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HIF-1α regulates bone formation after osteogenic mechanical loading

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ABSTRACT

HIF-1 is a transcription factor typically associated with angiogenic gene transcription under hypoxic conditions. In this study, mice with HIF-1α deleted in the osteoblast lineage (ΔHIF-1α) were subjected to damaging or non-damaging mechanical loading known to produce woven or lamellar bone, respectively, at the ulnar diaphysis. By microCT, ΔHIF-1α mice produced significantly less woven bone than wild type (WT) mice 7 days after damaging loading. This decrease in woven bone volume and extent was accompanied by a significant decrease in vascularity measured by immunohistochemistry against vWF. Additionally, osteocytes, rather than osteoblasts, appear to be the main bone cell expressing HIF-1α following damaging loading. In contrast, 10 days after non-damaging mechanical loading, dynamic histomorphometry measurements demonstrated no impairment in loading-induced lamellar bone formation in ΔHIF-1α mice. In fact, both non-loaded and loaded ulnae from ΔHIF-1α mice had increased bone formation compared with WT ulnae. When comparing the relative increase in periosteal bone formation in loaded vs. non-loaded ulnae, it was not different between ΔHIF-1α mice and controls. There were no significant differences observed between WT and ΔHIF-1α mice in endosteal bone formation parameters. The increases in periosteal lamellar bone formation in ΔHIF-1α mice are attributed to non-angiogenic effects of the knockout. In conclusion, these results demonstrate that HIF-1α is a pro-osteogenic factor for woven bone formation after damaging loading, but an anti-osteogenic factor for lamellar bone formation under basal conditions and after non-damaging loading.

Introduction

Hypoxia-inducible factor 1 (HIF-1) was initially reported as a nuclear factor for transcriptional activation in response to reduced O_2 concentration (hypoxia) [1]. Subsequently, it was identified as a basic helix–loop–helix heterodimeric protein consisting of two subunits designated HIF-1α and HIF-1β [2,3]. HIF-1α is expressed ubiquitously throughout the body. In normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin–proteasome pathway, but in conditions of hypoxia it remains stable [4]. HIF-1 stability is one of the methods by which all nucleated cells in the body sense and respond to O_2 availability [5]. As a result, HIF-1 has been called the "highly involved factor" [6], since it has a role in a wide variety of hypoxia-related processes required for development and homeostasis, including angiogenesis, erythropoiesis, and vasomotor control [7].

HIF-1α plays an important role in coupling angiogenesis and osteogenesis, particularly in skeletal healing and development [8–11]. During fracture healing, HIF-1 target genes such as VEGF, PGF, and SDF-1 are both spatially and temporally regulated [12–15]. VEGF in particular is also responsible for orchestrating the vascular invasion of hypertrophic cartilage that establishes the primary ossification center in developing mouse long bones [16,17]. Mice lacking HIF-1α in the osteoblast lineage have narrow, poorly vascularized long bones at 3 weeks of age, despite normal expression of HIF-1α in the surrounding tissues [18]. Moreover, these mice do not produce adequate vascularity to support woven bone induction during distraction osteogenesis [19], consistent with the requirement of angiogenesis in this process [20]. However, by 24 weeks of age, bones from mice lacking HIF-1α in the osteoblast lineage have normal cortical thickness and moment of inertia as well as significantly increased bone area compared with wild type controls [21]. Consistent with increased bone apposition in adult mice, these mice have enhanced periosteal lamellar bone formation when subjected to mild tibial mechanical loads [21]. Taken together, these results indicate that HIF-1α plays a complex role in postnatal osteogenesis.

In general, repetitive mechanical loading of the skeleton is a potent stimulus of bone formation. When applied at hyperphysiological strain levels for many cycles, mechanical loading produces fatigue damage that can progress to a non-displaced stress fracture and stimulate periosteal woven bone formation [22]. In this setting, woven bone formation is associated with the upregulation of angiogenic genes (*Vegf*, *Pecam1*, *Hif1α*) and a dramatic downregulation of the Wnt antagonist Sclerostin (*Sost*) [23,24]. Robust expression of HIF-1α has been observed in the inflammatory cells located in the expanded periosteal

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region as soon as 1 day after loading, with a peak in gene expression at day 7 [22,25]. Additionally, woven bone formation after stress fracture is preceded by increased periosteal vascularity [25,26] and is impaired by angiogenic inhibition [27]. In contrast to fatigue loading, mechanical loading applied near physiological strain levels for fewer cycles does not produce damage, and stimulates lamellar bone formation. Loading-induced lamellar bone formation is preceded by modest upregulation of angiogenic genes (*Vegf*, *Hif1α*) [25]. A small increase in vascularity is detectable after lamellar bone formation has occurred, but loading-induced lamellar bone formation does not depend on angiogenesis [28].

In this study, mice with HIF-1α selectively removed from the osteoblast lineage were subjected to either damaging or non-damaging ulnar mechanical loading that triggered the formation of woven or lamellar bone, respectively. Bone formation was assessed using microCT and dynamic histomorphometry, and vascularity was quantified by immunohistochemistry. The overall goal of this study was to determine the influence of osteoblastic HIF-1α on the postnatal formation of woven and lamellar bone using a single mechanical loading model.

Materials and methods

ΔHIF-1α mice

Mice with HIF-1α selectively removed from the osteoblast lineage with high efficiency (>90% by mRNA) have been previously described [18]. Briefly, mice with the second exon of HIF-1α flanked with loxP sites (floxed) [29] were crossed with mice expressing cre recombinase under the control of the osteocalcin (OC) promoter [30]. HIF-1α$^{fl/fl}$; OC-cre^{+} mice are referred to as ΔHIF-1α mice, and HIF-1α$^{fl/fl}$; OC-cre^{-} mice are considered wild type (WT) controls. PCR was used to determine expression of OC-cre using 5′-CAAATAGCCCTGGCAGATTC-3′ (forward) and 5′-TGATACAAGGGACATCTTCC-3′ (reverse) primers. Additionally, PCR was also used to identify the loxP site on the second exon of HIF-1α with 5′-TGATGTCCCTGCTGGTGTC-3′ (forward) and 5′-TTGTGTTGGGGCAGTACTG-3′ (reverse) primers.

Study design

Female mice were housed under standard conditions until 18–22 weeks of age, to ensure skeletal maturity [31,32] and allow approximate normalization of any cortical bone phenotype [21]. At the time of loading, there was no significant difference in weight between WT (23.1 ± 1.4 g) and ΔHIF-1α (22.6 ± 2.0 g) mice. The right forelimb of each mouse was mechanically loaded using one of two loading protocols designed to induce woven bone formation (WBF) or lamellar bone formation (LBF). For each animal, the contralateral (left) forelimb was used as a non-loaded control. Euthanasia was by CO_2 asphyxiation. WBF forelimbs were analyzed by microCT to assess woven bone volume and density, then decalcified and embedded in paraffin for histological analysis. LBF forelimbs were embedded in PMMA and sectioned for dynamic histomorphometry to assess measures of lamellar bone formation. All animal protocols were approved by the Animal Studies Committee of Washington University in St. Louis. Results are given as fold changes (loaded limb/non-loaded limb) and plotted as mean ± standard deviation. Statistical evaluation was performed using t-tests (Statview 5.0, SAS Institute Inc.) with p-value < 0.05 considered significant.

Mechanical loading protocols

Mechanical loading of the right ulna of each animal was performed as previously described [33]. Briefly, the right forelimb was axially compressed by placing the olecranon process and the flexed carpus into specially designed fixtures. Animals were anesthetized using isoflurane gas (1–3%) during loading. A material testing system (Instron ElectroPuls 1000) was used to apply force and monitor displacement. Similar to previous studies in mice [22] and rats [34], loading parameters were selected as a function of ultimate force and total displacement to fatigue fracture as measured during preliminary work. First, ultimate force was determined using axial monotonic compression to failure by displacement ramp (0.1 mm/s). Next, total displacement to fatigue fracture was determined using a cyclic haversine waveform of 3.5 N (80% of the ultimate force) at 2 Hz. Animals were euthanized immediately following either procedure. There were no significant differences between WT and ΔHIF-1α mice in ultimate force (4.38 ± 0.17 N vs 4.45 ± 0.21 N) or average total displacement to failure (0.89 ± 0.08 mm vs. 0.90 ± 0.13 mm), so loading parameters for subsequent survival experiments were the same for both genotypes.

For WBF loading of experimental animals, a 0.3 N compressive pre-load was applied followed by a cyclic haversine waveform of 3.5 N at 2 Hz until a total displacement of 0.5 mm relative to the 10th cycle was achieved. In this loading model, the magnitude of displacement increase is an external index of internal ulnar damage [22]. This amount of displacement was equivalent to 55% of the average total displacement to fatigue fracture (0.9 mm), reliably producing an ulnar stress fracture centered at 1 mm distal to the ulnar midpoint. Although the number of cycles required to reach 0.5 mm of displacement varied widely between animals (range: 743 to 10257 cycles), there was no significant difference between WT (6066 ± 2771 cycles) and ΔHIF-1α (4358 ± 3239 cycles) mice. For LBF loading, a 0.3 N compressive pre-load was applied followed by a cyclic rest-inserted trapezoidal waveform with a peak force of 3.0 N at 0.1 Hz for 100 cycles, similar to multi-day ulnar loading protocols used previously in the mouse [35–38]. The single loading bout protocol used here was modified from a similar procedure in rats [25], and stimulates strain-adaptive bone modeling via lamellar apposition. After loading, mice were given an intramuscular injection of analgesic (0.05 mg/kg buprenorphine) and allowed unrestricted cage activity. Mice were euthanized 3, 7, or 10 days after loading for subsequent analysis.

MicroCT analysis

Woven bone formation was analyzed using ex vivo micro computed tomography (μCT40, Scanco Medical AG) in WBF loaded animals 7 days after damaging loading, a timepoint when abundant woven bone is observed in this model [22]. The central 9 mm of each loaded ulna was scanned separately at 70 kV and 114 μA with 200 ms integration time. The scan tube diameter was 12.3 mm, and medium resolution was used to obtain a 12 μm voxel size. Scan slices were acquired in the transverse plane by placing the forelimb parallel to the z-axis of the scanner. Hand drawn contours (sigma = 1.2, support = 2, lower/upper threshold = 150/1000) were used to manually segment bone with Scanco imaging software. Woven bone volume was calculated by subtracting the original cortical bone volume from the total bone volume in the entire scan. Woven bone extent was quantified by measuring the axial length of woven bone formation along the ulna. Woven bone BMD was calculated by analyzing only woven bone in the middle 20 slices of the woven bone extent. Finally, the crack extent following WBF loading was measured as the axial length of apparent cortical cracking. Previous studies have demonstrated that microCT assessment of woven bone corresponds well with dynamic histomorphometric assessment [22], so separate histomorphometry was not performed for WBF groups.

Immunohistochemistry

HIF-1α expression and vascularity was visualized using immunohistochemistry in WBF loaded limbs at 3 and 7 days. Intact forelimbs were harvested and fixed overnight in 10% NBF, then decalcified in 14% EDTA for 14 days. Following this, each bone was embedded in paraffin to generate thin (5 μm) sections from 1 mm distal to the ulnar midpoint. Sections were deparaffinized in xylenes and rehydrated in graded ethanol solutions. Antigen retrieval was performed by overnight incubation in 0.33 M boric acid (Sigma, B6867) at 55 °C. A 20-minute incubation in