ORIGINAL ARTICLE

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Acute and severe hypobaric hypoxia-induced muscle oxidative stress in mice: the role of glutathione against oxidative damage

Abstract This study intended to analyze: (1) the effects of acute and severe hypoxia exposure on skeletal muscle oxidative stress and oxidative damage markers; (2) the protective role of the antioxidant glutathione against oxidative damage; and (3) the expression of heat shock protein 70 kDa (HSP70) induced by this hypoxic insult. Forty mice were divided into four groups: control + placebo (C+P), hypoxia + placebo (H+P), control + L-buthionine-[S,R]-sulfoximine (BSO, a GSH-depleting compound) (C+BSO) and hypoxia + BSO (H+BSO). Hypoxia groups were continuously exposed for 24 h to a hypobaric hypoxic environment equivalent to an altitude of 7000 m and sacrificed immediately after. Control groups were maintained at sea level during the experimental protocol. Analyzed biochemical parameters were: reduced (GSH) and oxidized (GSSG) glutathione, thiobarbituric acid reactive substances (TBARS), sulfhydryl protein groups (SH), N-acetyl-β-D-glucosaminidase (NAG) and HSP70 protein. Hypoxia (H+P) per se, compared to C+P, induced a significant increase in %GSSG (5.68 vs. 1.14%), TBARS (436.7 vs. 227.9 nM), NAG (4.49 vs. 3.35 U/mg) and HSP70 (178.7 vs. 100%). Compared with H+P, H+BSO showed a significant decrease in total glutathione (19.30 vs. 6.13 nmol/mg) and an additional increase in %GSSG (5.68 vs. 11.33%) and in HSP70 expression (178.7 vs. 202.2%). However, no further oxidative damage was observed in H+BSO. These data suggest that acute hypoxia per se might enhance oxidative stress; however, the glutathione system seems to have a modest role in skeletal muscle protection against hypoxia-induced oxidative stress. Moreover, hypoxia and BSO treatment is a sufficient stimulus to promote HSP70 overexpression.

Keywords L-Buthionine-[S,R]-sulfoximine (BSO) · Glutathione · HSP70 · Hypobaric hypoxia · Soleus muscle

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Introduction

Several environmental challenges are faced during high-altitude exposure, including a low oxygen (O_2) partial pressure, extreme cold, temperature shifts, increased ultraviolet radiation and disturbed daily nutritional diet, leading to physiological stress (Askew 2002). The reduced barometric pressure at altitude affects the so-called O_2 cascade, diminishing the ability of O_2 to diffuse from the atmospheric air to the blood and tissues, inducing hypoxia (Samaja 1997). This decrease in O_2 availability compromises body metabolism and promotes the loss of tissue and cell homeostasis. Moreover, most mammals have little tolerance to O_2 deprivation, which, among other acute and chronic responses, could result in the activation of some mechanisms of additional free radical production (Kehrer and Lund 1994; Bailey et al. 2001). In these hypobaric hypoxia conditions, the antioxidant body defense systems seem to be overwhelmed by the enhanced production of oxygen- and nitrogen-based reactive species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and peroxynitrite ($ONOO^-$), increasing oxidative stress (Kehrer and Lund 1994; Askew 2002). Despite some fundamental and vital cell mechanisms in which reactive oxygen species (ROS) are involved, such as the neutrophil respiratory burst and some cell signaling pathways (Thannickal and Fanburg 2000), increased ROS production related to an unbalanced response of the antioxidant system could result in unavoidable cell

damage. Usually, evidence of oxidative stress induced by hypobaric hypoxia is mainly based on indirect markers of tissue damage such as the levels of serum diene conjugation (Vasankari et al. 1997), 8-hydroxy-guanine (Moller et al. 2001), carbonyl group content (Radak et al. 1997), malondialdehyde (Sarada et al. 2002a), thiobarbituric reactive acid substances (TBARS) (Singh et al. 2001) and expired ethane gas (Risby et al. 1999). In fact, ROS can interact with membrane polyunsaturated fatty acids leading to lipid peroxidation, with protein thiol groups causing protein oxidation and with DNA bases generating DNA strand breaks and oxidative DNA damage (Halliwell and Gutteridge 1999).

Glutathione (γ-glutamylcysteinylglycine; GSH) is a known antioxidant tripeptide with a vital role in the protection of several tissues, namely skeletal muscle, from free radical production. Besides acting as an electron donor to neutralize hydrogen peroxide (H_2O_2) and lipoperoxide, GSH also scavenges oxygen- and nitrogen-based free radicals (Ji 1999). Based on these antioxidant properties, the effect of GSH has been extensively studied in many experimental models that induce tissue dysfunction by oxidative damage (Ji et al. 1994; Leichtweis and Ji 2001), namely acute exercise, ischemia-reperfusion and drug administration. With these experimental models, several authors have shown that the depletion of intrinsic GSH exacerbates tissue damage inflicted by many stimuli (Sen et al. 1993; Sen et al. 1994; Leeuwenburgh and Ji 1995).

However, to our knowledge, regarding hypoxia-induced enhanced free radical production in skeletal muscle, no data clearly indicate the effective role of GSH. In fact, a few authors have used GSH as a marker of oxidative stress to study the influence of dietary supplementation of anti-oxidants during oxidative stress induced by hypoxia (Sarada et al. 2002a, 2002b). Even though GSH depletion has been described in these hypobaric hypoxic conditions (Singh et al. 2001), and a reverse trend was reported with antioxidant supplementation, it is not sufficiently clear whether GSH has, as in exercise or in ischemia-reperfusion models, a determinant role against hypoxia-induced increase ROS production in skeletal muscle. Moreover, the above-referred experimental protocols were design using hypobaric hypoxia interspersed by long periods of normoxia (Singh et al. 2001; Sarada et al. 2002a, 2002b).

Therefore, the purposes of the present study were to analyze in skeletal muscle the effect of an acute period (24 h) of severe simulated-altitude hypobaric hypoxia (43 kPa): (1) on oxidative stress parameters (oxidized and reduced glutathione), on some oxidative damage markers (TBARS, sulfhydryl protein groups, SH) and lysosomal enzyme activity (N-acetyl-β-D-glucosaminidase, NAG), and (2) the antioxidant protective role of GSH on those parameters, using animals treated with L-buthionine-[S,R]-sulfoximine (BSO), a pharmacological GSH-depleting compound that inhibits γ-glutamylcysteine synthase (GCS), a rate-limiting step enzyme of the γ-glutamyl cycle, inducing reductions in cell glutathione content (Griffith 1982; Leeuwenburgh and Ji 1995). Additionally, and assuming that expression of the 70-kDa heat shock proteins (HSP70) is an inducible mechanism protecting proteins against cellular stress (for references see Feder and Hofmann 1999), another purpose of this study was to analyze, during acute altitude-hypoxia exposure, the expression of HSP70 as a marker of in vivo cellular stress. In fact, to our knowledge few available data have previously been reported regarding the role of these molecular chaperones on the intrinsic protection of skeletal muscle against systemic and physiological hypoxia.

Methods

Experimental design

Forty CD1 Charles River mice (30–35 g) were randomly divided into four groups ($n = 10$) for a 24-h experimental design protocol. Two control groups were injected, respectively, with 0.4 ml of placebo saline solution (C + P group) and with a single 4 mmol/kg dose of BSO (C + BSO group) in 0.4 ml solution and maintained at an atmospheric pressure of 101.3 kPa (760 mmHg) equivalent to sea level. Two experimental hypoxia groups (H + P and H + BSO), respectively injected as the above-mentioned groups, were exposed to a simulated atmospheric pressure of 43.2 kPa (324 mmHg) equivalent to an altitude of 7000 m in a hypobaric chamber. The depressurization to reach the simulated altitude of 7000 m and the pressurization until sea level conditions took 15 min (Fig. 1). All the animals of vehicle and BSO treatment groups were intraperitoneally (i.p.) injected 24 h before sacrifice and kept at a constant temperature (21–25°C) on a daily lighting schedule of 12 h light and 12 h dark with normal activity and food and water ad libitum. All the animals were sacrificed immediately after the end of the experiment. The Ethics Committee of the Scientific Board of Faculty of Sport Sciences approved the 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 total (TGSH), reduced (GSH) and oxidized (GSSG) glutathione, TBARS, protein sulfhydryl (SH), NAG activity and total protein content. The aliquots for glutathione assay were previously extracted in a medium containing perchloric acid at 5% (w/v).

Assays

TGSH, GSH and GSSG measurements were determined as previously described by Tietze (1969) by spectrophotometric techniques at 414 nm. Lipid peroxidation in the whole muscle homogenate was assayed spectrophotometrically according to the method described by Bertholf et al. (1987) and measured by the formation of TBARS. NAG activity was determined spectrophotometrically with a commercial kit (Boehringer Mannheim; cat no. 875406). Oxidative modification of protein SH groups was quantified by spectrophotometric measurement according to the method proposed by Hu (1990). Protein content was assayed spectrophotometrically using bovine serum albumin as standard according to Lowry et al. (1951). To determine the levels of HSP70 in the muscles, a certain volume of homogenate corresponding to 10 mg
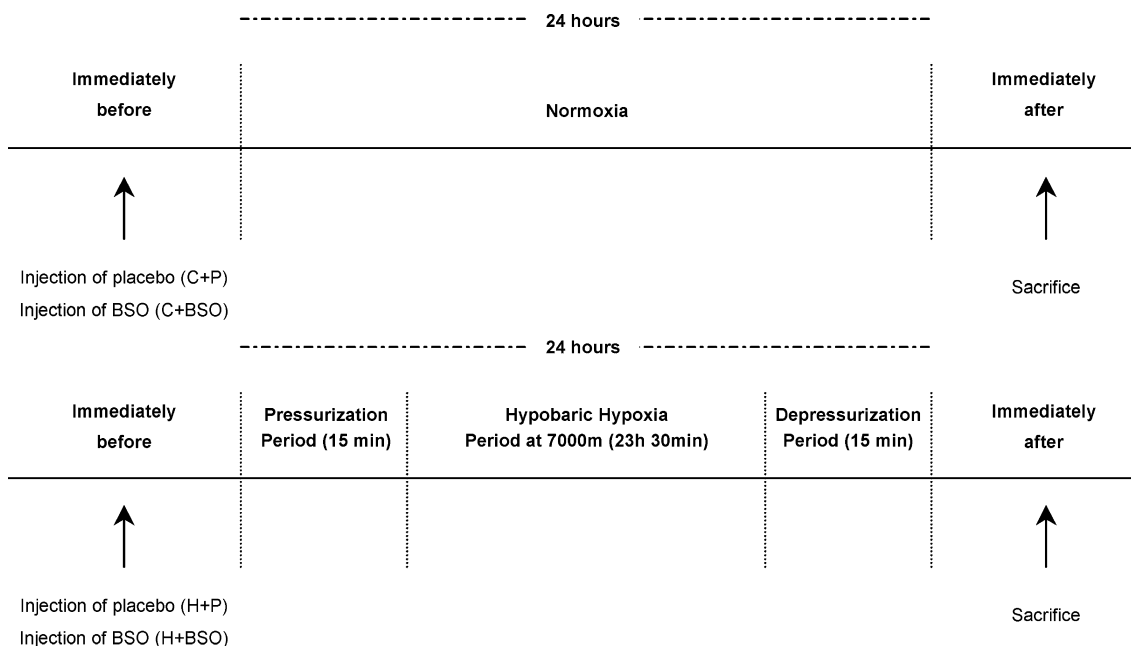
Fig. 1 Time course of the experimental protocol. C+P and C+BSO groups were injected with placebo and BSO, respectively, submitted to normoxia for 24 h and sacrificed immediately after. H+P and H+BSO groups were injected with placebo and BSO, respectively, immediately submitted to hypobaric hypoxia equivalent to 7000 m for 24 h and sacrificed immediately after

protein was resolved by SDS-PAGE (12.5% acrylamide gels of 1 mm thickness) as described by Laemmli (1970) and electroblotted onto nitrocellulose membranes according to Locke et al. (1990). The immunoblots were probed with 1:5000 dilution of monoclonal anti-Hsp70 (Sigma) and with 1:500 dilution of the secondary antibody (anti-mouse IgG peroxidase conjugate, Sigma, St. Louis, Mo., 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, Mo., USA). The films were analyzed with QuantityOne Software (Bio Rad). Optical density results are expressed as percentage variation of control values.

Statistical analysis

Mean and mean standard errors were calculated for all variables in each of the experimental groups. One-way ANOVA followed by the Bonferroni post-hoc test was used to compare groups. Statistical Package for the Social Sciences (SPSS, version 10.0) was used for all analysis. The significance level was set at 5%.

Results

Skeletal muscle glutathione contents are expressed in Figs. 2, 3, 4 and 5. There was a significant increase in GSSG ($p < 0.05$), TGSH ($p < 0.05$) and the GSSG/TGSH ratio ($p < 0.05$) with hypoxia exposure (C+P vs. H+P). However, no GSH content changes were found in the hypoxia exposure group (H+P) compared to C+P. BSO treatment induced a significant depletion in TGSH of approximately 40% (C+P vs. C+BSO; $p < 0.05$). However, no significant differences were found

in GSH, GSSG and the GSSG/TGSH ratio between the two control groups. The effect of GCS inhibition by BSO in animals submitted to hypoxic conditions was markedly evident between H+P vs. H+BSO, namely in TGSH, GSH and the GSSG/TGSH ratio. In this regard, there was a substantial fourfold decrease ($p < 0.05$) in GSH levels with altitude in the BSO-treated group, which seems to determine the TGSH content and %GSSG rather than the GSSG increment.

The levels of muscle TBARS, SH group and muscle NAG activity as indirect measures of lipid peroxidation, protein oxidation and lisosomic activity, respectively, are depicted in Table 1. Muscle lipid peroxidation increased in the altitude placebo group (H+P), while BSO administration did not induce additional levels of membrane damage in the muscle. With respect to protein SH content, no significant differences were found
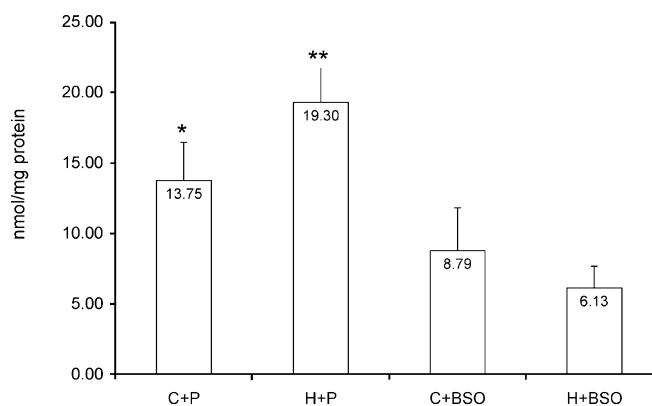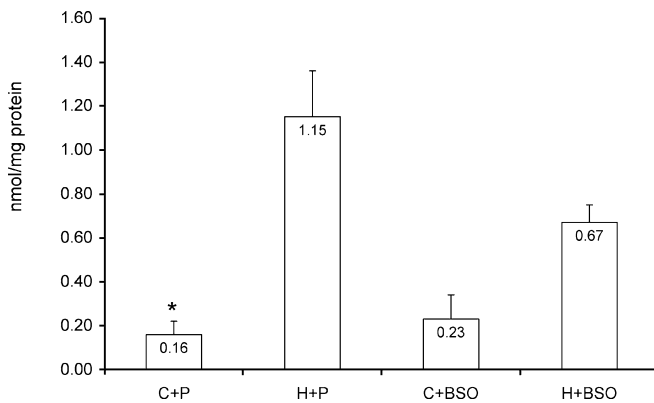

Fig. 2 Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and BSO treatment on soleus muscle total glutathione (*TGSH*). Values are mean (SEM, nmol/mg protein). *$p < 0.05$, C+P vs. H+P and C+BSO. **$p < 0.05$, H+P vs. H+BSO
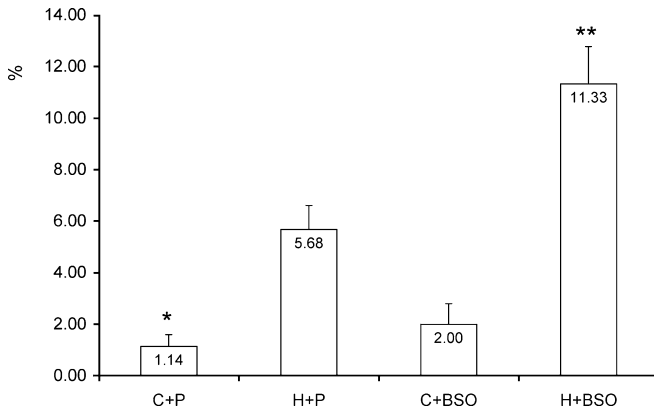
Fig. 3 Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and BSO treatment on soleus muscle GSSG/TGSH (*%GSSG*). Values are mean (SEM) (%). *$p < 0.05$, C + P vs. H + P, **$p < 0.05$, H + P and C + BSO vs. H + BSO
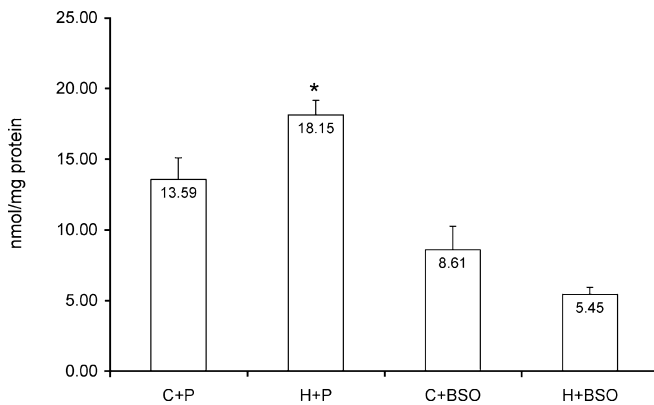
Fig. 4 Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and BSO treatment on soleus muscle reduced glutathione (*GSH*). Values are mean (SEM, nmol/mg protein). *$p < 0.05$, H + P vs. H + BSO

Fig. 5 Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and BSO treatment on soleus muscle oxidized glutathione (*GSSG*). Values are mean (SEM, nmol/mg protein). *$p < 0.05$, C + P vs. H + P

among groups, although an 18% decrease was observed between C + P and H + P. Simulated altitude exposure per se induced a significant and marked elevation in skeletal muscle NAG activity (C + P vs. H + P; $p < 0.05$); however, no additional increment in this enzyme activity was found in the animals submitted to hypoxia and treated with BSO (H + BSO).

Concerning HSP70 expression (Fig. 6), a significant increase was observed in the H + P group compared to the C + P group. BSO treatment induced a significant increase in HSP content (C + P vs. C + BSO) with no additional significant rise with hypoxia exposure (C + BSO vs. H + BSO), despite an almost 40% increase in HSP70 expression. Regarding hypoxic groups (H + P vs. H + BSO), although no significant difference was found, a slight increase (24%) was observed with BSO treatment.

Discussion

The overall picture of our results seems to confirm the increased level of tissue oxidative stress and muscle damage during exposure to acute and severe hypobaric hypoxia, which can be explained by a broad imbalance between oxidant production and the antioxidant's capacity to prevent oxidative injury. Indeed, in accordance with some other in vitro studies (Duranteau et al. 1998; Mohanraj et al. 1998) conducted in hypoxia/anoxia conditions and recent in vivo field (Simon-Schnass 2000; Moller et al. 2001) and laboratory (Joanny et al. 2001; Singh et al. 2001; Sarada et al. 2002a, 2002b) experiments related to human and animal hypobaric hypoxia exposure within the physiological range, our data support the assumption of enhanced free radical production during simulated-altitude hypobaric hypoxia exposure.

As an indicator of oxidative stress, the GSSG/TGSH ratio was increased after 24 h of hypoxia in the H + P group when compared to control (C + P), which may be explained by enhanced GSH oxidation (Sen et al. 1994). Although a decrease in the GSH content could be expected due to the substantial amount of GSH oxidation, unexpectedly a slight non-significant increase in GSH was observed in this placebo hypoxic group. Indeed, under skeletal muscle oxidative stress, GSH can be imported by muscle fibers from plasma via the γ-glutamyl cycle to cope with increased free radical production (Powers et al. 1999). In our study this hypothesis can be supported by the significant increase in TGSH content observed in the H + P group. Despite the significant increase in %GSSG in the H + P group, it is important to be aware that these results could be underestimated since an increase in glutathione reductase (GR) activity described to occur in hypobaric hypoxia (Singh et al. 2001) could enhance GSH *turnover* diminishing the evidences of oxidative stress.

The increased oxidative stress induced lipid peroxidation, yielding oxidation products that constitute TBARS, and oxidation of SH group with concomitant

Table 1 Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and L-buthionine-[S,R-L]-sulfoximine (*BSO*) treatment on soleus muscle thiobarbituric acid reactive substances (*TBARS*), protein sulfhydryl protein group (*SH*) content and *N*-acetyl-β-D-glucosaminidase (*NAG*) activity. All values are mean (SEM)

Conditions	TBARS (nM)		SH (mol/g protein)		NAG (U/mg protein)	
	Mean	SEM	Mean	SEM	Mean	SEM
C + P	227.9*	(49.9)	474.14	(48.34)	3.35**	(0.16)
H + P	436.7	(51.0)	391.47	(21.62)	4.49	(0.27)
C + BSO	411.8	(46.5)	456.80	(29.76)	3.22	(0.24)
H + BSO	409.4	(35.1)	433.86	(71.69)	3.90	(0.31)

*$p < 0.05$, C + P vs. H + P and C + BSO
**$p < 0.05$, C + P vs. H + P

changes in cell catabolism, namely activation of lysosomic function Table 1). The increase in TBARS is in accordance with some previous results obtained in studies of human plasma (Bailey et al. 2001; Joanny et al. 2001) and animal tissues submitted to hypoxia (Singh et al. 2001; Sarada et al. 2002b). As in other oxidative stress-inducible models (Venditti and Di Meo 1996), our results can be explained by peroxidative modification of membrane lipids, since polyunsaturated fatty acids seem to be extremely susceptible to increased oxidant production (Halliwell and Gutteridge 1999). However, lipid peroxidation did not increase significantly with hypoxia in rats exposed at 4000 m for 4 weeks (Radak et al. 1997) or in humans submitted to normobaric hypoxia conditions equivalent to low altitude ($F_IO_2 = 0.16$) (Bailey et al. 2000). An altitude-dependent enhanced oxidative stress effect (Joanny et al. 2001) may, at least in part, explain the lack of increase in TBARS in those studies.

Concerning the oxidation of protein thiol groups, our results revealed that there was slight but insignificant differences within C + P and H + P groups. Since, (1) GSH seems to be the most important electron donor under pro-oxidant redox conditions (Ji and Leeuwenburgh 1996) and (2) total protein represents a large quantity of absolute SH-containing compounds when compared to GSH, it is reasonable to hypothesize that a scaling effect could justify the absence of significant variations in protein SH content between the two groups. However, despite these protein thiol results, the enhanced NAG activity observed in hypoxia (H + P), reflecting increased functionality of lysosomes (Salminen and Marjomaki 1985), suggests the occurrence of protein damage. The inversely proportional variation exhibited in these groups concerning protein SH content and NAG activity suggests that an oxidative stress-induced mechanism could be responsible, at least in part, for lysosomal activation.

Compared to the C + P group, and as expected, BSO treatment significantly diminished muscle TGSH content in C + BSO group, which can be explained by GCS inhibition (Griffith and Meister 1979). On the other hand, GSH content decreased despite a non-significant increase in %GSSG. The slow rate of ROS production in the normoxic rest environment could explain these results. In fact, during these conditions an almost 30% GSH content decrease does not seem to be a sufficiently great stressor to disturb the GSH/GSSG turnover and thus raise %GSSG. However, when the BSO-treated animals were submitted to hypobaric hypoxia, a decrease of the TGSH and GSH content with a significant increase in %GSSG muscle status suggests an imbalance of GSH/GSSG turnover probably due to enhanced oxidative stress, which also explains the results between H + P and H + BSO. In fact, besides BSO-induced GCS inhibition, γ-glutamyl transpeptidase (GGT) activity can also be down-regulated by enhanced oxidative stress (Sen et al. 1993), impairing GSH membrane importation

Fig. 6 Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and BSO treatment on soleus muscle HSP70 expression. Values are mean (SEM) (%). A scan of representative Western blot for each group is immediately below the histogram. *$p < 0.05$, C + P vs. H + P, C + BSO and H + BSO
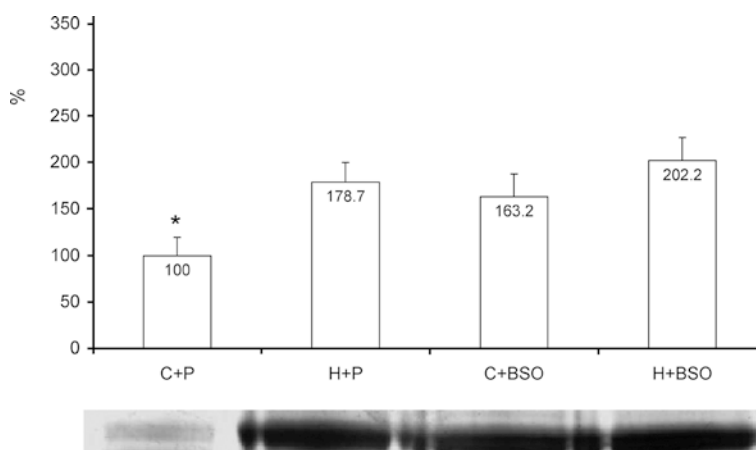
which exacerbates TGSH and GSH intracellular depletion (Powers et al. 1999). This decreased capability of intracellular GSH replacement under hypoxia-induced enhanced free radical production probably increases the rate of GSSG conversion and consequently augments the GSSG/TGSH ratio. Additionally, since these conditions can be potentially harmful to cell viability due to GSSG toxicity, its exportation to the circulation in order to maintain a constant and viable cell redox status (Sen 2001) might be a reasonable explanation for the lower GSSG content of the H + BSO compared to the H + P group.

As reported by others (Leeuwenburgh and Ji 1995; Mohamed et al. 2000), BSO treatment per se caused a marked enhancement in lipid peroxidation (C + P vs. C + BSO). Even though these results seem to conflict with %GSSG differences in these groups, they are in accordance with variations of TGSH content. Indeed, in contrast with %GSSG, which is closely related to the brisk redox changes, TBARS as well as TGSH could reflect some long-term process of cumulative cellular stress (Halliwell and Gutteridge 1999). Moreover, the TBARS content is the result of the accumulation of hydroperoxides in tissues via a turnover-dependent process involving the synthesis/degradation ratio (Halliwell and Gutteridge 1999). This is one hypothetical explanation to justify the absence of additional lipid peroxidation in animals exposed to hypoxia and treated with BSO (H + BSO vs. C + BSO), despite the enhanced oxidative stress signs provided by the glutathione system. However, another reason should be considered when accounting for the absence of enhanced muscle lipid peroxidation as well as protein oxidation and increased NAG activity in the H + BSO compared to the H + P group. In fact, conflicting with other in vivo stress models (Leeuwenburgh and Ji 1995), our results suggest that, in hypoxia-induced oxidative stress, antioxidant mechanisms other than the glutathione system seem to be protective against oxidative damage. Since glutathione depletion has been frequently reported (Leeuwenburgh and Ji 1995) as an additional deleterious cell redox disturbance, exuberant lipid peroxidation, protein oxidation and lysosomal activation levels in the H + BSO group would be expected, .

Concerning HSP70, our results are in accordance with other studies in which different stresses such as heat, acute exercise, exposure to oxidants and ischaemia/reperfusion have been shown to increase the expression of this highly conserved proteins (Locke et al. 1995; Noble 2002). Indeed, as molecular chaperones, HSP70 are reported to provide intrinsic protection to the tissues against deleterious stimuli, namely interacting with other proteins and minimizing the probability that these other proteins interact inappropriately with others, i.e., facilitating protein synthesis, folding and assembly (Feder and Hofmann 1999). Although the components of hypoxia and BSO treatment that are responsible for causing cellular HSP70 induction cannot be determined from the present study, the increased oxidative stress, among other possible cellular inducible signaling mechanisms, could explain, at least in part, HSP70 overexpression since the enhanced production of ROS has been described to be a signal for the up-regulation of heat shock proteins (Hamilton and Powers 2002).

In summary, the present study seems to confirm that hypoxia per se engenders increased oxidative stress. However, the exacerbated enhanced %GSSG with hypobaric hypoxia exposure in BSO-treated animals and the lack of concomitant increases in oxidative damage markers suggest a modest role for the glutathione system in cell protection against altitude-hypoxia-induced oxidative damage. Moreover, the physiological stress imposed by altitude-hypoxia exposure and BSO treatment is a sufficient stimulus to promote HSP70 induction and overexpression and seems to be, at least in part, explained by enhanced ROS production.

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