 cells per dish (35×10 mm cell culture dish with a 2 mm grid, Nalge Nunc International) in 1 ml 1.5% methylcellulose supplemented with 20% HI-FBS, and 1.0 ng/ml GM-CSF (R&D System, Minneapolis, MN). After 6 days, CFU-GM colonies (>40 cells) were counted.

Statistics

Data was analyzed with *t*-tests or two-way ANOVA followed by Bonferroni post-tests or paired *t*-tests as indicated in the text and individual figure legends.