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Amphiregulin lacks an essential role for the bone anabolic action of parathyroid hormone

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ABSTRACT

Although parathyroid hormone (PTH) has long been known to act as a bone anabolic agent when administered intermittently, the exact underlying mechanisms remain largely unknown. Amphiregulin (AREG), a ligand of the epidermal growth factor receptor, has been identified to be a PTH target gene *in vitro* and *in vivo*. Here, we used female global AREG knockout (AREG-KO) mice to explore the role of AREG in mediating the bone anabolic effects of PTH. AREG-KO mice were characterized by unchanged distal femoral cancellous bone mass and only subtle decreases in bone mineral density (BMD) and cortical thickness at the femoral midshaft at 3 and 8 months of age, relative to wildtype controls. AREG deficiency was associated with complex changes in the mRNA expression of other EGFR ligands in femoral cancellous bone osteoblasts *in situ* in 3-week-old mice. To examine the bone anabolic effects of PTH in the absence and presence of AREG, we injected 3-month-old AREG-KO females and wildtype control littermates with 80 μg/kg PTH or vehicle 5 times per week over 4 weeks. Intermittent PTH treatment of AREG-KO mice led to increases in femoral trabecular and cortical BMD, cortical thickness, endocortical and periosteal bone formation, cancellous bone formation rate, and serum osteocalcin, comparable to those observed in wildtype control mice. In conclusion, our data indicate that the bone anabolic effects of PTH do not require AREG, at least in 3-month-old female mice.

1. Introduction

Parathyroid hormone (PTH) is the main short-term hormonal regulator of bone remodeling and calcium homeostasis (Poole and Reeve, 2005). It is secreted by the parathyroid glands in response to low extracellular ionized calcium levels. PTH binds at its target cells to the PTH receptor, a G-protein-coupled receptor, and activates several intracellular signaling pathways (Swarthout et al., 2002). PTH acts directly on the bone to initiate bone resorption and on the kidneys to stimulate tubular calcium re-absorption and synthesis of 1,25-dihydroxyvitamin D_3, which then enhances the calcium uptake in the small intestine. Paradoxically, in addition to this classical bone catabolic action, PTH can also act as a bone anabolic agent depending on the way of administration. While continuous infusion of PTH causes bone loss, intermittent PTH injections stimulate bone formation and significantly increase bone mineral density (BMD) (Tam et al., 1982; Hock et al., 1988; Poole and Reeve, 2005). Teriparatide, a recombinant 1–34 peptide of PTH, is currently used successfully as a treatment for osteoporosis (Partridge et al., 2006).

Several pathways and molecules have been identified to play an important role in mediating this bone anabolic effect of PTH, including insulin-like growth factor-1 (Miyakoshi et al., 2001), c-fos (Demiralp et al., 2002), interleukin-18 (Raggatt et al., 2008), beta-arrestin 2 (Bouxsein et al., 2005) and sclerostin (Kramer et al., 2010). Additionally, microarray analyses were used to assess PTH-regulated genes in bone (Qin et al., 2003; von Stechow et al., 2004), and to compare gene expression profiles between continuous and intermittent PTH treatment (Onyia et al., 2005). It has been confirmed in several studies that not only genes associated with bone formation are required for the full anabolic effect of PTH, but also genes related to bone resorption and osteoclastogenesis

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(Koh et al., 2005; Li et al., 2007a, 2007b; Tamasi et al., 2013).

The epidermal growth factor receptor (EGFR, ERBB1, HER1) and its ligands have been reported to play an important role in bone biology and in mediating the anabolic actions of intermittent PTH (Schneider et al., 2009b). Microarray analysis identified amphiregulin (*Areg*), a ligand of the EGFR (Berasain and Avila, 2014; Schneider and Wolf, 2009), as a PTH-regulated gene in rat UMR 106-01 osteoblastic cells (Qin et al., 2003). Additional studies showed an increase of *Areg* mRNA levels in PTH-treated mouse MC3T3 cells and in rat calvarial primary osteoblastic cells, as well as in the femora of rats injected with PTH, confirming that *Areg* is a PTH target gene *in vitro* and *in vivo* (Qin et al., 2005). *Areg* is also a target gene for other bone anabolic factors, such as 1,25-dihydroxyvitamin D_3 and PGE_2. It stimulates proliferation and prevents differentiation and mineralization in osteoblastic cells, and female mice lacking AREG have been reported to have less trabecular bone than their control littermates at an age of four weeks (Qin et al., 2005).

Recently, it was shown that PTH-mediated release of AREG in osteoblastic cells promotes the migration of mesenchymal progenitors to the bone surface via PI3K/Akt and p38MAPK pathways (Zhu et al., 2012). Furthermore, the anabolic effect of PTH was blunted in mice with impaired EGFR activity in osteoblasts (Zhu et al., 2012). Thus, AREG seems to be the major EGFR ligand mediating the bone anabolic effect of PTH. However, it is still unclear whether these putative effects of AREG are an essential component of PTH's bone anabolic actions. In addition, the role of AREG in bone development and homeostasis *in vivo* has been only superficially investigated. Hence, to clarify to which extent AREG is required for the full anabolic action of PTH, global AREG-KO mice at an age of 12 weeks and their control littermates were intermittently injected with PTH, and the bone phenotype of these mice was evaluated in detail. We found that AREG deficient mice have only subtle changes in their skeletal phenotype, and that AREG is not essential for the bone anabolic actions of PTH.

2. Material and methods

2.1. Animals

Global AREG knockout (AREG-KO) mice (Luetteke et al., 1999) were obtained from The Mutant Mouse Regional Resource Center (MMRRC) Repository at the University of North Carolina, USA. The animals were maintained in a 129/C57BL/6 mixed background under specified pathogen free conditions in a closed barrier facility and had free access to a standard rodent diet (V1534; Ssniff, Soest, Germany) and tap water. Genotyping was performed using the primers AregDel#1 (5′ CTT TCC AGC TTT CTC CAC CTC AAG 3′), AregDel#2 (5′ ACA GTA ACC TCT GTT GCA TGC CAC 3′) and AregDel#3 (5′ CTG CAC GAG ACT AGT GAG ACG TGC 3′). Heterozygous AREG knockout mice were intercrossed to obtain homozygous knockout mice and wildtype (WT) control littermates. Only female mice were used for phenotyping of AREG-KO mice and PTH treatment. Before killing the animals, spontaneous urine was collected for measurement of bone resorption parameters. All experiments were approved by the Institutional Committee on Animal Care and carried out with permission from the responsible veterinary authority.

2.2. PTH treatment of mice

AREG-KO females at an age of 12 weeks and their WT littermates received either 80 μg/kg body weight (1–34) PTH (Bachem, Weil am Rhein, Germany) or vehicle (physiological saline) by subcutaneous injection once daily for five days per week over a period of four weeks. All mice were subcutaneously injected with alizarin complexone (30 mg/kg body weight) once at the beginning of the experiment and with calcein (20 mg/kg body weight) on days 4 and 2 before necropsy. Both fluorochromes were purchased from Sigma–Aldrich (Schnelldorf, Germany).

2.3. Serum and tissue collection

Blood samples were taken from the retroorbital plexus of anesthetized mice, serum was obtained by centrifugation and stored at −80 °C. The femora were removed and further processed as indicated below.

2.4. μ-CT analysis

In order to assess the trabecular and cortical bone microarchitecture of the left femur, quantitative microcomputed tomography was performed according to recent standards (Bouxsein et al., 2010), using a Scanco μCT35 (Scanco Medical AG, Brüttisellen, Switzerland).

In the femoral mid-diaphysis, a region of 0.8 mm in length, covering a total of 115 slices was analyzed. Voxel size was 7 μm (isotropic). For analysis of the distal femoral metaphysis, we used 170 slices starting 0.49 mm proximal from the growth plate. Image acquisition was performed at 70 kV and 114 μA with an angular increment of 0.18° between projections, and an isotropic voxel size of 3.5 μm. The grayscale images obtained were processed using a 3D Gaussian filter with $\sigma = 0.8$ to remove noise. A threshold of 361 mg hydroxyapatite/cm^3; was used to distinguish bone from the background (corresponding to a linear attenuation of $\mu = 1.6$). Unbiased 3D microstructural properties of cortical and trabecular bone were calculated using methods based on distance transformation of the binarized images as described previously (Schneider et al., 2012). Cross-sectional and 3D images were constructed using the volume rendering software Drishti (Limaye, 2012).

2.5. Bone histology and histomorphometry

Histological processing of the femurs and cancellous as well as cortical bone histomorphometry were performed as described previously (Erben, 1996; Reim et al., 2008; Schneider et al., 2009a). The right femur was fixed in 4%PFA in PBS at 4 °C for 24 h and washed with PBS for 24 h. The femur was cut in half and the distal part was dehydrated and embedded in methylmethacrylate (MMA). Four-μm-thick sections of the distal femoral metaphysis were cut using a Microm HM360 microtome with a tungsten carbide knife. Sections were stained with Kossa/McNeal and for tartrate resistant acid phosphatase (TRACP) enzyme activity according to standard protocols. For fluorochrome analysis, undeplasticized and unstained sections were mounted with Fluoromount (Serva, Heidelberg, Germany). For cortical histomorphometry, the left femur was embedded in MMA. 200-μm-thick cross sections of the femur shaft were taken using a precision band saw (Exakt, Norderstedt, Germany). The sections were subsequently ground to a final thickness of 20 μm with a microgrinding system (Exakt), and surface-stained with toluidine blue or left unstained for dynamic histomorphometry. Semiautomatic and automatic image analyses were performed using OsteoMeasure 3.0 (OsteoMetrics, Decatur, GA, USA) and AxioVision 4.6 (C. Zeiss, Jena, Germany) software, respectively. The area within 0.25 mm from the growth plates was excluded from the measurements in the distal femurs.