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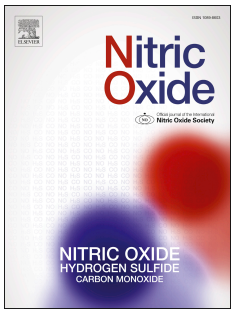

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Hypoxia causes increased monocyte nitric oxide synthesis which is mediated by changes in Dimethylarginine Dimethylaminohydrolase 2 expression in animal and human models of normobaric hypoxia

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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 8h 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.

Short Title

DDAH2 regulates immune cell nitric oxide synthesis in acute hypoxia.

Key words

Nitric Oxide

Hypoxia

Dimethylarginine Dimethylaminohydrolase

Asymmetric Dimethylarginine

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

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 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

Animal Models

Husbandry

Animals were housed in accordance with Home Office guidelines and procedures were performed under Project Licence (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.

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.

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.

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 hours prior to experiment in order to equilibrate medium partial pressure of oxygen with that of the hypoxic atmosphere.

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.

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 hour exposure to 12.0% oxygen in a hypoxic chamber with continuous observation of patient and environmental conditions. At 20 minutes 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.

Following completion of the eight hour exposure period, participants underwent a repeat cycle of testing including blood sampling, observations and cardiovascular assessment. Samples from both the pre and post exposure phases were prepared and stored immediately upon collection at -80°C.

Isolation of peripheral blood mononuclear cells

Blood collected from the patient was diluted with twice the volume of balanced salt solution and layered carefully over an equal volume of Ficoll-Paque Premium (GE Life Sciences, UK) separation medium to avoid mixing of the two liquids.

The sample was centrifuged at 400g at 18-20°C for 40mins in a swinging bucket centrifuge without break to facilitate separation of the sample into plasma/platelets, monocyte and erythrocyte/granulocyte layers

Following separation, the plasma portion of the separated blood was removed using manual pipetting and stored for later analysis. The mononuclear cell layer was removed without disruption of the Ficoll Medium and resuspended in RLT buffer for subsequent mRNA analysis or phosphate buffered saline with protease inhibitor for protein quantification and western blotting

Plasma sample preparation for analysis

Whole blood was collected in EDTA at 1.5mg/ml for analysis and stored on ice for subsequent preparation. Within 60mins of collection, the cells were removed from plasma by centrifugation for 10 minutes at 1,000-2,000g. Centrifugation for 15 minutes at 2,000g depletes platelets in the plasma sample. The separated plasma was stored separately at -80 °C pending subsequent analysis.

Sample preparation and analysis

Measurement of nitric oxide concentration in biological tissues: The Sievers NOA 280i (GE Analytical Instruments) was used to measure nitrate + nitrite (NOx) content of biological samples using a chemiluminescent technique. Further details can be found in the supplementary materials. Nitric oxide was re-derived from nitrites and nitrates (stable end-products of NO activity) by reduction in heated vanadium chloride. NO was detected and quantified in a gas-phase chemiluminescent reaction with ozone which emits in the red/infra-red spectrum. Tissue homogenate or plasma samples underwent protein extraction using methanol precipitation. The supernatant from the samples were run in triplicate, averaged and NO quantified by calculation against a standard curve of sodium nitrate (0-200µM).

Preparation of samples for Mass Spectrometric analysis

Deuterium^{7} labelled ADMA was used as an internal labelled control. A 50µL sample of supernatant from cell lysis or conditioned medium was collected and a known concentration of labelled deuterium standard was added. Following protein extraction with methanol the solution underwent centrifugation at 16000g for 10minutes. The sample was then dried and the residue re-suspended in mobile phase (0.1% formic acid) for analysis. A standard curve of ADMA samples of 10 known concentrations was prepared (0 to 100µM).

Statistics

Statistical analysis was performed using the Prism software package (GraphPad Inc, CA, USA). Normally distributed data was analysed using a t test or Analysis of Variance (ANOVA) with Bonferroni post-test comparison of groups as appropriate. Non parametric data was analysed using a Mann Whitney U test. In human studies, pre and post intervention samples are compared using paired analyses. All values were expressed as mean ± (SD). Significance was accepted for values of $p < 0.05$.

3. Results

Animal models of hypoxia

The synthesis of nitric oxide by isolated wild type primary murine monocytes exposed to hypoxic and normoxic conditions was determined. Intracellular nitrate plus nitrite concentration was higher in wild-type murine monocytes after hypoxia and reoxygenation than in normoxia-treated cells (mean(SD)) 13·2(2.4) pmols/mg protein *vs* 8·1(1·7) (p=0·009)(Fig 1A), and accumulation of extracellular nitric oxide (Nitrate+Nitrite) increased after hypoxic challenge (13·7(3.1)μM *vs* 1·9(0·18), p=0·002)(Fig 1B). This was associated with induction of the inducible form of nitric oxide synthase in monocytes, (mean (SD)) 4.0 (1.3) fold increase in iNOS mRNA (p=0.01) (Fig 1C). DDAH2 protein was 4·5-fold (SD 1·3) higher than in control cells (p=0·03) (Fig 1D) and Ddah2 mRNA was also increased by (mean(SD)) 3.6(0.12) fold over control cells (Fig 1E). This increase was associated with a 24% reduction in ADMA concentration (mean(SD) 0·33(0.04)pmols/mg to 0·24(0·03), p=0·002)(Fig 1F). There was no significant difference observed in intracellular L-arginine (Fig S1A, p=0.597) or L-NMMA (Fig S1B, p=0.74) concentrations following hypoxic incubation.

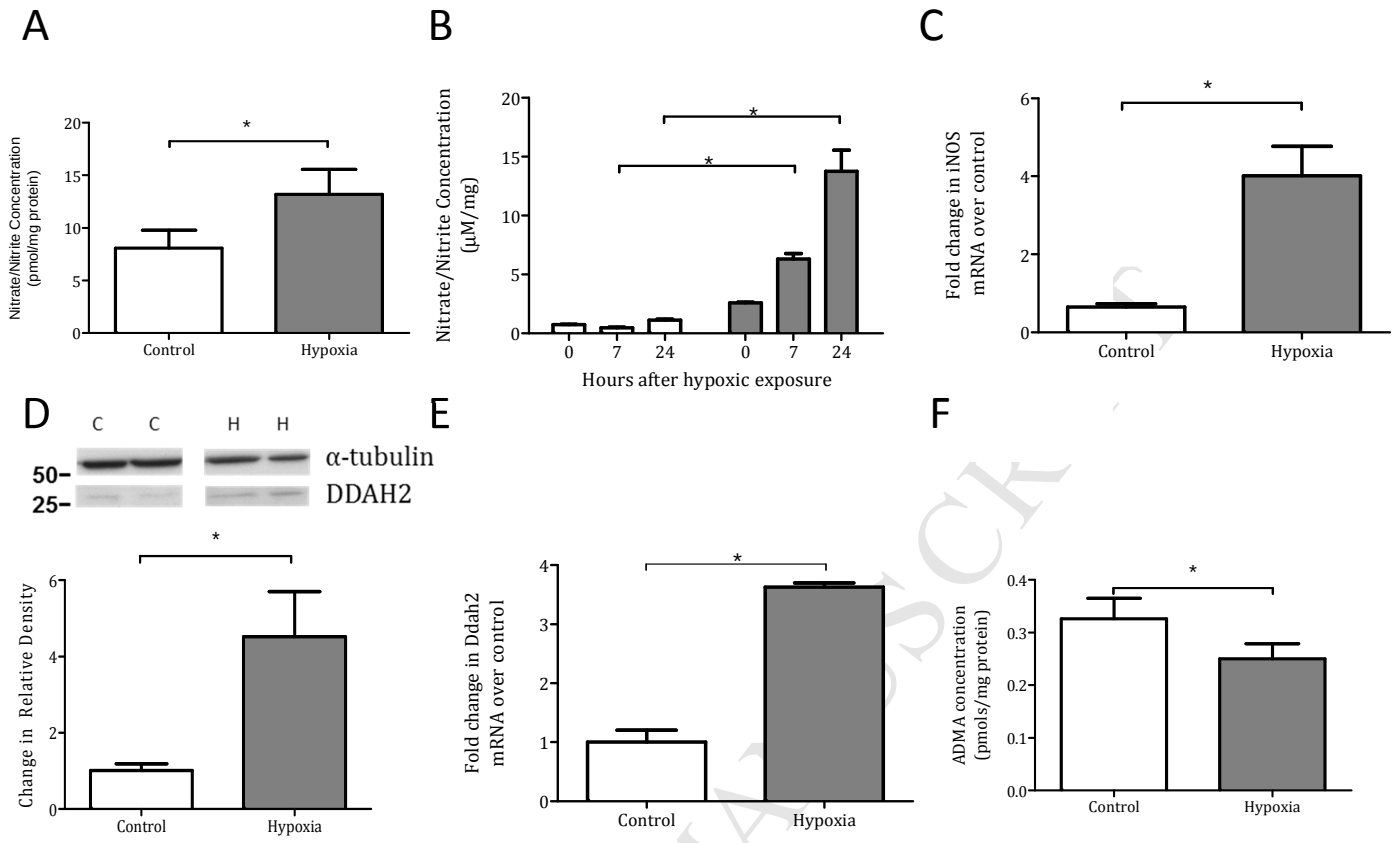

Figure 1: The impact of hypoxia on endogenous regulators of nitric oxide (NO) synthesis in primary peritoneal murine macrophages. A: Cell lysate NOx concentration at 24 hours of reoxygenation following 12 hours of hypoxic exposure (White bars: control cells not exposed to hypoxia, grey bars: hypoxia-treated cells, n=6 per group, * $p<0.05$, NOx concentration in culture medium corrected to cell lysate protein concentration at the termination of the experiment). B: Serial measurements of the accumulation of nitrate/nitrite (NOx) in culture medium during reoxygenation at a FiO_2 of 21% following hypoxic exposure for 12 hours at a FiO_2 of 3%. (White bars: control cells not exposed to hypoxia, grey bars: hypoxia-treated cells, n=6 per group, * $p<0.05$, NOx concentration in culture medium corrected to cell lysate protein concentration at the termination of the experiment). C: Quantitative PCR of inducible nitric oxide synthase mRNA expression in resident peritoneal macrophages from wild type (C57BL/6) mice: from control cells (white bar) and those exposed to 12 hours of hypoxia (grey bar). (n=6 per group, * $p<0.05$). D: Change in DDAH2 protein expression following 12 hour hypoxic exposure. Representative image of control (C) vs hypoxia (H) treated cells and quantification of n=6 per group, control (white) and hypoxia-treated (grey bar) (* $p<0.05$) All bars represent mean (+SEM).E: Quantitative PCR of *Ddah2* mRNA expression in resident peritoneal macrophages from wild type (C57BL/6) mice: from control cells (white bar) and those exposed to 12 hours of hypoxia. (n=6 per group, * $p<0.05$). F: Change in cell lysate asymmetric dimethylarginine (ADMA) concentration in murine primary peritoneal macrophages in control (white) and hypoxia-treated (grey) cells, corrected for lysate protein concentration (n=6 per group, * $p<0.05$).

Successful knockout of DDAH2 from primary peritoneal monocytes isolated from monocyte-specific *Ddah2* knockout mice (*Ddah2*$^{flox/flox}$ *LysM-cre*; Ddah2$^{M\Phi}$) was demonstrated by western blot and qPCR (Fig 2A). Comparison of NOx synthesis before and after hypoxic exposure was undertaken in peritoneal monocytes from the Ddah2$^{M\Phi-}$ mice and their Ddah2$^{flox/flox}$ litter mate controls. Cells deficient in DDAH2 displayed reduced intracellular NOx concentrations at baseline compared to controls, mean (SD) 5.15(0.61) vs 7.7(0.87) µM/mg protein (p=0.014) (Fig 2B). Following hypoxic exposure, Ddah2$^{flox/flox}$ cells displayed significant induction of NOx synthesis (11.6(0.94) µM/mg

protein (p<0.01). By contrast, peritoneal macrophages from Ddah2$^{MΦ-}$ mice displayed no significant increase in intracellular NOx following hypoxic exposure (p=0.10) (Fig 2B). This was associated with changes in intracellular ADMA in Ddah2$^{flox/flox}$ mice; cells showed a similar decrease in ADMA concentration to that seen in wild type peritoneal macrophages (p=0.01) (Fig 2C). In contrast to this, Ddah2$^{MΦ-}$ mice displayed a trend to increased ADMA level following hypoxia with concentrations of 0.13(0.04) µM/mg protein prior to exposure and of 0.28(0.03) µM/mg protein following hypoxic treatment (p=0.06) (Fig 2C). No significant differences were observed in L-arginine (Fig 2D) or SDMA (Fig 2E) concentrations between the monocytes derived from Ddah2$^{MΦ-}$ mice and their Ddah2$^{flox/flox}$ litter mate controls in their response to hypoxia.

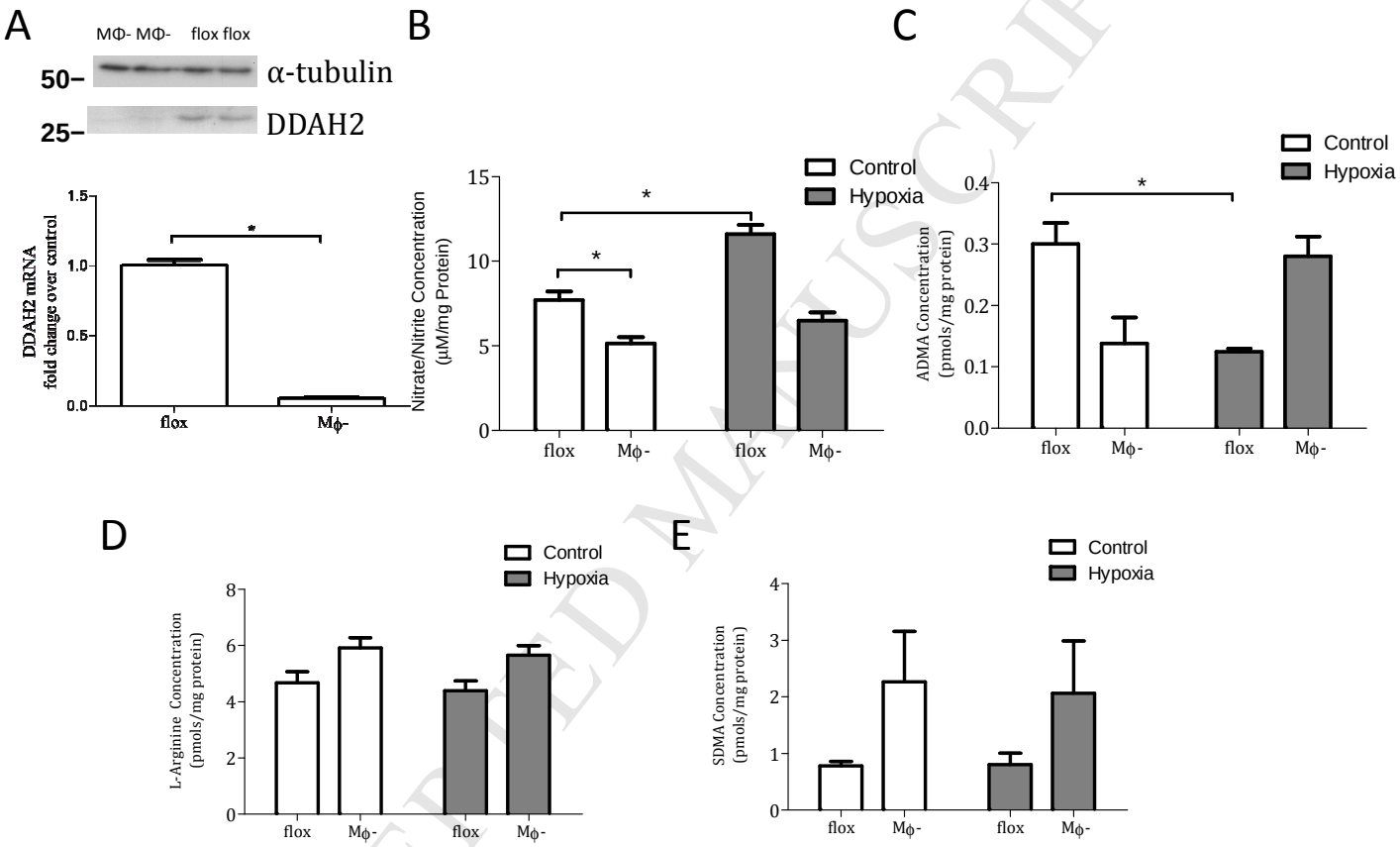

Figure 2: The impact of *Ddah2* knockout on endogenous regulators of NO synthesis in response to hypoxic exposure. A: Demonstration of *Ddah2* mRNA and protein knockout in peritoneal macrophages derived from *Ddah2* macrophage-specific knockout mice (*Ddah2*$^{MΦ-}$) or littermate flox/flox controls (*Ddah2*$^{flox/flox}$). Representative western blot and quantitative PCR analysis of *Ddah2* mRNA expression (n=6, * p<0.01). Panels B to E: analysis of *Ddah2*$^{MΦ-}$ or *Ddah2*$^{flox/flox}$ –derived macrophages following 12 hour hypoxic exposure and 24 hour reoxygenation. White bar: control cells, grey bar: hypoxia-treated cells. B: Change in intracellular NOx concentration (corrected for cell lysate protein concentration) (n=6 per group, *p<0.05). C: Change in intracellular ADMA concentration (corrected for cell lysate protein concentration) (n=6 per group, *p<0.05). D: Change in intracellular L-arginine concentration (corrected for cell lysate protein concentration) (n=6 per group). E: Change in intracellular SDMA concentration (corrected for cell lysate protein concentration) (n=6 per group). All bars represent mean (+SEM).

Human Hypoxia studies

Of fifteen participants, four exhibited subjective symptoms of acute mountain sickness with modified Lake Louise score of between 1 and 3 without significant objective symptoms. One volunteer developed significant nausea during the second half of the experimental period and was withdrawn from the hypoxic chamber. After a period of observation following extraction form the chamber there were no subjective or objective sequelae in this volunteer and follow up at 24 hours revealed no residual symptoms. The participant was excluded from the study and further analysis. Of the participants that completed the study, nine physiological, plasma and paired monocyte samples collection and analysis, a further three underwent plasma and physiological analysis only.

Hypoxic exposure in the fifteen participants led to an immediate reduction in arterial oxygenation to a mean(SD) value of 86(2.0)% which was sustained throughout the period of hypoxic chamber exposure (Fig 3A). No significant differences in indices of cardiovascular function were detected by non-invasive assessment of cardiovascular function, although a trend towards reduced systemic vascular resistance with compensatory increase in cardiac output was observed in the post prandial period (Fig 3B-F). Following completion of the hypoxia study protocol, participants were removed from the hypoxic chamber and arterial oxygen saturations assessed after 5mins of exposure to the normoxic environment. At this time, mean(SD) oxygen saturations were 97(1.0)% and heart rate was 71(10) bpm.

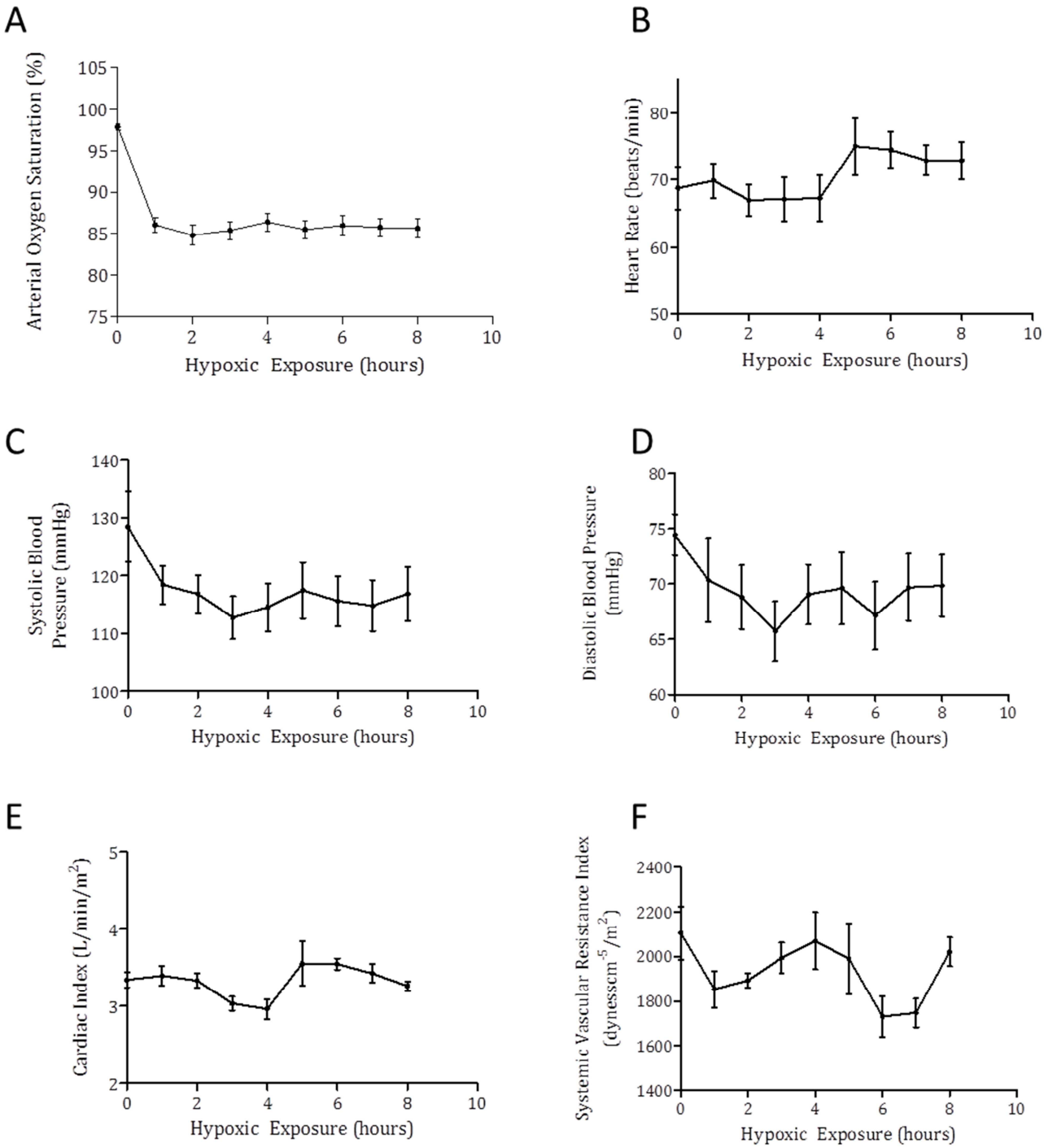

Figure 3: The impact of normobaric hypoxia on he althy volunteers exposed to an eight hour stimulus at 12% FiO2. A: The impact of exposure to 12% FiO2 on healthy volunteer peripheral arterial oxygen saturations measured at rest prior to entrance (0). Subsequent regular measurements are summarised hourly. (n=12) B: The impact of exposure to 12% FiO2 on healthy volunteer heart rate measured at rest prior to entrance (0). Subsequent regular measurements are summarised hourly. (n=12). C&D: The impact of exposure to 12% FiO2 on healthy volunteer systolic (C) and diastolic (D) blood pressure measured at rest prior to entrance (0) and following entry to the hypoxic chamber. (n=12) E&F: Change in cardiac index(E) and systemic vascular resistance index (F) following exposure to normobaric hypoxia at a FiO2 of 12% (n=12).

Eight hour hypoxic exposure led to a significant increase in plasma NOx concentration (Mean (SD)) 3.6 (1.8)µM vs 6.4 (3.2)µM p=0.01 (Fig 4A). This was associated with a significant reduction in plasma ADMA concentration from 0.42 (0.12)µM at baseline, to 0.29(0.05)µM after exposure ($p<0.01$) (Fig 4B). There were no significant differences in plasma arginine, SDMA or L-NMMA concentration (Fig S2A-C respectively). As a consequence of the reduction in ADMA concentration, plasma arginine:ADMA ratio was significantly reduced following hypoxia ($p<0.01$)(Fig 4C). Intracellular concentrations of methylarginines in peripheral blood mononuclear cells (PBMC) displayed a similar pattern with significant reduction in ADMA in paired samples from 3·6 pmols/mg

protein [0·27] to 3·15 [0·3] (p=0·0009)(Fig 4D) but no significant variation in intracellular arginine, SDMA or L-NMMA concentration (Fig S2D-F). Similarly, the intracellular Arginine:ADMA ratio was reduced (p<0.01) (Fig 4E). Consistent with previous observations of the relationship between plasma and monocyte ADMA concentrations[34], at baseline there was no apparent correlation (r^2 =0.08, p=0.44) (Fig S2G). However following hypoxia, a positive correlation between PBMC and extracellular ADMA level was observed (r^2=0.72, p=0.01) (Fig 4F).

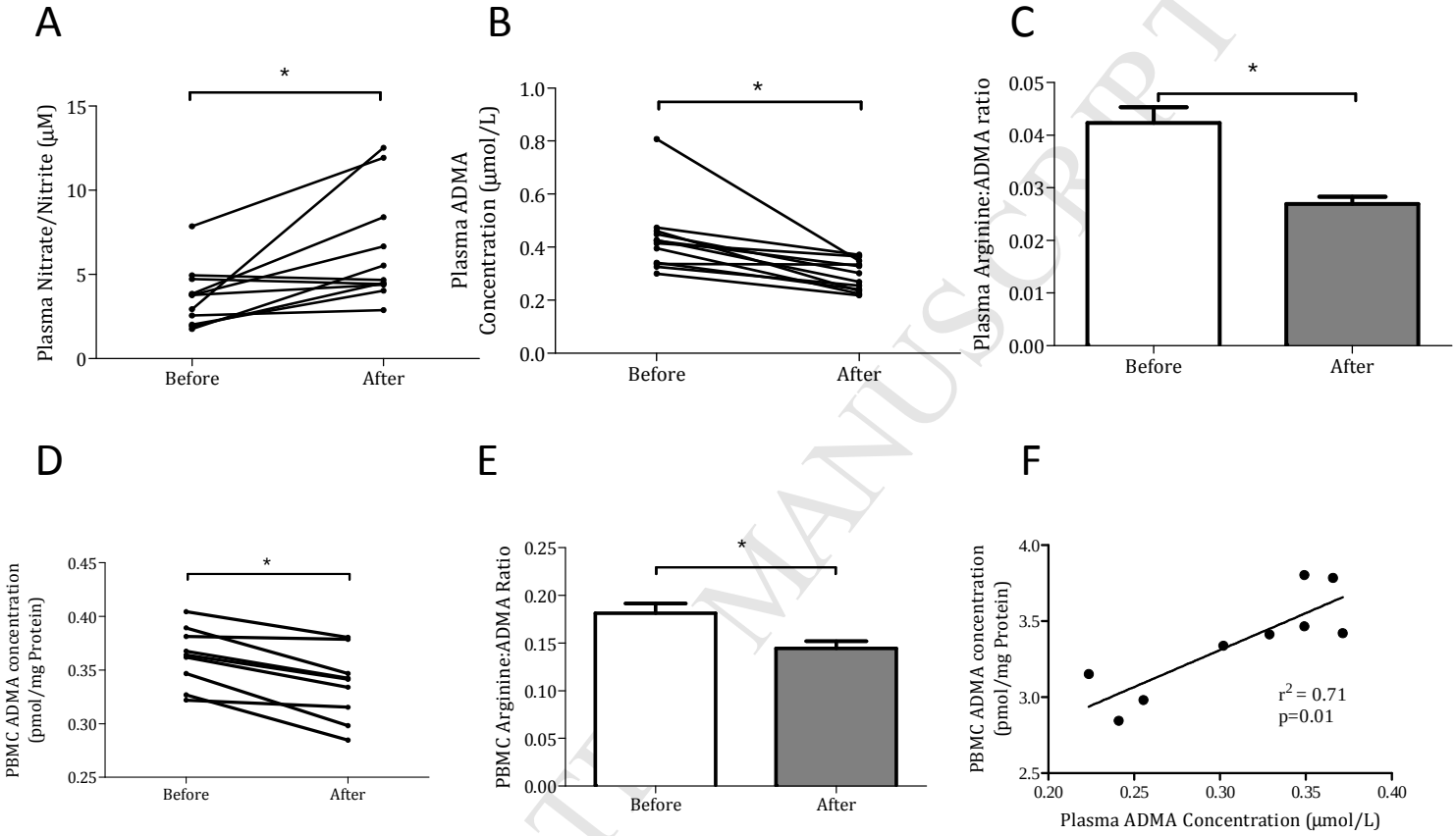

Figure 4: The impact of normobaric hypoxia at a FiO_2 of 12% on nitric oxide synthesis and methylarginine concentrations in plasma and peripheral blood mononuclear cells (PBMC) from a prospective observational healthy volunteer study. A: Change in plasma Nitrate/Nitrite (NOx) following 8 hour hypoxic exposure, each line represents plasma NOx concentration before and at the end of the exposure period, (n=12, * p<0.05). B: Change in plasma ADMA following 8 hour hypoxic exposure, each line represents plasma concentration before and at the end of the exposure period, (n=9, * p<0.05). C: Change in plasma L-arginine:ADMA ratio following 8 hour hypoxic exposure (n=9, * p<0.05). D: Change in PBMC ADMA concentration following 8 hour hypoxic exposure, each line represents intracellular concentration, corrected for cell lysate protein level before and at the end of the exposure period, (n=9, * p<0.05). E:Change in PBMC L-arginine:ADMA ratio following 8 hour hypoxic exposure, corrected for cell lysate protein level before and at the end of the exposure period, (n=9, * p<0.05). F: The relationship between PBMC (corrected for PBMC lysate protein) and plasma ADMA concentrations following 8 hour hypoxic exposure (n=9). All bars represent mean (+SEM).

In human subjects, the increase in NOx synthesis was not mediated by increased eNOS expression in PBMCs (Mean (SD)) change over baseline 0.68(0.28) fold (p=0.09)(Fig 5A). There was no discernible iNOS mRNA and iNOS protein was not detectable in human PBMCs (data not shown). Reduction in monocyte ADMA was associated with an increase in DDAH2 mRNA expression (Mean (SD) increase of 1.9(0.6) fold over baseline (p=0.03) (Fig 5B) DDAH2 protein (Mean (SD) fold increase 2.5(0.94, p=0.034)(Fig 5C).

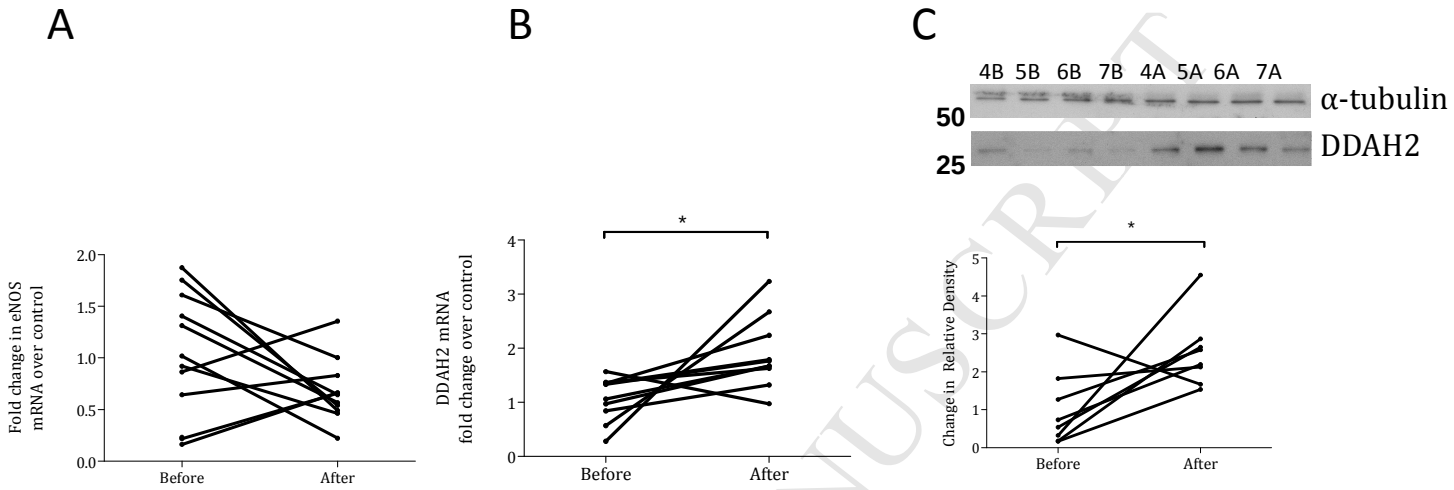

Figure 5: Impact of normobaric hypoxic exposure on regulators of nitric oxide synthesis in human peripheral blood mononuclear cells. A: Change in *eNOS* mRNA following 8 hour hypoxic exposure, each line represents *eNOS* mRNA expression before and at the end of the exposure period (n=10, * p<0.05). B: Change in *DDAH2* mRNA following 8 hour hypoxic exposure, each line represents *DDAH2* mRNA expression before and at the end of the exposure period, (n=10, * p<0.05). C: Representative western blot and quantification of DDAH2 protein expression following hypoxic challenge. Representative image: participant number (x) followed by B, Before or A, After hypoxic challenge. Quantification: Fold change in DDAH2 expression over the course of the study period (n=8, * p<0.05). Bars represent mean (+SEM).

4. Discussion

Inflammation either at the site of infection or systemically leads to an environment in which monocytes are exposed to grossly deranged conditions including hypoxia. It has been shown that acute hypoxia leads to delay in constitutive apoptosis in monocytes[35]. Hypoxia also has organism specific effects on bactericidal activity and the microenvironment itself[7]. The impact of acute hypoxic stress on regulators of NO synthesis by circulating immune cells has not been well elucidated and potentially has an impact both on immune cell function and also on the local microenvironment.

This study deepens our understanding of this process by exploring the impact of hypoxia on monocyte DDAH2 expression and regulation of NO synthesis. By using primary cells isolated from macrophage specific DDAH2 deficient mice and their relevant controls we have been able to elucidate the role this enzyme plays in regulating NO synthesis in hypoxia. Translating this work into humans has allowed us to demonstrate that this mechanism is preserved in humans following a clinically relevant degree of systemic hypoxia. It has previously been shown that in endothelial cells, DDAH2 is downregulated in Hypoxia. This suggests that DDAH2 regulation in hypoxia may be tissue specific and reflect differing adaptive responses to hypoxic stress.

We have previously shown that global and macrophage specific knockout of DDAH2 in murine models of severe sepsis leads to a significant excess mortality which is mediated by impaired macrophage phagocytosis, bactericidal ability and NO synthesis[31]. Sepsis is a complex physiological insult in which multiple processes contribute to organ dysfunction and death. This study shows that a cardinal feature of critical illness – hypoxia - leads to a significant increase in monocyte NO synthesis mediated by an increase in DDAH2 expression that reduces ADMA concentrations and facilitates increased NOS activity. The mechanism of hypoxia mediated induction of DDAH2 requires further elucidation however recent work has shown that NRF2 induces DDAH2 expression[36] which in turn, has been shown to be induced by hypoxia[37].

In macrophage specific knockout cells exposed to a hypoxic challenge, basal NOx is reduced and the increase in intracellular NOx seen in the floxed control cells upon exposure to hypoxia is not observed in the knockout cells. We postulate that this is one of the mechanisms by which animals deficient in DDAH2 demonstrate impaired monocyte function and elevated mortality in animal models of sepsis[31] and may in part explain the association between polymorphisms of the human *DDAH2* gene and outcome in patients with septic shock[38]

This study also highlights differing mechanisms in the synthesis of NO across species. In our murine studies, elevated iNOS was observed in isolated hypoxic primary murine macrophages consistent with previous studies of the impact of hypoxia on the inducible isoform of the enzyme[39]. By contrast, in the human samples, only a trend towards increased eNOS expression was observed. This is consistent with studies showing that in the absence of a pro inflammatory cytokine, iNOS induction is not observed in human hypoxia[40]. A number of studies have demonstrated previously that eNOS is present in human monocytes. There is limited evidence regarding the impact of hypoxia on immune cell eNOS expression, however a number of differing stimuli have been shown to regulate eNOS in isolated human monocytes[41]. Our data presented here suggest that regulation of eNOS activity in human macrophages by modulation of the concentration of competitive inhibitors contributes significantly to hypoxia-induced NO synthesis by these cells.

In summary, this study demonstrates that DDAH2 regulates ADMA mediated inhibition of NO synthesis in isolated murine primary monocytes and translates this observation into humans exposed to a clinically relevant model of normobaric hypoxia. We postulate that we have identified a novel mechanism, conserved between mice and humans that contributes to the monocyte response to hypoxia. It may also give insights into the mechanism by which polymorphisms in the human *DDAH2* gene functionally impact NO synthesis and lead to clinically relevant outcomes in humans with septic shock.

5. Conclusions

- The regulation of nitric oxide synthesis by immune cells is a key component of the immune response to pathophysiological stress and regulates both cell function and the microenvironment
- Here we show that acute hypoxia – a cardinal feature of pathological stress – leads to increased immune cell nitric oxide synthesis and that this is mediated by asymmetric dimethylarginine and the enzyme that metabolises it in monocytes, dimethylarginine Dimethylaminohydrolase 2.
- This translational study gives insights into the mechanism through which tissue hypoxia leads to local increases in nitric oxide level and offers avenues for further investigation of how this response may become pathological in some patients. Furthermore, DDAH2

modulation may possess therapeutic potential as a modulator of immune cell nitric oxide synthesis in response to pathological stress.

6. References

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Declaration of Interest

The authors declare no competing interests relating to the conduct of this study.

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Author Contribution

Experimental Design – SL, DM, MM, OB, JL.

Experimental Conduct – SL, BL, SP, JT, KV, OB, DM

Analysis – SL, DM, JL, KV, JT, SP

Preparation of Manuscript – SL, DM, BL, KV, JT, SP, MM, DM, JL

Highlights

- The regulation of nitric oxide synthesis by immune cells is a key component of the immune response to pathophysiological stress and regulates both cell function and the microenvironment
- Here we show that acute hypoxia – a cardinal feature of pathological stress – leads to increased immune cell nitric oxide synthesis and that this is mediated by asymmetric dimethylarginine and the enzyme that metabolises it in monocytes, dimethylarginine Dimethylaminohydrolase 2.
- This translational study gives insights into the mechanism through which tissue hypoxia leads to local increases in nitric oxide level and offers avenues for further investigation of how this response may become pathological in some patients. Furthermore, DDAH2 modulation may possess therapeutic potential as a modulator of immune cell nitric oxide synthesis in response to pathological stress.