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Acute and Chronic Exposition of Mice to Severe Hypoxia: The Role of Acclimatization against Skeletal Muscle Oxidative Stress

Abstract

The role of acclimatization and the effect of persistent severe hypoxia (7000 m) were analyzed in mice *soleus* muscle with respect to oxidative stress (glutathione redox status) and damage markers (TBARS and SH protein groups), NAG and SOD activities and HSP70 expression. Forty mice were divided into one normobaric-normoxic control group and four hypobaric-hypoxic experimental groups (n = 8). One experimental group (1 D) was acutely exposed to a simulated altitude of 7000 m in a hypobaric chamber for 1 day. Another experimental group (ACCL + 1 D) was exposed to a 3 days acclimatization period plus 1 day of hypoxia exposure at 7000 m. The third experimental group (ACCL + 8 D) was exposed to the same acclimatization protocol, remaining 8 subsequent days at 7000 m. The fourth experimental group (8 D) was chronically exposed without acclimatization. ACCL + 1 D showed a significant decrease ($p < 0.05$) in oxidative stress and damage compared to the 1 D group. Concerning chronic severe hypoxia, acclimatization was truly vital, since 8 D animals died after 5 days of exposure. Oxidative stress and damage markers in ACCL + 8 D tended to gradually increase throughout the 8 days of the hypoxic period. Total SOD activity did not change in 1 D compared to control; however, it increased significantly ($p < 0.05$) in ACCL + 1 D and ACCL + 8 D. HSP70 expression followed the observed oxidative stress and damage pattern, suggesting a protective role against hypoxia-induced oxidative stress. The present study supports the hypothesis that acclimatization attenuates oxidative stress and damage induced by acute hypoxia, although a trend to a gradually increased oxidative deleterious effect in skeletal muscle seems to occur during persistent severe hypoxia even after a previous acclimatization period.

Key words
Altitude · acclimatization · oxidative stress · oxidative damage · skeletal muscle · HSP70 expression

Introduction

Hypobaric-hypoxia resulting from acute high-altitude exposure has been described as a major organic challenging strain that could be, at least in part, mitigated by adequate altitude acclimatization [42]. Recently, growing evidences of acute hypoxia-induced oxidative stress and oxidative damage on proteins, lipids and DNA, through exacerbated increase in reactive oxygen species (ROS) production, have been reported in some studies conducted with animals [35] and humans [2] in simulated high-altitude conditions. However, during gradual exposure to high-altitude, a phenomenon of altitude/hypoxia severity-dependent pro-oxidant production [16] may be involved in some physiological adaptations that take place in response to hypoxia including ROS antioxidant defense [1]. In fact, a low concentration of ROS has been reported to act in the regulation of gene expression

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through the activation of certain transcription factors that are dependent on the cellular redox [10]. For example, ROS generated by mitochondria during brief hypoxia seem to be a triggering stimulus to initiate preconditioning protection in cardiomyocytes [19,38]. Moreover, intermittent [46] and continuous [40] chronic altitude-hypoxia exposure have been shown to respectively increase resistance and survival of rats from cardiac ischaemic injury by protective mechanisms related to heat shock protein (HSP70) expression.

Despite such a hypothetical pro-oxidant protective role induced by acclimatization, persistent signs of oxidative stress and oxidative damage have been found in some studies in which acclimatized humans were chronically submitted to simulated hypobaric-hypoxia [16] or climbers were "oxidatively stressed out" [2] during long sojourns at altitude [2,34]. In this sense, prolonged and continuous exposure to severe conditions of high-altitude hypoxia might be a physiological challenging stimulus that gradually promotes the overwhelming of total antioxidant capacity and consequently exacerbates conditions of oxidative stress. Indeed, some benefits of antioxidant supplementation regarding oxidative damage have been found in athletes [26] and animals [31] continuously and intermittently exposed to hypobaric-hypoxia, respectively. Nevertheless, those studies conducted in humans only reported blood oxidative stress and damage markers, which do not reflect chronic tissue adaptations such as those that may occur in skeletal muscle. In fact, skeletal muscle has been referred to as one of the most affected tissues after prolonged periods of hypobaric-hypoxia exposure in humans and animals (for refs. see [7]). On the other hand, skeletal muscle oxidative stress and damage in animal studies of Sarada et al. [31] and Singh et al. [35] were induced by a daily severe hypobaric-hypoxia exposure interspersed with a normoxia period (6 hours of hypoxia up to 7500 m followed by 18 hours of normoxia, for approximately four weeks). These experimental conditions might inhibit an adequate acclimatization process compromising the ability of antioxidant systems to cope with hypoxia-induced enhanced reactive oxygen and/or nitrogen species production.

Therefore, in the absence of consistent data concerning the explicit and specific role of acclimatization on hypoxia-induced oxidative stress, it seems reasonable to speculate that, analogous to preconditioning before prolonged ischemia/reperfusion in several tissues [18,20], acclimatization may confer some protection against oxidative stress, attenuating skeletal muscle harmful effects [18]. On the other hand, considering the organic deterioration and the reduced food intake often described after some time spent at extreme altitude [42], it seems also reasonable to hypothesize that even after an adequate acclimatization, the persistency of severe high-altitude exposure aggravates the oxidative stress and oxidative damage due to a time-dependent overall decrease in the organic antioxidant capacity.

In this regard, the main goals of our study were to analyze in mice *soleus* muscle 1) the specific role of short-term acclimatization on the repercussions of acute and chronic hypoxia and 2) the effect of persistent continuous and severe hypoxia in acclimatized animals. For these purposes oxidative stress markers (oxidized – GSSG and reduced – GSH glutathione), antioxidant enzyme activity (total superoxide dismutase activity – t-SOD), oxidative damage (thiobarbituric acid reactive substances – TBARS, sulfhydryl protein groups – SH) and lysossomic enzyme activity (N-Acetyl-β-D-glucosaminidase – NAG) were measured. Additionally, since expression of heat shock proteins of the 70-kDa family (HSP70) has been considered as an inducible mechanism protecting proteins against cellular stress (for refs see [28]), another purpose of this study was to analyze the relationship between *in vivo* acute and chronic altitude-hypoxia exposure and the expression of HSP70, in acclimatized and non-acclimatized animals, since to the best of our knowledge few data regarding this topic have been previously reported.

Material and Methods

Forty CD1 Charles River mice (10 – 12 weeks) were randomly divided into one normobaric-normoxic control group (C; 39.23 ± 0.7 g) and four hypobaric-hypoxic experimental groups (n = 8). One experimental group (1 D; 40.37 ± 0.74 g) was acutely exposed to a simulated atmospheric pressure of 43.2 kPa (324 mm Hg) equivalent to an altitude of 7000 m in a hypobaric chamber during 1 day. The depressurization period to reach the simulated altitude of 7000 m took 14 minutes, i.e. 500 m/min. Another experimental group, defined as acclimatization group (ACCL + 1 D; 40.12 ± 0.52 g), was exposed to a short acclimatization period of 3 days (following the first day of hypoxia exposure at 4000 m, altitude was incremented by 1000 m/day until 7000 m) plus 1 day of severe hypoxia exposure at 7000 m. The third experimental group (ACCL + 8 D; 41.24 ± 0.42 g) was exposed to the same short acclimatization conditions, however it remained in severe hypobaric-hypoxia at 7000 m continuously for 8 subsequent days. The fourth experimental group (8 D; 41.13 ± 0.68 g) was also chronically exposed, however it was not submitted to an acclimatization protocol, but it was acutely exposed to 7000 m and remained in these hypoxic conditions continuously during 5 of the 8 expected days. Indeed, in the night of the 5th day all the animals died and were found in *rigor mortis* the morning after, which prevented muscle sampling collection for later biochemical analysis. The control group was maintained in normoxia, at an atmospheric pressure equivalent to sea level conditions (± 101 kPa) during the course of the complete experimental protocol. For all the experimental groups, the pressurization period until sea level conditions took 15 minutes. All the animals were kept at constant temperature (21 – 25 °C) on a daily lighting schedule of 12 h of light vs. dark with normal activity and food and water *ad libitum*. The animals of each group were sacrificed immediately after the end of the experiment. Mice body mass was determined by weighing animals in a COBOS Precision C-300-SX scale to the nearest 0.01 g. *Soleus* muscles were weighed using a Kern 870 electronic scale to the nearest 0.00 001 g. Food consumption was measured with the aid of special feeders that allowed the recovery of spilled food and was expressed as a percentage of the control group food intake during the same corresponding periods.

The Ethics Committee of the Scientific Board of Faculty of Sport Sciences approved this study.

Tissue preparation

The animals were sacrificed by cervical dislocation. Both *soleus* muscles were excised and homogenized in tris (0.05 M) L-serine (0.03 M) borate (0.06 M) buffer (pH. 7.6) in a motor-driven Potter-glass homogenizer at 0 – 4 °C at low speed. The homogenized samples were separated into several aliquots and rapidly frozen at – 80 °C for later biochemical analysis of GSH, GSSG, TBARS, protein sulfhydryl groups and total protein content, t-SOD and NAG activities and HSP70 expression. The aliquots for glutathione assay were previously extracted in a medium containing percloric acid at 5% (w/v).

Assays

GSH and GSSG measurements were determined as previously described by Tietze [37] by spectrophotometric techniques at 414 nm. Lipid peroxidation on the whole muscle homogenate was assayed spectrophotometrically according to the method described by Bertholf et al. [6] and measured by the formation of TBARS. Oxidative modification of protein SH groups was quantified by spectrophotometric measurement according to the method proposed by Hu [14]. NAG activity was determined spectrophotometrically with a commercial kit (Boehringer Mannheim, Germany – cat no. 875 406). Total SOD activity was measured according to the protocol of Beauchamp and Fridovich [4] using the RANSOD kit (Randox Laboratories, UK). This method is based on the ability of t-SOD to prevent the formation of formazane from 2-(4-iodophenyl)-3-(4-nitro)-5-phenyltetrazolium chloride by superoxide radicals generated by xanthine oxidase/xanthine. To determine the levels of HSP70 in the muscles (n = 6 in each group), a certain volume of homogenate equivalent to 10 mg protein was resolved by SDS-PAGE (12.5% acrylamide gels of 1 mm thickness) as described by Laemmli [21] and electroblotted onto nitrocellulose membranes according to Locke et al. [23]. The immunoblots were probed with 1 : 5000 dilution of monoclonal anti-Hsp70 (Sigma, St. Louis, USA) and with 1 : 500 dilution of the secondary antibody (anti-mouse IgG peroxidase conjugate, Sigma, St. Louis, USA). The bands were visualized by treating the immunoblots with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshire, UK), according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software (Bio Rad, USA). Optical density results were expressed as percentage variation from control values. Protein content was assayed spectrophotometrically using bovine serum albumin as standard according to Lowry et al. [24].

Statistical analysis

The data were analyzed using the SPSS-PC 11.0 package for Windows. All the results given are expressed as means and mean standard error (SEM). Factorial ANOVA followed by Bonferroni *post-hoc* test was used to compare groups. The significant level was set at 5%.

Results

The following results concern all the groups, with exception of the one non-acclimatized that was submitted to a chronic hypoxic protocol (8 D group), since all the animals in this group died (100%) during the course of the experiment and consequently, no biochemical measurements were done. From a qualitative point of view, these animals revealed a progressive debilitating status throughout the 5 days that they were able to resist hypoxia, showing an obvious inadaptability to the experimental environmental conditions, low mobility and aggravated anorexia (98.5% reduced food intake) that culminated in their death.

Table **1** Mice (MW), soleus (SW) weight and protein content in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM

	MW *g*	*SW* *mg*	*Protein* *mg/ml*
C	40.07 ± 1.05	15.20 ± 0.34	0.426 ± 0.023
1 D	39.65 ± 0.85	15.87 ± 0.33	0.434 ± 0.023
ACCL + 1 D	38.36 ± 0.35	15.53 ± 0.6	0.424 ± 0.008
ACCL + 8 D	30.32 ± 0.86*	12.89 ± 0.66*	0.343 ± 0.017*

* $p < 0.05$, ACCL + 8 D vs. C, ACCL + 1 D and 1 D

Mice and *soleus* muscle weights as well as *soleus* total protein content are presented in Table **1**. There were significant differences in mice and *soleus* weights and in protein content between ACCL + 8 D and the remaining groups. When compared to the unexposed control group, the 1 D, ACCL + 1 D and ACCL + 8 D groups showed a decrease in food intake of 95.2%, 10.7% and 21.5%, respectively.

Skeletal muscle glutathione contents are depicted in Figs. **1** to **4**. Concerning total glutathione (TGSH) and GSH, no significant differences were found among groups throughout the experimental protocol. However, data related to GSSG/TGSH (%GSSG) and GSSG content showed a distinct profile. A significant difference was found in these parameters between control (C) and mice

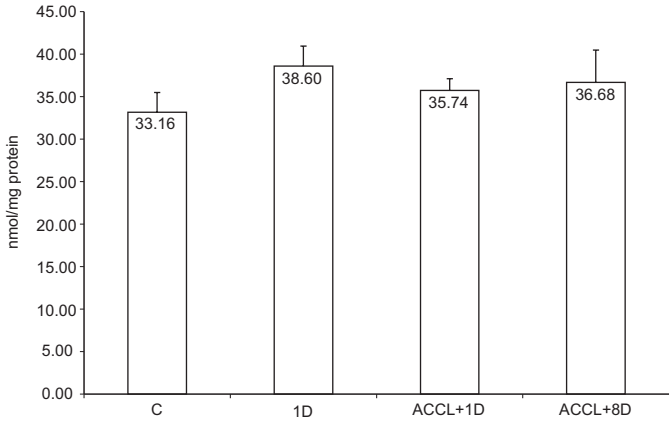

Fig. **1** S*oleus* muscle total glutathione (TGSH) in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM.

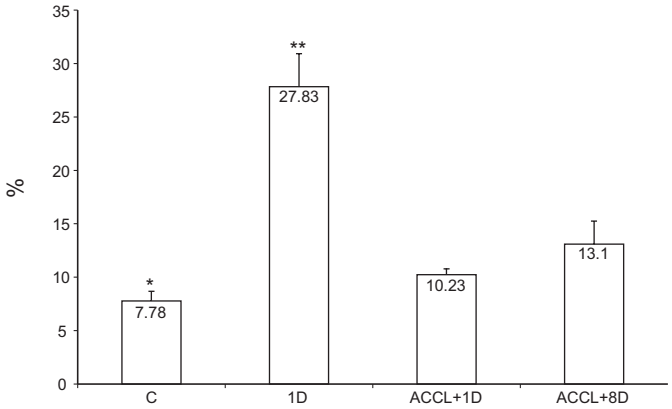

Fig. **2** So*leus* muscle GSSG/TGSH (% GSSG) in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. * p < 0.05, C vs 1 D; ** p < 0.05, 1 D vs. ACCL + 1 D and ACCL+ 8 D.

Table **2** *Soleus* muscle thiobarbituric acid reactive substances (TBARS) and protein sulfhydryl groups (SH) content and N-Acetyl-β-D-glucosaminidase (NAG) activity in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM

	TBARS *nM*	*SH* *mol/g prot*	*NAG* *U/mg prot*
C	288.4 ± 30.5*	292.00 ± 25.36 ‡	5.3 ± 0.27 ‡
1 D	552.7 ± 38.6**	202.63 ± 19.25	8.36 ± 0.33**
ACCL + 1 D	335.4 ± 18.6	265.12 ± 13.54	5.78 ± 0.14
ACCL + 8 D	383.6 ± 27.6	234.51 ± 18.25	6.09 ± 0.67

* p < 0.05, C vs. 1 D and ACCL+ 8 D; ** p < 0.05, 1 D vs. ACCL + 1 D and ACCL + 8 D; ‡ p < 0.05, C vs. 1 D

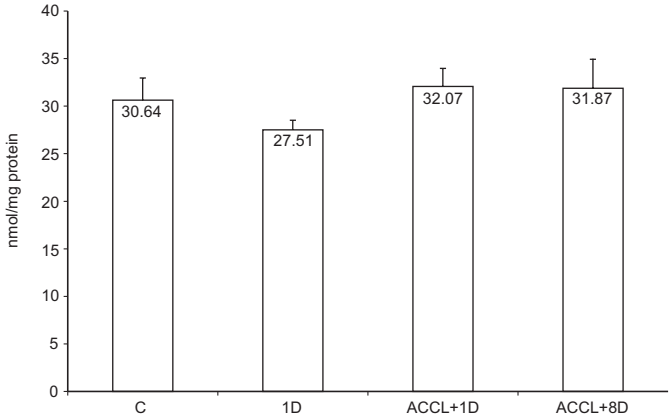

Fig. **3** So*leus* muscle reduced glutathione (GSH) in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM.

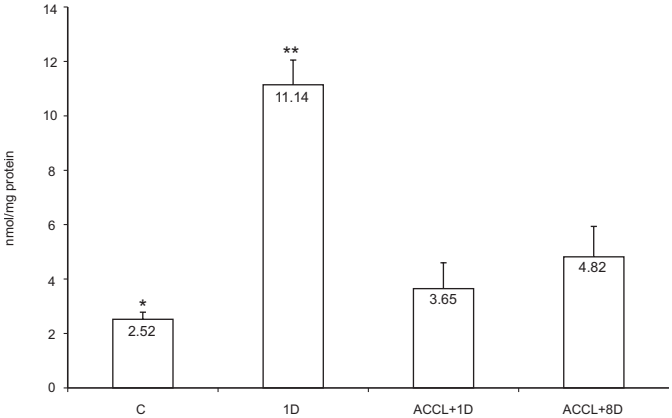

Fig. **4** So*leus* muscle oxidized glutathione (GSH) in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. * p < 0.05 C vs 1 D; ** p < 0.05 1 D vs ACCL + 1 D and ACCL + 8 D.

acutely exposed to 1 day of hypobaric-hypoxia (1 D), suggesting an increase in oxidative stress conditions during acute and severe hypoxic insult. On the other hand, the acclimatization period (ACCL + 1 D) seemed to inhibit hypoxia-induced oxidative stress since 1) no additional %GSSG or GSSG content was found in this group when compared to control (C) and 2) significant differences were found between this gradually exposed hypoxia group (ACCL + 1 D) and mice from the acutely exposed group (1 D). Regarding prolonged and continuous exposure after previous acclimatization (ACCL + 8 D), no significant enhancement of %GSSG or GSSG content was observed when compared to mice exclusively submitted to the acclimatization period (ACCL + 1 D). In this sense, persistent severe hypobaric-hypoxia exposure failed to increase oxidative stress conditions after an acclimatization period (ACCL + 1 D vs. ACCL + 8 D) despite a time-dependent trend to enhanced hypoxia-induced oxidative stress throughout the experimental protocol (C vs. ACCL + 1 D vs. ACCL + 8 D).

Regarding oxidative damage, the levels of muscle TBARS, protein sulfhydryl groups (SH) and NAG activity as indirect measures of lipid peroxidation, protein oxidation and lysossomic activity, respectively, are presented in Table **2**. Similarly to %GSSG and GSSG, TBARS content was higher in the group 1 D compared to control ($p < 0.05$). However, no significant difference was observed between ACCL + 1 D and C groups, demonstrating an absence of enhanced lipid peroxidation in response to acclimatization. Such a protective feature of acclimatization regarding lipid peroxidation was also suggested by the significant difference in TBARS content at 1 D when compared to ACCL + 1 D. Despite a significant increase in TBARS content in ACCL + 8 D when compared to controls, no further significant increase was found with persistent severe hypobaric hypoxia (ACCL + 8 D vs. ACCL + 1 D). Concerning protein sulfhydryl, our data showed that acclimatization (C vs. ACCL + 1 D) protects skeletal muscle from protein oxidation, which contrasts clearly with acute severe exposure (C vs. 1 D). On the other hand, prolonged severe hypoxia exposure in acclimatized mice failed to decrease protein oxidation significantly (ACCL + 8 D vs. ACCL + 1 D). NAG activity did not change

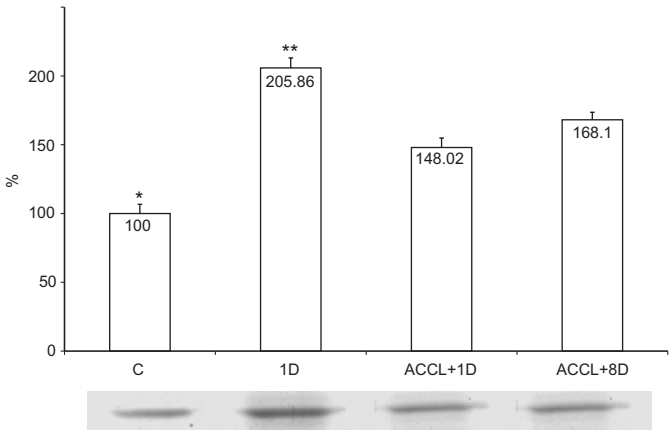

Fig. **5** So*leus* muscle HSP70 expression in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. A scan of representative Western blot for each group (n = 6) is immediately below the histogram. * p < 0.05 C vs. 1 D, ACCL + 1 D and ACCL + 8 D; ** p < 0.05 1 D vs. ACCL + 1 D and ACCL + 8 D.

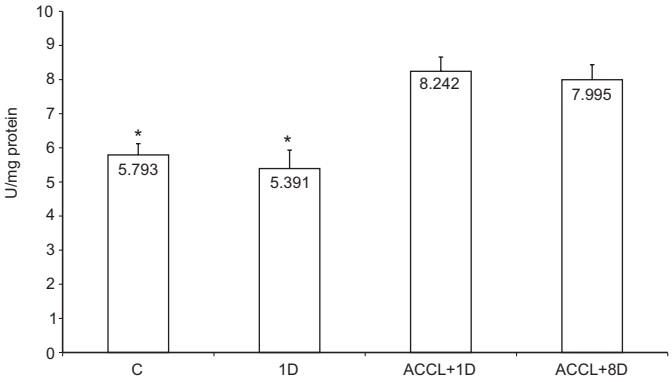

Fig. **6** So*leus* muscle total superoxide dismutase (t-SOD) activity in animals exposed to 1 day (1 D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL + 1 D) and 8 days (ACCL + 8 D) of hypoxia at 7000 m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. * p < 0.05 C and 1 D vs. ACCL + 1 D and ACCL + 8 D.

significantly in acclimatized mice (C vs. ACCL + 1 D); however, an up-regulation of this lysossomic enzyme was found in the acute non-acclimatized group (C vs. 1 D). Additionally, a clear significant difference was found between ACCL + 1 D and 1 D. Once again, no significant difference was found for this enzyme between ACCL + 1 D and ACCL + 8 D groups.

Regarding HSP70 expression, the results followed the same tendency observed for oxidative stress and damage markers (Fig. **5**). Significant differences were found between controls and the remaining groups. In addition, the 1 D group also revealed an increased content compared to ACCL + 1 D and ACCL + 8 D mice.

Total superoxide dismutase (t-SOD) activity increased significantly from C to ACCL + 1 D and ACCL + 8 D. However, no significant differences were found between controls and mice acutely exposed to 7000 m for 1 day (1 D). Moreover, significant differences were found between 1 D and chronically exposed groups (1 D vs. ACCL + 1 D and ACCL + 8 D) (Fig. **6**).

Discussion

Exposure to high altitude is a well-known environmental stressor with physiological and metabolic consequences. Such disruptions in cellular homeostasis elicit several acute and chronic organic adaptations designed to diminish the stress imposed by this hypoxic insult. These statements are consistent with the overall picture of our results, i.e., even a short-term period of acclimatization attenuates the oxidative stress and damage imposed by hypoxia insult.

After one day of acute hypoxia exposure (1 D) the GSSG/TGSH ratio increased when compared to controls, which could be explained by the enhanced GSH oxidation [33] and is corroborated by the significant increase in GSSG content. Furthermore, although a decrease in GSH content could be expected due to a substantial amount of GSH oxidation, only a slight but non-significant decrease was observed in this hypoxic group. This was probably due to muscle fibers' ability to import GSH from plasma via the γ-glutamyl cycle to cope with increased free radical production [27] which is consistent with the slight increase in the TGSH content. The enhanced oxidative stress in our mice acutely exposed to simulated high altitude is in accordance with data reported by Singh et al. [35] and confirms the skeletal muscle susceptibility to disturbances in redox status during acute and severe hypoxic insults. It is important to be aware that our results could be underestimated since an increase in glutathione reductase (GR) activity, described under hypobaric hypoxia [35], could enhance GSH *turnover* diminishing the evidence of oxidative stress. Among several invoked reasons, 1) the mitochondria reductive stress [9,17], 2) the leukocyte-endothelial adherence via nitric oxide depletion [44] and 3) the enhanced XO activity [13] seem to be potential sources of ROS that could, at least in part, explain the hypoxia-induced oxidative stress in skeletal muscle.

In accordance with these data, the markers related to oxidative damage were also significantly changed in the 1 D group when compared to controls. Indeed, as an indicator of lipid peroxidation, TBARS content increased almost two-fold in this experimental group (1 D). These results are in accordance with several other studies conducted in animals [35] and humans [3] and confirm the high susceptibility of polyunsaturated fatty acids to peroxidative disarrangement in hypoxia-induced oxidative stress. Similarly, SH protein groups decreased significantly after this hypoxic insult (1 D vs. C) suggesting a large oxidative modification in distinct cellular compounds due to an imbalance between ROS production and antioxidant capacity during acute high-altitude exposure.

On the other hand, the short-term acclimatization protocol (ACCL + 1 D) seems to diminish almost completely the levels of oxidative stress (C vs. ACCL + 1 D vs. 1 D), which is in clear contrast to the acute hypoxic insult. Among other possible explanations, such a protective effect of acclimatization might be due to the adaptive cardiovascular and ventilatory changes described in

animals and humans gradually submitted to high-altitude [11, 32, 41]. Hypoxia is a triggering stimulus, *per se*; to increase cellular ROS generation, the up-regulation of several systemic and metabolic processes might prevent an exacerbated decrease in intracellular oxygen tension diminishing blood and tissue hypoxia, which probably contributes to attenuate ROS formation. In fact, regarding the glutathione levels, ACCL + 1 D group did not differ from the control group. Moreover, concerning %GSSG and GSSG content, ACCL + 1 D animals were significantly different from those acutely submitted to severe hypoxia (1 D), which suggests a lower level of oxidant production and of GSH oxidation in the acclimatized mice. As previously mentioned, an inter-organ GSH transport mediated by increased GSH hepatic efflux and cellular importation [15] probably justifies the absence of differences in TGSH and GSH between the 1 D and the ACCL + 1 D groups. The protective capacity of short-term acclimatization against skeletal muscle oxidative stress in mice was also confirmed by oxidative damage data. In fact, at least regarding lipid and protein oxidative modification, no significant differences were found between the acclimatized (ACCL + 1 D) and the control group. On the other hand, clear and distinct oxidative deleterious changes in some cellular components were found between ACCL + 1 D and 1 D groups, reinforcing the idea that at least membrane phospholipids and proteins seem to be protected from hypoxia-induced oxidative stress after a short-term acclimatization protocol. In clear contrast to ACCL + 1 D, the enhancement of NAG activity observed in the 1 D group suggests an increase of muscle autophagic response [30] and supports the idea that muscle damage induced by hypoxia is attenuated by previous acclimatization. The protective role of acclimatization was also suggested by the reduced HSP70 expression observed in acclimatized animals (ACCL + 1 D vs. 1 D). Indeed, our results showed that these molecular chaperones (for refs. see [36]), commonly used as markers of cellular stress, followed the same trend observed in oxidative stress and damage markers, i.e., their enhanced expression parallel the increased oxidant stress and damage. Moreover, these data support the hypothesis that oxidative stress not only reduces glucose or glycogen levels and decreases intracellular pH [5, 28], but can also be an important stimulus to modulate the expression of skeletal muscle HSP70 in an altitude-hypoxia environment.

Besides adaptive cardiorespiratory changes [42] and an absence of inflammatory response [45] in animals and humans gradually submitted to high altitude, some genetic modulation might also be involved in the protective effect of acclimatization. Indeed, during the graded hypoxia stages of the acclimatization process, the hypoxia-induced dose-related increases in free radical production [9] could be, at least in part, responsible for the adaptive responses that explain the absence of significant enhanced oxidative stress and damage in acclimatized animals [8, 45].

In this sense, the slight increase in %GSSG (C vs. ACCL + 1 D and ACCL + 8 D) suggests that mild oxidative stress might induce skeletal muscle up-regulation of several protective mechanisms whose products exhibit antioxidant properties [8] counteracting the severe hypoxia-induced oxidative stress observed in the 1 D group.

This hypoxia-modulation effect on molecular strategies to cope with enhanced oxidative stress seems to be consistent with our data regarding the above-mentioned HSP70 expression and particularly, SOD activity throughout the experimental protocol. In fact, the increased SOD activity observed in the ACCL + 1 D and ACCL + 8 D groups, in response to the slight enhancement of oxidative stress, could be seen as a defensive strategy of muscle cells to attenuate additional oxidative stress and damage [10]. These adaptations probably result from cumulative effects of the graded altitude hypoxia-induced oxidative stress on gene expression of SOD, although the molecular basis underlying the signal transduction pathway in skeletal muscle is still unclear [10, 12]. Based on the assumption that the degree of oxidative stress is altitude dependent, our results are in clear contrast to those obtained by Radák et al. [29] in which Mn-SOD activity decreased and Cu,Zn-SOD remained unchanged after 6 months of intermittent exposure to 4000 m. The maintenance of SOD activity in the 1 D group could be explained either by enhanced protein degradation due to exacerbated oxidative stress and/or by insufficient time to complete protein synthesis and expression.

Concerning the effects of persistent severe hypoxia in acclimatized mice (ACCL + 1 D vs. ACCL + 8 D), our data showed a non-significant but consistent increase in oxidative stress and damage parameters. In fact, despite a non-significant variation in TGSH and GSH content between these two experimental groups (ACCL + 1 D vs. ACCL + 8 D), the GSSG/TGSH ratio and GSSG content increased for almost 30% in the ACCL + 8 D group. Measurements of muscle TBARS, SH content, NAG activity and HSP70 expression are consistent with glutathione data, suggesting an intrinsic and close relationship between severity/time-dependent hypoxia induced-oxidative stress and corresponding oxidative damage. Despite the non-significance, it is important to be aware that all these results concerning persistent severe hypoxia exposure are consistent and express a tendency to progressive muscle tissue deterioration, which is supported by the significant decrease of *soleus* muscle weight.

In order to explain this apparently progressive deterioration, some possible mechanisms related to the well-documented phenomenon of reduced food intake [25, 39] may be worth consideration. In fact, loss of appetite and consequently reduced food intake during long sojourns at extreme high altitude have frequently been reported [43] and have also occurred with the animals of our study exposed for a longer period. This can explain, at least in part, the significant decrease in mice weight (ACCL + 1 D vs. ACCL + 8 D) (Table **1**). In this sense, besides limiting glucose availability and indirectly affecting GSH turnover [22], the loss of appetite might directly result in deficient dietary ingestion of antioxidant compounds promoting changes of antioxidant enzyme status and exacerbated oxidative stress and damage in several tissues throughout the experimental protocol, particularly in skeletal muscle [22]. Under these conditions, ROS production is increased slightly and persistently and the antioxidant response may not be sufficient to reset the system to the original level of redox homeostasis resulting in progressive enhancement of oxidative stress [8] as confirmed by the %GSSG (ACCL + 1 D vs. ACCL + 8 D). However, future experimental approaches with a large number of animals or a longer hypoxic period should be consid-

ered in order to test this hypothetical deleterious hypoxic phenomenon.

In summary, the present study supports the concept that short-term acclimatization attenuates muscle oxidative stress and damage induced by an acute hypoxic insult. On the other hand, a trend to a gradually increased oxidative deleterious effect in mice skeletal muscle seems to occur during persistent severe hypoxia exposure even after a previous acclimatization period.

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