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Metabolic properties of the osteoclast

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Abstract

Osteoclasts are defined as cells capable of excavating 3-dimensional resorption pits in bone, and other mineralised tissues. They derived from the differentiation / fusion of promonocytic precursors, and are usually large, multinucleated cells. In common with other cells from this myeloid lineage such as macrophages and dendritic cells, they are adapted to function in hypoxic, acidic environments. The process of bone resorption is rapid and appears to be highly energy-intensive, since osteoclasts must actively extrude protons to dissolve hydroxyapatite mineral, whilst secreting cathepsin K to degrade collagen, as well as maintaining a high degree of motility. Osteoclasts are well known to contain abundant mitochondria but they are also able to rely on glycolytic (anaerobic) metabolism to generate the ATP needed to power their activity. Their primary extracellular energy source appears to be glucose. Excessive accumulation of mitochondrial reactive oxygen species in osteoclasts during extended periods of high activity in oxygen-poor environments may promote apoptosis and help to limit bone resorption - a trajectory that could be termed 'live fast, die young'. In general, however, the metabolism of osteoclasts remains a poorly-investigated area, not least because of the technical challenges of studying actively resorbing cells in appropriate conditions.

Introduction

Osteoclasts are bone-resorbing cells that are derived from promonocytic precursors present in marrow, spleen and circulating blood [67,68,16]; they are considered to be terminally differentiated cells that are incapable of self-replication. Osteoclasts excavate characteristic, scalloped pits and troughs in bone, dentine and other mineralised tissues by secreting acid and proteolytic enzymes (chiefly cathepsin K) into a sealed extracellular compartment to dissolve the mineral and collagenous organic matrix components, respectively.

Bone resorption by osteoclasts normally proceeds much more rapidly than bone formation by osteoblasts. In vitro, continuous resorption trails or fields are commonly observed when osteoclasts are formed on bone or dentine from marrow or blood precursor cells using MCSF and RANKL and exposed to the acidic conditions required for activation [51,7] (**Fig 1**). Activation of osteoclasts involves cell polarisation, with formation of a prominent ruffled border (**Figs 1 & 2**) and markedly increased expression of the machinery of bone resorption, including carbonic anhydrase 2, the vacuolar-type H^+ ATPase (v-ATPase), cathepsin K and tartrate-resistant acid phosphatase (TRAP). An activated osteoclast *in vitro* can resorb an estimated quantity of mineralised matrix equivalent to about twice its own volume per day [7]. This impressive work rate suggests that resorbing osteoclasts have high energetic requirements.

In addition to their clearly-defined state of activation, osteoclasts exhibit a number of other special features of interest with respect to energy metabolism. These include cell fusion and multinuclearity, abundant mitochondria (**Fig 2**), a high degree of cell motility, intracellular trafficking of waste products from resorption, the ability to function in both oxygenated and hypoxic environments, and susceptibility to apoptosis. Energy metabolism in osteoclasts has not been extensively investigated, particularly during the process of resorption pit formation. However, it is clear that osteoclasts display remarkable metabolic adaptability, reflecting the properties of their related cells in the monocytes / macrophage lineage [61].

This brief review discusses what is known of the metabolic characteristics of osteoclasts during the distinct phases of their lives: formation, activation and death.

Hypoxia and osteoclasts

Before discussing current knowledge of key pathways in osteoclast metabolism, it is worth considering a remarkable property of these cells, namely their responses to changes in ambient oxygen tension.

In most normal, resting tissues, oxygen tension (pO_2) is generally thought to be between about 90 mmHg (~12%) and 40 mmHg (~5%), the respective values for arterial and venous blood. The skeleton appears to be unusual in that it contains various hypoxic environments, as demonstrated by independent methods. For example, mean pO_2 values of 6.6% have been reported for bone marrow aspirates from normal human donors [21], whereas direct imaging measurements of pO_2 in the calvariae of live, young adult mice yielded the surprisingly low values of 6.3%, 3.9% and 1.5-2.5 % in periosteum, cortical bone and marrow, respectively [60]. An important corollary of these *in vivo* observations is that *in vitro* investigations of osteoclast function and metabolism performed in atmospheric oxygen (pO_2 = 21%, which is commonly considered to represent 'normoxia') are actually conducted in significantly hyperoxic conditions that constitute a 3 to 10-fold excess of oxygen, with respect to the physiological environment in bone. Pathological hypoxia in bone could result from numerous causes including inflammation, tumours, diabetes, anaemias, fractures and ageing [6].

Hypoxia was shown to strikingly increase the number and size of osteoclasts formed in cultures of RANKL/M-CSF-treated mouse marrow cells on ivory discs, resulting in large increases in resorption pit formation. Hypoxia also caused maximal increases in osteoclastic resorption in bone organ cultures of mouse calvariae. Optimal osteoclast formation occurred in 1-2% O_2 but osteoclastogenesis was elevated even in cultures gassed with 0.2% O_2, compared with 20% O_2 [5]. Comparable findings have been reported in other studies using human [31,65,48] and cat [46] osteoclasts. The resorptive function of osteoclasts was unaltered in pH-controlled, hypoxic conditions. This indicates that the primary action of hypoxia is to increase osteoclastogenesis, and that the increased resorption observed in hypoxic cultures reflects the presence of increased numbers of larger osteoclasts, rather than increases in their activity (which might result from hypoxia-induced acidification). However, osteoclast survival was significantly reduced in cultures exposed to severe and/or continuous hypoxia [5,31,65]. Periodic re-oxygenation (eg, at culture medium changes) is required for optimal osteoclastogenesis and survival, and the possibility cannot be excluded that it is re-oxygenation rather than hypoxia which primarily drives osteoclastogenesis [22]. Fluctuating, non-uniform oxygen levels may well reflect the *in vivo* microenvironments experienced by differentiating osteoclasts more accurately than steady state hypoxia [5,6,65,32,27]. The effect of hypoxia on osteoclastogenesis appears to reflect the trophic actions of low pO_2 on macrophage differentiation [26,9,33,61]. These findings may help explain why extravasation of monocytic precursors into relatively oxygen-deficient bone microenvironments could help drive osteoclast formation, and offer potential insights into the bone loss associated with the pathological hypoxia.

In contrast to the stimulatory effect of hypoxia on osteoclastogenesis, exposure to atmospheric oxygen levels (20% O_2) markedly inhibited formation of mouse and human

osteoclasts, with respect to 2-5% O_2, which roughly equates to average pO_2 values in extracellular environments of living bone [5,65]. Severe hyperoxia (95% - 100% O_2), both *in vitro* and *in vivo*, is reported to cause further inhibition of osteoclastogenesis, in comparison with atmospheric pO_2 [19,20]. Conversely, osteoblast function and bone formation are oxygen-dependent processes that are inhibited by hypoxia [64,6].

Glycolytic and oxidative metabolism in osteoclasts

General principles

In common with almost all animal cells, osteoclasts can generate ATP by both glycolysis and oxidative phosphorylation. Glycolysis is the cytosolic, oxygen-independent metabolic pathway that rapidly converts a molecule of glucose into pyruvate, releasing free energy that is used to generate 2 molecules of ATP. In the absence of oxygen, pyruvate is generally metabolised to lactate. When oxygen is present, pyruvate may theoretically be fully metabolised to CO_2 in the mitochondria via the tricarboxylic acid cycle, generating a potential maximum of 34 further molecules of ATP (**Fig 3**) [29]. Aerobic respiration is thus a far more effective way to generate energy than glycolysis. However, multiple cell types, including osteoblasts appear to metabolise glucose into lactate, even in the presence of sufficient oxygen, a process known as aerobic glycolysis.

Glycolytic metabolism of one glucose molecule yields 2 H^+, whereas its complete mitochondrial oxidation to CO_2 produces 6 H^+. Therefore, glycolysis theoretically generates 6 times more H^+ than oxidative phosphorylation per ATP molecule created [43]. This has potentially important consequences for the activation of osteoclastic resorption in hypoxic bone microenvironments subject to acidification due to glycolytic metabolism of resident (mainly non-osteoclastic) cells [6].

Mitochondria

It has long been recognised that osteoclasts contain plentiful mitochondria [12,15] (**Fig 2**). However, the biogenesis and function of mitochondria in osteoclasts has been relatively little studied. Estimates of mitochondrial mass in human osteoclasts suggest that they retain the mitochondria of their precursor cells [39], although increased expression of mRNAs for mitochondrial genes such as respiratory complexes I–V has been described during the formation of mouse osteoclasts [25]. Mitochondrial biogenesis in osteoclasts is reported to be regulated by peroxisome proliferator–activated receptor-c coactivator 1β (PGC-1β) and iron uptake (required for mitochondrial respiratory proteins), via the transferrin 1 receptor [25]. A subsequent study from another group obtained somewhat divergent results. Mitochondrial biogenesis was shown to be stimulated by RANKL but was

not affected by PGC-1β overexpression. Inactivating mutations in the NF-κB pathway proteins, RelB and NF-kB–inducing kinase (NIK) blocked bone resorption and the stimulatory action of RANKL on mitochondrial DNA, the expression of proteins related to oxidative phosphorylation and oxygen consumption [71].

The underlying reasons for the numerous mitochondria in osteoclasts are not entirely clear. One possibility is that they presumably help the cell to recover quickly following re-oxygenation after a high work rate in hypoxic conditions. A further explanation for the mitochondrial abundance may lie in their key role in the intrinsic apoptotic signalling pathway. Mitochondria act as a reservoir of pro-death factors, notably cytochrome c and Smac/DIABLO, which are released through stress-induced membrane pores to activate the caspase pathway [55]. Osteoclasts are well-known to be susceptible to apoptosis, and the triggering of apoptosis by mitochondria may constitute an effective mechanism for rapidly eliminating these highly destructive cells.

Metabolism in differentiating and activated osteoclasts

An early cytophotometric study of fresh human bone sections showed that osteoclasts associated with bone surfaces (and thus more likely to be actively resorbing) displayed a high capacity for the (mitochondrial) β-oxidation of fatty acids, a highly efficient energy source. In contrast, such osteoclasts were observed to be deficient in the enzymes associated with glycolysis and the anabolic pentose shunt pathway [14].

Subsequent metabolic investigations have relied on osteoclasts cultured *in vitro*. A study using multinucleated cells purified from the bones of egg laying hens provided the first direct evidence that D-glucose was the principal energy source for osteoclasts. Glucose consumption was shown to double when these osteoclast-like cells were in contact with (and degrading) powdered bone particles; lactate was also able to support bone degradation to a small extent [69]. Subsequent work showed that extracellular glucose upregulated mRNA for the v-ATPase [35] and quickly increased the intracellular ratio of ATP to ADP in the avian osteoclast-like cells [36]. Supraphysiological glucose concentrations of glucose inhibit osteoclastogenesis [70].

Studies with osteoclasts formed from mouse marrow cells on plastic (ie, non-resorbing cells) showed that RANKL treatment resulted in increased lactate production, via upregulation of lactate dehydrogenase and glucose utilisation, along with large increases in oxygen consumption, as well as significant extracellular acidification [30,1]. These findings suggest that both aerobic and anaerobic respiration occur during osteoclastogenesis. The same group also found that rotenone, an inhibitor of mitochondrial complex 1, strongly decreased in vitro osteoclastogenesis, together with the expression of mRNAs for key osteoclast-related factors such as c-Fos, NFATc1, TRAP and OSCAR [30]. Rotenone was subsequently

shown to block resorption pit formation in vitro and reduce LPS-induced bone loss in vivo [34]. The above findings contrast somewhat with a subsequent investigation which found that osteoclastogenesis was associated with increased expression of glycolytic genes, including hexokinase, phosphofructokinase and pyruvate kinase [24]. This study also reported increased expression of both glucose and glutamine transporters during osteoclast differentiation, and that depletion of glucose or L-glutamine suppressed osteoclast differentiation and function.

A recent study of osteoclasts derived from human peripheral blood cells cultured on plastic has also shown that treatment with MCSF and RANKL increased oxygen consumption, as well as the expression of mitochondrial respiratory chain enzymes. Rotenone and oligomycin (an ATP synthase inhibitor) both reduced oxygen consumption. When cells were cultured on collagen-coated surfaces, as an attempt to model bone resorption, replacement of glucose with galactose (which would be expected to reduce glycolytic flux) somewhat reduced collagen degradation, whereas rotenone slightly enhanced it. Confocal microscopy indicated that the glycolytic enzymes PKM2 and GAPDH localise at the sealing zone, close to the actin ring, suggesting the possibility that localised glycolysis could allow for rapid, effective generation and delivery of ATP for motility (and to power the vacuolar H^+ pump) during resorption. The overall conclusion was that oxidative phosphorylation was the main bioenergetic source for osteoclast formation, whereas 'bone resorption' mainly relied on glycolysis [40].

AMP-activated protein kinase (AMPK) is a key regulator of systemic and cellular energy homeostasis whose broad actions are to inhibit energy (eg, ATP) consumption and stimulate energy production; it may be considered as a sensor of metabolic stress. Somewhat conflicting results have been published on the role of AMPK in regulating osteoclast function. The AMPK activator, AICA-riboside strongly stimulated OC differentiation in vitro and caused profound bone loss in mice in vivo; however, germline deletion of AMPK subunits in mice reduced bone mass without any effects on osteoclast numbers in trabecular bone, suggesting that AMPK is not required for osteoclast formation [56]. In contrast, other groups reported that inactivation of AMPK by siRNA knockdown or deletion of its catalytic subunits increased osteoclastogenesis *in vitro* [38,28,17] and reduced bone mass *in vivo* [28], whereas chemical activation reduced osteoclast numbers and resorption *in vitro* [39] and *in vivo* [40]. Another 'energy sensor' of current interest in osteoclast biology is mTORC1 (mechanistic target of rapamycin complex 1), a highly conserved intracellular regulatory complex. Again, current understanding is far from clear. Inhibition of mTOR with rapamycin is reported to significantly reduce osteoclastogenesis [63]. In apparent contrast, genetically targeted inhibition or activation of mTORC1 in mouse osteoclast precursors are stated to strongly increase or decrease osteoclast differentiation and function, respectively [72].

Osteoclast regulation by products of metabolism

Various metabolic products or by-products are known to exert direct actions on osteoclast function. The most significant such factor appears to be H^+ ions, the universal by-product of cellular metabolism. Extracellular acidification has long been known to be an obligatory requirement for the activation of osteoclasts, which exhibit little or no resorption pit formation at normal blood pH (7.4) [3]. Acidification also acts as a signal to halt the precursor cell fusion during osteoclastogenesis [51]. The great sensitivity of osteoclasts to small pH changes poses technical challenges when assessing responses to other factors that themselves might affect local pH. The pH response of osteoclasts is also modulated by CO_2 and HCO_3^-, such that osteoclasts appear to show particular sensitivity to CO_2 acidosis [4].

Reactive oxygen species (ROS; predominantly H_2O_2 and superoxide) are produced in the mitochondria during generation of ATP [32,10] and play important roles in the function of cells in the monocyte-macrophage-osteoclast lineage. ROS is also generated by TRAP in osteoclasts and is thought to contribute to the vesicular degradation of organic matrix [66]. Numerous studies over the last quarter century have indicated that exogenous ROS [18,8] or endogenous ROS [62,37] may stimulate both the formation and resorptive activity of osteoclasts, possibly via activation of the NFκB pathway, although the details remain far from clear [10].

Pyruvate has been shown to boost oxygen consumption by non-resorbing osteoclasts formed from mouse marrow cells [30] and to stimulate the formation of osteoclast-like cells in RAW 264.7 cultures [17]. Lactate is reported to exert reprogramming effects on dendritic cells [47] but its effects on osteoclasts are unknown.

Lastly, the intracellular currency of energy, ATP (whose concentration inside cells is generally in the range 2-5 mM) is released in small amounts into the extracellular environment by osteoblasts and osteoclasts, where it acts on P2 receptors in an autocrine or paracrine fashion to modulate cell function [49,50,52,53]. At concentrations near the likely physiological range, ATP is a potent stimulator of both the formation and activity of rodent osteoclasts, whereas at higher concentrations, it is inhibitory [44,50,42].

Metabolic adaptation of osteoclasts to hypoxia

Cells respond to changes in pO_2 via oxygen-dependent degradation of hypoxia inducible transcription factors (HIFs), a heterodimer containing α and β subunits. In the presence of oxygen, conserved proline residues on HIF-1α, and the closely related HIF-2α are hydroxylated by prolyl hydroxylases, targeting them for proteasomal degradation. In the absence of sufficient oxygen, the prolyl hydroxylases are inactive and the proline residues

on HIF-α remain unmodified; HIF-α is stabilised and heterodimerises with its transcription partner, HIF-β. The HIF heterodimer binds hypoxia response elements in target gene promoters and initiates transcription of hundreds of hypoxia-regulated genes involved in a multiplicity of cellular processes [59,27].

Stabilised expression of both HIF-1α and HIF-2α, with nuclear translocation, has been described in response to hypoxia or the hypoxia mimetic, $CoCl_2$ in mature human osteoclasts and their mononuclear progenitor cells [65,32]. Knockdown of HIF-1α is reported to abolish resorption pit formation in normoxic cultures of osteoclast-forming mouse marrow cells [24]. HIF-1α knockdown has also been shown to inhibit resorption in hypoxic cultures of human peripheral blood mononuclear cells [31, 32], whereas induction of HIF-1α increased their resorptive activity [22]. However, the aforementioned study failed to show a significant regulatory action of HIF-1α on osteoclastogenesis. HIF-1α expression appears to be regulated by estradiol and estrogen receptor-α (ER-α). In hypoxic cultures of osteoclast-like RAW 264.7 cells, estradiol destabilised HIF-1α expression and, *in vivo*, osteoclast-specific HIF-1α inactivation antagonised bone loss in mice that were ovariectomised or lacking ER-α in their osteoclasts [41].

The HIF-mediated adaptation of cells to hypoxia normally necessitates switching to anaerobic metabolism to generate the ATP needed for their function and survival. The typical cellular responses to hypoxia entail: 1) increasing the efficiency of complex IV of the mitochondrial electron transport chain; 2) upregulation of the cytochrome c oxidase subunit COX4-2 at the expense of the COX4-1 subunit; 3) increased expression of glucose transporters and glycolytic enzymes; 4) inhibition of mitochondrial pyruvate dehydrogenase, thus blocking the tricarboxylic acid cycle; 5) initiation of mitophagy. A key reason for inhibition of mitochondrial ATP production in hypoxia is to reduce the associated generation of excess cytotoxic ROS [59,32].

The adaptive responses of osteoclasts to hypoxia are potentially somewhat less clear-cut because, like macrophages [58] they appear to exhibit a significant basal glycolysis rate during differentiation even in normoxic conditions [30,24,1], a situation analogous to the 'Warburg effect' in cancer cells [56,11]. In line with the striking increases in osteoclast formation and associated resorption pit formation observed in hypoxia, Knowles and colleagues showed that 24 hours exposure of human osteoclasts to a 2% oxygen atmosphere increased ATP production, with stimulation of both oxygen consumption and expression of COX4-2 mRNA (indices of mitochondrial activity), as well as increased glucose consumption and lactate production (indices of glycolytic flux). Glucose was necessary for the hypoxic increase in ATP production and also for cell survival in hypoxia. Overall, these atypical, HIF-1α -mediated metabolic responses were interpreted as facilitating the short-

term, high metabolic activity of osteoclasts, at the expense of long-term survival [45] – a process that could be summarised as 'live fast, die young'.

The evident reliance of osteoclasts on anaerobic metabolism suggests possibilities for new antiresorptives, and may help explain why imatinib, a c-Abl tyrosine kinase inhibitor that suppresses glycolysis can decrease bone resorption *in vitro* and *in vivo* [2,13,54].

Conclusions

Osteoclasts show impressive metabolic flexibility that enables them to function in diverse and sometimes hostile conditions. These remarkable cells are ultimately responsible for the majority of bone pathologies but our understanding their special metabolic characteristics remains quite limited. Although it seems clear that osteoclasts can switch to glycolysis in hypoxia, the extent to which they may also rely on anaerobic metabolism in 'normoxic' (or even hyperoxic) environments to support their formation, motility and resorptive activity is still unclear. The mechanism by which osteoclastogenesis is promoted by hypoxia additionally needs clarification, since it does not appear to be significantly dependent on HIF-1α (and, theoretically might even be driven by re-oxygenation). The reasons for the striking numbers of mitochondria in osteoclasts also need further examination.

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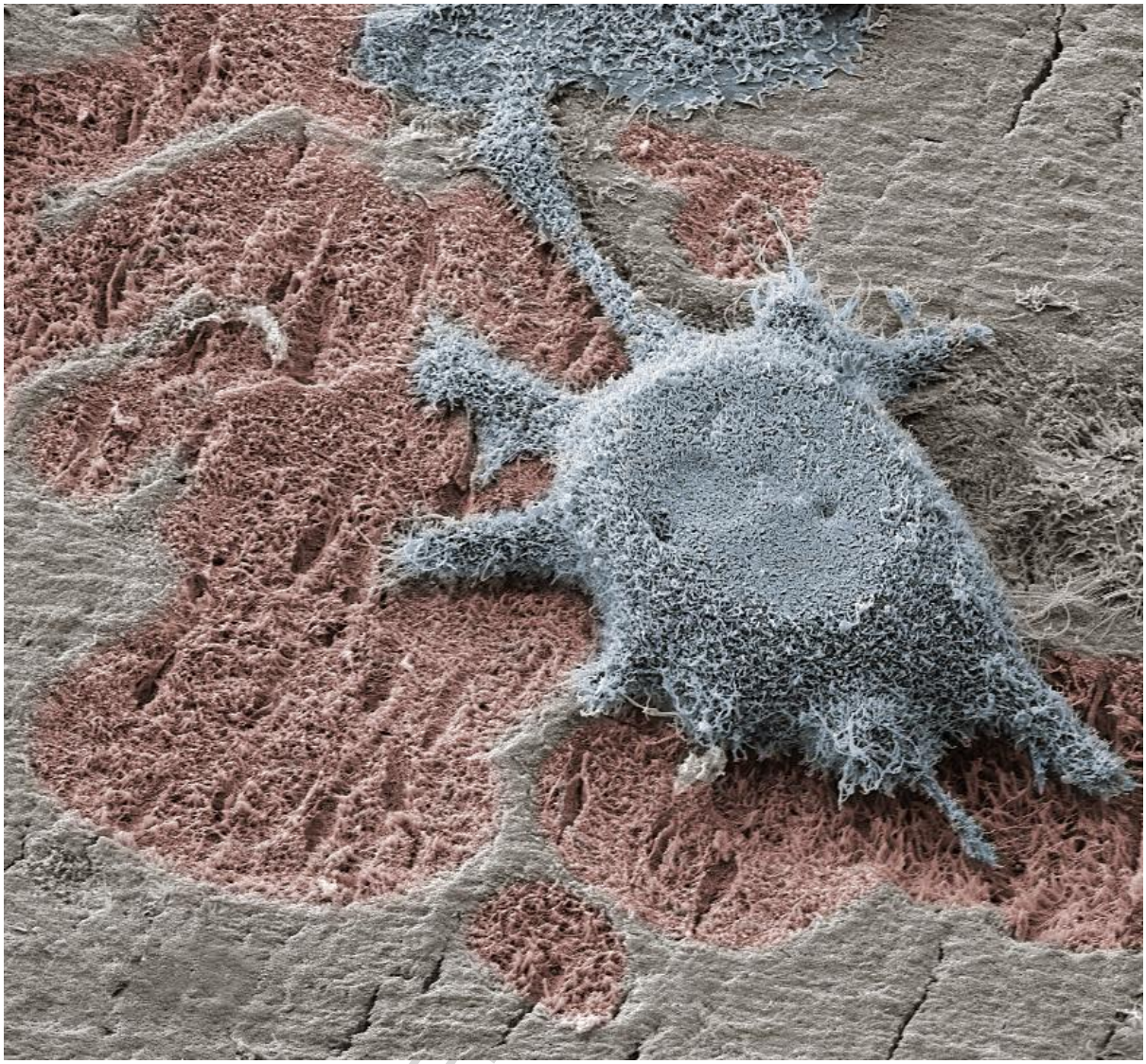

Fig 1. Scanning electron micrograph showing the extensive resorption area (pink) associated with a large osteoclast (blue) formed from mouse marrow cultured in the presence of M-CSF and RANKL on an ivory slice, and activated for 48 hours in acidified (pH 7.0) medium. Field width = 85 µm.

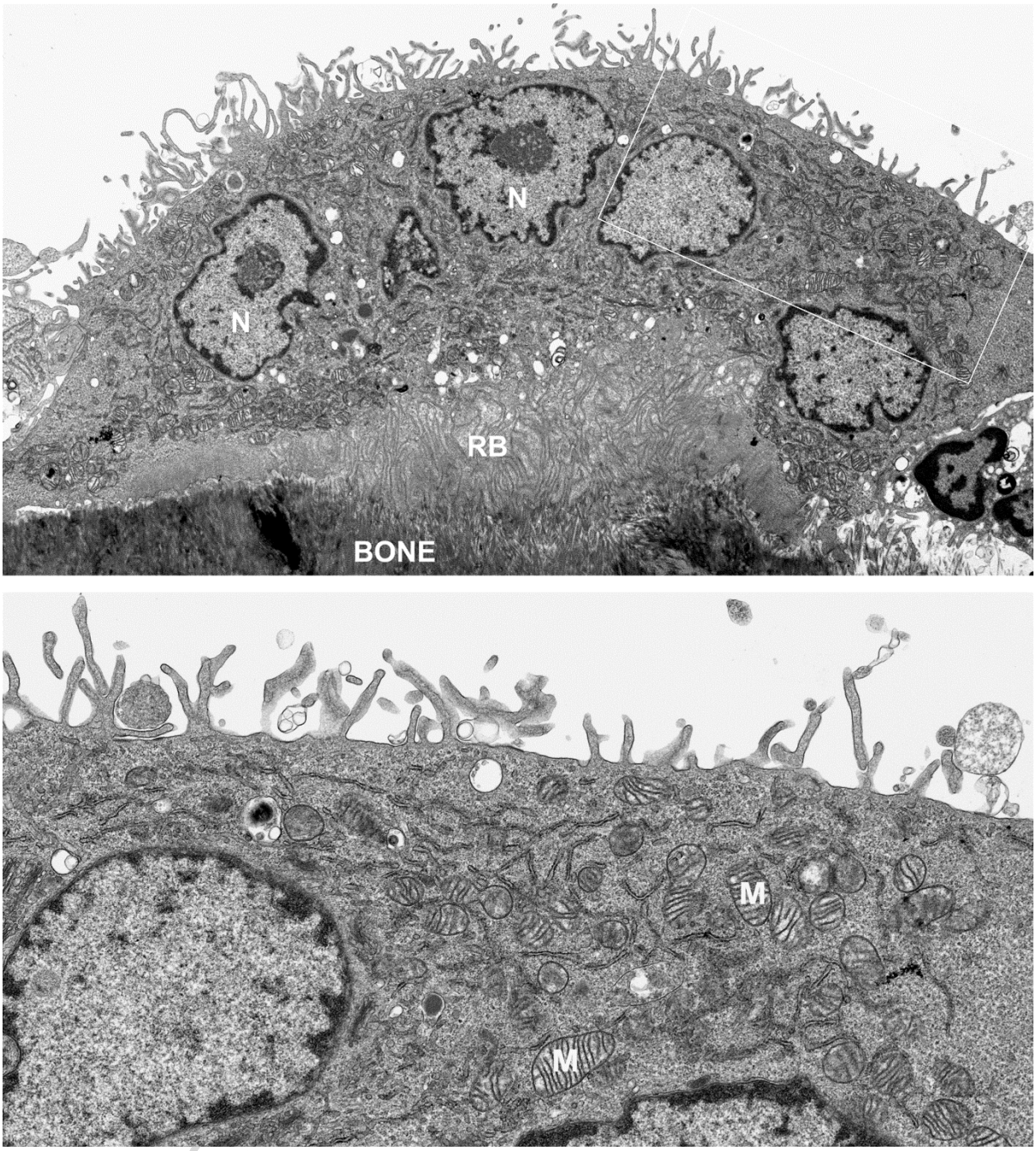

Fig 2. Transmission electron micrographs showing an osteoclast with 4 or 5 nuclei (N) and ruffled border (RB), actively resorbing bone. Lower panel is an enlargement of boxed area in upper panel, showing abundant mitochondria (M). Field width of upper panel = 35 µm. By kind permission of E. McDermott and M.H. Helfrich, University of Aberdeen.

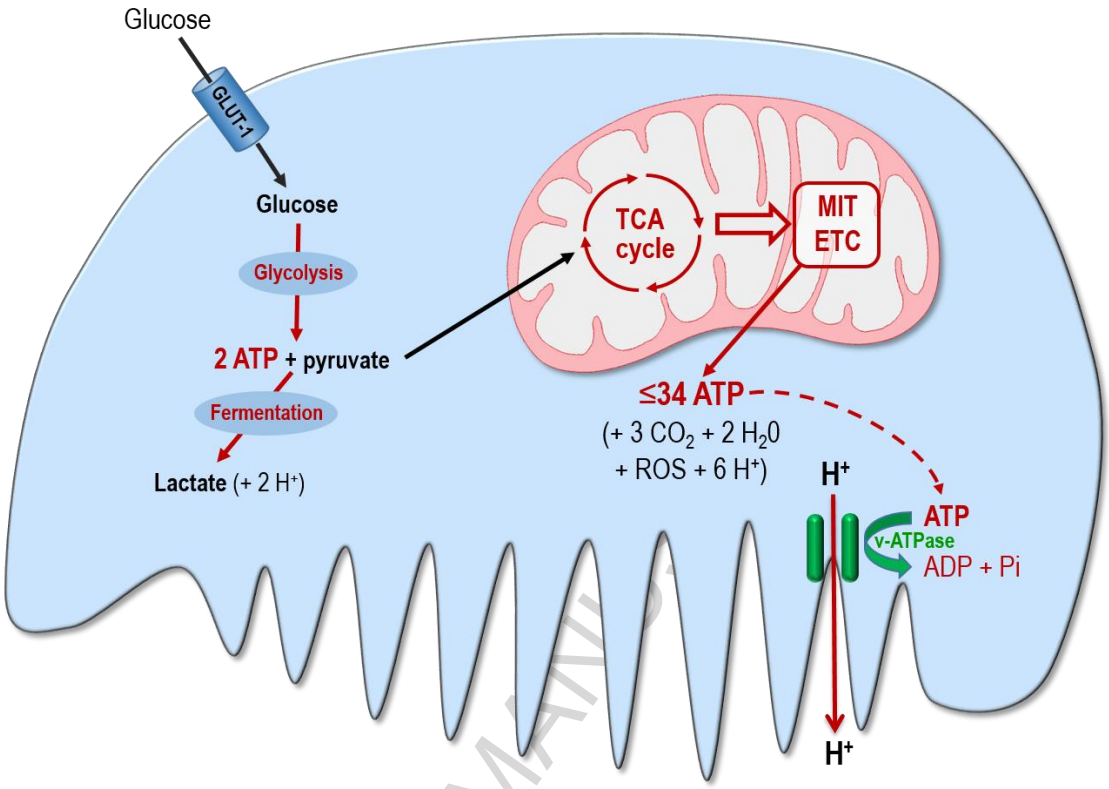

Fig 3. Simplified summary of aerobic and anaerobic metabolism in osteoclasts. GLUT-1, glucose transporter; TCA, tricarboxylic acid; MIT ETC, mitochondrial electron transport chain; ROS, reactive oxygen species; v-ATPase, vacuolar H^+ proton pump.

Highlights

- Resorption of bone by osteoclasts is rapid and energy-intensive (utilises glucose)
- Osteoclasts contain many mitochondria but also generate ATP anaerobically
- Resorptive function of osteoclasts is unimpaired in hypoxia
- Osteoclastogenesis (& thus resorption) is strongly enhanced by intermittent hypoxia
- Chronic hypoxia may promote osteoclast apoptosis via ROS build-up in mitochondria