High altitude pulmonary hypertension is associated with a free radical mediated reduction in pulmonary nitric oxide bioavailability

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High-altitude pulmonary hypertension is associated with a free radical-mediated reduction in pulmonary nitric oxide bioavailability

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High altitude (HA)-induced pulmonary hypertension may be due to a free radical-mediated reduction in pulmonary nitric oxide (NO) bioavailability. We hypothesised that the increase in pulmonary artery systolic pressure (PASP) at HA would be associated with a net transpulmonary output of free radicals and corresponding loss of bioactive NO metabolites. Twenty-six mountaineers provided central venous and radial arterial samples at low altitude (LA) and following active ascent to 4559 m (HA). PASP was determined by Doppler echocardiography, pulmonary blood flow by inert gas re-breathing, and vasoactive exchange via the Fick principle. Acute mountain sickness (AMS) and high-altitude pulmonary oedema (HAPE) were diagnosed using clinical questionnaires and chest radiography. Electron paramagnetic resonance spectroscopy, ozone-based chemiluminescence and ELISA were employed for plasma detection of the ascorbate free radical ($A^{\bullet-}$), NO metabolites and 3-nitrotyrosine (3-NT). Fourteen subjects were diagnosed with AMS and three of four HAPE-susceptible subjects developed HAPE. Ascent decreased the arterio-central venous concentration difference (a-cv_D) resulting in a net transpulmonary loss of ascorbate, α-tocopherol and bioactive NO metabolites ($P < 0.05$ *vs.* LA). This was accompanied by an increased a-cv_D and net output of $A^{\bullet-}$ and lipid hydroperoxides ($P < 0.05$ *vs.* sea level, SL) that correlated against the rise in PASP ($r = 0.56$–0.62, $P < 0.05$) and arterial 3-NT ($r = 0.48$–0.63, $P < 0.05$) that was more pronounced in HAPE. These findings suggest that increased PASP and vascular resistance observed at HA are associated with a free radical-mediated reduction in pulmonary NO bioavailability.

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Abbreviations AMS, acute mountain sickness; HA, high altitude; HAPE, high-altitude pulmonary oedema; HAPE-S, susceptible to high-altitude pulmonary oedema; HPV, hypoxic pulmonary vasoconstriction; LA, low altitude; PASP, pulmonary artery systolic pressure; PBF, pulmonary blood flow; PPF, pulmonary plasma flow; SL, sea level.

Introduction

Hypoxic pulmonary vasoconstriction (HPV) is a highly conserved adaptive response to alveolar hypoxia that serves to optimise ventilation–perfusion matching and gas exchange (Von Euler & Liljestrand, 1946). However, the prolonged systemic and alveolar hypoxia associated with high-altitude (HA) exposure can result in a potentially detrimental increase in pulmonary artery pressure and vascular resistance. When excessive, this can lead to high-altitude pulmonary oedema (HAPE),

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with potentially fatal consequences (Bartsch *et al.* 1991; Swenson *et al.* 2002).

Despite extensive research, the precise mechanisms underlying HA-induced pulmonary hypertension are not completely understood. Human studies suggest that this may be due to a functional imbalance favouring vasoconstriction through increased sympathetic activation of α-adrenergic efferent pathways (Duplain *et al.* 1999) with additional contributions from endothelin (Berger *et al.* 2009) and angiotensin II (Bartsch *et al.* 1988) in combination with reduced bioavailability of the endogenous vasodilator nitric oxide (NO) (Scherrer *et al.* 1996; Busch *et al.* 2001; Swenson *et al.* 2002; Berger *et al.* 2005; Ingram *et al.* 2010). At the cellular level, emerging evidence implicates the mitochondrial formation of reactive oxygen species (ROS) as the primary stimulus underlying HPV (Guzy & Schumacker, 2006; Waypa & Schumacker, 2008).

In support, there is now compelling evidence to suggest that hypoxia promotes systemic (Bailey *et al.* 2001, 2004, 2006*b*, 2009*a*) and focal cerebral (Bailey *et al.* 2009*b*) oxidative–nitrosative stress in humans. However, to what extent if indeed at all the pulmonary circulation through which the entire cardiac output must pass contributes to the intravascular accumulation of free radicals and subsequent implications for high-altitude pulmonary hypertension remains to be examined.

Therefore, our primary aim was to determine the temporal effects of hypoxia on the systemic and transpulmonary exchange of oxidative–nitrosative–inflammatory stress biomarkers during ascent to HA. The secondary aim was to examine if any subsequent relationships existed between exchange across the lungs and pulmonary hypertension. We hypothesised that compared to the sea level (SL) control condition, HA would promote the pulmonary formation and subsequent net output of free radicals (oxidative stress) and associated reactants (inflammatory stress) as indicated by a positive arterial–central venous concentration difference (a-cv_D). This would be accompanied by a net loss of bioactive NO metabolites and output of 3-nitrotyrosine (3-NT), reflecting oxidative inactivation of NO (Pacher *et al.* 2007) (nitrosative stress). Finally, we hypothesised that exchange would be directly proportional to both the absolute values and the increase in pulmonary artery systolic pressure (PASP) and subsequently more marked in those subjects who developed HAPE.

Methods

Subjects

Thirty-eight (32 male, 6 female) healthy non-acclimatised native lowlanders aged 37 (mean) $\pm$ 10 (S.D.) years old volunteered for the study, which had been approved by the Medical Faculty of the University of Heidelberg. Thirty subjects had previously climbed to altitudes above 3000 m. Of these, eight had previously developed moderate to severe acute mountain sickness (AMS) and eight had prior radiographic evidence of HAPE (classified as HAPE susceptible; HAPE-S). No subject spent more than four nights above 2500 m within 30 days before the study and there was no history of chronic daily headache, habitual antioxidant vitamin or anti-inflammatory supplementation. All subjects were encouraged to follow a low nitrate/nitrite diet prior to and throughout the duration of the study with specific instructions to avoid fruits, salads and cured meats (Wang *et al.* 1997).

Compliance

Four subjects withdrew following baseline control measurements, two of whom were HAPE-S. A further eight subjects were excluded (two HAPE-S, one of whom developed HAPE) due to failure to measure pulmonary blood flow (PBF) and/or obtain blood samples at all time points. Thus, the current data are based on full data sets for 26 subjects (aged 36 $\pm$ 10 years) with four classified as HAPE-S.

Experimental design

Baseline control measurements were conducted at sea level (SL) in Heidelberg, Germany (110 m). Between 2 and 4 weeks later, subjects travelled to Alagna, Valsesia, Italy. They were transported by cable car to 3200 m before trekking to 3611 m where they spent the night (the Gnifetti Hut). In the morning they were accompanied by a certified mountain guide while they ascended to 4559 m (Capanna Regina Margherita) within 4–6 h where they remained for the following 2 days.

Blood sampling

At SL subjects attended the laboratory following a 12 h overnight fast. A radial arterial catheter (RA 04020, Arrow International, Inc., Reading, PA, USA) was placed under local anaesthesia (2% lidocaine) and a central venous catheter (Cavafix Certodyn 375, B. Braun Melsungen AG, Melsungen, Germany) was inserted via an antecubital vein and advanced under electrocardiographic guidance into the superior vena cava (Corsten *et al.* 1994). Mixed venous samples would have been preferred, though this was not an option in light of the risks associated with pulmonary artery catheterisation. At HA, catheters were placed within 1 h of arrival to the Margherita Hut. Samples were obtained 3 h thereafter (HA-3h) to ensure that the baseline blood samples were not

contaminated by the combined stresses of catheterisation, exercise and/or post-prandial elevations in blood-borne biomarkers of oxidative–nitrosative–inflammatory stress. We were confident that given the short half-lives of the biomarkers employed that this 'recovery period' was sufficient to ensure an accurate baseline at HA. Catheters were kept patent overnight and the final sample was obtained within 1 h of waking at 07.00 h which equated to a 20 h exposure to 4559 m (HA-20h). With the exception of blood gas measurements, samples were centrifuged at 600 *g* (4°C) for 10 min and serum/plasma/red blood cells (RBC) immediately snap-frozen and stored in liquid nitrogen prior to analysis. Subjects remained at HA for a further 24 h having received medical treatment when necessary prior to guided descent.

Rescue medication

Subjects with clinical and radiographic signs of HAPE were treated with oral nifedipine (2×8 mg day^{-1}) and supplemental oxygen. Those subjects who developed AMS received symptomatic treatment with acetaminophen (500 mg) and/or ibuprofen (400 mg). All measurements were performed prior to pharmacological intervention and no further assessments were made thereafter. A more detailed description of the safety aspects and clinical management of subjects has been described by Swenson *et al.* (2002) and more recently in two separate publications that report further data from the same study described here (Berger *et al.* 2009; Dehnert *et al.* 2010).

Pulmonary haemodynamics

Echocardiography. Doppler echocardiography (Accuson Cypress, Siemens, Germany) was employed for the measurement of PASP according to the modified Bernoulli equation (Allemann *et al.* 2000). Measurements were performed on three cardiac cycles (mean recorded) by an experienced sonographer and subsequently evaluated off-line in random order by a separate sonographer blinded to the study. The inter- and intra-observer co-efficients of variation (C.V.) were both <15%.

Pulmonary flow. Pulmonary blood flow (PBF) was determined non-invasively (Innocor, Innovision A/S, Odense, Denmark) using an oxygen-enriched mixture of 0.5% nitrous oxide and 0.1% sulfur hexafluoride measured with photo-acoustic analysers over a 5-breath interval (Lang *et al.* 2007). Pulmonary plasma flow (PPF) was calculated as PBF × (1 – Hct). The potential limitations of this technique have been discussed by Berger *et al.* (2009) who performed identical measurements in a sub-set of subjects enrolled in the same study. The inter and intra-observer C.V. were both <5%.

Blood gases. Samples were immediately measured with a blood gas analyser (ABL 5, Radiometer, Copenhagen, Denmark) for the measurement of pH, haemoglobin (Hb), partial pressures of oxygen/carbon dioxide (P_{O_2}/P_{CO_2}) and oxyhaemoglobin saturation (S_{O_2}). Haematocrit (Hct) was determined manually following microcentrifugation. Calculation of the alveolar to arterial P_{O_2} difference [[A-a]O_{2diff}] (Fenn *et al.* 1946) assumed that P_{ACO_2} was equal to P_{aCO_2} and the respiratory quotient was 0.85.

Metabolic analyses

Oxidative stress. *Antioxidants.* For ascorbic acid, 900 μl of 5% metaphosphoric acid was added to 100 μl plasma and assayed via fluorimetry based on the condensation of dehydroascorbic acid with 1,2-phenylenediamine (Vuilleumier & Keck, 1993). Plasma α-tocopherol, was determined by HPLC (Catignani *et al.* 1983; Thurnham *et al.* 1988). The intra- and inter-assay C.V. for both assays were <10%.

Ascorbate free radical ($A^{\bullet -}$). Plasma (1 ml) was injected into a high-sensitivity multiple-bore sample cell (AquaX, Bruker Instruments Inc., Billerica, MA, USA) housed within a TM_{110} cavity of an electron paramagnetic resonance (EPR) spectrometer operating at X-band (9.87 GHz) (Bailey *et al.* 2009*a*). Samples were analysed using a modulation frequency of 100 kHz, modulation amplitude of 0.65 gauss (G), microwave power of 10 mW, receiver gain of 2×10^5, time constant of 41 ms, magnetic field centre of 3477 G and scan width of ±50 G for three incremental scans (Buettner & Kiminyo, 1992). After identical baseline correction and filtering, each of the spectral peak-to-trough line heights were normalised relative to $\sqrt{\bar{X}\text{line}}$ width and the mean considered a measure of the relative concentration of $A^{\bullet -}$. The intra- and inter-assay C.V. were both <5%.

Lipid hydroperoxides. Serum lipid hydroperoxides (LOOH) were determined using the ferrous iron/xylenol orange (FOX) assay (Wolff, 1994) with modification. The intra- and inter-assay C.V. were both <4%.

Inflammatory stress. *Cytokines.* Plasma tumour necrosis factor (TNF)-α and interleukin (IL)-6 were measured by ELISA (R&D Systems, Minneapolis, MN, USA). The intra/inter-assay C.V. were both <10%.

Nitrosative stress. *NO metabolites.* Plasma (200 μl) was injected into tri-iodide reagent for the measurement of nitrite (NO_2^-) + *S*-nitrosothiols (RSNOs) by ozone-based chemiluminescence (Model 280i, NOA, Sievers, Boulder, CO, USA) (Rassaf *et al.* 2004). Acidified sulphanilamide (5%) was added to a separate sample and left to incubate in the dark at 21°C for 15 min to permit

differentiation of NO_2^- from RSNOs. RBC (100 μl) were injected into modified tri-iodide (Rogers *et al.* 2005) for the measurement of total RBC-bound NO (NO_2^- + nitrosyl haemoglobin (HbNO) + *S*-nitrosohemoglobin (HbSNO)). All calculations were performed using Origin Peak Analysis software (OriginLab Corp., Northampton, MA, USA). The intra- and inter-assay C.V. for all NO metabolites were <10%.

3-Nitrotyrosine. Plasma 3-nitrotyrosine (3-NT) was measured by ELISA (Hycult Biotechnology, b.v., Uden, The Netherlands) and the intra- and inter-assay C.V. were both <10%.

Transpulmonary exchange kinetics. Transpulmonary exchange kinetics was determined according to the Fick Principle:

$$\text{Exchange} = \text{PBF (for RBC} - \text{bound NO) or PPF (plasma/serum} - \text{borne metabolites)} \times \text{a-cv}_\text{D}$$

By convention, a positive value reflects net output or gain and a negative value indicates net uptake or loss across the pulmonary vascular bed.

Altitude illness

HAPE was suspected clinically and confirmed by chest radiography (Vock *et al.* 1989) if at least one lung quadrant demonstrated patchy opacities compatible with interstitial or alveolar oedema. AMS was evaluated using the Lake Louise (LL-cumulative Self Report + Clinical Scores) (Roach *et al.* 1993) and Environmental Symptoms Questionnaire Cerebral (ESQ-C) (Sampson *et al.* 1983) scoring systems. Clinical AMS (moderate to severe) was confirmed by a combined total LL score of $\geq$5 points and ESQ-C score $\geq$0.7 points (Maggiorini *et al.* 1998) upon waking after the first night at HA (HA-20h).

Statistical analysis

Distribution normality was assessed using Shapiro–Wilk *W* tests (SPSS Version 17.0; SPSS Inc., Chicago, IL, USA). Exchange and a-cv_D data were analysed using a combination of one-factor (Location: SL *vs.* HA-3h *vs.* HA-20h) and two-factor (Location: SL *vs.* HA-3h *vs.* HA-20h × Sample Site: Radial Artery *vs.* Central Venous) repeated measures ANOVA with *post hoc* Bonferroni-corrected paired samples *t* test. Relationships were examined using a Pearson product moment correlation. Non-parametric equivalents were applied to TNF-α, IL-6 and ESQ-C data only. Significance was established at $P < 0.05$ and data presented as mean ± S.D. Where indicated, a main effect refers to a pooled (Arterial + Venous) difference between SL *vs.* HA-3h *vs.* HA-20h ($P < 0.05$) or pooled (SL + HA-3h + HA-20h) difference between arterial *vs.* venous ($P < 0.05$).

Results

Altitude illness

LL and ESQ-C scores increased from 1 ± 1 and 0.1 ± 0.2 points respectively at SL to 4 ± 3 and 0.8 ± 0.7 points at HA-3h ($P < 0.05$ *vs.* SL) to 6 ± 2 and 1.0 ± 0.8 points at HA-20h ($P < 0.05$ *vs.* SL). Fourteen subjects were diagnosed with AMS (AMS^+) by HA-20h, three of whom subsequently developed HAPE after 27 h, 37 h and 44 h at HA with radiographic scores of 7, 12 and 5 points, respectively, at the time of diagnosis. Three of the four subjects originally identified as HAPE-S at SL developed HAPE. The remaining 12 subjects reported only mild symptoms of AMS.

Pulmonary gas exchange and haemodynamics

As anticipated, HA was associated with marked hypoxaemia and respiratory alkalosis (Table 1) whereas PBF or PPF did not change relative to SL. PASP was elevated throughout the duration of HA and the increase (value at HA-20h minus the SL control value) was greater ($P < 0.05$) in HAPE subjects ($n = 3$, +27 ± 12 mmHg) whose individual PASP values were 66, 52 and 41 mmHg compared to the remaining (i.e. non-HAPE) controls ($n = 23$, +13 ± 6 mmHg).

Oxidative stress

HA was associated with a general reduction in ascorbate and α-tocopherol (Table 2) whereas no relationships were observed between transpulmonary loss (value at HA-20h minus the SL control value) and both the absolute values or increase in PASP ($P > 0.05$). HA was associated with a marked elevation in $A^{\bullet-}$ and LOOH, taken to reflect an increase in both the systemic and transpulmonary rates of free radical-mediated lipid peroxidation. The underlying reaction and typical EPR spectra of the $A^{\bullet-}$ ($g = 2.00518$; $a^{H4} \approx 1.76$ G) are illustrated in Fig. 1*A* and *B*. The latter provides a visual example of the differences observed in two separate subjects exposed to HA; one who remained healthy and the other diagnosed with HAPE. The increase (value at HA-20h minus the SL control value) in the transpulmonary gain of $A^{\bullet-}$ and LOOH was directly proportional to both the absolute PASP values ($r = 0.60$ and $r = 0.57$, $P < 0.05$) and the increase observed by HA-20h (Fig. 2*A*–*B*, $P < 0.05$). Furthermore, pulmonary gain was selectively elevated ($P < 0.05$) in HAPE subjects (+2132 ± 354 $AU^{\sqrt{G}}$/min+1.98±0.63 μmol min^{-1}) compared to controls (+351 ± 527 $AU^{\sqrt{G}}$/min+0.99 ± 0.67 μmol min^{-1}).

Table 1. Pulmonary gas exchange and haemodynamics

Location:	SL		HA-3h		HA-20h	
Sample site	Radial arterial	Central venous	Radial arterial	Central venous	Radial arterial	Central venous
P_{O_2} (mmHg)	95 ± 11	38 ± 5*	38 ± 3†	28 ± 2*†	41 ± 4†‡	29 ± 3*†
Main effects ($P < 0.05$): HA < SL + Arterial > Venous; interaction effect ($P < 0.05$)						
P_{CO_2} (mmHg)	40 ± 3	48 ± 4*	29 ± 3†	33 ± 4*†	28 ± 2†	32 ± 2*†
Main effects ($P < 0.05$) : HA < SL + Arterial < Venous; interaction effect ($P < 0.05$)						
S_{aO_2} (%)	97 ± 1	70 ± 6*	72 ± 5†	52 ± 5*†	74 ± 5†	50 ± 6*†
Main effects ($P < 0.05$): HA < SL + Arterial > Venous; interaction effect ($P < 0.05$)						
pH (units)	7.41 ± 0.03	7.36 ± 0.01*	7.48 ± 0.02†	7.46 ± 0.02*†	7.47 ± 0.02†‡	7.44 ± 0.02*†‡
Main effects ($P < 0.05$): HA > SL + Arterial > Venous; interaction effect ($P < 0.05$)						
[A-a]O_{2diff} (mmHg)	13 ± 7		10 ± 4		9 ± 5	
PBF (l min^{-1})	6.4 ± 1.4		6.9 ± 1.2		6.2 ± 1.0‡	
PPF (l min^{-1})	3.7 ± 0.8		3.9 ± 0.7		3.5 ± 0.5‡	
PASP (mmHg)	23 ± 4		39 ± 10†		38 ± 10†	

Values are means ± S.D.; SL, sea level; HA, high-altitude; P_{O_2}/P_{CO_2} partial pressure of oxygen/carbon dioxide; S_{O_2}, oxyhaemoglobin saturation; [A-a]$O_{2diff,}$ alveolar to arterial P_{O_2} difference; PBF/PPF, pulmonary blood/plasma flow; PASP, pulmonary artery systolic pressure. *$P < 0.05$ *vs*. radial arterial for given location; †$P < 0.05$ *vs*. SL for given sample site; ‡$P < 0.05$ *vs*. HA-3h for given sample site.

Table 2. Oxidative stress

Location	SL		HA-3h		HA-20h	
Sample site	Radial arterial	Central venous	Radial arterial	Central venous	Radial arterial	Central venous
Ascorbate (μmol l^{-1})	63.3 ± 9.7	62.2 ± 8.8	57.5 ± 7.7	60.2 ± 7.7	52.7 ± 5.5	55.2 ± 10.9
Main effect ($P < 0.05$): HA < SL						
a-cv_D (μmol l^{-1})	+1.1 ± 4.9		−2.7 ± 8.0†		−2.5 ± 8.3	
Exchange (μmol min^{-1})	+4.3 ± 16.9		−11.7 ± 25.8†		−10.1 ± 31.1	
α-Tocopherol (μmol l^{-1})	27.8 ± 8.0	28.4 ± 7.5	26.0 ± 6.6	27.4 ± 6.7	25.7 ± 7.6	26.6 ± 7.9
Main effect ($P < 0.05$): HA < SL						
a-cv_D (μmol l^{-1})	−0.6 ± 2.9		−1.3 ± 6.0		−0.9 ± 3.6	
Exchange (μmol min^{-1})	−2.4 ± 10.9		−5.3 ± 24.2		−3.9 ± 12.5	
$A^{\bullet-}$ (AU$\sqrt{G}$)	1572 ± 237	1536 ± 214	1982 ± 352†	1740 ± 257*†	1934 ± 387†	1736 ± 272*†
Main effects ($P < 0.05$): HA > SL + Arterial > Venous; interaction effect ($P < 0.05$)						
a-cv_D (AU$\sqrt{G}$)	+36 ± 111		+242 ± 270†		+198 ± 222†	
Exchange (AU$\sqrt{G}$ min^{-1})	+137 ± 398		+988 ± 960†		+715 ± 852†	
LOOH (μmol l^{-1})	0.49 ± 0.08	0.54 ± 0.09*	0.84 ± 0.29†	0.64 ± 0.15*†	0.95 ± 0.29†‡	0.69 ± 0.14*†
Main effects ($P < 0.05$): HA > SL + Arterial > Venous; interaction effect ($P < 0.05$)						
a-cv_D (μmol l^{-1})	−0.06 ± 0.07		+0.19 ± 0.20†		+0.26 ± 0.22†‡	
Exchange (μmol min^{-1})	−0.20 ± 0.21		+0.77 ± 0.77†		+0.91 ± 0.81†	

$A^{\bullet-}$, ascorbate radical expressed in arbitrary units (AU) $\sqrt{\text{linewidth}}$ in gauss (G); LOOH, lipid hydroperoxides; a-cv_D, radial arterial minus central venous concentration difference. *$P < 0.05$ *vs*. radial arterial for given location; †$P < 0.05$ *vs*. SL for given sample site; ‡$P < 0.05$ *vs*. HA-3h for given sample site.

Inflammatory stress

An increased transpulmonary gain of TNF-α was observed by HA-20h and the transpulmonary gradient of IL-6 shifted from net loss to gain by HA-20h (Table 3). No relationships were observed between changes (value at HA-20h minus the SL control value) in the output of any of these biomarkers and both the absolute values or increase in PASP ($P > 0.05$). Exchange was not different in HAPE subjects compared to controls ($P > 0.05$).

Nitrosative stress

A progressive decrease in arterial NO_2^- was observed at HA, which had the effect of suppressing transpulmonary

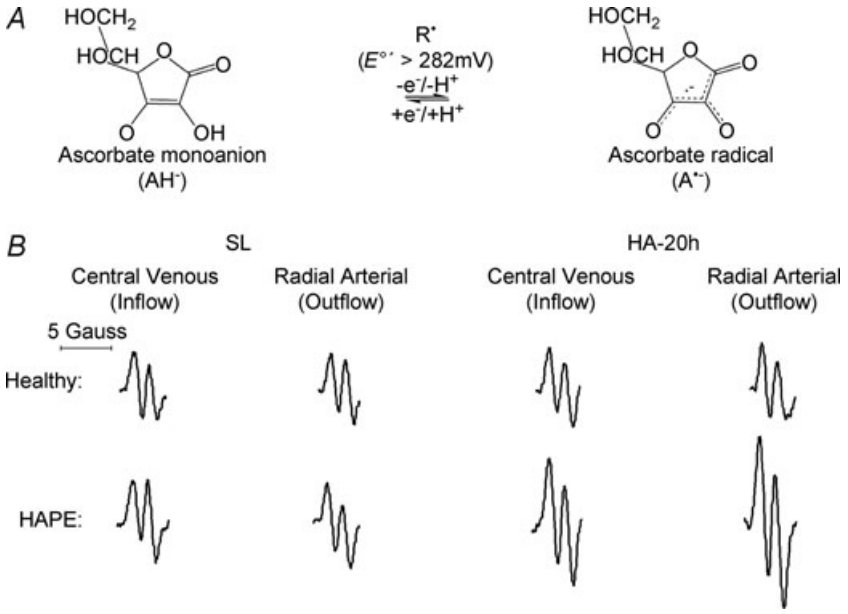

Figure 1
A, oxidation of the ascorbate monoanion (AH^-) by an initiating species ($R^\bullet$) with a one electron reduction potential ($E^{\circ\prime}$) greater than +282 mV to yield the domesticated ascorbate radical ($A^{\bullet-}$). The unpaired electron is delocalised over a highly conjugated tricarbonyl system rendering it resonance-stabilised, thus facilitating direct detection by electron paramagnetic resonance (EPR) spectroscopy. *B*, typical changes in the EPR spectral intensity of $A^{\bullet-}$ in a healthy control and subject with high-altitude pulmonary oedema (HAPE) who presented with the highest PASP value by HA-20h (66 mmHg) and most severe radiographic score (12 points) with oedema present in all 4 quadrants of the lungs.

gain (Table 4). The plasma RSNO and RBC-NO responses were generally antagonistic to that of NO_2^-. The arterial concentration of all bioactive NO metabolites decreased with increasing exposure to HA, which had the effect of shifting the transpulmonary gradient from net gain to loss. No relationships were observed between changes (value at HA-20h minus the SL control value) in the exchange of any of these biomarkers and both the absolute

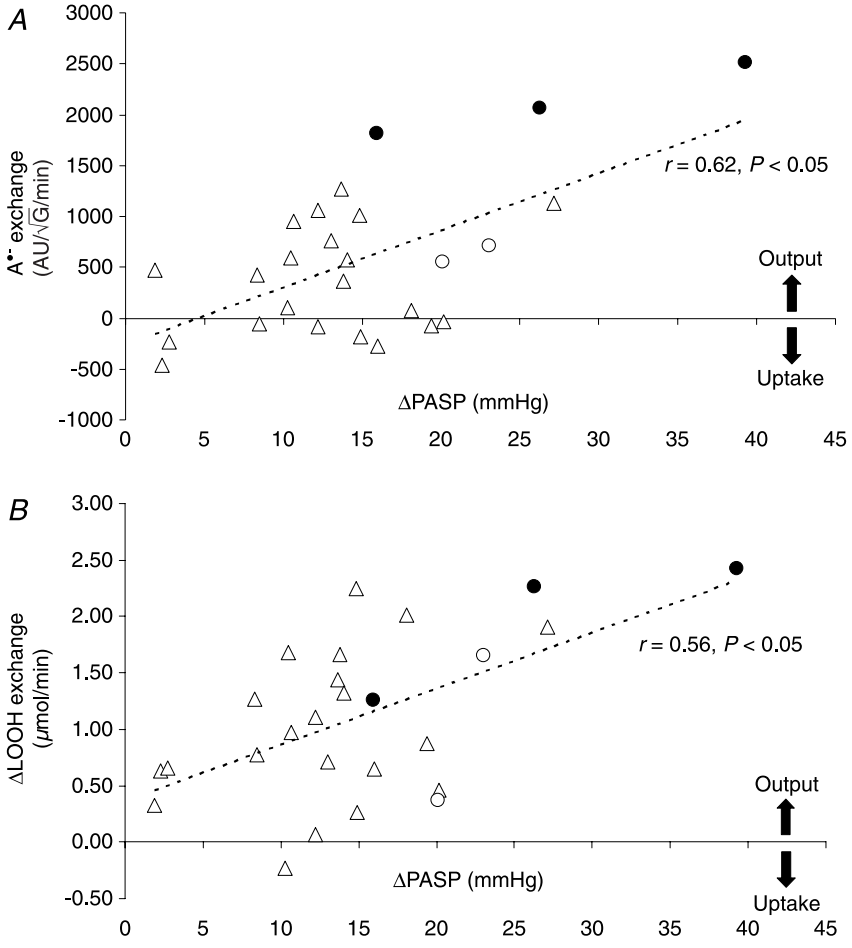

Figure 2. Relationship between changes (Δ, calculated as the value at HA-20h minus the SL control value) in pulmonary artery systolic pressure (PASP) and transpulmonary exchange of the ascorbate radical ($A^{\bullet-}$) (*A*) and lipid hydroperoxides (LOOH) (*B*)
Filled circles represent subjects who developed HAPE ($n = 3$, one of whom was not identified as HAPE susceptible (HAPE-S) at SL) and open circles represent additional subjects identified as HAPE-S but who did not develop HAPE ($n = 2$).

Table 3. Inflammatory stress

Location	SL		HA-3h		HA-20h	
Sample site	Radial arterial	Central venous	Radial arterial	Central venous	Radial arterial	Central venous
TNF-α (pg ml^{-1})	1.47 ± 1.54	1.35 ± 1.31	1.46 ± 1.23	1.38 ± 1.26	1.58 ± 1.24	1.40 ± 1.13*
a-cv$_D$ (pg ml^{-1})		+0.12 ± 0.36		+0.08 ± 0.17		+0.18 ± 0.42
Exchange (pg min^{-1})		+0.48 ± 1.48		+0.30 ± 0.69		+0.64 ± 1.37
IL-6 (pg ml^{-1})	0.50 ± 0.21	0.64 ± 0.38*	1.82 ± 1.29†	1.92 ± 1.23*†	3.28 ± 2.91†‡	3.06 ± 1.61*†‡
a-cv$_D$ (pg ml^{-1})		−0.14 ± 0.23		−0.10 ± 0.24		+0.22 ± 2.61‡
Exchange (pg min^{-1})		−0.58 ± 1.00		−0.36 ± 0.98		+0.77 ± 8.62‡

TNF, tumour necrosis factor; IL, interleukin. *$P < 0.05$ *vs.* radial arterial for given location; †$P < 0.05$ *vs.* SL for given sample site; ‡$P < 0.05$ *vs.* HA-3h for given sample site.

Table 4. Nitrosative stress

Location:	SL		HA-3h		HA-20h	
Sample site:	Radial arterial	Central venous	Radial arterial	Central venous	Radial arterial	Central venous
Plasma NO_2^- (nmol l^{-1})	432.8 ± 107.1	265.0 ± 52.9*	331.2 ± 56.3†	285.9 ± 68.2*	263.6 ± 61.2†‡	249.7 ± 84.5
Main effects ($P < 0.05$): HA < SL + Arterial > Venous; interaction effect ($P < 0.05$)						
a-cv$_D$ (nmol l^{-1})		+167.8 ± 103.8		+45.3 ± 68.1†		+14.0 ± 94.7†
Exchange (nmol min^{-1})		+609.5 ± 414.6		+163.1 ± 242.1†		+65.2 ± 331.0†
Plasma RSNO (nmol l^{-1})	2.3 ± 0.6	19.2 ± 3.8*	3.8 ± 0.6†	18.5 ± 4.6*	3.3 ± 1.0†‡	16.5 ± 5.6*†
Main effects ($P < 0.05$): HA < SL + Arterial < Venous; interaction effect ($P < 0.05$)						
a-cv$_D$ (nmol l^{-1})		−16.9 ± 3.7		−14.6 ± 4.4†		−13.2 ± 5.4†
Exchange (nmol min^{-1})		−62.7 ± 22.1		−57.8 ± 20.3		−45.0 ± 18.1†‡
RBC-NO (nmol l^{-1})	51.4 ± 30.7	106.5 ± 28.6*	90.8 ± 42.1†	183.8 ± 59.1*†	78.1 ± 41.0†	191.4 ± 62.7*†
Main effects ($P < 0.05$): HA > SL + Arterial < Venous; interaction effect ($P < 0.05$)						
a-cv$_D$ (nmol l^{-1})		−55.1 ± 44.9		−93.0 ± 37.5†		−113.3 ± 44.8†
Exchange (nmol min^{-1})		−143.4 ± 131.3		−277.7 ± 114.0†		−323.6 ± 153.6†
Total NO (nmol l^{-1})	486.5 ± 114.2	390.7 ± 66.6*	425.9 ± 84.3†	488.2 ± 113.5*†	345.0 ± 66.1‡	457.6 ± 117.7*†
Main effects ($P < 0.05$): HA < SL + Arterial < Venous; interaction effect ($P < 0.05$)						
a-cv$_D$ (nmol l^{-1})		+95.8 ± 112.5		−62.4 ± 83.9†		−112.6 ± 109.5†
Exchange (nmol min^{-1})		+620.6 ± 778.0		−451.9 ± 573.7†		−684.2 ± 661.2†

NO_2^-, nitrite; RSNO, *S*-nitrosothiols; RBC-NO (red blood cell-bound nitric oxide); Total NO refers to the cumulative concentration of (plasma) NO_2^- + RSNO + RBC-NO (excludes nitrate). *$P < 0.05$ *vs.* radial arterial for given location; †$P < 0.05$ *vs.* SL for given sample site; ‡$P < 0.05$ *vs.* HA-3h for given sample site.

values or the increase in PASP ($P > 0.05$). Likewise, exchange was not different in HAPE subjects compared to controls ($P > 0.05$). Arterial 3-NT (insufficient venous blood precluded exchange measurements) increased from 110.2 ± 136.0 nmol l^{-1} at SL to 145.5 ± 168.2 nmol l^{-1} at HA-3h ($P > 0.05$ *vs.* SL) and 225.0 ± 282.8 nmol l^{-1} at HA-20h ($P < 0.05$ *vs.* SL). The increase in 3-NT at HA-20h correlated with the increase in pulmonary $A^{\bullet-}$ ($r = 0.63$, $P < 0.05$) and LOOH ($r = 0.48$, $P < 0.05$) output and both the absolute values ($r = 0.55$, $P < 0.05$) and the increase in PASP ($r = 0.57$, $P < 0.05$). Furthermore, the increase in 3-NT was selectively elevated ($P < 0.05$) in HAPE subjects (+560.3 ± 545.7 nmol l^{-1}) compared to controls (+59.7 ± 75.9 nmol l^{-1}).

Discussion

The current study has highlighted several important findings and identifies the pulmonary circulation as a contributory source of oxidative–nitrosative–inflammatory stress at HA due in part to inadequate anti-oxidant defence. Consistent with our original hypothesis, HA increased the pulmonary output of free radicals that was accompanied by an equivalent output of lipid peroxidants, pro-inflammatory cytokines and loss of bioactive NO metabolites that correlated directly against both the absolute values and the increases observed in 3-NT and PASP. Together, these findings suggest that HA-induced pulmonary hypertension may be related

to a free radical-mediated reduction in pulmonary NO bioavailability.

Given the low reduction potential of the $A^{\bullet-}$/ascorbate monanion (AH^-) couple ($E^{\circ\prime} = 282$ mV) (Williams & Yandell, 1982), virtually every oxidising free radical that could arise within the pulmonary circulation will react with AH^- to form $A^{\bullet-}$ ($R^\bullet + AH^- \rightarrow A^{\bullet-} + R\text{-}H$) (Buettner, 1993). Thus, the EPR detection of $A^{\bullet-}$ and concomitant loss of ascorbate in the current study provides direct evidence for an increased transpulmonary flux of free radicals during hypoxia. The loss of α-tocopherol would have also been expected to constrain peroxidative stress since the phenolic-OH group located in its chromanol ring scavenges lipid-derived peroxyl radicals ($LOO^\bullet$) faster than the equivalent reaction with lipid side-chains ($\alpha - TOH + LOO^\bullet \xrightarrow[k\approx 8\times10^4\,M^{-1}s^{-1}]{\Delta E^{\circ\prime}\approx+500mV} LOOH + a{-}TO^\bullet$ *vs.* $LOO^\bullet + L-H \xrightarrow[k\approx 10\,M^{-1}s^{-1}]{\Delta E^{\circ\prime}\approx+400mV} LOOH + L^\bullet$) (Wang & Quinn, 1999). However, consumption of these chain-breaking donor antioxidants ultimately failed to terminate chain propagation as indicated by the increased pulmonary output of LOOH, one of the major reactants of lipid peroxidation.

Our data provide unique insight into the corresponding rates of pulmonary free radical-mediated lipid peroxidation, albeit within the constraints of a single arterio-venous transit. The arterial concentration of $A^{\bullet-}$ and LOOH increased by $363 \pm 356\,AU^{\sqrt{G}}$ and $0.46 \pm 0.28\,\mu mol\,l^{-1}$ respectively at HA-20h. Thus, assuming steady-state kinetics and dilution within the extracellular space (~12 litres) (Bailey *et al.* 2006*a*), the total amount of $A^{\bullet-}$ and LOOH formed during the sojourn at HA would have been substantial, equating to $4356\,AU^{\sqrt{G}}$ and $9.20\,\mu mol\,l^{-1}$ respectively implying that net pulmonary 'output' was ~197- and 119-fold greater than the respective rates of 'accumulation'.

These calculations emphasise the extraordinarily high turnover of free radicals across the hypoxic human pulmonary circulation, which is not surprising given the multiple potential sites of ROS formation and inherent susceptibility to peroxidation (Connolly & Aaronson, 2010). Likewise, similar kinetics have been observed across the hypoxic human brain (Bailey *et al.* 2009*b*) suggesting that transvascular free radical output is likely to be common to 'all' organ systems when challenged by hypoxaemia. While not disassociating cause from effect, the linear, albeit modest, relationships observed between oxidant output and rise in PASP are consistent with recent observations *in vitro*. Current evidence (Ward & McMurtry, 2009) suggests that cellular hypoxia triggers mitochondrial $O_2^{\bullet-}$ formation at complex III of the electron transport chain subsequent to increased electron transfer from $UQ^{\bullet-}$ to O_2 (Guzy & Schumacker, 2006). The resultant increase in ROS signalling is thought to initiate pulmonary arterial smooth muscle contraction through Ca^{2+} release from ryanodine-sensitive sarcoplasmic reticulum stores (Waypa & Schumacker, 2008).

Our findings suggest that a free radical-mediated reduction in pulmonary NO bioavailability may prove of equal significance by potentiating HPV. The metal-catalysed reductive decomposition of LOOH can yield secondary $LO^\bullet$ ($Fe^{2+} + LOOH \xrightarrow{k\approx1.5\times10^3\,M^{-1}s^{-1}} Fe^{3+} + OH^- + LO^\bullet$) that have previously been detected in hypoxic human blood (Bailey *et al.* 2004, 2006*b*, 2009*b*). In combination with $O_2^{\bullet-}$, these radicals have the potential to deplete pulmonary NO stores through a diffusion-controlled reaction that yields peroxynitrite ($LO^\bullet/O_2^{\bullet-} + NO^\bullet \xrightarrow{k\approx6-20\times10^9\,M^{-1}s^{-1}} ONOO^-$) (Nauser & Koppenol, 2002) which can promote nitration of tyrosine residues to form the stable reactant 3-NT, our molecular 'footprint' of ROS-mediated NO inactivation (Pacher *et al.* 2007).

Thus, the blunted systemic and pulmonary output of NO_2^- combined with the systemic rise in 3-NT in the current study suggests that NO bioavailability is likely to have been decreased at HA subsequent to oxidative inactivation. This finding agrees with previous reports documenting a reduction of NO in expired air (Duplain *et al.* 2000), loss of NO_2^- and nitrate in broncho-alveolar lavage fluid (Swenson *et al.* 2002) and impaired endothelium-dependent vasodilatation in the systemic circulation during hypoxia, especially in HAPE susceptible individuals (Berger *et al.* 2005). Additional NO_2^- loss may be attributable to reduced NO synthesis subsequent to limited O_2 substrate bioavailability (Le Cras & McMurtry, 2001) though this has recently been questioned (Steiner *et al.* 2002). Alternatively, it may reflect increased consumption catalysed by deoxyHb/deoxyMb and/or xanthine oxidoreductase/aldehyde oxidase-mediated reduction to NO and subsequent re-apportionment towards longer-lived species notably Hb-NO and Hb-SNO driven by the quite substantial differences in transpulmonary O_2 gradients (van Faassen *et al.* 2009). Whether the dynamic interchange of NO from the plasma to the RBC compartment serves to improve pulmonary oxygenation during hypoxia remains to be determined.

It remains to be established whether pulmonary oxidative–nitrosative stress was the cause or simply the consequence of HPV and HAPE. The early and progressive accumulation of oxidants–nitrosants lends tentative support for a causal role, which is not unreasonable given that these molecules have the thermodynamic potential to promote autocatalytic destruction of the pulmonary lipid membrane structure through

cross-linking polymerisation and breakage of fatty acid side chains (Roubal & Tappel, 1966). These species may be responsible for the degradation of proteogylcans and subsequent disruption of the endothelial basement membrane and pulmonary extracellular matrix observed in hypoxia (Miserocchi *et al.* 2001). When combined with elevated intravascular hydrostatic pressure, this can result in capillary stress failure, destruction of tissue matrix structures, increased alveolar–capillary membrane permeability and alveolar haemorrhage (Bartsch & Gibbs, 2007). Localised inflammation is likely to be a secondary event as suggested by the delayed pulmonary output of cytokines, which agrees with previous observations (Swenson *et al.* 2002).

Alternatively, these biomolecules have the capacity to serve as integral components of the signal transduction cascade that are physiologically essential in controlled though as of yet undefined amounts, capable of initiating protective adaptation in the face of hypoxic stress for the maintenance of homeostasis (Bailey *et al.* 2001; Guzy & Schumacker, 2006; Pryor *et al.* 2006). Though speculative, the oxidative-nitrosative stress response that may ultimately predispose to pulmonary hypertension at HA may represent a physiological attempt to regain homeostasis, a concept that warrants investigation in future studies.

In conclusion, these findings are the first to demonstrate a net pulmonary output and systemic accumulation of oxidative–nitrosative–inflammatory stress biomarkers at HA due in part to inadequate antioxidant defence. A free radical-mediated reduction in pulmonary NO bioavailability may prove the unifying mechanism underlying HA-induced pulmonary hypertension and subsequent susceptibility to HAPE which has broader implications for other human pulmonary diseases characterised by arterial hypoxaemia such as chronic obstructive pulmonary disorder (COPD). The improved clinical outcome of COPD patients given supplemental O_2 may be due to the suppression of hypoxia-mediated pulmonary ROS formation (Zouaoui Boudjeltia *et al.* 2009). Follow-up interventional studies need to consider combined ascorbate and α-tocopherol prophylaxis in order to optimise the chain-breaking antioxidant capacity of the pulmonary circulation at HA.

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