ELSEVIER

Life Sciences, Vol. 62, No. 15, pp. 1317-1322, 1998

0024-3205/98 $19.00 + .00

PII S0024-3205(98)00065-4

THE EFFECT OF HIGH ALTITUDE AND CALORIC RESTRICTION ON REACTIVE CARBONYL DERIVATIVES AND ACTIVITY OF GLUTAMINE SYNTHETASE IN RAT BRAIN

Zsolt Radák[1,4], Katsumi Asano[1], Yang Fu[1], Akihiro Nakamura[2], Hideko Nakamoto[2], Hideki Ohno[3] and Sataro Goto[2]

[1]Laboratory of Exercise Physiology, Institute of Sport and Health Sciences, University of Tsukuba, Tsukuba, [2]Department of Biochemistry, School of Pharmaceutical Sciences, Toho University, Funabashi, [3] Department of Hygiene, Defense Medical College, Tokorozawa, Japan

(Received in final form January 26, 1998)

Summary

Both exposure to high altitude (HA) and caloric restriction (CR) may influence free radical generation. The aim of the present study was to evaluate the effects of 4 wk chronic exposure to 4,000m of HA and CR (60% of CR of control (C) rats) on protein oxidation in brain. Eighteen rats with similar body mass were assigned to CR, HA and C rats. Reactive carbonyl derivatives (RCD), marker of protein oxidation, were measured by Western blot. In addition, the activity and protein content of glutamine syntethase (GS) were determined. The body mass of C rats was significantly higher ($P< 0.001$) than that of HA and CR groups. The quantified signal intensity of RCD was significantly stronger in C rats than in HA and CR rats. The activity of GS was significantly increased in CR rats, while the protein content of GS was decreased in HA rats compared to C group. The data suggest that both HA and CR decreases the accumulation of RCD in the brain, however the mechanism of the decrease seems to be different during HA and CR.

Key Words: free radicals, carbonyl derivatives, glutamine synthase, high altitude, caloric restriction

Cellular proteins are subjects of continuous turnover, especially abnormal proteins formed by oxidation of amino acid residues or other types of modifications potentially harmful to cells. An increase in reactive carbonyl derivatives (RCD) in amino acid residues is considered to be a result of metal ion-catalyzed oxidation (MCO) (1) and measurement of RCD is used to estimate the level of oxidative damage on proteins (2). Oxidative modification of amino acids has significant effect on cellular functions as oxidatively modified proteins lose their physiological activity and tend to be very sensitive to proteolysis (3,4). The enzyme of glutamine synthetase (GS) is very sensitive to oxidative stress as free radical-induced modification of its histidine residues leads to inactivation and proteolytic degradation of the enzyme (5). In addition, the amount of glutamine synthesized by GS, positively correlates with the rate of protein synthesis (6).

High altitude (HA) exposure depresses the rate of protein synthesis (7) and shifts the aerobic metabolism to anaerobic metabolism, which might induce changes in MCO (8). Moreover, exposure to HA sdecreases the formation of free radical species (9), which in some organs is associated with decreases in activity of antioxidant enzymes (10-12). Most studies done at HA concentrated on skeletal muscle (7, 11-13) however, exposure to HA in many cases might lead to neurological dysfunction, which is involved in mountain sickness and could be fatal. Therefore, the aim of the present investigation was to study the level of RCD and activity as

[4] Corresponding to: Zsolt Radák, Fax :+36-1156-6337, E-mail: radak@mte.hupe.hu

well as the content of GS following 4-week exposure to altitude of 4, 000m. Exposure to HA is usally associated with decreases in body weight, which itself could have a significant effect on the rate of protein synthesis. Thus, a group of caloric restricted (CR) animals was added, and the body weight of this group was matched with exposed rats. It was hypothesized that HA exposure causes oxidative stress, increases RCD, decreases the rate of protein synthesis and increases the activity of GS. On the other hand, it was suggested that CR decreases the accumulation of RCD and increases the activity of GS.

Methods

Animals. Eighteen male Wistar rats (8 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 assigned randomly into three groups: control (C), exposed to simulated high altitude of 4,000m (HA) and caloric restricted (CR).

Experimental Protocol. The C rats received water and food ad libitium. The rats of HA group were exposed to 4,000m of simulated high altitude in a hypobaric chamber throughout the four week experimental period. The chamber was daily recompressed for the time of cleaning and feeding. The rats were sacrificed by decapitation immediately after the last day of the 4-week exposure at the same age as other groups. The CR group food was restricted to 60 % of the C group, in order to achieve similar body weight as HA group. It cannot be ruled out, that the CR also resulted in some differences in antioxidant food intake, however the antioxidant food intake per body mass has changed only in a marginal level. The whole brain was excised, washed and frozen in liquid nitrogen, then stored at -80°C for later analysis. Subsequently, the samples were homogenized for various biochemical assays.

Assays. A recent paper by Lyras et al. (14) reports that, however the trichloroacetic acid (TCA) precipitation before and after the interaction of dinitrophenylhydrazine (DNPH) and proteins of homogenates give different base line of RCD level in proteins of brain, the relationship between the data obtained by differences in TCA precipitation is very significant. This obesrvation is very important when spectrophotometric measurement is used, however in the current study we used anti-DNPH antibodies to obtain specific data and the protein specific accumulation of RCD is also visible with this method. Therefore, accumulation of RCD in protein residues was measured by Western blot technique, using immunodetection of protein-bound 2,4-dinitrophenylhydrazones (DNPH) by the method of Levine et al.(15) modified by Nakamura and Goto (16). In brief, proteins precipitated with 20% (w/v) of TCA were suspended and incubated in a solution containing 10mM DNPH and 2 N HCl for 1 h at 15 °C. The resulting protein hydrazones were pelleted in a centrifuge at 11, 000 x 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 8M urea and 5% 2-mercaptoethanol using a sonicator for 10 min. Duplicate polyacrylamide gel electrophoresis of derivatized proteins were carried out in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Ten microgram of protein/lane were loaded. After the electrophoresis the proteins were transferred to nitrocellulose membranes. Then the membranes were soaked in PBS containing 3% skim milk, 0.05% Tween and 0.05% sodium azide and then treated with anti-DNPH antibodies, raised as described by Nakamura and Goto (16). After washing with buffer, 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., Japan). Quantitative analysis of protein bands was performed by densitometer. The Western blot data expressed in arbitrary units are a result of protein stain density per radioactive signal density. The activity of GS was measured by the method of Miller et al. (17). Assay mixture incubated at 37° for 60 min, contained in 1.0 ml: 50 mM imidazole.HCl, 50 mM NH_2OH, 100 mM L-glutamine, 25 mM potassium arsenate, 0.2 mM ADP, 0.5 mM $MnCl_2$ and samples with 500 μg protein content. Reactions were initiated by the addition of samples and terminated by 1.0 ml of 0.37 M $FeCl_3$/0.3 M trichloroacetic acid/0.6 M HCl. The precipitate was removed by centrifugation and the activity was determined at 505 nm. Parallel incubations of samples in reaction mixture lacking in ADP was done and served as a control. The activity of the GS was determined as described by Rowe et al. (18). The assay was performed with duplicate samples. The protein

concentration of the enzyme in the brain homogenates was measured by Western blot technique according to the method of antibody supplier (Transduction Lab. Lexington, USA). The catalog number of obtained monoclonal antibody :# G45020. The quantitative data were obtained similarly with RCD measurement, and the controls were normalized and set as 100% and the values of DR and HA group are related to them. The protein concentration was determined by the Lowry et al. (19) and the Pierce BCA method depending on the type of measurement.

Statistical analysis. The statistical significance of the data was assessed by ANOVA, followed by Scheffe's post-hoc test. When applicable, an unpaired Student's t-test was used. The significance was set at $P < 0.05$.

Results

The average body mass of the three groups was adjusted to the same level before the experiment and after the 4 wk experimantal period the body mass of C rats (499± 8 g) was significantly higher ($P< 0.001$) than that of HA (393±5 g) and CR (382± 5 g) groups. The data of the Figure 1 indicates that the accumulation of RCD occurs in protein specific manner and the differences in the RCD accumulation in different groups are also apparent. The BAS 2000 Bioimaging Analyzer quantified data showed that the radioactive signal intensity of DNPH anti-antibody was significantly stronger ($P < 0.05$) in C rats than in HA and CR rats (Fig. 2). The activity of GS was significantly higher in samples of CR rats (4.66±0.55 U/mg protein) than in C (3.49±0.45 U/mg protein) and HA rats (3.71±0.49 U/mg protein) (Figure 3.) The protein content of GS was significantly ($P< 0.05$) less in HA rats than in C and CR groups (Figure 4).

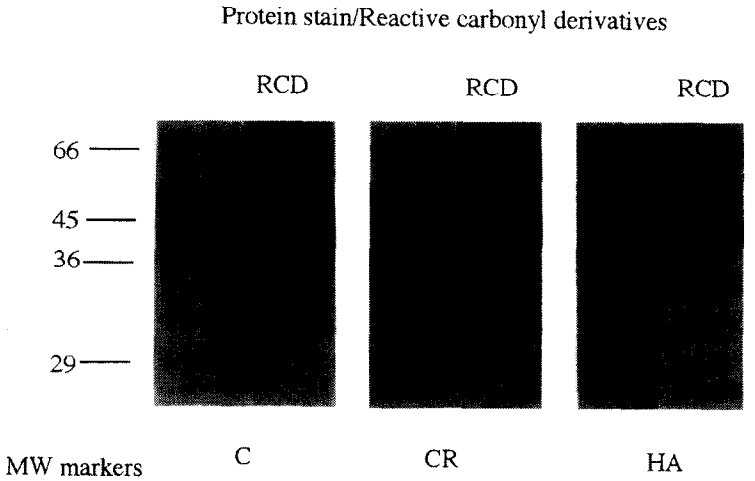

Fig. 1. Accumulation of reactive carbonyl derivatives (RCD) is significantly more enhanced in C rats than in CR and HA groups. Molecular weight markers are shown on the left. The RCD signals are displayed on the right of the Coomassie Blue panels.

Discussion

The present study is the first to show that short term exposure to HA and CR decrease the accumulation of oxidized proteins in the brain. This finding in HA rats is somewhat unexpected, because our previous studies showed increases in lipid peroxidation following 6 month intermittent exposure (11) and that physical training at HA also increased the level of RCD in skeletal muscle of rats (13). It is known that low oxygen pressure decreases the rate of free radical formation (9) and the activity of antioxidant enzymes decreases in some organs (10,11). Brain has a relatively low antioxidant enzyme activity and further decrease might jeopardize cell functions and increase oxidative damage (20). According to our hypothesis this

may happen during mountain sickness induced neurological dysfunctions. It seems unlikely that the decreases in accumulation of RCD was due to an increase in antioxidant enzyme activity as HA donwregulates the activity of antioxidant enzymes (10,11). Rather, it might be a result of increased proteolytic degradation of RCD, since the rate of oxidized protein accumulation depends on the proteolytic process (21). Hence, the accumulation of RCD in conditions which have the same level of free radical formation but induce very different level of proteolytic degradation might be substantially different. Therefore, decreases in accumulation of RCD do not always reflect a decrease in oxidative stress.

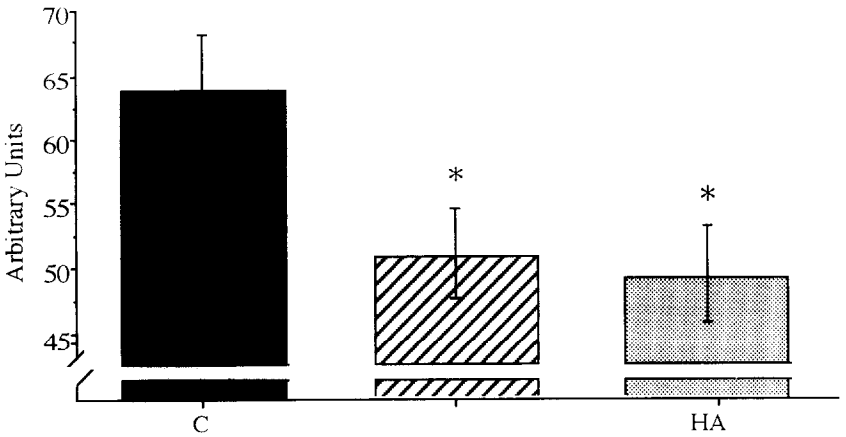

Fig. 2. The quantification of the accumulation of RCD was done as described in Methods section. The values are means+SD of five animals per group. *P <0.005 vs. C.

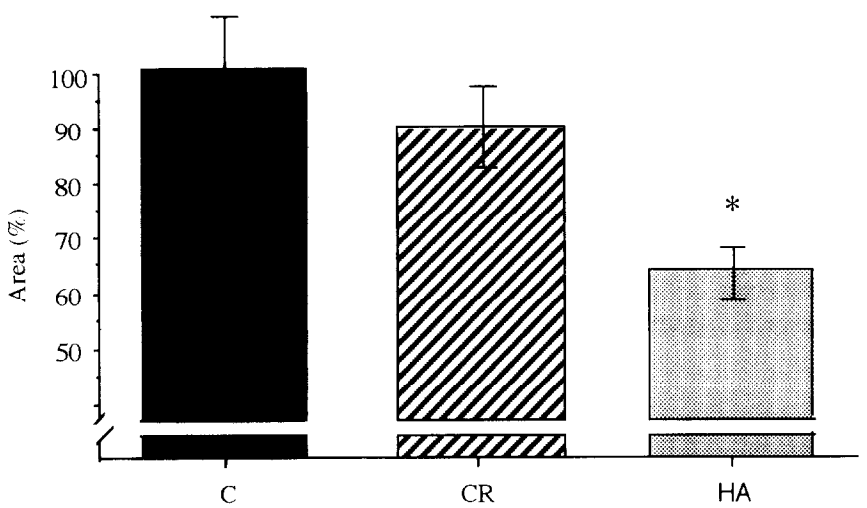

Fig 3. The activity of GS was significantly (*P <0.005 vs. C) higher in brain of CR rats than C rats. Values are means±SD of six animals per group. The assay was carried out with duplicate samples.

Rennie et al (7) reported that exposure to HA decreases the rate of protein synthesis and possibly increases the rate of protein degradation, and this process in some part could account for the decrease in body mass, which is associated with exposure to HA. Therefore, the possibility cannot be excluded that the decreases in accumulation of RCD in HA rats is rather a result of faster removal of "oxidative junk" than decrease in oxidative stress or increased level

of antioxidant protection. Decreases in protein content of GS in HA group might also be due to increases in proteolytic degradation and this process could support our hypothesis on the decreases in RCD. However, this suggestion needs further study.

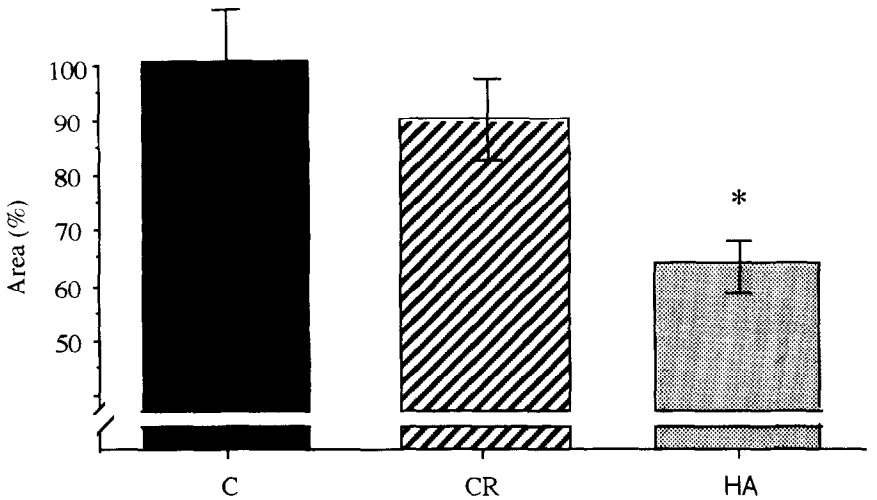

Fig. 4. The protein content of GS was measured by Western blot and the data was quantified as described in Methods. The content of GS decreased significantly in HA rats (*P <0.005 vs. C), which is an indication of increases in proteolytic degradation at HA. Values are means±SD of five animals per group.

CR decreases the basal metabolic rate and the need for ATP production. The intensity of electron transport is reduced and subsequently the rate of free radical formation could fall, as it is suggested from those studies in which the CR increased the life span (22-24). Moreover, CR increases the rate of protein turnover, so the rate of protein degradation is also altered. Indeed, Ishigami and Goto (25) reported that CR of mice decreases half-life of proteins in the cell in primary culture. A recent study by Kim et al. (26) showed that CR increased the activity of antioxidant enzymes and decreased the level of lipid peroxidation. In the present study, the decrease in RCD of CR and HA group was similar, however the mechanism might be different since the activity of GS inreased during CR compared with C rats. On the other hand, the protein content did not change significantly in CR rats unlike in HA group. This indicates that the decreased level of RCD in CR group is rather a result of decrease in an insult of radical species than increases in protein degradation. This suggestion is in accordance with the data of Kim et al. (26), possibly indicates an increase in scavenging activity of the antioxidant system.

GS is used as an oxidative stress marker on proteins (27). Glutamate plays a key role as neurotransmitter and is abundant in brain (17). Its concentration is regulated by the uptake by glial cells and its conversion to glutamine by GS. The activity of GS in the brain is significant compared with other organs, pointing out the importance of ammonia transport in central neurons system. During starvation there is an increase in ammonia formation, which is associated with increase in release of glutamine into the blood stream, due to the activity of GS in those organs that are responsible for glutamine synthesis (28). Skeletal muscle is considered to be a most important element in this system, however brain also plays similar but quantitatively less significant role. The activity of GS did not change significantly in HA rats, while the protein content of the enzyme decreased probably as a result of increase in proteolytic degradation (7). On the other hand, the increase in activity of GS in CR rats may be due to the increases in the protein turnover rate (GS plays a significant role in protein synthesis), which occurs during CR (19).

Therefore, it is suggested that both short term HA and CR decrease the accumulation of RCD in the brain. However, the mechanism behind the suppression of RCD might be different during exposure to HA and CR and this hypothesis needs further investigation. The activity of GS

increased during CR, which might be the effect of decrease in the insult of free radical species or it also could be a result of other physiological process(es) induced by CR.

Acknowledgements

The authors wish to gratefully acknowledge the advice of Professor Earl R. Stadtman and Dr. Abraham Z. Reznick.

References

1. A. AMICI, R.L. LEVINE, L. TSAI and E. STADTMAN, J. Biol. Chem. **264** 3341-3346 (1989).
2. E. STADMAN and C.N. OLIVER, J. Biol. Chem. **266** 2005-2008 (1991).
3. R.L. LEVINE, J. Biol. Chem. **258** 11823-11827 (1983).
4. R.L. LEVINE, J. Biol. Chem. **258** 11828-11833 (1983).
5. R.L. LEVINE, C.N. OLIVER, R.M. FULKS and E.R. STADTMAN, Proc. Natl. Acad. Sci. USA **78** 2120-2124 (1981).
6. M.J. RENNIE, H.S. HUNDEL, P. BABIJ, P. MACLENNAN, P.M. TAYLOR and P.W. WATT. Lancet **2** 1008-1011 (1986).
7. M.J. RENNIE, P. BABIJ, J.R. SUTTON, W.J. TONKINS, W.W. READ, R. FORD and D. HALLIDAY, In: J.R. Sutton, C.S. Houston, N.L. Jones (Eds), 317-323, A. R. Liss, New York (1983).
8. E.R. STADTMAN, Free Rad. Biol. Med. **9** 315-325, (1990).
9. H, DE GROOT, and A, LITTAUER, Free Rad. Biol. Med. **6** 541-551, (1989).
10. K. NAKANISHI, F. TAJIMA, A. NAKAMURA, S. YAGURA, T. OOKAWARA, H. YAMASHITA, K. SUZUKI, N. TANIGUCHI and H. OHNO, J. Physiol. **489** 869-876, (1995).
11. Z. RADAK, K. ASANO, M. INOUE, T. KIZAKI, S. OH-ISHI, K. SUZUKI, N. TANIGUCHI, and H. OHNO, Eur. J. Appl. Physiol. **72** 189-194, (1996).
12. I. SIMON-SCHNASS and L. KOMISZEWSKI, Int. J. Vitam. Res. **60** 26-34, (1990).
13. Z. RADAK, K. ASANO, K. LEE, H. OHNO, A. NAKAMURA, H. NAKAMOTO and S. GOTO, Free Rad. Biol. Med.**22** 1109-1114, (1997).
14. L. LYRAS, P.J. EVENS, P.J. SHAW, P.G. INCE and B. HALLIWELL, Free Rad. Res. **24** 397-406, (1996).
15. R.L. LEVINE, J.A. WILLIAMS, E.R. STADTMAN and E. SCHACTER, Methods of Enzymol. **37** 346-357 (1994).
16. A. NAKAMURA and S. GOTO, J. Biochem.**119** 768-774 (1996).
17. R.E. MILLER, R. HACKENBERG and H. GERSHMAN, Proc. Natl. Acad. Sci. USA **75** 1418-1422 (1978).
18. W.B. ROWE, R.A. RONZIO, V.P. WELLNER and A. MEISTER, Methods Enzymol **17a** 900-910 (1970).
19. O.H. LOWRY, N.J. ROSENBROUGH, A.L. FARR and T.D. RANDALL, J. Biol. Chem. **193** 265-275 (1951).
20. B. HALLIWELL, J. Neurochem. **59** 1609-1623 (1992)
21. S. GOTO, A. HASEGAWA, H. NAKAMOTO, A. NAKAMURA, R. TAKAHASHI and I.V. KUROCHKIN, in *Oxidative Stress and Aging,* R.G. Cutler, L. Packer, J. Bertram, A. Mori (Eds), 151-158, Birkhauser Verlag, Basel (1995)
22. R.S. SOHAL, H.H. KU, S. AGARMAL, M.J. FOSTER and H. LAL, Mech. Ageing. Dev. **74** 121-133 (1994).
23. W.C. ORR and R.S. SOHAL, Science **263** 1128-1130 (1994).
24. V.G. DESAI, R. WEINDRUCH, R.W. HART and R.J. FEUERS, Arch. Biochem. Biophys. **333** 145-151 (1996).
25. T. ISHIGAMI AND S. GOTO, Arch. Biochem. Biophys. **283** 362-366 (1990).
26. J.D. KIM, P.B, YU, R.J.M. McCARTER, S.Y. LEE and J.T. HERLIHY, Free Rad. Biol. Med. **20** 83-88 (1990).
27. C.N. OLIVER, P.E. STRARKE-REDD, E. R. STADTMAN, G.J. LIU, J. M. CARNEY, and R.A. FLOYD, Proc. Natl. Acad. Sci. USA **87** 5144-5147 (1990).
28. L. GOLDSTEIN, Fed. Proceed. **45** 2176-2179 (1986).