The deleterious effects of acute hypoxia on microvascular and large vessel endothelial function

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TITLE: The deleterious effects of acute hypoxia on microvascular and large vessel endothelial function

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NEW FINDINGS

What is the central question of this study?

Primarily to determine the effect of hypoxia on microvascular function, and secondarily whether superior cardiorespiratory fitness is protective against hypoxia-induced impairment in vascular function.

What is the main finding and its importance?

Hypoxia reduced endothelium-dependent microvascular function, but not endothelium-independent microvascular function. The extent of impairment was two-fold higher in the microcirculation compared to the large blood vessels. This study suggests individuals with superior cardiorespiratory fitness may preserve microvascular function in hypoxia. These findings highlight the sensitivity of the microvascular circulation to hypoxia.

ABSTRACT

Hypoxia is associated with diminished bioavailability of the endothelium-derived vasodilator, nitric oxide (NO). Diminished NO bioavailability can have deleterious effects on endothelial function. The endothelium is a heterogeneous organ; therefore, a comprehensive assessment of endothelial function is critical to understand the significance of hypoxia-induced endothelial dysfunction. We hypothesized that acute hypoxia would have deleterious effect on microvascular and large vessel endothelial function. Twenty-nine healthy adults (age: 24 (4) years) completed normoxic and hypoxic [inspired O_2 fraction (F_iO_2) = 0.209] trials in this double-blinded, counterbalanced crossover study. After 30 min, we assessed laser Doppler imaging-determined perfusion response to iontophoresis of acetylcholine (ACh) as a measure of endothelium-dependent microvascular function, and iontophoresis of sodium nitroprusside (SNP) as a measure of endothelium-independent microvascular function. After 60 min, we assessed brachial flow-mediated dilatation (FMD) as a measure of large vessel endothelial function. Thirty minutes of hypoxia reduced endothelium-dependent microvascular function determined by perfusion response to ACh ($\tilde{x}\Delta$ = -109%, {IQR: 542.7}, $P < 0.05$), but not endothelium-independent determined by perfusion response to SNP ($\tilde{x}\Delta$ 69%, {IQR: 453.7}, $P = 0.6$). In addition, 60 min of hypoxia reduced allometrically-scaled FMD compared to normoxia ($\bar{x}\Delta$-1.19 [-1.80, -0.58] %, $P < 0.001$). The decrease in microvascular endothelial function was associated with cardiorespiratory fitness ($r = 0.45$, $P = 0.02$). In conclusion, acute exposure to normobaric hypoxia significantly reduced endothelium-dependent vasodilatory capacity in small and large vessels. Collectively, these findings highlight the sensitivity of the microvascular circulation to hypoxic insult, particularly in those with poor cardiorespiratory fitness.

INTRODUCTION

Hypoxia can cause disturbances to vascular homeostasis (Tymko *et al.*, 2019), and is believed to be implicated in numerous stages of atherosclerosis development and progression, including endothelial dysfunction (Gautier-Veyret *et al.*, 2013; Bickler *et al.*, 2017; Marsboom & Rehman, 2018). A healthy endothelium maintains homeostasis by regulating vascular tone, coagulation and inflammation. Chronic and acute hypoxic

exposure has been shown to trigger endothelial damage and vascular inflammation (Tarbell *et al.*, 2020), increasing an individual's risk of vascular injury that can lead to adverse outcomes, such as cardiovascular disease (Lee *et al.*, 2019). Moreover, the progressive nature of cardiovascular disease is also proposed to exacerbate vascular hypoxia (Gupta & Zahid Ashraf, 2018), resulting in a reciprocal cycle. The endothelium plays a pivotal role in this cycle, and thus it is important to understand the deleterious effects of hypoxia on endothelial function.

Nitric oxide (NO) is recognised as an endothelium-derived vasodilator that plays a central role in maintaining vascular homeostasis (Sandoo *et al.*, 2010). The production of NO is limited during hypoxia due to the prevalence of oxidative stress. Overexpression of hypoxia-induced reactive oxygen species (ROS) is proposed to upregulate the scavenging of NO (Griendling *et al.*, 2000; Frey *et al.*, 2009) and downregulate the expression of endothelial nitric oxide synthase (eNOS) (Thompson & Dong, 2005; Janaszak-Jasiecka *et al.*, 2018). A reduction in the expression of NO can result in an imbalance between endothelium-derived vasoactive factors, contributing towards the development of endothelial dysfunction (Tymko *et al.*, 2019).

Flow-mediated dilatation (FMD) is a well-established technique that uses reactive hyperaemia to assess the endothelial NO vasodilatory system in large blood vessels (Green *et al.*, 2014). Previous research has shown that FMD responses decrease by as much as 45% during acute hypobaric hypoxia exposure (Bailey *et al.*, 2013; Lewis *et al.*, 2014, 2017). However, the authors also reported a decrease in endothelium-independent vasodilation, suggesting that impaired endothelial function did not fully account for the reduction in vasodilatory capacity. To better understand the underlying reason for these vascular impairments, it is important to also examine the microvascular responses to hypoxia, as evidence suggests that microvascular dysfunction precedes large vessel dysfunction (Krentz *et al.*, 2009). Peripheral microvascular endothelial dysfunction is an indicator of systemic endothelial dysfunction and atherosclerotic risk, and is considered a major cause of cardiovascular mortality (Anderson *et al.*, 1995; Widlansky *et al.*, 2003; Liew *et al.*, 2011). Furthermore, the microcirculation comprises a much larger surface area of the circulatory system which leads to greater ROS

production, therefore the risk of injury is significantly elevated in the microcirculation (Stokes & Granger, 2005). Iontophoretic application of acetylcholine (ACh) on human skin increases microvascular endothelium-dependent vasodilation (Furchgott *et al.*, 1987) and laser Doppler imaging (LDI) with simultaneous iontophoresis of ACh can be used to assess changes in cutaneous perfusion in response to the delivery of ACh.

Not only is it crucial to identify stimuli that may trigger the development or progression of impaired endothelial function, it is also important to understand how humans may be able to protect the endothelium against damage. Over the years, it has been established that lifestyle modifications including diets high in green leafy vegetables and increasing physical activity can prevent and reverse endothelial dysfunction (DeSouza *et al.*, 2000; Beck *et al.*, 2013; d'El-Rei *et al.*, 2016). However, despite the strong evidence to suggest that hypoxia can have a deleterious effect on endothelial function, there has yet to be a study that examines how these effects might be mitigated. As exercise intervention studies have already been shown to cause improvements in endothelial function (Beck *et al.*, 2013); prospective studies should consider examining the relationship between the fitness status and endothelial responses to hypoxia. Collectively, these studies might be able to highlight the importance of physical activity and fitness for individuals who have a higher risk of hypoxia-induced impairment in endothelial function.

To understand the systemic effect of hypoxia on the endothelium, it is important to assess endothelial function in different vasculatures (microvasculature and large vessels). The present double-blind, counterbalanced crossover study sought to determine the effect of hypoxia on microvascular and large vessel function. Our aims were to i) replicate the previous FMD findings reported by Lewis *et al*. (2017), and to assess and compare the effects of acute hypoxia on ii) endothelium-dependent microvascular function determined by perfusion response to iontophoresis of ACh, iii) endothelium-independent microvascular function determined by perfusion response to iontophoresis of sodium nitroprusside (SNP). Furthermore, we aimed to assess the relationship between cardiorespiratory fitness and the changes in endothelial function. We hypothesised that a degree of endothelial impairment was present in both

microvasculature and large vessels, but cardiorespiratory fitness would partly protect against the magnitude of the decline. However, as the risk of injury is increased for microvascular endothelial cells, we hypothesised that the magnitude of the decrease in function would be greater in the microcirculation.

METHODS

Ethical Approval

All participants were briefed on the nature and the purpose of the investigation before written consent was taken along with a short demographic questionnaire to ensure that they satisfied the study criteria. Ethical approval was granted by the Ethics Committee of the School of Sport, Health, and Exercise Sciences at Bangor University (Ethics ID: P19-16/17) and the study was performed in accordance with the guidelines of the WMA Declaration of Helsinki (2013), except for registration in a database.

Participants

Twenty-nine healthy adults (17 men) were recruited into the study (age: 24 (4) years). Participants had not travelled to altitude (≥ 1500 m) in the preceding six months, and had no medical contraindications to maximal exercise testing. Female participants were studied during the early follicular phase of their cycle, or the placebo phase of oral contraceptives.

Study design

The study followed a double-blind, repeated-measures, counterbalanced crossover design. Participants completed three separate laboratory visitations. During the first visit, individuals completed baseline health and fitness assessments, including a carotid intima-media thickness (cIMT) assessment and a maximal exercise test. Participants then completed normoxia [inspired O_2 fraction (F_iO_2) = 0.209] and poikilocapnic hypoxia (F_iO_2 = 0.120) experimental trials, separated by at least five days. Experimental trials consisted of 2 h exposure in a temperature (normoxia 24.7 (1.7)°C; hypoxia 24.4

(1.5)°C) and humidity (normoxia 42.6 (7.9)%; hypoxia 43.0 (5.7)%)-controlled environmental chamber (Hypoxico Inc.; NY). Ambient O_2 in the chamber was recorded at 30 min intervals throughout (normoxia 20.8 (0.1)%; hypoxia 12.2 (0.1)%). Both participants and researchers were blinded to condition (F_iO_2) as a separate researcher was responsible for setting and recording the F_iO_2 in the environmental chamber and all panels were covered during testing. Participants were randomly allocated to conditions in a counterbalanced order, using a computer-generated randomized list (Urbaniak & Plous, 2013). In experimental trials, participants rested supine for 20 min before manual BP, heart rate and blood saturation were recorded. These vital signs were measured every 30 min for the duration of the experimental trial. Whilst remaining in a supine position, vascular function of the small and large blood vessels was assessed after 30 and 60 min, respectively (separated by a minimum of 15 min). All participants abstained from strenuous exercise for 24 h before every study visit and procedure, abstained from food and caffeine 2 h before baseline procedures and overnight before experimental procedures. An overview of the protocol is depicted in Figure 1.

Baseline Procedures

Carotid intima-media thickness

Assessment of advanced but subclinical atherosclerosis was completed using cIMT. The right and left carotid arteries were imaged 1–2 cm proximal to the carotid bulb (Stein *et al.*, 2008), using a high-resolution ultrasound machine (Acuson X300, Siemens Healthcare GmbH; Erlangen: Germany) attached to a high frequency linear array transducer. Participants lay supine with a 45° tilt of the neck to align the carotid artery for scanning. Images were acquired at end-diastole, determined by the ECG R-peak. Three images were acquired for each side (left and right), with the cIMT measured in each and averaged across the three images for each side, and across both sides. Images were analysed to obtain cIMT measurements sing a semi-automated computerised offline analysis system; Artery Measurement System (AMS) (Wendelhag *et al.*, 1991). All images were acquired and analysed by GMKR (the between-day reliability of this technique is equal to coefficient of variation of 4.1%). Increased atherosclerotic risk was defined as having cIMT measurements greater than 1.0 mm in accordance with Simon *et al*.(2002).

Maximal exercise test

To determine cardiorespiratory fitness levels (VO_{2max}), participants completed a running test to exhaustion on a motorized treadmill (H-P-Cosmos, Sports & Medical GmbH; Nussdorf-Traunstein: Germany) with simultaneous online gas analysis (Cortex Metalyzer, Biophysik GmbH; Leipzig: Germany).

The test protocol was designed so that participants reached maximum between 10–15 min regardless of fitness level, using a similar method to da Silva and colleagues (2012). VO_{2max} was estimated using the Matthews equation (1999), and work rates were calculated using the ACSM metabolic equations for treadmill running. The test protocol began with an 8 min warm up at 50% estimated maximum and subsequent 2 min rest, followed by a ramped increase in work rate from 50% estimated maximum to 100% estimate maximum over 10 min, with the ramp of the slope continuing until exhaustion to obtain VO_{2peak}. After a 10 min rest, participants completed a validation stage at 110% of the work rate at exhaustion to obtain VO_{2max}. VO_{2max} was identified on the criterion the validation VO_2 had a greater than 3% negative discrepancy of the modelled 110% VO_{2peak} (Poole & Jones, 2017). All participants successfully met this criterion. Heart rate and Rating of Perceived Exertion (RPE assessed by the Borg CR100) (Borg & Borg, 2001) was recorded each minute of the test.

Experimental Procedures

Microvascular function: Laser Doppler Imaging (LDI)

Both endothelium-dependent (ACh) and endothelium-independent (SNP) microvascular function were assessed in normoxia and hypoxia after 30 min using laser Doppler imaging (LDI, moorLDI2, Moor Instruments, Devon, UK) with iontophoresis. All LDI assessments were completed under temperature-controlled conditions (25 (2) °C) and measured according to previously established methodology (Sandoo & Kitas, 2015). Simultaneous delivery of ACh (Miochol, Bausch & Lomb Inc. Berlin, Germany) and SNP (Rottapharm S.L., Barcelona, Spain) was performed using an iontophoresis controller (MIC2, Moor Instruments, Devon, UK) to assess endothelium-dependent and endothelium-independent cutaneous perfusion, respectively. Perfusion changes in

response to the delivery of both vasoactive drugs were assessed on the participant's volar aspect of the right forearm. The full protocol used for this study has been described in detail previously (Sandoo & Kitas, 2015). In summary, a baseline scan was performed before a series of ten scans with an iontophoresis charge of 30μA to administer 2.5ml of 1% ACh and 1% SNP. The iontophoresis current was administered continuously throughout the ten scans. ACh and SNP drugs were diluted with 0.9% saline and delivered simultaneously into the skin *via* anode (ACh) and cathode (SNP) internal electrode Perspex chambers (⌀22mm) (ION 6, Moor Instruments, Devon, UK). The scans were performed simultaneously with the iontophoresis protocol. Following ten scans with iontophoresis, two further recovery scans were performed without the delivery of the vasoactive drugs.

The exposure-time-response protocol took 15–20 min and all scans were performed in natural lighting conditions, with most of the ambient lighting restricted. Additionally, the settings of the laser Doppler imager (moorLDI2-IR, Moor Instruments, Axminster, Devon, UK) were kept consistent for all scans and acetate sheets labelled with anatomical markers were used to ensure the delivery site was consistent across trials. Measurements of perfusion were conducted offline using the moorLDI Review V6.1 software. Perfusion values were quantified for ACh and SNP calculating the median for each region of interest (Jadhav *et al.,* 2007). Results are presented as the percentage change in perfusion from the baseline scan collected immediately before the drug infusion, and was calculated as follows; ((Peak perfusion [AU] – Baseline perfusion [AU]) ÷ Baseline perfusion [AU]) × 100 = Change in perfusion [%AU].

Large vessel endothelial function: Flow-mediated Dilatation (FMD)

Large vessel endothelial function was assessed using FMD under temperature-controlled conditions (25 (2) °C) in normoxia and hypoxia after 60 min. The FMD procedure was performed as previously described in detail (Sandoo & Kitas, 2015). Briefly, a 2 min baseline ultrasound scan of the brachial artery was followed by 5 min of occlusion, achieved by inflating a blood pressure cuff placed around the wrist to suprasystolic pressures (220mmHg). After 5 min, the cuff was deflated rapidly to

induce reactive hyperaemia. To capture maximal dilation, a 3 min scan was performed following cuff deflation.

A Siemens Acuson X300 Ultrasound scanner was used with a multifrequency linear-array vascular probe set at 7.3MHz (Acuson X300, Siemens Healthcare GmbH; Erlangen: Germany) to perform the FMD procedure. B-mode images were captured at 15 frames per second to record a 120 s baseline and a 210 s clip following 5 min of occlusion. To capture the initial reactive hyperaemic response to cuff deflation, the recording was initiated 30 s before cuff release; therefore, only 180 s was used for the analysis. Images were analysed offline using an automated edge detection software (Brachial Analyser, Medical Imaging Applications, USA). The Brachial Analyser software is capable to detect the peak of the R-wave; therefore, this inbuilt feature was used to include only the images at the peak of the R-wave. The recommended image quality standard was set at a confidence threshold ≥70%. From the frames which were accepted the change in diameter from baseline to the peak was calculated as follows; ((Peak diameter [cm] – Baseline diameter [cm]) ÷ Baseline diameter (cm)) × 100 = FMD%. To account for the differences in baseline diameter, all the data was allometrically scaled as per the Atkinson and Batterham guideline (Atkinson & Batterham, 2013). The coefficient of variation for the sonographer (DTJ) is 8.5%, as previously reported (Jones *et al.*, 2019).

Statistical Analyses

The assumption of normality was examined with the Shapiro-Wilk test. For primary analysis (to determine the effect of hypoxia on vascular function), paired t-tests were applied on normally distributed data and Wilcoxon signed rank test was used for non-parametric data. P values < 0.05 were considered to indicate statistical significance. Also, effect sizes for paired t-tests (by Cohen's *d*) are presented as the mean difference divided by the pooled SD between both normoxic and hypoxic time points and can be interpreted as small (> 0.2), medium (> 0.5), and large (> 0.8). Alternatively, effect sizes for Wilcoxon signed rank test (by Rosenthal's *r*) are presented as the Z scores

divided by the square root of the sample size between both normoxic and hypoxic time points and can be interpreted as small (> 0.2), medium (> 0.3), and large (> 0.5).

A-priori sample size estimation for the primary analysis indicated that 10 participants were needed to produce an 80% chance of obtaining statistical significance at the 0.05 level for a 2-tailed design, based on a minimum important difference of 3.1 %, a standard deviation of the difference of 1.7 %, and an estimated average correlation of 0.5 (data from Lewis *et al*. (2017)). Results for all normally distributed data are presented as mean differences ($\Delta\bar{x}$) with 95% confidence intervals [95% CI]. The results of non-parametric analysis are presented as the median differences ($\tilde{x}\Delta$) and interquartile range (IQR). Due to poor image quality, three participants' scans were removed from the FMD analysis, and three different participants' scans were removed from the microvascular analysis. The removal of this data was performed before statistical analysis.

The effect of hypoxia on FMD was determined by a paired t-test comparing normoxia and hypoxia in the first instance. Additionally, the allometric scaling approach was used to adjust for baseline diameter in the calculation of FMD (Atkinson & Batterham, 2013). Briefly, baseline diameters and peak diameters were logarithmically transformed, and then a linear mixed model with repeated measures was performed in SPSS, where the baseline diameter was used as a covariate. Covariate adjusted means for diameter change were obtained from this SPSS model and then back-transformed.

To determine the relationships between the decrease in endothelial function with cardiorespiratory fitness (VO_{2max}), Pearson's correlations were used for parametric data and Spearmen's correlations for non-parametric data. For all correlational analyses, the strength of a relationship was determined by the correlation coefficient value, and *P* values < 0.05 were considered to indicate statistical significance.

RESULTS

Vascular Demographic: Carotid intima-media thickness (cIMT)

Baseline cIMT measurements were recorded to screen for any subclinical signs of atherosclerosis. For measurements of the right common carotid artery, the mean value was reported to be 0.46mm (SD = 0.07), and the left common carotid artery was measured to be 0.45mm (SD = 0.07) (Table 1). Carotid intima-media thickness measurements of <1.0mm are considered to be normal (Simon *et al.*, 2002).

Physiological Responses to 30 and 60-min Hypoxia

Resting physiological responses were recorded at 30 and 60 min during the trial. Hypoxia decreased SpO_2 compared to normoxia after 30 min ($\bar{x}\Delta$−19 [−20, −17] %) and 60 min ($\bar{x}\Delta$−18 [−20, −15] %; $P < 0.001$) exposure. Hypoxia significantly increased heart rate compared to normoxia after 30 min exposure ($\bar{x}\Delta$12 [8, 6] beats/min; $P <$ 0.001) and remained elevated after 60 min ($\bar{x}\Delta$11 [6, 16] beats/min; $P < 0.001$). Hypoxia increased mean arterial blood pressure compared to normoxia after 30 min ($\bar{x}\Delta$4 [1, 7] mmHg; $P = 0.02$), but had no effect on mean arterial blood pressure after 60 min ($\bar{x}\Delta$0 [−4, 4] mmHg; $P = 1.0$).

Effect of Hypoxia on Microvascular Function

Compared to normoxia, hypoxia did not affect baseline perfusion after 30 min in either chamber (ACh chamber $\tilde{x}\Delta = 0.3$, {IQR: 14.0}, $P = 0.13$; SNP chamber $\tilde{x}\Delta = 0.0$, {IQR: 10.8}, $P = 0.80$; Table 2). As expected, perfusion values increased in response to the iontophoresis of ACh and SNP during both trials. Compared to normoxia, endothelium-dependent (ACh) microvascular function was reduced after 30 min of exposure for 19/26 (73%) participants ($\tilde{x}\Delta$ = -109%, {IQR: 542.7}; $P = 0.05$) (Figure 2). Compared to normoxia, hypoxia did not affect endothelium-independent (SNP) microvascular function after 30 min of exposure, and 11/26 (42%) participants had lower responses during hypoxic trial ($\tilde{x}\Delta$ 69%, {IQR: 453.7}; $P = 0.6$).

Effect of Hypoxia on Flow-mediated Dilatation

In comparison to normoxia, hypoxia significantly increased baseline brachial diameter by 2.9% after 60 min ($\bar{x}\Delta$0.11 [0.03, 0.19] mm; $P = 0.01$). As baseline diameters were different between conditions, FMD results are presented as unscaled and allometrically

scaled responses (Figure 3). Compared to normoxia, hypoxia significantly reduced unscaled FMD responses in 22/26 (85%) participants after 60 min ($\bar{x}\Delta$-1.19 [-1.80, -0.58] %; $P < 0.001$). Compared to normoxia, hypoxia significantly reduced allometrically scaled FMD responses in 22/26 (85%) participants after 60 min ($\bar{x}\Delta$-1.21%; $P < 0.001$; relative −18.2%). Compared to normoxia, hypoxia had no effect on FMD time to peak ($\bar{x}\Delta$ -5.0 [-36.7, 26.8] s; $P = 0.75$).

The association between cardiorespiratory fitness and endothelial function

Cardiorespiratory fitness was not associated with endothelium-dependent (ACh) microvascular function (% change in perfusion) (r = -0.47; $P = 0.07$, Figure 4A), endothelium-independent (SNP) microvascular function (% change in perfusion) (r = 0.04; $P = 0.86$), or large vessel endothelial function (%FMD) (r = 0.06; $P = 0.76$, Figure 4B) in normoxia. Cardiorespiratory fitness was correlated with the magnitude of the hypoxia-induced decrease in endothelium-dependent microvascular function (r = 0.45; $P = 0.02$, Figure 4C). In contrast, cardiorespiratory fitness was not correlated with the magnitude of the decrease in endothelium-independent microvascular function (r = 0.1; $P = 0.35$) or large vessel endothelial function (r = -0.09; $P = 0.68$, Figure 4D).

DISCUSSION

The principal findings of this study are that 30 min of hypoxia reduced endothelium-dependent microvascular function (43% reduction in perfusion response to ACh), but did not affect endothelium-independent microvascular function (no change in perfusion response to SNP). Moreover, 60 min hypoxia reduced endothelium-dependent large vessel vasodilatation (18% reduction in FMD). Notably, the extent of the decrease was approximately two-fold higher in the microcirculation compared to the large vessels. Additionally, we are the first to demonstrate individuals with greater cardiorespiratory fitness preserve microvascular endothelial function during hypoxic exposure.

The present study is the first to our knowledge to examine the effect of hypoxia on microvascular and large vessel endothelial function in the same study. The difference in

the magnitude of the decrease between the different vessel sizes suggests that hypoxia may activate specific mechanisms, which effect endothelial function differently. Assessed separately, microvascular and large vessel function have been reported to decrease following acute hypoxia (Lewis *et al.*, 2014, 2017; Treml *et al.*, 2018). However, some studies have also reported increased vascular reactivity following hypoxic exposure (Lawley *et al.*, 2014). Differences in vascular stimulation methods and the length and type of hypoxic exposure make it difficult to compare these published findings. Therefore, when investigating the effects of acute hypoxia on endothelial function, it is important to consider assessing endothelial function in both small and large vessels for a comprehensive understanding of the underlying mechanisms. Furthermore, vascular assessments are highly sensitive and one should always acknowledge the potential influence of biological, environmental and methodological factors on inter-individual variability, which have been listed elsewhere (Bircher *et al.*, 1994; Charakida *et al.*, 2013). Despite the observed individual differences in the present study, we aimed to regulate most factors that can result in large inter-individual variability, including, physical exercise, caffeine, and the menstrual cycle. Additionally, we controlled for the observed individual differences by scaling our data correctly (Atkinson *et al.*, 2013) and performing appropriate analyses.

Using isocapnic hypoxia, Lewis *et al*. concluded that normobaric hypoxia-induced FMD reductions were more pronounced after 30 min of severe hypoxia ($P_{ET}O_2$ 50 mmHg) compared to mild hypoxia ($P_{ET}O_2$ 75 mmHg) (Lewis *et al.*, 2017). This finding suggests that hypoxaemia severity is associated with impaired endothelial function. However, the small range of SpO_2 that were recorded during hypoxia in the present study (range = 70–86%, SD = 5%) suggests that the hypoxic stimulus was relatively homogenous across participants, with most participants at a similar $P_{ET}O_2$ of ~42 mmHg. Thus, the minimal range makes it difficult to evaluate the relationship between hypoxaemia severity and decreased in vascular function. Nonetheless, our results do suggest that hypoxia has a greater deleterious effect on microvascular endothelial function than that of the large vessels, suggesting that the microvasculature endothelium may be more sensitive to hypoxia than larger blood vessels, highlighting the importance

of assessing both microvascular and large vessel endothelial function in hypoxia studies.

Most of the literature implies that hypoxia-induced decrease in endothelial function is linked to NO deficiency (Ten & Pinsky, 2002; Bonetti *et al.*, 2003). The synthesis of NO is an oxygen-dependent reaction, and therefore lower oxygen availability would imply a reduction in NO synthesis. In animal and human *in vitro* models, chronic hypoxia (> 24 h) has been proposed to downregulate the expression of eNOS, thus, blocking the synthesis of NO (Thompson & Dong, 2005; Janaszak-Jasiecka *et al.*, 2018). However, Prieto *et al*. suggested that acute hypoxic exposure (< 24 h) does not decrease eNOS protein expression, but rather, eNOS' capacity to produce NO is affected (Prieto *et al.*, 2011). L-arginine oxidation *via* eNOS is the primary source of NO in endothelial cells, but other enzymes including arginase-I and arginase-II also compete for the same substrate. Krotova *et al*. reported that the activation of hypoxia-inducible factor 1 (HIF-1) elevates the expression and activity of arginase-II in the human lung microvasculature, thus limiting the bioavailability of NO (Krotova *et al.*, 2010). To our knowledge, this finding has not been replicated in large blood vessels. Thus, the upregulation of arginase-II in the microvasculature could explain the more pronounced decrease in endothelial function in the microvasculature that we observed.

Hypoxia stimulates the activation and expression of HIF-1 and other transcriptional complex, which prompts metabolic changes within endothelial cells of small and large blood vessels. The changes in endothelial metabolism have been associated with nicotinamide adenine dinucleotide phosphate (NADH) oxidase-dependent increases in ROS, primarily, superoxide (Griendling *et al.*, 2000; Frey *et al.*, 2009). When an ample amount of superoxide is synthesised, it reacts rapidly with NO to produce peroxynitrite and thereby prevents NO's vasodilatory effect on vascular smooth muscle cells (Gryglewski *et al.*, 1986). In addition to the changes in endothelial metabolism, the interaction between HIF-1 and endothelial cells evokes proinflammatory reactions (Michiels *et al.*, 2000). The prevalence of adhesion molecules are proposed to be higher in microvascular endothelial cells compared to large vessel endothelial cells (Swerlick & Lawley, 1993). The overexpression of adhesion molecules makes the

microvasculature more susceptible to the infiltration of inflammatory molecules (Mendes *et al.*, 2018), which can activate endothelial cells and diminish NO bioavailability. Finally, acute hypoxia directly increases sympathetic outflow and in turn, attenuates NO-dependent vasodilation (Weisbrod *et al.*, 2001). Sympathetic excitation does not only stimulate vasoconstriction, but also increases retrograde shear rate, thus limiting FMD response (Dyson *et al.*, 2006; Padilla *et al.*, 2010). In summary, the available evidence suggests that acute hypoxia diminishes NO bioavailability by reducing eNOS activity, upregulating ROS and inflammation, and increasing sympathetic activity, and thus directly impairs the endothelial NO vasodilatory system. Further research is warranted to investigate the relative contribution of the aforementioned mechanisms of endothelial dysfunction between different vessel sizes.

Cardiorespiratory fitness is positively associated with cardiovascular health (Kaminsky *et al.*, 2019). Exercise interventions have been reported to significantly improve endothelial function (DeSouza *et al.*, 2000; Beck *et al.*, 2013) and prevent and restore age-related endothelial decline (DeSouza *et al.*, 2000). Moreover, exercise-induced improvements in endothelial function have been directly associated with increases in NO bioavailability (Beck *et al.*, 2013). However, independent of training interventions, resting FMD responses are not associated with fitness status in young adults. In the present study, while cardiorespiratory fitness was not associated with microvascular or large vessel endothelial function, the hypoxia-induced decrease in microvascular function was negatively correlated with cardiorespiratory fitness. Those with superior cardiorespiratory fitness had the smallest hypoxia-induced reduction in microvascular function. This moderate relationship is consistent with the interpretation that cardiorespiratory fitness may provide some protection against hypoxia-induced decrease in microvascular function. In contrast, we did not observe a similar relationship between cardiorespiratory fitness and FMD decline, possibly because the microvasculature is more sensitive to hypoxia-induced impairments. However, we acknowledge the limitations of a small sample size and a correlational analysis. Thus, our finding should not be considered conclusive evidence. Rather, this finding highlights the potential importance of physical fitness for microvascular function in hypoxia, which warrants future research in populations that suffer long-term hypoxia and vascular dysfunction.

Limitations

The laser Doppler imaging technique used in this study does not allow for continuous measurement, limiting the temporal resolution of the microvasculature's response to ACh and SNP. However, the technique does provide data from a larger area compared to some alternatives such as laser Doppler flowmetry, making it less sensitive to movement artefacts (Low *et al.*, 2020). A second limitation relating to the LDI procedure is that we did not obtain beat-by-beat blood pressure during the LDI measurement period. As such, we do not present our data as cutaneous vascular conductance, and therefore cannot be sure differences in flux are due to changes in vasomotor function, rather than changes in perfusion pressure (Roustit & Cracowski, 2013). Finally, the current applied during iontophoresis can elicit a vasodilation response independent of a drug response. We did not estimate the contribution of current effects, for example by conducting a separate LDI procedure administering only the vehicle (saline) using the same current and duration. However, though it is not possible to differentiate between current and drug-induced vasodilation, this has minimal consequence for our primary finding, since the same current and drug doses were used in both normoxia and hypoxia. Additionally, both drugs were dissolved in 0.9% saline to reduce the electrically induced iontophoretic artefacts (Ferrell *et al.*, 2002).

In addition to using baseline diameter for covariate-adjusted means, some researchers propose that FMD data should also be normalised for variation in the shear rate (Pyke & Tschakovsky, 2005, 2007). For the present study, shear rate was not recorded. However, Atkinson *et al*. suggested that normalising one variable (i.e. baseline diameter), by another variable (i.e. shear rate), is not good practice when analysing FMD data (Atkinson *et al.*, 2013). Furthermore, Atkinson *et al*. implied that scaling FMD to baseline diameter differences should outweigh the variation in shear rate.

CONCLUSION

To conclude, acute exposure to normobaric hypoxia reduced endothelium-dependent vascular function, in small and large vessels. The decline in microvascular endothelial function was approximately twice as large as that observed in the large blood vessel, demonstrating the sensitivity of the microvascular endothelium to hypoxia. Furthermore, our data suggests that superior cardiorespiratory fitness may be protective against the hypoxia-induced reduction in microvascular endothelial function, but this warrants further investigation. Collectively, these findings highlight the sensitivity of the microvascular circulation to hypoxic insult, particularly in those with poor cardiorespiratory fitness.

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Tables

	Minimum	Maximum	Mean	SD
Age (years)	20	39	24	4
Height (cm)	160	193	176	9
Body Mass (kg)	49	115	74	13
MAP (mmHg)	73	103	91	7
Haemoglobin (mmol/L)	7.45	10.43	9.06	0.68
Total Cholesterol (mmol/L)	2.88	5.65	4.05	0.82
LDL (mmol/L)	0.84	4.28	2.35	0.80
HDL (mmol/L)	0.98	2.49	1.67	0.43
Physical Activity (0-7)[1]	0	7	6	2
VO_{2max} (ml/min/kg)	35	79	50	10
Right CCA IMT (mm)	0.36	0.71	0.46	0.07
Left CCA IMT (mm)	0.35	0.58	0.45	0.07
Mean CCA IMT (mm)	0.37	0.56	0.46	0.06

Table 1. Participant Characteristics. Abbreviations: MAP, Mean Arterial Blood Pressure; LDL, Low-Density Lipoproteins; HDL, High-Density Lipoproteins; CCA, common carotid artery; IMT, intima-media thickness; SD, Standard Deviation; $VO_{2max,}$ maximal aerobic capacity. [1]Physical activity was measured using an instrument commonly used in VO_{2max} prediction models (Jackson et al., 1990; Matthews et al., 1999).

	Normoxia			Hypoxia			*P*
	Mean	SD	Median	Mean	SD	Median	
SBP (mmHg)	111	9	-	112	9	-	0.51
DBP (mmHg)	65	8	-	68	8	-	0.18
MAP (mmHg)	80	8	-	84	8	-	0.02*
Heart Rate (bpm)	61	10	-	73	12	-	<0.001***
SpO_2 (%)	98	1	-	79	5	-	<0.001***
Baseline flux (AU)							
ACh chamber	-	-	43	-	-	49	0.13
SNP chamber	-	-	38	-	-	43	0.80

Table 2. Physiological data at 30 min before LDI and FMD assessments.

Abbreviations: SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; MAP, Mean Arterial Blood Pressure; SpO_2, peripheral oxygen saturation; ACh, acetylcholine; SNP, sodium nitroprusside; SD, Standard Deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

FIGURES

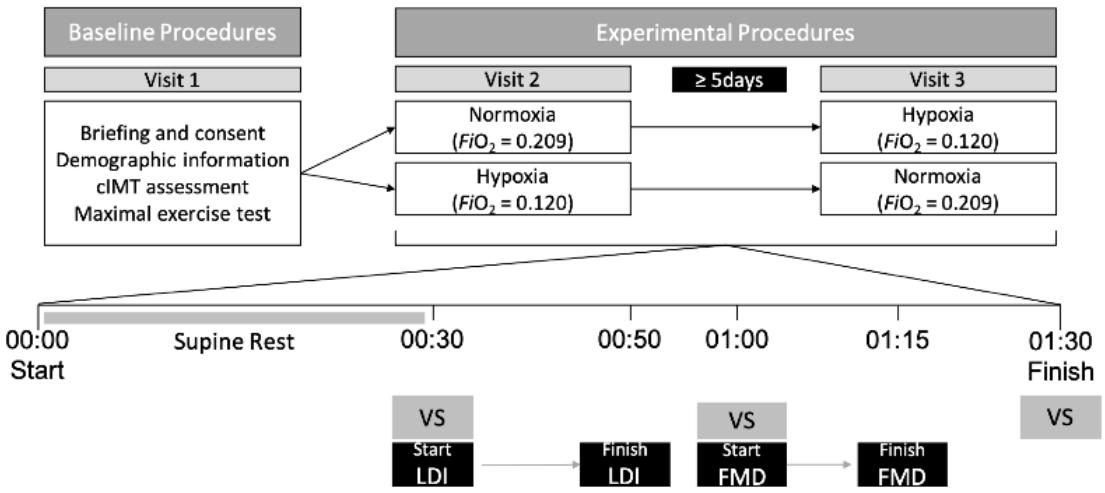

Figure 1. Overview of study protocol. Baseline characteristics were collected for all participants during visit 1. Participants then completed normoxia and poikilocapnic hypoxia experimental trials separated by at least five days. During each experimental trial vital signs were assessed every 30 min, LDI at 30 min and FMD at 60 min. cIMT, carotid intima-media thickness; F_iO_2, fraction of inspired oxygen; FMD, flow-mediated dilatation; LDI, laser Doppler imaging; VS, vital signs (blood pressure, heart rate, and blood saturation).

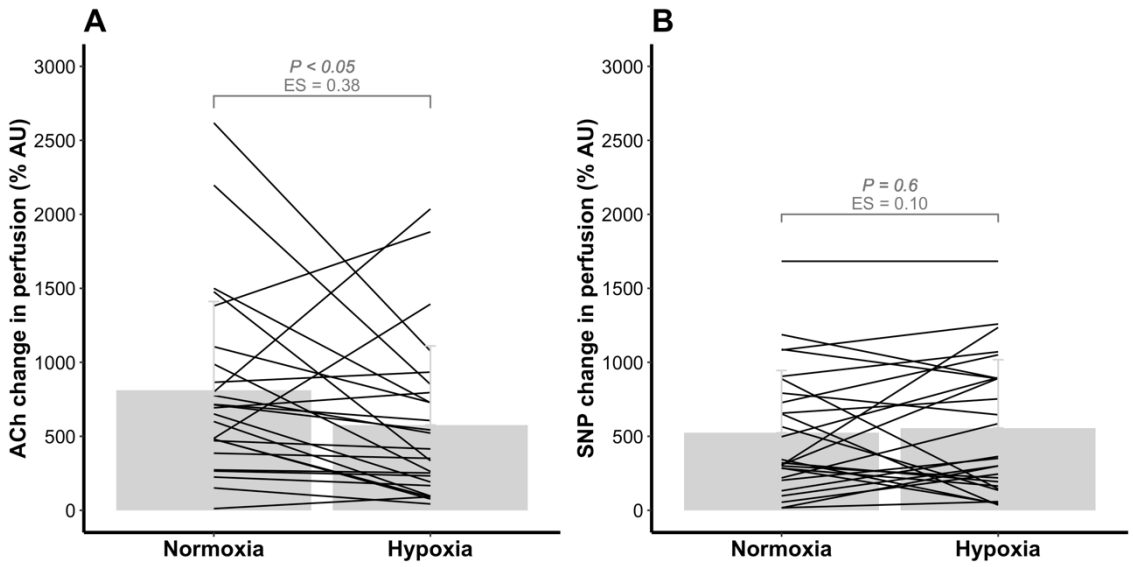

Figure 2. The effect of normoxia and hypoxia on microvascular function.

Date presented as median as well as individual responses (n = 26). (**A**) Microvascular response to acetylcholine (ACh) was significantly impaired during hypoxia. (**B**) Microvascular response to sodium nitroprusside (SNP) remained unchanged. Effects sizes (ES; by Cohen's d) can be interpreted as small (>0.2), medium (>0.5), large (>0.8). ACh, acetylcholine; SNP, sodium nitroprusside.

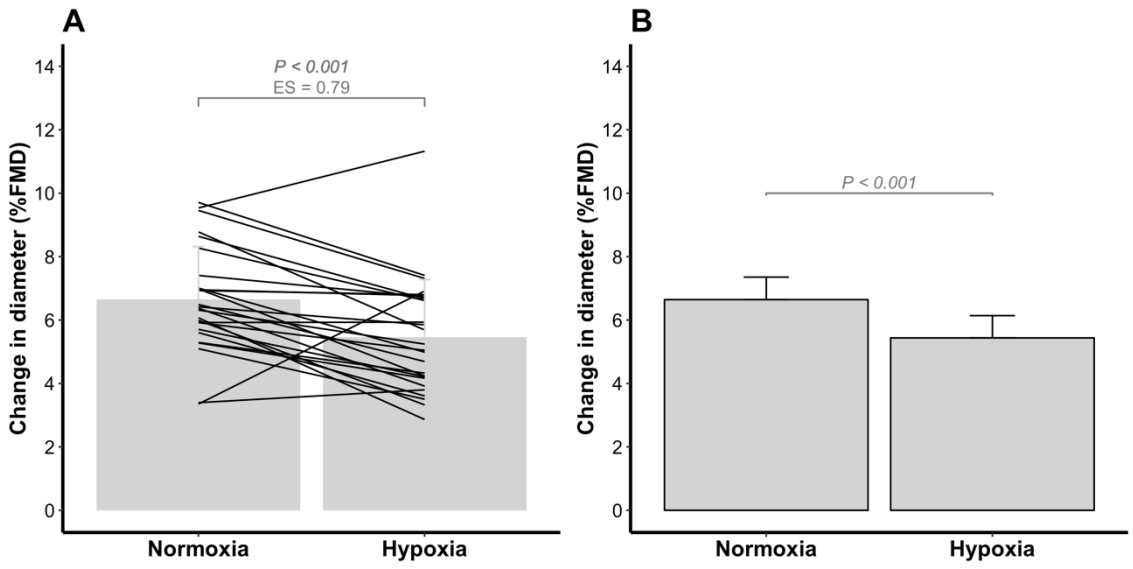

Figure 3. The effect of normoxia and hypoxia on flow-mediated dilatation.

Uncorrected data (**A**; paired t-test; presented as mean as well as individual responses) and allometrically scaled data for differences in baseline diameter (**B**; linear mix model; presented as mean (SD). Flow-mediated dilatation (FMD) response were significantly lower during hypoxia (n = 26). Effects sizes (ES; by Cohen's *d*) can be interpreted as small (>0.2), medium (>0.5), large (>0.8). FMD, flow-mediated dilatation.

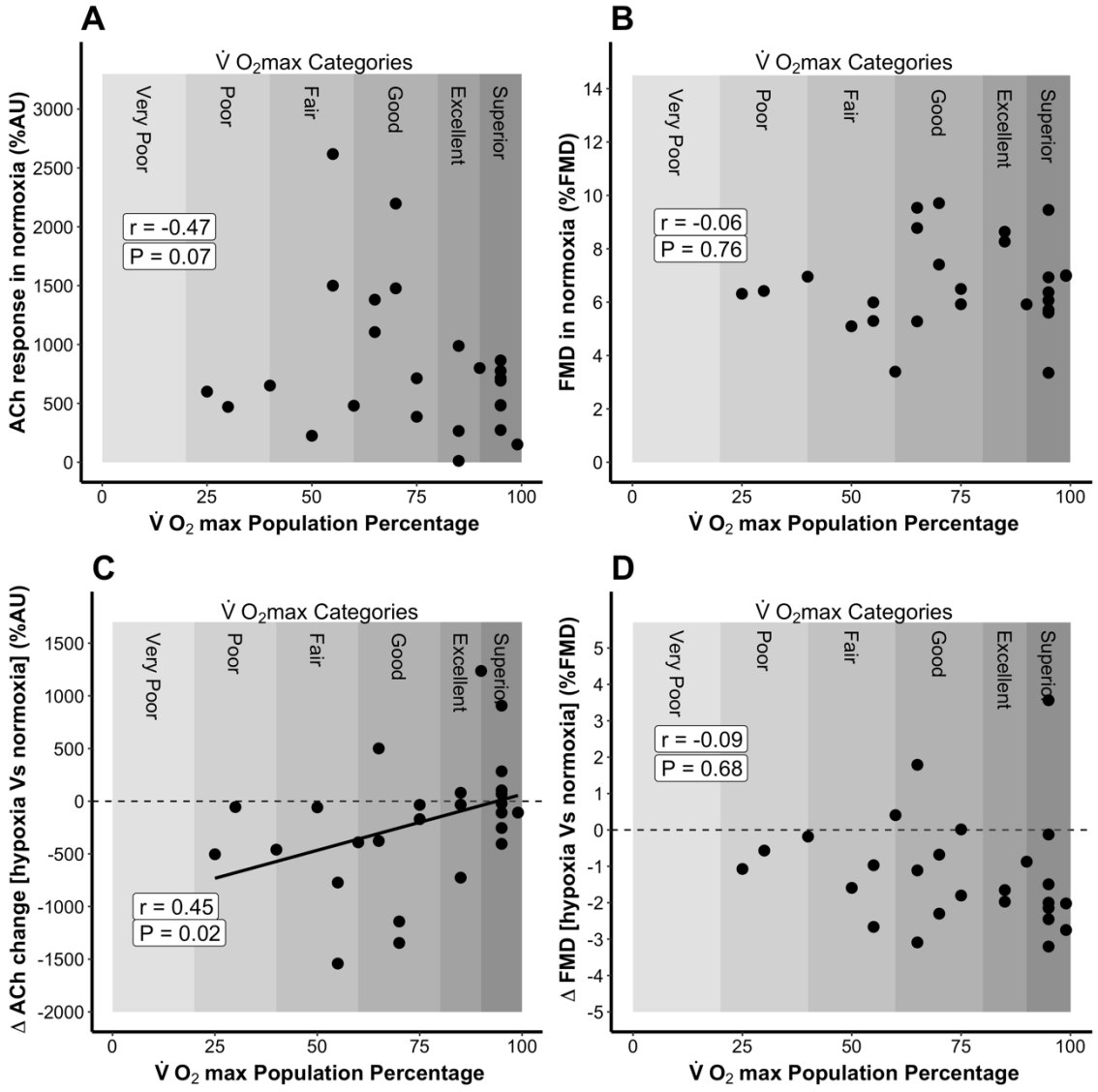

Figure 4. The association between cardiorespiratory fitness and endothelial function. During normoxia, cardiorespiratory fitness was not associated with (A) microvascular endothelial function (r = -0.47; P = 0.07) or (B) large vessel endothelial function (r = 0.06; P = 0.76). Higher cardiorespiratory fitness was associated with the decline between normoxia and hypoxia in (C) microvascular endothelial function (r = 0.45; P = 0.02), but was (D) not with large vessel endothelial function (r = -0.09; P = 0.68). Cardiorespiratory data is presented as the VO_{2max} score as a population percentage, according to the American College of Sports Medicine guidelines (American College of Sports Medicine, 2013). ACh, acetylcholine; FMD, flow-mediated dilatation.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.13525874.v2. Included as citation Rossetti et al. (2021).

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

The experiments were performed in the laboratory of the School of Sport, Health and Exercise Sciences, Bangor University, UK.

DTJ – Acquisition, analysis and interpretation of data for the work; Drafting of the work.

JHM – Conception and design of the work; Interpretation of data for the work; Revising the work critically for intellectual content.

AS – Conception and design of the work; Interpretation of data for the work; Revising the work critically for intellectual content.

SJO – Conception and design of the work; Interpretation of data for the work; Revising the work critically for intellectual content.

GMKR - Conception and design of the work; Acquisition, analysis and interpretation of data for the work; Drafting of the work.

All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the integrity of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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