Hypoxia increases the potential for neutrophil-mediated endothelial damage in COPD

Katharine M Lodge[1,2,$], Arlette Vassallo[1], Bin Liu[1], Merete Long[3], Zhen Tong[1], Paul R Newby[4], Danya Agha-Jaffar[2], Koralia Paschalaki[2], Clara E Green[4], Kylie BR Belchamber[4], Victoria C Ridger[3], Robert A Stockley[5], Elizabeth Sapey[4,5], Charlotte Summers[1], Andrew S Cowburn[2], Edwin R Chilvers[1,2], Wei Li[1,*], Alison M Condliffe[3,1,*,$]

Running title: Hypoxic neutrophil-mediated endothelial damage

[1]Department of Medicine, School of Clinical Medicine, University of Cambridge, Cambridge, UK

[2]National Heart and Lung Institute, Imperial College London, London, UK

[3]Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, UK

[4]Institute of Inflammation and Ageing, University of Birmingham, Queen Elizabeth Hospital, Birmingham, UK

[5]University Hospitals Birmingham NHS Foundation Trust, Queen Elizabeth Hospital Birmingham, Birmingham, UK

***Joint senior authors**

[$]Joint corresponding authors:

Dr Katharine Lodge, National Heart and Lung Institute, Imperial College London, ICTEM Building, Level 5 Vascular Science, Du Cane Road, London, W12 0NN; Email: k.lodge@imperial.ac.uk; Tel.: +44 (0)20 7594 6581

Professor Alison Condliffe, Department of Infection, Immunity and Cardiovascular Diseases, University of Sheffield Medical School, Beech Hill Road, Sheffield, S10 2RX; Email: a.m.condliffe@sheffield.ac.uk; Tel.: +44 114 215 9519

Author Contributions: KML, ERC, WL, AMC conceptualized the project and designed the methodology. KML recruited patients, collected samples, performed experiments and conducted data analysis. AV, BL, ML, ZT, PRB, DA-J performed experiments. CG, KB, ES recruited patients and collected plasma samples. KP, VCR, RAS, ES, CS, ASC provided resources and supervised experiments. WL, AMC supervised the project. KML, WL, AMC wrote the original manuscript. KML, ASC, ERC, WL, AMC revised the manuscript. All authors reviewed and edited the manuscript.

Funding: Wellcome Trust Research Training Fellowship for KML, BMA Foundation for Medical Research Josephine Lansdell 2017 grant for KML, Academy of Medical Sciences Starter Grant for Clinical Lecturers for KML, Cambridge NIHR BRC-GSK EMI Clinical Research Training Fellowship for AV, BHF Project Grant for KP, NIHR Imperial Biomedical Research Centre, MRC, and Imperial College NHLI Foundation.

Subject descriptor: 7.19 Immunology and inflammation: Neutrophils

Short summary:

Scientific knowledge on the subject: COPD is characterised by persistent neutrophilia in the setting of local and systemic hypoxia, and is associated with excess cardiovascular disease, even allowing for known risk factors. Neutrophils accumulating in areas of inflammation and microcirculatory impairment experience profound hypoxia, which prolongs their survival and increases their secretory responses. Thus, hypoxic neutrophils have increased potential to cause endothelial injury but their role in mediating the increased cardiovascular risk in COPD is poorly understood.

What this study adds to the field: Herein we show that hypoxia augments the ability of neutrophils to selectively secrete a subset of histotoxic proteins capable of damaging endothelial cells. Hypoxia further enhanced release of these proteins from exacerbating COPD patient neutrophils, with elevated levels also detected in patient plasma. This study suggests that hypoxic enhancement of neutrophil degranulation may contribute to increased cardiovascular risk in COPD and that the hypoxic neutrophil secretome proteins may represent new therapeutic targets to alleviate endothelial dysfunction in COPD.

Total word count: 3500

This article has an online data supplement which is accessible from this issue's table of content online at www.atsjournals.org

ABSTRACT

Rationale: Chronic obstructive pulmonary disease (COPD) patients experience excess cardiovascular morbidity and mortality, and exacerbations further increase the risk of such events. COPD is associated with persistent blood and airway neutrophilia, and systemic and tissue hypoxia. Hypoxia augments neutrophil elastase release, enhancing capacity for tissue injury.

Objective: To determine whether hypoxia-driven neutrophil protein secretion contributes to endothelial damage in COPD.

Methods: The healthy human neutrophil secretome generated under normoxic or hypoxic conditions was characterised by quantitative mass spectrometry, and the capacity for neutrophil-mediated endothelial damage assessed. Histotoxic protein levels were measured in normoxic *versus* hypoxic neutrophil supernatants and plasma from exacerbating COPD patients and healthy controls.

Main results: Hypoxia promoted PI3Kγ-dependent neutrophil elastase secretion, with greater release seen in neutrophils from COPD patients. Supernatants from neutrophils incubated under hypoxia caused pulmonary endothelial cell damage and identical supernatants from COPD neutrophils increased neutrophil adherence to endothelial cells. Proteomics revealed differential neutrophil protein secretion under hypoxia and normoxia; hypoxia augmented secretion of a subset of histotoxic granule and cytosolic proteins, with significantly greater release seen in COPD neutrophils. The plasma of COPD patients had higher content of hypoxia-upregulated neutrophil-derived proteins and protease activity, and vascular injury markers.

Conclusions: Hypoxia drives a destructive ‘hyper-secretory’ neutrophil phenotype conferring enhanced capacity for endothelial injury, with a corresponding signature of

neutrophil degranulation and vascular injury identified in COPD patient plasma. Thus, hypoxic enhancement of neutrophil degranulation may contribute to increased cardiovascular risk in COPD. These insights may identify new therapeutic opportunities for endothelial damage in COPD.

Abstract word count: 250

Keywords: cell degranulation, neutrophil elastase, vascular endothelium, cardiovascular disease

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by neutrophilic inflammation in the setting of tissue (and often systemic) hypoxia, and by increased risk of cardiovascular disease (CVD) and pulmonary hypertension (PH). Neutrophil elastase (NE) has been implicated in COPD pathogenesis (1), and we have previously shown that hypoxia markedly augments NE release from neutrophils to promote respiratory epithelial cell damage (2). However, the impact of hypoxia on the extended neutrophil secretome, and the potential for 'hypoxic neutrophils' to injure other disease-relevant cell types, are currently unknown. Identifying novel targets implicated in driving COPD morbidity may provide new therapeutic opportunities.

Even in health certain tissues, e.g. muscle, are hypoxic (3). This 'physiological hypoxia' may be compounded during exercise, inducing neutrophil phenotypic changes (4). In disease, profound 'pathological hypoxia' exists in inflamed/infected tissues and areas of microcirculatory impairment. Although patients with severe COPD are systemically hypoxic, significant tissue hypoxia (less than 1.3% oxygen) can occur even in mild disease, demonstrated in inflamed airways (5–7) and atherosclerotic vasculature (8), where neutrophils accumulate. Upregulation of hypoxia-inducible factors in neutrophils from patients with acute lung injury, including COVID-19 infection, indicates neutrophil exposure to hypoxia *in vivo* (9).

Neutrophil anti-microbial function depends on the fusion of pre-formed granules, containing cytotoxic proteins and proteases, with the pathogen-containing phagosome. However, highly activated neutrophils can release granule contents extracellularly (degranulation), with potential for collateral tissue damage (2). Platelet activating factor (PAF), a physiological priming agent capable of substantially

enhancing neutrophil degranulation in response to subsequent stimulation (2,10), has been implicated in COPD pathogenesis, and in endothelial damage and remote organ damage in the setting of hypoxia (11,12). Bacteria release formylated peptides (fMLP) which potently activate neutrophils; these peptides are present in cigarette smoke and have been implicated emphysema progression (13). COPD patients suffer recurrent infection-driven exacerbations but neutrophilic inflammation persists even in the absence of detectable infection, correlating with disease severity and progression (14). Despite evidence of NE-induced lung injury, translation of NE inhibitors has not led to significant benefit (15), perhaps reflecting the complex array of additional neutrophil-secreted proteins with damaging potential.

COPD patients have increased risk of cardiovascular morbidity and mortality even after adjusting for shared risk factors including smoking (16), particularly following exacerbation (17). Pulmonary endothelial dysfunction in COPD patients can induce PH, which correlates with hypoxia (18). Accumulating evidence indicates inflammation, oxidative stress and vascular tissue damage as key mechanisms linking COPD and CVD (19), with neutrophil degranulation identified as an important pathway (20). Circulating neutrophils primed for enhanced degranulation have been identified in exacerbating COPD patients (21), with potential to contribute to endothelial injury.

Herein we show that hypoxia promotes neutrophil degranulation and neutrophil-induced endothelial damage. Proteomic analysis reveals hypoxia-driven secretion of highly histotoxic proteins from healthy neutrophils, and a subset of these are further increased from COPD neutrophils. Supernatants from hypoxic COPD neutrophils enhance neutrophil-endothelial adhesion. Finally, we identify increased levels of corresponding ‘hypoxic neutrophil’ histotoxic granule proteins in COPD plasma,

together with endothelial injury biomarkers. Some results have been reported previously in abstract form (22–24).

MATERIALS AND METHODS

Ethics statement

Written informed consent was obtained from all healthy volunteers and COPD patients (06/Q0108/281, 08/H0308/281, 18/WM/0097, 20/SS/0085). All studies complied with the Declaration of Helsinki. All animal experiments were approved in accordance with Animals (Scientific Procedures) Act 1986 UK.

Human neutrophils

Venous blood neutrophils were isolated by centrifugation over plasma-Percoll® gradients (25). Neutrophils were re-suspended in 'normoxic' (atmospheric 21% O_2) or hypoxic (0.8% O_2 equating to media pO_2 of 3kPa (26), 5% CO_2, Baker Ruskinn or Whitley hypoxia workstation) Iscove's Modified Dulbecco's Medium (IMDM). At 4 hours (h), neutrophils ($11.1*10^6$/ml) were treated with PAF (1 μM, 5 min) then fMLP (100 nM, 10 min) (2). PI3K inhibitors were added prior to incubation: PI3Kγ-selective (AS605240, 3 μM), PI3Kδ-selective (CAL-101, 100 nM).

Murine neutrophils

Femoral bone marrow neutrophils were isolated by negative immunomagnetic selection from PI3Kδ-hyper-actived E1020K heterozygote (PPL 80/2248; P4802B8AC) (27), PI3Kδ-kinase-dead D910A homozygote (PPL 70/7661) (28) or PI3Kγ$^{-/-}$ (PPL 70/8100) mice, alongside age/strain-matched wildtype controls (E1020K/D910A: C57BL/6J; PI3Kγ$^{-/-}$: C57BL/E129). Neutrophils were re-suspended

in normoxic or hypoxic IMDM before treatment at 4h with cytochalasin B (5 µg/ml, 5 min) then fMLP (10 µM, 10 min).

Protein secretion

Neutrophil supernatant and plasma protein content were measured by ELISA, chemiluminescence immunoassay or activity assay. Plasma NE- and PR3-specific fibrinogen cleavage products were measured (1,29).

Neutrophil secretome preparation for Tandem Mass Tag-Mass Spectrometry (TMT-MS)

Neutrophils were re-suspended in normoxic or hypoxic IMDM (4h) containing EDTA (1 mM) and sivelestat (10 µM) and treated with PAF and fMLP as above. Concentrated protein supernatants underwent TMT-MS.

Endothelial cell survival

Confluent human pulmonary artery endothelial cells (hPAECs, Lonza) or human pulmonary microvascular endothelial cells (hPMECs, Promocell) were treated with neutrophil supernatants, with/without alpha-1-antitrypsin (α1AT, 46 µg/ml, 10 min). Cell detachment of rhodamine phalloidin- and DAPI-stained fixed hPAECs was assessed by immunofluorescence. Viability/apoptosis of unfixed hPAECs or hPMECs was assessed by MTT assay, or Annexin V positivity.

Endothelial–neutrophil interaction

Confluent hPMECs were treated with neutrophil supernatants. At 4h, hPMECs were perfused with neutrophils ($1*10^6$ cells/ml, 4 min, 0.1 Pa shear stress), and neutrophil adhesion/transmigration assessed.

Statistical Analysis

Data were analysed using GraphPad Prism v9 software, reported as mean ± SEM from (n) independent experiments. Gaussian data were analysed by t-test or two-way analysis of variance (ANOVA) with Sidak's correction. Non-Gaussian data were analysed by Mann-Whitney test. TMT-MS-generated p-values were adjusted by Benjamini-Hochberg procedure. A p-value of <0.05 was considered statistically significant.

For further information see online data supplement: **Expanded Materials and Methods.**

RESULTS

Exacerbating COPD patients have increased circulating evidence of neutrophil protease activity

Although neutrophil proteases are implicated in COPD lung parenchymal destruction, the extent of systemic release of neutrophil granule proteins and their potential role in endothelial injury are unclear. We show that exacerbating COPD patients (Table E1) have higher plasma levels of fibrinogen cleavage products AαVal^{360} and AαVal^{541} than age/sex-matched healthy controls (Fig 1A&B). These footprints specifically indicate increased activity of neutrophil azurophil granule proteases NE and proteinase 3, respectively, secreted upon neutrophil activation (which may occur in the circulation, during adherence to vascular endothelium or following migration into tissues) prior to inactivation by circulating anti-proteases, such as α1AT. Given this systemic signature of enhanced neutrophil protease activity during COPD exacerbation, established evidence of pathological hypoxia during inflammation, and our previous results

demonstrating hypoxia-augmented NE release from GM-CSF-primed neutrophils (2), we next examined the ability of inflammatory mediators relevant to COPD and hypoxia to influence neutrophil degranulation.

Hypoxia augments NE release from PAF-primed neutrophils in a PI3Kγ-dependent manner

Neutrophils treated with PAF and fMLP in combination (but not alone) released up to three-fold more active NE when incubated under hypoxia (0.8% O_2, 3 kPa) compared with normoxia (21 kPa) (Fig 2A). This enhanced secretion was not reversed by re-oxygenation (Fig 2B). Given the known role of the PI3K pathway in the hypoxic upregulation of degranulation from GM-CSF-primed neutrophils, and the aberrant chemotaxis of COPD patient neutrophils which could be corrected by PI3K inhibition (30), we explored whether inhibition of PI3K signalling pathways modulated the hypoxic response of PAF-primed neutrophils using PI3K isoform-selective small molecule inhibitors. PI3Kγ-selective inhibition abrogated the hypoxic uplift of NE release from PAF-primed neutrophils; this effect was not seen with the PI3Kδ-selective inhibitor (Fig 2C). These results were replicated in a cohort of COPD patients (Table E2, Fig E1). As PI3Kγ inhibition also markedly inhibited NE release from stimulated normoxic cells, we further explored whether PI3K signalling was simply essential for overall degranulation or had a specific role in hypoxia-mediated degranulation, using transgenic mice with abolished or enhanced activity of PI3Kγ/δ isoforms. As PAF did not elicit a detectable priming response in murine neutrophils (data not shown), these cells were treated with cytochalasin B and fMLP to liberate NE. The hypoxic increase in NE release from murine neutrophils deficient in PI3Kγ was abolished, with preserved ability to degranulate under normoxia (Fig 2D). In contrast, the hypoxic enhancement of NE release from murine neutrophils with either

activating or kinase-dead PI3Kδ mutations was unaffected (Fig 2E&F). Together, these data indicate that the augmented degranulation observed under hypoxia from human or murine neutrophils requires PI3Kγ but not PI3Kδ activity.

Hypoxia increases the capacity for neutrophil supernatants to damage endothelial cells

As COPD patients suffer increased cardiovascular morbidity compared to healthy controls and display a footprint of increased circulating protease activity (Fig 1), we investigated whether hypoxia increases the potential for neutrophil-mediated endothelial damage. We incubated supernatants from normoxic *versus* hypoxic activated (PAF/fMLP) neutrophils with hPAEC and hPMEC monolayers in the presence or absence of α1AT, and assessed cell integrity and survival. Supernatants from activated hypoxic neutrophils induced more endothelial detachment (Fig 3A&B) and death (Fig 3C&D and supplementary E2) than their normoxic counterparts, which was not completely rescued by co-incubation with α1AT (Fig 3D).

Hypoxia differentially regulates protein release from activated neutrophils

Since the anti-protease strategy did not completely mitigate neutrophil-induced endothelial damage (Fig 3D), and multiple neutrophil-derived granule products have potentially damaging actions, we investigated the effect of hypoxia on the total detectable proteome released by activated neutrophils. Mass spectrometry characterisation of the normoxic *versus* hypoxic neutrophil secretome revealed clear separation by principal component analysis (Fig 4A). TMT-MS identified 1245 proteins, 717 of which were present in all samples. Of these 717 proteins, 199 had a false discovery rate <0.01, and 63 were differentially regulated (adjusted p-value <0.05) between normoxia and hypoxia (Fig 4B). Of these 63 proteins, 35 were more abundant

in normoxic (Table 1) and 28 were increased in hypoxic (Table 2) neutrophil supernatants. The majority of proteins upregulated in hypoxic supernatants were granule proteins, whereas those more abundant in normoxic supernatants were predominantly cytoplasmic. However, some granule-associated proteins were increased in normoxic samples (e.g. leukocyte specific protein 1), and certain cytoplasmic (e.g. cyclophilin A) and nuclear (e.g. histone H4) proteins were increased by hypoxia. Selected hypoxia-upregulated protein targets with the potential to play a role in endothelial damage were biochemically validated using supernatants from independent healthy donors. Levels of the azurophil granule protein resistin (Fig 4C), the specific granule protein neutrophil gelatinase-associated lipocalin (NGAL, Fig 4D), and the cytoplasmic protein cyclophilin A (Fig 4E) were significantly elevated in hypoxic *versus* normoxic neutrophil supernatants.

Investigation of cytoplasmic and nuclear protein secretion

As cyclophilin A is cytoplasmic rather than granule-associated, we examined the release of neutrophil-derived microvesicles (NMVs), which contain components derived from parent cells, as a potential source of protein release in addition to degranulation. However, we found no difference in NMV numbers in hypoxic *versus* normoxic supernatants (Fig E3A) or in healthy *versus* COPD patient plasma (Fig E3B). Furthermore, there was no difference in the content of cyclophilin A between NMVs generated from normoxic *versus* hypoxic cells, and cyclophilin A was also detected in microvesicle-depleted neutrophil supernatants (Fig E3C&D).

Since the nuclear protein, histone H4, was increased in the hypoxic supernatant proteome (although other histones were not likewise increased), we also investigated the release of neutrophil extracellular traps (NETs), which release both nuclear and

granule proteins into the extracellular space. However, there was no difference in NETosis from normoxic *versus* hypoxic neutrophils (Fig E4). Overall, our data do not support a contribution of NMVs or NETs to the differential spectrum of cytoplasmic or nuclear proteins released from neutrophils under hypoxia.

Hypoxic neutrophils from exacerbating COPD patients release more histotoxic proteins

During COPD exacerbations (which are associated with excess cardiovascular morbidity), neutrophils are subject to intensified local and systemic hypoxia in addition to a markedly pro-inflammatory micro-environment. Since hypoxia enables even 'healthy' neutrophils to release multiple proteins capable of causing endothelial damage, we studied the effect of hypoxia on neutrophils isolated from exacerbating COPD patients (Table E1). COPD neutrophils were not basally shape-changed (indicating no priming/activation) and responded identically to healthy control neutrophils following fMLP stimulation (Fig 5A). Further, there was no difference in the release of NE from unstimulated neutrophils obtained from exacerbating COPD patients *versus* age/sex-matched healthy controls under normoxic or hypoxic conditions (Fig 5B). Together, these data indicate that, in our cohort 1, circulating COPD patient neutrophils were not primed during exacerbations. Despite this, when incubated under hypoxia, stimulated COPD neutrophils released up to three-fold more active NE than equivalent healthy control cells (Fig 5B). Likewise, secretion of selected granule and cytoplasmic protein targets identified by proteomics: NGAL (Fig 5C) and cyclophilin A (Fig 5D) was increased 1.5 and 5-fold, respectively, from stimulated hypoxic COPD patient *versus* healthy control neutrophils, with a similar pattern demonstrated for resistin release (Fig 5E). In contrast, although secretion of the azurophil granule protein, MPO, was consistently increased in hypoxia *versus*

normoxia, it was not further enhanced when comparing COPD and healthy control neutrophils (Fig 5F).

Hypoxia promotes endothelial-neutrophil interaction induced by COPD patient neutrophil supernatants

To investigate whether COPD patient neutrophils have increased capacity for endothelial cell injury/activation, we applied supernatants from normoxic or hypoxic activated COPD *versus* healthy neutrophils to hPMEC monolayers and assessed neutrophil recruitment (rolling, adhesion and transmigration) in a biologically relevant *in vitro* flow system (Fig 6A&B). Treatment with hypoxic COPD neutrophil supernatants resulted in a marked increase in neutrophil rolling and adhesion compared with both normoxic COPD supernatants and hypoxic healthy supernatants (Fig 6C).

Hypoxia may synergise with inflammatory mediators to promote upregulation of circulating histotoxic neutrophil granule proteins in COPD patients

Since hypoxia enhanced histotoxic protein release from COPD neutrophils (Fig 5) and supernatants from these cells promoted neutrophil-endothelial interaction (Fig 6), we examined whether there was a circulating signature of hypoxia-induced neutrophil protein secretion. We detected significantly increased levels of the neutrophil granule proteins: NE (Fig 7A), MPO (Fig 7B) and NGAL (Fig 7C) in exacerbating COPD patient *versus* healthy control plasma (derived from an independent cohort 3, Table E3), although there was no difference in the plasma content of resistin (Fig 7D). Despite observing increased release of the cytoplasmic protein, cyclophilin A, from isolated COPD neutrophils (Fig 5E), unexpectedly the plasma content of cyclophilin A was higher for healthy controls than COPD patients (Fig 7E). Biomarkers of vascular injury/activation: ICAM-1 (Fig 7F) and VCAM-1 (Fig 7G), and inflammation (Fig

E5A&B) were increased in exacerbating COPD patient plasma. In contrast, the COPD plasma content of angiogenesis biomarkers was predominantly unchanged compared to that of healthy controls (Fig E5C-J), suggesting a damage phenotype which may enhance endothelial-neutrophil interaction but without a corresponding increase in vascular regeneration ability.

DISCUSSION

Our work demonstrates that hypoxic neutrophils display a hyper-secretory phenotype with enhanced capacity to both activate and injure cultured endothelial cells. Hypoxia-driven release of histotoxic proteins was observed from healthy donor neutrophils and translated to a patho-physiologically relevant cohort of exacerbating COPD patients, confirming even further augmented histotoxic protein secretion under hypoxia, together with higher circulating levels of selected neutrophil-derived proteins and a plasma signature of increased neutrophil protease activity and vascular injury.

Our data from human neutrophils treated with PI3K isoform-selective inhibitors and from transgenic murine cells, support a non-redundant role for PI3Kγ in the hypoxic augmentation of NE release. Dysregulated PI3K signalling has previously been associated with COPD, with the impaired neutrophil migratory accuracy improved by PI3Kγ/δ inhibition (30), although we found no role for the δ isoform in hypoxic degranulation. Our results suggest that PI3Kγ is required for hypoxia-potentiated neutrophil degranulation, whether in response to tyrosine kinase- (2) or G-protein-coupled agonists, such as PAF. Consistent with a role for PI3Kγ-dependent PAF/hypoxia-mediated neutrophil degranulation in vascular insults, PI3Kγ/δ inhibition limited both PAF-induced hindlimb inflammation and ischaemic cardiac infarct size

(31), and PI3Kγ-null mice had improved cardiac recovery post ischaemia (32). Hence, this signaling pathway could conceivably be targeted to mitigate neutrophil-mediated endothelial damage in COPD and other diseases underpinned by hypoxia, inflammation and vascular damage.

Hypoxia enhances neutrophil degranulation but, surprisingly, hypoxia-upregulated proteins identified by proteomic analysis did not segregate precisely with discrete granule populations. We have previously shown that hypoxia promotes differential secretion from eosinophil granules (26), with similar results reported using mast cells (33). Our results imply a comparable “differential degranulation” may be occurring from neutrophils in the setting of hypoxia. A limited number of studies analysing the neutrophil secretome generated under normoxic conditions have shown variation in protein content according to the inciting stimulus (34), suggesting that the (potentially hypoxic) inflammatory environment can influence the precise composition of secreted granule proteins. This may also explain our observation that release of the azurophilic granule proteins, NE, resistin and MPO, from hypoxic COPD neutrophils did not fully mirror each other.

In addition to enhanced degranulation, our proteomic data further suggest active secretion of selected cytoplasmic and nuclear proteins under hypoxia. We provide the first description of neutrophilic secretion of the pro-inflammatory cytoplasmic protein cyclophilin A, aligning to a previous study demonstrating its hypoxia-driven secretion from cardiac myocytes (35). Our data also demonstrated increased release of the nuclear protein histone H4 (although no other histones) in hypoxic supernatants. The source of this protein remains unclear as we did not observe any difference in NETosis between normoxic and hypoxic neutrophils.

We have established that peripheral blood neutrophils from exacerbating COPD patients display markedly greater hypoxic release of NE, NGAL and cyclophilin A relative to matched healthy controls. These proteins have been previously been implicated in endothelial dysfunction or atherosclerosis (36,37), with raised circulating levels of NGAL associated with cardiovascular events, and with hypoxaemia in COPD (38,39). Although we found no relationship between admission or venesection oxygen levels and protein release (data not shown), this may have been confounded by prior exposure to supplemental oxygen. In keeping with a potential *in vivo* role for hypoxic augmentation of neutrophil degranulation, we found higher levels of the granule proteins NE, MPO and NGAL and a footprint of increased NE and PR3 activity in COPD *versus* healthy plasma, aligning with recent SARS-CoV-2 studies, where NGAL in particular was associated with mortality (40). Our results suggest that enhanced neutrophil degranulation in the setting of hypoxia may contribute to systemic endothelial injury in exacerbating COPD patients and potentially other conditions such as SARS-CoV-2 infection.

Primed circulating neutrophils with heightened potential for cellular damage have been identified in COPD patients (21). However, we did not observe significant shape change or enhanced degranulation (both features of priming) from unstimulated COPD neutrophils, suggesting that the enhanced hypoxic release of granule proteins from these cells is not simply a consequence of priming and may 'substitute' for this process in promoting damage. A potential explanation for enhanced protein release is a change in protein abundance in COPD neutrophils, with one study showing increased NE activity in COPD versus healthy neutrophil lysates (41). However, this result may represent a protease/anti-protease imbalance as leukocyte elastase inhibitor was

reduced, a finding consistent with our proteomic data, and which may explain the discrepancy between these results and the NE activity assay.

Our study has some limitations. Although our patients and controls were age- and sex-matched, the patients had a significant smoking history and more co-morbidities than the healthy volunteers. The COPD patients were studied during exacerbations, a high risk period for acute cardiovascular events (17), but with variation in terms of exacerbation aetiology and severity; however the use of two separate cohorts in different institutions mitigates this limitation. It is possible that acute or long-term medications could affect neutrophil function in our patient cohorts. For example, all COPD patients in cohort 1 were taking inhaled combination corticosteroid/long-acting beta agonists, and were treated with oral prednisolone during exacerbation with varying duration prior to venesection. However, although glucocorticoids are known to delay neutrophil apoptosis, this effect is lost in hypoxia (42), and inhaled corticosteroids have been shown not to affect neutrophil protein secretion (43). Given the variation in comorbidities (Table E4), and thus treatments, within our cohorts there is unlikely to be any consistent impact on neutrophil function.

Although our results are predominantly consistent with previous studies, we note that despite reports of enhanced circulating levels of resistin and cyclophilin A in COPD patients *versus* healthy controls (44,45), we did not detect such an increase in our COPD cohort. These discrepant results may reflect differences in the timing of sampling, medications, patient heterogeneity, or obscuration of a neutrophil-specific signal since both proteins have multiple cellular sources (46,47). Both cyclophilin A and its receptor have been detected at high levels in atherosclerotic plaques (48). Hence, in COPD, cyclophilin A may already be membrane-bound, preventing its detection in plasma. As COPD patients have increased atherosclerotic burden,

hypoxia within plaques may promote enhanced *local* release of cyclophilin A from neutrophils in this and other microenvironments. This would be consistent with our *in vitro* data and with a previous study demonstrating increased cyclophilin A expression in the lung tissue of COPD patients compared with either smoking or non-smoking controls (49).

Overall, our data demonstrate that exacerbating COPD patients have an enhanced footprint of circulating neutrophil protease activity, and that neutrophils from these patients exhibit a hypoxia-driven hyper-secretory phenotype with enhanced capacity for endothelial damage. We provide the first description of the ability of hypoxia to augment the secretion of histotoxic proteins from COPD neutrophils *in vitro*, and have identified a corresponding increase in the plasma levels of selected granule proteins and markers of vascular injury in COPD patients. Our findings may illuminate novel therapeutic targets in treatment-recalcitrant neutrophil-mediated inflammatory diseases such as COPD.

ACKNOWLEDGMENTS

The authors are grateful to Professor Klaus Okkenhaug and Dr Anne-Katrien Stark (Department of Pathology, University of Cambridge) for kindly providing E1020K heterozygote and D910A homozygote mice; Dr Helen Marriott (Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield) for kindly providing E1020K heterozygote mice; Dr Len Stephens and Dr Sabine Suire (Babraham Institute, Cambridge) for kindly providing PI3Kγ$^{-/-}$ mice; Dr Mairi MacLeod, Professor Gavin Donaldson and Professor Jadwiga Wedzicha (NHLI, Imperial College London) for facilitating access to blood samples from COPD patients at the Imperial

College London NIHR Biomedical Research Unit; Rosalind Simmons (Department of Medicine, University of Cambridge) for co-ordinating donor recruitment; Dr Mike Deery and Dr Marco Chiapello (University of Cambridge Centre for Proteomics) for TMT-MS; Keith Burling and NIHR Cambridge BRC Core Biochemistry Facility; Simon McCallum and NIHR Cambridge BRC Core Flow Cytometry; and all blood donors. COPD patients and healthy controls recruited from Birmingham were done so with the support of the NIHR Clinical Research Facility within UHB NHS Foundation Trust.

REFERENCES

1. Carter RI, Ungurs MJ, Mumford RA, Stockley RA. Aα-Val360: A marker of neutrophil elastase and COPD disease activity. Eur Respir J. 2013;41:31–8.

2. Hoenderdos K, Lodge K, Hirst R, Chen C, Palazzo S, Emerenciana A, et al. Hypoxia upregulates neutrophil degranulation and potential for tissue injury. Thorax. 2016;71:1030–8.

3. Nolte D, Steinhauser P, Pickelmann S, Berger S, Roger H, Messmer K. Effects of diaspirin-cross-linked hemoglobin (DCLHb) on local tissue oxygen tension in striated skin muscle: An efficacy study in the hamster. J Lab Clin Med. 1997;130:328–38.

4. van Staveren S, ten Haaf T, Klopping M, Hilvering B, Tinnevelt GH, de Ruiter K, et al. Multi-dimensional flow cytometry analysis reveals increasing changes in the systemic neutrophil compartment during seven consecutive days of endurance exercise. PLoS One. 2018;13:e0206175.

5. Mall MA, Harkema JR, Trojanek JB, Treis D, Livraghi A, Schubert S, et al. Development of chronic bronchitis and emphysema in β-epithelial Na+ channel-overexpressing mice. Am J Respir Crit Care Med. 2008;177:730–42.

6. Belton M, Brilha S, Manavaki R, Mauri F, Nijran K, Hong YT, et al. Hypoxia and tissue destruction in pulmonary TB. Thorax. 2016;71:1145–53.

7. Polosukhin V V., Cates JM, Lawson WE, Milstone AP, Matafonov AG, Massion PP, et al. Hypoxia-inducible factor-1 signalling promotes goblet cell hyperplasia in airway epithelium. J Pathol. 2011;224:203–11.

8. Joshi FR, Manavaki R, Fryer TD, Figg NL, Sluimer JC, Aigbirhio FI, et al.

Vascular imaging with 18F-Fluorodeoxyglucose Positron Emission Tomography is influenced by hypoxia. J Am Coll Cardiol. 2017;69:1873–4.

9. McElvaney OJ, McEvoy NL, McElvaney OF, Carroll TP, Murphy MP, Dunlea DM, et al. Characterization of the inflammatory response to severe COVID-19 illness. Am J Respir Crit Care Med. 2020;202:812–21.

10. Kitchen E, Rossi AG, Condliffe AM, Haslett C, Chilvers ER. Demonstration of reversible priming of human neutrophils using platelet-activating factor. Blood. 1996;88:4330–7.

11. Bitencourt CS, Bessi VL, Huynh DN, Menard L, Lefebvre JS, Levesque T, et al. Cooperative role of endogenous leucotrienes and platelet-activating factor in ischaemia–reperfusion-mediated tissue injury. J Cell Mol Med. 2013;17:1554–65.

12. Shukla SD, Muller HK, Latham R, Sohal SS, Walters EH. Platelet-activating factor receptor (PAFr) is upregulated in small airways and alveoli of smokers and COPD patients. Respirology. 2016;21:504–10.

13. Cardini S, Dalli J, Fineschi S, Perretti M, Lungarella G, Lucattelli M. Genetic ablation of the Fpr1 gene confers protection from smoking-induced lung emphysema in mice. Am J Respir Cell Mol Biol. 2012;47:332–9.

14. Stănescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM, et al. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. Thorax. 1996;51:267–71.

15. Kuna P, Jenkins M, O'Brien CD, Fahy WA. AZD9668, a neutrophil elastase

inhibitor, plus ongoing budesonide/ formoterol in patients with COPD. Respir Med. 2012;106:531–9.

16. Ye C, Younus A, Malik R, Roberson L, Shaharyar S, Veledar E, et al. Subclinical cardiovascular disease in patients with chronic obstructive pulmonary disease: a systematic review. QJM. 2017;110:341–9.

17. Kunisaki KM, Dransfield MT, Anderson JA, Brook RD, Calverley PMA, Celli BR, et al. Exacerbations of chronic obstructive pulmonary disease and cardiac events. A post hoc cohort analysis from the SUMMIT randomized clinical trial. Am J Respir Crit Care Med. 2018;198:51–7.

18. Dinh-Xuan AT, Higenbottam TW, Clelland CA, Pepke-Zaba J, Cremona G, Butt Y, et al. Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. N Engl J Med. 1991;324:1539–47.

19. Eickhoff P, Valipour A, Kiss D, Schreder M, Cekici L, Geyer K, et al. Determinants of systemic vascular function in patients with stable chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2008;178:1211–8.

20. Shi Y, Yang S, Luo M, Zhang W-D, Ke Z-P. Systematic analysis of coronary artery disease datasets revealed the potential biomarker and treatment target. Oncotarget. 2017;8:54583–91.

21. Oudijk E-JD, Gerritsen WBM, Nijhuis EHJ, Kanters D, Maesen BLP, Lammers J-WJ, et al. Expression of priming-associated cellular markers on neutrophils during an exacerbation of COPD. Respir Med. 2006;100:1791–9.

22. Lodge K, Hoenderdos K, Robbins A, Storisteanu D, Chilvers E, Li W, et al.

Hypoxia upregulates PI3Kinase-dependent neutrophil degranulation and neutrophil-mediated tissue injury. Thorax. 2016;71 Suppl 3:A27.

23. Lodge K, Hoenderdos K, Chilvers E, Li W, Condliffe A. Hypoxia upregulates PI3Kinase-dependent neutrophil degranulation and neutrophil-mediated tissue injury. Am J Respir Crit Care Med. 2017;195:A7058.

24. Lodge K, Hoenderdos K, Robbins A, Chilvers E, Li W, Condliffe A. Hypoxia drives neutrophil-mediated endothelial damage in COPD. Thorax. 2017;72 Suppl 3:A69–70.

25. Haslett C, Guthrie LA, Kopaniak MM, Johnston Jr RB, Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. Am J Pathol. 1985;119:101–10.

26. Porter LM, Cowburn AS, Farahi N, Deighton J, Farrow SN, Fiddler CA, et al. Hypoxia causes IL-8 secretion, Charcot Leyden crystal formation, and suppression of corticosteroid-induced apoptosis in human eosinophils. Clin Exp Allergy. 2016;47:770–84.

27. Stark A-K, Chandra A, Chakraborty K, Alam R, Carbonaro V, Clark J, et al. PI3Kδ hyper-activation promotes development of B cells that exacerbate Streptococcus pneumoniae infection in an antibody-independent manner. Nat Commun. 2018;9:3174.

28. Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, et al. Impaired B and T Cell Antigen Receptor Signaling in p110δ PI 3-Kinase Mutant Mice. Science (80-). 2002;297:1031–4.

29. Newby PR, Crossley D, Crisford H, Stockley JA, Mumford RA, Carter RI, et al.

A specific proteinase 3 activity footprint in α1-antitrypsin deficiency. ERJ Open Res. 2019;5:00095-2019.

30. Sapey E, Stockley JA, Greenwood H, Ahmad A, Bayley D, Lord JM, et al. Behavioral and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2011 May 1;183:1176–86.

31. Doukas J, Wrasidlo W, Noronha G, Dneprovskaia E, Fine R, Weis S, et al. Phosphoinositide 3-kinase γ/δ inhibition limits infarct size after myocardial ischemia/reperfusion injury. Proc Natl Acad Sci. 2006;103:19866–71.

32. Alloatti G, Levi R, Malan D, Del Sorbo L, Bosco O, Barberis L, et al. Phosphoinositide 3-kinase γ-deficient hearts are protected from the PAF-dependent depression of cardiac contractility. Cardiovasc Res. 2003;60:242–9.

33. Möllerherm H, Branitzki-Heinemann K, Brogden G, Elamin AA, Oehlmann W, Fuhrmann H, et al. Hypoxia modulates the response of mast cells to Staphylococcus aureus infection. Front Immunol. 2017;8:541.

34. Snäll J, Linnér A, Uhlmann J, Siemens N, Ibold H, Janos M, et al. Differential neutrophil responses to bacterial stimuli: Streptococcal strains are potent inducers of heparin-binding protein and resistin-release. Sci Rep. 2016;6:21288.

35. Seko Y, Fujimura T, Taka H, Mineki R, Murayama K, Nagai R. Hypoxia followed by reoxygenation induces secretion of cyclophilin A from cultured rat cardiac myocytes. Biochem Biophys Res Commun. 2004;317:162–8.

36. Zhang T, Zhang J, Ge H. Functions of cyclophilin A in atherosclerosis. Exp

Clin Cardiol. 2013;18:e118–24.

37. Wen G, An W, Chen J, Maguire EM, Chen Q, Yang F, et al. Genetic and pharmacologic inhibition of the neutrophil elastase inhibits experimental atherosclerosis. J Am Heart Assoc. 2018;7:e008187.

38. Lindberg S, Pedersen SH, Mogelvang R, Jensen JS, Flyvbjerg A, Galatius S, et al. Prognostic utility of neutrophil gelatinase-associated lipocalin in predicting mortality and cardiovascular events in patients with ST-segment elevation myocardial infarction treated with primary percutaneous coronary intervention. J Am Coll Cardiol. 2012;60:339–45.

39. Eagan TM, Damås JK, Ueland T, Voll-Aanerud M, Mollnes TE, Hardie JA, et al. Neutrophil gelatinase-associated lipocalin: A biomarker in COPD. Chest. 2010;138:888–95.

40. Aberst MS, Delmonte OM, Ricotta EE, Fintzi J, Fink DL, Almeida de Jesus AA, et al. An immune-based biomarker signature is associated with mortality in COVID-19 patients. JCI insight. 2020;144455:online ahead of print.

41. Shahriary A, Mehrani H, Ghanei M, Parvin S. Comparative proteome analysis of peripheral neutrophils from sulfur mustard-exposed and COPD patients. J Immunotoxicol. 2015;12:132–9.

42. Marwick JA, Dorward DA, Lucas CD, Jones KO, Sheldrake TA, Fox S, et al. Oxygen levels determine the ability of glucocorticoids to influence neutrophil survival in inflammatory environments. J Leukoc Biol. 2013;94:1285–92.

43. Culpitt S V, Maziak W, Loukidis S, Nightingale JA, Matthews JL, Barnes PJ. Effect of high dose inhaled steroid on cells, cytokines, and proteases in

induced sputum in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 1999;160:1635–9.

44. Kumor-Kisielewska A, Kierszniewska-Stepien D, Pietras T, Kroczynska-Bednarek J, Kurmanowska Z, Antczak A, et al. Assessment of leptin and resistin levels in patients with chronic obstructive pulmonary disease. Pol Arch Med Wewn. 2013;123:215–20.

45. Zhang M, Tang J, Yin J, Wang X, Feng X, Yang X, et al. The clinical implication of serum cyclophilin A in patients with chronic obstructive pulmonary disease. Int J COPD. 2018;13:357–63.

46. Park HK, Ahima RS. Resistin in rodents and humans. Diabetes Metab J. 2013;37:404–14.

47. Bukrinsky M. Extracellular cyclophilins in health and disease. Biochim Biophys Acta. 2015;1850:2087–95.

48. Wang C, Jin R, Zhu X, Yan J, Guohong L. Function of CD147 in atherosclerosis and atherothrombosis. J Cardiovasc Transl Res. 2015;8:59–66.

49. Hu R, Ouyang Q, Dai A, Tan S, Xiao Z, Tang C. Heat shock protein 27 and cyclophilin A associate with the pathogenesis of COPD. Respirology. 2011;16:983–93.

50. Lominadze G, Powell DW, Luerman GC, Link AJ, Ward RA, McLeish KR. Proteomic analysis of human neutrophil granules. Mol Cell proteomics. 2005;4:1503–21.

Table 1

Accession	Description	Adj. p value	FC	Location
Q5TCU8	Tropomyosin beta chain	0.001	11.855	CYT
P10599	Thioredoxin	0.017	3.909	CYT
E7EX29	14-3-3 protein zeta/delta	0.015	3.844	S/G
P52566	Rho GDP-dissociation inhibitor 2	0.004	3.802	CYT
P08670	Vimentin	0.006	3.766	CYT
O00299	Chloride intracellular channel protein 1	0.030	2.918	?
P11021	78 kDa glucose-regulated protein	0.009	2.873	CYT
E9PK25	Cofilin-1	0.023	2.753	CYT
E7EMB3	Calmodulin	0.026	2.488	?
P62993	Growth factor receptor-bound protein 2	0.004	2.478	CYT
P20700	Lamin-B1	0.004	2.435	NUC
Q32MZ4	Leucine-rich repeat flightless-interacting protein 1	0.004	2.337	NUC/ CYT
P06737	Glycogen phosphorylase, liver form	0.025	2.217	?
P32942	Intercellular adhesion molecule 3	0.015	2.149	S/G
Q9Y490	Talin-1	0.015	2.116	S/G
O15144	Actin-related protein 2/3 complex subunit 2	0.031	2.086	CYT
P06702	Protein S100-A9	0.013	2.084	CYT
P52209	6-phosphogluconate dehydrogenase, decarboxylating	0.013	1.950	CYT
P26038	Moesin	0.019	1.878	S
P33241	Leukocyte-specific protein 1	0.013	1.878	S/G
P18206	Vinculin	0.015	1.816	CYT
P30740	Leukocyte elastase inhibitor	0.006	1.813	A
Q96C19	EF-hand domain-containing protein D2	0.027	1.794	?
A6NIZ1	Ras-related protein Rap-1b-like protein	0.009	1.749	SV
P35579	Myosin-9	0.012	1.631	CYT
Q29963	HLA class I histocompatibility antigen, Cw-6 alpha chain	0.039	1.627	PM
P61247	40S ribosomal protein S3a	0.028	1.528	?
P02042	Hemoglobin subunit delta	0.049	1.492	?
E7EQR4	Ezrin	0.023	1.473	?
P08133	Annexin A6	0.035	1.458	?
P31146	Coronin-1A	0.034	1.451	S/G
Q15907	Ras-related protein Rab-11B	0.015	1.447	G
P46781	40S ribosomal protein S9	0.044	1.438	CYT
P46940	Ras GTPase-activating-like protein IQGAP1	0.049	1.411	G
P39687	Acidic leucine-rich nuclear phosphoprotein 32 family member A	0.043	1.358	?

Table 2

Accession	Description	Adj. p value	FC	Location
P62805	Histone H4	0.004	3.587	NUC
P05164	Myeloperoxidase	0.006	3.107	A
A6NC48	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	0.025	2.468	SV
O75083	WD repeat-containing protein 1	0.029	2.382	CYT
Q92820	Gamma-glutamyl hydrolase	0.020	2.143	S
P02788	Lactotransferrin	0.033	2.136	S
A5A3E0	POTE ankyrin domain family member F	0.015	2.074	CYT
Q0VD83	Apolipoprotein B receptor	0.025	1.993	S/G
P10124	Serglycin	0.012	1.904	CYT
Q9HD89	Resistin	0.004	1.898	A/S
P07737	Profilin-1	0.015	1.890	CYT
A0A087WXL1	Folate receptor gamma	0.029	1.877	S
P11215	Integrin alpha-M	0.004	1.872	S
P16035	Metalloproteinase inhibitor 2	0.034	1.853	G
X6R8F3	Neutrophil gelatinase-associated lipocalin	0.004	1.842	S
V9GYM3	Apolipoprotein A-II	0.049	1.769	?
G3V3D1	Epididymal secretory protein E1	0.007	1.765	A
P10153	Non-secretory ribonuclease	0.029	1.650	?
P04217	Alpha-1B-glycoprotein	0.015	1.594	?
P05107	Integrin beta-2	0.018	1.580	G
P01024	Complement C3	0.032	1.556	?
P20061	Transcobalamin-1	0.042	1.492	S
P78324	Tyrosine-protein phosphatase non-receptor type substrate 1	0.018	1.449	SV
J3KNB4	Cathelicidin antimicrobial peptide	0.039	1.446	S/G
P62937	Peptidyl-prolyl cis-trans isomerase A/Cyclophilin A	0.035	1.438	CYT
P30086	Phosphatidylethanolamine-binding protein 1	0.049	1.406	?
A0A075B6H6	Ig kappa chain C region	0.030	1.358	?
A0A075B6K9	Ig lambda-2 chain C regions	0.049	1.305	?

FIGURE LEGENDS

Figure 1. Exacerbating COPD patients have a circulating signature indicating increased protease activity. Plasma from exacerbating COPD patients or age/sex-matched healthy controls was assessed for content of NE-specific fibrinogen cleavage product $A\alpha Val^{360}$ (A) or PR3-specific fibrinogen cleavage product $A\alpha Val^{541}$ (B) by immunoassay (n=12 COPD, n=14 healthy; cohort 1). Results represent mean ± SEM, A: unpaired t test, B: Mann-Whitney test. * = $p<0.05$, ** = $p<0.01$.

Figure 2. Hypoxia increases elastase release from PAF-primed neutrophils in a PI3Kγ-dependent manner. A-C: Neutrophils from healthy human donors were incubated under normoxia or hypoxia in the presence or absence of PI3Kγ-selective inhibitor (AS605240, 3 μM) or PI3Kδ-selective inhibitor (CAL-101, 100 nM) as indicated. After 4h, cells were treated with PAF (1 μM, 5 min) and/or fMLP (100 nM, 10 min) or vehicle control as indicated. For re-oxygenation, unstimulated hypoxic cells were moved to normoxia with the addition of twice-volume normoxic media for 30 min prior to treatment with PAF and fMLP. Supernatant neutrophil elastase (NE) activity was measured and is expressed as fold change relative to hypoxic activated neutrophils. (A: n=5, B: n=4, C: n=4-6). D-F: Femoral bone marrow neutrophils were isolated from PI3Kγ-null ($PI3K\gamma^{-/-}$), PI3Kδ-hyper-active (E1020K), PI3Kδ-kinase dead (D910A), or wildtype mice from the relevant genetic background. After 4h, cells were treated with cytochalasin B (5 μg/ml, 5 min) and fMLP (10 μM, 10 min) or vehicle control. Supernatant NE activity was measured and is expressed as fold change relative to wildtype hypoxic activated neutrophils. (D: 3-4 mice per genotype per experiment, n=5 independent experiments; E-F: 3-4 mice per genotype per

experiment, n=3 independent experiments). Results represent mean ± SEM, two way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Figure 3. Supernatants from hypoxic activated neutrophils cause increased endothelial cell damage in a partially protease-dependent manner. Neutrophils from healthy donors were incubated under normoxia or hypoxia for 4h, then treated with PAF (1 μM, 5 min) and fMLP (100 nM, 10 min) or vehicle control. Supernatants from normoxic *vs* hypoxic, PAF/fMLP *vs* vehicle control-treated neutrophils were incubated with confluent hPAEC for 24h (A&B), 6h (C) or 48h (D) in the presence or absence of α1 antitrypsin (α1AT, 46 μg/ml) as indicated. A&B: hPAEC were fixed and stained with rhodamine-phalloidin and DAPI. Supernatants were from normoxic (NP) or hypoxic (HP) PAF/fMLP-treated neutrophils. Cell detachment was quantified using ImageJ, expressed as % detachment of whole field of view. Representative confocal images (A) from n=5 independent experiments (B), scale bars approximately 20μm. C: hPAEC were stained with FITC-AnV and PI for flow cytometric assessment of apoptosis with apoptotic (AnV^+PI^-) cells expressed as % of total population (n=4). D: Survival of hPAEC was measured by MTT assay (n=6-12). Results represent mean ± SEM, two way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Figure 4. Hypoxia selectively increases granule and cytoplasmic histotoxic protein secretion from activated neutrophils. Neutrophils from healthy donors were incubated under normoxia or hypoxia in the presence (A&B) or absence (C-E) of EDTA (1 mM) and sivelestat (10 μM) for 4h and then treated with PAF (1 μM, 5 min) and fMLP (100 nM, 10 min). A&B: Trypsin-digested supernatants were individually labelled with isobaric tags and subjected to MS/MS. A: PCA showed separation of normoxic *vs* hypoxic supernatant samples by PC1 (dashed line) with samples from individual donors indicated by dotted lines (n=5). B: Volcano plot representation of differential

protein expression between paired normoxic and hypoxic supernatants where vertical dotted line represents log2fold change (FC) of protein abundance = ±1, and horizontal dotted line represents adjusted p-value = 0.05 (n=5). C-E: Neutrophil supernatant content of resistin (C: n=17), NGAL (D: n=7) and cyclophilin A (E: n=7) was measured from independent samples by ELISA. Results represent mean ± SEM, B: paired t test with p-value adjusted by Benjamini-Hochberg procedure, C-E: paired t test. * = $p<0.05$, ** = $p<0.01$.

Figure 5. Hypoxia further augments histotoxic protein release from COPD *versus* healthy neutrophils. A: Neutrophils from healthy donors or exacerbating COPD patients were incubated under normoxia and treated with fMLP (100 nM, 30 min) or vehicle control. Shape change was assessed by flow cytometric analysis of forward scatter, expressed as % shape changed cells of total population (n=5-12). B-F: Neutrophils from healthy donors or exacerbating COPD patients were incubated under normoxia or hypoxia for 4h then treated with PAF (1 µM, 5 min) and fMLP (100 nM, 10 min) or vehicle control. Supernatant content of elastase (B: n=7-14), NGAL (C: n=7-12), cyclophilin A (D: n=3-7), resistin (E: n=7-12) and MPO (F: n=3-6) was measured by ELISA or activity assay. Supernatant NE activity is expressed as fold change relative to healthy hypoxic activated neutrophils. All samples were obtained from cohort 1. Results represent mean ± SEM, two way ANOVA. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

Figure 6. Hypoxia accentuates endothelial-neutrophil interaction induced by COPD *versus* healthy neutrophil supernatants. Neutrophils from healthy donors or exacerbating COPD patients were incubated under normoxia or hypoxia for 4h, then treated with PAF (1 µM, 5 min) and fMLP (100 nM, 10 min). Supernatants from normoxic (NP) or hypoxic (HP) PAF/fMLP-treated neutrophils were incubated with

confluent hPMEC for 4h in the presence of serum (2%). Washed hPMECs were perfused with healthy neutrophils. A: Representative images (original magnification, x100) showing arrested/rolling neutrophils (N) as bright phase and transmigrated neutrophils (TN) as dark phase. B: Endothelial-neutrophil interactions (total number of rolling, adhered and transmigrated neutrophils following bolus neutrophil injection) were captured with time lapse imaging (n=3). C: Quantification of neutrophil rolling and adherence was performed using ImagePro software (n=3). All neutrophil supernatant samples were obtained from cohort 1. Results represent mean ± SEM, two way ANOVA. * = $p<0.05$.

Figure 7. Plasma from COPD patients has increased content of hypoxia-upregulated histotoxic granule proteins and vascular injury biomarkers. Plasma from healthy donors or exacerbating COPD patients was assessed for content of NE (A), MPO (B), NGAL (C), resistin (D), cyclophilin A (E), ICAM-1 (F), VCAM-1 (G) by ELISA (NE, NGAL, cyclophilin A) or chemiluminescence immunoassay (MPO, resistin, ICAM-1, VCAM-1) (n=36 healthy, n=36 COPD; 4 samples from cohort 1 and 32 samples from cohort 3). Results represent mean ± SEM, Mann-Whitney test. * = $p<0.05$, ** = $p<0.01$, **** = $p<0.0001$.

TABLE LEGENDS

Table 1. Proteins significantly increased in normoxic neutrophil supernatants. Proteins significantly increased in supernatants from normoxic neutrophils (adjusted p value <0.05) which were present in all 10 samples with a FDR <0.01 are listed in order of the magnitude of the fold change (FC). Location data were compiled from (50) and the Uniprot database (www.uniprot.org). Abbreviations: azurophil (A), specific (S),

gelatinase (G) granules; secretory vesicles (SV); cytoplasm (CYT); nucleus (NUC); plasma membrane (PM). For some proteins, the location within neutrophils is currently uncertain/unknown (?).

Table 2. Proteins significantly increased in hypoxic neutrophil supernatants. Proteins significantly increased in supernatants from hypoxic neutrophils (adjusted p value <0.05) which were present in all 10 samples with a FDR <0.01 are listed in order of the magnitude of the fold change (FC). Location data were compiled from (50) and the Uniprot database (www.uniprot.org). Abbreviations: azurophil (A), specific (S), gelatinase (G) granules; secretory vesicles (SV); cytoplasm (CYT); nucleus (NUC); plasma membrane (PM). For some proteins, the location within neutrophils is currently uncertain/unknown (?).