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Review

Hypoxia, hypoxia-inducible factor (HIF) and bone homeostasis: focus on osteoclast-mediated bone resorption

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ABSTRACT

The growth, maintenance and repair of bone are regulated by homeostatic interactions between osteoclasts, which resorb bone, and osteoblasts, which produce bone. Disruption of this balance in favor of osteoclast over-activation, in the absence of a balancing amount of bone formation, results in pathological bone loss such as that which occurs in osteoporosis, primary bone cancer, cancer metastasis to bone and rheumatoid arthritis. Hypoxia is a major micro-environmental feature of these conditions which is predictive of disease progression and poor prognosis. There is currently considerable interest in the mechanisms whereby hypoxia, the hypoxia-inducible transcription factors HIF-1α and HIF-2α, and the HIF-regulating prolyl hydroxylase (PHD) enzymes affect bone re-modelling and bone pathologies. This review summarises the evidence for HIF-mediated stimulation of osteogenic-angiogenic coupling and the use of PHD inhibitors to stimulate new bone formation and prevent osteolytic disease. It then details the evidence for hypoxia-mediated regulation of osteoclast biology, including the role(s) of HIF in the differentiation of monocytic cells into multi-nucleated osteoclasts and in the activation of bone resorption by mature osteoclasts. Specific attention is paid to the unusual consequences of upregulation of both the glycolytic pathway and mitochondrial metabolism under hypoxic conditions, alongside the consequent generation of reactive oxygen species (ROS), in the context of osteoclast activity and survival. Evidence for the use of HIF inhibitors as potential therapeutic agents targeting bone resorption in osteolytic disease is discussed. Finally, it is considered how HIF induction and HIF inhibition could both be proposed as approaches to improve bone integrity in pathological osteolysis.

KEYWORDS: osteoclast, differentiation, bone resorption, glycolysis, reactive oxygen species (ROS), mitochondrial metabolism, osteoblast, hypoxia-inducible factor (HIF), hypoxia

INTRODUCTION

Bone is a highly dynamic organ that is continuously remodelled, initially during skeletal formation and then during development, in adaptation to mechanical use, for calcium homeostasis and during healing in response to traumatic events such as bone fracture. The bone remodelling process is regulated by a carefully co-ordinated balance between bone resorption, performed by osteoclasts, and bone formation, performed by osteoblasts.

Pathological bone loss occurs when the homeostatic relationship between osteoblasts and osteoclasts is disturbed in favour of osteoclast over-activation. This is directly responsible for the resorptive bone loss evident in rheumatoid arthritis, for example, which exhibits numerous osteoclasts within the rheumatic joint [1] associated with increased concentrations of circulating and urinary markers of bone erosion [2]. Similarly, excessive bone resorption by osteoclasts in the absence of the

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balancing amount of bone formation by osteoblasts contributes to bone loss in osteoporosis [3] and is the end result of colonisation of the skeleton when cancer metastasises to bone [4].

Hypoxia, defined here as a reduction in tissue oxygen concentration below normal physiological levels, is a micro-environmental characteristic of numerous pathological conditions that often correlates with disease progression and survival. Many of these hypoxic conditions also exhibit bone loss, including rheumatoid arthritis [5, 6], osteoporosis [7], cancer (primary bone tumours and cancer metastasis to bone) [8, 9] and bone fracture [10], as well as extra-skeletal conditions such as obstructive pulmonary disease [11].

Given the frequent pathophysiological association of hypoxia and bone loss, it is important to understand how hypoxia-regulated pathways affect bone homeostasis. This review will summarize the research into effects of hypoxia and the hypoxia-inducible factor (HIF) transcription factors on bone homeostasis, focusing specifically on the bone-resorbing osteoclasts and highlighting potential new targets for the development of anti-resorptive therapies.

Hypoxia-inducible factor (HIF)

The HIF transcription factors (HIF-1 and HIF-2) are the dominant mediators of cellular responses to hypoxia, initiating a complex program of gene expression that regulates processes including angiogenesis, apoptosis, glycolysis and pH regulation which are central to the survival and expansion of cells in an oxygen-deficient environment.

HIF is a heterodimeric transcription factor comprising a hypoxia-inducible alpha subunit (HIF-1α, HIF-2α) and a constitutively expressed beta subunit (HIF-β/ARNT). In normoxic conditions HIFα is post-translationally hydroxylated by the prolyl hydroxylase domain enzymes (PHD1-3), targeting it for interaction with the von Hippel-Lindau protein (pVHL), subsequent poly-ubiquitination and rapid proteasomal degradation [12, 13]. Under hypoxic conditions, lack of available oxygen reduces PHD enzyme activity, allowing HIFα protein to accumulate and translocate to the nucleus where it dimerizes with HIF-β and binds to the hypoxia-response element (HRE) of HIF target genes to initiate transcription.

HIF can also be stabilized by non-hypoxic stimuli, including oncogenic mutation and growth factor stimulation. The exact mechanism(s) mediating these effects are unclear, but likely involve the regulatory PHD enzymes. Src- and ras-induced HIF-1α is non-hydroxylated [14], for example, suggesting inhibition of PHD enzyme activity. On the other hand, enhanced translation of HIF-1α by insulin [15] or hepatocyte growth factor (HGF) [16] might present an increased substrate load exceeding PHD enzyme capacity.

Hypoxia, HIF and osteogenic-angiogenic coupling

Bone is a highly vascularized tissue and the processes of bone remodeling and bone repair are closely associated with new vessel formation, which allows supply of oxygen and nutrients to the newly formed bone. The link between new bone formation (osteogenesis) and angiogenesis, termed osteogenic-angiogenic coupling, and the role of hypoxia in this process has recently received considerable interest.

The oxygen tension (pO_2) of normal bone is reported as being between 54.9 mmHg and 71.4 mmHg (6.6-8.6% O_2), measured from bone marrow aspirates [17] and using polarographic needle electrodes [18]. Relatively hypoxic areas have been described as the driving force behind skeletal development and endochondral bone formation. The fetal growth plate is avascular and therefore centrally hypoxic [19], as is the endosteal surface of cortical bone due to factors such as the high degree of bone marrow cellularity and high levels of oxygen consumption by resident leucocytes [20, 21]. Diseased bone, on the other hand, is more generally hypoxic. In a rabbit model of bone fracture, haematoma pO_2 four days post-fracture was 0.8% O_2 and the pO_2 in new bone two weeks post-fracture was only 3.8% O_2 [22]. Similarly, osteonecrotic human mandibular bone records 3.9-1.6% O_2 [18].

A role for HIF in the hypoxic stimulation of new bone formation was first detailed by Wang *et al.* in 2007 [23]. Using mice with osteoblast-specific deletions in either *HIF-1α* itself or the von Hippel-Lindau gene (*VHL*), with consequent increased HIF expression, it was shown that hypoxia promotes osteoblast-mediated bone formation *in vivo* via

HIF-1α-dependent induction of the pro-angiogenic cytokine vascular endothelial growth factor (VEGF) [23]. Subsequent work confirmed that HIF plays a central role in stimulating angiogenesis and bone formation during skeletal development [24-26].

This body of work on osteogenic-angiogenic coupling, described in detail in two recent reviews [21, 27], led to the hypothesis that HIF pathway activation might be a therapeutic strategy to prevent pathological bone loss and/or to restore bone mass. Non-selective PHD enzyme inhibitors such as desferrioxamine (DFO), dimethyloxalyl glycine (DMOG), L-mimosine or $CoCl_2$, which prevent HIF degradation, have been shown to increase vascularity and improve new bone formation in murine models of bone regeneration post-fracture (either stabilised fracture or distraction osteogenesis models), whether injected into the fracture site [28-30] or applied to surgically introduced implants [31, 32]. Similar results have been found in ovariectomized (OVX) mice, which become depleted of estrogen and serve as a model of osteoporotic bone loss. Here PHD inhibitors improve bone mineral density, bone microarchitecture, bone mechanical strength and blood vessel formation in the OVX animals [33, 34].

Despite widespread concordance regarding the positive effects of HIF induction on bone formation, it is worth noting that no mention has been made of osteoclasts, the other major cell type responsible for regulation of bone remodelling. Indeed, of the above studies only Peng *et al*. mention the osteoclast response to chemical induction of HIF. They observed that the increase in both number of osteoclasts and the serum concentration of cross-linked C-telopeptide of type I collagen (CTXI), a marker of bone resorption, caused by ovariectomy was unaffected by treatment with DMOG [34]. This could imply that HIF induction within the bone microenvironment has the sole effect of increasing osteoblast activity and bone formation. However, genetic studies suggest that the situation is, unsurprisingly, more complex than this. Komatsu *et al*. studied bone regeneration following femoral fracture in HIF-$1\alpha^{+/-}$ mice, with the surprising result that HIF-$1\alpha^{+/-}$ fracture calluses are larger, stronger and stiffer than wild-type calluses. This was proposed to be due to decreased apoptosis of osteoblasts and chondrocytes in the HIF-$1\alpha^{+/-}$ mice [35]. However it is, of course, possible that effects of HIF knock-down on osteoclast formation and/or activity could contribute to the unexpected phenotype.

Given the relative absence of review literature on the effects of hypoxia and HIF on osteoclast-mediated bone resorption, this review will now summarize the current state of research into hypoxic regulation of osteoclast differentiation and bone resorption activity and its effect on bone homeostasis.

Osteoclastogenesis and bone resorption

Osteoclasts form by the fusion of $CD14^+$ monocyte or macrophage precursors, in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL), to produce mature multi-nucleated cells [36, 37]. Initially M-CSF, secreted from osteoblasts, interacts with the c-fms receptor on precursor cells stimulating them to proliferate, inhibiting apoptosis and inducing expression of the RANKL receptor, RANK [38]. RANKL, also produced by osteoblasts and stromal cells, then binds to RANK on osteoclast precursors to induce expression of osteoclastogenic factors such as nuclear factor of activated T cells, cytoplasmic 1 (NFATc1). NFATc1 is essential for osteoclastogenesis, interacting with transcription factors such as PU.1, cFos and microphthalmia-associated transcription factor (MITF) to promote transcription of osteoclastic genes such as tartrate-resistant acid phosphatase (TRAP), calcitonin receptor and cathepsin K and pro-fusion genes such as DC-STAMP [39]. Although M-CSF and RANKL are the primary cytokines driving osteoclastogenic differentiation, a growing number of other factors have been found to exhibit some level of osteoclastogenic potential [40-42].

Once formed, mature osteoclasts attach to mineralised bone via $\alpha_v\beta_3$-integrin and CD44 to isolate a resorptive compartment. Transporters in the bone apposing membrane, such as vacuolar H^+ ATPase (V-ATPase), then acidify the extracellular environment, causing release of bone minerals and exposing the organic matrix to resorption by secreted enzymes including cathepsin K, TRAP and matrix metalloproteinase-9 (MMP-9) [43, 44]. The degradation products are then endocytosed and transported across the cell for secretion at the plasma membrane.

For the *in vitro* study of monocyte-osteoclast differentiation, precursor cells are usually obtained either from the mixed cell population of murine bone marrow or from the peripheral blood mononuclear cell (PBMC) fraction of whole blood following Ficoll gradient separation. These cells are then differentiated by exogenous application of M-CSF and RANKL into mature multi-nucleated cells (cells with ≥3 nuclei which express an osteoclast marker such as TRAP or $\alpha_v\beta_3$-integrin). Resorption is normally measured by culturing osteoclasts on dentine (elephant ivory) discs and either quantifying the area of resorption tracks or assaying release of CTXI.

Effect of hypoxia on osteoclast formation, survival and activity

Initial studies on the effects of hypoxia on monocyte-osteoclast differentiation reported that exposure to hypoxia increased both the number of osteoclasts formed and the amount of bone resorbed per osteoclast, with maximal effect achieved at 2% O_2 [45-47]. However subsequent work revealed that exposure to constant 2% O_2 in a gloved hypoxic workstation completely abolished both osteoclast formation and bone resorption due to extensive cell death, whereas hypoxia–re-oxygenation promoted osteoclastogenesis [48]. Attention to the hypoxia protocol in the initial studies revealed that the cells were actually exposed to repeated hypoxia–re-oxygenation, rather than pure hypoxia as stated [49]. Hypoxia–re-oxygenation occurs during injury, ischaemia and reperfusion and is the more likely micro-environment to which monocytes and osteoclasts will be exposed *in vivo*.

The need for re-oxygenation during osteoclast differentiation is at least partially due to the relative sensitivity of osteoclasts to hypoxia-induced cell death. Whereas most cells continue to proliferate at 2% O_2, as do even primary monocytes and osteoblasts [50], osteoclast numbers fall after only 24 hours of exposure [48, 50].

Twenty-four hours at 2% O_2 also causes one fifth of the osteoclasts to exhibit compromised membrane integrity, which is reversed on re-oxygenation [48]. Various features of early cell death are known to be reversible including plasma membrane permeability [51, 52], plasma membrane phosphatidylserine exposure [53-55] and mitochondrial swelling [52, 56]. This sensitivity to hypoxia combined with the ability to rapidly recover from the early stages of hypoxia-induced cell death partially explains how hypoxia–re-oxygenation is able to stimulate osteoclastogenesis [45-48], whereas continuous hypoxic exposure is inhibitory [48].

Despite this relative sensitivity to hypoxia, acute hypoxic exposure (≤24 hours) increases the ability of mature osteoclasts to resorb bone. *In vitro* differentiated osteoclasts [46, 48, 50, 57] as well as primary human osteoclasts from the bone tumour giant cell tumour of bone (GCTB) [48] exhibit a 2- to 4-fold increase in resorption after 24 hours at 2% O_2.

The osteoclastogenic response to hypoxia, in terms of both differentiation and resorption, appears to be a cell intrinsic response given that it occurs both in osteoclasts derived from PBMCs, where there is effectively no stromal cell support [47, 48], and in osteoclasts derived from pure populations of $CD14^+$ monocytes [50, 57]. Obviously osteoclasts are not isolated *in vivo*, but co-exist with other cellular components of the bone microenvironment that will also experience local hypoxia. Co-culture experiments have revealed that osteoblasts, fibroblasts and cancer cells all increase secretion of pro-osteoclastogenic cytokines in response to hypoxia, including RANKL [58, 59], VEGF [58-61], M-CSF [61], insulin-like growth factor 2 (IGF-2) [62] and growth differentiation factor 15 (GDF-15) [63]. Hypoxia also inhibits the production of osteoprotegerin (OPG), a soluble decoy receptor for RANKL that prevents osteoclast formation and bone resorption [64].

In support of this *in vitro* data, the striking effect of hypoxia to promote osteoclastogenesis was confirmed *in vivo*. Bozec *et al*. showed that the bones of newborn mice lacking the Fos-related protein Fra-2 contained numerous giant osteoclasts [65]. This was associated with the presence of hypoxia in the long bones, a downstream effect of a placental defect also caused by the Fra-2 mutation [65]. The osteoclasts in the Fra-2 deficient bones also expressed high levels of HIF-1α due to reduced transcription of PHD2 downstream of the Fra-2 mutation.

HIF-1α and HIF-2α protein expression was first detected in human osteoclasts as recently as 2008 [60], the same year that Bozec *et al*. observed

HIF-1α expression in giant osteoclasts in mice [65]. It is therefore logical to consider whether HIF could contribute to the effects of hypoxia on monocyte-osteoclast differentiation and, separately, on osteoclast-mediated bone resorption.

Is there a role for HIF in osteoclastogenesis?

It was recently reported by Miyauchi *et al*. that expression of *HIF-1α*, but not *HIF-2α*, mRNA increases during differentiation of murine monocytes into osteoclasts [7]. However, they also demonstrated that HIF-1α is not required for normoxic osteoclast formation. Osteoclastogenesis was unaltered *in vitro* or *in vivo* in mice with an osteoclast-specific deletion of HIF-1α, created by crossing cathepsin K-Cre mice with *HIF-1α*-floxed mice [7].

We have found no reports in the literature that HIF protein is stabilised during monocyte-osteoclast differentiation. It also seems unlikely that induced expression of HIF alone is sufficient to emulate the stimulatory effect of hypoxia on cell autonomous osteoclastogenesis. Indeed, the majority of studies report HIF induction to inhibit osteoclastogenesis. Murine osteoclast formation was inhibited when HIF was stabilized by application of DFO or DMOG, or when monocytes were transfected with a constitutively active form of HIF-1α [66]. Similarly, differentiation of human PBMC into osteoclasts was inhibited in the presence of $CoCl_2$ [67]. However, other studies found that Co^{2+} stimulates murine osteoclast differentiation [68].

Zhao *et al*. used the murine RAW264.7 macrophage cell line, which can be induced to differentiate into osteoclast-like cells, to suggest a role for autophagy in the hypoxic stimulation of osteoclast formation. Specifically, they showed that 24 hours of exposure to hypoxia induced osteoclast differentiation, expression of HIF-1α and expression of the HIF-1α-dependent pro-autophagy gene BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3). Other markers of autophagy were also induced including LC3 induction and cleavage, detection of acidic vesicular organelles and expression of autophagy-related genes such as beclin 1 and Atg12-Atg5 [69]. Pharmacological inhibition of autophagy with 3-methyladine or transfection with dominant negative Atg5 prevented both the hypoxic increase in autophagic flux and the hypoxic increase in osteoclast differentiation [69]. Treatment with the HIF inhibitor YC-1 or siRNA-mediated knockdown of HIF-1α also inhibited hypoxic osteoclast differentiation via mechanisms dependent on the autophagy-regulating HIF target genes BNIP3 [69] and microRNA-20a [70]. It was proposed that autophagy might regulate the degradation and restructuring of the cytoplasmic components of pre-osteoclasts during osteoclastogenesis [69], although no role for autophagy could be detected during differentiation under normoxic conditions.

It would therefore seem that, while HIF might indeed play a role in increasing osteoclast differentiation under hypoxic conditions, its role in normoxic differentiation remains undefined.

HIF stimulates osteoclast-mediated bone resorption

Mature human monocyte-derived osteoclasts *in vitro* stabilize HIF-1α and HIF-2α in response to hypoxic exposure or hypoxia mimetics such as MG132, $CoCl_2$, DFO or DMOG [47, 48, 50, 57, 60]. Our lab has shown that normoxic inducers of HIF increase osteoclast-mediated bone resorption to the same magnitude as that induced by hypoxia [57], while siRNA targeting HIF-1α completely ablates the hypoxic increase in resorption [48, 57]. As no effect was observed with HIF-2α siRNA, this suggests HIF-1α as the primary factor driving the hypoxic increase in bone resorption by osteoclasts.

This data is supported by a number of *in vivo* studies. Most notably Miyauchi *et al*. showed that the osteoclasts in OVX mice express high levels of HIF-1α [7]. Osteoclast-specific inactivation of HIF-1α antagonized the bone loss in these mice, suggesting that HIF-1α is also responsible for promoting osteoclast activation and bone loss *in vivo*. In support of this theory, oral administration of 2-methoxyestradiol (2ME), an inhibitor of HIF-1α, protected OVX mice from osteoclast activation and bone loss [7].

2ME is an anti-tumour and anti-angiogenic agent that disrupts microtubule formation. A downstream effect of microtubule disruption is the inhibition of HIF translation and nuclear translocation [71]. 2ME has also been shown to protect against pathological bone loss in other osteolytic conditions. In murine models of osteolytic breast cancer, 2ME

induced apoptosis of osteoclasts and their precursors, protected against tumour-induced osteolysis and inhibited *in vitro* bone resorption [72, 73]. Similarly, in murine models of rheumatoid arthritis treatment with 2ME improved disease incidence and severity and reduced scores for subchondral bone erosion [74, 75]. Comparable results have been obtained in murine models of RA treated with endostatin, an anti-angiogenic agent which also down-regulates expression of HIF [76], and bortezomib, a proteasome inhibitor that also inhibits HIF transcriptional activity [77], as well as in OVX-induced osteoporosis treated with apigenin, an Hsp-90 inhibitor that also results in destabilisation of HIF-1α [78].

These studies combine to suggest that HIF inhibition might be a powerful method of reducing disease severity and preventing pathological osteolysis in a number of bone resorption conditions. This is of considerable potential therapeutic interest, given that HIF-positive osteoclasts have also been observed in human disease, including in the multi-nucleated giant cells associated with the primary bone tumour giant cell tumour of bone [60] and in resorbing osteoclasts within the rheumatoid synovium [79].

What are the downstream effects of HIF activation in osteoclasts?

Although hypoxia is known to stimulate secretion of numerous cytokines and growth factors, very little has been reported of its effects in osteoclasts in this regard. Hypoxic osteoclasts are known to secrete elevated concentrations of the osteoclastogenic cytokine VEGF [47, 48] and resorption-promoting angiopoietin-like 4 (ANGPTL4) [57]. HIF-1α drives hypoxic induction of ANGPTL4 in osteoclasts [57] and other cell types [80, 81], suggesting that local hypoxia would rapidly create a large local pool of the adipokine and generate an environment that promotes bone erosion. In support of this theory, serum concentrations of ANGPTL4 are elevated in the synovial fluid and serum of rheumatoid arthritis patients, with high serum concentrations being associated with elevated markers of bone resorption [79].

Other soluble factors secreted from surrounding osteoblasts and stromal cells will also be regulated by HIF and have the potential to contribute to the effects on osteoclast activity. However, this review will now focus on identifying other mechanism(s) by which HIF directly regulates increased bone resorption activity in osteoclasts.

Reactive oxygen species

Reactive oxygen species (ROS) are essential for the normal processes of osteoclast formation and bone resorption. During monocyte-osteoclast differentiation, RANKL signalling requires the generation of ROS to serve as second messengers in signalling pathways that induce expression of NFATc1 [39, 82, 83]. As part of the bone resorption process, cathepsin K-mediated degradation of TRAP inside transcytotic vesicles activates TRAP's ROS-generating activity. These ROS enable the final degradation of bone matrix resorption products during their transcytosis [84].

In addition to the normal level of ROS in resorbing osteoclasts, hypoxia specifically induces accumulation of mitochondrial ROS [50, 85, 86]. Mitochondrial ROS formation increases under hypoxia in a number of different mammalian cells [87]. This is normally an acute response, lasting little longer than 10 minutes, that is largely dependent on mitochondrial oxidative phosphorylation [88]. It is this ROS, especially that produced via complex III of the electron transport chain (ETC), that is responsible for mediating HIF-α subunit stabilisation under hypoxia [89, 90].

The hypoxic accumulation of mitochondrial ROS observed in osteoclasts is considerably prolonged in comparison with the acute response described above. Murine RAW264.7-derived osteoclasts exposed to 5% O_2 for 5 days exhibit elevated ROS as measured by electron paramagnetic resonance (EPR) spin trapping, which was prevented by treatment with the mitochondria-specific anti-oxidant MitoQ [86]. Similarly, human osteoclasts exposed to 2% O_2 for 24 hours demonstrated increased expression of superoxide dismutase 2 (SOD2), a marker of mitochondrial ROS formation [50].

This mitochondrial ROS is essential for hypoxic enhancement of osteoclast differentiation and resorption. MitoQ inhibited osteoclast formation and prevented hypoxic induction of key signalling molecules involved in osteoclast differentiation and activity such as NFκB, the calcineurin-NFAT pathway, adenosine 3' 5' cyclic monophosphate

response element binding protein (CREB) and HIF [86, 91]. This prolonged induction of mitochondrial ROS under hypoxia was proposed to be mediated by mitochondrial respiratory stress signalling, induced by hypoxic release of Ca^{2+} into the cytosol from the endoplasmic reticulum, which stimulates production of mitochondrial ROS [92].

The importance of mitochondrial ROS in osteoclasts has recently been demonstrated *in vivo* by Bartell *et al.* They showed that RANKL inhibits expression and transcriptional activity of the FoxO transcription factors, resulting in down-regulation of the FoxO target gene catalase, which is responsible for inactivation of H_2O_2. As a result, mice with a deficiency in either FoxO1, FoxO3 or FoxO4 within the monocyte/macrophage lineage, which includes osteoclasts, exhibit higher levels of H_2O_2 associated with reduced trabecular bone and increased amounts of resorption [93]. The importance of the intracellular location of this ROS was shown using mice which over-expressed mitochondria-targeted catalase, specifically in osteoclasts. These mice had increased bone mass due to a reduction in osteoclast formation and survival and were protected from ovariectomy-induced bone loss [93].

This data suggests that mitochondrial ROS are essential for osteoclast formation and bone resorption activity, providing support for the hypothesis that accumulation of mitochondrial ROS under hypoxia directly promotes bone resorption to some extent, potentially via activation of HIF.

ATP production in hypoxic osteoclasts

A key requirement of the bone resorption process is adenosine triphosphate (ATP). Once the resorptive compartment has been formed it is acidifed by active transport of protons across the bone-apposing membrane. ATP is specifically required in the active transport process, supporting the function of vacuolar H^+ ATPase (V-ATPase) as well as Na,K-ATPase, Ca-ATPase and gastric H,K-ATPase [94]. Osteoclasts are also highly motile, migrating along resorption tracks as they digest bone, and highly biosynthetic, producing the enzymes and secretory machinery necessary for resorption to proceed. These combined processes make osteoclast-mediated bone resorption an energy-intensive process [95, 96].

Most cells exhibit reduced concentrations of intracellular ATP on exposure to hypoxia [92]. However, Morten *et al*. showed that human monocyte-derived osteoclasts increase ATP production 1.5-fold after 24 hours under hypoxia. When cultured on dentine, a substrate which activates osteoclast resorption mechanisms, this increase could not be observed, suggesting that the extra ATP drives the hypoxic increase in bone resorption [50].

How then do osteoclasts achieve increased ATP production under hypoxic conditions? Conventionally, cellular adaptation to hypoxia necessitates a switch to anaerobic metabolism. This is thought to be a survival mechanism whereby inhibition of mitochondrial ATP production prevents accumulation of toxic levels of ROS [97, 98]. However, as has already been discussed, mitochondrial ROS are essential for osteoclast function.

The classical switch to anaerobic metabolism is a multi-step process, largely directed by HIF. Initially energy/redox homeostasis is maintained under hypoxia by increasing the efficiency of complex IV of the ETC with respect to the relative amounts of ATP and ROS produced. This is effected by a HIF-mediated switch in expression of cytochrome c oxidase subunits, from COX4-1 to COX4-2 [99]. Subsequently, the cell switches to purely glycolytic metabolism. A HIF-mediated increase in the expression of glucose transporters and glycolytic enzymes increases flux through the glycolytic pathway [100]. At the same time pyruvate dehydrogenase (PDH), the mitochondrial enzyme that converts pyruvate into acetyl CoA, is inhibited due to phosphorylation by PDH kinase (PDK), an enzyme that is also induced by HIF [101, 102]. This dramatically reduces mitochondrial metabolism, preventing further accumulation of ROS. As a final response HIF induces expression of BNIP3 to initiate mitochondrial autophagy [103].

Glycolysis

Although anaerobic metabolism produces less ATP than glycolytic plus mitochondrial metabolism, increased flux through the glycolytic pathway is more than sufficient to maintain cellular function under hypoxic conditions. Indeed the myeloid cell population, which includes osteoclast precursor cells, relies heavily on HIF-1α-mediated transcription of

glycolytic genes to produce ATP for normal function [104].

During monocyte-osteoclast differentiation, this high baseline glycolytic activity increases still further [105, 106]. This is because glucose is the main energy source required for bone degradation [106, 107], its availability being directly related to the intracellular energy status [108]. There is also a physical association between the two processes. Glucose activates transcription of the A-subunit of V-ATPase in osteoclasts [109], which interacts directly with the glycolytic enzyme phosphofructokinase-1 (PFK-1) [110]. This directly links glycolysis and bone resorption by micro-compartmentalizing glycolytic production of ATP at the required intracellular location. The importance of glycolysis is reiterated by studies describing the use of glycolytic inhibitors to ameliorate bone resorption in animal models of disease [111, 112]. There have even been case reports documenting induction of clinical remission in rheumatoid arthritis following treatment with imatinib mesylate, a tyrosine kinase inhibitor that reduces glycolytic activity [113, 114].

Exposure to hypoxia again elevates the glycolytic rate of osteoclasts. Increased expression of HIF-regulated glucose transporters (e.g. Glut-1) and glycolytic enzymes (e.g. *PGK1*, *PFKFB4*, *ALDOC*, *LDHA*) [50, 60, 79] results in a HIF-1α-dependent increase in glucose consumption under hypoxia [50]. This increased consumption apparently fuels the increased ATP production by hypoxic osteoclasts, as glucose depletion severely reduces intracellular ATP generation [50].

At first sight, it would therefore appear that osteoclasts follow the classical switch to anaerobic metabolism under hypoxia. However, Morten *et al*. observed that although glucose consumption and lactate production both increase in hypoxic osteoclasts, the ratio of glucose consumption to lactate production remains unchanged [50]. This is suggestive of a hypoxic increase in flux through the glycolytic pathway, but not necessarily of a switch to anaerobic glycolysis.

Mitochondrial metabolism

In addition to the absolute requirement for glucose, high mitochondrial metabolic activity usually drives ATP production in osteoclasts. Osteoclasts contain numerous mitochondria [115] and exhibit elevated expression of TCA cycle and oxidative phosphorylation enzymes [116] and high rates of oxygen consumption [105] compared with their monocytic precursors.

In addition to reduced concentrations of intracellular ATP, most hypoxic cells exhibit reduced mitochondrial metabolic flux [101, 102]. In direct contrast, Morten *et al*. observed both elevated concentrations of intracellular ATP and increased mitochondrial reductase activity in hypoxic osteoclasts. There was also no reduction in O_2 consumption via the ETC under hypoxia. This remained close to maximal and was comparatively more sensitive to ETC inhibition with rotenone than the corresponding normoxic cells [50]. This hypoxic increase in ETC activity was partially dependent on HIF-1α, being mediated by apparently selective utilisation of components of the classical HIF-mediated metabolic switch to anaerobic respiration that increase or maintain pathway activity (the COX subunit switch, increased glycolytic rate), while neither inhibiting PDH activity nor stimulating BNIP3 production [50].

It is not clear why PDH activity remains uninhibited in hypoxic osteoclasts. Hypoxia affected neither PDK1 expression nor PDH activity, neither were these altered by HIF-1α siRNA [50]. PDH can also be inhibited by the phosphorylation and activation of AMP-activated protein kinase (AMPK) and subsequent induction of PDK4 [117, 118]. However, AMPK phosphorylation did not occur in hypoxic osteoclasts [50]. As AMPK is also activated by reduced intracellular ratios of ATP:ADP [118, 119], it may be that the high levels of ATP in hypoxic osteoclasts over-ride hypoxic mechanisms of AMPK activation in favour of de-phosphorylation and inactivation. This would be necessary for hypoxic resorption to occur, as AMPK inhibits osteoclast differentiation and activity [120].

The classical hypoxic attenuation of PDH activity is therefore prevented in osteoclasts by blockade of at least two pathways that usually contribute to its inhibition, allowing continued mitochondrial metabolic flux. This could explain the significant accumulation of mitochondrial ROS observed in hypoxic osteoclasts [50, 85, 86]. Mitochondrial

respiratory stress signalling, proposed to be involved in hypoxic generation of mitochondrial ROS, might also contribute to the observed hypoxic increase in oxidative phosphorylation. Calcium uptake by mitochondria has been shown to activate isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase activity [88].

It therefore seems likely that the relative sensitivity of osteoclasts to hypoxia-induced cell death is a consequence of maintaining high rates of oxidative phosphorylation in a hypoxic environment. Despite mitochondrial ROS being essential for osteoclast activity, continued accumulation of ROS will eventually exceed their anti-apoptotic capacity. Interestingly, HIF-1α appears to regulate both aspects of this phenomenon. Morten *et al*. showed that HIF-1α siRNA prevents hypoxic induction of bone resorption, blocks the hypoxic increase in glucose consumption and reduces hypoxic mitochondrial ETC activity, but also rescues osteoclasts from cell death induced by chronic hypoxic exposure [48, 50]. This led to the hypothesis that, in hypoxic osteoclasts, functional HIF-1α-dependent pathways initially increase ATP production and bone resorption, but lack of activation of HIF-1α-dependent survival pathways eventually results in cell death. Permitting progressive accumulation of ROS under hypoxia may be an adaptive mechanism enabling rapid bone resorption in the short term, while ensuring that the process is halted in the absence of re-oxygenation [50].

What are the consequences of targeting HIF in bone?

The data compiled in this review now presents us with an interesting question. Is the best therapeutic strategy to improve bone architecture in diseases characterised by loss of bone that of HIF inhibition or HIF induction?

Intriguingly, intervention in either direction apparently tips the homeostatic balance back in favor of maintaining or improving bone integrity (Figure 1). Research tackling the problem from the angle of stimulating HIF-mediated induction of angiogenesis and osteogenesis supports HIF pathway activation by PHD enzyme inhibition as a strategy [28-34]. Conversely, research approaching from the angle of inhibiting osteoclast-mediated bone resorption supports the use of HIF inhibitors [7, 72-78]. Taken at face value this might suggest that, in the *in vivo* situation, osteoclast activity is more sensitive to HIF inhibition, and bone formation is more responsive to HIF activation. There is some support for this idea in the literature. Peng *et al.* used the PHD inhibitor DMOG to stimulate angiogenesis and osteogenesis in OVX mice. They also noted that DMOG had no significant effect on either osteoclast number or levels of bone resorption [34].

However, it is perhaps most likely that the reason for this apparent conundrum lies in the effects of cross-talk between the different types of resident bone cells. One such pathway was recently described by Wu *et al.*, who showed that mice with genetic inactivation of both PHD2 and PHD3 in cells of the osteoblast lineage (OSX-Cre; $Phd2^{fl/fl}$; $Phd3^{fl/fl}$), which therefore over-express HIF, have an increased trabecular bone volume that is not due to increased angiogenesis. Instead these mice have reduced numbers of osteoclasts, associated with increased expression of OPG mRNA and increased serum concentrations of OPG, the soluble receptor that inhibits RANKL signalling [25]. When co-cultured with wild-type bone marrow stromal cells, OSX-Cre; $Phd2^{fl/fl}$; $Phd3^{fl/fl}$ osteoblasts inhibited osteoclast formation in comparison with control osteoblasts. Wu *et al*. subsequently demonstrated that OPG is a direct target of HIF-2α; conditional deletion of HIF-2α in the OSX-Cre; $Phd2^{fl/fl}$; $Phd3^{fl/fl}$ mice completely abrogated the high bone phenotype [25].

However, even considered in isolation, OPG-mediated cross-talk is more complex than at first sight. In direct contrast to HIF-mediated induction of OPG in osteoblasts, chondrocytes show hypoxic inhibition of OPG expression, which is dependent on HIF-1α [64].

In order to untangle the complex and over-lapping effects of HIF manipulation in this system, it is therefore essential that the effects of HIF-modulating drugs *in vivo* are studied and reported in multiple cell types. This will provide a much needed pool of data regarding, for example, effects of HIF inhibition on osteoblast function and effects of PHD inhibition on osteoclast activity. Such information is essential to further our understanding of this complex micro-environment, so that appropriate steps can be taken to develop targeted agents to treat pathological bone loss conditions.

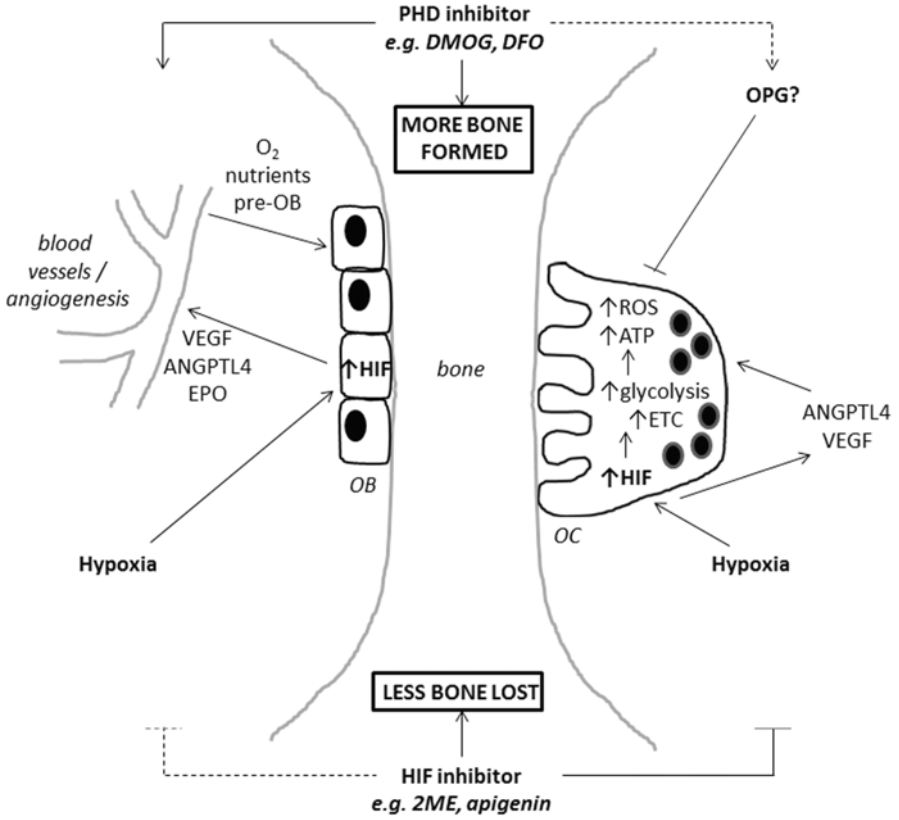

Figure 1. Beneficial effect of HIF inhibition and HIF induction on bone integrity. HIF induction results in an increase in osteoblast-mediated bone formation via stimulation of osteogenic-angiogenic coupling. Induction of HIF *in vitro* also enhances osteoclast-mediated bone resorption. Maybe the magnitude of the osteoclast response *in vivo* is less than the increase in bone formation? Or does cross-talk between cell types negate the intrinsic increase in osteoclast activity, for example by induction of OPG? HIF inhibition results in reduced bone loss via inhibition of osteoclast-mediated bone resorption. In the absence of HIF-mediated induction of angiogenesis this might be anticipated to also reduce the rate of bone formation. If this is the case, maybe the loss of osteoclast activity is greater than the reduction in bone formation *in vivo*? It is probable that cross talk between cells also modulates this response. OB = osteoblast; OC = osteoclast.

SUMMARY

HIF plays an integral role in the co-ordinated response of bone cells to hypoxia. It is central in directing increased hypoxic bone formation via VEGF-mediated induction of osteogenic-angiogenic coupling. It is also closely involved in monocyte-osteoclast differentiation and bone resorption by mature osteoclasts. HIF stimulates the expression of osteoclastogenic cytokines. It also elevates the glycolytic and mitochondrial metabolic rate, enabling osteoclasts to generate the requisite ATP to support increased bone resorption under hypoxia. Generation of mitochondrial ROS, a by-product of maintaining mitochondrial respiration in hypoxic conditions, is itself necessary to enable this high rate of resorption. This data suggests HIF as an attractive therapeutic target in bone disease, especially within the hypoxic micro-environment so closely associated with pathological osteolysis. However, further research is urgently needed in order to more fully understand the apparent dichotomy that both HIF inhibition and HIF activation can improve bone micro-architecture in these conditions.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflicts of interest.

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