The Effects of Acute Hypoxia on Tissue Oxygenation and Circulating Alarmins in Healthy Adults

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Summary

The binding of high-mobility group box-1 (HMGB-1) to the membrane receptor for advanced glycation end-products (mRAGE) is a key early mediator of non-infectious inflammation and its triggers include ischaemia/hypoxia. The effects of acute hypoxia on soluble RAGE (sRAGE) are unknown. Fourteen healthy adults (50 % women; 26.6±3.8 years) were assessed at baseline normoxia (T0), followed by four time-points (T90, 95, 100 and 180 min) over three hours of continuous normobaric hypoxia (NH, 4,450 m equivalent) and again 60 min after return to normoxia (T240). A 5-min exercise step test was performed during NH at T90. Plasma concentrations of HMGB-1, sRAGE VCAM-1, ICAM-1, VEGF IL-8 and IL-13 were measured using venous blood. Arterial and tissue oxygen saturations were measured using pulse oximetry (SpO_2) and near-infrared spectroscopy (StO_2), respectively. NH led to a significant reduction in SpO_2, StO_2, sRAGE and VEGF, which was compounded by exercise, before increasing to baseline values with normoxic restoration (T240). NH-exercise led to a paired increase in HMGB-1. sRAGE inversely correlated with HMGB-1 (r=-0.32; p=0.006), heart rate (r=-0.43; p=0.004) but was not linked to SpO_2 or StO_2. In conclusion, short-term NH leads to a fall in sRAGE and VEGF concentrations with a transient rise post NH-exercise in HMGB-1.

Key words

Acute hypoxia • Normobaric • Alarmins • sRAGE • Exercise

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Introduction

There is evidence to suggest that acute hypoxia can trigger a pro-inflammatory response, however the published data are inconsistent (Tamura *et al.* 2002), Rohm *et al.* 2015, Iglesias *et al.* 2015, Burki and Tetenta 2104). Research has tended to focus on more traditional infection-related inflammatory cytokines/biomarkers, yet hypoxia reflects a non-infectious stimulus (Burki and Tetenta 2014). Hence, the assessment of other 'sterile' pro-inflammatory mediators and their receptors may be more relevant to this environment (Rider *et al.* 2012).

The damage-associated molecular patterns (DAMPs; alarmins) are particularly attractive in this regard as they are associated with non-infectious inflammatory responses such as ischaemia, acute hypoxia and trauma as well systemic inflammatory conditions (Bianchi 2007, Feldman *et al.* 2015). They are released by stressed or tissue damaged cells leading to the early activation of endogenous danger signals and inflammatory responses (Bianchi 2007). One of the best known DAMPs is high mobility group box-1(HMGB-1).

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Its activation receptor includes the transmembrane, multi-pattern-recognition receptor for advanced glycation end products (mRAGE) (Sparvero *et al.* 2009 Alexiou *et al.* 2010). HMGB-1 binding to mRAGE leads to immediate and prolonged inflammatory response and increased expression of cellular adhesion molecules (e.g. vascular cell adhesion molecule-1 [VCAM-1] and selectins) and angiogenic factors (e.g. vascular endothelial growth factor [VEGF]). Limited available evidence suggests that HMGB-1 production is upregulated during hypoxia and that RAGE activation contributes the development of hypoxia-induced pulmonary hypertension (Jia *et al.* 2017). Membrane bound RAGE is very difficult to measure *in vivo*, however soluble forms of RAGE (sRAGE) also exist and possess a similar affinity for ligands to mRAGE. It is formed either by alternative splicing of RAGE mRNA or by proteolytic cleavage of full-length mRAGE protein. Whilst the actions of sRAGE have not been fully elucidated, it appears to elicit a counter-regulatory role by acting as a 'decoy receptor' to neutralise AGE-mediated inflammation and damage. Reduced levels of sRAGE have been identified across a number of known inflammatory conditions and appear to be inversely related to disease activity state (Raucci *et al.* 2008, Prasad 2014). Animal data has shown that sRAGE may be protective against ischaemia reperfusion injury and hypoxia-induced right ventricular pressure increase, raising several potential translational clinical applications (Farmer *et al.* 2014, Zeng *et al.* 2004).

In this study we aimed to investigate, for the first time, the effects of effect of acute hypoxia on sRAGE its relationship to tissue oxygenation and other pro- and anti-inflammatory mediators including HMGB-1 in healthy humans.

Methods

Study population

This was a prospective observational study of 14 fit and healthy serving British military adults. All participants were required to have abstained from caffeine, alcohol, non-steroidal anti-inflammatory drugs and smoking for >12 h prior to the study and to have avoided strenuous physical activity for 48 h prior to the experiment. Confirmation of health status was confirmed following a detailed health questionnaire and baseline echocardiogram to confirm suitability for inclusion. Pregnant women and persons suffering from an inter-current illness were excluded.

Ethics

The study was approved by the Ministry of Defence Research and Medical Ethics Committee and was conducted according to the standards of the declaration of Helsinki. All subjects underwent written informed consent for the study.

Study protocol

Subjects were examined across five separate continuous time points. The first baseline measured time point was at normoxic rest at near sea level (113 m) prior to acute nomobaric hypoxic (NH) exposure (T0). Four further time points were assessed during exposure to continuous NH in an experimental NH chamber at 90 (T90), 95 (T95), 100 (T100) and 180 (T180) min. The subjects were then studied for the final time 60 min post hypoxia (240 min, T240) under identical normoxic conditions to the baseline assessments.

The NH chamber (TISS, Alton, UK and Sporting Edge, Sherfield on Loddon, UK) was set to an FiO_2 of ~11.4 % (considering daily fluctuations of barometric pressure) which was equivalent to an altitude of 4,450 m (PiO_2 81.50 mm Hg) (Conkin 2011). In order to identify the potentially compounding effects of exercise, all subjects underwent a 5-min step test at minutes 90-95 of the 180 min during NH. SpO_2, StO_2 and only were obtained at end exercise (T95) to assess the intensity of exercise stimulus under NH. The chamber temperature was controlled at 21 °C throughout the study.

Physiological measurements

Resting recordings of peripheral arterial oxygen saturations (SpO_2) were performed from a sensor placed at the finger tip of either index finger using a Nellcor N-20P pulse oximeter (Nellcor Puritan Bennett, Coventry, UK). Tissue oxygen saturations (StO_2) were measured using near infrared spectroscopy as previously described (NIRS; INVOS, Somenetics, Michigan, USA) with sampling from the right frontal area of the brain and right deltoid muscle, two finger breadths above the muscle's insertion on the humerus (Scheeran *et al.* 2012).

Blood sampling

Venous blood samples were drawn from an indwelling cannula which was inserted into the antecubital fossa for the duration of the study. The samples were drawn into EDTA bottles and centrifuged at 10,000 rpm for 5 min immediately on site and the supernatant plasma was frozen at -80 °C for later analysis. Samples were analysed with enzyme linked immunosorbent assays (ELISAs) for the presence of HMGB-1 (Luminex ELISA, IBL International, Hamburg, Germany), sRAGE (DuoSet ELISA, R&D Systems, Oxford, UK) and for VCAM (Vascular cell adhesion molecule)-1, ICAM (Intercellular adhesion molecule)-1, VEGF (Vascular endothelial growth factor) and soluble E-Selectin. Multiplex technology was used to measure interleukin (IL)-8 and IL-13 using a commercial multiplex kit (R&D Systems). Standard curves for the ELISAs were produced using the spreadsheet program Excel (Microsoft). Plasma concentrations were then obtained by interpolation from these standard curves using the statistical software program Prism (GraphPad Software, San Diego, CA, USA).

Statistical methods

Data were analysed using GraphPad InStat version 3.05 and SPSS version 22. The figures were generated using GraphPad Prism version 4.00 for Windows. Data inspection and the Kolmogorov-Smirnov test was undertaken to assess normality of all continuous data which was presented as the mean ± standard deviation and as median ± inter-quartile range for all parametric and non-parametric data, respectively. Categorical variables were compared using Fishers Exact test. Comparison of unpaired data was performed using an unpaired T test and a Mann-Whitney test for parametric and non-parametric data, respectively. Paired two group comparisons of continuous data were performed using a paired test and Wilcoxon matched-pairs signed-ranks test for parametric and non-parametric data, respectively. The time-dependent changes in continuous data were assessed with repeated measures ANOVA for normally distributed data, with the Tukey post-test (comparing with baseline) for all significant results. Repeated measures of non-parametric continuous data were performed using the Friedman test with the Dunn post-test (comparing with baseline) for all significant results. If a significant overall main effect for time (hypoxia) was found in circulating biomarker concentrations an additional exploratory analysis was undertaken using a two-way factorial repeated measures ANOVA. This was done to discern whether there was an additional main effect for sex (men vs. women) on the changes in circulating markers (e.g. sRAGE) and any evidence of its potential interaction (sex x time/hypoxia) on their concentrations. Correlations were performed using Pearson and Spearman rank correlation (±95 % confidence interval, CI) for parametric and non-parametric data, respectively. A two-sided p value of <0.05 was considered significant.

Sample size calculation

In a previous acute hypoxia study Iglesias *et al.* (2015) observed a significant increase in a number of pro-inflammatory cytokines (e.g. hsCRP and TNFα) in 10 healthy subjects exposed to a simulated altitude of 4,000 m. Whilst there have been no healthy human studies that have assessed the effects of acute hypoxia on sRAGE levels, in a previous animal study Gopal *et al.* 2015) demonstrated a significant increase in sRAGE among 8 mice over 21 days of simulated hypoxia (FiO_2 down to 8 %). Hence, we estimated that a sample size of 14 subjects exposed to three hours of simulated hypoxia to an equivalent altitude of at least 4,000 m should provide both sufficient power and a hypoxic stimulus to detect a potential change in sRAGE if genuine. Given the recognised potential differences in expression and concentrations of various circulating biomarkers between men and women and in their physiological responses to hypoxia we aimed to recruit an equal number of men and women (Boos *et al*. 2016a, Planchard *et al*. 2009).

Results

The average age of the 14 Caucasian subjects was 26.6±3.8 (range 21-33) years, with an even number of men (n=7, 50 %) and women (n=7, 50 %). All participants completed the entire study and none were on regular medication. All women were on the oral contraceptive pill. The mean body mass index was 24.7±2.7 kg/m^2 and only one subject (7.1 %) was a current smoker.

Changes in physiological measures and oxygen saturation

Acute NH led to a significant increase in resting heart rate (Table 1). Heart rate significantly rose from 73.9±11.0 to 139.1±18.9/min with exercise (p<0.0001).

Frontal StO_2 was lower than deltoid StO_2 at all time points and were both lower than peripheral SpO_2 values (Fig. 1, Table 1). Peripheral SpO_2, deltoid and frontal lobe StO_2 all significantly fell during acute NH compared with baseline normoxia (T0). This fall was greatest immediately post exercise and all values returned to near baseline levels at T240 when normoxia was restored (Table 1). Peripheral SpO_2 correlated with both deltoid (r=0.53; 95 % CI: 0.37 to 0.66) and frontal (r=0.72; 95 % CI: 0.60 to 0.81: p<0.0001) StO_2. Deltoid and frontal StO_2 were significantly correlated (r=0.40; 0.21 to 0.55; p<0.0001).

Alarmin/cytokine level changes

There was a significant main effect for time (and hypoxia) on plasma sRAGE and VEGF concentrations, which significantly fell during acute NH and returned to near baseline values when normoxia was restored at T240 (Table 1, Figs 2 and 3). Although sRAGE concentrations tended to be higher and VEGF concentrations lower there was no significant main effect for sex (men versus women) or sex-time (hypoxic exposure) interaction on sRAGE and VEGF concentrations. There was a paired fall in IL-13 from T90 to T100 (p=0.04) and an increase in HMGB-1 from T90 to T100 (p=0.049) but again no main effect for sex. The plasma levels of IL-8, VCAM-1 and ICAM-1 did not change significantly over time (Table 1). sRAGE non-significantly increased (=15.4 %) with exercise (to 100 vs. T90) (Table 1, Fig. 2).

sRAGE inversely correlated with heart rate (r=-0.43; -0.66 to -0.14: p=0.004) and HMGB-1 concentrations (r=-0.32; -0.52 to -0.09: p=0.006). There was no significant correlation between sRAGE concentrations and peripheral or tissue oxygen saturation. SpO_2 inversely correlated with heart rate (r=-0.70; -0.81 to -0.55; p<0.0001).

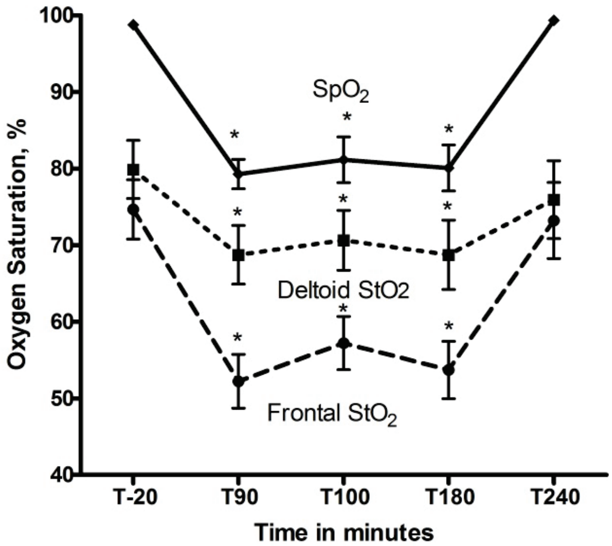

Fig. 1. Changes (mean, 95 % CI) in peripheral (SpO_2) and deltoid and frontal tissue oxygen (StO_2) Saturations with normoxia (T0 and T240) and 3 h of hypoxia (T90, T100 and T180); * significant change vs. baseline (T0).

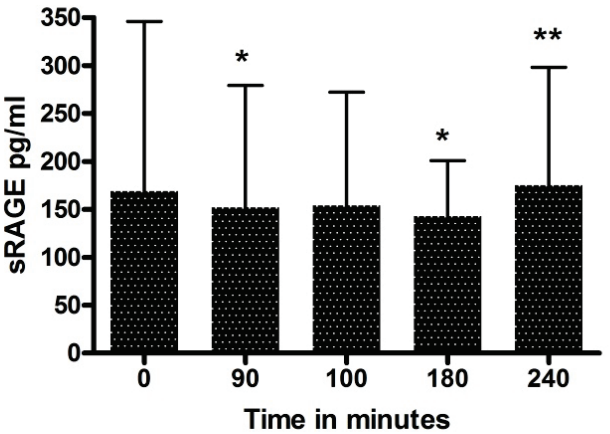

Fig. 2. Changes in sRAGE (median, interquartile range) with acute normobaric hypoxia (T90, T100 and T180); * significant change vs. baseline (T0); ** vs. T180.

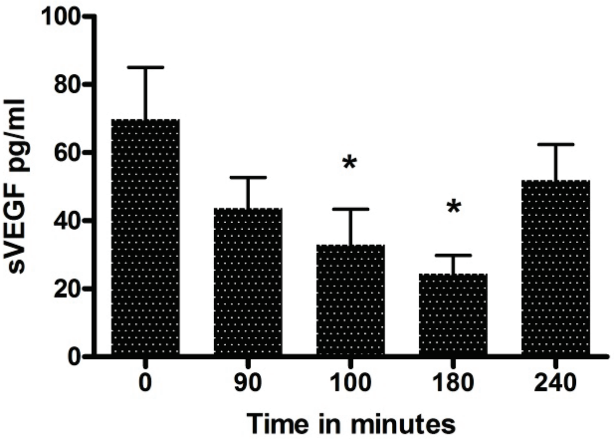

Fig. 3. Changes in VEGF (mean, standard deviation) with acute normobaric hypoxia (T90, T100 and T180); * significant change vs. baseline (T0).

Discussion

This is the first human study to assess the effect of acute NH on the levels of sRAGE and its relationship to peripheral and tissue oxygenation. NH led to a significant increase in heart rate and fall in peripheral arterial (SpO_2) and tissue oxygen (StO_2) saturation and a reduction in sRAGE and VEGF levels compared with baseline sea level normoxia. Brief exercise during NH was associated with a transient yet significant fall in IL-13 and an increase in HMGB-1 concentrations.

In this study, StO_2 was, as to be expected, consistently lower than SpO_2 with normoxia and acute NH. This reflects the fact that SpO_2 measures arterial oxygen saturation whereas StO_2 assesses tissue oxygen saturation and a degree of deoxygenated blood (Subudhi *et al.* 2007). Frontal (cerebral) StO_2 was, however, lower than that measured over the deltoid muscle at all time

Table 1. Changes in physiological parameters and vascular biomarkers with acute hypoxia.

	Normoxia	Normobaric hypoxia					Normoxia	P value
	T0	T90	5 min exercise	T95	T100	T180	T240	
Heart rate (min^{-1})	64.4 ± 7.3	73.9 ± 11.0		139.1 ± 18.9†	79.6 ± 11.0‡	82.3 ±7.9	65.3 ± 8.8	<0.0001 abcd
SpO_2 (%)	98.8 ± 0.4	79.3 ± 3.3		71.5 ± 4.8†	81.1 ± 5.1‡	80.1 ± 5.2	99.4 ± 1.2	<0.0001 abcd
STO_2 deltoid (%)	79.9 ± 6.6	68.7 ± 6.6		67.3 ± 8.0	70.6 ± 6‡	68.7 ± 7.8	75.9 ± 8.8	<0.0001 abcd
STO_2 frontal (%)	74.7 ± 6.7	52.2 ± 6.1		52.7 ± 6.1	57.2 ± 6.0‡	53.7 ± 6.0	73.2 ± 8.6	<0.0001 abcd
E-selectin (pg/ml)	2042	1796		-	2229	1976	1828	0.89
	[1636-2510]	[1003-2462]			[1254-2956]	[1746-2916]	[1485-2944]	
sRAGE (pg/ml)	169.0	152.0		-	154.3	142.8	175.3	<0.0001 a,d
	[144.3-346.0]	[134.3-279.2]			[114.7-272.4]	[91.6-200.7]	[131.3-298.1]	
HMGB-1 (pg/ml)	53.3	36.5		-	241.8†	44.6	135.8	0.56
	[0.0-606.1]	[0.0-227.2]			[0.0-701.9]	[0.0-648.2]	[0.0-912.2]	
VEGF (pg/ml)	65.9 ± 52.6	40.4 ± 32.2		-	37.5 ± 38.1	24.4 ± 18.7	51.9 ± 36.4	0.01 c,d
VCAM (pg/ml)	410.8 ± 158.5	435.1 ± 161.0		-	401.0 ± 113.4	375.7 ± 96.9	402.7 ± 102.4	0.45
ICAM (pg/ml)	256.7 ± 89.1	290.2 ± 62.3		-	263.7 ± 57.4	251.9 ± 54.0	256.8 ± 56.2	0.24
IL-8 (pg/ml)	2.0	3.2		-	2.8	2.4	2.8	0.70
	[0.0-7.0]	[0.40-7.40]			[0.0-6.80]	[0.0-7.4]	[0.0-6.40]	
IL-13 (pg/ml)	1.80	2.15		-	1.60‡	3.20	2.30	0.21
	[0.0-7.0]	[0.0-8.0]			[0.0-6.0]	[0.0-7.90]	[0.0-5.8]	

Results of post-test vs. baseline (T0) vs.: a T90, b T95, c T100, d T180, e T240; significant paired differences: † vs. T90, ‡ vs. T95.

points. This finding supports the limited published comparative data and likely reflects the greater oxygen extraction in cerebral versus deltoid muscle tissue (Rupp *et al.* 2013). Despite generating a significant hypoxic stimulus we failed to observe a significant increase in any of the inflammatory markers measured with NH, apart from a borderline rise in HGMB-1 following brief exercise. This finding was unexpected given the cited links between hypoxia and inflammation (Tamura *et al.* 2002, Rohm *et al.* 2015, Iglesias *et al.* 2015). However, there is contrary data where a hypoxia-inflammatory link has not been demonstrated (Tamura *et al.* 2002, Burki and Tetenta 2014, Woodside *et al.* 2014, Ylimaz *et al.* 2016). These differences in study findings could be explained by dissimilarities in study population (age and sex), methodology including the duration, type (normobaric versus hypobaric hypoxia) and severity of hypoxia and in the biomarkers studied. In fact, the most consistent data have been with terrestrial high altitude, where a number of confounding factors may be more relevant (Boos *et al*. 2016b, West 2012). These include the greater physical (e.g. cold) and exercise challenge, sleep deprivation and increased psychological stress (Boos *et al.* 2016b*,* West 2012).

In this study we introduced a short duration of exercise using a 5-min step test over the 90-95th min of NH. This was undertaken in order to assess the relative impact of exercise during acute NH on arterial/tissue oxygenation and vascular inflammation. SpO_2 fell further on exercise whereas tissue oxygenation (both deltoid and frontal StO_2) was maintained. This would appear to suggest preservation of tissue oxygenation and its microcirculation compared with arterial oxygenation during exercise (Ide and Secher 2000). By five minutes post exercise and hence its early recovery (T100) both SpO_2 and StO_2 had significantly increased compared with the values at end exercise (T95). This mild increase in both SpO2 and StO_2 post exercise could reflect local vasodilatation or a shift in the oxygen dissociation curve to repay an oxygen debt accumulated during exercise.

RAGE is a multi-ligand, pattern recognition receptor, allowing it to act as an early sensor of DAMPs and act as an early trigger receptor in acute inflammation (Lin *et al.* 2006, Bapp *et al.* 2008). Once activated, it initiates transcription factor pathways and expression of various pro-inflammatory cytokines. While the full RAGE is membrane bound, it is the extracellular portion that is converted to the soluble form in the circulation (sRAGE) by cleavage and shedding from the membrane bound form. This occurs at a steady background rate but is increased by ligand binding (Raucci *et al.* 2008). Reduced plasma levels of sRAGE have been reported in a number of acute and chronic 'sterile' inflammatory conditions and have been linked to disease severity (Prasad 2014, Maillard-Lefebvre *et al.* 2009). However, the data does appear to be conflicting and depends on the study population, disease state and its chronicity (Nakamura *et al.* 2011, Prasad 2014).

We observed a fall in sRAGE levels with acute hypoxia compared with baseline normoxia. This fall was not different among men and women. RAGE engages numerous ligands and its signalling is highly complex and influenced by a multitude of different factors including ligand identity/type and concentration, cell type as well as the surface concentration of RAGE. Furthermore, sRAGE reflects both cleaved and endogenous secretary RAGE and the relative proportion and activity of each can be highly variable (Tang *et al.* 2009). Soluble RAGE is known to neutralize AGE-mediated damage by acting as a decoy and competitive inhibitor of ligand-RAGE interaction and downstream inflammatory cascades (Lindsey *et al.* 2009, Kalea *et al.* 2009). Hence, we speculate that the reduction in sRAGE observed in this study could reflect sequestering of AGEs, reduced VEGF expression and might explain the failure of HGMB-1 and other pro-inflammatory levels to rise (Prasad 2014, Keirdorf and Fritz 2014).

The exercise stimulus in this study as brief but intensive and led to a 100 % increase in heart rate and further arterial desaturation and was associated with a transient but non-significant increase in sRAGE. These findings are consistent with a recent study by Danzig *et al.* (2005), in which sRAGE was shown to non-significantly but similarly increase following brief high-intensity exercise (bicycle) in both healthy controls (n=22) and participants with a previous history of coronary disease. Similar to our study they measured sRAGE levels at five minutes post exercise. There are no previous comparative studies with acute hypoxia in healthy humans. In one of the only studies of hypoxia on sRAGE levels it was shown that sustained hypoxia (21 days) led to an increased gene expression of RAGE in lung tissue and a rise in circulating sRAGE of mice (Gopal *et al.* 2015). In contrast to this study, we studied a much shorter period of hypoxia and thus cannot discount the possibility that sRAGE levels could have risen with longer much longer hypoxic exposure. The fact that sRAGE levels fell with acute hypoxia before increasing to near baseline concentrations on return to normoxia does support a genuine short term effect of NH on sRAGE.

VEGF is best known as a mitogen, acting mainly on the vascular endothelium. It is responsible for both pathological and physiological angiogenesis, vasodilatation and capillary hyperpermeability in response to localised hypoxia (Ferrara 2009). VEGF has been shown to act on multiple different inflammatory cells; mediating their survival, proliferation and differentiation (Maharaj and d´Amore 2007). Its precise function as an inflammatory mediator and cytokine is unknown but hypoxia is known to be one of the most potent stimuli for VEGF expression (Maharaj and d´Amore 2007). Increased free plasma VEGF levels have been linked to worsening hypoxia and to the development of acute mountain sickness in a previous study (Tissot van Patot *et al.* 2005). However the wider published data has been inconsistent with several studies reporting a rise and others either a fall or no change in circulating VEGF following acute hypoxia (Pavlicek *et al.* 2000, Oltmanns *et al.* 2005, Gunga *et al.* 1999).

We identified a fall in VEGF with NH which became significant by 100 min of NH, with restoration of baseline values on return to normoxia. This change was not influenced by the subject's sex. There are several potential mechanisms to explain this observed reduction in VEGF with NH. The fact that we measured short term hypoxia and unbound circulating VEGF may be important. It has been proposed that a decrease in VEGF with acute hypoxia might reflect simultaneous upregulation of the soluble VEGF receptor (sFlt-1) which traps soluble VEGF as well as inhibiting its formation (Oltmanns *et al.* 2005). Another proposed mechanism could be hypoxia induced glucose intolerance, which has been reported to inhibit VEGF generation (Oltmanns *et al.* 2004). Unfortunately, we did not measure glucose concentration during this study. One of the key functions of VEGF is to stimulate the mobilisation of endothelial progenitor cells (EPC) from bone marrow to support angiogenesis. It has been recently shown that acute NH (equivalent to 4,100 m) led to a reduction in EPCS and increased EPC apoptosis and markers of oxidative stress in 10 healthy adults, which became significant by ≥30 min of NH exposure (Colombo *et al.* 2012). These changes may be related to a reduction of VEGF with acute NH.

This study has additional strengths and limitations that should be acknowledged. The fact that we studied an equal number of men and women of similar age, a broad range of biomarkers and their relationship to both peripheral and tissue oxygenation are major strengths of this study. However, the sample size in this study is relatively small with significant variance around several measured markers. Hence we cannot exclude the fact that we may not have appreciated a genuine difference due to the sample size. However, our sample size is similar or larger than majority of published work and was sufficient to identify significant differences in sRAGE and VEGF with hypoxia across a number of measured time points. Whilst we did measured three time points at T90, T100 and T180 during acute NH is could be argued that an earlier acute change could have been missed as the first sampling time point was 90 min into acute NH exposure and arguably during a more steady state.

In conclusion, acute NH led to a significant reduction in both peripheral and arterial tissue oxygen saturation and an associated fall in sRAGE and VEGF concentrations. Brief exercise during hypoxia led to a transient fall in IL-13 and increase in HMGB-1 concentrations and an increase in both peripheral and tissue oxygen saturation.

Conflict of Interest

There is no conflict of interest.

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