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ORIGINAL ARTICLE

Moderate altitude but not additional endurance training increases markers of oxidative stress in exhaled breath condensate

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Abstract Oxidative stress occurs at altitude, and physical exertion might enhance this stress. In the present study, we investigated the combined effects of exercise and moderate altitude on redox balance in ten endurance exercising biathletes, and five sedentary volunteers during a 6-week-stay at 2,800 m. As a marker for oxidative stress, hydrogen peroxide (H_2O_2) was analyzed by the biosensor measuring system Ecocheck^{TM}, and 8-iso prostaglandin F2α (8-iso PGF2α) was determined by enzyme immunoassay in exhaled breath condensate (EBC). To determine the whole blood antioxidative capacity, we measured reduced glutathione (GSH) enzymatically using Ellman's reagent. Exercising athletes and sedentary volunteers showed increased levels of oxidative markers at moderate altitude, contrary to our expectations; there was no difference between both groups. Therefore, all subjects' data were pooled to examine the oxidative stress response exclusively due to altitude exposure. H_2O_2 levels increased at altitude and remained elevated for 3 days after returning to sea level ($p \leq 0.05$). On the other hand, 8-iso PGF2α levels showed a tendency to increase at altitude, but declined immediately after returning to sea level ($p \leq 0.001$). Hypoxic exposure during the first day at altitude resulted in elevated GSH levels ($p \leq 0.05$), that decreased during prolonged sojourn at altitude ($p \leq 0.001$). In conclusion, a stay at moderate altitude for up to 6 weeks increases markers of oxidative stress in EBC independent of additional endurance training. Notably, this oxidative stress is still detectable 3 days upon return to sea level.

Keywords Hypoxia · Lung · Hydrogen peroxide · 8-iso prostaglandin F2α · Glutathione

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Introduction

Altitude training is frequently used by endurance athletes to improve competition performance at sea level (Levine and Stray-Gundersen 1997). Three to four weeks at moderate altitude is the minimal recommended period required to

maximize the erythropoietic response (Chapman and Levine 2007; Heinicke et al. 2005). However, not all aspects of altitude acclimatization may be beneficial for competing athletes. In particular, it is known that exposure to altitude combined with exercise (Araneda et al. 2005; Bailey et al. 2001; Chao et al. 1999; Pfeiffer et al. 1999; Schmidt et al. 2002), and even exposure to altitude in resting conditions (Bailey et al. 2001), increases reactive oxygen species (ROS) levels/formation, especially in lung and blood. On the other hand, acute physical work per se is a contributor to oxidative stress where mitochondria represent the major source of free radicals (Bailey et al. 2001; Bailey et al. 2004; Sen 1995). Apart from exercise, additional stressors increase oxidative stress at altitude, such as exposure to ultra violet light, lack of dietary antioxidants, cold climate, increased xanthine oxidase activation and catecholamine production, as well as increased reductive stress and generation of free radicals through anoxia/reoxygenation (Askew 2002). Excessive generation of ROS can induce both, a detrimental effect on lipids, proteins, and DNA as well as an adaptive response. Several studies demonstrate the pathophysiological significance of oxidative stress molecules such as hydrogen peroxide (H_2O_2) and 8-iso prostaglandin F2α (8-iso PGF2α) in relation to cellular inflammation and lung diseases (Makris et al. 2007; Montuschi 2005; Psathakis et al. 2006). H_2O_2 is produced after converting superoxide anions $O_2^{\cdot -}$ to H_2O_2 by superoxide dismutase and is less stable than 8-iso PGF2α that is a product of arachidonic acid oxidation by ROS. Collection of exhaled breath condensate (EBC) offers the opportunity to measure these markers in the lower respiratory tract in a noninvasive manner (Horvath et al. 2005). In addition, maintenance of redox state depends on the efficiency of antioxidant defense systems and the amount of ROS generated. As such, reduced glutathione (GSH) is one of the most important cellular antioxidants that plays a central role in the regulation of redox sensitive signal transduction (Sen 1999).

Despite its impact, there is little knowledge as to whether oxidative stress increases during a prolonged period of exercise at moderate altitude. We hypothesized that a cumulated effect of exercise and hypoxia exists that causes up-regulation of free radical production, and consequently, an increase in oxidative stress markers. Therefore, using EBC and blood we determined oxidative stress response parameters upon exercise or rest during a 6-week-sojourn at moderate altitude (2,800 m).

Methods

Subjects and protocol

Ten healthy, endurance trained biathletes, and five sedentary, control subjects, all 15 volunteers being members of the Chilean Military "Escuela de Montaña", who usually live at sea level, were investigated during a 6-week-training camp or stay at moderate altitude (2,800 m) in Portillo, Chile. The subjects gave their written informed consent to participate in this study, which was approved by the Ethical Committee for Humans from the Faculty of Medicine, University of Chile, Santiago and supported by the headquarter of the Chilean Military. The daily training time of the athletes [four females and six males; mean age (SD), 24.7 ± 1.3 years, 167.2 ± 9.5 cm, 63.1 ± 8.0 kg, BMI 22.5 ± 1.5 kg/m^2] was 4–6 h with one rest day per week. Training included extensive cross country skiing, strength training, and shooting technique training. The sedentary control subjects (five males, 30.2 ± 3.3 years, 168.2 ± 5.3 cm, 79.8 ± 7.1 kg, BMI 28.2 ± 1.7 kg/m^2) performed mainly office work and regular daily life tasks with no heavy work load. Diet composition and caloric intake was monitored 1 week before and throughout the study. Dietary characteristics were balanced and identical for both test groups. All test subjects were members of the army, a fact that helped to standardize the experimental conditions. EBC (see below) was collected initially 2 weeks prior at sea level, on day 2 (24 h after arrival at altitude) and day 44 at altitude as well as at sea level 3 days after returning from altitude. Capillary blood samples for determination of hemoglobin concentration ([Hb]) and cubital-venous blood samples for reduced GSH were taken initially 2 weeks prior at sea level (670 m), and on days 2 (24 h after arrival at altitude), 13, 24, 34 and 44 at altitude (2,800 m). All data were collected in the morning after a minimum period of 12 h rest for the test subjects.

Exhaled breath condensate (EBC) collection and analysis

Exhaled breath condensate was collected with a self-constructed device as described earlier (Araneda et al. 2005). In brief, prior to the sampling procedure, the subject's mouth was rinsed with distilled water and the nostrils were blocked by cotton stoppers. Expired air was led from a face mask with attached heated tube (40°C) into a glass collector placed in ice at −5°C. Using a trap, saliva was collected in the bottom of the face mask that was not connected to the condenser. About 1.5 ml EBC was collected during a period of 20 min and immediately stored in liquid nitrogen until analyzed.

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide concentration was measured in EBC with the Ecocheck^{TM} (Flit GmbH, Berlin, Germany) by using a biosensor as described previously (Gerritsen et al. 2005). In brief, 500 µl EBC are mixed with 500 µl dilution

buffer to adjust the phosphate concentration. H_2O_2 is reduced enzymatically by a peroxidase on the sensor. The electrons are transferred to the sensor electrode by a redox pair where they induce a voltage change, which is proportional to the H_2O_2 concentration.

8-iso prostaglandin F2α (8-iso PGF2α)

8-iso PGF2α concentration was determined by an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA), previously tested for its utility to assess 8-iso PGF2α in EBC (Montuschi et al. 2000). The test kit was modified according to the manufacturer's instructions to reach a lower limit of detection of 1 pg/ml.

Blood analysis

Capillary blood samples were immediately analyzed with the GEM Oxygenation Portable Laboratory (OPL), (Instrumentation Laboratory, Munich, Germany) to measure [Hb].

Reduced glutathione (GSH)

GSH was determined in whole blood by analyzing the concentration in erythrocytes that is about 500 times higher than in plasma (Jones 2002). Hundred microliter venous blood was added to 800 µl of precipitation solution containing: 1.67 g of glacial metaphosphoric acid, 0.2 g Na_2EDTA and 30 g NaCl in 100 ml ddH_2O. Samples were left at room temperature for 10 min to reach complete hemoglobin denaturation and were then frozen in liquid nitrogen.

Ellman's reagent (DTNB) was used to evaluate the nonprotein thiol GSH as described earlier (Tietze 1969). After centrifugation (15 min at 5,000*g*), supernatants were assayed for nonprotein thiols. GSH was measured using a Lambda 25 UV/VIS spectrometer (Perkin Elmer). Absorption of complexes of GSH with DTNB was measured at 412 nm and a calibration curve was constructed for each experiment using a commercially available GSH standard (Sigma).

Statistics

Statistical analysis was carried out using GraphPad InStat software version 3.06 (San Diego, CA). The nonparametric Friedman test for repeated measures was performed to compare groups by time followed by Dunn's post-hoc test. Comparisons between the single groups (trained vs. sedentary) were analyzed by applying the Mann–Whitney test. The level of statistical significance was set at $p \leq 0.05$.

Results

Increased levels of oxidative stress markers were found at altitude in both groups, trained and sedentary (Table 1). However, we did not detect any significant differences in H_2O_2, 8-iso PGF2α and GSH levels between the trained and sedentary group at sea level or at altitude. Therefore, the subjects' data were pooled to examine the effect of hypoxia on redox balance. As depicted in Fig. 1, H_2O_2 significantly increased after 44 days at altitude from 60 nmol/l (median), 40–106 nmol/l (interquartile range) at sea level to 131, 118–167 nmol/l, $p \leq 0.05$. The level of H_2O_2 remained elevated even 3 days after returning to sea level (129, 92–194 nmol/l, $p \leq 0.05$) when compared to sea level (-pre) values. 8-iso PGF2α showed a tendency to increase during the stay at altitude (day 2 vs. day 44, from 2.48, 1.90–3.24 to 3.17, 2.37–4.31, pg/ml, Fig. 2) and significantly decreased upon returning to sea level (1.84, 1.58–2.23, pg/ml, $p \leq 0.05$). Finally, Fig. 3 demonstrates that an acute exposure to hypoxia resulted in significantly

Table 1 Effects of prolonged hypoxia and exercise on oxidative stress markers and antioxidative capacity

	Days	H_2O_2 (nmol/l)		8-iso PGF2α (pg/ml)		GSH (µmol/g Hb)	
		Trained	Sedentary	Trained	Sedentary	Trained	Sedentary
Sea level (-pre) 2,800 m altitude	−14	84.30 ± 42.44	45.40 ± 19.79	2.77 ± 0.65	2.95 ± 0.95	4.44 ± 0.90	4.97 ± 0.66
	2	99.20 ± 38.91	93.60 ± 42.47	2.77 ± 0.89	2.60 ± 0.94	5.30 ± 0.93	5.60 ± 0.50
	13	–	–	–	–	4.18 ± 1.06	4.73 ± 0.56
	24	–	–	–	–	4.60 ± 0.71	4.66 ± 1.12
	34	–	–	–	–	3.96 ± 1.05	3.67 ± 0.91
	44	128.20 ± 53.53	169.60 ± 75.33	3.45 ± 1.73	3.65 ± 1.08	3.98 ± 0.94	3.81 ± 0.55
Sea level (-post)	+3	154.10 ± 90.42	150.00 ± 66.35	1.79 ± 0.48	2.06 ± 0.51	–	–

Exhaled breath condensate and blood samples were obtained from all volunteers after a resting period. Values are mean ± SD

H_2O_2 hydrogen peroxide, *8-iso PGF2α* 8-iso prostaglandin F2α, *GSH* reduced glutathione, *trained* endurance athletes training at moderate altitude ($n = 10$), *sedentary* control subjects living at moderate altitude ($n = 5$)

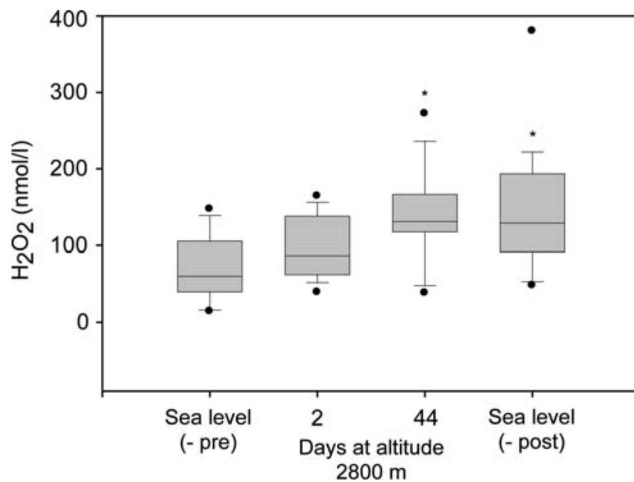

Fig. 1 Changes in hydrogen peroxide (H_2O_2) in response to prolonged hypoxia. Sea level (-pre) values, 2 weeks prior to altitude exposure; sea level (-post) values, 3 days post altitude exposure. Values are given as *box plots* indicating the median (center horizontal line), interquartile range from the 25th to the 75th percentile (*box*), and the 10th and 90th percentiles (*whiskers*). Outliers are presented as *circles*. *Asterisk* indicates significant differences from sea level (-pre) values, $p \leq 0.05$. Pooled data from ten trained and five sedentary control subjects are shown

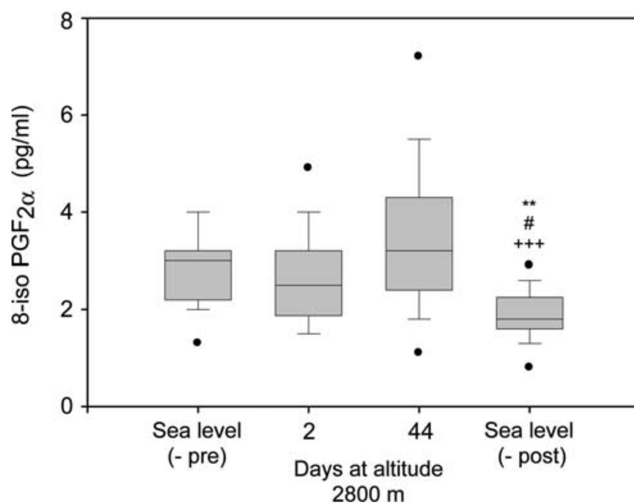

Fig. 2 Changes in 8-iso prostaglandin F2α (8-iso PGF2α) in response to prolonged hypoxia. Sea level (-pre) values, 2 weeks prior to altitude exposure; sea level (-post) values, 3 days post altitude exposure. Values are given as *box plots* indicating the median (center horizontal line), interquartile range from the 25th to the 75th percentile (*box*), and the 10th and 90th percentiles (*whiskers*). Outliers are presented as *circles*. *Asterisk* indicates significant differences from sea level (-pre) values, *hash* different from altitude values—day 2, *plus* different from altitude values—day 44, $p \leq 0.05$. Pooled data from ten trained and five sedentary control subjects are shown

elevated GSH levels [sea level (-pre) vs. day 2, from 4.82, 4.12–5.33 to 5.38, 5.01–6.08, μmol/g Hb, $p \leq 0.05$]. GSH levels significantly decreased throughout the stay at

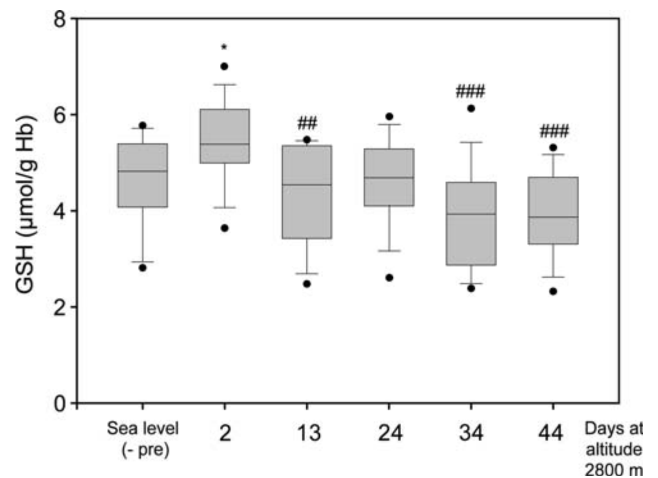

Fig. 3 Time course of reduced glutathione (GSH) at moderate altitude. Sea level (-pre) values, 2 weeks prior to altitude exposure; sea level (-post) values, 3 days post altitude exposure. Values are given as box plots indicating the median (center horizontal line), interquartile range from the 25th to the 75th percentile (*box*), and the 10th and 90th percentiles (*whiskers*). Outliers are presented as *circles*. *Asterisk* indicates significant differences from sea level (-pre) values, $p \leq 0.05$; *hash* different from altitude values—day 2, $p \leq 0.05$, *double hash* $p \leq 0.01$, *triple hash* $p \leq 0.001$. Pooled data from ten trained and five sedentary control subjects are shown

altitude (day 2 vs. day 44, from 5.38, 5.01–6.08 to 3.87, 3.32–4.66 μmol/g Hb, $p \leq 0.001$).

Discussion

Contrary to our hypothesis, the present study determined that the effects of two combined oxidative stimuli, chronic hypoxia and training, did not demonstrate a cumulative effect on the redox state in endurance trained athletes compared to sedentary controls. We observed, however, altitude-induced alterations with significantly increased levels of H_2O_2 in EBC at altitude and after return to sea level, a tendency to increased 8-iso PGF2α levels in EBC at altitude as well as a depletion of the antioxidant GSH in blood. Our results suggest that living at moderate altitude for an extended period of time is accompanied by a long-term increase in lung oxidative stress independent of strenuous physical exertion in trained subjects.

Earlier studies have shown increased levels of oxidative stress associated with physical work at moderate altitude over a shorter period of time than our study, 11–24 days (Araneda et al. 2005; Chao et al. 1999; Pfeiffer et al. 1999; Schmidt et al. 2002; Vasankari et al. 1997), and 12 hypoxic training sessions during 6 weeks (Pialoux et al. 2006). However, in those studies no sedentary control groups were investigated at altitude and the objective was mainly to investigate the influence of antioxidant supplementation.

It is known that acute exercise in hypoxia (Araneda et al. 2005; Bailey et al. 2001; Joanny et al. 2001; Moller et al. 2001; Pialoux et al. 2006) and normoxia (Bailey et al. 2001; Bailey et al. 2004) increases oxidative stress compared to resting conditions. On the other hand, regular intermittent hypoxic training was leading to attenuated increases in lipid peroxidation and reduced plasma malondialdehyde due to mobilization of α-tocopherol compared with normoxic training (Bailey et al. 2001). The observation that there was no difference between sedentary and endurance trained subjects might be explained by the lower exercise-induced oxidative stress after intermittent hypoxic training. This suggests that controlled ROS generation is required to initiate adaptation to physical stress.

There is evidence that normoxic exercise training results in increased cellular concentrations of antioxidants (Powers et al. 1999). Endurance training can elevate antioxidant enzyme activities in erythrocytes (Miyazaki et al. 2001). It also seems to be capable of reducing DNA damage in white blood cells after exhaustive exercise (Niess et al. 1996), and strengthens GSH dependent tissue antioxidant defense by increasing cellular concentrations of GSH in skeletal muscles (Powers et al. 1999; Sen 1999). However, we found no differences in GSH sea level values between our trained and sedentary subjects. At moderate altitude, both groups followed the same antioxidant pattern. Hypoxic exposure did lead to an increase in GSH-concentration after 24 h; and, GSH levels decreased to normoxic levels during the stay at altitude. Earlier we described a rapid increase in mouse erythrocytes GSH levels on 0.5% O_2 hypoxic exposure in vitro (Bogdanova et al. 2003), and others showed an increase in mouse muscle reduced GSH with acute hypoxia equivalent to an altitude of 7,000 m (Magalhaes et al. 2004), a result that correlates with the observation of suppressed ROS production in hepatocytes primary cultures in response to acute hypoxia (Bogdanova et al. 2005). Chronic hypoxic exposure reduces erythrocytes GSH content (Singh et al. 2001) and triggers activation of glutathione cycle related enzyme activity (Magalhaes et al. 2005) leading to improved antioxidant capacity of blood.

Permanent residence at high altitude was accompanied by increased oxidative stress (Jefferson et al. 2004), and 1 day of high altitude hypoxia in sedentary lowlanders was associated with increased steady-state levels of oxidative DNA damage (Moller et al. 2001). However, prolonged hypoxic exposure in lowlanders showed an adaptive response to oxidative stress. After 8 weeks at high altitude, similar oxidative DNA damage was found as it was seen at sea level (Lundby et al. 2003). Moreover, after 13 months, the antioxidant defense was upregulated, e.g. as identified by elevated whole blood GSH levels (Vij et al. 2005).

In our study, oxidative stress persisted upon returning to sea level for 3 days. Indeed, there are reports of continued increased levels of lipid peroxidase (Joanny et al. 2001), increased levels of H_2O_2 (Araneda et al. 2005) after a prolonged stay at high altitude, and increased polarity of the erythrocyte membrane bilayer surface with increased formation of malondialdehyde after a short-term stay at moderate altitude (Gonzalez et al. 2005).

In conclusion, 6 weeks of endurance training at moderate altitude did not result in an additional long-term increase in lung oxidative stress in trained compared to sedentary subjects. In addition, oxidative stress remained elevated upon returning to sea level. These observations suggest that the increased oxidative stress is due to exposure to moderate altitude. Our data imply that a protective adaptation to physical stress occurred in athletes that adjusts the systemic homoeostasis. However, the physiological consequences of increased lung oxidative stress at moderate altitude remain to be elucidated.

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