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Brief Communication

HIGH ALTITUDE TRAINING INCREASES REACTIVE CARBONYL DERIVATIVES BUT NOT LIPID PEROXIDATION IN SKELETAL MUSCLE OF RATS

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Abstract—The oxidative stress related consequences of physical training at high altitude are not known. The hypothesis was tested that physical training and exposure to high altitude have adverse effects on free radical generation and activities of antioxidant enzymes. The present results showed that 4 weeks of exercise at an altitude of 4000 m increased the activity of Mn-SOD in both white and red types of skeletal muscle. The activities of Cu,Zn-SOD, catalase, and glutathione peroxidase, as well as the level of lipid peroxidation measured by TBARS and lipid hydroperoxides, did not change significantly. In contrast, the level of reactive carbonyl derivatives measured by anti-2,4-dinitrophenylhydrazone antibodies and spectrophotometry showed an increase in both types of muscle of altitude trained rats compared with sea level trained and control groups. It was suggested that the oxidative modification of certain amino acids is due to the increasing gap between activity of SOD and peroxide scavenging enzymes, which results in increases in the number of hydrogen peroxide molecules. Thus, since the mechanism of generation and/or the mode of action of radicals resulting in lipid peroxidation and protein oxidation appears to be different in vivo, both processes should be studied during oxidative stress.

Keywords—Exercise, Free radical, High altitude, Reactive carbonyl derivatives, Lipid peroxidation, Antioxidant enzymes

INTRODUCTION

It is speculated that about 10^{12} O_2 molecules enter into rat cells each day, yielding about 2×10^{10} superoxide and hydrogen peroxide molecules per day per cell.[1] During physical exercise, the oxygen flux into the mitochondria of skeletal muscle may increase up to 100-fold, most probably resulting in an extensive increase in free radical generation. Thus, acute physical exercise results in oxidative stress, which is associated with increases in lipid peroxidation (LIPOX)[2–4] and protein oxidation.[5,6] On the other hand, it appears that physical training increases the activity of antioxidant enzymes,[7] which enhance protection against free radical species. Treadmill running, which increased the level of LIPOX in skeletal muscle of untrained rats, did not cause membrane damage in the muscle of trained rats.[8] However, reactive carbonyl derivatives (RCD), an indicator of oxidative modifications of proteins, increased in hindlimb muscle of rats after training.[9]

Physical exercise and exposure to high altitude cause inverse (increase[2–4,9] vs. decrease[10–13]) effects on free radical formation and activity of antioxidant enzymes. Low oxygen pressure, such as exposure to high altitude, decreases the generation of free radical species[10] and activity of SOD.[11–13] Furthermore, it was proposed by Stadtman[14] that partial oxygen pressure influences the rate of protein turnover and oxidative modifications of amino acids.

High altitude training is often used by athletes to increase the number of red blood cells, which is be-

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lieved to increase endurance performance. However, the oxidative stress related consequences of high altitude training are not known. It was shown that long-term intermittent exposure to an altitude of 4000 m decreases the activity and content of Mn-SOD and increases the level of LIPOX.[15] Moreover, pentane exhalation at high altitude also showed a significant increase in humans.[16] Therefore, it was hypothesized that physical training at high altitude would induce oxidative stress that cannot be completely curbed by antioxidant enzymes. Consequently, the purpose of the present study was to elucidate the effects of high altitude training on the antioxidant enzyme system and on the levels of LIPOX and RCD in skeletal muscle of rats.

MATERIALS AND METHODS

Thirty male Wistar rats (6 weeks old) were obtained from Clea Japan, Inc. (Tokyo) and were cared for according to the "Guiding Principles for the Care and Use of Animals." The rats were randomly assigned to three groups: control (C), sea level trained (ST), and simulated high altitude (4000 m) trained (HT).

Training protocol

All rats (except for the C group) were introduced to treadmill running with 6–8-min exercise bouts, at 12–27 m/min, for 6 days in order to accustom them to running. Then, the training intensity of ST increased in the first week and was maintained for another 3 weeks at a running speed of 27 m/min for 1 h on an incline of 10%. The rats of the HT group were exposed to 4000 m simulated high altitude in a hypobaric chamber throughout the 4-week experimental period. The chamber was recompressed for the time of cleaning and feeding. After recompression, the rats were transferred to another chamber and exposed to 4000 m simulated altitude. HT rats trained five times a week at a running speed of 15 m/min with a duration of 1 h. The training intensities of HT and ST rats are assumed to be similar.[17] To eliminate diurnal effects, the experiments were performed at the same time. One day after the last training session the rats were sacrificed.

Tissue collection

The deep red (RQ) and white superficial part (WQ) of quadriceps muscle were excised and frozen in liquid nitrogen and stored at −80°C for later analysis. Subsequently, the samples were homogenized for various biochemical assays.

Assays

The total SOD (EC 1.15.1.1) activity was determined by the method of Crapo et al.[18] Mitochondrial Mn-SOD activity was measured by adding potassium cyanide because the cyanide inhibits Cu,Zn-SOD activity but not Mn-SOD. Cu,Zn-SOD activity was calculated by subtracting Mn-SOD activity from total SOD activity. Total activity of GPX (EC 1.11.1.9) was assayed with cumene hydroperoxide as a substrate according to Tappel.[19] The activity of CAT (EC 1.11.1.6) was measured by the method of Aebi.[20] For estimation of malondialdehyde, a peroxidation marker, the TBARS method was used according to Ohkawa et al.[21] To obtain a more reliable index of lipid peroxidation, lipid hydroperoxide levels were determined as described by Matsushita et al.[22] Carbonylazation of protein residues was measured by the Western blot technique, using immunodetection of protein-bound 2,4-dinitrophenylhydrazones (DNPH) by the method of Levine et al.[23] as modified by Nakamura and Goto.[24] In brief, proteins precipitated with trichoroacetic acid were suspended and incubated in a solution containing 10 mM DNPH and 2 N HCl for 1 h at 15°C. The resulting protein hydrazones were pelleted in a centrifuge at 11,000 × *g* for 5 min. The pellets were washed three times with ethanol-ethyl acetate (1:1) and then once with acetone. The final precipitates (1 mg protein) were dissolved in 1 ml buffer containing 8 M urea and 5% 2-mercaptoethanol using a sonicator for 10 min. Du-

Table 1. Cytosolic and Mitochondrial SOD Activities of White (WQ) and Red (RQ) Portions of Quadriceps Muscles

	Cu,Zn-SOD (U·mg protein^{-1})		Mn-SOD (U·mg protein^{-1})	
Group (n = 6)	WQ	RQ	WQ	RQ
Control	9.06 ± 0.7	11.5 ± 0.5	5.24 ± 0.5	8.18 ± 0.3
Sea level	9.16 ± 0.4	14.9 ± 1.1	8.31 ± 0.7*	10.3 ± 0.8
High altitude	9.46 ± 0.6	13.5 ± 1.3	8.48 ± 0.6*	11.9 ± 1.08*

Values are means ± SE.
* $p < .05$ vs. control group.

Table 2. Activities of CAT and GPX in White (WQ) and Red (RQ) Portions of Quadriceps Muscles

Group (n = 6)	CAT ($K \times 10^{-4}$ mg protein^{-1})		GPX (U·mg protein^{-1})	
	WQ	RQ	WQ	RQ
Control	1.56 ± 0.1	1.83 ± 0.1	7.23 ± 0.9	16.7 ± 1.2
Sea level	1.54 ± 0.1	1.96 ± 0.2	8.03 ± 0.7	17.8 ± 0.8
High altitude	1.62 ± 0.2	2.01 ± 0.3	5.80 ± 0.5	16.0 ± 1.1

Values are means ± SE.

plicate polyacrylamide gel electrophoresis of derivatized proteins was carried out on 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Then the membranes were soaked in phosphate-buffered saline containing 3% skim milk, 0.05% Tween, and 0.05% sodium azide and then treated with anti-DNPH antiserum (Sigma D-9656). After washing in buffer without antibodies, the membranes were treated with ^{125}I-Protein A (0.02 μCi/ml). Finally, the radioactive signals were quantified by BAS 2000 Bioimaging Analyzer (Fuji Film Co., Tokyo, Japan). Spectrophotometric measurement of RCD was performed according to Levine et al.[23] to confirm the data obtained by Western blot analysis. The protein concentration was determined by the Lowry et al.[25] method.

Statistical analysis

The statistical significance of the data was assessed by ANOVA followed by Scheffé's post-hoc test. When applicable, an unpaired Student's t-test was used. Significance was set at $p < .05$.

RESULTS

Antioxidant enzymes

There was no significant change in cytosolic SOD activity (Table 1). The activity of Mn-SOD showed a significant increase in WQ muscle in ST and HT groups. Moreover, Mn-SOD activity also increased in the RQ muscle of HT rats. The activities of CAT and GPX remained significantly unchanged in all groups (Table 2).

Lipid peroxidation

The TBARS and lipid hydroperoxide levels of skeletal muscle did not change significantly in either group (Table 3).

Reactive carbonyl derivatives

The RCD content, measured by anti-2,4-dinitrophenylhydrazone antibodies, increased in RQ and WQ samples of HT rats compared with C and ST groups (Fig. 1). Moreover, the accumulation of RCD was considered to be more enhanced in the red type of skeletal muscle than in the white type. The quantitative data from the Western blots (protein stain density per radioactive signal density) as well as spectrophotometry showed significant ($p < .05$) increases in RCD in both types of skeletal muscle of HT rats compared with C and ST groups (Table 4).

DISCUSSION

The present results appear to provide the first demonstration of immunoreactive measurement of carbonyl groups in skeletal muscle. The pattern and intensity of Western blot signals and Coomassie brilliant blue staining were similar in C and ST groups, indicating that the accumulation of RCD depends on the amount of protein. On the other hand, the intensity of the DNPH signal increased noticeably in WQ and RQ muscles of HT rats as compared with C and ST rats. Strong DNPH signals appeared in proteins having molecular weights of around 41,000 kDa. The present study does not allow naming the modified protein(s), as this needs further investigation. However, our unpublished observations (Radak et al., Nakamura and Goto) suggest that

Table 3. Lipid Peroxidation Levels of White (WQ) and Red (RQ) Portions of Quadriceps Muscles

Group (n = 6)	TBARS (nmol·mg protein^{-1})		Lipid Hydroperoxides (nmol·min^{-1}·mg protein^{-1})	
	WQ	RQ	WQ	RQ
Control	0.34 ± 0.01	0.36 ± 0.01	0.52 ± 0.06	0.58 ± 0.09
Sea level	0.38 ± 0.02	0.35 ± 0.01	0.62 ± 0.08	0.61 ± 0.09
High altitude	0.41 ± 0.03	0.39 ± 0.03	0.57 ± 0.05	0.56 ± 0.06

Values are means ± SE.

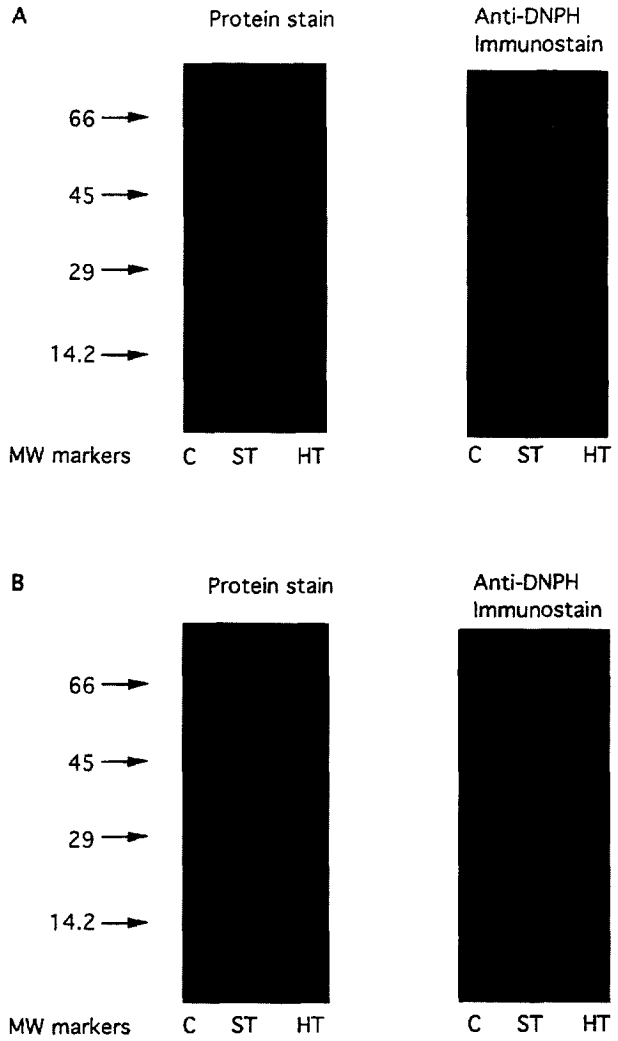

Fig. 1. Reactive carbonyl derivatives in red (A) and white (B) types of skeletal muscle of control (C), sea level trained (ST), and high altitude trained (HT) rats. Molecular weight markers are shown on the left of the Coomassie Blue protein stain panel. The signal of DNPH antibodies in both types of muscle of HT rats is stronger than that of C and ST rats. Western blot analysis was carried out three times with three samples per group, and a randomly chosen data set is shown.

actin might be a possible candidate because of its abundance at the given molecular weight.

DNPH activity of proteins is postulated to indicate the presence of RCD by free radical-initiated reactions of side chains of amino acid residues.[26,27] Agglomeration of RCD, at amino acid residues, is possibly due to oxidative stress or to a lower rate of degradation of proteins.[28] Massive oxidative stress might increase both lipid peroxidation and protein oxidation levels. In the present study, however, the level of lipid peroxidation, which is similar to other data,[3] was not altered, indicating that amino acids are more sensitive to oxidative modification than polyunsaturated fatty acids and/or that the underlying mechanism of these two processes is different. This observation might indicate that the results of the Davies and Goldberg[29] in vitro study are also applicable to in vivo conditions. Our data suggest that increases in activity of SOD, especially Mn-SOD, as a result of adaptative response to exercise-induced oxidative stress and/or mitochondrial reproduction, could cope with the generation of superoxide anions. Hence, the increasing gap between the activities of SOD and peroxidase scavenger enzymes might lead to an accumulation of H_2O_2 molecules in skeletal muscle of HT rats, and these molecules may not be elicited by free radicals, whereas LIPOX usually involves a free radical chain reaction. Thus, the discrepancy of LIPOX and RCD generation may be accounted for by these related but separate mechanisms. The reaction of H_2O_2 with Fe(II)–protein complexes could induce conversion of certain amino acid residues to RCD.[14,26,30] The definite source of Fe(II) for metal-catalyzed oxidation (MCO) in the present conditions is not known; however, different sources are possible, such as hemoglobin, ferritin, or the intracellular mobile iron pool.[31] Moreover, myoglobin also could be a possible source, since high altitude training increases the myoglobin content more significantly than training at sea level does,[32] and its heme ring can serve as an iron donor after the damaging attack of H_2O_2.[33] The role of H_2O_2 is indispensable for MCO: administration of CAT has been shown to prevent inactivation of proteins, which is believed to be the first signal of MCO.[34] In addition, H_2O_2 alone does not induce LIPOX,[35] which did not increase in this study either, but it does increase the oxidative damage of proteins.[36]

The present results, in part, support the hypothesis of Witt et al.[9] that increases in RCD in proteins of skeletal muscle after training are due to inadequate protection of the antioxidant defense system. It is speculated that a lack of response of CAT and GPX to the possible increases in H_2O_2 formation could result in an increase in RCD in skeletal muscle of HT rats. The other possible mechanism underlying the increases in RCD content in skeletal muscle of HT rats might be the hypoxia/reoxygenation phenomenon due to daily recompression of the hypobaric chamber. The lower rate of protein degradation can also lead to accumulation of RCD. However, exposure to high altitude suppresses the rate of protein synthesis in skeletal muscle,[37] so it seems unlikely that the rate of protein degradation decreases. Witt et al.[9] reported increases in RCD (as measured by spectrophotometry) in hindlimb muscle of rats after a 12 weeks of training at sea level. In the present study, the RCD level of ST and C rats was not dif-

Table 4. Reactive Carbonyl Derivative Contents of White (WQ) and Red (RQ) Portions of Quadriceps Muscles

	RCD by Spectrophotometry (nmol carbonyl·mg protein^{-1}) (n = 6)		RCD by Western Blot (arbitrary units) (n = 3)	
Group	WQ	RQ	WQ	RQ
Control	0.745 ± 0.31	0.90 ± 0.11	4635 ± 190	4214 ± 110
Sea level	1.10 ± 0.15	0.938 ± 0.106	4231 ± 227	3981 ± 175
High altitude	2.42 ± 0.14*	2.67 ± 0.38*	3526 ± 258*	2995 ± 239*

Values are means ± SE.

* $p < .05$ vs. control and sea level trained groups. The Western blot quantitative data expressed in arbitrary units are a result of protein stain density per radioactive signal density.

ferent, which might be due to differences in training protocols.

Physical training had a significant stimulating effect on mitochondrial SOD activity even at an altitude of 4000 m. It is thus conceivable that the stimulating effects of physical exercise on Mn-SOD activity are probably more significant than the downregulating effects of exposure to high altitude.

Taken together, the observations of the present study suggest that physical training at high altitude increases the RCD in amino acids of skeletal muscle. The current results indicate that lipid peroxidation and protein oxidation have different mechanisms in vivo; therefore, it seems to be important to determine both processes.

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ABBREVIATIONS

C—control
CAT—catalase
DNPH—2,4-dinitrophenylhydrazine
GPX—glutathione peroxidase
HT—high altitude trained
LIPOX—lipid peroxidation
MCO—metal-catalyzed oxidation
RCD—reactive carbonyl derivatives
TBARS—thiobarbituric acid-reactive substances
ST—sea level trained
SOD—superoxide dismutase