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Vascular reactivity and biomarkers of endothelial function in healthy subjects exposed to acute hypobaric hypoxia

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ABSTRACT

Aims: The aim of this study was to evaluate the effects of acute hypobaric hypoxia (HH) on vascular reactivity and biochemical markers associated with endothelial function (EF).

Main methods: Ten healthy subjects were exposed to a simulated altitude of 4,000 meters above sea level for 4 hours in a hypobaric chamber. Vascular reactivity was measured by the flow-mediated vasodilatation (FMVD) test. Endothelin-1, high sensitive-C reactive protein (hsCRP), vascular cell adhesion molecule 1, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), paraoxonase and adiponectin levels, and FMVD were evaluated before and after the exposure.

Key findings: Subjects were young (age: 32 ± 6 years), lean [body mass index: 23.9 ± 2.0 kg/m^2, waist circumference: 77(IQR: 72–80) cm], and presented normal clinical and biochemical parameters. No significant changes were evidenced in FMVD in response to HH (pre: 0.45 (0.20–0.70) *vs.* during: 0.50 (0.20–1.22) mm; $p = 0.594$). On the other hand, endothelin-1 (+54%, $p < 0.05$), hsCRP (+37%, $p < 0.001$), IL-6 (+75%, $p < 0.05$), TNF-α (+75%, $p < 0.05$), and adiponectin (−39%, $p < 0.01$) levels were significantly altered post-HH. FMVD was increased in 7 subjects, and it was decreased in 3 individuals during HH exposure. Interestingly, when EF biomarkers were compared between these two subgroups of subjects, only post exposure-adiponectin levels were significantly different (49 ± 5 *vs.* 38 ± 6 μg/ml, respectively, $p < 0.05$).

Significance: HH exposure had an effect on endothelin-1, adiponectin, hsCRP, IL-6, and TNF-α concentration. However, adiponectin was the only biomarker associated with an altered vascular reactivity.

1. Introduction

Annually, more than 100 million tourists are attracted by the mountainous areas around the world [1]. Acute exposure to altitude increases the risk of high altitude related illnesses such as Acute Mountain Sickness, High Altitude Pulmonary Edema (HAPE) and High Altitude Cerebral Edema. However, the exact mechanism responsible for these pathologies remains unclear, and no physiological variables have been fully recognized. In fact, major independent risk factors include history of acute mountain sickness, fast ascent, and lack of previous acclimatization [2,3].

It has been well demonstrated that hypoxia at high altitude is responsible for an increase in the risk of acute mountain sickness [4,5]. In this regard, Goerre et al. [6] reported that the degree of hypoxia-induced acute pulmonary hypertension was associated with increased plasma endothelin-1 (ET-1) levels in healthy mountaineers. Moreover, they described that the increase in ET-1 would be part of an acute response, since ET-1 levels were most markedly increased 2 hours after arrival at high altitude. In agreement, the ACME-1 study demonstrated that the use of ET-1 antagonist at high altitudes effectively controls the hypoxia-induced increase in pulmonary blood pressure [7]. Nevertheless, it is currently not clear whether high ET-1 plasma levels are mediators of the disease or only markers [8].

Altered endothelial response to hypobaric hypoxia (HH) might also contribute to the development of high altitude complications [9,10]. Indeed, Berger et al. showed that people susceptible to acute HAPE featured altered vascular reactivity assessed by the acetylcholine vasodilatation test [9]. Moreover, HAPE patients featured lower nitric oxide concentration in comparison with healthy controls [10]. However, inconsistent results have been obtained when vascular reactivity was assessed in subjects exposed to acute HH [11–13].

Endothelial function involves the concerted action of a wide spectrum of vasoactive substances including nitric oxide, inflammatory cytokines, namely, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, as well as the adipocyte-derived adiponectin [14,15]. Taking into

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consideration that high TNF-α and IL-6, and low adiponectin concentration were associated with an altered vascular reactivity, the circulating levels of these molecules have been proposed as biomarkers of endothelial function.

Evaluation of vascular reactivity could be of relevance when counseling mountaineering activities and especially in individuals who will perform mountaineering activities for the first time [16]. As history of acute mountain sickness is not known in these cases, the assessment of vascular and cardio-respiratory responses to hypoxia could help to identify subjects at risk of high altitude related illnesses. In this context, the aim of the present study was to assess the vascular reactivity, measured by the flow-mediated vasodilation (FMVD) test and by different biochemical markers in healthy subjects exposed to HH. We hypothesized that hypoxia would lower FMVD and that altered levels of endothelial function biomarkers would adequately identify subjects with an altered vascular response.

2. Materials and methods

2.1. Subjects

Volunteers were invited to participate in this interventional study through an advertisement published in the website of the Argentine Society of Mountain Medicine and in a local mountain journal. Inclusion criteria were male gender, mountaineers or sportsmen, age between 21 and 40 years old, normal body mass index (BMI), absence of any cardiovascular risk factor, and not under chronic treatment [17,18].

It is important to note that for people born or living at sea level, the altitude acclimatization is a reversible physiological process, which is held or maintained while the individual is exposed to hypoxia, either permanently or occasionally. However, once the individual (including mountaineers) descends to sea level, the altitude acclimatization phenomenon disappears quickly in 2–3 weeks, leaving a residual effect for up to 3 months [19]. Importantly, in our study, we enrolled subjects who had not been exposed to altitude (including flights) in the 6 months prior to the study. In this sense, the individuals studied could be considered as naive for new exposures. At the same time, based on their previous experiences, it was much unlikely that they could suffer from any severe adverse event in the chamber, which would have prevented carrying out the study.

Thirty-eight subjects applied for the study and ten of them met all the inclusion criteria [age: 32 ± 6 years; BMI: 23.9 ± 2.0 kg/m^2; and waist circumference: 77 (72–80) cm]. We used these strict inclusion criteria to narrow the well-known inter- and intra-subject variability of FMVD [20–22].

Confirmation of the health status was undertaken via a detailed medical history, clinical examination, chest X-ray, electrocardiogram (ECG), cardiopulmonary exercise test, and biochemical studies.

Two independent medical ethics committees approved the study: The Institute of Aerospace Medicine (IMAE) depending on the Argentine Air Force and the Foundation for Neurological Disease Control of Children (FLENI). All participants gave and signed their informed written consent.

2.2. Simulated altitude assay

All volunteers avoided drinking alcohol, performing exercise training, and using any drug 72 hours previous to the study. The study was carried out in a cylindrical 8 seat hypobaric chamber (HC); three seats were removed to place a stretcher to perform the ultrasound test. The group of 10 subjects was exposed to 4,000 meters above sea level in the HC for about 4 hours in two stages of 5 volunteers each one [23,24]. Barometric pressure in the HC was reduced at a rate of 1,000 meters every 5 minutes in accordance with the protocol of Ohrui et al. [23]. Inside the HC, the group of volunteers was accompanied by two investigators who breathed enriched oxygen via a facemask during all the study. Every hour, high altitude symptoms were assessed by a brief clinical check-up (including blood pressure, heart rate, breathing rate, and finger pulse oximetry) and by performing Lake Louise acute mountain sickness scores. Recompression was made at the rate of 1,000 meters every 5 minutes until reaching sea level altitude.

2.3. Anthropometric and fitness-related measurements

Height and weight were measured with subjects wearing light clothing and without shoes. After the participant had been seated for at least 5 minutes, a trained physician recorded blood pressure using a random-zero sphygmomanometer.

Oxygen saturation and heart rate were measured by a pulse finger oximeter (Onyx II 9500, Plymouth, USA). ECG records were made with a 12 lead ECG AT-3 device (Schiller, Baar, Switzerland). Cardiopulmonary exercise test was performed with Medgraphics CPX-D (Saint Paul, Minnesota, USA) using Bruce protocol.

2.4. Endothelial function assessment

FMVD in the brachial artery was assessed by the same trained professional (DI) using two dimension-ultrasound with portable Sonosite Titan device (Bothell USA) and a 5–10 MHz 25 mm lineal array transducer according to the technique described by Corretti et al. [25]. Briefly, the subject was positioned supine with the arm in a comfortable position for imaging the brachial artery. The brachial artery was imaged above the antecubital fossa in the longitudinal plane. A segment with clear anterior and posterior intimal interfaces between the lumen and vessel wall was selected for continuous 2D grey-scale imaging. To create a flow stimulus in the brachial artery, a sphygmomanometer (blood pressure) cuff was first placed above the antecubital fossa. A baseline rest image was acquired, and blood flow was estimated by time-averaging the pulsed Doppler velocity signal obtained from a mid-artery sample volume. Thereafter, arterial occlusion was created by cuff inflation to suprasystolic pressure. The cuff was inflated to at least 50 mm Hg above systolic pressure to occlude arterial inflow for a standardized length of time. The longitudinal image of the artery was recorded continuously from 30 seconds before to 2 minutes after cuff deflation. A mid-artery pulsed Doppler signal was obtained upon immediate cuff release and no later than 15 seconds after cuff deflation to assess hyperemic velocity.

FMVD was evaluated at baseline (normoxia) the same day of the protocol and exactly before exposure to HH, and then the measurement was repeated during the last minutes of exposition to HH which lasted 4 hours.

2.4.1. Blood sampling and biochemical determinations

Baseline biochemical characteristics were recorded a week before exposition to acute HH. The day of the study, blood samples were drawn from the antecubital vein before and immediately after exposure to HH. Serum and plasma were separated and samples were stored at 4 °C until some aliquots were frozen at −70 °C in a period of time no longer than 8 hours from the first extraction. These samples were used for the assessment of endothelin-1, high sensitivity C reactive protein (hsCRP), vascular cell adhesion molecule 1 (VCAM-1), IL-6, TNF-α, and adiponectin plasma levels.

Complete blood count, glucose, urea, creatinine, lipid profile, and homocysteine were determined employing standardized methods in a Coulter® GEN-S™ autoanalyzer (Beckman Coulter, Fullerton, CA, USA.) and in a Cobas® C501 autoanalyzer (Hitachi, Tokyo, Japan). HsCRP concentration was assayed by an automatized immunoturbidimetric method (Roche Diagnostics, Mannheim, Germany) and endothelin-1, VCAM-1, IL-6, TNF-α, and adiponectin levels employing ELISA assays (R&D Systems, Minneapolis, USA). The activity of the antioxidative enzyme paraoxonase 1 (PON-1) was measured using paraoxon as substrate by the kinetic method described by Furlong et al. [26].

Table 1
Biochemical characteristics from the studied population (n = 10).

	Mean (median)	SD (IQR)
Hb (g/dl)	14.9	0.8
Hematocrit (%)	43	2
WBC (10^3/μl)	6.9	1.6
Glucose (mg/dl)	95	7
Urea (mg/dl)	30	8
Creatinine (mg/dl)	1.02	0.10
TG (mg/dl)	61	23
TC (mg/dl)	175	31
LDL-C (mg/dl)	(93)	(86–117)
HDL-C (mg/dl)	50	15
hsCRP (mg/l)	0.5	0.2
Homocysteine (μmol/l)	9.7	1.5

SD, standard deviation; IQR, interquartile range; Hb, hemoglobin; WBC, white blood cells; TG, triglycerides; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; hsCRP, high sensitivity C reactive protein. Variables are expressed as mean, SD or median, IQR for parametric and non-parametric distribution.

2.4.2. Statistical analysis

Variable distribution was assessed by Shapiro–Wilk's test. Data were expressed as mean ± SD or median [interquartile range (IQR)] for parametric and non-parametric distribution, respectively. FMVD test results were expressed as percentages. Pre- and post-HH differences were evaluated by the T-test or Wilcoxon test for paired samples according to variable distribution. Paired differences were expressed as mean (95% confidence interval). Bivariate correlations were calculated employing the Spearman correlation test. p values < 0.05 were considered significant in the bilateral situation. All the statistical analyses were performed with the INFOSTAT® statistical software (Infostat Group, National University of Córdoba, Argentina) and SPSS® 17.0 (IBM, Illinois, USA).

3. Results

In the present study, 10 young lean men who presented good/above-average maximal oxygen consumption (VO2 max: 52 ± 7 l/min) were exposed to HH. General biochemical characteristics are shown in Table 1. As it may be observed, hematological parameters, markers of renal function, lipid and lipoprotein profile, hsCRP, and homocysteine levels were within reference values.

During the simulated altitude protocol, no serious adverse events took place inside the chamber apart from a mild to moderate ear pain in two volunteers. Importantly, Lake Louis score, blood pressure, and heart rate assessment showed no clinical data of relevance to acute deterioration on the health condition. Cardiovascular health-related parameters at baseline and during acute HH are shown in Table 2. As it was expected, systolic blood pressure and heart rate significantly increased while oxygen saturation decreased during HH (Table 2).

Table 2
Basal and intra-chamber cardiovascular health-related parameters from the studied population (n = 10).

Hypobaric hypoxia				
	Basal	Intra-chamber[a]	Paired differences[b]	p[c]
DBP (mmHg)	76 ± 7	78 ± 8	2 (−4 to 7)	0.278
SBP (mmHg)	122 ± 11	130 ± 9	8 (1–16)	<0.05
Heart Rate (bpm)	64 ± 10	78 ± 9	13 (5–21)	<0.005
O_2 Sat (%)	98 ± 1	80 ± 3	−18 [−20 to (−16)]	<0.001

Data are expressed as mean ± standard deviation or as median (IQR), according to variable distribution. DBP, diastolic blood pressure; SBP, systolic blood pressure; O_2 sat, oxygen saturation. Variables are expressed as mean ± SD or median (IQR) for parametric and non-parametric distribution.
[a] Intra-chamber: Measurements done in the last 30 minutes of exposure.
[b] Paired differences are expressed as mean (95% CI) and calculated as the difference between during and previous to hypobaric hypoxia.
[c] Shown p corresponds to that of T-paired test or Wilcoxon test for paired samples according to the variable parametric or non-parametric distribution, respectively.

Table 3
Flow-mediated vasodilatation test from the studied population (n = 10).

Baseline pre-hyperemia anterior–posterior diameter (mm)			
Pre-exposure	Intra-chamber[a]	Paired differences[b]	p[c]
3.99 ± 0.38	4.01 ± 0.38	0.02 (−0.02 to 0.06)	0.343
Baseline pre-hyperemia flow velocity (m/sec)			
Pre-exposure	Intra-chamber[a]	Paired differences[b]	p[c]
54 ± 8	59 ± 11	4.8 (−2.8 to 12.3)	0.184
Post-hyperemia change in anterior-posterior diameter (mm)			
Δ Pre-exposure	Δ Intra-chamber[a]	Paired differences[b]	p[c]
0.45 (0.20–0.70)	0.50 (0.20–1.22)	0.13 (−0.38 to 0.65)	0.594
Post-hyperemia change in flow (m/sec)			
Δ Pre-exposure	Δ Intra-chamber[a]	Paired differences[b]	p[c]
11.5 (5.5–19.2)	16.0 (8.2–24.3)	4.1 (−6.8 to 15.0)	0.414

Data are expressed as mean ± standard deviation or as median (IQR), according to variable distribution.
[a] Intra-chamber: Measurements done in the last 30 minutes of exposure.
[b] Paired differences are expressed as mean (95% CI) and calculated as the difference between during and previous to hypobaric hypoxia.
[c] Shown p corresponds to that of t-paired test or Wilcoxon test for paired samples according to the variable parametric or non-parametric distribution, respectively.

No significant differences in pre-hyperemia artery diameter and flow velocity were observed before and during HH (Table 3). Then, we compared the magnitude of the baseline and during HH changes (Δ) that occurred post-hyperemia. Vasodilator and flow velocity response after hyperemia were similar before and during HH (Table 3). However, FMVD was increased in 7 subjects (Δ artery diameter: 0.41 ± 0.23 and 0.89 ± 0.44 mm, previous and during HH, respectively; p = 0.027), while it was reduced in three individuals in response to HH exposure (Δ artery diameter: 0.67 ± 0.44 and −0.10 ± 0.16 mm, previous and during HH, respectively; p = 0.106).

Biochemical markers of endothelial function were affected by HH (Fig. 1). HH induced a significant increase in endothelin-1, hsCRP, IL-6, and TNF-α levels while no modification was observed in VCAM-1 (Fig. 1). Furthermore, adiponectin, the only adipokine with cardioprotective actions, resulted to be significantly reduced after exposure to HH (Fig. 1). Finally, HH provoked a slight non-significant decrease in

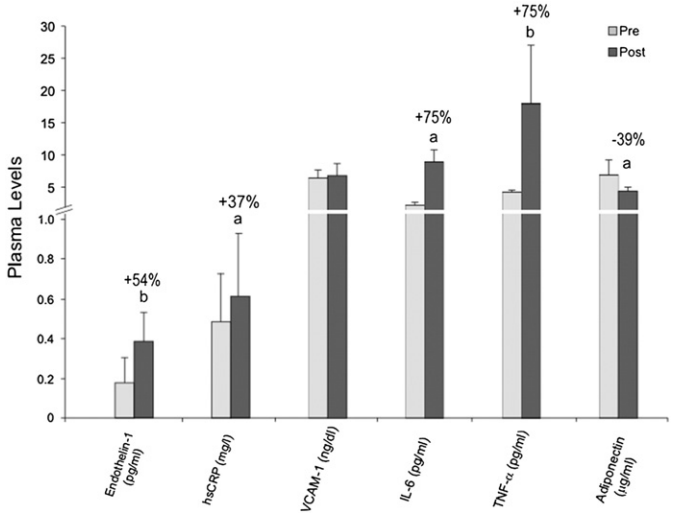

Fig. 1. Biomarkers of endothelial function and adiponectin levels in healthy volunteers exposed to hypobaric hypoxia (n = 10). [a]p < 0.05; [b]p < 0.005. hsCRP, high sensitivity C reactive protein; VCAM-1, vascular cell adhesion molecule 1; IL-6, interleukin 6; TNF-α, tumor necrosis factor- α.

PON-1 activity [Pre: 480 (329–652) *vs.* Post: 401 (279–562) nmol/ml.min; paired difference: −73 (−158 to 10) nmol/ml.min; $p = 0.060$].

When endothelial function biomarkers were compared between the subjects in which FMVD increased *vs.* those in which FMVD decreased, only post-exposure adiponectin levels were significantly different (49 ± 5 *vs.* 38 ± 6 μg/ml, respectively; $p = 0.014$). Furthermore, baseline adiponectin levels were positively correlated with basal maximum oxygen consumption ($r = 0.75$, $p = 0.01$) and negatively with triglycerides ($r = -0.62$, $p < 0.05$) and low density lipoprotein-cholesterol ($r = -0.74$, $p = 0.01$) levels. Changes in adiponectin levels following HH (Δ adiponectin) were correlated with baseline FMVD ($r = -0.66$, $p < 0.05$).

4. Discussion

The most important findings of the present study were the significant reduction observed in adiponectin concentration after HH and its association with an altered vascular reactivity as assessed by the FMVD test. In addition, increments in endothelin-1, hsCRP, IL-6, and TNF-α levels were detected. The strict protocol design used strongly suggests that such changes in endothelial function biomarkers were directly related to the reduction in oxygen supply.

The decrease observed in adiponectin levels is in accordance with recent concepts about the roles played by adipokines in the regulation of vascular tone [27]. Recent data showed that adiponectin-deficient mice developed vascular remodeling, inflammation and pulmonary hypertension, confirming the influence of this adipokine on the vasculature [28]. Finally, in accordance with our results, *in vitro* studies showed that hypoxia (and more specifically the activation of the hypoxia inducible factor-1 α) suppresses adiponectin expression by adipocytes [29]. Nonetheless, other studies conducted in humans had not detected a reduction in adiponectin after acute HH. Adiponectin concentration was reported to remain unchanged and even to increase after longer stays at different altitudes [30,31]. Interestingly, correlation analyses suggest that subjects with better endothelial response at baseline were the ones whose adiponectin levels were less affected after HH exposure. Further on, post-exposure levels of adiponectin were higher in subjects with a vasodilator response than those with a vasoconstrictor response. Therefore, adiponectin levels could represent a novel marker of abnormal endothelial response to hypoxia.

FMVD test, which is a clinical tool to assess endothelial function, was not altered by acute HH. In agreement with our results, Boos et al., who employed a similar protocol, did not find changes in arterial stiffness in response to acute HH [11]. On contrary, biochemical markers of endothelial function were markedly modified by HH. Endothelin-1, which is a gene activated by the hypoxia inducible factor-1 α, [32] was increased after HH. Therefore, endothelin-1 might be of importance in the regulation of the vascular tone in response to acute HH [33]. Depending on the physiological context, endothelin-1 can feature vasoconstrictor or vasodilator effects [34]. According with our results obtained from the FMVD test, it would seem that endothelin-1 exerted a vasodilator effect rather than a vasoconstrictor one. In fact, low levels of endothelin-1, as the ones seen in the subjects studied, may favor vasodilator over vasoconstrictor effects. Therefore, the increment in endothelin-1 levels observed would not be enough to cause a significant vasoconstriction. Other possible explanations for these findings would be that the increase in endothelin-1 had differentially affected particular arterial beds [11,35] increasing cardiac output by the increase of cardiac contractility.

Similar to the findings regarding endothelin-1, hsCRP, IL-6, and TNF-α were also increased after exposure to HH. An increment in IL-6 secretion in response to hypoxia has been demonstrated in a variety of cell types [36,37]. Moreover, Ali et al. [10] showed that hypoxia induced the generation of radical oxygen species in endothelial cells, which increased IL-6 and lead to changes in vascular permeability. On the other hand, Wang et al. [38] showed that chronic intermittent hypoxia stimulated NF-κB-mediated TNF-α generation, which in turn, down-regulated eNOS expression, impairing endothelium-dependent vasodilatation. Besides inflammation, oxidative stress seems to contribute to impaired endothelial function in hypoxia [39]. In this regard, even if we did not directly evaluate markers of oxidative stress, the tendency of paraoxonase-1 to decrease after HH could be interpreted as an oxidative inactivation of the enzyme.

Longer exposure time and higher altitude could have enhanced the significant changes observed. However, the experimental conditions were, in part, adopted because of safety reasons according to recommendations of the ethical committees. Another limitation of this study could be the sample size, which was not increased due to the limited capacity of the HC. Nonetheless, our sample size allowed us to detect differences with an effect size of 1.0 with a significance level of 0.05 and with a 0.80 power similar to other studies [11].

5. Conclusion

In conclusion, the present study describes the impact of acute HH exposure on vascular reactivity and biomarkers of endothelial function. The identification of adiponectin levels as a marker of vascular compliance in response to hypoxia is novel and may represent another clinical utility for the measurement of adiponectin. The need of further knowledge in this field is highlighted by the fact that current tools for risk assessment are insufficient, especially in those subjects with no history of exposure to altitude. New methods for identifying subjects at risk will help to prevent altitude-related sickness and to safely recommend mountaineering activities for health improvement.

Disclosure statement and acknowledgements

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