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Title: Acute hypoxemia and vascular function in healthy humans

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Running Title: Acute hypoxic exposure and the NO-vasodilator system

Abstract: Vascular function is impaired at high altitude and following one hour of comparably severe normobaric hypoxia (~FIO_2=0.11). Whether vascular function is impaired during milder hypoxia is unknown. We examined the hypothesis that vascular function would be impaired following acute exposure to mild (74{plus minus}2 mmHg $P_{ET}O$**l**) and moderate (50{plus minus}3 mmHg $P_{ET}O$**l**) normobaric hypoxia. Brachial endothelium-dependent flow mediated dilation (FMD) was assessed at baseline and following 30-minutes of hypoxia (n=12) or normoxia (time control trial; n=10). Endothelium-independent dilation (via glyceryl trinitrate; GTN) was assessed following the hypoxic FMD test, and in normoxia on a separate control day (n=8). Compared to normoxic baseline, allometrically correcting for baseline diameter and FMD shear rate under the curve, FMD and GTN-induced dilation were reduced following mild hypoxia (FMD: 6.4{plus minus}1.0 vs. 5.9{plus minus}1.0%; GTN: 16.4{plus minus}4.0 vs. 14.3{plus minus}4.0%; P{less than or equal to}0.02) and moderate hypoxia (FMD: 6.6{plus minus}1.0 vs. 4.5{plus minus}1.0%; GTN: 16.4{plus minus}4.0 vs. 12.9{plus minus}4.0%; {less than or equal to}0.02). The

normoxic time-control data, however, revealed a ~8% decline in FMD (comparable with the FMD decline during mild hypoxia), indicating that 30 minutes of recovery for repeated FMD assessments is insufficient. Considering the methodological effects of repetitive FMD testing, endothelial dilation is unaltered following mild hypoxia exposure, yet it is significantly impaired during more moderate hypoxia. Graded impairments in smooth muscle function is evident following mild and moderate hypoxia, and this has implications for individuals acutely exposed to hypoxia.

New Findings: Endothelial dilation is impaired following an acute moderate hypoxia stimulus; therefore, the central question of this study is to investigate whether this impairment in endothelial dilation is evident following a mild hypoxic exposure, and if smooth muscle dilation is impaired following acute hypoxic exposure. Vascular smooth muscle cells sensitivity to a NO is impaired following mild and moderate hypoxia equivalent to ~2000m and ~5000m respectively. Unlike following moderate hypoxia exposure, it appears endothelial dysfunction is not impaired following mild hypoxia. These findings have important implications for individuals with pre-exiting medical conditions, especially those who are rapidly exposed to hypoxia.

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Acute hypoxemia and vascular function in healthy humans

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New Findings (100 words)

What is the central question of this study?

Endothelial dilation is impaired following an acute moderate hypoxia stimulus; therefore, the central question of this study is to investigate whether this impairment in endothelial dilation is evident following a mild hypoxic exposure, and if smooth muscle dilation is impaired following acute hypoxic exposure.

What is the main findings and its importance?

Vascular smooth muscle cells sensitivity to a NO is impaired following mild and moderate hypoxia equivalent to ~2000m and ~5000m respectively. Unlike following moderate hypoxia exposure, it appears endothelial dysfunction is not impaired following mild hypoxia. These findings have important implications for individuals with pre-exiting medical conditions, especially those who are rapidly exposed to hypoxia.

Abstract (250)

Vascular function is impaired at high altitude and following one hour of comparably severe normobaric hypoxia (~F_IO_2=0.11). Whether vascular function is impaired during milder hypoxia is unknown. We examined the hypothesis that vascular function would be impaired following acute exposure to mild (74±2 mmHg $P_{ET}O_2$) and moderate (50±3 mmHg $P_{ET}O_2$) normobaric hypoxia. Brachial endothelium-dependent flow mediated dilation (FMD) was assessed at baseline and following 30-minutes of hypoxia (*n*=12) or normoxia (time control trial; *n*=10). Endothelium-independent dilation (via glyceryl trinitrate; GTN) was assessed following the hypoxic FMD test, and in normoxia on a separate control day (*n*=8). Compared to normoxic baseline, allometrically correcting for baseline diameter and FMD shear rate under the curve, FMD and GTN-induced dilation were reduced following mild hypoxia (FMD: 6.4±1.0 vs. 5.9±1.0%; GTN: 16.4±4.0 vs. 14.3±4.0%; P≤0.02) and moderate hypoxia (FMD: 6.6±1.0 vs. 4.5±1.0%; GTN: 16.4±4.0 vs. 12.9±4.0%; ≤0.02). The normoxic time-control data, however, revealed a ~8% decline in FMD (comparable with the FMD decline during mild hypoxia), indicating that 30 minutes of recovery for repeated FMD assessments is insufficient. Considering the methodological effects of repetitive FMD testing, endothelial dilation is unaltered following mild hypoxia exposure, yet it is significantly impaired during more moderate hypoxia. Graded impairments in smooth muscle function is evident following mild and moderate hypoxia, and this has implications for individuals acutely exposed to hypoxia.

Introduction

The nitric oxide (NO)-vasodilator system is important in the maintenance of vasoregulation and vascular health and its function is a marker of cardiovascular risk. Endothelium-dependent flow mediated dilation (FMD) assesses conduit artery vasodilatory capacity following a reactive hyperemia stimulus (shear stress). The latter component of the NO-dilator cascade endothelium-independent NO-mediated smooth muscle relaxation can be assessed by administering glyceryl trinitrate (GTN)(Corretti *et al.*, 2002). Therefore, the assessment of both FMD and GTN measures within subjects provides complimentary information regarding the locus of change in vascular function *in vivo* (Celermajer *et al.*, 1992).

The effect of acute normobaric or hypobaric hypoxia on basal FMD and GTN-induced dilation is unclear. Some studies have reported a significant decline following hypoxia (Bailey *et al.*, 2013; Lewis *et al.*, 2014), others an absence of change (Frick *et al.*, 2006; Frobert *et al.*, 2008; Bailey *et al.*, 2013). These discrepancies are perhaps not surprising, since studies are often confounded by 1) pathology such as metabolic syndrome (Frick *et al.*, 2006), chronic mountain sickness (Bailey *et al.*, 2013) and cardiovascular disease (Frobert *et al.*, 2008); or 2) methodological limitations e.g., different definition of acute hypoxic exposure (5 minutes vs. 1 hour vs. 3 days), different acute hypoxic stimuli (hypobaric hypoxia vs. normobaric hypoxia) (Frobert *et al.*, 2008; Lewis *et al.*, 2014), different population groups (native highlanders vs. lowlanders; (Bailey *et al.*, 2013), and/or inappropriate FMD and GTN data collection and analysis protocols [cuff placement, period of data collection, non-use of edge detection software; (Frobert *et al.*, 2008)].

By employing international guidelines for the assessment of FMD and endothelium-independent NO-mediated smooth muscle relaxation (Thijssen *et al.*, 2011), we recently documented a 14% decline in both FMD and GTN-induced dilation following three days of hypobaric hypoxia (5050m) in healthy individuals (Lewis *et al.*, 2014). These findings suggest that endothelial and vascular smooth muscle dysfunction both contribute to a decline in the NO-vasodilator system with hypobaric hypoxia. In a follow-up study, we discovered that a substantial larger and sustained decline in FMD (~28%) occurs as a result of 60-minutes of exposure to normobaric hypoxia (FIO_2=0.11; a hypoxic level stimulating ~5000m). These marked reductions in FMD were abolished following sympathetic nerve activity (SNA) blockade (Lewis *et al.*, 2014). However, it is currently unclear whether GTN-induced dilation is impaired to the same degree within 60-minutes following normobaric hypoxia. Furthermore, it is unknown whether the impairment in FMD and potential decline in GTN-induced dilation following acute (<60-minutes) normobaric hypoxia is sensitive to distinct levels of hypoxia.

The primary purpose of this study was to examine the effect of acute (<60 minutes) exposure to mild (end-tidal oxygen $P_{ET}O_2$ = 75 mm Hg; ~2000m) and moderate (end-tidal $P_{ET}O_2$ = 50 mm Hg; ~4600m) isocapnic hypoxia on brachial FMD and GTN-induced dilation. We hypothesized that FMD and GTN-induced dilation would be impaired following mild hypoxia and more so following moderate hypoxia. We intentionally chose this mild exposure as a comparable PO_2 to that encountered during commercial air travel (Smith *et al.*, 2012), during trekking, and ski vacation sites in North America. To ensure that there were no repetitive influences of the FMD testing, we conducted a normoxic time-control study to quantify the effect of 30 minutes of supine normoxic rest on FMD. Based on previously published guidelines (Corretti *et al.*, 2002;

Barton *et al.*, 2011), we reasoned that FMD and related hemodynamic variables would be unaltered following 30 minutes of normoxic supine rest.

Materials and Methods

Participants: Twelve healthy normotensive volunteers (7 men, 5 women; mean ± SD: age, 26 ± 6 years; body mass, 71 ± 12 kg; height, 176 ± 8 cm; body mass index, 23± 3 kg/m^2) participated in this randomized counter-balanced experiment. The study was approved by the Human Ethics Committee of the University of British Columbia and conformed to the standards set by the Declaration of Helsinki. All volunteers provided written informed consent. Participants were non-smokers, had no previous history of cardiovascular, cerebrovascular, or respiratory diseases, and were not taking any medications, other than the contraceptive pill. Females were tested during the either the pill withdrawal/placebo phase, or in the earlier follicular phase of the menstrual cycle of consecutive cycles. All experimental testing took place at the University of British Columbia (altitude 344 m).

Study design: Participants attended the laboratory on four occasions (one familiarisation session and three experimental session). The experimental sessions were separated by >7 day and each session commenced between 8:00-9:00 A.M. Experimental testing followed a minimum of 12 h abstinence from alcohol, caffeine, and strenuous exercise, and an overnight fast. Experimental session one and two consisted of 20 minutes of supine rest following which, cardiorespiratory measures were monitored for 5 minutes and the assessment of FMD was undertaken under normoxic conditions. Participants were then rapidly exposed to isocapnic hypoxia. Following 30 minutes of isocapnic hypoxia exposure cardiorespiratory measures and the assessment of FMD

were repeated and following 60 minutes of isocapnic hypoxia GTN-induced dilation assessed. The level of isocapnic hypoxia experience in each session was randomized and counter-balanced. Using end-tidal forcing, in the separate visits, the participant's end-tidal oxygen ($P_{ET}O_2$) was rapidly reduced down to 75 mm Hg (mild-hypoxia) or 50 mm Hg (moderate-hypoxia) following baseline assessments. End-tidal carbon dioxide ($P_{ET}CO_2$) was clamped as baseline levels.

During experimental session three, following 20 minutes of supine rest the assessment of GTN-induced dilation was made in normoxic conditions, in eight of the twelve participants who completed experimental sessions one and two. The GTN-dilation assessment was made on a separate day from the two hypoxic tests due to the half-life of GTN being approximately four hours and the potential interference with other measures if conducted at normoxia in experimental sessions one or two.

Experimental Measures and Data Analysis

Brachial artery vascular function: A 10 MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (Terason 3000, Teratech) was used to image the brachial artery in the right arm. Blood flow velocity was measured as peak blood flow velocity of the Doppler shift, with the sample gate begin placed in the centre of the lumen.

Endothelium-dependent FMD. FMD was assessed according to international guidelines (Thijssen *et al.*, 2011). With the occluding cuff placed distal to the ultrasound probe, 1 minute of brachial diameter and blood flow velocity recordings preceded forearm cuff inflation to 220

mmHg for 5 minutes. Brachial diameter and blood flow velocity recordings resumed 30 s prior to cuff deflation and continued for 3 minutes thereafter.

Endothelium-independent FMD (GTN). Following 20 minutes of rest, brachial diameter and blood flow velocity recordings were made for 1 minute prior to participants receiving a sublingual dose of glyceryl trinitrate (GTN; 400 μg spray). Brachial diameter and blood flow velocity recordings were taken continuously for a 10-minute period thereafter.

Custom-designed edge-detection and wall-tracking software, which is largely independent of investigator bias, was utilised for the analysis of FMD and GTN (Woodman *et al.*, 2001; Black *et al.*, 2008; Thijssen *et al.*, 2011). This software provides continuous and simultaneous diameter, blood flow velocity at 30Hz. From this synchronized diameter and velocity data, blood flow (the product of lumen cross-sectional area and Doppler velocity and shear rate (SR [4 times velocity divided by diameter]) (Pyke *et al.*, 2004; Pyke & Tschakovsky, 2007) are calculated at 30 Hz. This semi-automated software provides higher reproducibility of diameter measurements and reduces both observer error and bias with a reported intra-observer CV for FMD% of 6.7% (Woodman *et al.*, 2001). Baseline diameter, blood flow, and SR patterns were calculated as the mean of data acquired across the minute preceding the cuff inflation period. Peak diameter after cuff deflation was automatically detected according to an algorithm that identified the maximum bracket of data, and FMD% was calculated as the percentage rise of this peak diameter from the preceding baseline diameter. The time to peak diameter (in seconds) was calculated from the point of cuff deflation to the maximum post-deflation diameter and SR area under curve (SR_{AUC}) was calculated for the FMD stimulus up to peak diameter (Black *et al.*, 2008). Recent evidence has highlighted that FMD% can under some circumstances fail to consider the difference in

baseline artery diameter following an intervention or between groups (Atkinson & Batterham, 2013; Atkinson *et al.*, 2013). Therefore, as outlined in detail (Atkinson & Batterham, 2013; Atkinson *et al.*, 2013), we adopted an allometric scaling approach to adjust for baseline diameter in the calculation of FMD and GTN-induced dilation. Also, where necessary we also adjusted the FMD and GTN dilation for changes in SR_{AUC}. These results are presented as 'allometrically corrected' FMD%. Oscillatory shear index, an indicator of the magnitude of shear oscillation, was defined as: (|retrograde SR|) / (|antegrade SR| + |retrograde SR|). We also calculated FMD/GTN ratio, to correct the FMD for potential differences in GTN-induced dilation.

Cardiorespiratory Measures: Beat-to-beat blood pressure (BP) was measured by finger photoplethysmography (Finometer PRO, Finapress Medical Systems, Amsterdam, Netherlands) and normalized to manual cuff measurements of the brachial artery. Stroke volume (SV) and cardiac output (CO) were calculated from the BP waveform obtained from the finger photoplethysmography using the Modelflow method, incorporating age, sex, height, and weight (BeatScope 1.0 software; TNO TPD; Biomedical Instruments). Heart rate was measured (HR) via three-lead electrocardiogram (ML132; ADInstruments, Colorado Springs CO). All measures were monitored for 5 minutes prior to FMD assessment, where minute 4 to 5 was used as a representation of baseline values. Peripheral oxygen saturation (Sp_{O2}; Pulse Oximeter MD300K1; Vacumed, Ventura, CA) was measured immediately prior to the FMD assessment.

For measurement of $P_{ET}CO_2$ and $P_{ET}O_2$, subjects breathed through a mouthpiece connected to a two-way non-rebreathing valve. Respired gas pressures were sampled at the mouth by securing a sample line connected to a calibrated online gas analyzer (model ML206, AD Instruments,

Colorado Springs, CO) into the mouthpiece. Respiratory flow was measured at the mouth using a pneumotachograph (model HR 800L, HansRudolph, Shawnee, KS). $P_{ET}CO_2$, $P_{ET}O_2$ and inspiratory and expiratory tidal volume were determined for each breath online using specifically designed software (LabView, Austin, TX). $P_{ET}CO_2$ and $P_{ET}O_2$ were controlled via end-tidal forcing system (Tymko *et al.*, 2015). This system uses independent gas solenoid valves for oxygen, carbon dioxide and nitrogen and controls the volume of each gas delivered to the inspiratory reservoir through a mixing-and-humidification chamber. With use of feedback information regarding $P_{ET}CO_2$, $P_{ET}O_2$, and inspiratory and expiratory tidal volume, the system prospectively targets the inspirate to bring end-tidal gas to the desired level. Gas control was fine-tuned using a feedback control and error reduction algorithm. Clamped $P_{ET}CO_2$ levels were determined as the values measured during the last 5-minutes of normoxic measurements.

Normoxic Time-Control Study

Participants: Ten healthy normotensive volunteers, (9 men, 1 women; mean ± SD: age, 27 ± 2 years; body mass, 77 ± 8 kg; height, 180 ± 1 cm; body mass index, 23± 2 kg/m^2) participated in this study. All participant pre-experimental considerations were the same as described for the hypoxia studies.

Experimental Design and Methods: All participants were familiarised with the FMD protocol, and attended the laboratory for one experimental session. Here, FMD was assessed in normoxic conditions prior to (FMD one) and following 30 minutes of supine rest (FMD two). All methodological and data analysis procedures were performed as outlined for the hypoxia studies.

Statistical Analysis: All data were analysed using SPSS (version 21, IBM, Surrey, UK) and expressed as mean±SD. Statistical significance was defined as $P \leq 0.05$ and distribution normality confirmed using repeated Shapiro–Wilk *W* tests.

Study 1: To examine the interaction between the experimental intervention (normoxia vs. hypoxic stimuli) and the experimental condition ($P_{ET}O_2$ 75 mmHg (mild-hypoxia) trial vs. $P_{ET}O_2$ 50 mmHg (moderate-hypoxia)) a two-way repeated measures ANOVA was used; to further exposure any significant interaction effect, two-tailed paired *t* tests were used to quantify the effect of the hypoxic stimuli on the measures of interest. For the assessment of GTN-induced dilation and related variables, a one-way repeated measures ANOVA was used to compare the trial difference between normoxia and the two hypoxic stimuli. Pearson's correlation analysis was used to examine the relationship between selected measures. *Normoxic time-control study* To examine the interaction between normoxic baseline and following 30 minutes of supine rest a two-tailed paired *t* tests were used, unless stated otherwise. *Hypoxia and Normoxic time-control studies*: A linear mixed model for repeated measures was used to allometrically correct FMD and GTN-dilation for baseline diameter and SR_{AUC} as covariates.

Results

Effect of hypoxia on cardiorespiratory variables

Per the study design, a significant interaction between the experimental intervention (normoxia vs. hypoxia) and experimental condition ($P_{ET}O_2$ 75 mmHg trial vs. $P_{ET}O_2$ 50 mmHg trial) was evident for $P_{ET}O_2$ ($P<0.01$). No difference in baseline (normoxia) $P_{ET}O_2$ was evident between experimental conditions; however, as desired, $P_{ET}O_2$ was lower following hypoxia exposure in the $P_{ET}O_2$ 50 mmHg (50 ± 3 mm Hg) versus the $P_{ET}O_2$ 75 mmHg trial (74 ± 2 mm Hg; $P<0.01$;

Table 1). There were no differences in $P_{ET}CO_2$ during the experimental intervention or condition (Table 1).

A significant interaction between the experimental intervention and experimental condition was evident for ventilation ($P<0.01$; Table 1). Compared to normoxia, ventilation was increased following exposure to $P_{ET}O_2$ 75 mmHg (+3.1 ± 3.5 %; $P=0.01$) and $P_{ET}O_2$ 50 mmHg (+10.0 ± 5.8 %; $P<0.01$); the increase in ventilation was greater following exposure to $P_{ET}O_2$ 50 mmHg ($P<0.01$). Likewise, a significant interaction between the experimental intervention and experimental condition was evident for SaO_2 ($P=0.001$; Table 1). Compared to normoxia, the reductions in SaO_2 were greater at $P_{ET}O_2$ 50 mm Hg compared with 75 mmHg (-15 ± 3 % vs -5 ± 2 %; $P<0.01$).

A main effect for the experimental intervention was evident for mean arterial blood pressure (MAP) independent of hypoxic stimulus; compared to normoxia, MAP increased in both hypoxic trials by 6 ± 2 mmHg ($P=0.001$, Table 1), respectively. An interaction between experimental intervention and experimental condition was evident for HR ($P=0.01$) and CO ($P=0.03$; Table 1). The increase in HR was greater (6 ± 4 beats·min^{-1}; $P<0.01$; Table 1) in the $P_{ET}O$ 50 mmHg trial compared with the 75 mmHg trial. Likewise, the increase in CO was greater in the $P_{ET}O_2$ 50 mmHg trial (0.6 ± 0.7 L·min^{-1}; $P=0.03$; Table 1). No difference was evident in the SV response following exposure to hypoxia (n= 10).

Effect of hypoxia on brachial artery baseline measures

A significant interaction between the experimental intervention (normoxia vs. hypoxia) and experimental condition ($P_{ET}O_2$ 75 mmHg trial vs. $P_{ET}O_2$ 50 mmHg trial) was evident for baseline brachial arterial diameter (P=0.01). Following exposure to $P_{ET}O_2$ 50 mmHg, arterial diameter increased by 0.02 ± 0.02 cm (relative 4%; P=0.01). In contrast, no diameter changes were evident following exposure to $P_{ET}O_2$ of 75 mmHg (P=0.80; Table 2).

Compared to normoxia, independent of the level of hypoxic stimulus, significant main effects were evident for reductions in baseline peak blood velocity (-3.6 ± 2.9 $cm \cdot s^{-1}$; [relative ~-42%] P<0.01), baseline peak blood flow (-13.2 ± 9.3 $ml \cdot min^{-1}$ [relative, ~-39%; P=0.01], baseline mean SR (-37 ± 12 s [relative, ~-43%; P<0.01], baseline antegrade SR (-24 ± 7 s [relative, ~ -21%; P=0.01], and an increase in baseline retrograde SR (+ -13 ± 5 s [relative, ~ +48%; P=0.01) and oscillatory shear index (+ 0.1 ± 0.0 [relative, ~+54%; P=0.01; Table 2 and Figure 1). A significant interaction between intervention (normoxia vs. hypoxia) and experimental condition ($P_{ET}O_2$ 75 mmHg trial vs. $P_{ET}O_2$ 50 mmHg trial) was evident for baseline retrograde SR and oscillatory shear index (P≤0.01); the intervention change following exposure to $P_{ET}O_2$ 50 mmHg was greater (retrograde SR: +-18 ± 9 s; oscillatory shear index +0.12 ± 0.0) than exposure to $P_{ET}O_2$ 75 mm Hg (retrograde SR: +-8 ± 2 s; oscillatory shear index: 0.07 ± 0.0; P≤0.03; Figure 1).

Effect of hypoxia on brachial artery FMD (n=12)

A significant interaction between the experimental intervention and experimental condition was evident for FMD (P<0.01; Figure 2 A). Compared to normoxia, FMD was significantly reduced

following exposure to $P_{ET}O_2$ 75 mmHg (-1.1 ±1.1% [relative, ~ -17%]); P=0.005) and $P_{ET}O_2$ 50 mmHg (-3.1 ±1.7 % [relative 45%]; P<0.01); the decline in FMD was greater following exposure to $P_{ET}O_2$ 50 mmHg by 2 ±1 % [relative 63%; P<0.01]. A significant main effect for intervention was evident for SR_{AUC} (P=0.01). Here, compared to normoxia, SR_{AUC} (25855 ± 9699 AUC) was reduced following hypoxia exposure (19441 ± 10386 AUC) independent of hypoxic stimulus (Table 2). Following allometric scaling of FMD and accounting for the decline in SR_{AUC} as a covariate, a significant interaction between experimental intervention and experimental condition was evident (P<0.01). Compared to normoxia, FMD was significantly reduced following exposure to $P_{ET}O_2$ 75 mmHg (-0.5% [relative -8%]), but the decline following the 30 minutes exposure to $P_{ET}O_2$ 50 mmHg was greater (-2.1% [relative, ~-32%]; Figure 2B).

Effect of hypoxia on brachial artery GTN (*n*=8; Table 3)

One-way ANOVA revealed that GTN-induced dilation was reduced following hypoxic exposure (P=0.01); Figure 3A). Compared with normoxia, GTN-induced dilation was significantly decreased following exposure to $P_{ET}O_2$ 75 mmHg (-2.1 ± 2.5 % [relative -12%] and $P_{ET}O_2$ 50 mmHg (-4.2 ± 4.0 % [relative -25%]). The decline with $P_{ET}O_2$ 50 mmHg was greater than the decline observed with $P_{ET}O_2$ 75 mmHg by 2.1 ± 2.6 %; [relative 14%; P=0.06; Figure 3). Following allometric scaling for baseline diameter, GTN-dilation was still significantly decreased following exposure to $P_{ET}O_2$ 75 mmHg (-2.1% [relative -13%] and even more so following $P_{ET}O_2$ 50 mmHg (-3.5% [relative -22%]; P=0.02; Figure 3B). Compared to normoxia, the FMD:GTN ratio was significantly decreased following exposure to $P_{ET}O_2$ 75 mmHg (-0.05 ± 0.03 % [relative 11%] and $P_{ET}O_2$ 50 mmHg (-0.16 ± 0.04 % [relative 35%] P<0.01). The decline

in the FMD:GTN ratio was significantly greater following exposure to $P_{ET}O_2$ 50 mmHg than $P_{ET}O_2$ 75 mmHg (P=0.02; Table 3).

Normoxic time-control study

Baseline MAP, HR, and SaO_2 % were 80 ± 6 mmHg, 56 ± 8 beats·min^{-1}, 98 ± 1 %. No significant difference in baseline diameter was evident following 30 minutes of supine rest (Table 4). Compared to baseline (pre-FMD one), however, reductions in baseline peak blood flow velocity (-5.8 ± 1.2 cm·s^{-1}; [relative ~36%] P=0.01), peak blood flow (-0.98 ± 0.32 ml·min^{-1} [relative, ~37%; <0.01]), were evident following 30 minutes of supine rest (Table 4). Reduction in baseline mean SR (~-37 ± 8 s [relative, ~-34%]; P<0.04) and baseline anterograde SR (-32 ± 8 s [relative, ~ -27%]; P=0.06) were evident following FMD one and 30 minutes of supine rest, retrograde SR and oscillatory SR index were not significantly changed (P=0.19; Table 4). Compared to FMD one, FMD was significantly reduced following 30 minutes of supine rest by -0.62 ± 0.28 % (relative, ~ -8.4%; P=0.02). No significant difference in FMD SR_{AUC} was evident between the two FMDs (P=0.13; Table 4). Following allometric scaling of FMD, where baseline diameter and SR_{AUC} were considered as a covariate, the decline in FMD following 30 minutes of supine rest still evident (P=0.05, Figure 4).

Discussion

The primary aim of study one was to examine the acute effects (<60 minutes) of a mild ($P_{ET}O_2$ 75 mm Hg; ~2000m) and moderate ($P_{ET}O_2$ 50 mm Hg; ~5000m) isocapnic normobaric hypoxic stimulus on the NO-vasodilator system via the assessment of FMD and GTN-induced dilation in the brachial artery. The novel findings were: 1) Compared to normoxia, FMD and GTN-induced

341 dilation were reduced following moderate hypoxia and, to a lesser extent, following mild
342 hypoxia. 2) FMD SR_{AUC} was reduced during both moderate and mild hypoxic conditions;
343 however, when the decline in FMD was corrected for the decline in SR_{AUC}, the decline in FMD
344 with mild and moderate hypoxia were attenuated. 3) Following exposure to both mild and
345 moderate hypoxia there was a decline in baseline blood flow and anterograde SR, and an
346 increase in retrograde SR. The increase in retrograde SR was greater during moderate hypoxia.
347 The main findings of the normoxic time-control study were that baseline blood flow and blood
348 flow velocity along with FMD were all reduced following 30 minutes of supine normoxic rest.
349 Such findings indicate that 30 minutes of recovery time for repeated FMD assessments is
350 insufficient. Based on these findings, the following discussion outlines putative mechanisms that
351 likely underpin hypoxia-induced declines in vascular function, including: 1) methodological
352 considerations of repetitive FMD testing and data interpretation; 2) hypoxic-induced declines in
353 FMD SR_{AUC}; 3) an increase in oscillatory shear; and 4) impaired endothelial function and
354 smooth muscle vasodilation.
355
356 ***Methodological considerations of repetitive FMD testing*:** The initial finding of this study
357 revealed an acute decline in FMD following 30-minutes of isocapnic hypoxia, which appears to
358 be dependent on the severity of the hypoxic stimulus. FMD was reduced by (relative) ~17% and
359 ~45% following 30 minutes of mild ($P_{ET}O_2$ 75 mm Hg, $Sa0_2$ 93%) and moderate ($P_{ET}O_2$ 50 mm
360 Hg, $Sa0_2$ 83%) hypoxia, respectively. The SR_{AUC} component of the FMD provides an estimation
361 of the shear stress stimulus created upon cuff release, which ultimately provokes the production
362 and release of NO from the endothelium. In both hypoxic trials, FMD SR_{AUC} decreased by
363 ~25%, a finding which was not evident in our normoxic time-control trial. Although not

statistically significant, SRA_{UC} has previously been reported to be reduced by ~21% following 60-minutes of hypoxia, and appears to recover to pre-hypoxic levels following ~6-hours of hypoxic exposure (Lewis *et al.*, 2014). When we accounted for the decline in FMD SR_{AUC} in our covariate analyses, we found the relative decline in FMD with mild hypoxia (-17% to -8%) and moderate hypoxia (-45% to -32%) was attenuated by ~9%. These results suggest the decline in FMD with hypoxia is partly due to a decline in FMD SR_{AUC}. Although the mechanisms influencing the decline in FMD SR_{AUC} with acute hypoxia are unknown, we speculate the possibility that forearm sympathetic constraint following hypoxic (Weisbrod *et al.*, 2001) exposure may have hindered the ischemic response to FMD cuff occlusion, and resulted in a lower reactive hyperemic response on cuff release. Nevertheless, as discussed next, other mechanism(s) also appear to affect the decline in FMD with moderate hypoxia.

A strength of our study was that we conducted a normoxic time-control trial to rule out any influence of repetitive FMD testing. Had we not have done this control, we would have falsely concluded a major influence of mild hypoxia on FMD. Our data indicates that a repeated assessment of FMD following 30 minutes of supine rest is reduced by 8%. Although not mentioned in the recent FMD guidelines (Thijssen *et al.*, 2011), the original International Brachial Artery Reactivity Task Force (Corretti *et al.*, 2002) states that at least 10 minutes of supine rest is needed after reactive hyperemia before another assessment is conducted. More recently, it was reported that repeated measures of FMD in the brachial artery may be taken after a minimum of 5 minutes or as soon as the vessel has returned to its baseline diameter (Barton *et al.*, 2011). In light of these studies, 30 minutes of supine rest between FMD assessment in the current studies should have been conservative recovery period, especially since baseline arterial

diameter was unchanged in the time-control study or following mild hypoxia exposure. Arterial diameter was larger following moderate hypoxia exposure; however, this was likely due to the effect of moderate hypoxia and has been accounted for in our interpretations of the data (allometric scaling). In summary, at least in our experimental study with a highly experienced (>1000 FMD tests and established high reducibility) vascular scanner it appears 30 minutes of recovery for repeated FMD assessments is insufficient, and should be considered in future research.

Given that the relative decline in FMD following mild hypoxia exposure (-8%) was comparable to that seen in the time-control study it is likely that the decline in FMD following acute mild exposure was due to lasting effect of the baseline (normoxic) FMD assessment. Nevertheless, even if we consider the 8% decline in FMD due to the negative impact of repeated measures, a relative decline of 24% in FMD is still present following moderate hypoxia. We have previously (Lewis *et al.*, 2014) documented a ~28% decline in FMD following 60-minutes of normobaric hypoxia (FIO_2=0.11; SaO_2 79%), supporting the current findings of an acute impairment in FMD following moderate hypoxia.

Alterations in baseline blood flow and oscillatory shear patterns: Pre-FMD baseline blood flow velocity and blood flow were reduced by ~42% and ~39%, respectively, following hypoxic exposure. Declines in brachial artery blood flow (-11%) and blood flow velocity (-2%) have previously been reported following 10 minutes of moderate hypoxia (FIO_2 0.12) (Iwamoto *et al.*, 2015). Given that the decline in blood flow velocity / blood flow was comparably reduced following hypoxic exposure (39-42%) and in our normoxic time control trial (~36-37%)), it is

possible long lasting effects of forearm ischemia from the baseline FMD may have altered blood velocity hemodynamics and explain this decline in blood flow prior to repeated assessment of FMD. The topics require further investigation as it clearly have important methodological considerations in the design of related vascular function experiments.

Significant change in baseline SR patterns were evident with hypoxia, with a decrease in antegrade SR (~21%) and an increase in retrograde SR (~48%), and oscillatory shear index (~54%). No significant changes in SR patterns were evident in the normoxic time-control study, therefore, it appears the alteration in SR were an effect of hypoxia. This is supported by others who have reported an increase in retrograde SR (>39%) and oscillatory shear index (>35%) following 10 minutes of hypoxia (FIO_2 0.12) (Iwamoto *et al.*, 2015; Katayama *et al.*, 2016). Although hypoxia causes net vasodilation (Heistad & Wheeler, 1970), sympathetic excitation within 5-10 minutes of isocapnic hypoxia (SaO_2 85%) exposure has been shown to mask the vasodilation effects of hypoxia in the resistant vessels of the forearm (Weisbrod *et al.*, 2001; Weisbrod *et al.*, 2004). Additionally, acute excitation and elimination of sympathetic nerve activity on forearm vascular resistant has been shown to increase and reduce retrograde and oscillatory SR patterns respectively, in the brachial artery (Thijssen *et al.*, 2009; Padilla *et al.*, 2010; Casey *et al.*, 2012; Padilla *et al.*, 2014). Therefore, it is possible that heightened sympathetic vasoconstrictor activity with acute hypoxia (Dinenno *et al.*, 2003) and subsequently hypoxic vasodilation constraint in the forearm (Weisbrod *et al.*, 2001) could have increased downstream resistance vessel tone, and altered SR blood flow patters.

The increase in retrograde and oscillatory SR patterns in the current study was significantly larger following exposure to moderate vs. mild hypoxic exposure. Acute and progressive increases in baseline retrograde and oscillatory SR patterns in the brachial artery have been shown to elicit a dose-dependent impairment in brachial FMD (Thijssen *et al.*, 2009). Furthermore, graded reductions in hypoxia have been shown to elicit a graded increase in MSNA (Rowell *et al.*, 1989), and graded increase in MSNA have been was associated with an incremental increase in retrograde and oscillatory SR patterns (Padilla *et al.*, 2010). Therefore, it is possible that a greater increase in SNA with moderate hypoxic exposure possibly explains the larger increase in retrograde and oscillatory SR patterns in this condition and the significant impairment in FMD, this concept warrant future investigation.

Impaired vascular smooth muscle and endothelial vasodilation: The GTN-induced dilation in the current study was reduced by (relative) ~13% and ~22% following 60-minutes of mild and moderate hypoxia, respectively. We have previously reported a decline (relative: ~14%) in GTN-induced dilation following 3-days at 5050m (Lewis *et al.*, 2014); however, as far as we are aware, this is the first report of acute effects of hypoxia on GTN-induced vasodilation. Given that the assessment GTN-induced dilation represents vascular smooth muscle cell sensitivity to NO (Corretti *et al.*, 2002; Maruhashi *et al.*, 2013), the findings of the current study supports the notion of impairment in vascular smooth muscle function following hypoxic exposure (Lewis *et al.*, 2014). This reduction in GTN-induced dilation undoubtedly influence the impairment observed in FMD responses, especially following moderate hypoxia. However, currently what level of impairment in smooth muscle function is required to hinder upon FMD measures is currently unknown.

455 The acute decline in FMD following 60-minutes of normobaric hypoxia has previously been
456 shown to be partially reversed following an α1-adrenoreceptor blockade, suggesting
457 sympathoexcitation is one of the mechanisms by which FMD is impaired following acute
458 hypoxic exposure (Weisbrod *et al.*, 2004; Lewis *et al.*, 2014). Although the effect of hypoxic-
459 induced sympathoexcitation on the acute impairment in GTN-induced dilation has not been
460 reported, it is likely a key mechanism for reductions in FMD (Saito *et al.*, 1988; Rowell *et al.*,
461 1989) i.e., via increasing vascular smooth muscle tone and impairing vascular smooth muscle
462 cell ability to relax in response to NO.
463
464 Previous work has reported a ~20% increase in muscle sympathetic nerve activity (MSNA)
465 following 5-minutes of isocapnic hypoxia (FIO_2=0.10; SaO_2 82%) (Somers *et al.*, 1988).
466 Moreover, Rowell et al., (1989) reported an inverse relationship between graded reductions in
467 FIO_2 and elevations in MSNA. For example, after 20-minutes of hypoxia at FIO_2 0.12 and FIO_2
468 0.10, MSNA was elevated by ~90% and 250%, respectively (Rowell *et al.*, 1989). The duration
469 that MSNA remains elevated during an acute hypoxic insult, and its potential effect on GTN-
470 induced dilation and FMD are currently unknown and warrant investigation. Furthermore, since
471 the magnitude of hypoxic-induced elevations in MSNA seems to be dependent on the severity of
472 the hypoxic stimulus, this could also explain why the degree of FMD and GTN-induced dilation
473 impairment were larger following the moderate hypoxic exposure compared to the mild hypoxic
474 exposure in the current study. Future studies combining MSNA measures with and without SNA
475 blockade are needed to clearly test this hypothesis.
476

When we corrected our assessments of FMD with the changes in GTN induced dilation with hypoxia, we found that FMD-to-GTN% was decreased by ~ 35% from normoxia moderate hypoxic exposure. The FMD-to-GTN ratio represents global NO-dependent vasodilator function (Spence *et al.*, 2013; Lewis *et al.*, 2014); thus, following 30-minutes of moderate hypoxia it appears the decline in FMD is partly due to endothelial dysfunction in addition to vascular smooth muscle dysfunction.

Implications

It has been estimated that a 1% absolute reduction in FMD is associated with a 9% increase in cardiovascular disease risk (Green *et al.*, 2012); thus, 2% absolute decline in FMD with moderate isocapnic Keephypoxia in the current study is potentially associated with an elevation in cardiovascular disease risk. This may potentially have some health implications for individuals acutely exposed to moderate hypoxia, such as Heli hikes / skiing activities. Acute impairment in smooth muscle dilation may potentially have implications for individuals exposed to mild and moderate hypoxia during air travel. Medical issues during air travel are estimated at about 350 per day worldwide, and currently aircraft carrying passengers are pressurized and maintain a cabin altitude between 1525m to 2438 m (Sohail & Fischer, 2005). One study investigated the change in SpO_2 levels in healthy flight-crew members during 22 scheduled flights, and found mean SpO_2 nadir levels fell from 97% (preflight) to 88.6% at cruising altitude (Cottrell *et al.*, 1995). Therefore, air travel has the potential to exacerbate risk for passengers with underlying cardiovascular conditions, and increase the risk of medical events. Furthermore, although factors in addition to the mild hypoxemia likely also play a role (e.g. diet, shift work,

499 sleep patterns) flight attendants have a 3.5 fold increase risk of developing cardiovascular disease
500 compared to the general public (McNeely *et al.*, 2014).
501
502 **Conclusion**
503 In light of the methodological effects of repetitive FMD testing, there does not appear to be an
504 impairment in endothelial function following mild hypoxia exposure. However, there is
505 significant endothelial impairment following moderate hypoxic exposure and our data indicate
506 that this impairment is potentially influenced by adverse changes in SR patters and increase in
507 oscillatory SR with graded increases in hypoxia. Graded impairments in smooth muscle cell
508 sensitivity to NO is evident following mild and moderate hypoxia, and has important
509 implications for individuals acutely exposed to mild and moderate hypoxia, especially those with
510 cardiovascular risk factors.
511
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647 **Additionally Information**

648 **Author Contribution:** 1) Conceived and designed research; 2) Performed experiments,
649 Analyzed data, Interpreted results of experiment; 3) Drafted manuscript, Edited and revised
650 manuscript for important intellectual content; 4) Approved final version of Manuscript. 5)
651 Agreed to be accountable for all aspects of the work. 6) Qualify for authorship.
652

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654 **PN**: 1,2,3,4,5,6
655

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Figure Captions

Figure 1: Effect of normoxia (baseline) and acute isocapnic hypoxia ($P_{ET}O_2$ 75 mmHg and $P_{ET}O_2$ 50 mmHg) on pre-FMD baseline shear rate (SR) patterns and oscillatory shear index. * Significant main effect for intervention (normoxia vs hypoxia), P=0.01. † Significant interaction between intervention and condition, P=0.04, the increase from normoxic baseline in retrograde SR and oscillatory shear index with hypoxia was greater in the $P_{ET}O_2$ 50 mmHg trial compared to $P_{ET}O_2$ 75 mmHg.

Figure 2: The effect of normoxia and acute isocapnic hypoxia ($P_{ET}O_2$ 75 mmHg and $P_{ET}O_2$ 50 mmHg) on FMD. A) Uncorrected and B) corrected for significant changes in baseline arterial diameter and shear rate area under the curve. * Significant main effect for intervention

(normoxia vs hypoxia), P<0.01. † Significant main effect for condition ($P_{ET}O_2$ 75 mmHg vs $P_{ET}O_2$ 50 mmHg), P<0.01. ‡ Significant interaction between intervention and condition, P<0.01.

Figure 3: The effect of normoxia and acute isocapnic hypoxia ($P_{ET}O_2$ 75 mmHg and $P_{ET}O_2$ 50 mmHg) on GTN dilation; A) uncorrected and B) corrected for significant changes in pre-GTN arterial diameter. * Significant main effect for intervention (normoxia vs hypoxia), P=0.05. † Significant main effect for condition ($P_{ET}O_2$ 75 mmHg vs $P_{ET}O_2$ 50 mmHg), P<0.01. ‡ Significant interaction between intervention and condition, P=0.01.

Figure 4: Mean and SD uncorrected and corrected (for baseline arterial diameter and shear rate area under the curve). * (paired t-test) † (linear mix model) Post 30-min significantly different from baseline; P≤0.05.

705 **Tables**

706

707 **Table 1: Effect of normoxia and acute isocapnic hypoxia ($P_{ET}O_2$ 75 mmHg and $P_{ET}O_2$ 50**
708 **mmHg) on cardiorespiratory variables.**
709

Experimental Condition	**$P_{ET}O_2$ 75 mmHg**		**$P_{ET}O_2$ 50 mmHg**		
Experimental Intervention	Normoxia	Hypoxia	Normoxia	Hypoxia	
Ventilation (L·min)	12.4 ± 2.7	15.1 ± 3.8	12.8 ± 2.1	22.3 ± 6.7	* † ‡
$P_{ET}O_2$ (mmHg)	92.0 ± 4.7	74.0± 1.6	92.6 ± 5.8	50.0 ± 2.8	* † ‡
$P_{ET}CO_2$ (mmHg)	40.8 ± 2.2	40.9± 2.0	41.3 ± 2.4	41.0 ± 2.4	
MAP (mmHg)	85 ± 14	93 ± 16	85 ± 13	90 ± 15	†
SBP (mmHg)	106 ± 22	108 ± 27	107 ± 27	117 ± 25	†
DBP (mmHg)	64 ± 8	69 ± 8	64 ± 9	65 ± 11	†
HR (beats·min^{-1})	58 ± 13	57 ± 12	64 ± 13	64 ± 11	* † ‡
SV (ml)	103 ± 27	106 ± 30	107 ± 21	106 ± 23	
CO (L·min)	5.5 ± 1.1	6.0 ± 1.3	5.6 ± 1.1	6.7 ± 1.4	† ‡
SaO2 (%)	97 ± 1	93 ± 2	97 ± 1	83 ± 3	* † ‡

710

711 Values expressed as mean ± SD: End-tidal oxygen ($P_{ET}O_2$), end-tidal carbon dioxide ($P_{ET}CO_2$),
712 mean arterial blood pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure
713 (DBP), heart rate (HR), stroke volume (SV, n=10), cardiac output (CO, n=10), oxygen saturation
714 (SaO_2). * Significant main effect for intervention (normoxia vs hypoxia), P≤0.04. † Significant
715 main effect for condition ($P_{ET}O_2$ 75 mmHg vs. $P_{ET}O_2$ 50 mmHg), P≤0.04. ‡ Significant
716 interaction between intervention and condition, P≤0.02.

Table 2: Effect of normoxia (baseline) and acute isocapnic hypoxia on FMD related variables.

Experimental Condition	$P_{ET}O_2$ 75 mmHg		$P_{ET}O_2$ 50 mmHg		
Experimental Intervention	Normoxia	Hypoxia	Normoxia	Hypoxia	
FMD					
Baseline diameter (mm)	3.86 ± 0.73	3.85 ± 0.73	3.94 ± 0.70	4.12 ± 0.77	†‡
Baseline peak blood flow velocity ($cm \cdot s^{-1}$)	8.4 ± 4.4	5.5 ± 2.8	8.6 ± 4.9	4.4 ± 2.6	*
Baseline peak blood flow ($ml \cdot min$)	66.2 ± 52.9	43.5 ± 32.5	70.7 ± 57.1	40.5 ± 34.4	*
Peak diameter (mm)	4.12 ± 0.73	4.07 ± 0.75	4.21 ± 0.71	4.25 ± 0.78	
Time to peak diameter (s)	62 ± 27	46 ± 13	57 ± 27	56 ± 31	
SR_{AUC} (AUC)	26546 ± 10249	20199 ± 9886	25163 ± 11096	18683 ± 11947	*

Values expressed as mean ± SD: Shear rate area under the curve (SR_{AUC}); Flow mediated dilation (FMD). * Significant main effect for intervention (normoxia vs hypoxia), $P<0.01$. † Significant main effect for hypoxic condition (75 mm Hg vs. 50 mm Hg), $P=0.01$. ‡ Significant interaction between intervention and condition, $P<0.001$.

Table 3: Effect of normoxia and acute isocapnic hypoxia on GTN related variables

Experimental Condition	**Normoxia**	**$P_{ET}O_2$ 75 mmHg**	**$P_{ET}O_2$ 50 mmHg**	
GTN				
Baseline diameter (mm)	3.95 ± 0.69	3.99 ± 0.77	4.13 ± 0.72	*
Peak diameter (mm)	4.61 ± 0.73	4.54 ± 0.77	4.63 ± 0.72	
Time to peak diameter (s)	450 ± 95	472 ± 82	453 ± 62	
FMD:GTN ratio	0.45 ± 0.20	0.40 ± 0.18	0.29 ± 0.17	*

Values expressed as mean ± SD: Shear rate area under the curve (SR_{AUC}); Flow mediated dilation (FMD); Endothelium-independent FMD (GTN). * Significant main effect for intervention (normoxia vs hypoxia), P<0.0

Table 4: FMD related variables prior to and following 30-minutes of supine normoxic rest.

	Baseline	Post 30-min	
Baseline diameter (mm)	4.46 ± 0.42	4.39 ± 0.39	
Baseline blood flow velocity ($cm \cdot s^{-1}$)	16.0 ± 7.1	10.2 ± 5.9	*
Baseline peak blood flow (ml·min)	162 ± 106	96 ± 70	*
Baseline Mean SR (s)	111 ± 49	74 ± 42	†
Baseline Anterograde SR (s)	120 ± 46	87 ± 38	
Baseline Retrograde SR (s)	-8 ± 8	-13 ± 13	
Baseline Oscillatory SR Index	0.08 ± 0.09	0.13 ± 0.12	
Peak diameter (cm)	4.79 ± 0.35	4.69 ± 0.33	*
Time to peak diameter (s)	61 ± 22	64 ± 30	
SR_{AUC} (AUC)	30948 ± 10303	277 68± 11846	

Values expressed as mean ± SD: Shear rate area under the curve (SR_{AUC}); Flow mediated dilation (FMD); Shear rate (SR). * †(Wilcoxon test) Post 30-min significantly different from baseline; P<0.03.

Figure 1

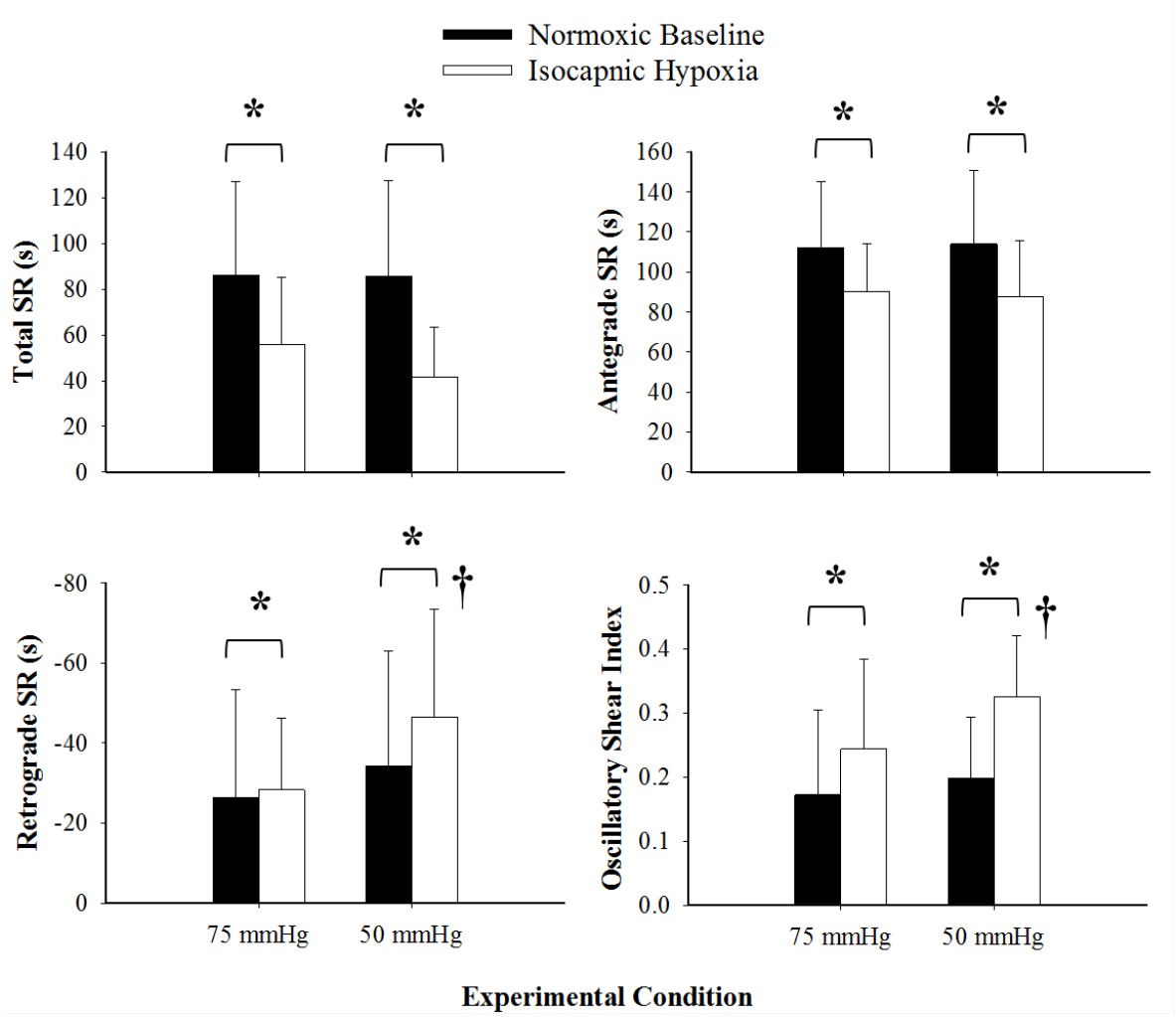
Normoxic Baseline
Isocapnic Hypoxia
Total SR (s)
Antegrade SR (s)
Retrograde SR (s)
Oscillatory Shear Index
75 mmHg
50 mmHg
Experimental Condition

Figure 2

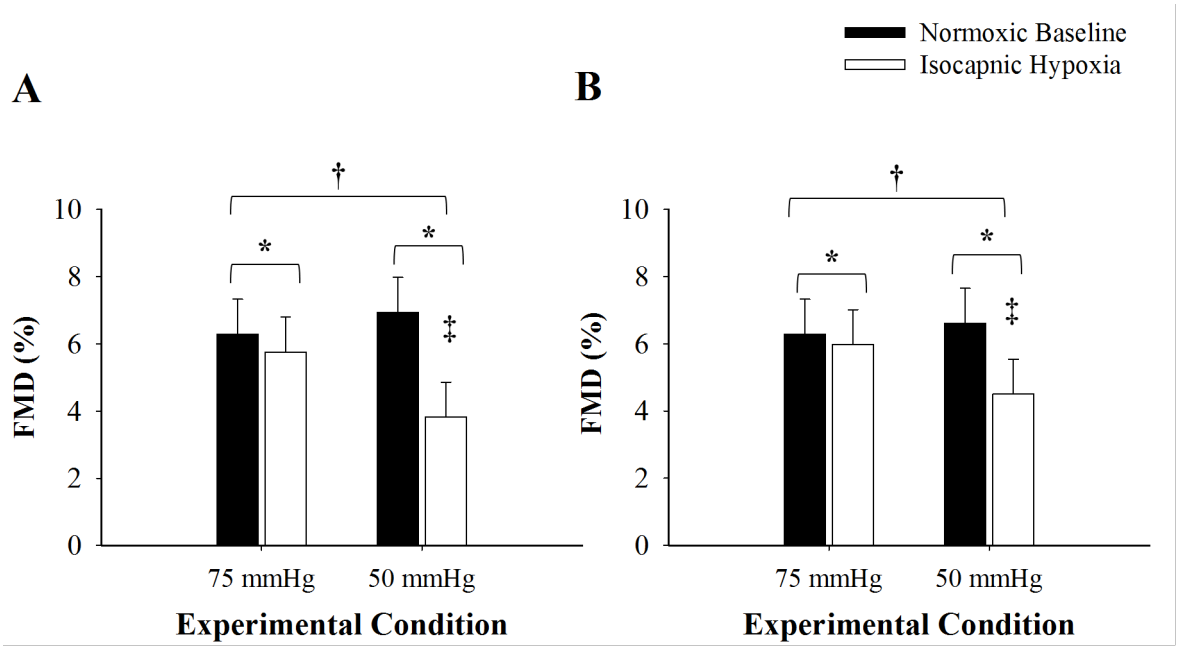
A
B
Normoxic Baseline
Isocapnic Hypoxia
FMD (%)
10
8
6
4
2
0
†
*
*
‡
75 mmHg
50 mmHg
Experimental Condition

Figure 3

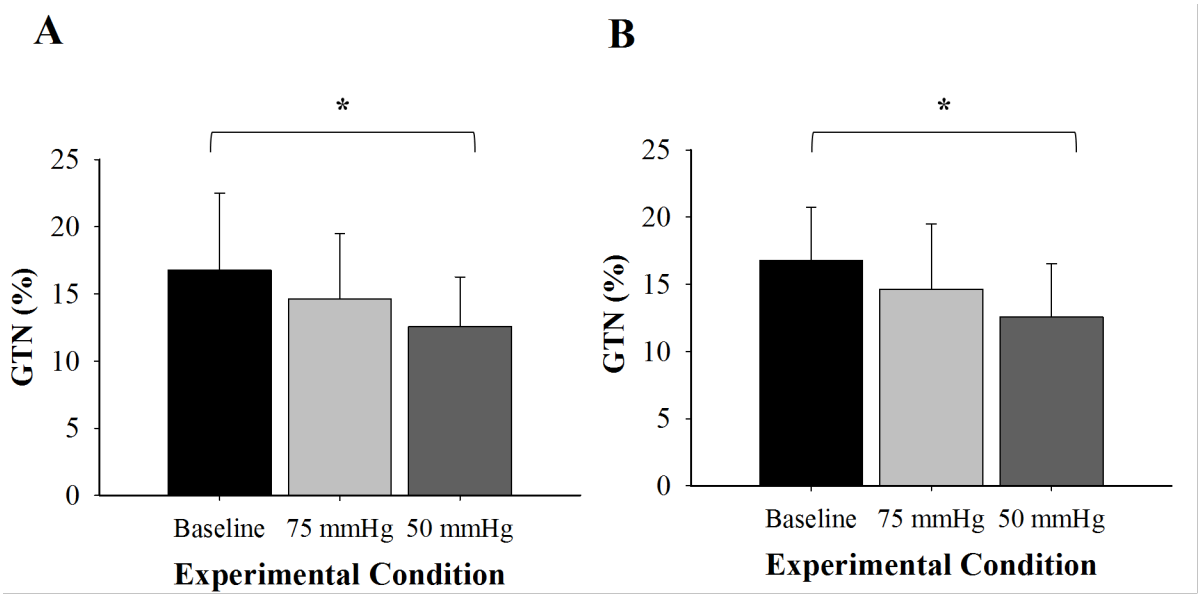
A
*
25
20
15
10
5
0
GTN (%)
Baseline
75 mmHg
50 mmHg
Experimental Condition
B
*
25
20
15
10
5
0
GTN (%)
Baseline
75 mmHg
50 mmHg
Experimental Condition

Figure 4