Molecular Regulation of Bone Resorption by Hypoxia

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In normal body tissues, the partial pressure of oxygen (pO_2) varies greatly. The mean pO_2 of bone marrow aspirated from healthy subjects is 51.8-54.9mm Hg (or 6.8 – 7.2% O_2 v/v).[1, 2] In pathological lesions of osseous tissues, including inflammation, fracture, and tumors, pO_2 is evidently lower.[3, 4] Low O_2 can alter bone homeostasis, leading to osteolysis. Patients exposed to long-term hypoxic states are at risk for accelerated bone loss. Decreased vascular flow to the lower extremities correlates with an increased rate of bone loss at the hip and calcaneus.[5] Chronic respiratory failure patients with lower blood pO_2 show significant time-dependent loss of bone mass density.[6] This phenomenon can also be seen in experimental animals. Bone mass is significantly reduced in Wistar rats exposed to hypobaric atmosphere.[6]

Bone is continuously resorbed and reformed by osteoclasts and osteoblasts, respectively. Formation and function of these cells depends on a 'normal' bone microenvironment. Reduced oxygen tension causes a variety of biological responses. Low oxygen conditions are potent inducers or suppressors of differentiation in numerous cell types including osteoblasts, chondrocytes, and osteoclasts. In this article we review the hypoxic regulation of osteoblasts, chondrocytes, and osteoclasts, and mechanisms relevant to these responses. Furthermore, we include our recent measurements of the effects of hypoxia on osteoclasts and related actions of nickel and cobalt ions.

HYPOXIA AND OSTEOBLASTS/STROMAL CELLS

Osteoblasts are bone-forming cells originating from mesenchymal stem cells (MSC). In addition to osteoblasts, these progenitor cells can differentiate into chondrocytes, adipocytes, myoblasts, and fibroblasts by different stimulating factors.[7] Mild hypoxic condition (8% O_2, *vs* the 20% O_2 v/v in a typical tissue culture incubator) accelerates MSC differentiation toward both osteogenic and adipogenic phenotypes.[8] Differentiation of MIAMI cells is inhibited by 3% O_2, thus favoring stemness over osteogenic differentiation.[9] Rat osteoblasts cultured under 2-5% O_2 form fewer mineralized bone nodules, and express less alkaline phosphatase (ALP), while anoxia (0.2% O_2) almost abolishes nodule formation.[10] The inhibitory effects of hypoxia are due to decreased osteoblast proliferation and differentiation.[10, 11] Cultures exposed to 2% O_2 decrease osteoblast formation by reducing expression of Runx2, a transcription factor essential for osteoblast differentiation. Expression of type I collagen, osteocalcin, and ALP in human MG3 cells is reduced by low oxygen tension in a time-dependent manner.[11] Salim *et al* report that brief exposure to anoxia, but not hypoxia (2% O_2) inhibits osteoblast differentiation by down-regulating BMP2 and Runx2 levels.[12] Collagen fibrils are less organized in osteoblasts cultured in 2% O_2 and much less abundant than in 20% O_2.[10] In mice, haploinsufficiency of hypoxia inducible factor-1α (HIF-1α), a transcriptional activator that regulates O_2 homeostasis, results in decreased chondrocytic and osteoblastic apoptosis, and fracture callus exhibits greater mineralization and is larger, stronger, and stiffer in HIF-1$\alpha^{+/-}$ than in wild-type mice.[13]

HYPOXIA AND CHONDROCYTES

Endochondral bone is formed in a low oxygen environment. The cartilaginous growth plate at the end of long bone is avascular, thus hypoxic.[14] Low oxygen tension affects chondrocyte energy metabolism. Culture of chondrocytes under hypoxia causes a proportional increase in glucose utilization and elevated level of lactate synthesis.[15] The limitations in the oxygen supply regulate chondrocyte growth arrest and survival in the growth cartilage.[14] Under hypoxia, MSC preferentially differentiate into chondrocytes and synthesize cartilage matrix.[16, 17] Real-time PCR of RNA isolated from pluripotent C3H10T1/2 cells reveals hypoxia-induced changes in the expression of Sox9 and its downstream targets aggrecan and col2α.[16] Although the growth plate is avascular, it requires blood vessel invasion and resorption of calcified cartilage by osteoclasts to be replaced by bone. An important mediator of angiogeneis, vascular endothelial growth factor A (VEGFA), is upregulated by hypoxia. VEGFA plays a significant role in both early and late stages of cartilage vascularization. Knock-out of VEGFA in mice shows delayed invasion of blood vessels into primary ossification centers and delayed removal of terminal hypertrophic chondrocytes.[18]

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HYPOXIA AND OSTEOCLASTS

Bone-resorbing osteoclasts form by fusion of hematopoietic monocyte/macrophage precursors.[19, 20] Osteoclast formation is enhanced directly or indirectly by hypoxia. Culture of mouse bone marrow macrophage cells (BMM) on ivory discs in reduced oxygen tension caused progressive increase in the formation of multinucleated osteoclasts.[21] An indirect effect of hypoxia in BMM-stromal cell co-cultures increases osteoclast formation through upregulation of IGF2 in the stromal cells.[22] In addition, *in vitro* hypoxia induces formation of bigger osteoclasts,[21, 23] which contain several hundred nuclei and can be so large as to be seen with the naked eye.[23]

In mouse and feline osteoclasts, reducing O_2 from 20% to 2% increases the mean resorption area by approximately 21-fold and 13-fold, respectively.[21, 23] Significant stimulation of osteoclast formation and resorption is observed even in severely hypoxic cultures gassed with 0.2% oxygen. Osteoclast products required for resorptive activity, including TRAP, cathepsin K and proton pump enzymes, are also significantly increased.[23]

BIOCHEMICAL MECHANISM OF OXYGEN SENSING

Hypoxia promotes the increase of a key transcription factor, hypoxia inducible factor-alpha (HIF-α), in virtually all cell types. In normoxia, HIF-1α is rapidly destroyed ($t_{1/2}$ =5 min) after its hydroxylation by HIF prolyl hydroxylases (PHDs). PHD1, PHD2 and PHD3 are a family of oxygen sensors that require molecular O_2 and Fe^{2+} ion for hydroxylation of proline residues (Pro-564 and Pro-402) in HIF-1α. Hydroxyproline allows Von Hippel-Lindau tumor suppressor protein (pVHL) to recognize and bind HIF-1α, leading to ubiquitination and, ultimately, proteasomal degradation. Unlike collagen prolyl hydroxylases, which have high affinity for O_2 and hydroxylate collagen efficiently in hypoxia, HIF-PHDs are sensitive to the change in oxygen concentration.[24] Decreased oxygen levels reduce the hydroxylation activity of the PHDs, thus stabilizing HIF-1α and mediating the translocation of HIF-1α from cytoplasm into the nucleus. HIF-1α then binds HIF-1β on hypoxia response elements (HRE) in gene promoter sequences, thus elevating the expression of up to several hundred genes. HIF transcriptionally regulates genes for angiogenesis, vascular remodeling, cellular metabolism, cell proliferation and viability [25-27] (Fig. 1).

IMPLANTS, METAL CATIONS, AND HYPOXIA

Hypoxic responses can also be induced by iron chelators such as desferrioxamine or metal ions such as cobalt (Co^{2+}) and nickel (Ni^{2+}).[28-33] Fe, Ni, and Co are group 8 transition metals. Ni^{2+} and Co^{2+} can replace iron (Fe^{2+}) in the catalytic site, leading to inhibition of the PHD enzymes.[26, 30] Ni^{2+} can also deplete ascorbate, a cofactor for PHD.[34] Direct binding of Co^{2+} to the oxygen-dependent degradation domain of HIF-2α interferes with hydroxylation by PHD.[35] Gene expression profiling in human hepatocellular carcinoma cells has shown that a shared group of core genes is upregulated by all four treatments: hypoxia, desferrioxamine, Co^{2+}, and Ni^{2+}.[36] Taken together, these metal ions can promote hypoxic-like responses, and can therefore mimic low pO_2.

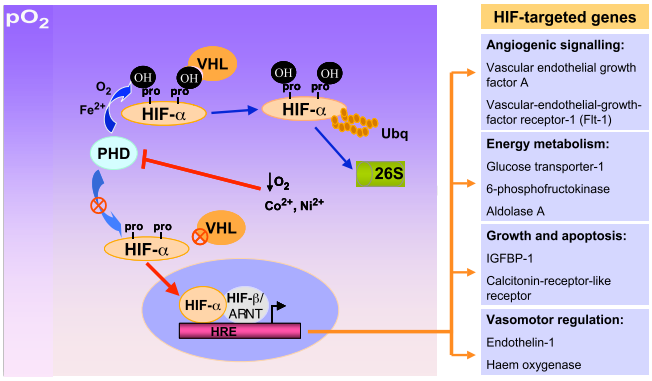

Figure 1. Biochemical mechanism of oxygen sensing. With adequate concentrations of O_2 and cofactors (Fe^{2+}, ascorbate, 2-oxo-glutarate), PHDs hydroxylate two proline residues on HIF-α (Pro-402 and Pro-564). Hydroxylated HIF-α allows VHL binding, subsequently triggering ubiquitination and 26S proteasomal degradation. Under hypoxia, or in the presence of transition metals such as Co^{2+} and Ni^{2+}, the enzymatic function of PHDs is inhibited, leading to the stabilization of HIF-α. HIF-α translocates into the nucleus, pairs with constitutive HIF-β, and binds to HRE in the promoter region of HIF-target genes. Right panel: examples of known HIF-responsive genes. Oxygen concentration is designated by the intensity of the blue background. Dark blue color represents high pO_2 level (normoxia), while decreasing color from top to bottom indicates limited pO_2 (hypoxia).

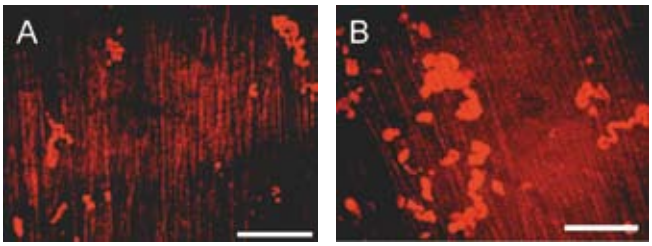

Figure 2. Resorption pits on mineralized dentin. Osteoclasts were cultured in normoxia on sperm whale dentin slices and then stained with TRITC-wheat germ agglutinin (WGA). Each pit formed by a single osteoclast appears as a unilobulated or multilobulated excavation. (A) Control. (B) 10 μM Ni^{2+} treatment for 24 hr. Bar = 200 μm.

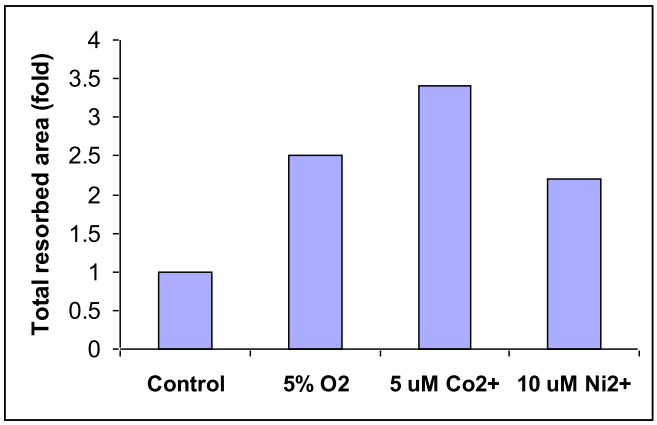

Figure 3. Hypoxia, Co^{2+}, and Ni^{2+} significantly increase resorption activity of osteoclasts. Bone marrow macrophages were incubated in α-MEM supplemented with macrophage colony stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL) on sperm-whale dentin slices for 5 days in 20% O_2. $CoCl_2$ or $NiCl_2$ were added at 5 and 10 μM, respectively, for the last 24 hr. Some dentin slices were transferred to an hypoxic incubator containing 5% O_2 for the last 2 d. After removal of osteoclasts, resorption pit areas were analyzed by ImageJ version 1.33 (NIH). Data represent total resorbed area as fold change relative to control.

EFFECTS OF COBALT AND NICKEL IONS ON OSTEOCLAST PROPERTIES

Nitinol and CoCr alloys, widely used in medical devices including orthopaedic and dental implants, stents, and surgical tools, contain high concentrations of Ni and/or Co. Passive release studies indicate that Ni is released from nitinol both *in vivo*[37] and *in vitro*.[38] Ni^{2+} serum concentration (0.026 μM) increases significantly after nitinol-based implantation.[37] In addition, elevated level of Co^{2+} (10 μM) is observed in the synovial fluid of patients who received CoCr implants.[39] Bone tissue accumulation of Ni^{2+} and Co^{2+} ions released from implants can cause adverse effects. *In vitro*, osteoblasts exposed to Ni^{2+} (10 μM) show significantly suppressed ALP activity.[40] We are investigating the effects of Ni^{2+} and Co^{2+}, as well as hypoxia, on osteoclasts.

Mouse bone marrow macrophage cells (BMM) were isolated from the femurs and tibias of Black6 mice as described by Abu-Amer.[41] BMMs were cultured on sperm whale dentin slices under osteoclastogenic conditions. Mature osteoclasts form pits in bone and dentin by H^+-mediated dissolution of mineral and proteolysis of collagen and other organic matrix components. After removal of osteoclasts from dentin slices, resorption pits are quantitated by light microscopy. Ni^{2+} (10 μM) significantly increases the resorbed area compared to untreated control (Fig. 2 and 3). In work submitted for publication, Co^{2+} (5 μM) and hypoxia also increase osteoclast resorptive activity (Fig. 3), and these metal ions trigger an hypoxic response in osteoclasts and their mononuclear macrophage precursors.[42]

SUMMARY

Direct Co^{2+} and Ni^{2+} stimulation of osteoclast formation and activity occurs through the hypoxia pathway and may contribute to orthopaedic implant loosening and failure. However, osteoclasts stimulated by Ni^{2+} and Co^{2+} are not necessarily harmful, as osteoclasts play an essential role in bone remodeling, vascularization, and osseointegration. The understanding of this pathway is clearly important for the bioengineering of implants and long-term retention without complications.

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