Neutrophils and tissue damage: is hypoxia the key to excessive degranulation?

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Neutrophils are the first innate immune cells recruited into sites of infection and inflammation, and although they are critical for host defence against microbial pathogens, they have also been associated with local tissue damage. Predominant neutrophilic inflammation is a key hallmark of many respiratory diseases and the release of proteinases and reactive oxygen species (ROS) from primed neutrophils has been implicated in mediating the damage to surrounding tissue.[1] Inflammation is further accompanied by severe hypoxia (lack of oxygen) within the affected tissue, and is a characteristic of both acute and chronic lung diseases, such as acute respiratory distress syndrome (ARDS),[2] chronic obstructive pulmonary disease (COPD),[3] and cystic fibrosis (CF) .[4] Considering that tissue hypoxia is part of a normal inflammatory response, innate immune cells such as neutrophils and macrophages possess important cellular and molecular mechanisms that enable them to function effectively at low oxygen concentrations.[5]

There is considerable interplay between the molecular pathways that regulate hypoxia and inflammation. In previous studies, mice exposed to hypoxic conditions (5% O_2 for 1 hour) demonstrated increased levels of IL-6, TNF and IL-1 in both serum and isolated macrophages.[6] In humans, increased levels of IL-6, IL-1RA and C-reactive protein were detected in serum after volunteers experienced three overnight stays at altitude.[7] Moreover, infiltrating neutrophils directly increase their oxygen consumption in order to assemble the NADPH oxidase complex that generates ROS and, in conjunction with superoxide dismutase and myeloperoxidase, hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO), thereby depleting local levels of oxygen.[8, 9] Neutrophils are also thought to influence the tissue microenvironment during episodes of hypoxia. For example, neutrophil migration across epithelia increases the transcriptional activity of hypoxia-inducible genes in epithelial cells, which in turn influence the resolution of inflammation.[10]

Neutrophils have therefore been implicated in the pathogenesis of several inflammatory lung diseases associated with tissue hypoxia.[5] However, the mechanisms by which neutrophils mediate tissue damage under hypoxic conditions are less clear. Under homeostatic conditions neutrophils are short-lived cells that readily undergo apoptosis in order to avoid the release of toxic-granule contents. However, under hypoxic conditions neutrophil survival is prolonged, which is thought to delay the resolution of inflammation and promote tissue damage, potentially through the release of destructive proteinases or ROS.[11] The study by Hoenderdos *et al.* in this issue of *Thorax* aims to explore the functional link between hypoxia and the regulation of neutrophil degranulation in the context airway inflammation.

Compared to neutrophils incubated under normoxic conditions (atmospheric O_2 concentration), this study demonstrated that neutrophils incubated under hypoxic conditions (0.8% O_2, 3 kPa for 4 hours) released significantly elevated levels of neutrophil elastase (NE), myeloperoxidase (MPO), lactoferrin and metalloproteinase-9 (MMP-9), thereby indicating degranulation of azurophil (primary), specific (secondary) and gelatinase (tertiary) granules. This effect was largely dependent on priming with granulocyte-macrophage colony stimulating factor (GM-CSF) and activation with formylated peptide (fMLP), as little degranulation occurred at basal levels in either normoxic or hypoxic conditions. These findings support previous studies demonstrating that neutrophils isolated from human volunteers, following a period of hypoxemia, released increased levels of NE after fMLP stimulation,[12] and neutrophils from healthy volunteers exposed to hypoxia *ex vivo* (3 kPA for 1-4 hours) released NE.[13] However, these two studies reported opposing effects on ROS production.

Previous studies under hypoxia have sometimes given conflicting results, which is likely related to differing experimental conditions that affect neutrophil function. There may therefore be some merit in standardising methodologies across laboratories, to enable direct comparison of studies within the scientific community. It is also important to consider whether experimental hypoxic conditions mirror those found in the lung during episodes of inflammation. The lung is normally an oxygen-rich organ, with an alveolar PO_2 ~110 mm Hg compared to other systemic organs (3-20 mm Hg). Therefore, oxygen tensions in a hypoxic lung are higher than other organs (~50 mm Hg compared to less than 10 mm Hg).[14] The current study exposed neutrophils to hypoxic conditions of 3 kPa, which is equivalent to ~20 mm Hg, and therefore attempts to recapitulate the physiological conditions within the airway epithelium. It is argued that similar mechanistic *ex vivo* studies should also reflect these unique conditions. More challenging will be to recapitulate hypoxic conditions within tissue niches or foci of inflammation, such as those that occur in the small airway epithelium in COPD and CF.

The release of neutrophil proteinases has been strongly implicated in promoting tissue damage within inflamed lung.[15] In this study, hypoxic supernatants from GM-CSF and fMLP stimulated neutrophils increased the detachment and death of epithelial cells compared to normoxic neutrophils. Treatment with alpha-1-anti-trypsin further revealed that the toxic effects were mediated by proteinases, supporting a role for activated neutrophils in mediating tissue damage. The enhanced toxic effects of hypoxic neutrophil supernatants were apparent when epithelial cells were cultured in 2D monolayers. However, recapitulating these findings in 3D culture systems or, more importantly *in vivo*, remain a considerable challenge.

It is important to fully appreciate the significant effect that hypoxia has on downstream signalling pathways in both tissue resident cells and infiltrating leukocytes. Eukaryotic cells have evolved several oxygen sensing pathways, of which the principal regulators are hypoxia-inducible transcription factor-1 and -2 (HIF-1 and HIF-2).[16] Under hypoxic conditions HIFs are stabilised, allowing them to translocate to the nucleus and regulate HIF-response genes.[17] Interestingly, the current study reports that hypoxia-induced degranulation of neutrophils is not primarily mediated by HIF-1. Treatment of neutrophils

with either dimethyloxalyl glycine (DMOG) or desferrioxamine (DFO), which are both HIF-1 stabilising agents, did not affect NE release. Furthermore, hypoxia did not increase the expression of NE but did increase the expression of BNIP-2, both of which are putative HIF-1 response genes. Taken together these data suggest that NE release following hypoxia-induced degranulation may be HIF-1 independent, although the contribution of HIF-2 was not directly explored in this study.

Neutrophil degranulation is also thought to be dependent on actin cytoskeletal rearrangements. Indeed, hypoxia caused focal actin polymerisation in fMLP stimulated neutrophils. However, inhibition of actin polymerisation using cytochalasin B increased NE release, while treatment with jasplakinolide (which promotes actin polymerisation) had no significant effect on NE release. Actin remodelling is necessary for neutrophil degranulation, as well as polarisation and migration in response to exogenous stimuli such as fMLP and IL-8.[18] Cytoskeletal rearrangements are thought to be a prerequisite for the intracellular movement of granules within the cytoplasm, while actin disassembly is necessary for access to the inner surface of the plasma membrane. It is therefore plausible that cytochalasin B relaxes the actin cytoskeleton and thereby promotes granule fusion with the plasma membrane, in a similar way that immune synapses form during polarised secretion of cytotoxic T cell granules.[19]

Degranulation is a tightly regulated process involving the activation of G-protein coupled receptors (GPCRs), intracellular calcium mobilisation and ATP-dependent phosphorylation of downstream signalling molecules.[18] Treatment with a phospholipase C (PLC) inhibitor or EGTA, in order to block intracellular Ca^{2+}, inhibited NE release under hypoxic conditions. Furthermore, treatment with thapsigargin, which is a Ca^{2+} ATPase inhibitor that elevates intracellular Ca^{2+} levels, caused an increase in NE release. These studies therefore confirm that Ca^{2+} mobilisation is an important signalling event during neutrophil degranulation under hypoxic conditions. However, the specific downstream targets of Ca^{2+} during neutrophil degranulation remain largely unknown.

Considering that hypoxia-induced neutrophil degranulation was independent of HIF signalling, an alternative PI3K signalling pathway was investigated. A pan PI3K inhibitor reduced neutrophil survival under hypoxic conditions, while a pan PI3K inhibitor and a PI3Kγ inhibitor completely abolished NE release from GM-CSF and fMLP stimulated neutrophils. Inhibition of PI3Kγ also inhibited the downstream phosphorylation of Akt, suggesting that the PI3K-Akt pathway may play a role in hypoxia-mediated neutrophil degranulation. It will be interesting to explore whether other known regulators of neutrophil degranulation, such as Src family kinases, guanosine triphosphatases (GTPases such as Ras and Rho) and MAP kinases are also regulated by hypoxia. In addition, hypoxia, PI3K/Akt and NF-κB pathways interact to promote pro-inflammatory and HIF-target gene expression. Although not explored in the current study, the interplay between hypoxia-mediated signalling pathways and NF-κB mediated pathways are likely to be functionally important in driving neutrophilic inflammation. [11]

In summary, the current study by Hoenderdos *et al.* in this issue of *Thorax* highlights the importance of hypoxia in regulating neutrophil degranulation. The continued release of neutrophil-derived proteinases is likely to contribute to the tissue damage observed in acute and chronic lung diseases, such as ARDS, CF and COPD. Studying neutrophil function in hypoxic conditions *ex vivo* may therefore reveal novel, drug-targetable cellular and molecular processes. The future challenge will be to reproduce the physiological conditions that occur within an injured lung as closely as possible.

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