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Chronic hypobaric hypoxia mediated skeletal muscle atrophy: role of ubiquitin–proteasome pathway and calpains

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Abstract The most frequently reported symptom of exposure to high altitude is loss of body mass and decreased performance which has been attributed to altered protein metabolism affecting skeletal muscles mass. The present study explores the mechanism of chronic hypobaric hypoxia mediated skeletal muscle wasting by evaluating changes in protein turnover and various proteolytic pathways. Male Sprague–Dawley rats weighing about 200 g were exposed to hypobaric hypoxia (7,620 m) for different durations of exposure. Physical performance of rats was measured by treadmill running experiments. Protein synthesis, protein degradation rates were determined by ^{14}C-Leucine incorporation and tyrosine release, respectively. Chymotrypsin-like enzyme activity of the ubiquitin–proteasome pathway and calpains were studied fluorimetrically as well as using western blots. Declined physical performance by more than 20%, in terms of time taken in exhaustion on treadmill, following chronic hypobaric hypoxia was observed. Compared to 1.5-fold increase in protein synthesis, the increase in protein degradation was much higher (five-folds), which consequently resulted in skeletal muscle mass loss. Myofibrillar protein level declined from 46.79 ± 1.49 mg/g tissue at sea level to 37.36 ± 1.153 ($P < 0.05$) at high altitude. However, the reduction in sarcoplasmic proteins was less as compared to myofibrillar protein. Upregulation of Ub-proteasome pathway (five-fold over control) and calpains (three-fold) has been found to be important factors for the enhanced protein degradation rate. The study provided strong evidences suggesting that elevated protein turnover rate lead to skeletal muscle atrophy under chronic hypobaric hypoxia via ubiquitin–proteasome pathway and calpains.

Keywords Hypobaric hypoxia · Muscle atrophy · Protein turnover · Ubiquitin–proteasome pathway

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Introduction

It has now become a well known fact that physical performance of people decreases on ascending to high altitude [1]. People ascending to high altitude expose themselves to a number of challenging environmental conditions such as low air humidity, low temperature, high UV radiations, and most importantly hypoxia. High altitude hypoxia has been reported to be an important factor in skeletal muscle atrophy at moderate altitudes [2]. High altitude mediated hypobaric hypoxia initiates many physiological changes with loss of body mass and protein stores being the most inevitable changes [3]. The decreased physical performance observed at high altitude [4–6] is likely to be a consequence of the dwindled protein stores. Sustained exposure to severe hypoxia has detrimental effects on muscle structure [7]. Some studies showed that muscle cross-sectional area in the thigh decreased by 10% after sojourns went to the Himalayas. Morphologically this loss in muscle mass appeared as a decrease in muscle fiber size mainly due to a loss of myofibrillar proteins [8]. Muscle lipofuscin, a degradation product of lipid peroxidation and

an indicator of muscle fiber damage, has been shown to increase by more than two-fold after an expedition to the Himalayas [9]. Human subjects climbing to Peak Lenin (7,134 m) showed a significant decrease in fat-free mass after their return [10]. The study by Bigard et al. [11], clearly demonstrate that hypobaric hypoxia decreases growth rate in rats and increasing the dietary protein intakes in rat had no effect on the depression of muscle growth related to high altitude but had deleterious effects on glycogen deposition in liver and fast muscle.

Decreased protein synthesis is suggested as the underlying mechanism for the loss of skeletal muscle mass [12–14]. Since these studies are acute, presumably, the short duration exposure to hypoxia does not allow sufficient time for relevant hypoxic responses to occur and thus does not determine whether and how the chronic hypoxia affects protein synthesis rate. Only a limited number of chronic studies reporting protein turnover under hypobaric hypoxia have been published. Most of these studies have reported attenuated protein synthesis rate based on indirect measurements such as reduction in mammalian target of rapamycin (mTOR), a marker of protein synthesis after hypobaric hypoxic exposure [15].

Thus, declined protein synthesis has been shown to be detrimental for skeletal muscle mass in numerous studies. However, contrasting results have also been reported. Imoberdorf and co-workers [16] assessed muscle protein synthesis rate after exposure to high altitude and showed that in a group of subjects, investigated acutely after active ascent to high-altitude, muscle protein synthesis rate was higher compared to in a group that was flown to the altitude and compared to the rate at sea level. Similarly, resting skeletal muscle myocontractile protein synthesis rate was shown to be concomitantly elevated by high-altitude induced hypoxia [17].

Increased excretion of proteins in the urine of native high landers [18] provides a clue for enhanced muscle protein catabolism at high altitude. Skeletal muscle atrophy under various catabolic conditions such as cachexia, burn, and sepsis has been linked to upregulation of different proteolytic pathways like ubiquitin–proteasome pathway [19, 20], lysosomal pathways [21], and calpains [22, 23]. However, the role of these pathways is still not clear in the hypobaric hypoxia mediated skeletal muscle loss.

Inconsistency of the earlier results and lack of conclusive evidences for the mechanism of hypobaric hypoxia induced skeletal muscle atrophy led us to design the present study. The purpose of this study was to evaluate the effect of chronic hypobaric hypoxia on skeletal muscle mass and protein turnover and to find out the exact mechanism of loss of skeletal muscle mass under these conditions.

Materials and methods

Ethical approval

The study was approved by the Animal Ethical Committee of our institute in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) of the government of India.

Experiments were conducted on Male Sprague–Dawley rats, weighing about 180–200 g. Rats were maintained at 25 ± 2°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 rats were randomly allocated to two groups. The groups were control: C and hypoxia treated: H. Hypoxia group was further divided into three batches: hypoxia exposure for 3, 7, and 14 days. These batches ($n = 12$) were exposed to hypoxia at a simulated altitude of 7,620 m in a hypobaric chamber. Since food intake decreases during hypoxia exposure, one group was pair fed to the intake of hypoxia exposed rats. Control group rats were maintained in the normoxic condition within the same laboratory.

Physical performance measurements: treadmill exercise

Before starting the exposure experiments, all the animals were familiarized for 2 weeks on a motorized treadmill (0% grade) at 25 m/min for 45 min daily. Fatigue time of rats on the treadmill was recorded before and after exposure of rats to hypoxia.

Histology

The animals ($n = 12$) were anesthesized by injection of sodium pentobarbital (50 mg/kg, i.p.), and perfused intracardially with 0.1 M PBS (pH 7.4) followed by 4% formaldehyde. The muscles were then carefully dissected out and postfixed in the same fixative overnight. Paraffin blocks were then prepared after dehydration, clearing, and wax impregnation. Sections of 5 μm were cut with a rotary microtome, deparaffinized in xylene, and stained with Hematoxylin and Eosin.

Protein synthesis and protein degradation rates

Rats were killed by cervical dislocation and hind limb gastrocnemius muscles were excised and immediately placed in Krebs-Henseleit bicarbonate buffer for incubation as described [24, 25]. 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 rate was measured as previously described [25, 26]. Muscles were first preincubated at 37°C for 30 min. After the preincubation period, fresh Krebs-Henseleit bicarbonate buffer supplemented with 0.10 μCi/ml ^{14}C-leucine were added to the skeletal muscle, and incubated for a further 60 min. At the end of the incubation, muscles were removed from the incubation buffer, washed with cold buffer and homogenized in 10% (w/v) ice-cold TCA. The homogenate was centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant was decanted and the pellet was suspended in 1 M NaOH and incubated at 37°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 h as described previously [24, 25]. Because tyrosine is neither synthesized nor degraded by muscles, its release from muscle into the incubation medium reflects the net protein degradation rate. Tyrosine was assayed fluorometrically [27]. The rate of protein degradation was expressed as nmoles of tyrosine released per 2 h per milligram of muscle protein.

Protein degradation pathways

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 [28]. The fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) served as substrate for the chymotrypsin-like activity. 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-MCA, 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 amidomethylcoumarin was monitored in a Perkin-Elmer fluorometer at excitation 380 nm, emission 460 nm. Chymotrypsin-like enzyme activity was expressed arbitrary units per minute per microgram of muscle protein.

Calpain assay

Calpains, calcium activated proteases, were measured in the homogenate using *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) as a substrate [29]. A stock solution of 50 mM SLY-AMC was prepared in dimethyl sulfoxide and stored at −20°C. Muscles were homogenized in buffer having 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 10 mM NaH_2PO_4, 1% nonidet P-40, and 0.4 mM sodium orthovanadate. Homogenate was then centrifuged at 13,000×*g* for 15 min at 4°C. The following procedure was used for measuring calpain activity in muscle extracts: 30 μl 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. After addition of 5 ml of the substrate solution, buffer was added to adjust the volume of the assay to 2 ml. Fluorescence of the liberated AMC was monitored in a Perkin-Elmer fluorimeter (LS-45) at excitation 380 nm, emission 460 nm. Calpain activity, Ca^{2+} dependent cleavage of SLY-AMC, was expressed as picomole AMC released per microgram of muscle protein.

Lysosomal enzymes assay

Acid phosphatase activity, marker enzyme of lysosomes, was determined using the *p*-nitrophenyl-phosphate method [30]. An aliquot of 20 μl of the homogenate was incubated at 37°C for 10 min in 2.5 mM sodium acetate buffer, pH 5.0 and 0.5 mM *p*-nitrophenyl phosphate. The reaction was stopped by adding 0.2 ml NaOH (5 N) and the absorption was read at 405 nm using UV–Vis spectrophotometer, (BioRad, USA). One unit of the enzyme was defined as the amount of enzyme that liberates 1 mol of *p*-nitrophenol per hour.

Total protein estimation

Total Protein in skeletal muscle homogenate (10% w/v in 150 mM KCl) was assayed using Lowry's method [31]. Results were expressed as milligram of protein per gram wet tissue weight.

Myofibrillar and sarcoplasmic protein content

Myofibrillar and Sarcoplasmic fractions from rat skeletal muscle were obtained by slight modification in the method described earlier [32]. In brief, muscle samples were homogenized in a 5% ice-cold buffer containing 0.25 M sucrose, 2 mM EDTA, and 10 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at low speed (600×*g*) and the pellet containing myofibrillar protein was collected. From the supernatant, the sarcoplasmic protein fraction was isolated after centrifugation at 100,000×*g* for 60 min at 4°C. Protein content in both the fractions was assayed using Lowry's method [31].

Estimation of hormones

IGF-1 and catecholamines in rat skeletal muscle homogenate and plasma, respectively, were estimated using

commercially available kits (Rat/Mouse IGF-1 ELISA, Novozymes, UK and 2-CAT ELISA, LDN, Germany, respectively).

CPK activity

Creatine Phosphokinase (CPK) activity was measured in rat skeletal muscle homogenate using commercially available kit (Randox CK-NAC, UK) as per manufacturer's instructions. CPK activity was expressed as mIU per mg tissue weight.

Glutaminase enzyme activity

Glutaminase enzyme activity in rat muscle homogenate was measured as described earlier [33]. In brief, GDH (Sigma; 42 units/mg) was incubated in buffer containing L-Glutamine (100 mM), oxoglutarate (50 mM), phosphate buffer (1 M), EDTA (2 mM), and NADH for 5 min at 25°C. Initial absorbance was read at 340 nm. Tissue homogenate was added to it and incubated again for 2 min at 25°C. Final absorbance was again read at 340 nm. Activity was expressed as micromole per minute per milligram muscle protein.

Glutamine synthetase activity

Glutamine synthetase activity in rat muscle homogenate was measured as described earlier [34]. In brief, 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 min at 37°C. Reaction was terminated by adding ferric chloride (0.37 M) and absorbance was read at 540 nm using UV–Vis spectrophotometer, (BioRad, USA). Glutamine synthetase activity was expressed as katal units per minute per milligram of muscle protein.

Western blot: ubiquitin, calpain and HIF-1α expressions

The time dependent expression of ubiquitinated proteins, calpain, and HIF-1α on exposure to hypobaric hypoxia was determined by western blot. Primary anti-ubiquitin antibody was obtained from Santa Cruz and anti-μ-calpain antibody and anti-HIF-1α antibody were purchased from Sigma. Muscles were dissected at 4 °C on completion of hypoxia exposure. Ten percent tissue homogenate was prepared in ice cold- lysis buffer (10 mM Tris–HCl, 100 mM NaCl, 0.1 mM dithiothreitol, 1 mM EDTA, 0.1% NaN_3, 100 µg/ml PMSF, protease inhibitor cocktail, pH 7.6). The homogenate was centrifuged at 1,000×g for 10 min at 4°C and the supernatant was used for further studies. Total protein content was estimated by Lowry's method. Fifty µg of sample protein was then resolved by SDS-PAGE and transferred to nitrocellulose membrane pre-soaked in transfer buffer (20% methanol, 0.3% Tris and 1.44% glycine) using a semidry transblot module (BIORAD). The membranes were blocked with 5% non-fat milk, washed with PBST (0.01 M phosphate buffer saline, pH 7.4, 1 ml of 0.01% Tween 20), and probed with primary antibody (1:1000 dilution) for 3 h at room temperature. The membranes were then washed with PBST and incubated for 2 h at room temperature in secondary antibody (Santa Cruz) diluted in 3% non-fat milk. Membranes were then finally washed with PBST and the bands were developed on X-ray film using chemiluminescent substrate (Sigma). The bands thus obtained on the films were quantified by densitometry to determine expression of the protein.

Oxidative stress markers

Free radical generation

The production of free radicals was determined by using 2,7-dichlorofluoroscein diacetate (DCFH-DA) as described earlier [35]. In brief, 150 µl of muscle homogenate was incubated with (10 µl) 100 µM DCFH-DA for 30 min in dark. Fluorescence was read using a fluorimeter (Perkin Elmer, UK) with excitation at 485 nm and emission at 535 nm. Percentage change in free radical generation was expressed as result.

Lipid peroxidation

Malondialdehyde (MDA), a marker for lipid peroxidation, was measured in muscle tissue homogenates as described by Buege and Aust [36]. In brief, 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). Results were expressed as percentage change in lipid peroxidation.

Statistical analysis

All the results are presented as mean ± SEM. The experiments were conducted on two different occasions and the data was analyzed using ANOVA followed by Student–Newman–Keuls (SNK) 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). Multi regression analysis was also performed between the physical performance and other biochemical parameters to understand the dependency of these variables.

Results

Effect of chronic hypobaric hypoxia on physical performance of rats

Exposure of rats to chronic hypobaric hypoxia decreased their ability to perform physical activity as shown by decline in their fatigue time on treadmill running. The decline in physical activity was time dependent, showing a significant reduction after 3 days of exposure, continuing to 7 and 14 days. A significant reduction of 24% ($P < 0.05$) in the fatigue time was observed in rats exposed to 14 days of hypoxia (Table 1). Pair fed group did not show significant difference as compared to the control group (data not shown).

Effect of chronic hypobaric hypoxia on rat gastrocnemius muscle weight/tibial length

With increasing duration of exposure to hypobaric hypoxia, the ratio of rat gastrocnemius muscle weight/tibial length decreased significantly. The ratio decreased by 25% ($P < 0.05$) following 7 days of exposure and the decrement moved up to 34% ($P < 0.05$) following 14 days of hypoxia exposure (Table 2).

Histological effects of chronic hypobaric hypoxia on skeletal muscle

Histological examination of sections from gastrocnemius muscles were used to evaluate the effects of chronic hypobaric hypoxia on their integrity. Muscles were removed from hypobaric hypoxia-treated and control rats and analyzed for the potential presence of histopathological signs. The 40× High power photomicrograph of muscle biopsy showed transverse cut muscle fibers with uniform size and shape revealing the thin, delicate endomysial connective tissue, fibers of uniform size, and the peripherally placed nuclei. After exposure to hypoxia for 3 days the muscle fibers were uniform in size and shape. However, the fibers were smaller in size and had relatively more space between them as compared to control fibers. The photomicrograph of muscle tissue from rats exposed to 7 days of hypoxia exposure showed both atrophic and hypertrophic muscle fibers. Photomicrograph of muscle tissue from rat exposed to 14 days of hypobaric hypoxia, showed further atrophy of muscle fibers with irregularity in fiber size, which had relatively more space between them, compared to 7 days exposed groups. However, no necrotic fiber or cell splitting could be observed in any of the micrographs (Fig. 1).

Effect of chronic hypobaric hypoxia on total protein, myofibrillar and sarcoplasmic protein content

A reduction in protein levels is expected under conditions where skeletal muscle atrophy is observed. Predictably, total protein content in the skeletal muscle homogenates of rats exposed to chronic hypobaric hypoxia of different durations showed a significant decrease. Following 3 days of exposure, 13% ($P < 0.05$) reduction is obtained in the total protein content of skeletal muscle homogenates of rats followed by 18 and 22% ($P < 0.05$) reduction over the control rats after 7 and 14 days, respectively (Fig. 2a).

On subfractionation of the total protein, we found a significant decrease of 30% ($P < 0.05$) in the myofibrillar protein content in the 14 days exposed rats (Fig. 2b). The 7% decrease in sarcoplasmic protein (Fig. 2b), was much less as compared to the loss of myofibrillar protein.

Table 1 Effect of chronic hypobaric hypoxia on physical performance of rats on treadmill

Parameter	Control rats	3 days hypoxia	7 days hypoxia	14 days hypoxia
Fatigue time on treadmill running (min)	96.28 ± 3.01	86.56 ± 2.5[a]	80.07 ± 3.04[a, b]	73.85 ± 3.57[a, b, c]

[a] $P < 0.05$ versus control group

[b] $P < 0.05$ versus 3 days

[c] $P < 0.05$ versus 7 days

Table 2 Effect of chronic hypobaric hypoxia on rat gastrocnemius muscle weight/tibial length ratio

Parameter	Control rats	3 days hypoxia	7 days hypoxia	14 days hypoxia
Muscle weight/tibial length ratio (mg/mm)	41.729 ± 0.2	35.16 ± 0.3[a]	30.91 ± 0.2[a, b]	27.729 ± 0.2[a, b, c]

[a] $P < 0.05$ versus control group

[b] $P < 0.05$ versus 3 days

[c] $P < 0.05$ versus 7 days

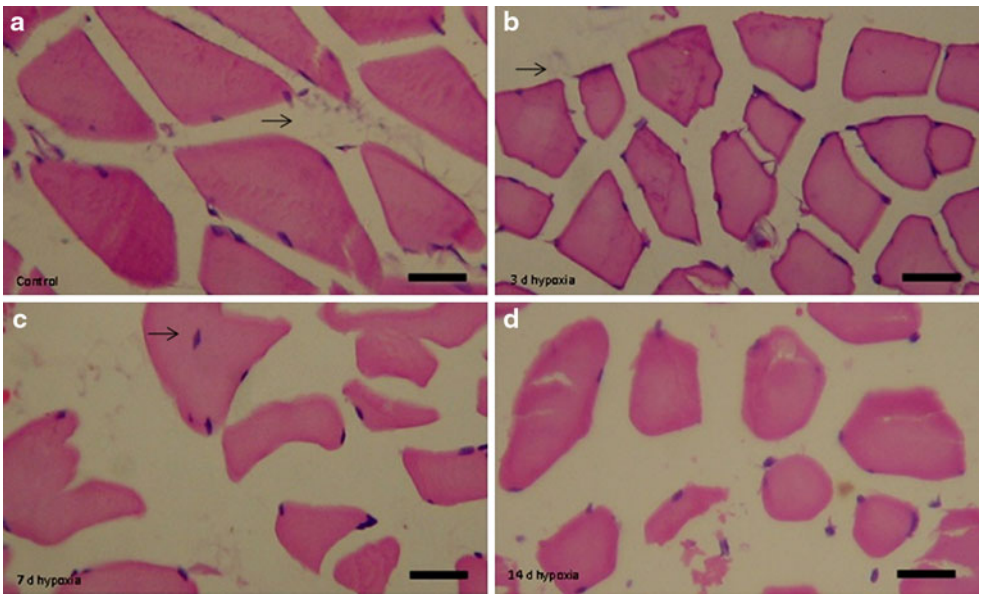

Fig. 1 Hematoxylin and Eosin staining of muscle cells in rat gastrocnemius muscle. Hematoxylin and Eosin staining of muscle cells in rat gastrocnemius muscle: **a** Control group showing transverse cut muscle fibers having uniform size and shape with peripheral nuclei. The fibers are closely set with little space between them; **b** 3 days hypoxia exposed group fibers are smaller in size and have relatively more space between them; **c** 7 days exposed group showing centralized nuclei (*arrow*), and irregularity of fiber size; **d** 14 days exposed fibers show further atrophy. Thin endomysium is visible only in the control group (*arrow*) and 3 days hypoxia group (*arrow*). A change in shape is observed in all the hypoxia exposed groups, however no cell splitting is visible in any group. *Scale bar* 10 μm

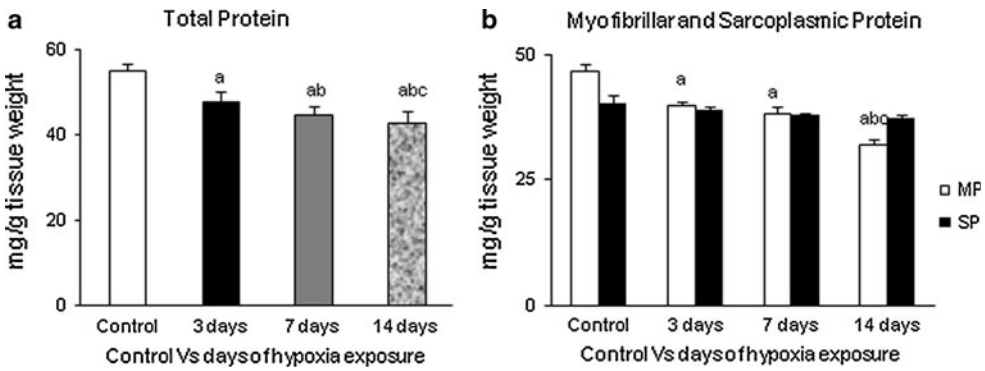

Fig. 2 Effect of chronic hypobaric hypoxia on **a** total protein, **b** myofibrillar protein and sarcoplasmic protein in rat gastrocnemius muscle ("a" indicates $P < 0.05$ versus control group, "b" indicates $P < 0.05$ versus 3 days, "c" indicates $P < 0.05$ versus 7 days)

Effect of chronic hypobaric hypoxia on protein turnover

Muscle proteolysis, represented by the release of tyrosine from hind limb muscles, was compared between control and hypoxia exposed groups (Fig. 3a). The hypoxic exposure led to about 2.5-Fold ($P < 0.05$) and three-fold ($P < 0.05$) increase in muscle proteolysis following 3 and 7 days exposure, respectively. The proteolysis further increased up to five-fold ($P < 0.05$) after 14 days of hypoxia exposure. Protein synthesis rates in rat hind limb muscle were determined by measuring ^{14}C-leucine incorporation into newly synthesized proteins. These analyses revealed that synthesis rate also increased in skeletal muscle of rats exposed to chronic hypobaric hypoxia as compared to control animals (Fig. 3b). Though the increment was not significant till 3 days of hypoxia exposure, the augmented rate of protein synthesis (1.2-fold, $P < 0.05$ following 7 days hypoxia and 1.5-fold, $P < 0.05$ following 14 days of hypoxia) was obtained on subsequent durations of hypobaric hypoxia exposure.

However, increase in protein degradation rate was significantly higher in comparison to synthesis rate (Fig. 3c), leading to overall enhanced protein depletion.

Effect of chronic hypobaric hypoxia on Ub-proteasome pathway, calpain activity, and lysosomal pathway

In accordance with the proteolytic rate, the chymotrypsin-like activity of Ub-proteasome pathway (Fig. 4a) was

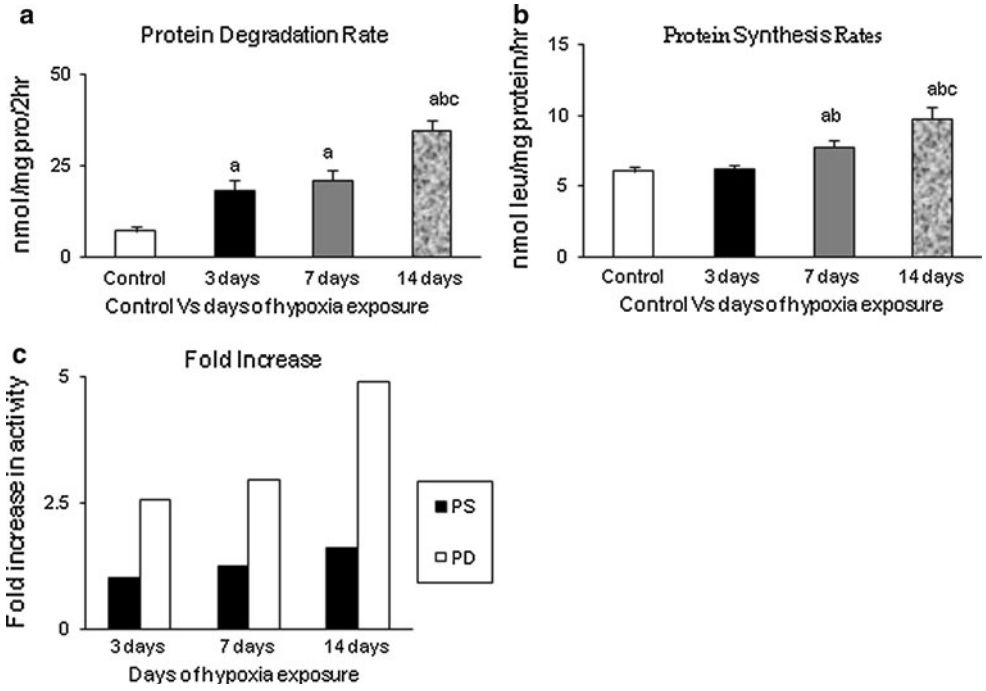

Fig. 3 Effect of chronic hypobaric hypoxia on **a** *PD* protein degradation rate, **b** *PS* protein synthesis rates, and **c** fold increase over control in protein synthesis and degradation rate n rat gastrocnemius muscle ("a" indicates $P < 0.05$ versus control group, "b" indicates $P < 0.05$ versus 3 days, "c" indicates $P < 0.05$ versus 7 days)

increased in the hypoxia exposed group compared with the control group. The increment was approximately five-fold ($P < 0.05$) over control in the 14 days hypoxia exposed group. Similarly, calpain activity also showed a three-fold increase ($P < 0.05$) over control (Fig. 4b). Acid phosphatase activity was measured as a marker for lysosomal pathway. No significant change was observed in the acid phosphatase activity after 3 and 7 days of exposure, but the activity increased by about 20% after 14 days of hypoxia exposure (Fig. 4c).

Effects of chronic hypobaric hypoxia on IGF-1 and catecholamine

IGF-1 is an anabolic hormone while catecholamine inhibits skeletal muscle proteolysis. Chronic exposure to hypobaric hypoxia does not result in any significant change in the IGF-1 level and epinephrine (Table 2). However, an increase in nor-epinephrine levels was observed after 3 and 7 days of hypoxia exposure but the increase was not significant (Table 3).

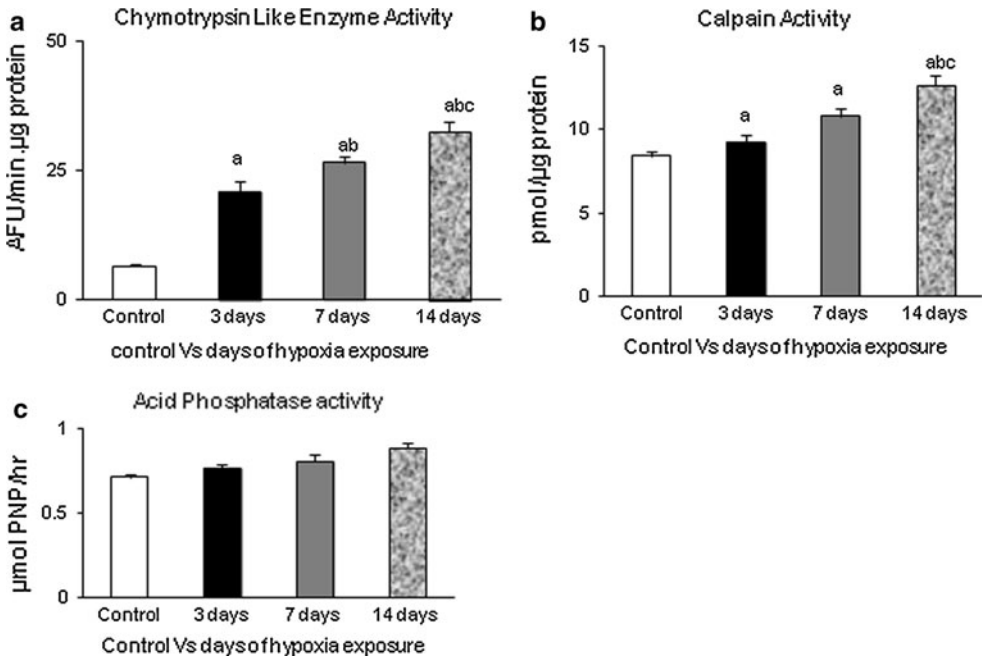

Fig. 4 Effect of chronic hypobaric hypoxia on protein degradation pathways **a** chymotrypsin-like enzyme activity, **b** calpain activity, **c** acid phosphatase activity in rat hind limb muscle ("a" indicates $P < 0.05$ versus control group, "b" indicates $P < 0.05$ versus 3 days, "c" indicates $P < 0.05$ versus 7 days)

Table 3 Effect of chronic hypobaric hypoxia on different hormones

Hormone	Control rats	3 days hypoxia	7 days hypoxia	14 days hypoxia
IGF-1 (ng/g muscle)	21.42 ± 1.5	20.92 ± 1.5	21.33 ± 1.7	21.5 ± 1.8
Nor-epinephrine (pg/ml)	494 ± 8.2	506 ± 5.5	508 ± 8.3	491 ± 10.3
Epinephrine (pg/ml)	78.88 ± 3.8	86.66 ± 7.5	82.77 ± 6.9	80.55 ± 6.7

[a] $P < 0.05$ versus control group

[b] $P < 0.05$ versus 3 days

[c] $P < 0.05$ versus 7 days

Effects of chronic hypobaric hypoxia on enzymes CPK, glutaminase, and glutamine synthetase

CPK levels were measured as an index of muscle damage under hypobaric hypoxia. With increase in duration of hypoxia exposure, the CPK content of muscle homogenates decreased significantly. There was two-times decrease in the CPK activity ($P < 0.05$) in the 14 days hypoxia exposed group as compared with the control group (Fig. 5a). Glutamine is known to play a very important role in protein metabolism and muscle mass accounts for about 90% of all glutamine synthesized in the body. Glutaminase and glutamine synthetase are therefore measured for conclusive study of protein turnover in skeletal muscle. A time dependent increase in the glutaminase activity supports the observed increment in skeletal muscle proteolysis. Following 3 days of exposure, significant change was observed in the Glutaminase activity. A significant increase of 3.5-fold ($P < 0.05$) and four-fold ($P < 0.05$) was obtained after 7 and 14 days of hypoxia exposure, respectively, Fig. 5b. Similar trend was observed in glutamine synthetase enzyme activity. It showed a significant increase (Fig. 5c) of 1.5 times over control following 14 days of hypoxia exposure ($P < 0.05$).

Effects of chronic hypobaric hypoxia on expressions of ubiquitinated proteins and calpain

Expressions of ubiquitin, calpain were studied using western blot (Fig. 6a). The results showed that there was an increased expression of ubiquitinated proteins after hypoxic exposure (Fig. 6b). Exposure to 3 days lead to approximately three-fold increase over control ($P < 0.05$) which gradually increased to 3.5-fold ($P < 0.05$) after 7 days exposure and up to five-fold increase ($P < 0.05$) over control in 14 days exposed rats. Similarly, μ-calpain expression also increased in hypobaric hypoxia exposed animals with about 2.7-fold increase ($P < 0.05$) over control after 14 days of hypoxia exposure (Fig. 6c).

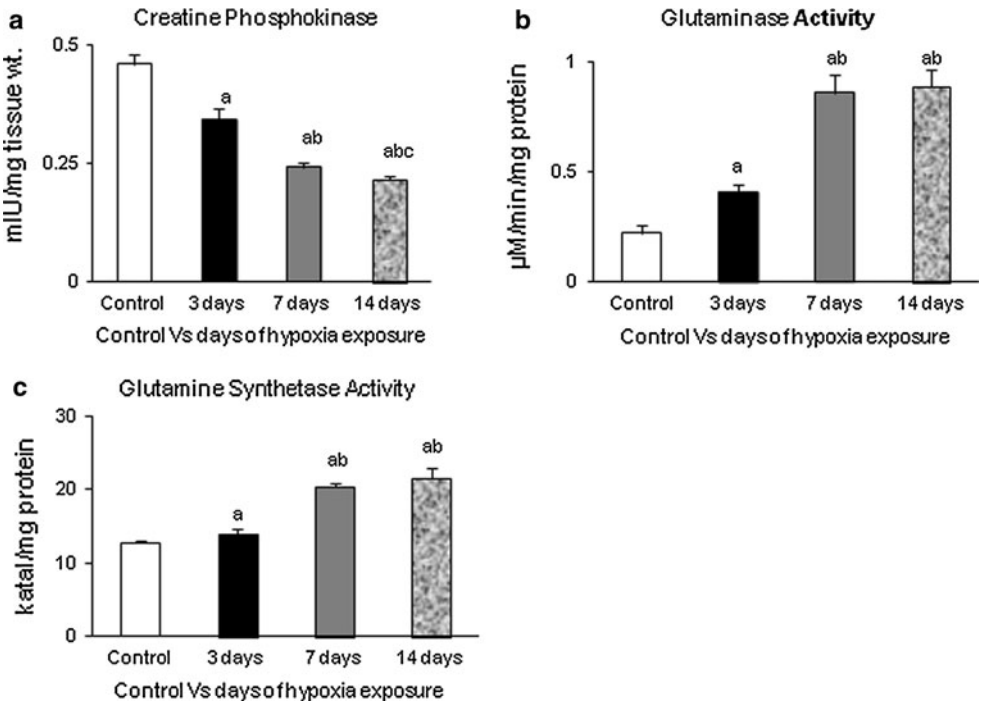

Fig. 5 Effect of chronic hypobaric hypoxia on **a** creatine phosphokinase, **b** glutaminase activity and **c** glutamine synthetase activity in rat hind limb muscle ("a" indicates $P < 0.05$ versus control group, "b" indicates $P < 0.05$ versus 3 days, "c" indicates $P < 0.05$ versus 7 days)

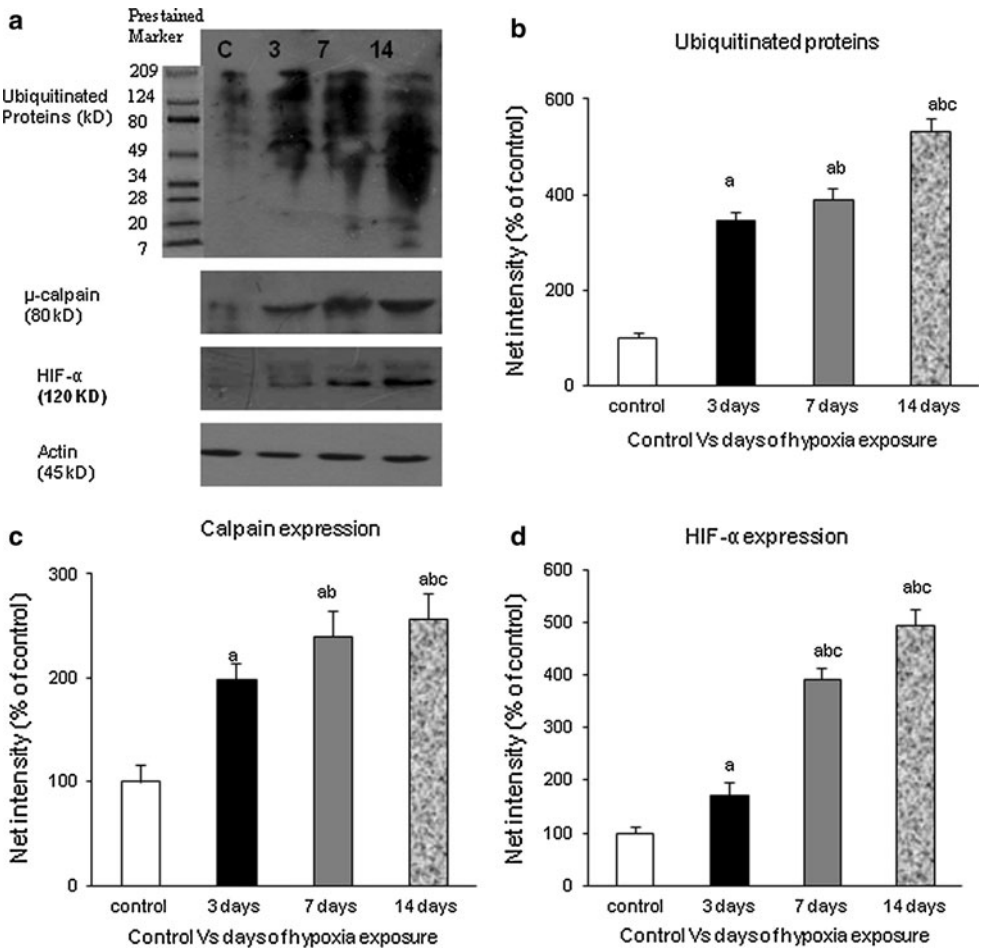

Fig. 6 Effect of chronic hypobaric hypoxia on **a** ubiquitinated proteins, μ-calpain and HIF-1α as determined by western blotting. Blots showing increasing band intensities at different hypoxia durations represent upregulated ub-proteasome pathway, calpains and HIF-1α, **b** densitometry of ubiquitinated proteins, **c** densitometry of calpain and **d** Densitometry of HIF-1α ("a" indicates $P < 0.05$ versus control group, "b" indicates $P < 0.05$ versus 3 days, "c" indicates $P < 0.05$ versus 7 days)

Effects of chronic hypobaric hypoxia on HIF-1α and oxidative stress markers

HIF-1α is an important transcription factor for the expression of hypoxia responsive genes. Hence to assess the magnitude of hypobaric hypoxic stress, expression of HIF-1α was studied using western blot. The results showed a time dependent increase in expression of HIF-1α in rat skeletal muscle homogenate with maximum increase of five-fold over control observed in the 14 days exposed rats (Fig. 6d).

Free radical generation and lipid peroxidation were measured as markers of oxidative stress. A significant increase of 1.5-fold over control was observed in free radical generation after 3 days of hypoxia exposure ($P < 0.05$). Free radical generation increased further with 2.4-fold increase ($P < 0.05$) over control after 7 days of hypobaric hypoxia and up to 3.5-fold increase ($P < 0.05$) over control after 14 days of hypobaric hypoxia exposure (Fig. 7a). Similar results were obtained in lipid peroxidation with a maximum increase of 80% over control ($P < 0.05$) in 14 days hypoxia exposed rats (Fig. 7b).

Discussion

Although the atrophic phenomenon at altitude has been studied for decades, the underlying mechanisms remain unresolved and only few data on protein turnover in response to chronic hypobaric hypoxia exist. High altitude affects the human body because of oxygen deprivation. A consistent consequence of severe altitude exposure where hypoxia is inevitable, such as during an expedition to the Himalayas, is a loss of body mass and a similar loss of muscle volume [7, 8]. The main findings in the present study are: (i) chronic hypobaric hypoxia lead to elevated skeletal muscle protein synthesis rate. It proves that synthesis rate is not a factor responsible for loss of skeletal muscle mass under chronic exposure. (ii) The fold increase in protein degradation rate is much higher than fold increase in protein synthesis rate with chronic hypobaric hypoxic exposure leading to overall decreased protein turnover. (iii) upregulation of ubiquitin–proteasome pathway and calpain activity are responsible for the enhanced protein degradation following chronic hypobaric hypoxia.

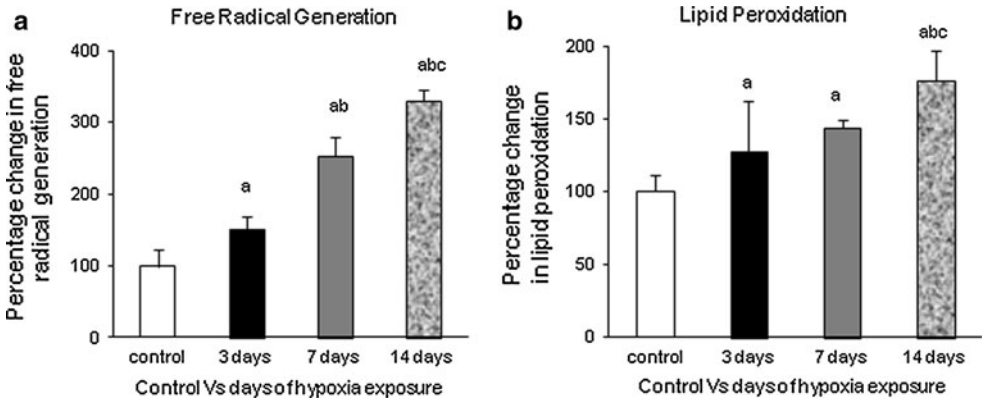

Fig. 7 Effect of chronic hypobaric hypoxia on **a** free radical generation, **b** lipid peroxidation in rat hind limb muscle ("a" indicates $P < 0.05$ versus control group, "b" indicates $P < 0.05$ versus 3 days, "c" indicates $P < 0.05$ versus 7 days)

We studied various aspects of skeletal muscle alterations under the conditions of hypobaric hypoxia by assessing physical, biochemical, and histological parameters in rats after exposing them to different durations of hypobaric hypoxia. As shown in our results, chronic hypobaric hypoxia decreases physical performance which is indicated by a significant decrease in the fatigue time in treadmill running. Earlier studies from our lab have demonstrated that exposure to intermittent hypobaric hypoxia for 7 days results in decreased muscle performance [37]. They used electrical stimulation to induce six tetanic muscular contractions in the gastrocnemius muscle after completion of exposure. Percentage mean performed work (PW), time of decay to 50% peak force of contraction ($T50$), and peak force of contraction (F_{peak}) were measured during titanic contractions. F_{peak} was reduced in the hypoxia exposed group at the second, third, fourth, fifth, and sixth titanic contractions as compared with respective forces in the unexposed control group. Similarly, T50 was also reduced in the fifth and sixth titanic contractions as compared with respective forces in the unexposed control group. They also observed a reduction in PW in the third, fourth, and sixth contractions when compared with respective values of the unexposed group. Fluctuations in body weight as occur with aging make body weight an unreliable reference for normalizing muscle weight. We measured the effect of hypobaric hypoxia on skeletal muscle mass by measuring the ratio of gastocnemius muscle weight to tibial length. Predictably, chronic hypobaric hypoxia resulted in significant reduction in gastrocnemius muscle/tibial length ratio. A decline in muscle to tibial length ratio indicates muscle atrophy under such conditions which might serve as an important factor responsible for hampered physical activities of people ascending to high altitude. Histological results also showed a time dependent skeletal muscle atrophy in the hypoxia exposed rats. Pair fed groups did not show any significant difference in any parameter when compared to the control groups and therefore its data is not shown. But this confirms that the changes observed are restricted to hypobaric hypoxia exposure.

Our current results indicate that prolonged periods of hypobaric hypoxia has severely adverse effects on skeletal muscle protein status. Total protein content of skeletal muscle decreased significantly after hypoxia exposure. The decrease is mainly because of depletion of myofibrillar proteins. This decline in myofibrillar protein may be due to a marked increase in the rate of skeletal muscle protein degradation. Multiple regression analysis (using SPSS 15) was also carried out between the physical performance (PP) and muscle mass (MM), myofibrillar proteins (MP), and degradation rate (DR) to understand the dependency of these parameters. The multiple regression equation between these parameters is $PP = 55.05 - 0.673\ DR + 0.09\ MW + 0.810\ MP$. The equation is significant at $P < 0.001$ with $r^2 = 0.95$. Since maximum changes in all the parameters were observed after 14 days of hypoxia exposure, the above equation depicts the dependency of the parameters in that group. This further signifies the role of these biochemical parameters in decreasing physical performance under chronic hypobaric hypoxia.

The most interesting and unpredictable outcome in our study is that chronic hypobaric hypoxia also lead to an increase in the protein synthesis rate which could possibly be explained as an adaptive response under these conditions. However, the fold increase in hypoxia exposed muscle over control, for protein degradation rate is much higher than fold increase in protein synthesis resulting in an overall decreased skeletal muscle mass.

As protein degradation emerged to be the cause of skeletal muscle loss under chronic hypobaric hypoxia, we further studied various proteolytic pathways. Our results indicate that Upregulation of the ubiquitin and calpains results in amplified activities of these two pathways which eventually lead to increased degradation of skeletal muscle proteins. The Ub-proteasome pathway has also been shown to account for the majority of skeletal muscle degradation in cancer cachexia where hypoxia is encountered [38]. We have found in this study that both the calpains and ubiquitin–proteasome pathways are implicated simultaneously leading to muscle atrophy during hypobaric hypoxia.

Recent evidences also point toward interactive involvement of these systems in proteolysis under different catabolic conditions. Since oxidization of proteins lead to their degradation, hypobaric hypoxia induced skeletal muscle protein oxidation might play a role in upregulation of these proteolytic pathways.

It is also believed that the observed protein turnover is not driven by changes in hormones as we and others [16, 39] have not been able to detect any significant changes in the hormonal levels.

In this study, we also studied enzymatic activities which could affect protein metabolism under chronic hypobaric hypoxia. 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. Similarly, increased glutamine synthetase enzyme activity may be a factor responsible for the enhanced protein synthesis. These results are in accordance with the results of previous study carried by Vats et al. [40].

CPK is the key energy reservoir in skeletal muscles. Lower levels of CPK in skeletal muscle of rats exposed to chronic hypobaric hypoxia are also a contributing factor to the hampered physical activity under these conditions. Hypobaric hypoxia leads to increased muscle permeability which results in leakage of the CPK from muscle to the bloodstream. Thus, the decreased creatinine phosphokinase activity in skeletal muscle homogenates, as shown in our results, is an indicator of the muscle permeability under chronic hypobaric hypoxia.

To assess the magnitude of hypoxic stress, expression of HIF-1α was studied. Our results show that with increase in hypoxia duration HIF-1α expression also increased. Chronic hypoxia also induced oxidative stress which was indicated by a significant increase in the free radicals and malondialdehyde levels. This increased oxidative damage may be a trigger for the increased protein degradation under chronic hypoxia. Several studies show that oxidative stress upregulate ubiquitin proteasome pathway in different conditions such as in lens epithelial cells exposed to H_2O_2 [41], retinal endothelial cells exposed to H_2O_2 [42], coronary atherosclerosis [43], and in C2C12 myotubes exposed to $FeSO_4$ and H_2O_2 [44]. The reactive oxygen species is also known to activate NF-Kb [45] which may have a role in upregulation of the proteasome pathway. Inhibition of ubiquitin–proteasome activity has been shown to down-regulate NF-kB-mediated inflammatory pathways [46] and vice versa inhibition of NF-kB resulted in decreased Ub-proteasome activity [37].

Detailed mechanism of chronic hypobaric hypoxia induced skeletal muscle atrophy has been explained diagrammatically in Fig. 8.

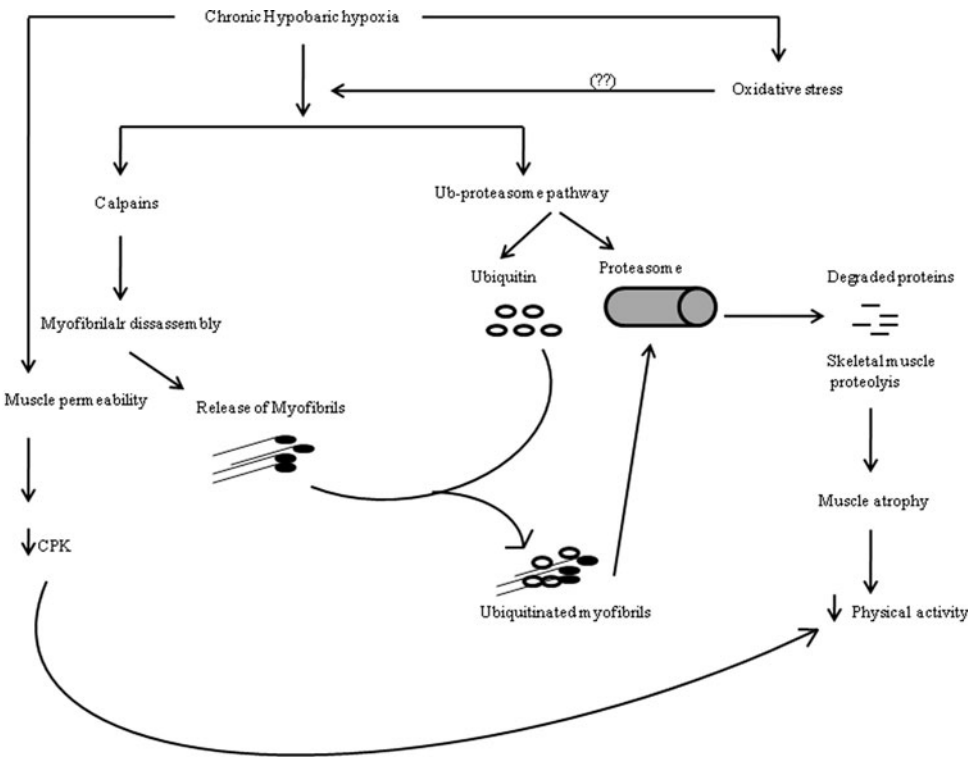

Fig. 8 Mechanism of chronic hypobaric hypoxia induced skeletal muscle atrophy

Conclusion

Excessive protein degradation during exposure to chronic hypobaric hypoxia resulted in skeletal muscle atrophy which could be detrimental for performing any physical task under these conditions. The majorly affected protein is myofibrillar protein and the pathways responsible for this loss of skeletal muscle mass are Ub-proteasome pathway and calpains. Attenuation of these pathways could be significant for preventing or impeding the observed skeletal muscle atrophy at high altitude and under various catabolic conditions mimicking the symptoms of high-altitude maladies such as COPD. Our results also concluded that increased oxidative stress induced at high altitude may also be important factor responsible for the skeletal muscle loss.

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