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

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Edited by: David Edwards

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 tissue; therefore, a comprehensive assessment of endothelial function is crucial to understand the significance of hypoxia-induced endothelial dysfunction. We hypothesized that acute hypoxia would have a deleterious effect on microvascular and large vessel endothelial function. Twenty-nine healthy adults [24 (SD = 4) years of age] completed normoxic and hypoxic [inspired O2 fraction = 0.209] trials in this double-blinded, counterbalanced crossover study. After 30 min, we assessed the laser Doppler imaging-determined perfusion response to iontophoresis of ACh as a measure of endothelium-dependent microvascular function and iontophoresis of sodium nitroprusside as a measure of endothelium-independent microvascular function. After 60 min, we assessed brachial flow-mediated dilatation as a measure of large vessel endothelial function. Thirty minutes of hypoxia reduced endothelium-dependent microvascular function determined by the perfusion response to ACh (median difference (x̄Δ) = −109% [interquartile range: 542.7], P < 0.05), but not endothelium-independent microvascular function determined by the perfusion response to sodium nitroprusside (x̄Δ = 69% [interquartile range: 453.7], P = 0.6). In addition, 60 min of hypoxia reduced allometrically scaled flow-mediated dilatation compared with normoxia (x̄Δ = −1.19 [95% CI = −1.80, −0.58 (Confidence Intervals)]%, 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.

KEYWORDS
cardiorespiratory fitness, endothelium, iontophoresis, nitric oxide, vasodilatation

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1 | INTRODUCTION

Hypoxia can cause disturbances to vascular homeostasis (Tymko et al., 2019) and is believed to be implicated in numerous stages of the development and progression of atherosclerosis, including endothelial dysfunction (Bickler et al., 2017; Gauthier-Veyret et al., 2013; 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; therefore, it is important to understand the deleterious effects of hypoxia on endothelial function.

Nitric oxide (NO) is recognized 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 owing to the prevalence of oxidative stress. Overexpression of hypoxia-induced reactive oxygen species (ROS) is proposed to upregulate the scavenging of NO (Frey et al., 2009; Griendling et al., 2000) and downregulate the expression of endothelial nitric oxide synthase (eNOS) (Janaszak-Jasiecka et al., 2018; Thompson & Dong, 2005). A reduction in the expression of NO can result in an imbalance between endothelium-derived vasoactive factors, contributing to 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 exposure to hypobaric hypoxia (Bailey et al., 2013; Lewis et al., 2014, 2017). However, the authors also reported a decrease in endothelium-independent vasodilatation, suggesting that impaired endothelial function did not fully account for the reduction in vasodilatory capacity. To gain a better understanding of the underlying reason for these vascular impairments, it is also important to examine the microvascular responses to hypoxia, because 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; Liew et al., 2011; Widlansky et al., 2003). Furthermore, the microcirculation makes up a much larger proportion of the 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 ACh on human skin increases microvascular endothelium-dependent vasodilatation (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.

New Findings

- **What is the central question of this study?**
  The aim was 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 but not endothelium-independent microvascular function. The extent of impairment was twofold higher in the microcirculation compared with the large blood vessels. This study suggests that individuals with superior cardiorespiratory fitness might preserve microvascular function in hypoxia. These findings highlight the sensitivity of the microvascular circulation to hypoxia.

Not only is it crucial to identify stimuli that might trigger the development or progression of impaired endothelial function, but is also important to understand how humans might 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 (Beck et al., 2013; d’El-Rei et al., 2016; DeSouza et al., 2000). 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. Given that 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 as follows: (1) to replicate the previous FMD findings reported by Lewis et al. (2017); and to assess and compare the effects of acute hypoxia on (2) endothelium-dependent microvascular function, determined by the perfusion response to iontophoresis of ACh, and (3) endothelium-independent microvascular function, determined by the perfusion response to iontophoresis of sodium nitroprusside.
Baseline characteristics were collected for all participants during visit 1. Participants then completed experimental trials in normoxia and poikilocapnic hypoxia separated by ≥5 days. During each experimental trial, vital signs were assessed every 30 min, LDI at 30 min and FMD at 60 min. Abbreviations: 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).

Furthermore, we aimed to assess the relationship between cardiorespiratory fitness and the changes in endothelial function. We hypothesized that a degree of endothelial impairment was present in both microvasculature and large vessels, but that cardiorespiratory fitness would partly protect against the magnitude of the decline. However, given that the risk of injury is increased for microvascular endothelial cells, we hypothesized that the magnitude of the decrease in function would be greater in the microcirculation.

2 | METHODS

2.1 | Ethical approval

All participants were briefed on the nature and the purpose of the investigation before giving written consent and filling out 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 World Medical Association Declaration of Helsinki (2013), except for registration in a database.

2.2 | Participants

Twenty-nine healthy adults (17 men) were recruited into the study [24 (SD = 4) years of age]. Participants had not travelled to high altitude (≥1,500 m) in the preceding 6 months and had no medical contraindications to maximal exercise testing. Female participants were studied during the early follicular phase of their menstrual cycle or the placebo phase of oral contraceptives.

2.3 | Study design

The study followed a double-blind, repeated-measures, counterbalanced crossover design. Participants completed three separate laboratory visits. 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 ≥5 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., New York, NY, USA). 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 conditions (\( F_{iO_2} \)), because 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 blood pressure (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, and abstained from food and caffeine for 2 h before baseline procedures and overnight before experimental procedures. An overview of the protocol is depicted in Figure 1.

2.4 | Baseline procedures

2.4.1 | 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, 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.
were analysed to obtain cIMT measurements using a semi-automated computerized offline analysis system, Artery Measurement System (AMS) (Wendelhag et al., 1991). All images were acquired and analysed by G.M.K.R. (the between-day reliability of this technique is equal to a coefficient of variation of 4.1%). Increased atherosclerotic risk was defined as having cIMT measurements > 1.0 mm in accordance with Simon et al. (2002).

2.4.2 | Maximal exercise test

To determine cardiorespiratory fitness levels [maximal aerobic capacity (\(\dot{V}_\text{O}_2\max\)), participants completed a running test to exhaustion on a motorized treadmill (H-P-Cosmos; Sports & Medical, Nussdorf-Traunstein, Germany) with simultaneous online gas analysis (Cortex Metalyzer; Biophysik, Leipzig, Germany).

The test protocol was designed such that participants reached a maximum between 10 and 15 min regardless of fitness level, using a similar method to that of da Silva and colleagues (2012). The \(\dot{V}_\text{O}_2\max\) was estimated using the equation from the study by Matthews et al. (1999), and work rates were calculated using the American College of Sports Medicine 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% estimated maximum over 10 min, with the ramp of the slope continuing until exhaustion to obtain peak oxygen uptake. After a 10 min rest, participants completed a validation stage at 110% of the work rate at exhaustion to obtain \(\dot{V}_\text{O}_2\max\). The \(\dot{V}_\text{O}_2\max\) was identified on the following criterion, the validation oxygen uptake had a > 3% negative discrepancy of the modelled 110% peak oxygen uptake (Poole & Jones, 2017). All participants successfully met this criterion. Heart rate and rating of perceived exertion (RPE assessed by the Borg centiMax® Scale (CR100)) (Borg & Borg, 2001) was recorded each minute of the test.

2.5 | Experimental procedures

2.5.1 | Microvascular function: Laser Doppler imaging

Both endothelium-dependent (ACh) and endothelium-independent (SNP) microvascular function was assessed in normoxia and hypoxia after 30 min using LDI (moorLDI2; Moor Instruments, Axminster, Devon, UK) with iontophoresis. All LDI assessments were completed in temperature-controlled conditions (25 ± 2°C) and measured according to previously established methodology (Sandoo & Kitas, 2015). Simultaneous delivery of ACh (Miochol; Bausch & Lomb, Berlin, Germany) and SNP (Rottapharm, Barcelona, Spain) was performed using an iontophoresis controller (MIC2; Moor Instruments, Axminster, Devon, UK) to assess endothelium-dependent and endothelium-independent cutaneous perfusion, respectively. Changes in perfusion in response to the delivery of both vasoactive drugs were assessed on the volar aspect of the participant’s 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 10 scans with an iontophoresis charge of 30 µA to administer 2.5 ml of 1% ACh and 1% SNP. The iontophoresis current was administered continuously throughout the 10 scans. The ACh and SNP were diluted with 0.9% saline and delivered simultaneously into the skin via anode (ACh) and cathode (SNP) internal electrode Perspex chambers (22 mm in diameter; ION 6; Moor Instruments). The scans were performed simultaneously with the iontophoresis protocol. After 10 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) were kept consistent for all scans, and acetate sheets labelled with anatomical markers were used to ensure that the delivery site was consistent across trials. Measurements of perfusion were conducted offline using the MoorLDI Review v.6.1 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 (in arbitrary units (a.u.)) from the baseline scan collected immediately before the drug infusion, calculated as follows: \(\frac{[\text{peak perfusion (in arbitrary units)} - \text{baseline perfusion (in arbitrary units)}]}{\text{baseline perfusion (in arbitrary units)}} \times 100 = \text{change in perfusion (arbitrary units).}\)

2.5.2 | Large vessel endothelial function:
Flow-mediated dilatation

Large vessel endothelial function was assessed using FMD in 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 a suprasystolic pressure (220 mmHg). After 5 min, the cuff was deflated rapidly to induce reactive hyperaemia. To capture maximal dilatation, a 3 min scan was performed after cuff deflation.

A Siemens Acuson X300 ultrasound scanner was used with a multifrequency linear-array vascular probe set at 7.3 MHz to perform the FMD procedure. B-Mode images were captured at 15 frames/s to record a 120 s baseline and a 210 s clip after 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 automated edge-detection software (Brachial Analyser; Medical Imaging Applications, USA). The Brachial Analyser software is capable of detecting 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 that were accepted, the change in diameter from baseline to the peak was calculated as follows: [(peak diameter (in centimetres) – baseline diameter (in centimetres)) ÷ baseline diameter (in centimetres)] × 100 = Δ%FMD. To account for the differences in baseline diameter, all the data were allometrically scaled according to the guidelines of Atkinson & Batterham (2013). The coefficient of variation for the sonographer (D.T.J.) is 8.5%, as previously reported (Jones et al., 2019).

2.6 | 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), Student’s paired t-tests were applied on normally distributed data, and the Wilcoxon signed rank test was used for non-parametric data. Values of P < 0.05 were considered to indicate statistical significance. Also, effect sizes for Student’s 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 two-tailed design, based on a minimum important difference of 3.1%, an SD 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 (x̄Δ) with 95% confidence intervals [95% CI]. The results of non-parametric analysis are presented as the median differences (kdΔ) and interquartile range (IQR). Owing to poor image quality, the scans of three participants were removed from the FMD analysis, and the scans of three different participants were removed from the microvascular analysis. The removal of these data was performed before statistical analysis.

The effect of hypoxia on FMD was determined by Student’s 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 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA), 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 (V̇O₂ max), Pearson’s correlations were used for parametric data and Spearman’s correlations for non-parametric data. For all correlational analyses, the strength of a relationship was determined by the correlation coefficient value, and values of P < 0.05 were considered to indicate statistical significance.

3 | RESULTS

3.1 | Vascular demographic: Carotid intima–media thickness

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

Table 1: Participant characteristics

| Characteristic          | 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)a| 0       | 7       | 6     | 2   |
| VO2 max (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|

Abbreviations: CCA, common carotid artery; HDL, high-density lipoproteins; IMT, intima–media thickness; LDL, low-density lipoproteins; MAP, mean arterial blood pressure; VO2 max, maximal aerobic capacity.

aPhysical activity was measured using an instrument commonly used in VO2 max prediction models (Jackson et al., 1990; Matthews et al., 1999).

3.2 | Physiological responses to 30 and 60 min hypoxia

Resting physiological responses were recorded at 30 and 60 min during the trial. Hypoxia decreased peripheral oxygen saturation (SpO2) compared with normoxia after 30 min (kdΔ = −19 [−20, −17%]) and 60 min (kdΔ = −18 [−20, −15%]; P < 0.001) exposure. Hypoxia significantly increased heart rate compared with normoxia after 30 min exposure (kdΔ = 12 [8, 16] beats/min; P < 0.001), and it remained elevated after 60 min (kdΔ = 11 [6, 16] beats/min; P < 0.001). Hypoxia increased mean arterial blood pressure compared with normoxia after 30 min (kdΔ = 4 [1, 7] mmHg; P = 0.02) but had no effect on mean arterial blood pressure after 60 min (kdΔ = 0 [−4, 4] mmHg; P = 1.0).
TABLE 2 Physiological data at 30 min before laser Doppler imaging and assessment of flow-mediated dilatation

| Parameter          | Normoxia |          |          | Hypoxia |          |          | P-value |
|--------------------|----------|----------|----------|---------|----------|----------|---------|
|                    | 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***|
| \text{SpO}_2 (%)   | 98       | 1        | 43       | 79      | 5        | –        | <0.001***|
| Baseline flux (a.u.) | ACh chamber | – | – | 43 | – | – | 49 | 0.13 |
|                    | SNP chamber | – | – | 38 | – | – | 43 | 0.80 |

Abbreviations: DBP, diastolic blood pressure; MAP, mean arterial blood pressure; SBP, systolic blood pressure; SNP, sodium nitroprusside; \text{SpO}_2, peripheral oxygen saturation.

\*P < 0.05; ***P < 0.001.

FIGURE 2 The effects of normoxia and hypoxia on microvascular function. Data are presented as the median and as individual responses (n = 26). (a) The microvascular response to ACh was significantly impaired during hypoxia. (b) The microvascular response to sodium nitroprusside (SNP) remained unchanged. Effect sizes (ES; by Cohen’s d) can be interpreted as small (> 0.2), medium (> 0.5) and large (> 0.8).

3.3 Effect of hypoxia on microvascular function

In comparison to normoxia, hypoxia did not affect baseline perfusion after 30 min in either chamber (ACh chamber: kΔ = 0.3, [IQR: 14.0], P = 0.13; SNP chamber: kΔ = 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. In comparison to normoxia, endothelium-dependent (ACh) microvascular function was reduced after 30 min of exposure for 19 of 26 (73%) participants (kΔ = −109%, [IQR: 542.7]; P = 0.05; Figure 2). In comparison to normoxia, hypoxia did not affect endothelium-independent (SNP) microvascular function after 30 min of exposure, and 11 of 26 (42%) participants had lower responses during the hypoxic trial (kΔ = 69%, [IQR: 453.7]; P = 0.6).

3.4 Effect of hypoxia on FMD

In comparison to normoxia, hypoxia significantly increased baseline brachial diameter by 2.9% after 60 min (kΔ = 0.11 [0.03, 0.19] mm; P = 0.01). Given that the baseline diameters were different between conditions, FMD results are presented as unscaled and allometrically scaled responses (Figure 3). In comparison to normoxia, hypoxia significantly reduced unscaled FMD responses in 22 of 26 (85%) participants after 60 min (kΔ = −1.19 [−1.80, −0.58%]; P < 0.001). In comparison to normoxia, hypoxia significantly reduced allometrically scaled FMD responses in 22 of 26 (85%) participants after 60 min (kΔ = −1.21%; P < 0.001; relative −18.2%). In comparison to normoxia, hypoxia had no effect on FMD time to peak (kΔ = −5.0 [−36.7, 26.8] s; P = 0.75).

3.5 The association between cardiorespiratory fitness and endothelial function

Cardiorespiratory fitness was not associated with endothelium-dependent (ACh) microvascular function (percentage change in perfusion) (r = −0.47; P = 0.07; Figure 4a), endothelium-independent (SNP) microvascular function (percentage 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 3** The effects of normoxia and hypoxia on flow-mediated dilatation (FMD). (a) Uncorrected data are presented as the mean and as individual responses, with *P*-values by Student’s paired *t*-test. (b) Allometrically scaled data for differences in baseline diameter, presented as the mean (SD); linear mixed model. The FMD responses were significantly lower during hypoxia (*n* = 26). Effect sizes (ES; by Cohen's *d*) can be interpreted as small (> 0.2), medium (> 0.5) and large (> 0.8).

**FIGURE 4** The association between cardiorespiratory fitness and endothelial function. (a,b) During normoxia, cardiorespiratory fitness was not associated with microvascular endothelial function (*r* = −0.47; *P* = 0.07; a) or large vessel endothelial function (*r* = 0.06; *P* = 0.76; b). (c,d) Higher cardiorespiratory fitness was associated with the decline between normoxia and hypoxia in microvascular endothelial function (*r* = 0.45; *P* = 0.02; c), but not with large vessel endothelial function (*r* = −0.09; *P* = 0.68; d). Cardiorespiratory data are presented as the maximal aerobic capacity (VO₂ max) score as a population percentage, according to the American College of Sports Medicine guidelines (American College of Sports Medicine, 2013). Abbreviation: FMD, flow-mediated dilatation.
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 \((r = -0.09; P = 0.68; \text{Figure 4d})\).

4 | 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 of hypoxia reduced endothelium-dependent large vessel vasodilatation (18% reduction in FMD). Notably, the extent of the decrease was approximately twofold higher in the microcirculation compared with the large vessels. Additionally, we are the first to demonstrate that 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 might activate specific mechanisms, which effect endothelial function differently. Assessed separately, microvascular and large vessel function have been reported to decrease after acute hypoxia (Lewis et al., 2014, 2017; Treml et al., 2018). However, some studies have also reported increased vascular reactivity after 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 the observed individual differences by scaling our data correctly (Atkinson et al., 2013) and performing appropriate analyses.

Using isocapnic hypoxia, Lewis et al. (2017) concluded that reductions in FMD induced by normobaric hypoxia were more pronounced after 30 min of severe hypoxia [end-tidal partial pressure of oxygen \((P_{ET,\text{O}_2})\) of 50 mmHg] compared with mild hypoxia \((P_{ET,\text{O}_2}\) of 75 mmHg). This finding suggests that the severity of hypoxemia is associated with impaired endothelial function. However, the small range of \(S_{\text{O}_2}\) values that were recorded during hypoxia in the present study \((\text{range} \sim 70–86\%, \text{SD} = 5\%)\) suggests that the hypoxic stimulus was relatively homogeneous across participants, with most participants at a similar \(P_{ET,\text{O}_2}\) of \(-42 \text{ mmHg}\). Thus, the minimal range makes it difficult to evaluate the relationship between the severity of hypoxaemia 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 might 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 the hypoxia-induced decrease in endothelial function is linked to NO deficiency (Bonetti et al., 2003; Ten & Pinsky, 2002). The synthesis of NO is an oxygen-dependent reaction; hence, 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, blocking the synthesis of NO (Janaszak-Jasiecka et al., 2018; Thompson & Dong, 2005). However, Prieto et al. (2011) suggested that acute hypoxic exposure (<24 h) does not decrease eNOS protein expression but that the capacity of eNOS to produce NO is affected. Oxidation of L-arginine via eNOS is the primary source of NO in endothelial cells, but other enzymes, including arginase 1 and arginase 2, also compete for the same substrate. Krotova et al. (2010) reported that the activation of hypoxia inducible factor 1 (HIF1) elevates the expression and activity of arginase 2 in the human lung microvasculature, thus limiting the bioavailability of NO. To our knowledge, this finding has not been replicated in large blood vessels. Thus, the upregulation of arginase 2 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 HIF1 and other transcriptional complexes, which prompts metabolic changes within endothelial cells of small and large blood vessels. The changes in endothelial metabolism have been associated with NADH oxidase-dependent increases in ROS, primarly superoxide (Frey et al., 2009; Griending et al., 2000). When an ample amount of superoxide is synthesized, it reacts rapidly with NO to produce peroxynitrite and thereby prevents the vasodilatory effect of NO on vascular smooth muscle cells (Gryglewski et al., 1986). In addition to the changes in endothelial metabolism, the interaction between HIF1 and endothelial cells evokes pro-inflammatory reactions (Michiels et al., 2000). The prevalence of adhesion molecules is proposed to be higher in microvascular endothelial cells compared with 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 vasodilatation (Weisbrot et al., 2001). Sympathetic excitation not only stimulates vasoconstriction, but also increases retrograde shear rate, thus limiting the 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, thereby impairing the endothelial NO vasodilatory system directly. 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 improve endothelial function significantly (Beck et al., 2013; DeSouza et al., 2000) and to 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, although cardiorespiratory fitness was not associated with microvascular or large vessel endothelial function, the hypoxia-induced decrease in microvascular conductance 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 might provide some protection against the 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.

4.1 | Limitations

The LDI technique used in this study does not allow for continuous measurement, limiting the temporal resolution of the response of the microvasculature to ACh and SNP. However, the technique does provide data from a larger area in comparison 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; therefore, we cannot be sure that differences in flux are attributable to changes in vasomotor function rather than changes in perfusion pressure (Roustit & Cracowski, 2013). Finally, the current applied during iontophoresis can elicit a vasodilatory response independent of a drug response. We did not estimate the contribution of effects of the current, for example by conducting a separate LDI procedure with administration of only the vehicle (saline) using the same current and duration. However, although it is not possible to differentiate between current- and drug-induced vasodilation, this has minimal consequence for our primary finding, because 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 normalized for variation in the shear rate (Pyke & Tschakovsky, 2005, 2007). For the present study, shear rate was not recorded. However, Atkinson et al. (2013) suggested that normalizing one variable (i.e., baseline diameter) by another variable (i.e., shear rate) is not good practice when analysing FMD data. Furthermore, Atkinson et al. (2013) implied that scaling FMD to baseline diameter differences should outweigh the variation in shear rate.

4.2 | Conclusion

To conclude, acute exposure to normobaric hypoxia reduced endothelium-dependent vascular function in both small and large vessels. The decline in microvascular endothelial function was approximately twice as large as that observed in the large blood vessels, demonstrating the sensitivity of the microvascular endothelium to hypoxia. Furthermore, our data suggest that superior cardiorespiratory fitness might 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.

ACKNOWLEDGEMENTS

We would like to thank Kevin Williams and Dr Jason Edwards for their technical assistance in the completion of this work. We would also like to thank Dr Tim Van Reissen, Hannah Davies, Morgan Gregory, Dr Kate Harding, Dr Holly Burton, Matthew Rogan, Joseph Smith, Katy Pearce, Natasha Farmer, Joshua Dautzenberg, Samuel Wynne and Harrison Simms for their help with data collection.

AUTHOR CONTRIBUTIONS

The experiments were performed in the laboratory of the School of Sport, Health and Exercise Sciences, Bangor University, UK. Conception and design: J.H.M., A.S., S.J.O. and G.M.K.R. Acquisition, analysis and interpretation of data: D.T.J. and G.M.K.R. Interpretation of data: J.H.M., A.S. and S.J.O. Drafting of the manuscript: D.T.J. and G.M.K.R. Revising the manuscript critically for intellectual content: J.H.M., A.S. and S.J.O. 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.

COMPETING INTERESTS

None declared.
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