Changes in Acute Pulmonary Vascular Responsiveness to Hypoxia During a Progressive Ascent to High Altitude (5,300 m)

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New Findings

What is the central question of this study? Do the pulmonary vascular responses to hypoxia change during progressive exposure to high altitude and can alterations in these responses be related to changes in concentrations of circulating biomarkers that affect the pulmonary circulation.

What are the main finding and its importance? In our field study with healthy volunteers, we demonstrate changes in pulmonary artery pressure suggestive of remodeling in the pulmonary circulation, but find no changes in the acute responsiveness of the pulmonary circulation to changes in oxygenation during two weeks of exposure to progressive hypoxia. Pulmonary artery pressure changes were associated with changes in erythropoietin, 8-isoprostane, nitrite and cGMP.

Abstract

We sought to determine whether changes in pulmonary artery pressure responses to hypoxia suggestive of vascular remodeling occur during progressive exposure to high altitude and whether such alterations are related to changes in concentrations of circulating biomarkers with known or suspected actions on the pulmonary vasculature during ascent. We measured tricuspid valve transvalvular pressure gradients (TVPG) in healthy volunteers breathing air at sea level (London, UK) and under hypoxic conditions simulating the P_IO_2 at two locations in Nepal, Namche Bazaar (NB, elevation 3,500 m) and Everest Base Camp (EBC, elevation 5,300 m). During a subsequent thirteen day trek, TVPG was measured at NB and EBC while volunteers breathed air and hyperoxic or hypoxic mixtures simulating the P_IO_2 at the other locations. For each location, we determined the slope of the relationship between TVPG and arterial oxygen saturation (S_aO_2) to estimate the pulmonary vascular response to hypoxia. Mean TVPG breathing air was higher at any S_aO_2 at EBC than at sea level or NB, but there was no change in the slope of the relationship between S_aO_2 and TVPG between locations. Nitric oxide availability remained unchanged despite increases in oxidative stress (elevated 8-isoprostane). Erythropoietin, pro-ANP and IL-18 levels progressively increased on ascent. Associations with TVPG were only observed with erythropoietin, 8-isoprostane, nitrite and cGMP. While the increased TVPG for any given S_aO_2 at EBC suggests pulmonary vascular remodeling may occur during 2 weeks of progressive hypoxia, the lack of change in the slope of the relationship between TVPG and S_aO_2 indicates that the acute pulmonary vascular responsiveness to changes in oxygenation does not vary within this time frame.

Abbreviations: **FIO_2: inspired fraction of oxygen;** HPV: hypoxic pulmonary vasoconstriction; IVC: inferior vena cava; PA: Pulmonary artery; P_AO_2: Alveolar partial pressure of oxygen; PVR: Pulmonary vascular resistance; RAP: right atrial pressure; S_aO_2: arterial oxygen saturation; TR: tricuspid regurgitation; TVPG: transvalvular pressure gradient;

Introduction

Hypoxic pulmonary vasoconstriction (HPV) occurs in response to a decrease in the alveolar partial pressure of oxygen (P_AO_2) and serves to maintain adequate ventilation-perfusion relationships and thereby preserve gas exchange in response to localized processes, such as pneumonia, that affect the lung in a heterogeneous manner. However, following ascent to high altitude, P_AO_2 is decreased throughout the lung (due to the fall in barometric pressure and therefore ambient PO_2) leading to generalized HPV, which in conjunction with increased cardiac output, leads to a rise in pulmonary artery pressure. (Swenson, 2013)

There are many well-understood features of this response. For example, it is known that the initial phase of HPV in humans occurs within minutes of exposure to hypoxia with a further increase continuing over the next 8 hours (Dorrington *et al.*, 1997; Talbot *et al.*, 2005) and that individual pulmonary artery pressure responses vary substantially in magnitude with some people demonstrating large responses that may predispose to complications when traveling at high altitude. (Grunig *et al.*, 2000; Dehnert *et al.*, 2005) There are several aspects of HPV, however, that are not well understood.

First, it is unclear how the response changes over time with continued exposure to hypoxic conditions. In other words, as an individual acclimatizes to high altitude, are there changes in the magnitude of the acute pulmonary artery (PA) pressure response to hypoxia? Baggish et al. (Baggish *et al.*, 2010) examined pulmonary artery pressure responses to hypoxia following acute chamber exposure to hypobaric hypoxia (P_B ~ 460 mmHg) and during exposure to terrestrial high altitude (P_B ~ 460 mmHg) following 7 days at moderate altitude (P_B 548 mm Hg). They noted much smaller increases in mean

PA pressure with exposure to hypobaric hypoxia following the extended period at moderate elevation (25 $\pm$ 4 vs. 37 $\pm$ 8 mmHg, $P < 0.001$). Ghofrani et al. (Ghofrani *et al.*, 2006) noted a lower PA systolic pressure (PASP) following an 8-day trek to Everest Base Camp (EBC; 27.1 mmHg (95% CI 24.1 – 30)) compared to that following acute exposure at sea level to inspired oxygen tensions simulating EBC (P_IO_2 70 mmHg; PASP 30.5 mmHg (95% CI 26 – 35)). Other studies have also found evidence of changes in the pulmonary vasculature with more prolonged hypoxic exposures. Groves et al. (Groves *et al.*, 1987) exposed healthy volunteers to steadily decreasing barometric pressure in a hypobaric chamber simulating an ascent of Mt. Everest over 40 days and noted that PA pressure and pulmonary vascular resistance remained above sea level baseline values when volunteers breathed 100% oxygen during the last 20 days of their time in the chamber. However, the use of 100% oxygen breathing as a test to eliminate HPV as a contributor, may itself introduce other changes that confuse the issue, such as reduction of elevated high sympathetic nervous activation. (Hansen & Sander, 2003)

Another unanswered question is whether observed changes in PA pressure with progressive ascent to high elevation can be related to changes in the plasma or exhaled breath concentrations of vasoactive substances (biomarkers) known to affect the pulmonary circulation. While these biomarker concentrations have been measured at final altitudes following ascent, only a few studies have examined how these concentrations change with progressive ascent and how such changes relate to changes in pulmonary artery pressure. Donnelly et al (Donnelly *et al.,* 2011) found that changes in exhaled nitric oxide did not correlate with PASP in an ascent to 5050 m. Mellor et al (Mellor *et*

al., 2014) studied trekkers at 3833, 4450 and 5129 m and showed that B-type natriuretic peptides (BNP) and cardiac troponin T elevations were correlated with PASP.

Intended to address these questions, our study had two aims. First, recognizing that HPV demonstrates a dose-response relationship, whereby greater degrees of hypoxia are associated with greater rises in pulmonary artery pressure, we sought to determine whether the PA pressure responses over a range of hypoxic conditions also change following prolonged exposure to high altitude. We hypothesized that the slope of the dose-response relationship tested at several altitudes during a trek to the Everest Base Camp (EBC) in Nepal would decrease due to acclimatization or remodeling. Second, we sought to measure a much larger number of biomarkers known or suspected to affect the pulmonary circulation repeatedly during an ascent to high altitude and determine whether the observed changes in these biomarkers were associated with observed changes in resting PA pressure. We hypothesized that biomarkers related to nitric oxide metabolism, oxidative stress, and inflammation, as well as endothelin, natriuretic peptides and erythropoietin would be associated with the changes in PA pressure at altitude.

Methods

Ethical Approval and Recruitment

After obtaining approval from Research Ethics Committee at University College London, we recruited volunteers from a group of healthy individuals, aged 18-65 years, participating in Caudwell Xtreme Everest, an observational cohort investigating human adaptation to progressive environmental hypoxia during a trek to EBC. (Levett *et al.*, 2010) In London, we screened 32 of the 198 participants in the larger study for the presence of tricuspid regurgitation (TR) using echocardiography (ACUSON Cypress Ultrasound System, Siemens, Munich, Germany). Individuals with measureable tricuspid regurgitation were eligible to participate and provided written informed consent, which conformed to the standards of the Declaration of Helsinki.

Testing Protocol

For each eligible volunteer, an additional set of at least 10 valid spectral waveforms for TR was measured while breathing ambient air. Volunteers then breathed two hypoxic gas mixtures designed to simulate the average inspired PO_2 (P_IO_2) at locations where they would undergo further testing in Nepal, Namche Bazaar (NB; elevation 3,500 m, F_IO_2 0.135) and EBC (elevation 5,300 m, F_IO_2 0.104, Table 1). Gas mixtures were administered by tight-fitting mask with a Hans-Rudolph valve to prevent re-inhalation of expired air or entrainment of ambient air. Volunteers inhaled gas from a 120-L Douglas bag filled from an external tank containing the pre-specified gas concentration (BOC Medical Gases, Manchester, United Kingdom). Gas mixtures were administered in the same order to each volunteer.

While supine, volunteers breathed each gas mixture for 45 minutes with collection of spectral waveforms of TR during the last 15 minutes of each exposure. Arterial oxygen saturation (S_aO_2) was measured by pulse oximetry (Mini-Torr Plus ®, Smiths Medical UK, Kent United Kingdom) every minute, and average values were calculated for the entire 45-minute period and for the 15 minutes during which echocardiography measurements were made. Volunteers breathed ambient air for 10 minutes between gas exposures.

In Nepal, all volunteers followed the same itinerary from the Lukla (elevation 2800 m) to EBC (Figure 1). At NB, S_aO_2 was measured every 60 seconds for 5 minutes while supine, at rest and breathing ambient air. After measuring spectral waveforms of TR repeatedly over an additional 10-minute period breathing air, volunteers breathed a hyperoxic gas mixture simulating the P_IO_2 at sea level (F_IO_2 0.326) and then a hypoxic gas mixture simulating the P_IO_2 at EBC (F_IO_2 0.16; Table 1). Volunteers breathed each mixture for 45 minutes with repeated measurements of the spectral waveforms of TR over the last 15 minutes of each exposure. Volunteers breathed ambient air for 5 minutes between mixtures.

A similar protocol was employed at EBC. Echocardiography measurements were made with volunteers breathing ambient air, hyperoxic gas mixtures simulating the P_IO_2 at sea level (F_IO_2 0.425), NB (F_IO_2 0.273), and a hypoxic gas mixture equivalent to the P_IO_2 at Camp 1 (elevation 5900 m) on Mt. Everest (F_IO_2 0.18) (Table 1).

At NB and EBC, gas mixtures were administered in the same order to all volunteers. The apparatus for administering the gas mixtures, pulse oximeters and echocardiography

system were the same as used in London. All testing took place at least 3 hours following exercise to allow resolution of any exercise-induced changes in PA pressure.

Due to time considerations, testing took place over a two-day period at each testing location. Testing occurred in NB on the third or fourth day of the trek while testing occurred at EBC on the twelfth or thirteenth day of the trek. Volunteers tested on Day 3 at NB were tested on the Day 12 at EBC and those tested on the Day 4 at NB were tested on Day 13 at EBC. Testing took place at approximately the same time of day in each location to control for diurnal variations in physiologic responses.

To minimize the effects of acclimatization to hypoxia, volunteers abstained from exposure to simulated or terrestrial high altitude for 3 months prior to departure for Nepal. Volunteers were not allowed to take prophylactic medications to prevent altitude illness and were treated for altitude illness according to standardized protocols.

Echocardiography Measurements

TR measurements were performed using two-dimensional and Doppler echocardiography. Data were obtained using an apical 4-chamber view or a modified parasternal short axis view that permitted visualization of the regurgitant jet across the tricuspid valve. The same sonographer, who was not blinded to the testing conditions, obtained all sea level and altitude measurements. Data from all testing sessions were stored on external disks and transported to Seattle, Washington where an experienced sonographer blinded to the study conditions determined the peak TR velocities by identifying the highest coherent boundary of the spectral waves (Siemens Cypress Viewer, Siemens, Munich Germany).

The transvalvular pressure gradient (TVPG) was estimated using the simplified Bernoulli equation: TVPG = $4V^2$ where V is the peak TR jet velocity in m/sec. We chose to report the TVPG rather than right ventricular or PA systolic pressure because the latter measurements require an estimate of right atrial pressure and logistical factors prevented storage and transport of videos of respiratory variation in inferior vena cava (IVC) diameter necessary for estimation by the blinded observer. Reported values for TVPG are mean $\pm$ SD of the highest 10 values obtained during each measurement period.

Blood Collection and Biomarker Measurements

Blood samples were collected from volunteers at each study location prior to the echocardiography protocol. All samples were drawn from the antecubital vein into EDTA containers (BD Vacutainer). Whole blood samples were centrifuged at 800 x g for 15 minutes and the plasma separated. Plasma samples prepared in London were placed in -40°C commercial cryostorage, while those samples prepared in Nepal were immediately frozen in 1 ml aliquots in liquid nitrogen and maintained at liquid nitrogen temperatures for the duration of the expedition including transport back to Kathmandu. Samples were transported from Kathmandu to London on dry ice and stored in -40°C commercial cryostorage until analysis.

Nitric oxide metabolite concentrations were quantified immediately following thawing of frozen plasma aliquots in the presence of N-ethylmaleimide (NEM, in PBS; 10 mM final concentration). To determine circulating S-nitrosothiol concentrations, aliquots of NEM-treated EDTA plasma were directly injected into a triiodide-containing reaction chamber, and the NO produced from the reduction of protein nitroso species was

quantified using gas phase chemiluminescence (CLD 77sp, EcoMedics); the difference in NO detected from untreated samples and aliquots subjected to pretreatment by mercuric chloride served to calculate the mercury-displaceable proportion of NO bound to thiols. (Rassaf *et al.*, 2002) To determine nitrate and nitrite concentrations, NEM-treated samples were deproteinized with ice-cold methanol (1:1 v/v), cleared by centrifugation and subjected to analysis by high pressure liquid chromatography using a nitrite/nitrate analyzer (ENO20, Eicom). Samples were processed in a staggered manner to ensure reproducible processing times, with daily calibrations and internal quality controls. Reported values are corrected for background contaminant levels of nitrite and nitrate.

Pro-atrial natriuretic peptide (pro-ANP 1-98) and brain natriuretic peptide fragment (NT-proBNP 8-29) concentrations were determined by commercial enzyme immunoassay kits (ALPCO Diagnostics, Salem, NH). Erythropoietin (EPO) and guanosine 3',5'-cyclic monophosphate (cGMP) concentrations were measured by ELISA (Bender MedSystems GmbH, Vienna, Austria; R&D Systems, Abingdon, United Kingdom). Endothelin-1 was measured by enzyme immunoassay (Endothelin-1 kit, Enzo Life Sciences, Farmingdale, NY). 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane) was quantified using direct competitive enzyme immunoassay (Assay Designs, Ann Arbor, MI). Plasma cytokines, including IL-1ß/IL-1ra, IL-6, IL-8, IL-18, macrophage migration inhibitory factor (MIF) and TNF-α were measured in a multiplexed fashion using a premixed human cytokine panel and xMAP technology (Bio-Plex Pro™ and Bio-Plex 200, Bio-Rad, Hemel Hempstead, Hertfordshire, UK).

Biochemical data are only reported for 10 out of the 11 individuals who completed the study due to missing data in some of the markers.

Statistical Analysis

Data are presented as mean values (± the standard deviation), 95% confidence intervals and medians with interquartile ranges (IQR) where appropriate. Cytokine responses were skewed so were log transformed for analysis. Given the multiple experimental conditions, linear regression models were used to assess the impact of each experimental condition on TVPG (location and simulated F_IO_2 conditions). Repeated measures linear regression models using generalized estimating equations (GEE) were used to analyze the data. GEE allowed us to address repeated measures and the associated within-individual correlation within the model while generating population and individual level slopes; this latter aspect of the model provides distinct advantages over repeated measures ANOVA. The models were first formulated as follows: $Y= \beta_0 + \beta_1$(location), where Y equals TVPG in separate models with additional models including a second term [β_2 (S_aO_2)]. An interaction term was used to assess where the relationship between oxygen saturation and location (surrogate for altitude) to test whether the slope of the relationship between S_aO_2 and TVPG differed by location. Similar models were used to assess the differences with each experimental condition by location (Y = to oxygen saturation).

Because hypoxic pulmonary vasoconstriction is thought to occur in response to changes in alveolar PO_2, we originally intended for the exhaled PO_2, a surrogate measure of the alveolar PO_2, serve as β_2. However, logistical considerations in Nepal prevented use of a breath-by-breath cardiopulmonary exercise system necessary to measure this parameter. We opted, therefore, to use arterial oxygen saturation (S_aO_2) as the surrogate

measure. We feel this is valid given that none of the volunteers had underlying lung disease or new respiratory issues during the trek that could lead to poor association of alveolar PO_2 with S_aO_2.

For assessments of the biomarker relationships to location, each analyte was modeled as the dependent variable with location as the independent variable. For all models, standardized residuals were plotted to assess assumptions of linear models. Because of the concern that errors from the regression models did not have the same distribution across all observation points (heteroscedasticity), Huber/White-corrected standard errors were employed (White, 1980). Power calculations were not done because the number of volunteers was limited by logistical constraints of the Caudwell Xtreme Everest protocol. No adjustment was made for multiple comparisons. A two-sided p-value of ≤ 0.05 was determined to be significant. Analyses were performed with Stata 12.0 (StataCorp, College Station, TX).

Results

Eleven volunteers (8 men, 3 women, mean age 47 $\pm$12 years) completed the entire protocol. Five volunteers met criteria for acute mountain sickness (AMS) at NB (defined as a Lake Louise Acute Mountain Sickness score $\geq$ 3) and five volunteers met criteria at EBC. Only two met criteria for AMS at both locations. All of the volunteers used non-steroidal anti-inflammatory drugs or acetaminophen for various purposes at some point during their trek. These medications do not have an effect on pulmonary artery pressure. (Naeije *et al.*, 1987) No volunteers used acetazolamide, dexamethasone, nifedipine or a phosphodiesterase inhibitor before testing was completed. No volunteers developed or required treatment for high altitude pulmonary edema or cerebral edema.

Table 2 displays the mean S_aO_2 and TVPG for all volunteers breathing ambient air at each location.

Table 3 displays the mean S_aO_2 achieved during the time period when echocardiography was performed under each testing condition at each location. In general, the S_aO_2 achieved under each testing condition closely approximated that measured when volunteers breathed ambient air at the location the testing conditions were intended to simulate. The largest observed differences were seen with attempts to simulate NB conditions in London and attempts to simulate EBC conditions in both London and NB.

Figure 2 displays the TVPG as a function of S_aO_2 for all volunteers at each testing location. Best-fit lines through each set of data points represent the dose response relationship between S_aO_2 and TVPG. There were no statistically significant differences in the slopes of the best-fit lines between London and EBC. The slope of the best-fit line in NB showed a significant difference relative to London (mean difference 0.15, 95% CI 0.08 to 0.23, $P< 0.001$) and a non-significant difference relative to EBC (mean difference 0.09, 95% CI -0.20 to -0.16, $P=0.09$).

Table 4 displays the mean TVPG at each testing location for different S_aO_2. At 70%, 80% and 90% S_aO_2, the mean TVPG was higher at EBC than at London or NB ($p<0.001$).

Figure 3 displays the steady-state concentrations of relevant plasma biomarkers at the three testing locations. A similar profile of changes in response to environmental hypoxia as that reported for members of the Core Team of the 2007 Caudwell Xtreme Everest expedition(Levett *et al.*, 2011) was seen in the participants of the current study. Ascent

to EBC was accompanied by progressive elevation of circulating erythropoietin concentrations. Consistent with the notion of increased oxidative stress at high altitude, 8-isoprostane concentrations were higher at NB and EBC than London, ($P< 0.001$ for both comparisons). Yet, this was likely not a consequence of local or generalized inflammation as evidenced by the lack of significant changes in IL-1ß/IL-1ra, IL-6, IL-8 and TNF-α. Those alterations were accompanied by modest, non-significant increases in nitrite and nitrate concentrations at NB; while S-nitrosothiols were lower at NB than London ($P=0.002$). While mean plasma cGMP levels remained largely unchanged between testing locations the inter-individual variability in circulating cGMP concentrations was markedly reduced on ascent to EBC. Taken together, this data suggests that systemic nitric oxide (NO) availability was maintained irrespective of the degree of hypoxia. No statistically significant differences were observed in the concentrations of endothelin-1 and pro-BNP between testing locations whereas levels of pro-ANP, interleukin-18 (IL-18) and macrophage migration inhibitory factor (MIF) tended to be higher at NB and EBC compared to sea-level.

Mean TVPG at rest at each location was also examined as a function of biomarker concentrations. Statistically significant associations were seen between TVPG and EPO, 8-isoprostane, nitrite and cGMP concentrations but not with any of the other biomarkers (Table 5). Mechanistically, this pattern of associations confirms the dependence of PA pressure on environmental oxygen availability and suggests that TVPG is dependent, at least in part, on the degree of systemic oxidative stress and nitric oxide availability.

Discussion

Our study is one of the first attempts to examine changes in pulmonary vascular responses to hypobaric hypoxia in a concentration-response manner during progressive exposure to high altitude and demonstrates two key findings: (1) the mean TVPG at any given S_aO_2 is higher after 12-13 days at high altitude than following acute exposure to hypoxia at sea-level or shorter duration of exposure at high altitude and (2) the dose response to hypoxia over a range from sea level to 5,300 m does not change with 12-13 days of progressive ascent to high altitude. Taken together, these findings suggest that while some hypoxic hypertensive remodeling may occur during this time frame, the pulmonary circulation still displays an undiminished sensitivity and response to acute changes in alveolar PO_2 as indirectly reflected by the surrogate S_aO_2.

Evidence of Time-Dependent Hypoxic Remodeling of the Human Pulmonary Circulation

Our finding that TVPG is higher at any given S_aO_2 following a few-week stay at high altitude is similar to results demonstrated in previous studies measuring PA pressure responses to acute and sustained hypoxia. In their simulated ascent of Mt. Everest over 40 days, Groves et al. (Groves *et al.*, 1987) noted a decrease in mean PA pressure when volunteers received supplemental oxygen (F_IO_2 = 1.0) at barometric pressures of 347, 282 and 240 mm Hg, but the PA pressure at each time point remained above the mean PA pressure measured breathing ambient air at sea-level. Similarly, Maggiorini et al. (Maggiorini *et al.*, 2001) found that PA pressure after several days at 4,559 m decreased in normal and HAPE-susceptible individuals following administration of an F_IO_2 1.0, but

remained higher than PA pressure measured breathing ambient air at 490 m prior to the ascent.

The fact that TVPG or PA pressure does not return to sea-level values with inhalation of supplemental oxygen enough to recreate the sea level inspired PO_2 suggests that pulmonary vascular remodeling may occur during prolonged hypoxic exposure at high altitude in the range of several days to weeks. With addition of supplemental oxygen, cardiac output declines to sea-level values (Groves *et al.*, 1987; Maggiorini *et al.*, 2001) and, as a result, any remaining increase in the TVPG or PA pressure would be expected to result from higher pulmonary vascular resistance (PVR). Given that supplemental oxygen should raise the P_AO_2 and eliminate HPV, this implies that other factors account for the higher PVR. We did not measure cardiac output and, as a result, cannot confirm that the higher TVPG is due to higher PVR rather than residual increases in cardiac output in our study, but Groves et al. (Groves *et al.*, 1987) did measure cardiac output and found that PVR remained elevated despite supplemental oxygen.

In considering these results further, both Groves et al. (Groves *et al.*, 1987) and Maggiorini et al. (Maggiorini *et al.*, 2001) administered an F_IO_2 of 1.0 to study participants at each barometric pressure whereas we administered only enough supplemental oxygen to raise the P_IO_2 to sea-level values. This is significant as an F_IO_2 of 1.0 would raise P_aO_2 far higher than in our study and could alter sympathetic tone and other aspects of both left and right-sided hemodynamics in a different manner than that seen with our approach. Because in both studies, cardiac output fell considerably with 100% oxygen possibly as a result of withdrawal of sympathetic tone, (Hansen & Sander, 2003) it is not possible to be certain that the lack of change in calculated PVR is

consistent with loss of HPV, even as early as several days at high altitude. Ideally HPV would be better assessed by return of the subject to a normoxic arterial PO_2 rather than a very hyperoxic PO_2.

Two studies (Dorrington *et al.*, 1997; Fatemian *et al.*, 2016) in which volunteers exposed to 8 hours of normobaric isocapnic hypoxia to maintain an end-tidal PO_2 of 50 mmHg (S_aO_2 of roughly of 80%) suggest an even shorter duration of time to initiate remodeling. These two studies demonstrated that when subjects returned to breathing normal ambient sea-level air, their PA systolic pressure did not fall completely back to baseline. This could be taken as evidence that even eight hours is sufficient to generate some remodeling. However, given that systemic blood pressure displays a diurnal variation with an increase from morning to afternoon/evening, what these authors found may have been simply a diurnal variation in PA pressures. They did not examine this possibility by using a suitable control group not exposed to hypoxia over the same timeframe and in our search of the literature; we found no studies of the diurnal behavior of the pulmonary circulation in healthy subjects.

Despite the fact that pulmonary vascular remodeling may occur after just a short time at high altitude, our data suggest that the acute pulmonary vascular responsiveness to changes in P_AO_2 is unchanged over at least the first several weeks at high altitude. This conclusion is based on the fact that the slope of the relationship TVPG and S_aO_2 was unchanged between EBC and London (Figure 2). There was a statistically significant difference in slope between London and NB but this was not robust to changes in parameterization and thus is not considered materially significant. If the small differences we found in the slopes in NB and London were indicative of a changing

behavior of the pulmonary vasculature to longer duration hypoxia, we would predict an even greater difference between EBC and London. This was not evident in our study.

In support of our finding of preservation in responsiveness of the pulmonary circulation, Hilty et al. (Hilty *et al.*, 2016) studied healthy volunteers by right heart catheterization after three weeks at 3600 m. They found that while PA pressures were mildly elevated, when the pulmonary circulation was challenged with a brief 2.5 l/min increase in cardiac output by transient thigh cuff occlusion there was no increase in PA pressure. In this regard, our findings of maintained responsiveness to changes in inspired oxygen are similar and indicate that the pulmonary vascular pressures, while greater over this time, do not become permanently fixed at a higher resistance. More work will be necessary to further delineate the time course and whether the rate of remodeling varies based on the duration of stay and rate of ascent at high altitude.

The lack of change in pulmonary vascular responsiveness to changes in alveolar PO_2 as reflected by changes in S_aO_2 is different than we originally hypothesized based on data from Baggish et al., (Baggish *et al.*, 2010) in which PA pressure at terrestrial 4,300 m was lower following a 6-day stay at an intermediate altitude (2,200 m) when compared to acute exposure to simulated 4,300 m. We do not feel that their findings refute the observations in our study as our approach was different from that used in their investigation. Whereas Baggish et al. only estimated PA pressure breathing ambient air at 4,300 m and, as a result, did not assess the dose response to hypoxia, we estimated TVPG under multiple conditions at the same elevation and, as a result, were better able to define the relationship. The differences in results between our studies may also be due to the fact

the volunteers in our study spent much longer time at high altitude than the volunteers examined by Baggish et al.

Biomarker Measurements

The mechanism by which preservation of acute pulmonary vascular responsiveness to hypoxia remains unchanged despite the fact that baseline pulmonary vascular resistance may be elevated because of hypoxic remodeling is unclear We measured various circulating mediators and biomarkers of pathways known to affect the pulmonary circulation to explore what vasoactive mediators and potential mechanisms may be involved. With the exception of EPO, 8-isoprostane, nitrite and cGMP, we did not find any statistically-significant associations between resting TVPG at each location and the concentration of the measured biomarkers. These changes are consistent with the notion that human exposure to hypoxia alters systemic redox status towards a higher oxidative poise (indicated by increased levels of circulating lipid oxidation products such as 8-isoprostanes) and that an elevation of nitric oxide production and availability (as reflected by enhanced plasma nitrite and cGMP levels) is important to allow adequate adjustment of pulmonary vascular tone to enable ventilation perfusion matching that is fit for purpose. While we do observe changes in biomarkers of nitric oxide metabolism that are clearly known to affect PA pressure, more sophisticated measurements of production and consumption are needed to ascertain whether they are driving changes in TVPG and PA pressure in a fundamental manner.

The association of EPO with HPV changes may not imply causality because EPO also rises with increasing altitude exposure as the kidneys themselves experience a fall in

their local PO_2. Furthermore, there are no data that EPO causes pulmonary vasoconstriction (Kuriyama *et al.*, 2014; Berendsen *et al.*, 2016). Our failure to find any associations of the other chosen biomarkers with PA pressure changes may relate to the fact that other possibly relevant vasoactive mediators also known to alter HPV, such as several products of prostaglandin metabolism and microRNAs, might be more relevant.

Another issue that may have affected the results is the fact that these biomarkers were assessed at each location while volunteers breathed ambient air prior to our testing protocol rather than during or shortly after breathing the different gas mixtures. As a result, the measured circulating concentrations of these markers may not reflect the true plasma levels (or more relevant local tissue concentrations) during the different hypoxic or hyperoxic exposures, assuming that they would rapidly change in response to altered inspired oxygen concentration. Nevertheless, some of the changes observed are informative inasmuch as they document that neither circulating concentrations of endothelin nor the systemic availability of NO, both important modulators of pulmonary vascular tone, changed considerably during ascent. The latter is particularly remarkable in view of the oxidative stress that typically accompanies acute and chronic hypoxic exposures, as verified by the rise in circulating levels of the lipid oxidation product 8-isoprostane, and is consistent with earlier observations. (Levett *et al.*, 2011)

Other biomarker changes are noteworthy in the context of the known association between pulmonary diseases and ventricular diastolic dysfunction. Consistent with a stimulation of ANP secretion as part of the normal acclimatization process to hypoxia, (Chen, 2005) we documented a trend towards higher plasma concentrations of pro-ANP at NB and EBC compared to sea level, with concomitant rises in IL-18 and MIF. The

latter is an oxygen-sensitive archetypical cytokine that has been proposed to mediate hypoxia-induced pulmonary hypertension (Zhang *et al.*, 2012) and also plays a role in several aspects of cardiovascular disease; (Zernecke *et al.*, 2008) the former has been implicated in the diastolic dysfunction that results from global hypoxic vasoconstriction and consecutive increases in right ventricular afterload. (Larsen *et al.*, 2008) While brain natriuretic peptide is often elevated with pulmonary hypertension, we found no changes in NT-BNP with time and increasing altitude. Our findings are consistent of those with Toshner et al., (Toshner *et al.*, 2008) but differ from Mellor et al. (Mellor *et al.*, 2014) who reported increased concentrations in trekkers ascending to EBC. To the best of our knowledge, ours is the first study to document variations in plasma concentrations of these cytokines and other modulators of pulmonary vascular tone in conjunction with a functional assessment of the pulmonary circulation at high altitude. We find variations in these biomarkers occur as part of the normal physiologic adaptation to reduced inspired oxygen concentrations in healthy individuals, but do not in any simple way appear to be linked to the pulmonary vascular response.

Study Limitations

Owing to the constraints of the study design with its strict continuous ascent profile, we recognize that a true steady-state was not likely achieved at each of the two high altitude test sites. Thus other unmeasured effects of varying time, different individual rates of acclimatization, and differences in individual time-averaged hypoxia ‘dose’ could weaken our claim to have found no differences in the acute HPV responsiveness over this

altitude span. These weaknesses would need to be addressed in a separate study allowing subjects to remain longer at each altitude and to vary the order of altitude exposure.

Another limitation is the fact that we did not measure exhaled gas concentrations or arterial blood gases to determine the P_aCO_2 or estimate the P_AO_2. We originally intended to estimate P_AO_2 and P_ACO_2 by measuring exhaled gas concentrations with the integrated gas sensors of a commercial breath-by-breath cardiopulmonary exercise system (Metamax 3b, Cortex, Leipzig, Germany), but logistical issues prevented use of the system during testing in Nepal and this plan was aborted. The lack of such data is potentially important for several reasons. Because P_aCO_2 affects pulmonary vascular tone (Balanos *et al.*, 2003; Swenson, 2013), the lack of information on this parameter prevented us from controlling for the effect of inter-individual differences in ventilation that may have affected pulmonary vascular responses. Because P_AO_2 rather than the P_aO_2 is the dominant stimulus for HPV, we cannot confirm that we achieved exactly similar alveolar oxygen tensions across volunteers under each testing condition and, therefore, achieved similar stimuli for changes in PA pressure. Instead, we used S_aO_2 as a surrogate measure. We lack information on the alveolar-arterial oxygen difference to know if similar S_aO_2 corresponded to similar P_AO_2, but the saturation data and the fact that all volunteers had no preexisting lung disease and no evidence of HAPE based on clinical assessment would suggest they had normal gas exchange and, as a result, the expected P_AO_2.

While the S_aO_2 achieved under each testing condition generally approximated the S_aO_2 measured when volunteers breathed ambient air at the location the testing conditions were intended to simulate, differences were observed with attempts to

simulate NB conditions in London and attempts to simulate EBC conditions in both London and NB. Despite the measured differences in the average saturations under these conditions, we achieved a large enough spread between S_aO_2 at each testing location that it is still feasible to define and compare the slopes of the concentration response relationship between the TVPG and S_aO_2 between locations.

Another limitation is that we did not measure right atrial pressure (RAP) and, as a result, report the TVPG rather than the PA pressure. While guidelines for estimating PA pressures recommend estimating RAP by assessing collapsibility of the IVC through the respiratory cycle (Rudski *et al.*, 2010) many studies assume a normal value of 5 or 7 mm Hg in healthy individuals. (Grunig *et al.*, 2000; Maggiorini *et al.*, 2006) Logistical issues prevented assessment of IVC collapsibility by our blinded observer upon completion of the study and we opted not to use the latter approach. We do not feel this limits the findings of the study, as the primary goal was to determine the concentration response relationship to hypoxia and, as a result, we are less dependent on the absolute pressure measurements and more interested in how pressures change in response to changes in oxygenation. Given that we were studying healthy euvolemic individuals at each testing location, we have no reason to suspect large differences in RAP that would have systematically affected this relationship.

Other confounding factors in assessing changes in pulmonary vasoreactivity to oxygen by changes in TVPG are the effect of cardiac output and changes in hematocrit (viscosity) (Grant & Canty, 1989; Hoffman, 2011). We did not measure cardiac output, but data from many other studies show at rest that acclimatized volunteers have very little change (5-7% fall) in cardiac output up to altitudes of 6000 m and over the changes in

inspired oxygen fraction we tested. (Groves *et al.*, 1987; Ghofrani *et al.*, 2006) The impact on TVPG and, therefore, pulmonary artery systolic pressure (PASP) with these small changes in cardiac output would be to reduce PASP by about 2% (Grant & Canty, 1989) and thus would not likely to have influenced our conclusions.

The mean (and standard deviation) hematocrit of the volunteers was 44.1 % (+ 1.7) in London, 46.7.1 % (+ 1.9) at NB, and 49.2 % (+ 2.2) at EBC. These small differences between hematocrit at each location were statistically significant different from each other ($p < 0.05$). The imprecision that the increases in hematocrit and viscosity from London to EBC adds to the tricuspid regurgitant velocity as a surrogate for pulmonary vascular resistance would be an approximate 3% underestimation of the true TVPG. (Cinar *et al.*, 1999; Giardini, 2011; Hoffman, 2011) Recalculating TVPG values taking the hematocrit changes into account did not change the slopes of the TVPG vs. S_aO_2 at each altitude shown in Figure 2.

Conclusion

This study of healthy trekkers traveling to 5,300 m in elevation demonstrated that the mean TVPG at any given S_aO_2 is higher after 12-13 days at high altitude than following acute exposure to hypoxia at sea-level or shorter duration of exposure at high altitude and that the slope of the relationship between TVPG and S_aO_2, reflective of the acute pulmonary vascular responsiveness to hypoxia, does not change over the first 12-13 days of progressive high altitude exposure. Further studies including estimates of cardiac

output and end-tidal CO_2 tensions along with other vasoactive mediators not measured in this study are warranted to confirm these findings and further evaluate the time course and mechanisms of pulmonary vascular remodeling and acute responsiveness to oxygen with progressive hypoxic exposure.

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Additional Information

Competing Interests / Disclosures:

At the time the project was conducted, Dr. Martin was a Critical Care Scholar of the London Clinic, and Dr. Levett was a Fellow of the Association of Anaesthetists of Great Britain and Ireland. Drs. Luks, Feelisch, Goss, Grocott, and Swenson have no conflicts of interest to report.

Author Contributions

All persons designated as authors qualify for authorship and all those who qualify for authorship are listed. All authors have approved the final version of the manuscript.

Dr. Luks contributed to the conception or design of the work, acquisition, analysis or interpretation of data for the work and drafting and revising the work.

Drs. Levett and Martin contributed to the conception or design of the work and revising the work critically for important intellectual content.

Drs. Fernandez and Feelisch conducted the biochemical marker analysis while at the University of Warwick and revised the work critically for important intellectual content while at the University of Southampton.

Kay Mitchell served as Project Manager for the Caudwell Xtreme Everest Expedition, provided critical logistical support for the data collection phase of the study. She contributed to the conception or design of the work and revising the work critically for important intellectual content.

Dr. Goss conducted the statistical analysis and drafted and revised the work critically for important intellectual content.

Drs. Grocott and Swenson contributed to the conception or design of the work, interpretation of data for the work and drafting and revising the work for important intellectual content.

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Table 1: Estimated Inspired Gas Concentrations Used During Echocardiography Measurements at Each Testing Location

Testing Location	Altitude (m)	Mean Barometric Pressure (mm Hg)	Inspired Gas Concentrations	Estimated P_IO_2 (mm Hg)
London	75	754	0.210	149
			0.135	95
			0.104	74
Namche Bazaar	3500	505	0.326	149
			0.210	96
			0.160	73
Everest Base Camp	5300	404	0.425	152
			0.273	97
			0.210	75
			0.180	64

Table 2: Arterial Oxygen Saturation and Transvalvular Pressure Gradient Breathing Ambient Air at Each Testing Location

Testing Location	Mean S_aO_2 (%, 95% CI of Mean)	Mean Transvalvular Pressure Gradient (mm Hg, 95% CI of Mean)
London	96.6 (95.9-97.3)	21.5 (19.8 to 23.3)
Namche Bazaar	87.3 (86.0- 88.7)	28.2 (25.9 to 30.6)
Everest Base Camp	73.0 (70.3-75.5)	38.0 (34.5 to 41.4)

Table 3: Mean Arterial Oxygen Saturation During Echocardiography Measurements at Each Testing Location

Experimental Condition	Testing Location	Mean S_aO_2 Under Experimental Condition (%)	Mean Difference in S_aO_2 Relative To Ambient Air at Testing Location*	95% CI of Mean Difference
London	London	96.9	N/A	N/A
	NB	97.0	0.46 #	0.153 to 0.772
	EBC	96.6	.001	-0.161 to 0.164
Namche Bazaar (NB)	London	84.9	-2.46 #	-1.476 to -3.436
	NB	87.3	N/A	N/A
	EBC	87.5	0.19	-0.413 to 0.798
Everest Base Camp (EBC)	London	67.4	-2.79 #	-1.937 to -3.643
	NB	68.8	-4.05 #	-2.828 to -5.270
	EBC	72.9	N/A	N/A

* Mean differences are based on a repeated measures regression model that differs slightly from the actual mean values at each side based on differing numbers of experimental assessments at each site. Site measurements range from 5 (during ambient air measurements) to 15 (while breathing gas mixtures) saturation assessments under each experimental condition. # $P < 0.05$

Table 4: Mean Transvalvular Pressure Gradient (TVPG) at Each Location For Different Arterial Oxygen Saturations

Testing Location	TVPG adjusted for S_aO_2 90% (Mean, 95% CI)	TVPG adjusted for S_aO_2 80% (Mean, 95% CI)	TVPG adjusted for S_aO_2 70% (Mean, 95% CI)
London	25.4 (22.9 – 27.9)	29.4 (26.6 – 32.1)	33.6 (30.5 – 36.7)
Namche	26.3 (23.4 – 29.2)	30.5 (26.8 – 34.2)	34.7 (30.5 – 38.9)
EBC	29.4 (27.1 – 31.8) †	33.6 (30.7 – 36.5)	37.8 (34.5 – 40.1)

† $P < 0.001$ when comparing EBC transvalvular pressure gradient to either London or Namche. Comparisons between London and Namche did not demonstrate statistically significant differences.

Table 5: Relationship Between Biomarkers and Mean Transvalvular Pressure Gradient (mm Hg) While Breathing Air at Each Location

Cytokine	Coefficient for estimated TVPG	95% CI for Coefficient	P value
Hypoxia/Oxidative Stress			
Erythropoietin [mIU/mL]	**0.171**	**0.022 to 0.320**	**0.024**
8-isoprostanes [ng/mL]	**-0.284**	**-0.381 to -0.187**	**<0.001**
Endothelin/Natriuretic Peptides			
Endothelin-1 [pg/mL]	0.109	-0.019 to 0.236	0.095
pro-ANP [nmol/mL]	1.864	-0.718 to 4.447	0.157
NT-BNP [nmol/mL]	0.007	-0.005 to 0.0175	0.248
Nitric Oxide Pathway			
Nitrite [µM]	**-24.88**	**-43.4 to -6.35**	**0.009**
Nitrate [µM]	0.033	-0.053 to 0.120	0.456
S-nitrosothiols [nM]	0.080	-0.0284 to 0.188	0.148
N-nitrosamines [nM]	0.150	-0.255 to 0.556	0.417
cGMP [pmol/mL]	**0.068**	**0.014 to 0.122**	**0.014**
Inflammatory Cascade			
IL-1β[pg/mL]	-1.490	-17.813 to 14.833	0.858
IL-1ra [pg/mL]	-0.030	-0.094 to 0.033	0.344
IL-6 [pg/mL]	-0.540	-1.400 to 0.320	0.218
IL-8 [pg/mL]	-0.097	-0.390 to 0.195	0.513
TNFα [pg/mL]	-0.042	-0.236 to 0.152	0.633
IL-18 [pg/mL]	0.075	-0.034 to 0.184	0.176

MIF [ng/mL]	0.0008	-0.0002 to 0.0019	0.126

Note: Using a repeated measures GEE regression model, Mean Transvalvular Pressure Gradient was the independent variable with cytokine levels each at dependent variables. Coefficient interpretation: for every 1 mm Hg increase in mean TVPG estimate, the biomarker changed by its specific unit. Statistically significant associations are highlighted in bold text.

Figure Legends

Figure 1. Ascent profile. All volunteers followed the same itinerary. Testing was performed on either the second or third day at Namche Bazaar and Everest Base Camp.

Figure 2. The transvalvular pressure gradient (TVPG) as a function of S_aO_2 for all volunteers at London (black squares), Namche Bazaar (open diamonds) and Everest Base Camp (grey triangles). Best-fit lines are drawn through each set of data points and denote the concentration response relationship between S_aO_2 and the TVPG at each location. The mean slope and 95% confidence interval of the best fit lines for the relationship between transvalvular pressure gradient and arterial oxygen saturation at each location are as follows: London: 2.1 (1.5-2.6); Namche Bazaar: 2.2 (1.6-2.8); Everest Base Camp: 2.1 (1.6-2.6). The slope represents the change in TVPG for a 5% decrease in oxygen saturation.

Figure 3. Circulating biomarker concentrations at different testing locations. Hypoxia (erythropoietin; EPO) and oxidative stress (isoprostanes; 8-iso$PGF_{2\alpha}$) markers demonstrate robust increases upon ascent to Namche Bazaar ($p < 0.05$) and Everest Base Camp (EBC) compared to London ($p < 0.001$). The nitric oxide metabolites nitrite and nitrate increased slightly at Namche Bazaar and showed trends towards a decrease at EBC. Bioactive S-nitrosothiols decreased significantly at Namche Bazaar ($p < 0.05$) and recovered to near-basal levels at EBC ($p < 0.05$ vs. Namche Bazaar). Systemic NO availability (cyclic GMP concentrations) remained unchanged at all altitudes. Atrial

natriuretic peptide (proANP) remained unchanged at Namche Bazaar but increased modestly at EBC (n.s.). The proinflammatory cytokines IL-18 and MIF tended to increase upon ascent to EBC ($p < 0.05$ for MIF; n.s. for IL-18). Data are presented as mean ± SEM. (* $p < 0.05$ vs. London, ** $p < 0.001$, † $p < 0.05$ vs. Namche Bazaar).