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Parathyroid hormone regulation of hypoxia-inducible factor signaling in osteoblastic cells

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ABSTRACT

Osteoblasts perceive and respond to changes in their pericellular environment, including biophysical signals and oxygen availability, to elicit an anabolic or catabolic response. Parathyroid hormone (PTH) affects each arm of skeletal remodeling, with net anabolic or catabolic effects dependent upon duration of exposure. Similarly, the capacity of osteoblastic cells to perceive pericellular oxygen has a profound effect on skeletal mass and architecture, as mice expressing stable hypoxia-inducible factor (HIF)-1α and -2α demonstrate age-dependent increases in bone volume per tissue volume and osteoblast number. Further, HIF levels and signaling can be influenced in an oxygen-independent manner. Because the cellular mechanisms involved in PTH regulation of the skeleton remain vague, we sought whether PTH could influence HIF-1α expression and HIF-α-driven luciferase activity independently of altered oxygen availability. Using UMR106.01 mature osteoblasts, we observed that 100 nM hPTH(1–34) decreased HIF-1α and HIF-responsive luciferase activity in a process involving heat shock protein 90 (Hsp90) and cyclic AMP but not intracellular calcium. Altering activity of the small GTPase RhoA and its effector kinase ROCK altered HIF-α-driven luciferase activity in the absence and presence of PTH. Taken together, these data introduce PTH as a regulator of oxygen-independent HIF-1α levels through a mechanism involving cyclic AMP, Hsp90, and the cytoskeleton.

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1. Introduction

The mammalian skeleton undergoes continuous remodeling throughout its lifetime. This occurs in response to mechanical demands, the accumulation of microdamage within bone, and in response to hormonal factors like parathyroid hormone (PTH). PTH (Forteo™) remains the only current FDA-approved therapeutic agent that is skeletally anabolic when given intermittently; although extremely effective at mitigating senile osteoporosis [1], its use is limited to 18 months because of the emergent increased risk of osteosarcoma and other skeletal neoplasms documented in rodent models treated for longer periods. PTH binding to its cognate receptor activates distinct Gα subunits to synthesize cyclic AMP, activate phospholipase C to cause release of calcium from intracellular stores, and disrupt the actin cytoskeleton [2,3]. Each of these signaling pathways is implicated in mediating osteoblast mechanosensitivity [4,5], and the anabolic and catabolic effects of PTH on the skeleton [6].

The cells that form tissues and organs and comprise an organism demonstrate a remarkable capacity to respond to changes in their extracellular milieu, enabling them, and ultimately the whole organism, to adapt to altered nutritional, biophysical or other conditions. For example, cells and tissues must adapt to changes in oxygen bioavailability arising from physiological or pathological conditions. Adaptive responses to decreased oxygen tension (hypoxia) are rectified by the transcriptional induction of genes that promote angiogenesis and anaerobic glycolysis, a phenomenon mediated in part by the hypoxia-inducible factor (HIF) family of transcription factors [7]. Constitutively expressed HIF-1β (also arylhydrocarbon receptor nuclear translocator, ARNT) binds to one of the three distinct HIF-alpha isoforms (HIF-1α, HIF-2α, and HIF-3α) to initiate gene expression. Oxygen levels regulate HIF-responsive gene expression. Under normoxic conditions, HIF alpha subunits are hydroyxlated by proline hydroxylase-domain proteins (PHDs), targeted for ubiquitination by the E3 ligase complex von Hippel–Landau (Vhl) and are ultimately degraded by the 26*S* proteasome. Under hypoxic conditions, prolyl hydroxylation of HIF-α is inhibited, enabling HIF-α levels to accumulate, translocate into the nucleus, complex with HIF-β/ARNT, and finally bind to HIF-response elements (HRE) within target genes.

During embryonic development, endochondral bone formation is accompanied by vascular invasion of the cartilaginous anlage. Inhibition

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of angiogenic growth factor VEGF with a decoy receptor prevents vessel invasion and trabecular bone growth [8]. The interdependence between osteogenesis and angiogenesis is further supported by tissue engineering approaches, wherein the combination of angiogenic and osteogenic factors increase in vivo bone formation compared to delivery of either factor alone [9]. Deletion of *Vhl* in osteoblasts and osteocytes constitutively stabilizes HIF-1α and -2α, increases VEGF expression, endothelial sprouting, and enhances long bone volume, bone formation rate, and osteoblast number [10]. Similarly, deletion of HIF-1α and -2α in osteoblasts and osteocytes exerts distinct and overlapping effects on the skeleton: HIF-1α deletion decreases trabecular bone volume, mineral apposition rate, and proliferation, whereas both HIF-1α and -2α contribute to vessel number and volume and hypoxic induction of *Vegf* [11]. Thus, regulation of HIF signaling has direct effects on the skeleton.

In addition to the well-characterized oxygen-dependent regulation of HIF-α members, mammalian cells also demonstrate the capacity for oxygen-independent regulation of HIF stability and signaling. Insulin and IGF-I increase HIF-1α protein levels through PI3K/mTOR [12,13] without altering expression of endogenous inhibitors of HIF-α stability [13], although there are contradictory reports on whether IGF-I directly affects HIF-1α mRNA levels [13,14]. Additionally, heat shock protein 90 (Hsp90) serves as an endogenous oxygen-independent stabilizer of HIF-1α protein levels: competition between binding of HIF-1α by Hsp90 versus receptor for activated C-kinase 1 (RACK1) influences HIF-1α presentation to ElonginC and its subsequent oxygen-independent degradation [15,16].

Since the cellular mechanisms whereby PTH exerts anabolic or catabolic effects remain unknown and because of emerging interest in the role of HIF in bone, we examined the influence of PTH on HIF signaling, in osteoblasts. Here, we demonstrate that PTH inhibits HIF-1α protein levels under normoxic conditions, and decreases HIF-dependent signaling. This process is mimicked with the Hsp90 antagonist geldanamycin, involves cyclic AMP, and requires RhoA function. These results attribute a novel function for PTH in oxygen-independent regulation of HIF-1α.

2. Materials and methods

2.1. Materials

Human PTH(1–34), human PTH(1–31), and bovine PTH(3–34) were purchased from Bachem, reconstituted at 100 μM stock, aliquoted and frozen at −80 °C. The Rho inhibitor exoenzyme C3 transferase and the Rho Activator II (CN-03) were each purchased from Cytoskeleton. 8-bromo-cAMP was from Calbiochem, and geldanamycin was from Sigma.

2.2. Cell culture

UMR106.01 mature osteoblasts [17] (kindly provided by Dr. Alexander G. Robling, Indiana University School of Medicine) were cultured in MEM with Earle's Salts (*Invitrogen*) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin and streptomycin (P&S; Invitrogen). Cells were maintained in a standard humidified incubator at 37 °C with a 95% air and 5% CO_2 environment, and were routinely sub-cultured when 70–80% confluent with 0.05% trypsin (Gibco Life Technology).

2.3. Transient transfections and luciferase assays

A pGL3-HIF reporter element (pVEGF-Luc) vector driving expression of firefly luciferase (*Photinus pyralid*), containing the promoter region of human vascular endothelial growth factor (hVEGF) from −1005 to +379, was used as described previously [18]. Alternately, a firefly luciferase plasmid containing three hypoxia response elements from the *Pgk1* gene was used; pPGK1-luciferase was a gift from Dr. Navdeep Chandel (Addgene plasmid #26731, [19]). Renilla luciferase, under control of the thymidine kinase promoter (pRL-TK; Promega), was co-transfected as a control for transfection efficiency. Cells were seeded at 20,000 cells/well in 48-well plates and transfected the next day in Opti-MEM with 300 ng pVEGF-Luc or pPGK1-Luc, 300 ng pRL-TK, and 1.2 μL of X-TremeGENE HD (Roche). Reagents were added the following day, and wells were collected in 50 μL passive-lysis buffer 24 h later unless otherwise indicated. Luciferase activity in recovered lysates was determined using the Dual-Luciferase Reporter Assay System (Promega) and a Turner Designs Model 20/20 Luminometer. Within each sample, firefly luciferase activity was normalized to pRL-TK to compensate for potential variations in transfection efficiency or cell number. For transfection of RhoA mutants, GFP-RhoA was a gift from Dr. Channing Der (Addgene plasmid #23224, [20]), while dominant negative pRK5-myc-RhoA-T19N (Addgene #12963, unpublished) and constitutively active pcDNA3-EGFP-RhoA-Q63L (Addgene plasmid #12968, [21]) were gifts from Dr. Gary Bokoch (The Scripps Research Institute); cells were seeded and transfected as described above.

2.4. Site-directed mutagenesis of pPGK1-Luc

Inverse PCR was performed to generate the HRE-deleted pPGK1-Luc plasmid. The following phosphorylated primers were used in a 50 μL reaction with PFU turbo (Strategene): (F) 5′-AGCTCGAGATCCGGCCCC-3′; (R) 5′-ACAGAGCTCGGTACCTCCC-3′. Following 24 cycles of PCR, the product was digested with *DpnI*, ligated and transformed into DH5alpha competent cells (Life Technologies). Mini-prep of colonies, followed by PCR, identified deletion mutants (249 bp vs 320 bp for wildtype plasmid) which were confirmed by sequencing. The following primers were used for deletion identification and sequencing: (F) 5′-GCATTC TAGTTGTGGTTTGTCC-3′; (R) 5′-ACGCTGTTGACGCTGTTAAGC-3′.

2.5. Western immunoblotting

For protein analysis, samples were cultured as described above and lysed in 0.1% Triton X-100, 10 mM Tris–HCl, pH 8, 1 mM EDTA, supplemented with a protease and phosphatase inhibitor cocktail (Pierce-ThermoFisher). Samples were resolved in 10% Bis–Tris gels (Invitrogen), transferred onto 0.2 μm nitrocellulose membranes, and blocked in non-fat milk in a Tris-buffered saline supplemented with 0.1% Tween-20. Antibodies against HIF-1α (Millipore) and Hsp90 (Cell Signaling) and α-tubulin (Cell Signaling) were added at recommended concentrations overnight, then probed at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch). Immunoreactive bands were visualized using enhanced chemiluminescence (Bio-Rad) and a ChemiDoc MP Imaging System (Bio-Rad).

2.6. Statistical analysis

Each experiment consisted of a minimum of 3 independent trials, with each independent trial composed of samples in triplicate or quadruplicate. Unless otherwise noted, Luciferase data were normalized to internal control pRL-TK, and then to vehicle control to account day-to-day transfection variability, and are presented as mean +/− SEM. Immunoreactive bands for western blotting were normalized to internal control α-tubulin, and then to vehicle. Statistical significance was assessed by two-tailed Student's *t* test or ANOVA for non-repeated measurements followed by a Dunnett post-hoc analysis compared to vehicle control. A $p < 0.05$ was considered statistically significant. Statistically similar columns are noted with the same letters whereas statistically distinct columns are denoted by different letters.

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