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Effect of acute hypobaric hypoxia on skeletal muscle protein turnover

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Abstract: *Objective:* Present study was designed to investigate the effects of altitude mediated acute hypobaric hypoxia on protein turnover in skeletal muscle. *Background:* Skeletal muscle wasting is considered to be an important factor leading to decrease in physical performance at high altitude. Changes in protein turnover and the mechanism underlying the loss of skeletal muscle mass under these conditions are not very well understood. *Method:* Male Sprague Dawley rats (n=12 each group) weighing about 180-200 g were exposed to hypobaric hypoxia (9144 m) for six hours. Skeletal muscle homogenates were prepared for carrying out various studies including proteolytic pathways. *Result:* Acute exposure to hypobaric hypoxia has deleterious effects on skeletal muscle mass which was reflected by one third attenuation in protein synthesis and more than 50 % increase in protein degradation. Hypobaric hypoxia resulted in upregulation of Ub-Proteasome pathway and calpain whereas lysosomal mediated proteolysis remained unchanged. Glutaminase activity, protein carbonyl status, oxidative stress markers were also found to be elevated in hypoxia exposed rats. *Conclusion:* These results are indicative of excessive protein degradation and decreased protein synthesis during acute hypobaric hypoxia. Moreover increased protein oxidation may also be an important contributor for the skeletal muscle loss.
Keywords: hypobaric hypoxia, protein turn over, skeletal muscle atrophy, oxidative stress

Introduction

Exposure of sea level residents to high altitude leads to a number of physical and physiological alterations. Hypobaric hypoxia, which prevails at high altitude causes loss of body mass, decreased growth and increased oxidative stress inducing various deleterious cellular effects [1]. It also leads to changes in metabolic processes especially in protein metabolism in skeletal muscle [2]. Consalazio et al [3] observed negative nitrogen and water balance after four week exposure to 4300 m. Hypobaric hypoxia has been documented to be an important factor in skeletal muscle atrophy even at moderate altitudes [4]. Furthermore, skeletal muscle glutaminase and glutamine synthetase, the main enzymes of protein metabolism, increased following hypobaric hypoxic exposure [2]. A few studies suggest that acute exposure to hypoxia decreases skeletal muscle protein synthesis rate [5-6]. However, Imoberdorf et al [7] have observed an increase in fractional protein synthesis rate in human volunteers after 19-23 h of exposure after active ascent to high altitude. Skeletal muscle atrophy may be an adaptive response to widely divergent stimuli that could be mechanistically explained by the decrease of protein synthesis and increase of protein breakdown [8]. The ubiquitous calcium activated proteases calpains m and μ are assumed to play a key role in the disassembly of sarcomeric proteins that occurs in muscle atrophy as well as in the necrosis process accompanying muscular dystrophies [9].

Earlier investigators have also observed a major role of the ubiquitin-proteasome dependent proteolysis as a primary mediator of muscle remodeling in various types of atrophy [8]. Hypoxia induced oxidative stress and protein oxidation may be an important factor mediating enhanced protein degradation under hypobaric hypoxic condition. It has been observed that high altitude hypoxia increases reactive protein carbonyls in skeletal muscle of rats [10], which become substrate for the proteasome [11-12]. Despite a number of studies reporting skeletal muscle atrophy as an important consequence of exposure to

hypobaric hypoxia, the underlying mechanism is still to be uncovered. Since the knowledge of the mechanisms of muscle mass loss will be vital to the design of rational therapeutic or nutritional intervention, the present study was designed to elucidate the mechanism behind hypobaric hypoxia mediated skeletal muscle loss by evaluating changes in protein turnover.

Material and Methods

Animals and experimental design: Experiments were conducted on Male Sprague-Dawley rats, weighing about 180-200 g. Rats were maintained at 25±2 ^{o}C in Animal facility, DIPAS, India, and given food and water ad-libitum. The animals were housed three rats per cage, and maintained on a 12 h, day–night cycle. The study was approved by the Institute's Animal Ethical Committee and confirms to National guidelines on the care and use of laboratory animals. The rats were randomly allocated to two groups. The groups were control: 'C 'and hypoxia treated: 'H'. Each group consisted of twelve rats. Rats in 'H' group were exposed to hypobaric hypoxia at a simulated altitude of 9144 m in a hypobaric chamber for 6 h. Control group rats were maintained in the normoxic condition within the same laboratory. Rats were killed by cervical dislocation and hind limb muscles were excised and immediately placed in Krebs-Henseleit bicarbonate buffer for incubation as described [13-14]. The muscles were quickly rinsed and incubated in Krebs-Henseleit buffer consisting of 120 mM NaCl, 4 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$ (pH 7.4), supplemented with 5 mM glucose, 5 mM HEPES, 0.1% (w/v) BSA, 0.17 mM leucine, 0.20 mM valine, and 0.10 mM isoleucine.

Protein Synthesis and Protein Degradation Rates: Protein synthesis rate was measured as previously described [13,15]. Muscles were first preincubated at 37 ^{o}C for 30 min. After the preincubation, fresh Krebs-Henseleit bicarbonate buffer supplemented with ^{14}C-leucine (0.10 µCi/ml) was added to the skeletal muscle, and incubated for a further 60 min. Muscles were removed from the incubation buffer and homogenized in 10% (w/v) ice-cold TCA. The homogenate was centrifuged at 10,000 g for 10 min at 4 ^{o}C. The supernatant was decanted and the pellet was suspended in 1M NaOH and incubated at 37 ^{o}C for 30 min. Aliquots of this mixture were used to quantify the radioactivity based on liquid scintillation counting of β emission. The rate of protein synthesis was expressed as nmoles of leucine incorporated per hour per milligram of muscle protein. Protein degradation rate was determined by the release of tyrosine over a period of 2 hr as described previously [13-14]. Tyrosine was assayed fluorometrically [16]. The rate of protein degradation was expressed as nmoles of tyrosine released per 2 hour per milligram of muscle protein.

Protein Degradation Pathways

Calpain Assay: Calpains were measured in the homogenate using *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) as a substrate [17]. A stock solution of 50 mM SLY-AMC was prepared in dimethyl sulfoxide and stored at -20°C. Muscle extract was incubated for 60 min at 37°C in a buffer solution (pH 7.4) containing 25 mM HEPES (pH 7.5), 0.1% CHAPS, 10 % sucrose, 10 mM DTT, 0.1 mg/ml ovalbumin and substrate. Fluorescence of the liberated AMC was monitored in a Perkin-Elmer fluorimeter (LS-45) at excitation 380 nm, emission 460 nm.

20 S Proteasome Activity of Ub- Proteasome pathways: The ubiquitin proteasome pathway was studied by assaying the chymotrypsin-like enzyme activity of 20 S Proteasome, as described earlier [18]. The muscle extracts containing 60 µg protein were incubated for 30 min at 37°C in 50 µl of a buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM DTT, 5 mM $MgCl_2$, 1 mM Suc-LLVY-AMC, 2 mg/ml ovalbumin, and 0.07% SDS. The reaction was terminated by 25 µl of 10% SDS and diluted by 2 ml of 0.1 M Tris-HCl (pH 9.0). Fluorescence of the liberated AMC was monitored in a Perkin-Elmer fluorimeter at excitation 380 nm, emission 460 nm.

Lysosomal Enzymes Assay: Acid Phosphatase activity: Acid phosphatase, marker enzyme of lysosomes, was determined using the p-nitropheno-phosphate method [19]. Muscle homogenate was incubated at 37°C for 10 min in 2.5 mM sodium acetate buffer, pH 5.0 and 0.5 mM p-nitrophenol phosphate. The reaction was stopped by adding 0.2 ml NaOH (5N) and the absorption was read at 405 nm using UV-Vis spectrophotometer (BioRad, USA).

Oxidative Stress Markers

Protein Carbonyl Estimation: Protein carbonyl content was measured as described previously [20]. Briefly, muscle homogenate was incubated with DNPH for 1 hour. Tricarboxylic acid (20 %) was then added to the homogenate and centrifuged at 11000 g for 3 minutes. After washing the pellet thrice with ethanol: ethylacetate, pellet was redissolved in guanidine HCl (6 M) and absorbance was then read at 366 nm using UV-Vis spectrophotometer, (BioRad, USA).

Free Radicals (ROS) Estimation: The production of free radicals was determined by using 2,7-dichlorofluoroscein diacetate (DCFH-DA) as described earlier [21]. Briefly, 150 µl of muscle homogenate was incubated with (10 µl) 100 µM DCFH-DA for 30 minutes in dark after which fluorescence was read at excitation at 485 nm and emission at 535 nm.

GSH Estimation:
Reduced glutathione was measured as described by Butler et al [22] with slight modifications. Briefly, muscle homogenate was incubated with precipitating reagent (5M metaphosphoric acid, 5 mM EDTA and 5 M NaCl) at room temperature for 5 minutes and then centrifuged at 6500 g for 15 min. Supernatant was added to Na_2HPO_4 (0.3M) and DTNB (1M) in sodium citrate. Absorbance was read at 412 nm using UV-Vis spectrophotometer, (BioRad, USA).

MDA Estimation: Malondialdehyde (MDA) was measured in muscle tissue homogenates as described earlier [23]. Briefly, 100 mg tissue was homogenized in 15 % (w/v) TCA and 0.355 % (w/v) TBA and then incubated in boiling water bath for 30 min. It was then centrifuged and absorbance was read at 535 nm using UV-Vis spectrophotometer, (BioRad, USA).

Total Protein Estimation: Total Protein in skeletal muscle homogenate was assayed using Lowry's method [24].

Glutaminase enzyme activity: Glutaminase enzyme activity in rat muscle homogenate was measured as described earlier [25]. Briefly, GDH was incubated in buffer containing L-Glutamine (100 mM), oxoglutarate (50 mM), phosphate buffer (1 M), EDTA (2mM) and NADH for 5 minutes at 25°C. Initial absorbance was read at 340 nm. Tissue homogenate was added to it and incubated again for 2 minutes at 25°C. Final absorbance was again read at 340 nm.

Glutamine synthetase activity: Glutamine synthetase activity in rat muscle homogenate was measured as described earlier [26]. Briefly, tissue homogenate was incubated with buffer containing Tris (0.1 M), $MgSO_4$ (20 mM), sodium glutamate (80 mM), hydroxylamine (6 mM) and ATP (8 mM) for 5-15 minutes at 37°C. Reaction was terminated by adding ferric chloride (0.37 M) and absorbance was read at 540 nm.

Statistical Analysis: All the results are presented as mean ± SEM. The experiments were conducted on two different occasions and the data was analyzed using Student t-test. Significance level was set at $p<0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc Version 15.0)

Results

Effect of acute hypobaric hypoxia on Protein turnover and total protein: Protein synthesis rates analyses revealed that synthesis rates decreased by 32 % ($P<0.05$) in skeletal muscle of rats exposed to hypobaric hypoxia for 6 hours as compared to control animals (Fig.1a). Muscle proteolysis was compared between control and hypoxia exposed groups (Fig.1a). The hypoxic exposure led to about 57 % increase in the rate of protein degradation compared to control rats. Total protein content in the skeletal muscle homogenates of rats exposed to acute hypobaric hypoxia showed a significant decrease of about 13% ($p<0.05$) over the control rats (Fig 1b).

Fig-1: Effect of acute hypobaric hypoxia on (a) Protein Turnover rate (PS: Protein Synthesis Rate expressed in nmol/hr/mg protein and PD: Protein Degradation Rates expressed in nmol/2hr/mg protein), (b) Total Protein Content in rat hind limb muscle (*$P<0.05$ versus control group)

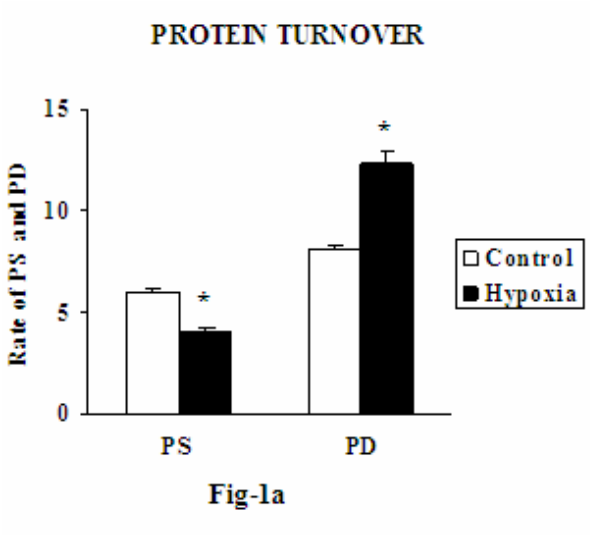


Fig-1a

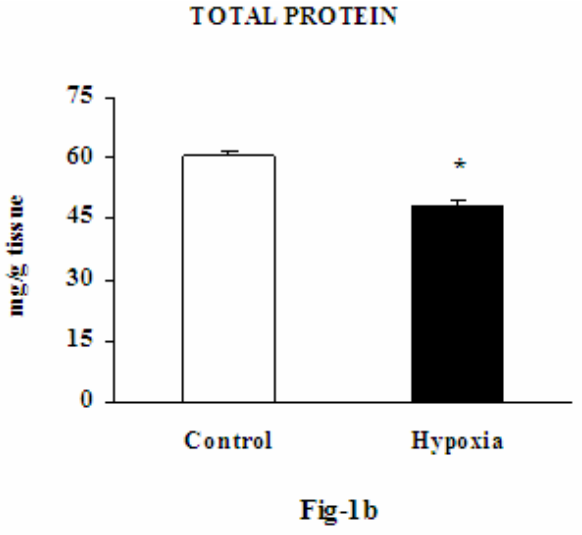


Fig-1b

Effects of acute hypobaric hypoxia on enzymes Glutaminase and Glutamine synthetase: Exposure of animals to acute hypobaric hypoxia caused a significant increase in glutaminase activity (Fig. 2a). Whereas glutamine synthetase enzyme activity showed a significant decrease (Fig. 2b) following acute hypobaric hypoxia exposure.

Fig-2: Effect of acute hypobaric hypoxia on (a) Glutaminase Activity and (b) Glutamine Synthetase Activity in rat hind limb muscle (*P<0.05 versus control group)

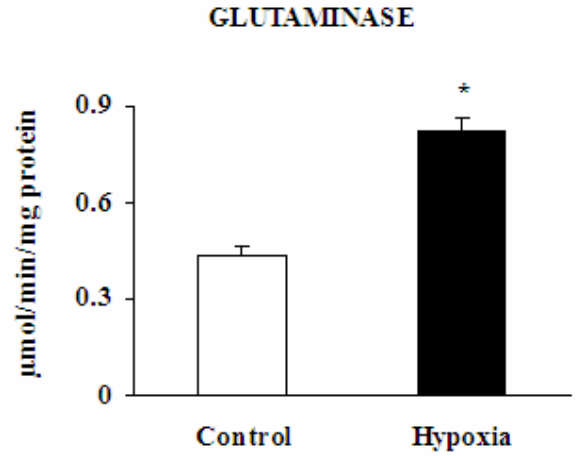


Fig. 2a

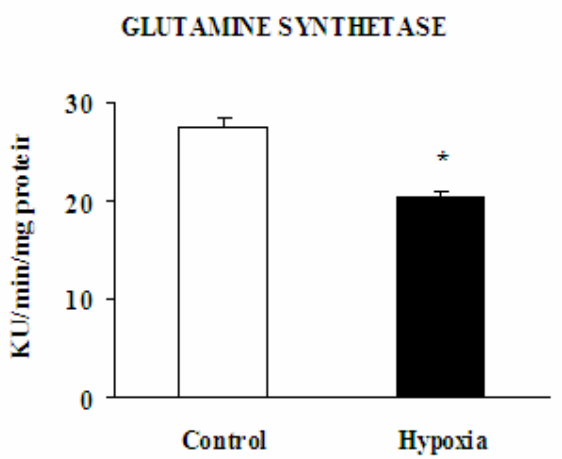


Fig. 2b

Effects of acute hypobaric hypoxia on Ub-proteasome pathway, Calpain Activity and Lysosomal Acid Phosphatase Activity: The chymotrypsin-like activity of Ub-proteasome pathway (Fig. 3a) was increased by approximately 60% ($p<0.05$) over control in the exposed group. Similarly, calpain activity also showed a 40% increase ($p<0.05$) over control (Fig. 3b). However, no significant change was observed in the acid phosphatase activity (Fig.3c) after exposure to acute hypobaric hypoxia.

Fig-3: Effect of acute hypobaric hypoxia on protein degradation pathways (a) Chymotrypsin like enzyme activity, (b) Calpain activity, (c) Acid phosphatase activity in rat hind limb muscle (*P<0.05 versus control group)

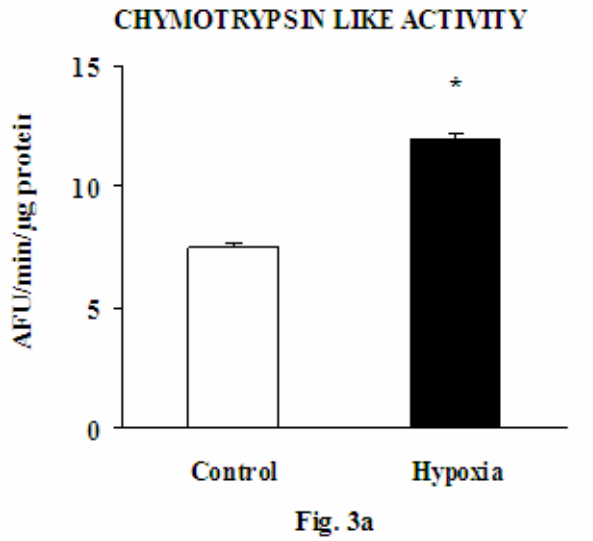


Fig. 3a

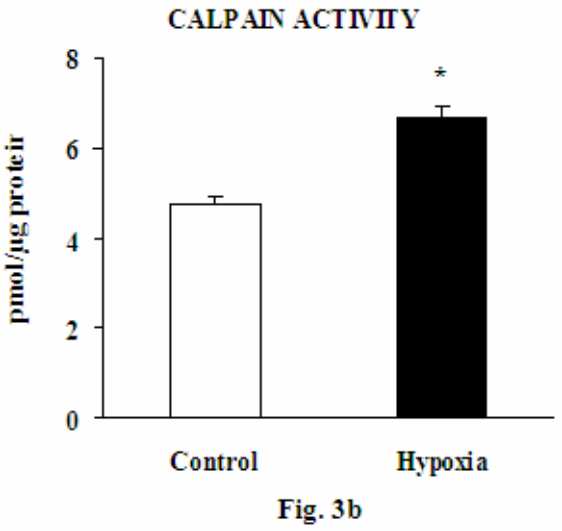


Fig. 3b

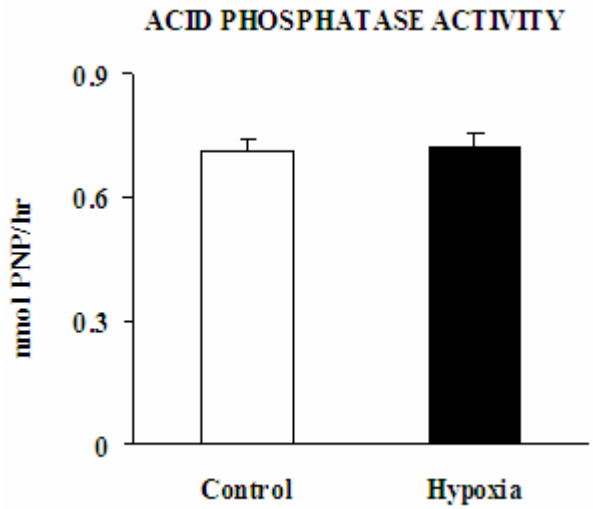


Fig. 3c

Effects of acute hypobaric hypoxia on Oxidative stress markers: A significant increase in the levels of Protein carbonyl, MDA and ROS was observed in animals after exposure to acute hypoxia. Levels of reduced glutathione (GSH), an antioxidant, showed a significant decrease over control (Table 1).

Table-1: Effect of acute hypobaric hypoxia on oxidative stress markers in rat hind limb muscle

Oxidative stress marker	Control group	6 h hypoxia exposed group
Protein carbonyl (nmol/mg protein)	2.72±0.45	6.04±0.28*
ROS (AFU/g tissue)	51.29±0.07	72.25±0.09*
MDA (nmol/g tissue)	20.14±1.39	33.03±2.11*
GSH (µmol/g tissue)	2.9±0.13	2.36±0.12*

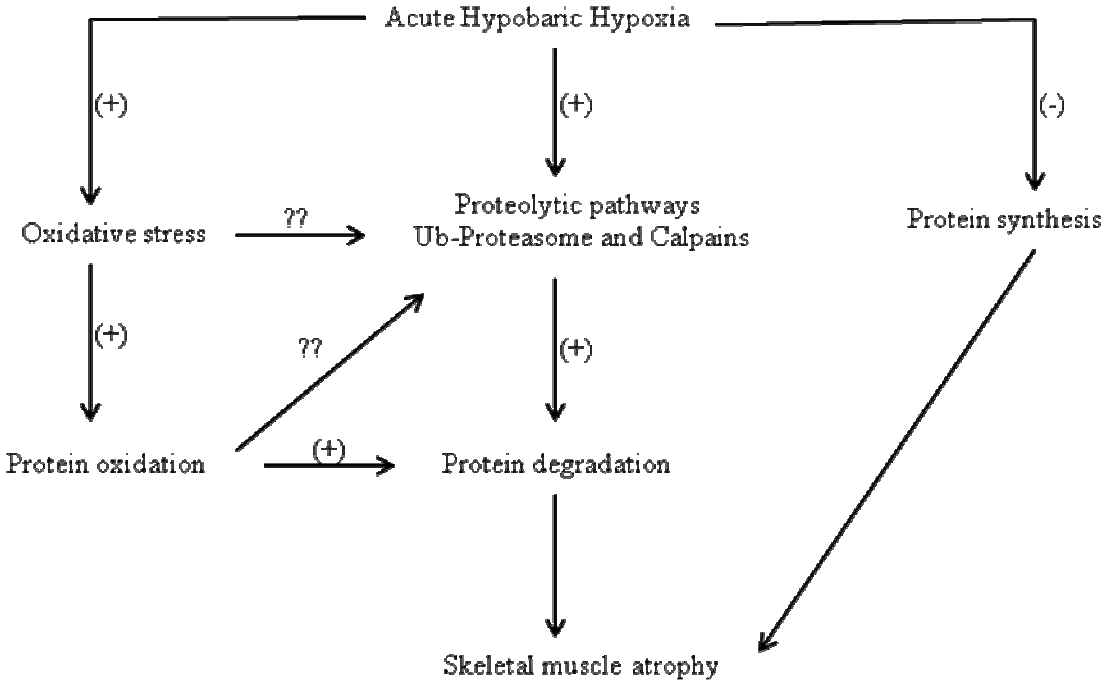
*P<0.05 versus control group

Discussion

The present study demonstrated the effect of acute hypoxia on the skeletal muscle protein turn over. The exposure of animals to acute hypoxia led to a significant increase in protein degradation leading to a loss of muscle protein. Through our experiments, we have established that acute hypobaric hypoxia leads to loss of lean body mass by exhibiting a decrease in muscle protein synthesis rate and a concomitant increase in protein degradation rate. The decreased protein synthesis after acute hypoxic exposure, as observed by us, is in accordance with earlier studies [5-6]. An increase in glutaminase enzyme activity supports the observed increase in protein degradation rate as glutaminase catalyzes the breakdown of glutamine residue resulted from proteolysis of skeletal muscle proteins. A decrease in glutamine synthetase enzyme may be a factor responsible for the decreased protein synthesis.

Skeletal muscle has three different proteolytic pathways which are responsible for the protein degradation under different catabolic conditions. These include the ubiquitin-proteasome pathway, calpains (calcium activated proteases) and the lysosomal enzymes. Our results indicate that upregulation of ubiquitin-proteasome pathway and calpains may be responsible for enhanced protein degradation in the skeletal muscle under hypobaric hypoxic conditions. The Ub-proteasome pathway has also been shown to

account for the majority of skeletal muscle degradation in cancer cachexia where hypoxia is encountered [27]. Acute hypobaric hypoxia led to increased protein oxidation as indicated by enhanced protein carbonyl level, which is in accordance with earlier study [10]. Hypoxia induced increased oxidative stress was indicated by a significant increase in the free radicals and malondialdehyde status alongwith a significant decrease in the GSH level. The reactive oxygen species is known to activate NF-Kb [28] which may have a role in upregulation of the proteasome pathway. The inhibition of ubiquitin-proteasome activity is associated with down-regulation of NF-kB mediated inflammatory pathways [29] and vice versa inhibition of NF-kB resulted in decreased Ub-proteasome activity [30].



In summary, excessive protein degradation and decreased protein synthesis during exposure to acute hypobaric hypoxia resulted in loss of skeletal muscle mass. Hypobaric hypoxia altered the protein synthesis machinery as indicated by decrease in glutamine synthetase enzyme activity. At the same time up-regulation in activity of Ub-proteasome pathway and Calpains led to enhanced protein degradation in skeletal muscle. Our results also concluded that increased protein oxidation as a result of oxidative stress induced at high altitude may also be important factor responsible for the skeletal muscle loss.

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