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Hypoxia causes increased monocyte nitric oxide synthesis which is mediated by changes in dimethylarginine dimethylaminohydrolase 2 expression in animal and human models of normobaric hypoxia

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ABSTRACT

Background: Tissue hypoxia is a cardinal feature of inflammatory diseases and modulates monocyte function. Nitric oxide is a crucial component of the immune cell response. This study explored the metabolism of the endogenous inhibitor of nitric oxide production asymmetric dimethylarginine(ADMA) by monocyte dimethylarginine dimethylaminohydrolase 2(DDAH2), and the role of this pathway in the regulation of the cellular response and the local environment during hypoxia.
Methods: Peritoneal macrophages were isolated from a macrophage-specific DDAH2 knockout mouse that we developed and compared with appropriate controls. Cells were exposed to 3% oxygen followed by reoxygenation at 21%. Healthy volunteers underwent an 8 h exposure to normobaric hypoxia with an inspired oxygen percentage of 12%. Peripheral blood mononuclear cells were isolated from blood samples taken before and at the end of this exposure.
Results: Intracellular nitrate plus nitrite(NOx) concentration was higher in wild-type murine monocytes after hypoxia and reoxygenation than in normoxia-treated cells (mean(SD) 13·2(2·4) *vs* 8·1(1·7) pmols/mg protein, $p = 0·009$). DDAH2 protein was 4·5-fold (SD 1·3) higher than in control cells ($p = 0·03$). This increase led to a 24% reduction in ADMA concentration, 0·33(0.04) pmols/mg to 0·24(0·03), $p = 0·002$). DDAH2-deficient murine monocytes demonstrated no increase in nitric oxide production after hypoxic challenge. These findings were recapitulated in a human observational study. Mean plasma NOx concentration was elevated after hypoxic exposure (3·6(1.8)μM *vs* 6·4(3·2), $p = 0·01$), which was associated with a reduction in intracellular ADMA in paired samples from 3·6(0.27) pmols/mg protein to 3·15(0·3) ($p < 0·01$). This finding was associated with a 1·9-fold(0·6) increase in DDAH2 expression over baseline($p = 0·03$).
Discussion: This study shows that in both human and murine models of acute hypoxia, increased DDAH2 expression mediates a reduction in intracellular ADMA concentration which in turn leads to elevated nitric oxide concentrations both within the cell and in the local environment. Cells deficient in DDAH2 were unable to mount this response.

1. Introduction

Hypoxia is a cardinal feature of critical illness of many aetiologies [1] It arises as a consequence of both increased metabolic demand [2] and also changes in the microcirculation that impair delivery of oxygen to the tissues [3]. Pro- and anti-inflammatory activation is also a major component of the response to critical illness [4,5]. Mediated in large part by immune cells [6], the interaction between hypoxia and monocytes has been shown to play a role in the immune response [7] and may give insights to the pathological responses seen in some patients in whom exaggerated systemic inflammation leads to organ failure and death.

Nitric oxide (NO) is an important regulator of a broad range of physiological processes [8]. In addition, it plays an important role in the response to infection [9–12]. NO synthesised in response to

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Abbreviations

Ddah2	Dimethylarginine Dimethylaminohydrolase 2 gene
DDAH2	Dimethylarginine Dimethylaminohydrolase 2 protein
$Ddah2^{flox/flox}$	LoxP positive Cre negative litter mate controls
$Ddah2^{M\Phi-}$	Ddah2flox/flox LysM-cre Monocyte specific DDAH2 knockout animals
PBMC	peripheral blood mononuclear cells
PRMT	Protein Arginine Methyltransferases
ADMA	asymmetric dimethylarginine
SDMA	symmetric dimethylarginine
L-NMMA	monomethyl-L-arginine
DDAH	dimethylarginine dimethylaminohydrolase
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
LC-MS/MS	liquid chromatographic assay with tandem mass spectrometric detection
NOx	nitrate and nitrite
FiO_2	Fraction of inspired oxygen

infection has diverse functions including bactericidal and phagocytic function by monocytes [13] and the regulation of the macro [14] and microcirculation [15,16]. The interaction between nitric oxide signalling and hypoxia is critically important in regulating the immune response to infection [17].

Synthesised by the two constitutive and one inducible isoforms of nitric oxide synthase (NOS) [18], NO production is regulated in part by the methylarginines asymmetric dimethylarginine (ADMA) and Monomethyl-L-arginine (L-NMMA) [19].

Methylarginines are produced by post translational methylation of certain arginine residues in proteins by the family of Protein Arginine Methyltransferases (PRMTs). In mammals there are three methylarginine species, ADMA, symmetrical dimethyarginine (SDMA) and L-NMMA. ADMA and L-NMMA competitively inhibit arginine binding to NOS and reduce NO production [20,21]. SDMA does not inhibit the activity of the NOS enzymes [22]. Elevated circulating concentrations of ADMA have been associated with poor outcomes in a variety of conditions including cardiovascular disease [23,24], metabolic disorders [25] and sepsis [26].

ADMA is metabolised by dimethylarginine dimethylaminohydrolase (DDAH) to dimethylamine and citrulline [27]. The two isoforms of DDAH have different tissue distributions [27,28] which lead to differing roles in both basal and pathological states. DDAH1 knockout or pharmacological inhibition leads to a hypertensive phenotype [29] and is protective in animal models of septic shock [30] whereas knockout of DDAH2 leads to minimal cardiovascular disturbance but profound immune dysfunction and excess mortality in sepsis [31].

Recently we have demonstrated that in pulmonary endothelial cells hypoxia induces miRNA-mediated reduction in DDAH1 expression that results in increased ADMA concentration and reduced nitric oxide synthesis that is associated with pulmonary hypertension [32]. The role of DDAH2 – the only isoform found in immune cells – in regulating the synthesis of NO in response to acute hypoxia has not been elucidated. Here we examine for the first time the impact of normobaric hypoxia on NO synthesis, ADMA level and DDAH2 expression in murine models and human healthy volunteers. Our data provide novel insights into the pathways by which hypoxia regulates NO synthesis following acute hypoxic stress.

2. Materials and methods

2.1. Animal models

2.1.1. Husbandry

Animals were housed in accordance with Home Office guidelines and procedures were performed under Project License (70/7049) and Personal License (76/26000). Throughout the care and experimental phases animals were kept in standard environmental conditions with free access to food and water.

2.1.2. Development of genetically modified animals

$DDAH2^{flox/flox}$ LysMCre animals employed the LoxP Cre recombinase model with tissue specificity delivered via Cre expression at the Murine M Lysozyme promoter using a previously established technique [33]. We have previously shown that immune cells express only DDAH2 but not DDAH1 [28, 31]. See Supplementary material for further details.

2.1.3. Isolation of resident peritoneal macrophages

Isolation of primary macrophages was undertaken using a peritoneal washout technique. Further details can be found in the Supplementary materials.

2.1.4. Hypoxic chamber incubation

To determine the impact of subacute hypoxia on isolated primary macrophages Cells were incubated for varied amounts of time in a sealed hypoxic incubator at 92% nitrogen, 3% oxygen and 5% CO_2 at 37 °C. Culture medium (High Glucose DMEM with Glutamine) was placed in the chamber at least 12 h prior to experiment in order to equilibrate medium partial pressure of oxygen with that of the hypoxic atmosphere.

2.1.5. Human normobaric hypoxia study

Ethical Approval was received from the University College London Ethical review panel on 4th March 2014 ref: 2416.001 for conduct of a prospective observational study into the effects of acute normobaric hypoxia on endogenous regulators of nitric oxide synthesis on healthy volunteers.

2.1.6. Normobaric hypoxia

In order to study the relationship between acute hypoxia and methylarginine regulation, a healthy volunteer study was designed that explored the effect of moderate normobaric hypoxia on plasma methylarginine concentrations, monocyte DDAH2 expression and indices of haemodynamic function. In brief, healthy male volunteers aged between 18 and 60 were recruited and consent obtained. Following baseline haemodynamic and clinical observations, patients underwent phlebotomy and samples of blood were taken for plasma separation and isolation of peripheral blood mononuclear cells (PBMCs). Cardiovascular assessment was undertaken before entry to the hypoxic chamber.

Participants then underwent an 8 h exposure to 12.0% oxygen in a hypoxic chamber with continuous observation of patient and environmental conditions. At 20 min after chamber entry and after each successive hour of hypoxic exposure, volunteers underwent haemodynamic and oxygenation assessments and completed a Lake Louise acute mountain sickness assessment modified to exclude the sleep assessment. This ensured that features of acute mountain sickness could be detected early and participants removed from the hypoxic chamber in this eventuality. Details of the hypoxic chamber, monitoring and safety can be found in the Supplementary materials.