Universidade do Porto

Faculdade de Ciências do Desporto
e de Educação Física

Oxidative Stress and Damage induced by Hypobaric Hypoxia

Effects of the acute and chronic exposure to simulated and real high-altitude environments in blood and skeletal muscle of humans and mice

José Fernando Magalhães Pinto Pereira

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II.

Magalhães, J., Ascensão, A., Viscor, G., Soares, J., Oliveira, J., Marques, F., Duarte, J. (2004). Oxidative stress in humans during and after 4 hours of hypoxia at a simulated altitude of 5500m. *Aviat Space Environ Med* 75: 16-22.

III.

Magalhães, J., Ascensão, A., Soares, J., Ferreira, R., Neuparth, M., Oliveira, J., Amado, F.; Marques, F.; Duarte, J. (2004). Acute and chronic exposition of mice to severe hypobaric hypoxia: the role of short-acclimatization against skeletal muscle oxidative stress and damage. *Int J Sports Med* Published online July 30. 2004; DOI: 10.1055/s-2004-817858.

IV.

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V.

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Abstract

Exposure to simulated or real high altitude conditions of hypobaric hypoxia is a serious physiological challenge that predisposes the body to innumerous systemic and tissue deleterious effects. Among other hypothetical mechanisms involved in some of these organic constrains, the increased production of reactive oxygen and nitrogen-based species (RONS) and the resulting cellular condition of oxidative stress and damage have recently been addressed. In this dissertation, a series of studies (6) comprising distinct experimental designs and involving humans and animals exposed to acute and chronic simulated or real high altitude conditions, was used to analyze the impact of severe hypobaric hypoxia on several distinct markers of oxidative stress and damage in plasma, erythrocytes and skeletal muscle.

In study I, II and III, the main objectives were to investigate, (i) the influence of acute and chronic simulated hypobaric hypoxia exposure in mice skeletal muscle oxidative stress and damage markers, (ii) the influence of the post-hypoxic reoxigenation period induced by pressurization until sea level conditions in human, and (iii) the role of the gradually staged process of acclimatization and of the persistency under that severe conditions of hypobaric hypoxia in both non-acclimatized and acclimatized animals. Various biochemical parameters were assayed to achieve those purposes, including the content of reduced and oxidized glutathione, total antioxidant status (TAS), thiobarbituric acid reactive substances (TBARS), protein sulfhydryl groups (-SH) and 70-kDa heat shock proteins, and the activity of N-acetyl-b-D-glucosaminidase and superoxide dismutase (SOD). In study IV and V, the effects of a high altitude expedition to a Himalayan peak on (i) plasma and erythrocyte antioxidant profile, and on (ii) skeletal muscle microvasculature ultrastructure were analyzed in humans using biochemical markers, such as the activities of the enzymes SOD, glutathione peroxidase and glutathione reductase, TAS content, tissue-type plasminogen activator and plasminogen activator inhibitor type 1 activities, and skeletal muscle ultrastructure morphometrical analysis (thickness of the vascular and fiber basement membrane). In study VI, the influence of an acute and severe hypobaric hypoxia exposure in skeletal muscle mitochondrial functionality and cell fate was analyzed in mice. The rates of oxygen consumption in state 3, state 4 and with the addition of olygomycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP) as well as the respiratory control ratio (RCR) and ADP/O (state 3/state 4) were obtained to study mitochondrial respiratory function. Additionally, aconitase activity, malondialdehyde (MDA), CGs and –SH content, 60-kDa heat shock protein expression and markers of the triggering of cellular apoptosis (Bax/Bcl-2) were also measured.

In general, data collected from the all the studies present an interesting scenario where RONS may actually play an important role during hypobaric hypoxia exposure by modulating cell redox status and homeostasis in a hypoxic cell, tissue and organism. In fact, the exposure of animals and humans to acute or chronic hypobaric hypoxia, under simulated or real high altitude conditions, engenders an increased oxidative stress and damage in blood and in skeletal muscle, with biochemical, functional and histological consequences at distinct levels of cellular organization.

Keywords: High altitude, hypobaric hypoxia, oxidative stress and damage, acclimatization, skeletal muscle, blood, humans, animals.

Resumo

A exposição a condições de hipóxia hipobárica em ambientes simulados ou reais de elevada altitude é um sério desafio fisiológico que predispõe o organismo a inúmeros efeitos deletérios a nível sistémico e tecidual. Entre outros mecanismos hipoteticamente envolvidos nesta perda da homeostasia celular e orgânica, têm sido recentemente sugeridos a produção acrescida de espécies reactivas de oxigénio e nitrogénio (RONS) e a concomitante condição celular de stress e lesão oxidativa. Nesta dissertação, são apresentados seis estudos com protocolos experimentais distintos, envolvendo humanos e animais de laboratório expostos de forma aguda ou crónica a condições reais ou simuladas de elevada altitude. A partir destes estudos é analisado o impacto da hipóxia hipobárica severa a nível plasmático, eritrocitário e muscular esquelético, em distintos marcadores de stress oxidativo e de lesão celular.
Os principais objectivos dos estudos I, II e III foram investigar: (i) a influência da exposição aguda e crónica à hipóxia hipobárica simulada em marcadores de stress oxidativo e de lesão celular no músculo esquelético de ratinhos; ii) a influência em humanos do período de reoxigenação pós-hipóxia hipobárica; e iii) o papel da aclimatação e da persistência nessas condições severas de hipóxia hipobárica, em animais aclimatados e não-aclimatados. Para estes fins foram analisados vários parâmetros bioquímicos a nível plasmático e muscular esquelético, incluindo o estado antioxidante total (TAS), o conteúdo de glutationa reduzida e oxidada, de substâncias reactivas ao ácido tiobarbitúrico (TBARS), de grupos sulfídrilicos proteicos (-SH), de proteínas de choque térmico de 70-kDa, bem como, a actividade das enzimas N-acetil-b-D-glucosaminidase e da superóxido dismutase (SOD). Nos estudos IV e V foram analisados, em humanos, os efeitos de uma expedição de elevada altitude a um cume dos Himalaias (i) no perfil antioxidante plasmático e eritrocitário e (ii) na ultraestrutura microvascular do músculo esquelético, utilizando marcadores bioquímicos, tais como a actividade das enzimas SOD, glutationa peroxidase e glutationa reductase, o TAS, as actividades do activador do plasminogénio tipo-tecidual e do inibidor do activador do plasminogénio tipo-tecidual tipo1 e, ainda, a análise morfométrica da ultraestrutura do músculo esquelético (espessura das membranas basais da fibra e dos capilares). No estudo VI foi analisada, em ratinhos, a influência da exposição aguda e severa à hipóxia hipobárica na funcionalidade mitocondrial do músculo esquelético e na apoptose celular. Para estudar a funcionalidade respiratória a nível mitocondrial foram analisadas as taxas de consumo de oxigénio em estado 3, estado 4, após adição de oligomicina, carbonyl cyanide m-chlorophenylhydrazone (CCCP) bem como, o ratio de controlo respiratório (RCR) e o ADP/O (estado 3/estado 4). Adicionalmente foram medidas a actividade da enzima acotinase, o conteúdo mitocondrial de malondialdeído (MDA), de CGs e de –SH, a expressão das proteínas de choque térmico 60-kDa e marcadores de apoptose celular (Bax/Bcl-2). Em conclusão, os resultados sugerem um cenário interessante no qual os RONS podem ter um papel importante durante a exposição à hipoxia hipobárica modelando o estado redox celular e a homeostasia da célula, do tecido e do organismo hipóxicos. De facto, a exposição aguda e crónica de animais e humanos a estas condições induz, a nível sanguíneo e muscular esquelético, um acréscimo de stress oxidativo e de lesão celular com repercussões bioquímicas, funcionais e histológicas em diferentes níveis de organização celular.

Palavras-chave: altitude, hipoxia hipobárica, stress e lesão oxidativa, aclimatação, músculo esquelético, sangue, humanos, animais

Résumé

L'exposition à des conditions d'hypoxie hypobarique dans des milieux simulés ou réels de grande altitude est un sérieux défi physiologique qui prédispose l'organisme à des nombreux effets délétères au niveau systémique et au niveau des tissus. Parmi d'autres mécanismes hypothétiquement impliqués dans cette perte de l'homéostasie cellulaire et organique, la production accrue d'espèces réactives d'oxygène et de nitrogène (RONS) et la concomitante condition cellulaire de stress et de lésion oxydatif ont été récemment suggérées. Dans cette dissertation, nous présentons six études avec des protocoles expérimentaux distincts, utilisant des humaines et des animaux de laboratoire exposés d'une forme aiguë ou chronique à des conditions réelles ou simulées d'une grande altitude. À partir de ces études nous analysons l'impact de l'hypoxie hypobarique sévère au niveau plasmatique, érythrocytaire et musculaire squelettique, dans différents marqueurs de stress oxidative et de lésion cellulaire.
Les principaux objectifs des études I, II et III ont été investiguer: (i) l'influence de l'exposition aiguë et chronique à l'hypoxie hypobarique simulée dans des marqueurs de stress oxidative et de lésion musculaire dans le muscle squelettique de souris; (ii) l'influence dans des humaines de la période de ré-oxygénation pos-l'hypoxie hypobarique; (iii) le rôle de l'acclimatation et de la persistance dans ces conditions sévères de l'hypoxie hypobarique, dans des animaux acclimatés et non- acclimatés. Pour atteindre ces objectifs, nous avons analysé plusieurs paramètres biochimiques au niveau plasmatique et musculaire squelettique, en incluant l'état antioxydant total (TAS), le contenu de glutathione réduite et oxydée, de substances réactives à l'acide tiobarbiturique (TBARS), de groupes sulphydryl des protéines (-SH), des protéines de choc thermique de 70-kDa, ainsi comme, l'activité des enzymes N-acetil-b-D-glucosaminidase et de la superoxide dismutase (SOD). Dans les études IV et V nous avons analysé, dans des humaines, les effets d'une expédition de grande altitude à une cime des Himalayas (i) dans le profil antioxydant plasmatique et érythrocytaire et (ii) dans l'ultrastructure microvasculaire du muscle squelettique, utilisant des marqueurs biochimiques, comme l'activité des enzymes SOD, glutathione réductase, le TAS, les activités de l'activateur du plasminogène tissulaire et de l'inhibiteur l'activateur du plasminogène tissulaire type 1 et, encore, l'analyse morphométrique de l'ultrastructure du muscle squelettique (épaisseur des membranes basales de la fibre et des capillaires). Dans l'étude VI nous avons analysé, dans des souris, l'influence de l'exposition aiguë et sévère à l'hypoxie hypobarique dans fonctionnalité mithochondrial du muscle squelettique et dans l'apoptose cellulaire. Pour étudier la fonctionnalité respiratoire au niveau mythochondriale, nous avons analysé les taxes de consommation d'oxygène en état 3, état 4, après l'addition d'oligomycine, de carbonile cyanure m-chlorophenylhydrazone (CCCP) ainsi comme la ratio de contrôle respiratoire (RCR) et l'ADP/O (état 3/état 4). Additionnellement, nous avons mesuré l'activité de l'enzyme acotinase, le contenu mithochondrial de malondialdéhyde (MDA), de CGs et de –SH, l'expression des protéines de choc thermique de 60-kDa et des marqueurs d'apoptose cellulaire (Bax/Bcl-2). En conclusion, les résultats suggèrent un scénario intéressant dans lequel les RONS peuvent avoir un rôle important pendant l'exposition à l'hypoxie hypobarique modelant l'état redox cellulaire et l'homéostasie de la cellule, du tissu et de l'organisme hypoxiques. En fait, l'exposition l'influence de l'exposition aiguë et chronique d'animaux et d'humaines à ces conditions là mène, au niveau sanguin et musculaire squelettique, à un accroissement de stress oxidative et de lésion cellulaire avec de répercussions biochimiques dans différents niveaux d'organization cellulaire.

Mots-clés: altitude, hypoxie hypobarique, stress et lésion oxidative, acclimatation, muscle squelettique, sang, humaines, animaux

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General Introduction

High altitude exposure has been considered as a major challenging strain for the organism compromising cardiorespiratory, endocrine, metabolic, nutritional and thermal homeostasis (Grissom and Elstad, 1999; Hackett, 1999; Hochachka *et al.*, 1999; Bonnon *et al.*, 2000; Karakucuk and Mirza, 2000; Moore, 2000; Severinghaus, 2000; Ward *et al.*, 2000; Hoppeler and Vogt, 2001; Rupert and Hochachka, 2001). In fact, distinct environmental stimuli are present during high-altitude exposure including extreme cold, temperature shifts, very low absolute humidity, increased ultraviolet (UV) radiation, and particularly low barometric pressure, leading to an exacerbated physiological stress (West, 1996; Hultgren, 1997; Ward *et al.*, 2000; Askew, 2002). Barometric pressure decreases with altitude because the higher we go, the less atmosphere there is above us pressing down by virtue of its weight. Nevertheless, it is important to be aware that barometric pressure also varies both with latitude and with the season of the year, which means that the geographic location and the climate are major determinants to consider regarding the physiological effects induced by a high-altitude hypobaric hypoxic exposure (West, 1999). This reduced barometric pressure at altitude results in the decrease of the partial pressure of inspired oxygen, affecting the so-called "oxygen cascade" and diminishing the ability of the oxygen (O_2) to diffuse from the atmospheric air to the blood and tissues, i.e., inducing systemic and local hypoxia (Hultgren, 1997; Samaja, 1997). Molecular oxygen is vital to the survival of all mammalian cells serving as the terminal acceptor in the oxidative process that provides the usable energy necessary for life. Thus, a compensatory fine tuning of the hypoxia sensing and signal transduction pathway cascades, eliciting central respiratory, circulatory and several peripheral processes is triggered in such conditions to counteract the limit O_2 availability (Hochachka *et al.*, 1999; Hochachka and Rupert, 2003). However, depending on the severity, the duration and the rapidity of the onset of hypoxia, the decreased levels of O_2 might severely compromise body metabolism, promoting reversible or irreversible loss of tissue and cell homeostasis and leading to organic and functional decay. Given that, even the acclimatized body remains hypoxic at certain severe altitudes (West, 2003), i.e., the oxygen unavailability to oxidative energetic pathways continues to affect cellular homeostasis, an organic deterioration is a condition that is often described in animals and humans after some time spent at extreme altitude (Bigard *et al.*, 1996; Zamboni *et al.*, 1996; Ward *et al.*, 2000). In fact, altitudes above 5000m are considered extremely deleterious to organic homeostasis and altitudes higher than 8000m have been called "the death zone", i.e., the body degradation is so severe and fast that even for well-acclimatized climbers, summit bids on peaks over this height are wisely planned so as to spend as short time as possible in this zone. This deterioration process is frequently attributed to several factors usually experienced by dwellers in high-altitude sojourns, such as dehydration, starvation, physical exhaustion and extreme cold (West, 2003). However, in the absence of those factors, it seems that hypobaric hypoxia *per se,* if sufficiently severe, brisk or persistent, plays a major role causing mental and physical deterioration. In fact, hypobaric hypoxia exposure seems to result in significant weight loss (particularly free-fat mass), skeletal muscle degradation, poor appetite, slow recovery from fatigue, lethargy, irritability, an increasing lack of willpower to start new tasks (Houston, 1997; Abraini *et al.*, 1998; de

Glisezinski *et al.*, 1999; Nicolas *et al.*, 1999; Bouquet *et al.*, 2000; Caquelard *et al.*, 2000; Westerterp *et al.*, 2000), and, ultimately, in a benign illness related to neurological and respiratory symptoms that even might culminate in a life-threatening high-altitude cerebral (Hackett, 1999) or pulmonary edema (Bartsch, 1999), respectively. Nevertheless, despite the scientific worldwide efforts to find out and better understand the specific mechanisms underlying these hypoxia-mediated deterioration occurrences, there are still many doubts and unanswered questions.

Amongst several potential mechanisms suggested to explain the different physiological constrains associated with high altitude exposure, an oxidative-based aetiology has been advanced in the last years. Indeed, increasing scientific evidences of blood lipid peroxidation, protein oxidation and oxidative DNA damage associated to an enhanced production of reactive oxygen and nitrogen based-species (RONS) have been reported in many studies conducted with humans in real high-mountains environment (Pfeiffer *et al.*, 1999; Bailey *et al.*, 2000a; Simon-Schnass, 2000; Moller *et al.*, 2001). These blood *redox* status-based disturbances suggest an apparently paradoxical phenomenon of hypobaric hypoxia-induced oxidative stress that might contribute, at least in part, to some of the organic deleterious effects perpetrated by a high-altitude hypoxic insult. In these hypobaric hypoxic conditions, the antioxidant body defense systems seem to be overwhelmed by the enhanced production of RONS, like superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot-}$) and peroxinitrite ($ONOO^-$), which probably affects cellular *redox* equilibrium (Kehrer and Lund, 1994; Askew, 2002). Nevertheless, in these high-altitude field-based studies, besides hypoxia, other factors like intense UV radiation, physical activity and brisk air temperature shifts may also be related to RONS formation leading to enhanced oxidative stress (Simon-Schnass, 2000). In this sense, to dissociate hypobaric hypoxia from other possible "oxidative stressors", some acute, chronic and intermittent hypoxia exposure studies carried out with animals (Chang *et al.*, 1989; Singh *et al.*, 2001; Sarada *et al.*, 2002a) and humans (Bailey *et al.*, 2000a; Bailey *et al.*, 2000c; Joanny *et al.*, 2001) in conditions of hypobaric or normobaric hypoxia that simulate high-altitude environments, had confirmed the role of hypoxia *per se* as an independent modulator of cell and tissue *redox* status leading to oxidative stress and damage. However, according to the definition of oxidative stress, i.e., a shift of prooxidant/antioxidant balance in favor of the former, besides the enhanced capacity of RONS production, the efficiency of the cellular antioxidant defense system is of vital importance in the extent of oxidative stress and cell or tissue damage (Sies, 2000; Ji, 2002). In fact, in an attempt to counteract this RONS-mediated deleterious impact on several cellular constituents, such as lipids, proteins and DNA, mammals are 'equipped' with an efficient antioxidant defense system consisting in antioxidant vitamins, glutathione and other sulfhydryls compounds, and antioxidant enzymes (Ji, 1995; Fridovich, 1998; Sen and Goldfarb, 2000). According to these authors, each of these antioxidant systems plays a unique role but also complements each other functionality. In general, antioxidant vitamins (e.g. vitamin E, C and ß-carotene) are particularly involved in the direct trapping of free radicals and singlet oxygen. On the other hand, glutathione and other thiol sources play an important role maintaining the cellular oxidoreductive status, and antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT) and GSH peroxidase (GPX), catalyze the one-electron reduction of ROS. Nevertheless, their functions are supported by a number of other enzymes involved in the supply of reducing powers (i.e., NADPH), such as

glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH). In addition, GSH-sulfur transferase (GST) is capable of conjugating GSH with a variety of xenobiotic compounds including organic and lipid peroxides contributing to diminish the impact of increased RONS production on cells (Ji, 1995; Fridovich, 1998; Sen and Goldfarb, 2000). However, it is important to be aware that RONS, being involved in fundamental and vital cell mechanisms such as the neutrophil respiratory burst within the scope of an inflammatory response and many cellular signaling and transcription pathways, including some related to hypoxia physiological and metabolic modulation (Baar *et al.*, 1999; Thannickal and Fanburg, 2000; Finkel, 2001; Droge, 2002), might also be important mediators in cell viability and survival.

Regardless some evidences suggesting that a period of systemic hypoxia related to simulated high-altitude conditions might contribute *per se* to increased RONS production and oxidative damage at different levels of cell organization, very little is known concerning the influence of the post-hypoxic reoxigenation period until sea level conditions in the RONS production and concomitant oxidative stress. In fact, despite the large amount of literature dealing with the physiological and pathophysiological responses to intermittent hypoxia exposure (for refs see Neubauer, 2001), the influence of the *in-vivo* post-hypoxic reoxygenation induced by pressurization on oxidative stress levels is not well understood and, to our knowledge, has not been previously demonstrated in humans after a hypobaric hypoxic insult. Similarly to an ischemia/reperfusion (I/R) model (Kadambi and Skalak, 2000; Li and Jackson, 2002), during post-hypoxic reoxygenation, an increased production of RONS might occur with the sudden wide modification in the levels of oxygen masking and/or exacerbating the cellular conditions of oxidative stress measured after returning to sea level conditions. In accordance to current literature (Walker, 1991; Granger and Korthuis, 1995; Jassem *et al.*, 2002), a tissue submitted to I/R undergoes significant structural and functional disorders that could be, at least in part, attributed to enhanced free radical production, particularly during the reperfusion (reoxygenation) period. In fact, while reactive oxygen species may be generated in a small extent during ischemia, far greatest production of these compounds occurs after reintroduction of oxygen during the period of reperfusion (Walker, 1991; Ferrari, 1995; Franko *et al.*, 1999). Accordingly, ultrastructural and metabolic cellular disturbances associated with the decreased oxygen delivery during ischemia and aggravated oxidative-mediated tissue harmful effects during the reperfusion period have been referred in many studies conducted in several tissues (Walker, 1991; Ferrari, 1995; Franko *et al.*, 1999). Increased capillary permeability (induced by microvascular alterations and subsequent edema), endothelial reactive oxygen species production (triggered by the xanthine dehydrogenase conversion into an oxidase form in the ischemic period), and polymorphonuclear leukocytes mobilization (with endothelial adherence and tissue infiltration) have been found in post-ischemic reperfused tissues (Appell *et al.*, 1997; Duarte *et al.*, 1997; Schlag *et al.*, 2001). Therefore, one can argue that exacerbated oxidative stress and damage could be expected after the reoxygenation period subsequent to a hypobaric hypoxic stimulus. Being true, this additional post-hypoxic oxidative stress and damage might exacerbate, at least in some studies, the real extent and severity of the *redox* disturbances that elapse from hypobaric hypoxia.

Taking into account that the organic challenging strain resulting from an acute hypobaric hypoxia exposure could be, at least in part, attenuated by adequate altitude

acclimatization (Orizio *et al.*, 1994; Basnyat *et al.*, 1999; Cymerman *et al.*, 2000; Green, 2000; Ricart *et al.*, 2000; Ward *et al.*, 2000; Zielinski *et al.*, 2000), another inevitable question concerns the influence of an adequate process of altitude acclimatization on the extent and severity of this oxidant insult. Altitude acclimatization is the physiological process that takes place on going gradually to altitude. It comprises a series of responses by different systems in the body, which collectively mitigate the effects of the diminished oxygen partial pressure so that the tissues are defended against this fall to a remarkable degree (West, 1993; Houston, 1998). This wide spread physiological adaptive processes, that take place when sea-level dwellers are gradually submitted to high-altitude, improve their ability to increase oxygen up-take from the hypoxic environment and to transport and to use it in cells diminishing blood and tissue hypoxia (reviewed in West, 1991; Hultgren, 1997). Indeed, although there is no sharp line separating acute and acclimatory phases of hypoxia exposure, it is clear that most hypoxia response systems do not have time to go to completion during acute hypoxia (Hochachka *et al.*, 1999). Moreover, these acclimatory responses to hypobaric-hypoxia do not depend exclusively on preexisting macromolecular machinery, since there is ample time for restructuring and reorganizing. In fact, preexisting macromolecular components may be up- or down-regulated allowing the orchestration of responses that are simply unattainable in acute hypoxia (Hochachka, 1996; Hochachka *et al.*, 1999). In this sense, during gradual and staged exposed conditions to high-altitude, in the context of an acclimatization process, a presumable phenomenon of altitude/hypoxia severity-dependent pro-oxidant production (Joanny *et al.*, 2001) may also be involved in some physiological adaptations that take place in response to hypoxia, including RONS antioxidant defense (Askew, 2002). As referred above, at high concentrations, reactive oxygen and nitrogen species are hazardous for living organisms and damage all major cellular constituents. However, at moderate concentrations, nitric oxide (NO·), superoxide anion, and related RONS seem to play an important role as regulatory mediators in signaling processes (Droge, 2002). In fact, depending on the magnitude of the cellular *redox* changes, those substances also seem to act as specific signaling mediators of certain transcription factors that are involved in the genetic regulatory expression of several molecules related to distinct physiological functions (Halliwell and Gutteridge, 1999; Turpaev, 2002) protecting cells against oxidative stress and helping them to reestablish "*redox* homeostasis". For instances, reactive oxygen species generated by mitochondria during brief hypoxia seem to be a triggering stimulus to initiate preconditioning protection in cardiomyocytes (Vanden Hoek *et al.*, 1998; Kulisz *et al.*, 2002). Furthermore, under hypoxia, mitochondrial reactive oxygen species generated at Complex III caused accumulation of the subunit hypoxia-inducible factor-1□ (HIF-1□) (Chandel *et al.*, 2000). This protein is responsible for initiating the expression of a luciferase reporter construct under the control of a hypoxic response element, and required for the induction of a variety of genes coding for proteins that stimulate the formation of new blood vessels as well as glycolytic enzymes that can produce energy from glucose without the aid of oxygen (Baar *et al.*, 1999; Marx, 2004).

Despite this hypothetical protective role induced by an adequate process of acclimatization against oxidative stress, persistent signs of blood oxidative damage have been found in some studies in which acclimatized humans were chronically submitted to simulated hypobaric hypoxia (Joanny *et al.*, 2001) or in which climbers were "oxidatively stressed out" (Bailey *et al.*, 2000a) during long sojourns at altitude (Bailey *et al.*, 2000a;

Simon-Schnass, 2000). In these circumstances, the antioxidant protective system seems to be gradually overwhelmed or depleted of its capacity to withstand the enhanced RONS production imposed by the severe and persistent hypoxic insult. In fact, data from several studies dealing with antioxidant supplementation on humans (Chao *et al.*, 1999; Pfeiffer *et al.*, 1999; Bailey and Davies, 2001; Schmidt *et al.*, 2002) or animals (Ilavazhagan *et al.*, 2001; Sarada *et al.*, 2002a; Sarada *et al.*, 2002b) submitted to chronic hypoxia conditions showed notable benefits concerning distinct oxidative damage markers. On the other hand, considering the organic deterioration and the reduced food intake often described after some time spent at extreme altitude (Kayser, 1992; Bailey *et al.*, 2000b; Ward *et al.*, 2000; Askew, 2002), it seems 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. 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 might occur in skeletal muscle. In fact, besides being considered relatively resistant to long-term ischemia (Appell *et al.*, 1998), skeletal muscle has been also referred as one of the most adaptive tissues after prolonged periods of hypobaric-hypoxia exposure in humans and animals (for refs. see Cerretelli and Hoppeler, 1996; Hoppeler and Vogt, 2001; Fluck and Hoppeler, 2003; Howald and Hoppeler, 2003).

Besides the abovementioned general considerations regarding hypoxia-induced oxidative stress, new insights will be needed at distinct levels of cellular organization to understand the basic mechanisms responsible' for this apparently paradoxical phenomenon of hypoxia-induced oxidative stress and damage. Although several possible causes to explain the increased oxidative stress under hypoxic conditions have been advanced, (i) the presence of an inflammatory process resulting in increased leukocyte endothelium adherence via nitric oxide depletion and leukocyte emigration into perivascular space (Wood *et al.*, 1999, 2000) (ii) the enhanced xanthine oxidase (XO) activity probably due to cellular energetic and metabolic inefficiency and excessive calcium loading in hypoxic conditions (Hoshikawa *et al.*, 2001), and (iii) the higher levels of circulating epinephrine due to enhanced adrenal medullary secretion (Seals and Jones, 2001) increasing epinephrine spontaneous oxidation (Jones, 1985; Alessio, 2000) could be potential sources of RONS that might affect cellular *redox* equilibrium in several tissues under hypobaric hypoxia. However, the reduced oxygen availability to terminally accept electrons from oxidative phosphorylation resulting in the accumulation of reducing equivalents throughout the electron transport chain (ETC) – the so-called condition of reductive stress (Kehrer and Lund, 1994; Duranteau *et al.*, 1998) - might constitute a major and determinant physiological mechanism to elicit hypoxia-induced oxidative stress.

Mitochondria are powerhouse cellular organelles of eukaryotic cells where the energy required to drive the endergonic and vital biochemical processes of cell life is produced through a well-coupled mechanism of oxidative phosphorylation (Cadenas, 2004). Moreover, coupled with ATP synthesis, mitochondria are also critical organelles involved in the modulation of osmotic regulation, cell *redox* status and pH control, signal transduction, and in the establishment of cellular Ca^{2+} homeostasis (Wallace *et al.*, 1997). Nevertheless, mitochondria respiratory function has been considered a relevant mechanism involved in cellular oxygen toxicity under distinct conditions of depressed oxygen levels (Vanden Hoek *et*

al., 1998; Kulisz *et al.*, 2002). Indeed, reducing equivalents seem to accumulate throughout the mitochondria ETC due to an inability to transfer electrons to oxygen. This elicits the impairment of the respiratory chain activity, the increase in the so-called electron leakage to oxygen and the production of reactive oxygen species, and ultimately, of $ONOO^-$ (Dawson *et al.*, 1993; Kehrer and Lund, 1994; Chandel *et al.*, 1998; Duranteau *et al.*, 1998; Mohanraj *et al.*, 1998; Schild *et al.*, 2003b). Additionally, a decrease in V_{max} of cytochrome *c* oxidase during hypoxia also seems to contribute to an increase in the reductive state of mitochondrial electron carriers upstream of cytochrome aa_3 (Duranteau *et al.*, 1998) favoring electron leakage and increased univalent reduction of oxygen with formation of reactive oxygen species. Consequently, mitochondria themselves may become oxidative targets resulting in the peroxidation of there membrane lipids, protein oxidation and cleavage of mitochondrial DNA (Kowaltowski and Vercesi, 1999; Petrosillo *et al.*, 2001; Choksi *et al.*, 2004), which could culminate in the down-regulation of the respiratory function (Richter, 1997; Kokoszka *et al.*, 2001; Wei and Lee, 2002), impaired ATP generation (Arai *et al.*, 1999), mitochondrial calcium overload and, eventually, in cellular decay (Kokoszka *et al.*, 2001; Childs *et al.*, 2002; James and Murphy, 2002). In fact, some studies reported that under the synergistic effects of oxidative stress and deregulated cytosolic free Ca^{2+}, mitochondria can become severely dysfunctional (reviewed in Crompton, 2004). For example, decreased activity of some citric acid-cycle enzymes and/or ETC complexes through free radical-mediated protein oxidation (Yen *et al.*, 1999) or by RONS-induced inner membrane phosphoslipid peroxidation, including cardiolipin (Paradies *et al.*, 2000; Paradies *et al.*, 2004) seem to correlate well with decreased mitochondrial respiratory functionality. Moreover, depending on the magnitude of the insult, alterations in membrane permeability might result in important metabolic consequences, namely (i) the collapse of the mitochondrial transmembrane potential, (ii) the uncoupling of the respiratory chain, (iii) the hyperproducion of superoxide anions, (iv) the disturbance of mitochondrial biogenesis, (v) the outflow of matrix calcium and glutathione, (vi) the release of soluble intermembrane proteins, and (vii) a burst of mitochondrial oxygen consumption, among other effects. Finally, in a cellular dimension, the mitochondrial dysfunction might entail a bioenergetic catastrophe that could culminate in the disruption of plasma membrane integrity (necrosis) and/or in the activation of specific cysteine apoptogenic proteases (caspases), by mitochondrial proteins (cytochrome *c*, apoptosis-inducing factor) that leak into the cytosol through the outer membrane and trigger the intrinsic pathway of apoptosis (reviewed in Kroemer *et al.*, 1998; Hengartner, 2000). Considering the distinct abovementioned oxidative-mediated mechanisms responsible for mitochondria dysfunction, it could be argued that tissue oxidative stress induced by hypobaric hypoxia might also impair mitochondria functionality. In accordance, ultrastrutural data obtained from rat (Amicarelli *et al.*, 1999) and human (Hoppeler *et al.*, 2003) skeletal muscle exposed to chronic hypobaric hypoxia revealed significant mitochondria morphological changes, namely significant swelling and cristae degeneration, which have also been described in several other tissues (Kowaltowski *et al.*, 2000; Santos *et al.*, 2002; Schild *et al.*, 2003a) as being associated to abnormal mitochondrial functionality and to cellular death fate. However, presently, no hypobaric hypoxia experimental data can support this assumption.

As reported above, in addition to mitochondria, leukocyte-endothelial interactions (Wood *et al.*, 1999) and enhanced endothelial XO activity (Hoshikawa *et al.*, 2001) could also be considered important origin sites of RONS production in several tissues during

hypoxia exposure. In this sense, besides myocytes, RONS might also target endothelial cells compromising, consequently, its structural and biochemical features. Indeed, systemic enhanced oxidative stress induced by several pathologies, such as diabetes (Jakus, 2000) or cardiovascular diseases (reviewed in Brown and Hu, 2001) have been described as having aggressive and deleterious effects in capillary walls. Thus, due to their intrinsic hypoxic status-mediated XO activation (Hoshikawa *et al.*, 2001) and to a close vicinity relationship with such a pro-oxidant circulatory pool (Frei *et al.*, 1988; Houston *et al.*, 1999; Wood *et al.*, 1999), endothelial cells might be simultaneously oxidative source and target during sustained high-altitude insult. Moreover, plasma concentration of some substances related to coagulation and fibrinolytic systems are directly dependent from the level of endothelial cell function (Pearson, 1993). After chemical or mechanical stress, endothelial cells alter their functional pattern, characterized, among other events, by the exposition of cell adhesion molecules, by the reduction of prostacyclin and nitric oxide production and by the release of *tissue-type* plasminogen activator and plasminogen activator inhibitor type 1 to blood circulation (Pearson, 1993) with impact in leukocyte-endothelium interaction and in the regulation of coagulation and fibrinolytic systems. In fact, few studies dealing with acute hypobaric hypoxia in humans suggest a transient down-regulation of the fibrinolytic system (Bendz *et al.*, 2000; Mannucci *et al.*, 2002) that could be, at least in part, related to reactive oxygen species modulate hypoxia inducible factor–1 mediated plasminogen activator inhibitor-1 expression (Gorlach *et al.*, 2003). Accordingly, animal studies showed that systemic hypoxia modify endothelial cells physiology inducing a generalized and rapid microvascular inflammatory response characterized by increased RONS levels, leukocyte-endothelial adherence and emigration, and increased vascular permeability (reviewed in Gonzalez and Wood, 2001). In this sense, the question arises to whether sustained high-altitude chronic hypoxia-induced oxidative stress also induces microvascular damage and leads to endothelial morphologic and biochemical changes.

Additionally, another target of hypobaric hypoxia-induced oxidative stress could be the progenitor erythrocytes nucleated cells, located in bone narrow, which could modify the antioxidant features of newly formed erythrocytes. In fact, as a hallmark of the above referred collectively adaptive response to chronic hypoxia, bone narrow is severely stressed-out through erythropoietin-mediated regulation to increase erythropoiesis in an attempt to minimize the physiological impairment related to diminished tissue oxygen tension (Samaja, 2001). Indeed, an increased red cell mass allows more oxygen to be transported *per* liter of blood from lung to tissues at any given oxygen saturation counteracting the fall in arterial saturation and preserving, at least in part, blood oxygenation (Grover and Bartsch, 2001). Under normal conditions, reactive oxygen species are continuously generated in the erythrocyte, but well-developed antioxidant defense mechanisms usually prevent their deleterious effects on cellular components (Tamer *et al.*, 2004). However, during increased prolonged conditions of systemic RONS generation, such as those that presumably occur in chronic high-altitude exposure, red blood cells (RBC) might also be considered as potential oxidative stress targets. They transport high concentrations of a potentially pro-oxidant haem protein (haemoglobin) and oxygen inside a membrane rich in polyunsaturated fatty acid side-chains (Halliwell and Gutteridge, 1999), which is a prone metabolic condition to RONS interaction and to oxidative damage. Moreover, due to RBCs lack of DNA, they have limited molecular repair mechanisms, which compromise their ability to regenerate from

an oxidative deleterious insult against several cellular constituents. However, despite this limited adaptive response of mature erythrocytes, the sustained and systemic pro-oxidant *redox* status induced by hypoxia might also directly affect bone narrow erythrogenic cells contributing to the modulation of the newly formed erythrocytes. Being true, this condition would possibly allows the genesis of qualitatively different RBCs in order to cope with this aggressive systemic environment.

Taking into account the abovementioned scientific context concerning the hypothetical condition of enhanced *oxidative stress and damage induced by hypobaric hypoxia*, the main purpose of this dissertation was to analyze biochemical, functional and histological deleterious consequences at distinct levels of cellular organization, of acute or chronic hypobaric hypoxia exposure, under simulated or real high altitude conditions, in humans and mice, in blood and in skeletal muscle, and to associate those harmful effects with markers of oxidative stress.

This goal is supported by all the specific aims of the papers published on the compass of this dissertation that were, respectively:

Paper I

To analyze:

(i) the effects of acute and severe hypoxia exposure on mice skeletal muscle oxidative stress and oxidative damage markers;

(ii) the protective role of the antioxidant glutathione against oxidative damage;

(iii) the expression of muscle heat shock protein 70kDa (HSP70), induced by hypoxic insult, as a marker of *in-vivo* cellular stress.

Paper II

To investigate:

(i) whether or not the acute exposure of non-acclimatized humans to simulate acute, severe and short-term hypobaric hypoxia increases plasma oxidative stress and oxidative damage;

(ii) the influence of post-high-altitude reoxygenation period induced by pressurization until sea level conditions in plasma oxidative stress and oxidative damage biomarkers.

Paper III

To analyze:

(i) in mice *soleus* muscle the specific role of short-term acclimatization on the repercussions of acute and chronic hypoxia regarding oxidative stress and damage markers;

(ii) the effect of persistent continuous and severe hypoxia in those acclimatized animals;

(iii) the relationship between *in-vivo* acute and chronic altitude-hypoxia exposure and the expression of HSP70, in acclimatized and non-acclimatized animals.

Paper IV

To investigate:

(i) whether acute and severe hypobaric hypoxia induces mitochondrial oxidative damage and, thus, influence the qualitative features of mitochondrial functionality;

(ii) the potential protective effect afforded by Vitamin E against the hypothetical free radical-mediated mitochondrial dysfunction associated to this acute and severe hypoxic insult;

(iii) the consequence of acute and severe hypoxia on skeletal muscle cellular apoptotic fate.

Paper V

(i) To analyze whether or not a 4 weeks high-altitude expedition to a Himalayan peak (Pumori, 7161m) induces skeletal muscle ultrastructural and plasma biochemical changes (Plasminogen activator inhibitor type 1 and tissue-type plasminogen activator) suggestive of microvascular dysfunction.

Paper IV

(i) To analyze whether or not a 4 weeks high-altitude expedition to a Himalayan peak (Pumori, 7161m) induces plasma antioxidant defense changes as well as qualitative erythrocyte alterations (antioxidant enzyme activity, membrane fatty acid profile) in addition to the commonly used quantitative haematological variables.

Experimental Work

PAPER I

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

José Magalhães[1], António Ascensão[1], José M.C. Soares[1], Maria J. Neuparth[1,2], Rita Ferreira[1], José Oliveira[1], Francisco Amado[2], José A. Duarte[1]

[1] Department of Sport Biology
Faculty of Sport Sciences and
Physical Education
University of Porto
Portugal

[2] Department of Chemistry
University of Aveiro
University Campus of Santiago
Aveiro
Portugal

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Abstract

This study intended to analyze (i) the effects of acute and severe hypoxia exposure on skeletal muscle oxidative stress and oxidative damage markers, (ii) the protective role of the antioxidant glutathione against oxidative damage and (iii) the expression of heat shock protein 70kDa (HSP70) induced by this hypoxic insult. Forty mice were divided into four groups: control + placebo (C+P), hypoxia + placebo (H+P), control + L-buthionine–[*SL*]-sulfoximine (BSO, a GSH depleting compound) (C+BSO) and hypoxia + BSO (H+BSO). Hypoxia groups were continuously exposed for 24h to a hypobaric hypoxic environment equivalent to an altitude of 7000m 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-ß-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, 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

BSO, Glutathione, Hypobaric hypoxia, , HSP70, soleus muscle,

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 ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot -}$) and peroxinitrite ($ONOO^-$), 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 unbalance response of 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-hydroxyguanine (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 scavengers 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 exacerbate tissue damage inflicted by many stimulus (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 had used GSH as a marker of oxidative stress to study the influence of dietary supplementation of antioxidants during oxidative stress induced by hypoxia (Sarada *et al.*, 2002a; Sarada *et al.*, 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 or not 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; Sarada *et al.*, 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 (43kPa): (i) on oxidative stress parameters (oxidized and reduced glutathione), on some oxidative damage markers (TBARS, sulfhydryl protein groups - SH) and lysossomic enzyme activity (N-

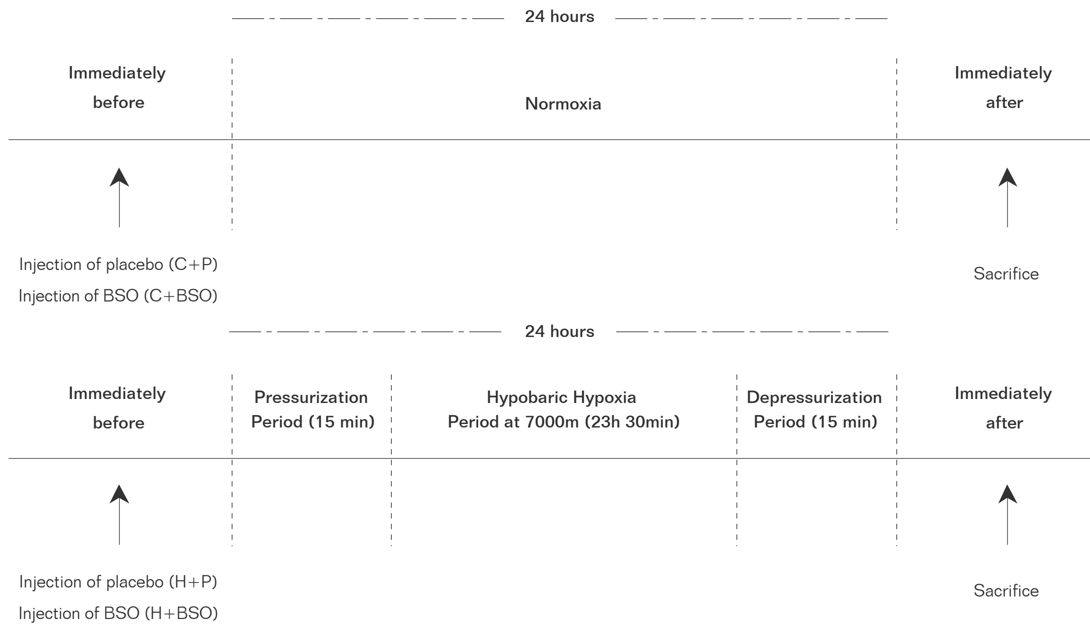

Fig. 1. Time course of the experimental protocol. C+P and C+BSO groups were injected with placebo and BSO, respectively, submitted to normoxia during 24 hours and sacrificed immediately after. H+P and H+BSO groups were injected with placebo and BSO, respectively, immediately submitted to hypobaric hypoxia equivalent to 7000 m during 24 hours and sacrificed immediately after.

Acetyl-ß-D-glucosaminidase - NAG), and (ii) 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 γ-glutamyl cycle, inducing reductions in cell glutathione content (Griffith, 1982; Leeuwenburgh and Ji, 1995). Additionally, and assuming that expression of heat shock proteins 70-kDa family (HSP70) is an inducible mechanism protecting proteins against cellular stress (for refs 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

Fourty CD1 Charles River mice (30-35 g) were randomly divided into four groups (n=10) for a 24 hours experimental design protocol. Two control groups were injected, respectively, with 0,4 ml of placebo saline solution (C+P group) and with a single 4mmol/kg dose of L-buthionine-*SR*-sulfoximine (C+BSO group) in 0,4ml 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 simulated atmospheric pressure of 43.2 kPa (324 mmHg) equivalent to an altitude of 7000m in an hypobaric chamber. The depressurization to reach the simulated altitude of 7000m and the pressurization until sea level conditions took 15 min (Fig.1). All the animals of vehicle and BSO treatment groups were intraperitonially (i.p.) injected 24 h before sacrifice and kept at a constant temperature (21-25ºC) on

a daily lighting schedule of 12 h of light vs. 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* muscle were excised and homogenized in tris (0.05M) – L-serine (0.03M) – borate (0.06M) buffer (pH. 7.6) in a motor-driven Potter-glass homogenizer at 0-4 ºC at low speed. The homogenized samples were separated into several aliquots and rapidly frozen at –80ºC 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 percloric acid at 5% (w/v).

Assays

TGSH, GSH and GSSG measurements were determined as previously described by Tietze (1969) by spectrophotometric techniques at 414nm. Lipid peroxidation on 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 correspondent to 10mg protein was resolved by SDS-PAGE (12.5% acrylamide gels of 1mm thickness) as described by Laemmli (1970) and electroblotted onto nitrocellulose membranes according to Locke et al. (1990). The immunoblotts were probed with 1:5 000 dilution of monoclonal anti-Hsp70 (Sigma) 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 immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine, 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 analysed 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 Bonferroni *post-hoc* test was used to compare groups. The Statistical Package for the Social Sciences (SPSS Inc. version 10.0) was used for all analysis. The significant level was set at 5%.

Results

Skeletal muscle glutathione contents are expressed in figures 2, 3, 4 and 5. There was a significant increase in GSSG ($p<0.05$), TGSH ($p<0.05$) and 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 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 BSO-treated group, which seems to determine the TGSH content and %GSSG rather

than GSSG increment.

The levels of muscle TBARS, SH groups and muscle NAG activity as indirect measures of lipid peroxidation, protein oxidation and lissosomic activity, respectively, are depicted in Table I. 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 among groups, although an 18% decrease was observed between C+P and H+P. Simulated altitude exposure *per se* induced a significant and markedly 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 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 in 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 slightly increase (24%) was observed with BSO treatment.

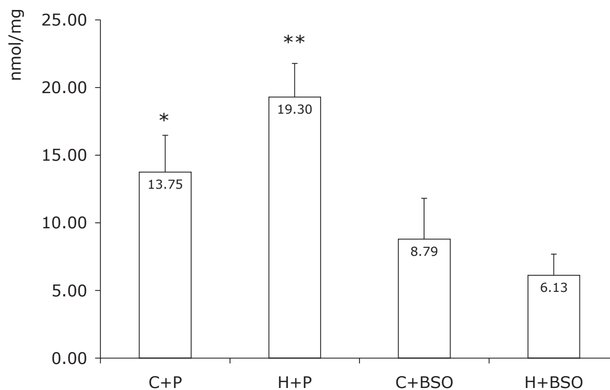

Fig. 2. Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000m and BSO treatment on *soleus* muscle total glutathione (TGSH). Values are mean±SEM (nmol/mg protein); *p<0.05, C+P vs H+P and C+BSO; **p<0.05, H+P vs H+BSO.

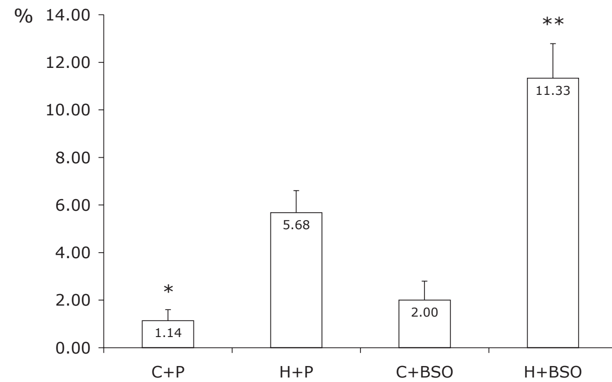

Fig. 3. Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000m and BSO treatment on *soleus* muscle GSSG/TGSH (%GSSG). Values are mean ± SEM (%); *p<0.05, C+P vs H+P; **p<0.05, H+P and C+BSO vs H+BSO.

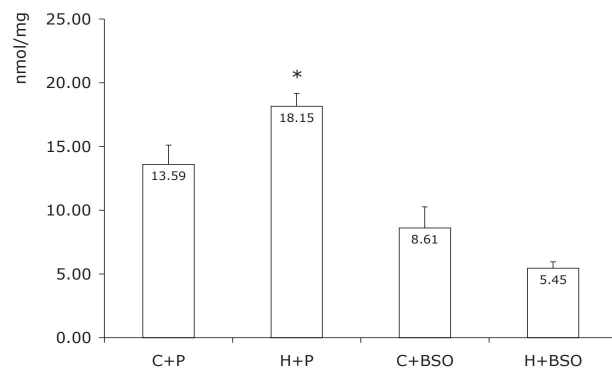

Fig. 4. Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000m and BSO treatment on *soleus* muscle reduced glutathione (GSH). Values are mean ± SEM (nmol/mg protein); *p<0.05, H+P vs H+BSO.

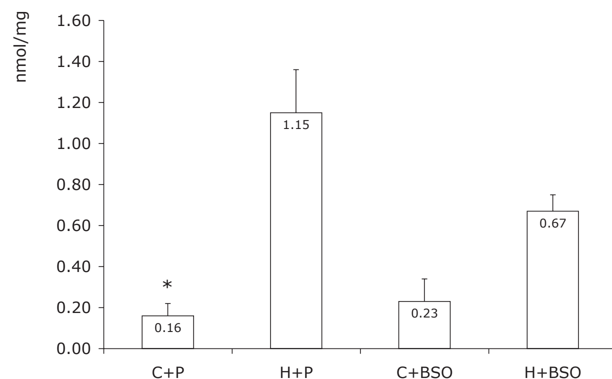

Fig. 5. Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000m and BSO treatment on *soleus* muscle oxidized glutathione (GSSG). Values are mean ± SEM (nmol/mg protein); *p<0.05, C+P vs H+P

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; Sarada *et al.*, 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 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).

Table I. Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and BSO treatment on *soleus* muscle tiobarbituric reactive substances (TBARS), protein SH content and N-Acetyl-ß-D-glucosaminidase (NAG) activity. All values are mean ± SEM.

	TBARS	-SH	NAG
	nM	mol/g prot	U/mg prot
C+P	227.91 ± 49.9 *	474.14 ± 48.3	3.35 ± 0.2 **
H+P	436.75 ± 51.0	391.47 ± 21.6	4.49 ± 0.3
C+BSO	411.83± 46.5	456.80 ± 29.8	3.22 ± 0.2
H+BSO	409.43 ± 35.1	433.86 ± 71.7	3.90 ± 0.3

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

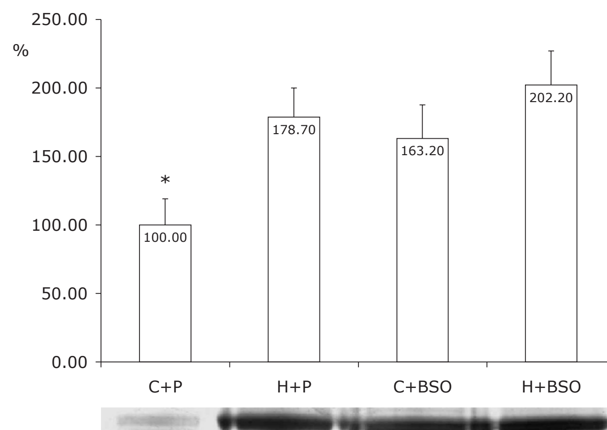

Fig. 6. Effect of acute hypobaric hypoxia exposure equivalent to an altitude of 7000 m and BSO treatment on *soleus* muscle HSP70 expression. Values are mean ± SEM (%). A scan of representative Western blot for each group is immediately below histogram. * $p<0.05$, C+P vs H+P, C+BSO and H+BSO.

In our study this hypothesis can be supported by the significant increase in TGSH content observed in H+P group. Despite the significant increased 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 changes in cell catabolism, namely activation of lysossomic function can be observed in Table I. The increased 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 increased significantly with hypoxia in rats exposed at 4000m for four 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 (i) GSH seems to be the most important electron donor under pro-oxidant redox conditions (Ji and Leeuwenburgh, 1996) and (ii) total protein represent 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 lysossomes (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 sufficiently 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 the 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 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 group compared to the H+P.

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 group compared to 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 oxida-

tion and lysossomic 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 to 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, the 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 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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PAPER II

Oxidative stress in humans during and after 4 hours of hypoxia at a simulated altitude of 5500 m

José Magalhães[1], António Ascensão[1], Ginés Viscor[2], José M.C. Soares[1], José Oliveira[1], Franklin Marques[3], José A. Duarte[1]

[1] Department of Sport Biology
Faculty of Sport Sciences and
Physical Education
University of Porto
Portugal

[3] Departament of Clinical Analysis
Faculty of Pharmacy
Institute for Molecular and Cell Biology
University of Porto
Portugal

[2] Department of Physiology
Faculty of Biology
University of Barcelona
Spain

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Abstract

Background: High-altitude hypoxia may induce oxidative stress in humans. However, the effect of acute, severe and non-acclimatized short-term hypobaric hypoxia exposure in humans has not been described. Additionally, little is known regarding the confounding role of reoxygenation in the extent of oxidative stress and damage markers in hypoxia. Our goals were to analyze the effect of hypobaric hypoxia and reoxygenation in plasma oxidative stress and oxidative damage. Methods: There were six male volunteers exposed to a simulated altitude of 5500m (52,52 kPa) in the INEFC-UB hypobaric chamber over 4 h and returned to sea level (SL) in 30 min. Data were collected at baseline sea level (SL), at 1h and 4h of hypoxia at 5500m and immediately after return to sea level (RSL). Results: Elevated scores of acute mountain sickness (13) and significant changes in arterial oxygen saturation (97.5±0.5; 53.3±1.9; 97.1±0.3%, $p<0.05$ at SL, 4h and RSL, respectively) were observed. Significant reductions ($p<0.05$) on total glutathione (TGSH) content were measured from SL and 1h vs. 4h and RSL. The percentage of oxidized glutathione (%GSSG) as an indicator of redox oxidative changes increased significantly (SL vs. 1h; 1h vs. 4h and RSL). Lipid peroxidation (TBARS), protein oxidation (SH protein groups) and total antioxidant status (TAS) followed the redox changes suggested by the glutathione system throughout the protocol. Conclusions: Hypobaric hypoxia increased the burden of plasma oxidative stress and damage markers all through the hypoxia period. However, no additional changes were observed with reoxygenation at the end of the reoxygenation period.

Keywords

Hypobaric hypoxia, reoxigenation, oxidative stress, plasma, humans

Introduction

High-altitude exposure has been considered a major cardiorespiratory, endocrine, metabolic, nutritional, thermal and psychological strain for human body. The atmospheric low oxygen partial pressure affects oxygen (O_2) cascade inducing metabolic adaptations, such as changes in cell oxidative metabolism. Recently, growing evidence of blood enhanced markers of lipid peroxidation and oxidative DNA damage have been reported in some chronic studies conducted in real high-mountains environments suggesting an apparently paradoxical effects of altitude hypoxia-induced oxidative stress (Pfeiffer *et al.*, 1999; Bailey *et al.*, 2000a; Simon-Schnass, 2000; Moller *et al.*, 2001). Additionally, some benefits of antioxidant supplementation has been found in athletes (Pfeiffer *et al.*, 1999) and climbers (Bailey *et al.*, 2000a; Simon-Schnass, 2000) "oxidatively stressed out" (Bailey *et al.*, 2000a) during long sojourns at altitude. Nevertheless, during high-altitude field-based studies, oxidative stress cannot be easily ascribed to only one specific environmental factor such as hypoxia. In those conditions it is difficult to isolate the several factors that could contribute to enhanced free radical production. Besides hypoxia, other factors like UV radiation, intense physical activity and acute temperature shifts may be considered as potential sources of pro-oxidant formation leading to increased oxidative stress in high-altitude conditions (Simon-Schnass, 2000). To dissociate hypobaric hypoxia from other environmental oxidative stressors some acute, chronic or intermittent hypoxia exposure studies have been carried out with animals in hypobaric chambers simulating high-altitude hypoxia conditions (Chang *et al.*, 1989; Sarada *et al.*, 2002). Regarding human experiments, Bailey et al. (2001a) examined the independent effect of acute moderate normobaric hypoxia and of physical exercise on metabolic indices of lipid peroxidation in physically active males. Likewise, the effect of progressive, prolonged and severe simulated hypobaric hypoxia exposure on lipid peroxidation and antioxidant defense systems was investigated in humans during Operation Everest III (Joanny *et al.*, 2001). However, to our knowledge, no data have been published concerning the effect of acute, severe and non-acclimatized short-term simulated high-altitude hypobaric hypoxia exposure on human blood oxidative stress and oxidative damage markers. Furthermore, despite a remarkable amount of scientific information regarding physiological and pathophysiological responses to intermittent hypoxia exposure (for refs see Neubauer, 2001), the influence of *in vivo* post-hypoxic reoxygenation induced by pressurization until sea level conditions on oxidative stress is not well understood and, to our knowledge, has not been previously demonstrated in humans after acute and severe high-altitude hypoxic insult. Indeed, like in ischemia/reperfusion (I/R) (Kadambi and Skalak, 2000), during post-hypoxic reoxygenation a similar phenomenon might occur with the sudden wide modification in tissue oxygenation. Therefore, it could be hypothesized that additional oxidative stress and damage are expected in the reoxygenation period after a hypoxic high-altitude stimulus. If so, the additional post-hypoxic oxidative stress induced by the reoxygenation might exacerbate the real extent of oxidative stress in hypoxia.

Thus, the aim of the present study was to investigate whether or not the acute exposure of non-acclimatized humans to simulate acute, severe and short-term hypobaric hypoxia increases plasma oxidative stress and oxidative damage. In addition, to testing the above hypothesis, another purpose of the study was to analyze the influence of the post-high-altitude reoxygenation period induced by pressurization on plasma oxidative stress and oxidative damage biomarkers. For those purposes several plasma biochemical parameters were measured, namely oxidized, reduced glutathione and total antioxidant status (as markers of oxidative stress), thiobarbituric acid reactive substances and sulfhydryl protein groups (as markers of lipid peroxidation and protein oxidation, respectively). Simultaneously, clinical (acute mountain sickness symptoms) and physiological (HR and arterial blood

oxygen saturation) parameters were measured to confirm the intensity of hypoxic stress insult experienced by the subjects.

Material and methods

Experimental Design

There were six male non-smoker volunteers (age: 36.1 ± 2.0 yrs; weight: 74.0 ± 2.3 kg; height: 173.6 ± 1.6 cm; fat mass: 14.6 ± 0.9 %) sea-level residents took part in this study. No women participated in the protocol since gender differences in oxidative stress responses have been reported elsewhere (Ginsburg *et al.*, 2001). All the subjects were sedentary and had not experience high-altitude conditions within a period of, at least, 6 mo. All the experimental procedures and the possible risks involved in this study were explained to the subjects whose written consent was obtained. The study protocol was approved in advance by the Ethics Committee of the Scientific Board of Faculty of Sport Sciences, University of Porto and was designed in accordance to the recommendations of the Declaration of Helsinki. The study was conducted in the hypobaric chamber of the Universitat de Barcelona at the Institut Nacional d'Educació Física de Catalunya located at sea level in Barcelona, Spain.

The six subjects were exposed to 4 h to acute hypobaric hypoxia conditions at an atmospheric pressure of 52.52 kPa equivalent to an altitude of 5500m. During this period the subjects were allowed to develop the normal activities feasible to confinement in a steel pressure chamber. The depressurization period from sea level to 52.52 kPa was reached in 15 min and the pressurization period back to sea level conditions following the 4 h of hypobaric hypoxia took 30 minutes. During all the experimental protocol food and fluid ingestion was allowed *ad libitum*. Inside the chamber and after 4 h of hypoxia exposure at simulated altitude of 5500m all the subjects were scored for acute mountain sickness (AMS) according to the Lake Louise Consensus scoring system (Roach *et al.*, 1993). The presence of AMS was defined as a cumulative score of >5 points for the total Lake Louise score (self-report questionnaire, clinical assessment and functional score). However, item 5 from self-report (sleeping difficulty) was not considered since the subjects did not sleep during the 4h and 45 minutes of chamber confinement. A physician undertook the clinical assessment (CA) and the functional score inside the chamber after the subjects had answered the self-report (SR) questionnaire.

Antioxidant vitamins were not allowed 4 weeks prior to protocol and subjects were instructed to maintain their normal dietary and routine daily-life habits for 2 weeks before the experimental protocol. Moreover, participants were asked to abstain from taking any prophylactic AMS medication.

Blood sampling and preparations

Blood samples (5mL) were withdrawn four times during the experimental protocol from the antecubital vein. The first blood sample was drawn at sea level conditions immediately before the subjects were confined into the hypobaric chamber. The next two blood samples were taken inside the chamber after 1 and 4 h of exposure at 5500m, and the last one was collected outside the chamber immediately after returning to sea level conditions. During blood drawning, no tourniquet constriction was used in order to minimize potentially enhanced oxidative stress induced by an ischaemia-reperfusion maneuver. All the venous blood samples were taken using plastic syringes, placed in lithium heparinized tubes and immediately centrifuged for 5 minutes at 4000 rpm. The plasma was separated into several aliquots and rapidly frozen at –80ºC for later biochemical analysis of reduced (GSH) and oxidized (GSSG) glutathione, total antioxidant status (TAS), thiobarbituric acid reactive substances (TBARS) and sulfhydryl protein groups (SH). The aliquots for glutathione assay were previously extracted in perchloric acid at 5% (w/v).

Cardiovascular and Hematological Assays

HR was measured at rest with a downloadable heart rate monitor (Polar Vantage, NV) recording every 15 s during three intervals of 15 min: (i) immediately before the subjects got into the chamber, (ii) before the pressurization phase (at 3.45h-4h of hypoxia at 5500m) and (iii) at sea level after the reoxygenation period. SaO_2 was measured at rest before the subjects entered in the hypobaric chamber, after 4 h of hypoxia exposure at 5500m and immediately after returning to sea level conditions using a fingertip pulsoximeter (Pulsox 5P, Minolta, Japan) on the left index finger.

Biochemical Assays

GSH and GSSG measurements were determined in plasma using spectrophotometric techniques at 414nm as previously described by Tietze (1969). TAS was determined spectrophotometrically at 600 nm with a commercial kit (Randox - cat nº NX2332). Lipid peroxidation was assayed spectrophotometrically according to the method described by Bertholf et al. (1987) 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 (1990). Protein content was assayed spectrophotometrically using bovine serum albumin as standard, according to Lowry et al. (Lowry *et al.*, 1951).

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. The ANOVA for repeated measures was used to test differences within different moments of the experimental protocol. The Pearson correlation coefficient was used to analyze the correlation between SaO_2 and markers of oxidative stress and damage at SL and 4h of hypoxia exposure and between 4h and the RSL. The significant level was set at 5%.

Results

AMS symptoms were obtained after 4 h of hypobaric hypoxia exposure at 5500m with all the subjects having severity scores ranging from 11-18 in the total AMS Lake Louise scoring system (Table I). As depicted in Table II, HR increased significantly from SL until 4h of hypobaric hypoxia and significantly returned toward baseline after the reoxygenation period (RSL). SaO_2 after 4h of simulated hypobaric hypoxia equivalent to an altitude of 5500m was markedly decreased ($p<0.01$) from 97.5±0.5% to 53.3±1.9%.

Table I. Effect of hypoxia exposure on individual Lake Louise Consensus Acute Mountain Sickness (AMS) scores. Results were scored inside the hypobaric chamber after 4h of hypoxia exposure at simulated altitude of 5500m.

Subjects	SR * Score	CA‡ Score	Functional Score	AMS Score
S1	7	4	2	13
S2	6	3	2	11
S3	7	4	2	13
S4	6	4	2	12
S5	10	5	3	18
S6	7	4	2	13
m	7.16	4.00	2.16	13.33
SEM	0.60	0.25	0.16	0.98

* Self Report, ‡ Clinical Assessment
Note: Results were scored inside the hypobaric chamber after 4 h of hypoxia exposure at simulated altitude of 5500m.

Immediately after the reoxygenation period SaO_2 rose again until it reached 97.1±0.3%.
The total plasma glutathione (TGSH) content and percentage of GSSG expressed as the ratio between oxidized form and total glutathione content (GSSG/TGSH) can be observed in Table II. TGSH content at 1h of hypoxia did not differ significantly from the control values at sea level. However, a significant decrease in TGSH content, compared with SL and 1h of hypoxia, was observed after 4h of hypoxia exposure at 5500 m and remained low after RSL. At sea level conditions, the GSSG/TGSH ratio was 9.17%. After 1h of hypoxia exposure this parameter

Table II. Effect of hypoxia exposure and reoxygenation on heart rate (HR), arterial oxygen saturation (SaO2), plasma total (TGSH), reduced (GSH) and oxidized (GSSG) glutathione, GSSG/TGSH ratio (%GSSG), thiobarbituric reactive substances (TBARS), on protein SH content, total antioxidant status (TAS) and total protein content. Values are mean ± SEM of the six subjects at each time point.

Variables	SL	1h	4h	RSL
HR (bpm)	66 ± 2,9 [a]	NA	106 ± 5,2 [b]	71,4 ± 2,6
SaO_2 (%)	97,5 ± 0,5	NA	53,3 ± 1,9 [c]	97,1 ± 0,3
TGSH (nmol/mg prot)	0,200 ± 0,01 [d]	0,203 ± 0,005 [e]	0,160 ± 0,007	0,153 ± 0,018
GSH (nmol/mg prot)	0,181 ± 0,007 [d]	0,157 ± 0,005 [e]	0,103 ± 0,005	0,100 ± 0,003
GSSG (nmol/mg prot)	0,019 ± 0,004 [f]	0,046 ± 0,004	0,057 ± 0,009	0,053 ± 0,003
%GSSG	9,17 ± 0,81 [f]	22,4 ± 2,13 [e]	34,57 ± 2,11	35,74 ± 1,21
TAS (mmol/L)	1,147 ± 0,02 [f]	1,081 ± 0,02	1,043 ± 0,01	1,053 ± 0,02
TBARS ($M*10^6$)	7,87 ± 1,3 [a]	10,71 ± 1,95	11,83 ± 0,41	10,04 ± 1,47
SH (mol/g prot)	9,08 ± 0,49 [d]	8,98 ± 0,49 [b]	7,71 ± 0,39	7,24 ± 0,17
Protein (mg/ml)	57,73 ± 2,29 [d]	59,53 ± 2,77 [e]	67,92 ± 2,85	67,84 ± 3,85

SL - Sea level; 1h - after 1 hour of hypoxia at 5500m; 4h - after 4 hours of hypoxia at 5500m; RSL - immediately after return to sea level; NA - not available.
($p<0,05$) [a] SL vs 4h; [b] 1h vs RSL; [d] SL vs 4h and RSL; [e] 1h vs 4h and RSL; [f] SL vs 1h, 4h and RSL
($p<0.01$) [c] 4h vs SL and RSL

increased significantly to 22.4% and after 4h at 5500m had markedly risen to 34.58%. Following RSL, the GSSG/TGSH ratio remained elevated showing a non-significant increase to 35.74%. GSSG/TGSH ratio at 4h and RSL were significantly different from the values obtained at sea level and after 1h of hypoxia exposure.

Data concerning GSH, GSSG and TAS plasma content during the experimental protocol are presented in Table II. The effect of hypoxia and RSL on GSH was similar to that obtained for TGSH. Plasma GSSG content was significantly higher after 1h and 4h of simulated hypobaric hypoxia exposure and following reoxygenation induced by RSL. However, changes in GSSG content failed to prove significant differences between 4h and RSL. Regarding TAS, our data showed a significant decrease throughout the hypoxia period (1h and 4h) compared to baseline sea level conditions. The reoxygenation caused by the pressurization period did not induce additional changes in TAS when compared with 4h.

Lipid peroxidation levels measured by the formation of TBARS are given in table II. TBARS enhanced significantly from SL to 4h and was maintained with no significant further increase with reoxygenation after RSL. With respect to plasma protein SH content (Table II) a slight and non-significant decrease after 1h of hypoxia could be observed. However, a significant decrease was found between SL control content and the values obtained after 4h and following the reoxygenation period (RSL). Despite a significant decrease in protein SH content observed between 1h of hypoxia exposure and the RSL, no additional oxidative protein modification was found with reoxygenation when compared with the values obtained after 4h of hypoxia at 5500m.

Regarding total protein content (Table II) a significant increase was observed after 4h (17.6%)

of hypoxia exposure and at RSL (17.5%) compared with SL control values. Compared with plasma total protein content at 1h, a significant increase was also found after 4h and at RSL. However, no significant difference was found in total protein content with reoxygenation when compared with the values obtained after 4h of hypoxia. The range of GSH changes (-13% at 1h, -43% at 4h and -44.7% at RSL) as well as GSSG (142% at 1h, 200% at 4h and 178% at RSL) when compared with SL exceed those of protein content, which suggests the absence of a cause-effect relationship between the variations of these two parameters.

Regarding the hypoxic period (SL and 4h) SaO_2 correlated significantly with %GSSG ($r = -0.85$, $p<0.001$) and with TBARS ($r = -0.66$, $p<0.05$). Despite being non-significant, the correlation between SaO_2 and protein SH groups is close to the significant level ($r = 0.54$, $p=0.06$). Concerning the reoxygenation period (4h and RSL) no significant correlations were found between SaO_2 and %GSSG ($r = 0.05$, $p=0.87$), TBARS ($r = -0.25$, $p=0.43$) and SH content ($r = -0.34$, $p=0.45$).

Discussion

Acute hypobaric hypoxia

To our knowledge this is the first *in vivo* study regarding plasma oxidative stress and oxidative damage markers in humans exposed to hypoxic stress induced by acute, severe and non-acclimatized short-term simulated high-altitude exposure.

A remarkable decrease in SaO_2 to dramatic low values were observed in our study during the acute hypobaric hypoxic insult (table II) suggesting that the participants were exposed to a considerably high systemic and tissue hypoxic stress. A plausible explanation for these lower values compared with findings of other outdoor (Tannheimer *et al.*, 2002) and hypobaric chamber high-altitude protocols (Schoene *et al.*, 1990), was certainly the absence of an acclimatization period. In fact, this process invokes marked hematological and cardiorespiratoy responses in order to increase SaO_2 and minimize hypoxic stress. Accordingly, with this severe arterial oxygen desaturation after 4 h of exposure at 5500 m, elevated rest HR probably resulted from a compensatory protective mechanism against decreased oxygen flux to tissues through adrenergic activation (Ward *et al.*, 2000). The severity of this hypoxic insult was also confirmed by the high extent of the AMS symptoms since all the subjects had scores above 11 in a maximal of 15 points. These exacerbated AMS scores observed in our subjects were clearly above the usual AMSs reported in other studies during high-altitude expeditions (Bailey *et al.*, 2000b) and acclimatized hypobaric chamber exposures (Nicolas *et al.*, 1999) and were probably due, as referred above, to the severity of the hypoxic stress protocol aggravated by the absence of a previous acclimatization period. Moreover, because accumulated evidence suggests that, at least in part, enhanced free radical production may contribute to the complex pathophysiology of AMS (Bailey *et al.*, 2001b), elevated AMS scores obtained in our study may be partly explained by the increased oxidative stress. In fact, a field study with trekkers in Nepal at altitudes up to 5400 m (Roncin *et al.*, 1996) reported a marked effectiveness of *Gingko biloba* extract (Egb761), a well documented oxygen-derived free radical-quenching (for refs see Roach and Hackett, 2001), in preventing AMS. The authors reported that none of the Egb761 treated subjects developed AMS compared with 41% in the placebo-treated group, which could be, at least in part, explained by the possible role of this antioxidant in preventing endothelial damage that could cause blood-brain barrier opening in AMS pathophysiology (Roach and Hackett, 2001). This conjecture is consistent with our data regarding oxidative stress and oxidative damage markers. Indeed, in accordance with other studies (Wood *et al.*, 1999; Joanny *et al.*, 2001), evidence of augmented reactive oxygen species production and increased oxidative stress during the hypoxic exposure seem to be pointed out by the founded correlations, emphasizing a relationship between the individual hypoxic stress and the magnitude of the oxidative stress markers.

Despite apparently being a paradoxical physiological condition, mitochondria reductive stress (Duranteau *et al.*, 1998), leukocyte-endothelial adherence via nitric oxide depletion (Wood *et al.*, 1999) and enhanced xanthine oxidase (XO) activity (Hoshikawa *et al.*, 2001) could be potent sources of ROS production and inducible-oxidative stress in several tissues during hypoxia that could, at least in part, explain our results. Moreover, in hypobaric hypoxia, higher levels of circulating epinephrine due to increased adrenal medullar secretion, probably increased epinephrine spontaneous oxidation generating superoxide radical and thus contributing to elicit oxidative stress (Jones, 1985).

Plasma glutathione content has been widely used to evaluate the levels of oxidative stress in humans (Halliwell and Gutteridge, 1999) and animals (Chang *et al.*, 1989). Besides acting as an electron donor to neutralize hydrogen peroxide (H_2O_2) and lipoperoxide, GSH is also a scavenger of oxygen and nitrogen based free radicals (Halliwell and Gutteridge, 1999). Considering that %GSSG is a sensitive indicator of enhanced oxidative stress closely related to brisk cellular redox changes, the significant increase from SL to 1h and 4h seems to demonstrate additional free radical production and the overwhelming of antioxidant capacity in the body induced by acute and severe hypoxia in our non-acclimatized subjects. Concerning the increase in %GSSG observed at 1h, our data suggest that during this initial hypoxic stress period, despite tissues' GSH oxidation and compensatory GSH importation from plasma to protect cells from enhanced free radical production, hepatic exportation of GSH to circulation was sufficient to maintain its concentrations and consequently plasma TGSH almost unchangeable. In this sense, increased %GSSG at 1h was mostly related to GSSG exportation from the tissues, due to its toxic effect and destabilizing action on cell redox status when present at high concentrations (Sies, 1985). At 4h, the rise in %GSSG might be explained both by a marked reduction in plasma GSH content and by the significant increase in plasma GSSG. Indeed, continuous exposure to hypobaric hypoxia-induced tissue oxidative stress, besides a probable aggravated increase in tissue GSSG exportation (Sies, 1985), seem to led to an incapacity of the liver for keeping up sufficient GSH exportation to maintain plasma levels and consequently plasma redox status. In accordance, the total hepatic non-protein-bound sulfhydryl content, which is largely GSH, was found to be decreased 25% in rats exposed to 7% of O_2 (Jaeger and Cote, 1982). These data are consistent with the results provided by TAS analysis during the hypoxia period. In fact, as an integrated parameter that considers the cumulative status of all the different antioxidants present in plasma and provide an insight into the delicate *in vivo* balance between oxidants and antioxidants (Ghiselli *et al.*, 2000), the decrease in TAS throughout the hypoxia period (1h and 4h) correlates well with the kinetics of the glutathione system, and reinforces the idea of an exacerbated oxidative stress during this severe hypoxia insult.

Our data regarding oxidative stress are in accordance with findings in Operation Everest III (Joanny *et al.*, 2001) concerning the impact of hypoxia-induced oxidative stress in the glutathione system, despite some important methodological differences such as the duration and severity of the hypoxia exposure. In our study, the hypoxic stress led to an imbalance between antioxidant capacity and oxidant production that could also be testified by some biomarkers of oxidative damage. In fact, increased TBARS content, as well as, decreased SH protein groups (Inayama *et al.*, 2002) content are commonly used as markers of an ongoing pathophysiological process associated to oxidative stress. Concerning TBARS and SH content, our data showed that lipid peroxidation and protein oxidation follows an incremental and degenerative course related to time of hypoxia exposure. Compared with sea level normoxic conditions, TBARS and SH content were significantly different only after 4h of hypoxic stress at 5500m (SL vs 4h; $p<0.05$). These results suggest an increased time-dependent cumulative peroxidative modification and protein oxidation in hypoxia-mediated oxidative stress. In fact, plasma TBARS content is the result of a degenerative hydroperoxides accumulation, both from endothelial

and circulating blood cell and from tissues leakage, which is a long term turnover-dependent process related to its synthesis/degradation ratio (Halliwell and Gutteridge, 1999). The increase in these by-products of lipid peroxidation is in accordance with some previous results in human plasma (Bailey *et al.*, 2001a; Joanny *et al.*, 2001) and in animal tissues submitted to hypoxia (Sarada *et al.*, 2002) and reflect the high susceptibility of polyunsaturated fatty acids to peroxidative modification during increased oxidant production (Halliwell and Gutteridge, 1999).

Concerning protein modification, some data suggest that redox status of sulfhydryl groups seems to be related to that of low-molecular-weight thiols, such as GSH (Inayama *et al.*, 2002). Hence, increased GSH exportation by the liver in order to maintain plasma redox status might, at least in part, explain the preservation of plasma SH groups at 1h. After 4h of hypoxia exposure at 5500m, a failure in the liver's exportation capacity to preserve plasma GSH content, might justify the increased oxidation of plasma protein thiols (Jones, 1985). Since protein-bound sulfhydryl groups are highly unstable and likely to be oxidized under oxidative stress (Inayama *et al.*, 2002), a decrease in SH protein groups has also been reported in other studies dealing with different oxidative stress-inducible models such as exercise (Inayama *et al.*, 2002).

Reoxygenation induced by the return to sea level (RSL)

Despite these and other evidence suggesting that systemic physiological hypoxia induced by simulated high-altitude exposure might exacerbate free radical production, very little is known concerning the influence of post hypoxic *in vivo* reoxygenation in humans. In classic ischaemia/reperfusion (I/R) models, the literature points out that oxidative stress and cellular injury is dramatically aggravated during reperfusion (reoxygenation). However, our results, in opposition to that model showed that reoxygenation induced by post-systemic hypoxia seems to maintain, at least in plasma, oxidative stress and oxidative damage levels, suggesting fundamental differences in the mechanisms responsible for redox status during situations of physiological hypoxia-reoxygenation vs. I-R. This statement can be supported by the absence of significant correlations between SaO_2, oxidative stress and damage markers during the reoxygenation period. In fact, after RSL, despite the SaO_2 recovery, plasmatic glutathione levels (%GSSG, TGSH, GSH and GSSG), were maintained compared with 4h, suggesting the absence of additional oxidative stress during the reoxygenation period. A reasonable explanation might be that in systemic physiological hypoxia, in contrast with ischemia, hypoxanthine content resulting from the breakdown of adenine nucleotides due to oxidative phosphorylation is not sufficiently high to exacerbate the pro-oxidant activity of XO during the reoxygenation period inducing enhanced oxidative stress. Moreover, Kadambi and Skalak (2000) suggested that in skeletal muscle ischemia-reperfusion injury, conversion of xanthine dehydrogenase in the pro-oxidant oxidase form is dependent to the severity of the ischemic period. In this sense, despite some data reporting increased XO activity even during the hypoxic period (Hoshikawa *et al.*, 2001), perhaps systemic physiological hypoxia is not a sufficiently severe enough hypoxic stress to induce an exacerbated response of XO activity during the post-hypoxic reoxygenation period. Accordingly, in our physiological hypoxia/reoxygenation model, a pro-oxidant production imbalance between the hypoxia mechanisms of ROS production, mostly mitochondria reductive stress (Duranteau *et al.*, 1998), and those related to reoxygenation, mainly XO activity (Kadambi and Skalak, 2000) could favored the former resulting in the lack of additional free radical production during the reoxygenation period. In fact, Wood *et al* (1999) in a study conducted with rats submitted *in vivo* to a protocol of normobaric hypoxia (10% O_2) vs normoxic recovery period in order to examine changes in ROS generation in the mesenteric microcirculation found a ROS production pattern clearly distinct from the observed during I/R. Indeed, using measurement of dihydrorhodamine 123 fluorescence signals, Wood et al (1999) showed that ROS generation increased significantly during

hypoxia when a physiological O_2 delivery deficit to the tissues was imposed but did not increased further, it even decreased, during the recovery reoxygenation period. Since in our study, blood samples related to the reoxygenation period were collected immediately after the subjects returned to SL conditions, our biochemical parameters were probably not sufficiently time-sensitive to detect redox status changes in such a short time period and, thus, no decreased oxidative stress was observed. Nevertheless, our results are in accordance with the study conducted by Joanny et al (2001) in which GSH/GSSG erythrocytes percentage, as a marker of oxidative stress, was similar after returning to sea level compared with the values obtained during extreme hypoxia (8848 m). Plasma TAS after RSL, once more showed a clear relationship with the glutathione redox status. This parameter suggests that arterial blood oxygen re-saturation did not induce enough free radical production to further overwhelm antioxidant status and confirms the absence of additional oxidative stress after the reoxygenation period.

Concerning oxidative damage, lipid peroxidation and protein oxidation follow the same trend as oxidative stress. Like in hypoxia, TBARS and SH content, compared with 4h, was maintained after the reoxygenation period reinforcing the straight physiological relationship between plasma oxidative stress and oxidative damage biomarkers.

In summary, our results suggest that in high altitude-hypoxic stress conditions, the burden of plasma oxidative stress and oxidative damage increases during the time spent at altitude. However, in clear contrast with our hypothesis and with some other authors suggestions (Neubauer, 2001), a thirty minutes period of reoxygenation after severe physiological hypobaric hypoxia insult does not seem to increase further oxidative stress or oxidative damage induced by previous simulated altitude exposure, at least at the end of the reoxygenation period.

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PAPER III

Acute and chronic exposition of mice to severe hypoxia: the role of acclimatization against skeletal muscle oxidative stress

José Magalhães[1], António Ascensão[1], José M.C. Soares[1], Rita Ferreira[1], Maria J. Neuparth[1,2], José Oliveira[1], Francisco Amado[2], Franklim Marques[3], José A. Duarte[1]

[1] Department of Sport Biology
Faculty of Sport Sciences and
Physical Education
University of Porto
Portugal

[2] Department of Chemistry,
University of Aveiro
Portugal

[3] Departament of Clinical Analysis
Faculty of Pharmacy
Institute for Molecular and Cell Biology
University of Porto
Portugal

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original fac-simile on appendix 3

Abstract

The role of acclimatization and the effect of persistent severe hypoxia (7000m) 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 (1D) was acutely exposed to a simulated altitude of 7000m in a hypobaric chamber for 1 day. Another experimental group (ACCL+1D) was exposed to a 3 days acclimatization period plus 1 day of hypoxia exposure at 7000m. The third experimental group (ACCL+8D) was exposed to the same acclimatization protocol, remaining 8 subsequent days at 7000m. The fourth experimental group (8D) was chronically exposed without acclimatization. ACCL+1D showed a significant decrease ($p<0.05$) in oxidative stress and damage compared to the 1D group. Concerning chronic severe hypoxia, acclimatization was truly vital, since 8D animals died after 5 days of exposure. Oxidative stress and damage markers in ACCL+8D tended to gradually increase throughout the 8 days of hypoxic period. Total SOD activity did not change in 1D compared to control however, it increased significantly ($p<0.05$) in ACCL+1D and ACCL+8D. 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 gradual increased oxidative deleterious effect in skeletal muscle seems to occur during persistent severe hypoxia even after a previous acclimatization period.

Keywords
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 (West, 1996). 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 (Singh *et al.*, 2001) and humans (Bailey *et al.*, 2000) in simulated high-altitude conditions. However, during gradual exposure to high-altitude, a phenomenon of altitude/hypoxia severity-dependent pro-oxidant production (Joanny *et al.*, 2001) may be involved in some physiological adaptations that take place in response to hypoxia including ROS antioxidant defense (Askew, 2002). In fact, low concentration of ROS has been reported to act in the regulation of gene expression through the activation of certain transcription factors that are dependent on the cellular redox (Halliwell and Gutteridge, 1999). For example, ROS generated by mitochondria during brief hypoxia seem to be a triggering stimulus to initiate preconditioning protection in cardiomyocytes (Vanden Hoek *et al.*, 1998; Kulisz *et al.*, 2002). Moreover, intermittent (Zhong *et al.*, 2000) and continuous (Wen *et al.*, 2002) chronic altitude-hypoxia exposure have been shown to respectively increase resistance and survival of rats from cardiac ischaemic injury by protecting 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 (Joanny *et al.*, 2001) or climbers were "oxidatively stressed out" (Bailey *et al.*, 2000) during long sojourns at altitude (Bailey *et al.*, 2000; Simon-Schnass, 2000). 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 exacerbate conditions of oxidative stress. Indeed, some benefits of antioxidant supplementation regarding oxidative damage have been found in athletes (Pfeiffer *et al.*, 1999) and animals (Sarada *et al.*, 2002) continuous 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 Cerretelli and Hoppeler, 1996). On the other hand, skeletal muscle oxidative stress and damage from animal studies of Sarada *et al*. (2002) and Singh *et al*. (2001) were induced by a daily severe hypobaric hypoxia exposure interspersed with a normoxia period (6 hours of hypoxia up to 7,500m 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 (Kohin *et al.*, 2001; Laclau *et al.*, 2001), acclimatization may confer some protection against oxidative stress, attenuating skeletal muscle harmful effects (Kohin *et al.*, 2001). On the other hand, considering the organic deterioration and the reduced food intake often described after some time spent at extreme altitude (West, 1996), 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 (i) the specific role of short-term acclimatization on the repercussions of acute and chronic hypoxia and (ii) 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-ß-D-glucosaminidase - NAG) were measured. Additionally, since expression of heat shock proteins of 70-kDa family (HSP70) as been considered as an inducible mechanism protecting proteins against cellular stress (for refs see Powers *et al.*, 2001), 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

Experimental Design

Forty CD1 Charles River mice (10-12 weeks) were randomly divided into one normobaric-normoxic control group (C; 39.23±0.7g) and four hypobaric-hypoxic experimental groups (n=8). One experimental group (1D; 40.37±0.74g) was acutely exposed to a simulated atmospheric pressure of 43.2 kPa (324 mmHg) equivalent to an altitude of 7000m in a hypobaric chamber during 1 day. The depressurization period to reach the simulated altitude of 7000m took 14 minutes, i.e. 500m/min. Another experimental group, defined as acclimatization group (ACCL+1D; 40.12±0.52g), was exposed to a short acclimatization period of 3 days (following the first day of hypoxia exposure at 4000m, altitude was incremented by 1000m/day until 7000m) plus 1 day of severe hypoxia exposure at 7000m. The third experimental group (ACCL+8D; 41.24±0.42g) was exposed to the same short acclimatization conditions, however it remained in severe hypobaric hypoxia at 7000m continuously for 8 subsequent days. The fourth experimental group (8D; 41.13±0.68g) was also chronically exposed, however it was not submitted to an acclimatization protocol, but it was acutely exposed to 7000m 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 (21-25ºC) 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 weighted using a Kern 870 electronic scale to the nearest 0.00001 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* muscle were excised and homogenized in tris (0.05M) – L-serine (0.03M) – borate (0.06M) buffer (pH. 7.6) in a motor-driven Potter-glass homogenizer at 0-4 ºC at low speed. The

homogenized samples were separated into several aliquots and rapidly frozen at –80ºC 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 (1969) by spectrophotometric techniques at 414nm. Lipid peroxidation on the whole muscle homogenate was assayed spectrophotometrically according to the method described by Bertholf et al. (1987) 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 (1990). NAG activity was determined spectrophotometrically with a commercial kit (Boehringer Mannheim - cat nº 875406). Total SOD activity was measured according to the protocol of Beauchamp and Fridovich (1971) using the RANSOD-kit (Randox Laboratories). 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 10mg protein was resolved by SDS-PAGE (12.5% acrylamide gels of 1mm thickness) as described by Laemmli (1970) and electroblotted onto nitrocellulose membranes according to Locke et al. (1990). The immunoblotts were probed with 1:5 000 dilution of monoclonal anti-Hsp70 (Sigma) 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 immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine, 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). 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. (1951).

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 (8D group), since all the animals in this group died (100%) during the course of the experience 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 to hypoxia, showing an obvious inadaptability to the experimental environmental conditions, low mobility and aggravated anorexia (98.5% reduced food intake) that culminated in their dead.

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+8D and the remaining groups. When compared to the unexposed control group, the 1D, ACCL+1D and ACCL+8D 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.

Table 1. Mice (MW), soleus (SW) weight and protein content in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. 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	15.2 ± 0.34	0.426 ± 0.023
1D	39.65 ± 0.85	15.87 ± 0.33	0.434 ± 0.023
ACCL+1D	38.36 ± 0.35	15.53 ± 0.6	0.424 ± 0.008
ACCL+8D	30.32 ± 0.86 *	12.89 ± 0.66 *	0.343 ± 0.017 *

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

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 acutely exposed to 1 day of hypobaric hypoxia (1D) suggesting an increase in oxidative stress conditions during acute and severe hypoxic insult. On the other hand, the acclimatization period (ACCL+1D) seemed to inhibit hypoxia-induced oxidative stress since (i) no additional %GSSG or GSSG content was found in this group when compared to control (C) and (ii) significant difference were found between this hypoxia gradually exposed group (ACCL+1D) and mice from the acutely exposed group (1D).

Regarding prolonged and continuous exposure after previous acclimatization (ACCL+8D), no significant enhancement of %GSSG or GSSG content was observed when compared to mice exclusively submitted to the acclimatization period (ACCL+1D). In this sense, persistent severe hypobaric hypoxia exposure failed to increase oxidative stress conditions after an acclimatization period (ACCL+1D vs ACCL+8D) despite a time-dependent trend to enhanced hypoxia-induced oxidative stress throughout the experimental protocol (C vs ACCL+1D vs ACCL+8D).

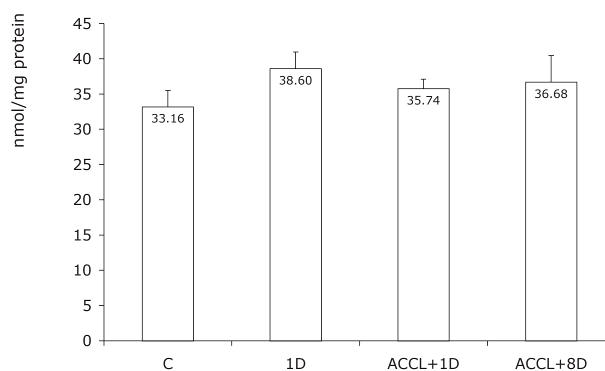

Fig.1. So*leus* muscle total glutathione (TGSH) in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to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

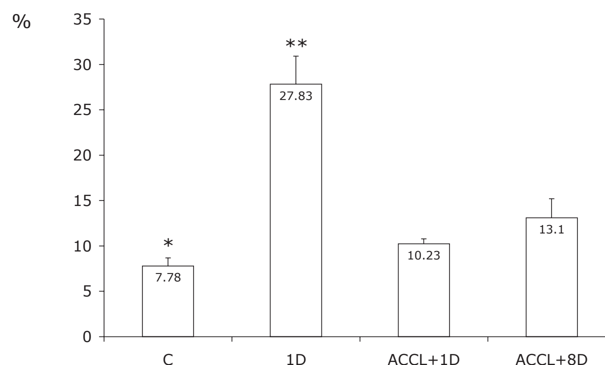

Fig.2. So*leus* muscle GSSG/TGSH (%GSSG) in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. * p<0,05, C vs 1D; ** p<0,05, 1D vs ACCL+1D and ACCL+8D

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 1D compared to control (p<0.05). However, no significant difference was observed between ACCL+1D and C groups, demonstrating an absence of enhanced lipid peroxidation in response to acclimatization. Such protective feature of acclimatization

regarding lipid peroxidation was also suggested by the significant difference in TBARS content at 1D when compared to ACCL+1D. Despite a significant increase in TBARS content in ACCL+8D when compared to control, no further significant increase was found with persistent severe hypobaric hypoxia (ACCL+8D vs ACCL+1D). Concerning protein sulfhydryl groups, our data showed that acclimatization (C vs ACCL+1D) protects skeletal muscle from protein oxidation, which contrasts clearly with acute severe exposure (C vs 1D). On the other hand, prolonged severe hypoxia exposure in acclimatized mice failed to decrease significantly protein oxidation (ACCL+8D vs ACCL+1D). NAG activity did not change significantly in acclimatized mice (C vs ACCL+1D) however, an up-regulation of this lysossomic enzyme was found in the acute non-acclimatized group (C vs 1D). Additionally, a clear significant difference was found between ACCL+1D and 1D. Once again, no significant difference was found in this enzyme between ACCL+1D and ACCL+8D groups.

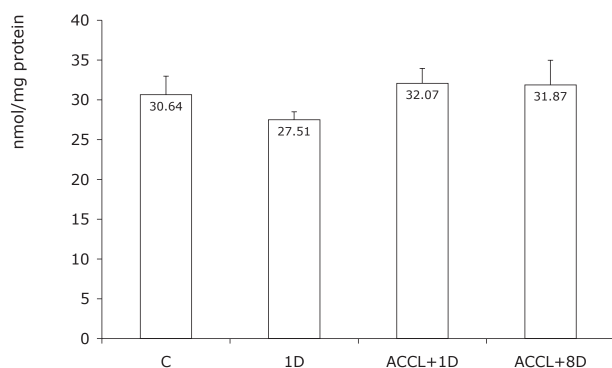

Fig.3. So*leus* muscle reduced glutathione (GSH) in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to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

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

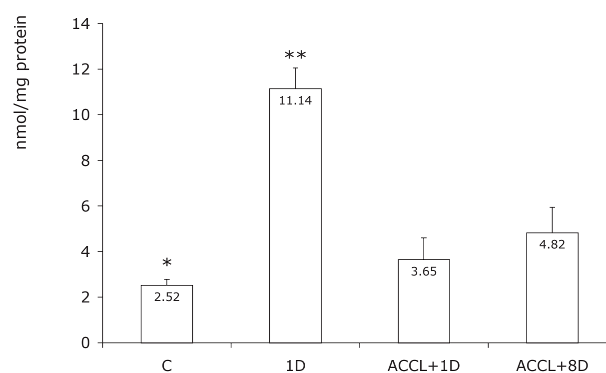

Fig.4. So*leus* muscle oxidized glutathione (GSH) in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. * $p<0{,}05$ C vs 1D; ** $p<0.05$ 1D vs ACCL+1D and ACCL+8D

Table 2. *Soleus* muscle thiobarbituric reactive substances (TBARS) and protein sulfhydryl groups (SH) content and N-Acetyl-ß-D-glucosaminidase (NAG) activity in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. 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 ‡
1D	552.7 ±38.6 **	202.63 ± 19.25	8.36 ± 0.33 **
ACCL+1D	335.4±18.6	265.12 ± 13.54	5.78 ± 0.14
ACCL+8D	383.6±27.6	234.51 ± 18.25	6.09 ± 0.67

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

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

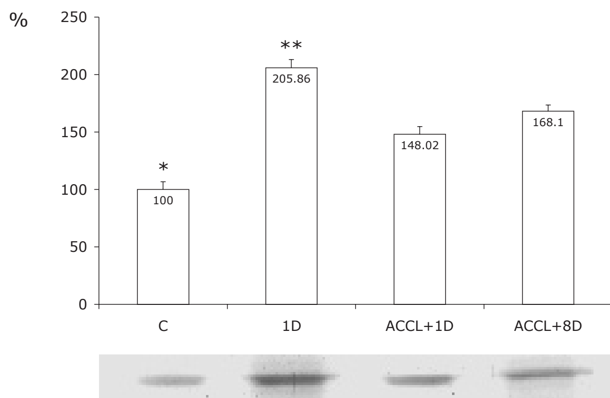

Fig.5. So*leus* muscle HSP70 expression in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. A scan of representative Western blot for each group (n=6) is immediately below histogram. *p<0,05 C vs 1D, ACCL+1D and ACCL+8D; **p<0.05 1D vs ACCL+1D and ACCL+8D.

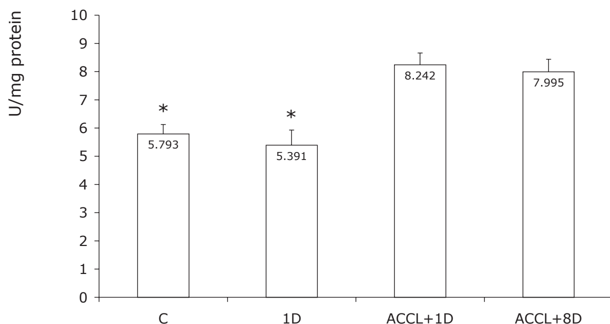

Fig.6. So*leus* muscle total superoxide dismutase (t-SOD) activity in animals exposed to 1 day (1D) of acute hypoxia and to an acclimatization period (three progressive days) plus 1 (ACCL+1D) and 8 days (ACCL+8D) of hypoxia at 7000m. Control animals (C) were submitted to a normobaric normoxic environment equivalent to sea level. Values are mean ± SEM. *p<0,05 C and 1D vs ACCL+1D and ACCL+8D

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 (1D) the GSSG/TGSH ratio increased when compared to control, which could be explained by the enhanced GSH oxidation (Sen *et al.*, 1994) 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 -glutamyl cycle to cope with increased free radical production (Powers *et al.*, 1999) 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. (2001) 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 (Singh *et al.*, 2001), could enhance GSH *turnover* diminishing the evidences of oxidative stress. Among several invoked reasons, (i) the mitochondria reductive stress (Kehrer and Lund, 1994; Duranteau *et al.*, 1998), (ii) the leukocyte-endothelial adherence via nitric oxide depletion (Wood *et al.*, 1999a) and (iii) the enhanced XO activity (Hoshikawa *et al.*, 2001) 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 1D group when compared to control. Indeed, as an indicator of lipid peroxidation, TBARS content increased almost two-fold in this experimental group (1D). These results are in accordance with several other studies conducted with animals (Singh *et al.*, 2001) and humans (Bailey *et al.*, 2001) and confirms

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 (1D 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+1D) seems to diminish almost completely the levels of oxidative stress (C vs ACCL+1D vs 1D), which is in clear contrast with 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 (Schoene *et al.*, 1990; West, 1993; Heinicke *et al.*, 2003). Hypoxia is a triggering stimulus *per se* to increase cellular ROS generation, the up-regulation of several systemic and metabolic processes might prevent 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+1D group did not differ from the control group. Moreover, concerning %GSSG and GSSG content, ACCL+1D animals were significantly different from those acutely submitted to severe hypoxia (1D), which suggests a lower level of oxidant production and of GSH oxidation in the acclimatized mice. As previously mentioned, an inter-organs GSH transport mediated by increased GSH hepatic efflux and cellular importation (Ji and Leeuwenburgh, 1996) probably justifies the absence of differences in TGSH and GSH between the 1D and the ACCL+1D 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+1D) and the control group. On the other hand, clear and distinct oxidative deleterious changes in some cellular components were found between ACCL+1D and 1D 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 with ACCL+1D, the enhancement of NAG activity observed in 1D group suggests an increase of muscle autophagic response (Salminen, 1985) 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+1D vs 1D). Indeed, our results showed that these molecular chaperones (for refs see Thomason and Menon, 2002), 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 (Benjamin *et al.*, 1990; Powers *et al.*, 2001), but can also be an important stimulus to modulate the expression of skeletal muscle HSP70 in altitude-hypoxia environment.

Besides adaptive cardiorespiratory changes (West, 1996) and absence of an inflammatory response (1999b) 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 (Duranteau *et al.*, 1998) 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 (Wood *et al.*, 1999b; Droge, 2002).

In this sense, the slight increase in %GSSG (C vs ACCL+1D and ACCL+8D) suggests that mild oxidative stress might induce skeletal muscle up-regulation of several protective mechanisms whose products exhibit antioxidant properties (Droge, 2002) counteracting the severe hypoxia-induced oxidative stress observed in 1D group.

This hypoxia-modulation effect on molecular

strategies to cope with enhanced oxidative stress seems to be consistent with our data regarding the above-referred HSP70 expression and particularly, with SOD activity throughout the experimental protocol. In fact, the increased SOD activity observed in the ACCL+1D and ACCL+8D 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 (Halliwell and Gutteridge, 1999). 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 yet unclear (Halliwell and Gutteridge, 1999; Hollander *et al.*, 2001). Based on the assumption that the degree of oxidative stress is altitude dependent, our results are in clear contrast with those obtained by Radák et al. (1994) in which Mn-SOD activity had decreased and Cu,Zn-SOD remained unchanged after 6 months of intermittent exposure to 4000m. The maintenance of SOD activity in the 1D 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+1D vs ACCL+8D), 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+1D vs ACCL+8D), the GSSG/TGSH ratio and GSSG content increased for almost 30% in ACCL+8D 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 correspondent 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 (Vats *et al.*, 1999; Norese *et al.*, 2002) may be worth of consideration. In fact, loss of appetite and consequent reduced food intake during long sojourns at extreme high-altitude have frequently been reported (Westerterp-Plantenga *et al.*, 1999) 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 wet weight (ACCL+1D vs ACCL+8D) (Table 1). In this sense, besides limiting glucose availability and indirectly affects GSH turnover (Leeuwenburgh and Ji, 1996), 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 (Leeuwenburgh and Ji, 1996). 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 in oxidative stress (Droge, 2002) as confirmed by the %GSSG (ACCL+1D vs ACCL+8D). However, future experimental approaches with a large number of animals or a longer hypoxic period should be considered 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 gradual 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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PAPER IV

Acute and severe hypobaric hypoxia impairs mitochondrial functionality in mice skeletal muscle: the protective role of vitamin E

José Magalhães[1], António Ascensão[1], José M.C. Soares[1], Rita Ferreira[1], Maria J. Neuparth[1], Franklim Marques[2], José A. Duarte[1]

[1] Department of Sport Biology
Faculty of Sport Sciences and
Physical Education
University of Porto
Portugal

[2] Departament of Clinical Analysis
Faculty of Pharmacy
Institute for Molecular and Cell Biology
University of Porto
Portugal

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Abstract

The effect of an *in-vivo* acute and severe hypobaric hypoxic insult (48h at a pressure equivalent to 8500m) in Vitamin E supplemented (60mg.kg^{-1} i.p., 3 times/wk for 3 wks) and non-supplemented mice was analyzed in mitochondria isolated from skeletal muscle using markers of oxidative damage, respiratory function and of the triggering of cellular apoptosis. Forty male mice were randomly divided into 4 groups: control+placebo (C+P), hypoxia+placebo (H+P), control+Vitamin E (C+V) and hypoxia+Vitamin E (H+V). Compared to C+P, the increased content of protein carbonyl (CGs), and the decreased sulfhydryl groups (SH) found in H+P were in accordance with the highest HSP60 expression. Mitochondrial respiration was significantly impaired in H+P as demonstrated by diminished state 3, respiratory control ratio and ADP/O, and by enhanced state 4 with both complex I and II-linked substrates. Using malate+pyruvate (MP), hypoxia decreased respiratory rate with CCCP and increased olygomycin-inhibited respiration. Bcl-2/Bax ratio decreased as a result of hypoxia exposure. Vitamin E treatment attenuated the effect of hypoxia in the levels of SH, CGs, state 3 with MP, RCR, ADP/O, state CCCP, aconitase activity and HSP60 expression. In conclusion, our data suggest that hypobaric hypoxia exposure induces mitochondrial oxidative damage, impairs mitochondrial respiratory function, and triggers the intrinsic pathway to apoptotic cell death, and that Vitamin E attenuated the impact of this hypoxic oxidant insult.

Keywords:
apoptosis, oxidative stress and damage, antioxidants, heat shock proteins, high altitude

Introduction

High-altitude exposure is known for a long time as an aggressive physiological stress inducing wide cellular deleterious effects. Recently, increasing evidences (Bailey *et al.*, 2001; Moller *et al.*, 2001; Singh *et al.*, 2001; Magalhaes *et al.*, 2004a; Magalhaes *et al.*, 2004b) have also pointed out that severe high-altitude hypoxia seems to be a triggering stimulus for enhanced cellular oxidative stress and damage to lipids, proteins and DNA. Among others, mitochondria reductive stress (Duranteau *et al.*, 1998) has been suggested as a free radical source involved in this apparently paradoxical phenomenon of hypoxia-induced oxidative stress.

Mitochondria are powerhouse's cellular organelles of eukaryotic cells that produce the energy required to drive the endergonic and vital biochemical processes of cell life through a well-coupled mechanism of oxidative phosphorylation (Cadenas, 2004). Nevertheless, the hypoxia-related reduced oxygen availability to terminally accept electrons seems to result in the accumulation of reducing equivalents throughout the mitochondria electron transport chain (ETC). This elicits the impairment of the respiratory chain activity, the increase in the so-called electron leakage to oxygen (O_2), and the consequent enhanced production of reactive oxygen and nitrogen-based species (RONS) (Dawson *et al.*, 1993; Kehrer and Lund, 1994; Chandel *et al.*, 1998; Duranteau *et al.*, 1998; Mohanraj *et al.*, 1998; Schild *et al.*, 2003b). Due to this enhanced mitochondrial production of RONS, mitochondria themselves may become oxidative targets leading to peroxidation of its membrane lipids, protein oxidation, DNA cleavage, and consequently impaired ATP generation (Arai *et al.*, 1999). In fact, the vicinity of the free radical formation to mitochondrial membranes makes phospholipids components rich in polyunsaturated fatty acids (Paradies *et al.*, 1999), and respiratory chain proteins particularly vulnerable and prone to oxidative damage, which might lead to ETC malfunction (Petrosillo *et al.*, 2001; Paradies *et al.*, 2002; Rafique *et al.*, 2004) and mitochondrial dysfunction (Amicarelli *et al.*, 1999). In accordance, ultrastrutural data obtained from rat (Amicarelli *et al.*, 1999) and human (Hoppeler *et al.*, 2003; Magalhaes *et al.*, *in press*) skeletal muscle exposed to hypobaric hypoxia revealed significant mitochondria morphological changes, namely significant swelling and, in some cases, cristae degeneration, which have been described in several other tissues (Kowaltowski *et al.*, 2000; Santos *et al.*, 2002; Schild *et al.*, 2003a) as being associated to abnormal mitochondrial functionality and to cellular apoptotic fate.

Studies of isolated mitochondria have been widely used to assess mitochondrial functionality in several normal and pathological conditions (Tonkonogi and Sahlin, 1997). In fact, *in-vitro* measurements of oxygen consumption can provide valuable information concerning the efficiency of mitochondria oxidative energy transfer and the degree of respiratory control, which are considered important parameters regarding mitochondria function (Tonkonogi and Sahlin, 1997). Early data from Kramer and Pearlstein (1983) showed that isolated mitochondria submitted to *in-vitro* severe hypoxic oxygen (O_2) concentrations had low phosphorylation efficiency (ADP/O ratio), presumably reflecting an increased electron flux directly from the complex III to O_2. However, *in-vivo* studies revealed conflicting data in mitochondria isolated from cerebral cortex (Chavez *et al.*, 1995), liver (Costa *et al.*, 1988) and heart (Costa *et al.*, 1988; Luo *et al.*, 1998), regarding respiratory properties such as state 3 and state 4 respiration, as well as respiratory control ratio (RCR). Furthermore, despite previous studies from our lab (Magalhaes *et al.*, 2004a) evidenced signs of skeletal muscle oxidative stress and damage after acute hypobaric hypoxia, it is not sufficiently clear whether mitochondria contribute to these tissue *redox* disturbances. Additionally, to our knowledge, no available information has been published concerning the effect of *in-vivo* acute and severe simulated high-altitude hypoxia exposure on rat skeletal muscle mitochondria function. In these sense, and based on previous studies in which oxidative stress was elicited by other stimuli

rather than severe hypoxia (Leichtweis *et al.*, 1997; Paradies *et al.*, 1999; Tonkonogi *et al.*, 2000; Santos *et al.*, 2002), it seems reasonable to hypothesize that hypoxia-induced oxidative stress and damage in skeletal muscle correlates with disturbances of mitochondrial respiratory functionality.

Therefore, the aim of this study was to investigate whether acute and severe hypobaric hypoxia induces mitochondrial oxidative damage and, thus, influences the qualitative features of mitochondrial functionality. Moreover, and considering the effectiveness of Vitamin E supplementation in preventing tissue oxidative damage inflicted by high altitude stress (Ilavazhagan *et al.*, 2001) we complementarily intended to analyze the potential protective effect afforded by Vitamin E against the hypothetical free radical-mediated mitochondrial dysfunction associated to this acute and severe hypoxic insult. Furthermore, since it is now well established that mitochondrial functionality plays a key role in cellular apoptosis (Childs *et al.*, 2002), another purpose of this study was to analyze the consequence of acute and severe hypoxia on skeletal muscle cellular apoptotic fate involving the intrinsic pathway.

Methods

Experimental Design

Forty male Charles River CD1 10 wk aged mice were randomly divided into four groups (n=10) according to the following independent variables: Non-hypoxic control (C), Hypoxia (H), Vitamin E (V) and Placebo (P). Two groups (C+V and H+V) were intra-peritoneally (i.p.) supplemented with cumulative doses (three times a week during three weeks, 60mg/Kg) of α-tocopherol acetate (Vitamin E) and the other two groups (C+P and H+P) received the correspondent vehicle placebo solution (sterilized soybean oil) during the same period (Appell *et al.*, 1997).

The animals of the C+P and C+V groups were maintained at an atmospheric pressure of 101.3 kPa (760 mmHg) equivalent to sea level. On the other hand, and one at a time, the animals of the hypoxic groups (H+P and H+V) were acutely exposed to a simulated atmospheric pressure of 35.2 kPa (265 mmHg) equivalent to an altitude of 8500m in a hypobaric chamber and kept at that pressure during 48 hours. The depressurization period to reach the simulated altitude of 8500m and the pressurization period until sea level conditions took 30 minutes. All the animals were kept at constant temperature (21-25ºC) on a daily lighting schedule of 12 hours of light vs. dark with normal activity and food and water *ad libitum* during the experimental protocol and were sacrificed 49 hours after the last injection. The Ethics Committee of the Scientific Board of Faculty of Sport Sciences approved the study.

Preparation of skeletal muscle mitochondria

The animals were sacrificed by cervical dislocation and hindlimb muscles (*soleus*, *gastrocnemius*, *tibialis anterior* and *quadriceps*) were extracted for preparation of isolated mitochondria. Skeletal muscle mitochondria were prepared by conventional methods of differential centrifugation, as previously described by Tonkonogi and Sahlin (1997). Briefly, the overall muscles specimen were immediately excised and minced in ice-cold isolation medium containing 100mM sucrose, 0.1mM EGTA, 50mM Tris-HCl, 100mM KCl, 1mM KH_2PO_4 and 0.2% BSA, pH 7.4. The minced blood-free tissue was rinsed and suspended in 10 ml of fresh medium containing 0.2 mg/ml bacterial proteinase (Nagarse E.C.3.4.21.62, Type XXVII; Sigma) dissolved immediately prior to use and stirred for 2 minutes. Thereafter, the sample was carefully homogenized with a tightly fitted Potter-Elvehjen homogenizer and a Teflon pestle. After homogenization, three volumes of Nagarse-free isolation medium were added to the homogenate, which was then fractionated by centrifugation at 700x*g* for 10 minutes. The resulting pellet was removed and the supernatant suspension centrifuged at 10 000x*g* during 10 minutes. The supernatant was decanted and the pellet was gently resuspended in isolation medium (1.3mL per 100mg

of the initial tissue) and centrifuged at 7000x*g* for 3 minutes. The supernatant was discarded and the final pellet, containing the mitochondria, was gently resuspended (0.4µl.mg^{-1} initial tissue) in a medium containing 225mM mannitol, 75mM sucrose, 10mM Tris and 0.1mM EDTA, pH 7.40. All the mitochondria isolation procedures were performed at 0-4ºC. Mitochondrial protein concentration was estimated spectrophotometrically according to the method of biuret using bovine serum albumin as standard. The mitochondrial suspensions were used within 4 hours after the excision of the muscles and were maintained on ice (0-4ºC) throughout this period. There was no significant alteration of the mitochondrial RCR between the first and last measurements within each mouse.

An aliquot of the resulting mitochondria suspension was taken for immediate measurement of the aconitase enzyme activity. On the other hand, other aliquots of the mitochondria suspension were separated and rapidly frozen at –80ºC for later biochemical analysis of sulfhydryl protein groups (SH), heat shock protein of the 60 kDa family (HSP60), and the pro- and anti-apoptotic Bax and Bcl2 proteins, respectively.

Measurement of mitochondrial respiratory activity

Mitochondrial respiratory function was measured polarographically, at 25ºC, using a Clark-type oxygen electrode (Hansatech DW 1, Norfork, UK). Reactions were conducted in a 0.75ml closed thermostated and magnetically stirred glass chamber containing 0.5mg of mitochondrial protein in a reaction buffer of 225mM mannitol 75mM sucrose, 10mM Tris, 10mM KCl, 10mM K_2HPO_4 and 0.1mM EDTA, pH 7.5 in accordance to Tonkonogi *et al* (2000). After 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5mM) plus malate (2mM) or succinate (10mM) plus rotenone (4µM). Conventional state 3 respiration (V3) was determined after adding ADP to a final concentration of 200µM; state 4 respiration (V4) was measured as the rate of oxygen consumption in the absence of ADP, i.e., after state 3, when all the ADP was exhausted. The respiratory control ratio (RCR), i.e., the ratio between V3 and V4, and the ratio between phosphorylated ADP and oxygen consumed (ADP/O), which reflects the efficiency of mitochondria oxidative phosphorylation, were calculated according to Estabrook (1967), using 235 nmolO_2/ml as the value of solubility of oxygen at 25ºC.

To test mitochondria inner membrane permeability and the maximal rate of uncoupled oxidative phosphorylation, oligomycin and carbonyl cyanide m-chlorophenylhydrazone (CCCP), respectively, were consecutively added in state 3 during mitochondria respiration with saturated amount of ADP (final concentration 1mM). The state oligomycin and the state CCCP were measured as the rate of oxygen consumption after adding the proton translocation inhibitor of Fo-F1, oligomycin (final concentration 1.5µg.ml^{-1}) and the protonophorous uncoupler that permeabilizes the membrane to H^+, CCCP (final concentration 2µM), respectively.

Biochemical assays in mitochondria extracted fraction

Vitamin E concentration

α-tocopherol was determined by high performance liquid chromatography (HPLC) based on Vatassery et al. (2004) method. Briefly, to an aliquot of 100µl mitochondrial extract, 2ml ethanol containing 0.025% (w/v) butylated hydroxytoluene (BHT) and 0.1ml of 30% (w/v) ascorbic acid was added. Tubes were cooled and 2ml of water was added followed by 2ml of hexane containing 0.025% (w/v) BHT. The hexane phase was separated out and evaporated down under a stream of nitrogen at 40ºC. The residue was redissolved in mobile phase and analyzed by HPLC using the following conditions: Column=Inertsil, 5 Si, 250x3mm (Varian); mobile phase=n-hexane with 3% dioxane; flow rate=0.7ml/min. The α-tocopherol were detected fluorimetrically, $\lambda_{emission}$: 290nm, $\lambda_{absortion}$: 330nm.

Aconitase enzyme activity

The activity of aconitase was assayed in isolated mitochondria as an index of superoxide radical generation (Li *et al.*, 2001). Immediately prior to aconitase activity measurement, the mitochondrial fraction was diluted in 0.5ml buffer containing 50mM Tris-HCl and 0,6mM $MnCl_2$ (pH 7.4), and sonicated for 2 seconds. Aconitase activity was immediately measured spectrophotometrically by monitoring at 240nm the formation of *cis*-aconitate after the addition of 20mM isocitrate at 25ºC according to Krebs and Holzach (1952). One unit was defined as the amount of enzyme necessary to produce 1μM *cis*-aconitate *per* minute (ε_{240}= 3.6mM^{-1}cm^{-1}).

Analysis of HSP 60, protein carbonylation, Bax and Bcl-2

Equivalent amounts of proteins were electrophoresed on a 15% SDS-PAGE gel, followed by blotting on a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech). After blotting, non-specific binding was blocked with 5% nonfat dry milk in TTBS [Tris-buffered saline (TBS) with Tween 20] and the membrane was incubated with either anti-Bcl-2 (1:500; sc-7382 mouse monoclonal IgG; Santa Cruz Biotechnology) or anti-Bax (1:500; sc-493 rabbit polyclonal IgG; Santa Cruz Biotechnology) or HSP60 (1:2000; 386028 mouse monoclonal IgG; Calbiochem) antibodies for 2 hours at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:1000; Amersham Pharmacia Biotech) for 2 hours.

For protein carbonyl derivatives (CGs) assay, a certain isolated mitochondrial volume (V) containing 20μg of protein was derivatized with dinitrophenylhydrazine (DNPH). Briefly, the sample was mixed with 1 V of 12% SDS plus 2 V of 20mM DNPH 10% TFA, followed by a 30 minutes of dark incubation, after which 1,5 V of 2M Tris / 18.3% of -mercaptoethanol were added. A negative control was simultaneously prepared for each sample. After diluting the derivatized proteins in TBS to obtain a final concentration of 0.001μg/μL, a 100ml volume was slot-blotted into a Hybond-PVDF membrane. Immunodetection of carbonyls was then performed using rabbit anti-DNP (DAKO) as the first antibody (1:2000 dilution), and anti-rabbit IgG-Peroxidase (Amersham Pharmacia) as the second antibody (1:2000 dilution).

For both referred assays, the bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine, 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). Optical density results were expre ssed as percentage variation of control values.

Analysis of sulfhydryl protein groups (SH groups)

The mitochondrial content of oxidative modified sulfhydryl protein groups was quantified by spectrophotometric measurement according to the method proposed by Hu (1990). Briefly, the colorimetric assay was performed after the reaction of 50μl aliquot of mitochondrial extract with 10μl of 5,5'-dithio-bis(2-nitrobenzoic acid) (10mM) in a medium containing 150μl of Tris (0,25M) and 790μl methanol, at 414nm against a blank test. SH content was expressed in nmol/mg of mitochondrial protein (ε_{414} = 13,6 mM^{-1}cm^{-1}).

Preparation and biochemical assays in total muscle homogenate

Immediately before the mitochondrial isolation procedure, one homogenized sample was separated into aliquots and rapidly frozen at –80ºC for later biochemical analysis of total (TGSH) and oxidized (GSSG) glutathione and total protein content. TGSH and GSSG measurements were determined as previously described by Tietze (1969). The aliquots for glutathione assay were previously extracted for

a medium containing percloric acid at 5% (w/v) in order to precipitate proteins. Regarding TGSH, after neutralization with potassium hydrogen carbonate (0,76M), samples were centrifuged for 1 min at 13,000*g*. Then, a supernatant aliquot was incubated for 15 min at 30ºC in a microtiter plate with a reagent solution containing NADPH (1,68 mM) and 5,5'dithio-bis(2-nitrobenzoic acid) (0,7mM). Immediately after the addition of glutathione reductase 20U/ml, a kinetic analysis was performed at 412nm. For GSSG content evaluation, a 2-vinylpyridine addition, to a final concentration of 5% (v/v), was made prior to the neutralization step in order to inactivate the sulfhydryl groups. The remained assay was analogous to the total glutathione measurement. TGSH and GSSG concentrations (nmol/mg protein) were established based on calibration curves assembled with several commercial standards dilutions. Protein content was assayed spectrophotometrically using bovine serum albumin as standard according to Lowry *et al*. (1951).

Statistical analysis

Mean and mean standard errors were calculated for all variables in all groups. Two-way ANOVA was used to analyze the effect of severe hypobaric hypoxia and of vitamin E supplementation. A Bonferroni *post-hoc* test was done to further evaluate differences between group pairs. The Statistical Package for the Social Sciences (SPSS Inc. version 10.0) was used for all analysis. The significant level was set at 5%.

Results

Mitochondrial Vitamin E content

As a result of the intraperitoneally cumulative administration of Vitamin E, skeletal muscle mitochondria from control supplemented mice (C+V) showed a significant higher content (+85.80%) of Vitamin E than C+P (Fig. 1). Moreover, mice from both hypoxic groups (H+P and H+V) showed a significant decrease in Vitamin E levels (81,37 and 61,79%, respectively) when compared to their counterparts (C+P and C+V). Nevertheless, the Vitamin E content in H+P was significantly lower than in H+V.

Mitochondrial respiratory rates

The state 3 and state 4 respiration rates as well as the RCR presented in this study were comparable to other data reported in skeletal muscle isolated mitochondria (Brandao *et al.*, 2003) showing effective respiratory intrinsic properties with both malate+pyruvate and succinate+rotenone.

As illustrated in tables I and II, the acute and severe hypoxic insult induced significant impairment in the intrinsic respiratory properties of skeletal muscle isolated mitochondria energized with malate+pyruvate (M-P) and succinate+rotenone (S-R). The acute and severe hypoxic insult significantly decreased state 3 respiration by 40.50% with M-P and by 27.74% with S-R when compared with non-hypoxic control mitochondria. On the other hand, hypoxic mice (H+P) showed a 26.77% and 35.76% significant increase in state 4 respiration with M-P and S-R, respectively compared with C+P. The respiratory control ratio was also significantly

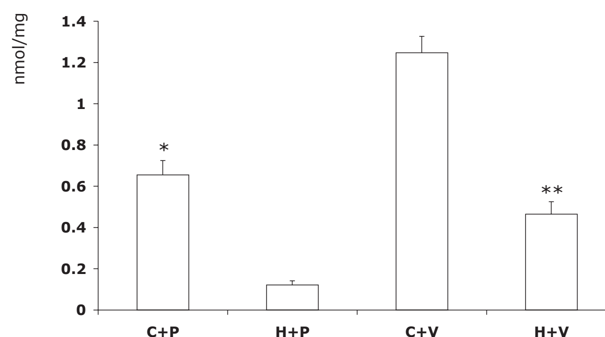

Figure 1. Mitochondrial α-tocopherol (Vitamin E) content in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil. Values represent mean ± SEM and are expressed as nmol/mg mitochondrial protein. *p<0.05, C+P vs C+V and H+P; **p<0.05, H+V vs C+V and H+P.

affected by this experimental procedure. In fact, RCR decreased 53.60% using M-P and 46.51% with S-R as respiratory substrates when compared to control. With both M-P and S-R, the ADP/O ratio was also significantly impaired. Indeed, the ratio of the ADP to oxygen consumption significantly decreased 28.19% with M-P and 25.80% using S-R as substrates.

Vitamin E supplementation induced a significant protective effect in state 3 respiration both with M-P and S-R (tables I and II). Indeed, Vitamin E supplemented animals were able to counteract the significant impairment in state 3 induced by hypobaric hypoxia in their counterparts (H+P vs H+V). However, regarding state 4 respiratory rates, our data showed that Vitamin E supplemented animals did not differed from their non-supplemented counterparts after the hypoxic insult both with M-P and S-R. In what concerns to RCR and ADP/O both with M-P and S-R, our data showed that Vitamin E supplementation was helpful against the deleterious effect perpetrated by the acute and severe hypoxia on these respiratory parameters. Moreover, concerning RCR and taking into account the above-referred effect of Vitamin E supplementation in state 3 and state 4 respiratory rates both with M-P and S-R, data revealed that the effective protection of this antioxidant derived essentially from the attenuated decrease in state 3.

After the hypoxic insult, mitochondria energized with malate-pyruvate and stimulated with saturated ADP showed a respiratory rate with oligomycin (Fig. 2) significantly increased (31%) compared to C+P. On the other hand, Vitamin E supplementation inhibited completely the increased inner membrane permeability induced by hypobaric hypoxia to 9.58% compared to control (H+P vs H+V). Mitochondrial respiratory uncoupling by CCCP (Fig. 2) showed a marked reduction in O_2 consumption in H+P compared to C+P (43.77%). Vitamin E supplemented animals presented an attenuated decrease in CCCP rate (18.33%) after the acute and severe hypoxia compared with their counterparts (H+V vs H+P). Nevertheless, the uncoupled CCCP rate revealed by H+V was still significantly distinct from the one obtained in Vitamin E control mice (C+V).

Acute and severe hypobaric hypoxia-induced muscle oxidative stress, mitochondrial stress, superoxide production and oxidative damage

Skeletal muscle glutathione contents are expressed in table III. There was a significant reduction in TGSH and GSH in the H+P compared to C+P. Moreover, a remarkable increase in GSSG concentration and %GSSG was also found in these animals when compared to non-hypoxic control. Vitamin E (H+P vs H+V) attenuated significantly the impact of the hypoxic insult on TGSH, GSH, GSSG and %GSSG. Nevertheless, for all the above-referred parameters, Vitamin E supplemented animals presented values substantially distinct from control (H+V vs C+V)

Table I. Mitochondrial respiratory rates in state 3 (V3) and state 4 (V4), RCR and ADP/O with malate+pyruvate in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil.

	V3 $nmolO_2.min^{-1}.mg.^{-1}$	V4 $nmolO_2.min^{-1}.mg.^{-1}$	RCR	ADP/O
C+P	153,27 ± 3,54	23,12 ± 0,91	6,66 ± 0,35	2,27 ± 0,04
H+P	91,19 ± 5,7 *	29,31 ± 0,88 #	3,09 ± 0,18 *	1,63 ± 0,10 *
C+V	164,40 ± 4,54	25,41 ± 0,89	6,47 ± 0,05	2,22 ± 0,02
H+V	120,09 ± 4,64 **	29,38 ± 0,55	4,08 ± 0,15 **	1,95 ± 0,05 **

Values represent mean ± SEM; *p<0.05, H+P vs C+P and H+V; **p<0.05, H+V vs C+V; #p<0.05, H+P vs C+P.

Table II. Mitochondrial respiratory rates in state 3 (V3) and state 4 (V4), RCR and ADP/O with succinate+rotenone in in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil.

	V3	V4	RCR	ADP/O
	$nmolO_2.min^{-1}.mg.^{-1}$	$nmolO_2.min^{-1}.mg.^{-1}$		
C+P	322,08 ± 11,14	101,80 ± 2,34	3,16 ± 0,11	1,55 ± 0,03
H+P	232,72 ± 8,90 *	138,21 ± 7,62 #	1,69 ± 0,12 *	1,15 ± 0,07 *
C+V	324,39 ± 7,32	105,60 ± 3,28	3,08 ± 0,09	1,64 ± 0,08
H+V	298,89 ± 9,32	124,97 ± 14,31	2,40 ± 0,09 **	1,43 ± 0,02 **

Values represent mean ± SEM; *$p<0.05$, H+P vs C+P and H+V; **$p<0.05$, H+V vs C+V; #$p<0.05$, H+P vs C+P.

after the acute and severe hypoxic insult.

As can be depicted in Fig. 3, the aconitase enzyme activity was severely affected by the experimental hypoxic protocol. In fact, in H+P animals the activity of this superoxide-sensitive enzyme decreased significantly (34.94%) when compared to C+P. Vitamin E significantly attenuated the inactivation of aconitase activity (H+P vs H+V) induced by the hypoxic insult, however the mitochondrial aconitase activity found in the hypoxic supplemented mice was still significantly affected compared to control (17.91%).

Regarding the markers of mitochondrial oxidative damage (table IV), our data showed that the hypoxia exposure inflicted substantial oxidative deleterious effects on protein oxidation. With respect to protein SH content, a significant 53.57% decrease was found in H+P compared to C+P. Despite the significantly antioxidant protective effect of Vitamin E (H+P vs H+V), supplemented hypoxic mice (H+V) also showed significant diminished levels of sulfhydryl protein groups compared to C+V. Concerning CGs , a significant increase was found in H+P when compared to C+P (74.26 and 109.37%, respectively). Vitamin E protected mitochondria from CGs formation induced by hypobaric hypoxia, however supplemented mice (H+V) still differed from their counterparts (C+V).

Concerning HSP60 expression (Fig. 4), our data showed that acute and severe hypoxia exposure induced a significant mitochondria HSP60

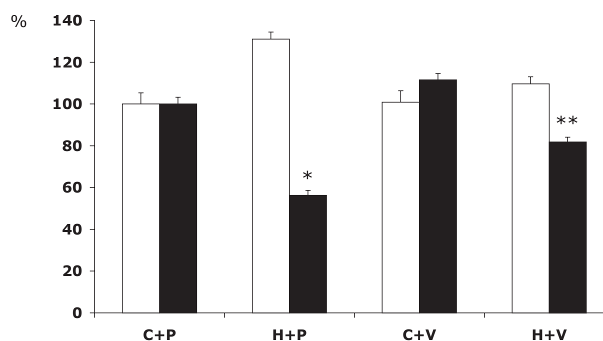

Figure 2. Respiratory rates of malate-pyruvate energized mitochondria with oligomycin (open bars) and CCCP (close bars) in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil. Values represent mean ± SEM and are expressed as percentage of the Vitamin E non-supplemented control (C+P). *$p<0.05$, H+P vs C+P and H+V; **$p<0.05$, H+V vs C+V.

overexpression (86.42%) when compared to non-hypoxic conditions (H+P vs C+P). The hypoxic animals supplemented with Vitamin E also showed a significant overexpression (34.60%) of HSP60 compared to C+P, however significantly distinct from their non-supplemented counterparts (H+P vs H+V).

Impact of mitochondrial dysfunction after acute and severe hypobaric hypoxia on skeletal muscle cell fate

In what concerns the expression of proapoptotic protein Bax (Fig. 5), our data showed a significant

Table III. Skeletal muscle glutathione content in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either with Vitamin E or a placebo solution of sterile soyabean oil.

	TGSH	GSH	GSSG	%GSSG
	nmol/mg	nmol/mg	nmol/mg	%
C+P	18,48 ± 0,95	18,13 ± 0,96	0,34 ± 0,02	1,90 ± 0,19
H+P	10,12 ± 0,60 *	8,13 ± 0,51 *	1,99 ± 0,11 *	19,72 ± 0,61 *
C+V	22,45 ± 1,03	22,13 ± 1,04	0,32 ± 0,02	1,46 ± 0,16
H+V	14,57 ± 0,70 **	13,68 ± 0,65 **	0,89 ± 0,06 **	6,13 ± 0,20 **

Values represent mean ± SEM and are expressed as nmol/milligram of protein (nmol/mg); Total glutathione (TGSH), reduced glutathione (GSH), Oxidized glutathione GSSG) and GSSG/TGSH ratio (%GSSG); *p<0.05, H+P vs C+P and H+V; **p<0.05, H+V vs C+V.

increase in the animals submitted to acute and severe hypoxia compared with both C+P (32.02%) and their Vitamin E supplemented counterparts (22.54%). There was no significant difference in the levels of Bax between C+P, C+V and H+V. On the other hand, the levels of the antiapoptotic protein Bcl-2 did not differed significantly within all groups independently of the conditions. Therefore, the Bcl-2 / Bax ratio (table V) revealed a significant decrease in hypoxic animals compared to C+P. Vitamin E supplementation was able to counteract the triggering of the apoptotic pathway induced by acute and severe hypobaric hypoxia.

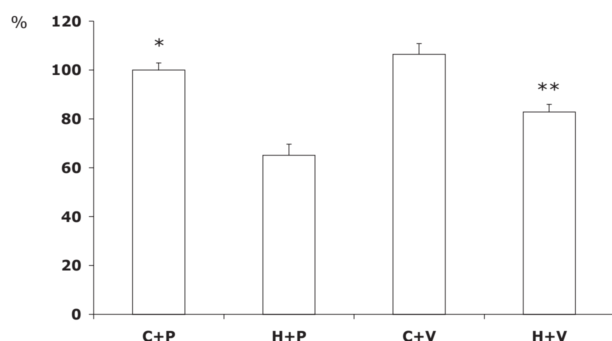

Figure 3. Activity of the mitochondrial enzyme aconitase in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil. Values represent mean ± SEM and are expressed as percentage of the Vitamin E non-supplemented control (C+P). *p<0.05, H+P vs C+P and H+V; **p<0.05, H+V vs C+V.

Discussion

Overview of the principal findings of this study

Results obtained in the present study provided new insights into the biochemical and functional effects of the *in-vivo* acute and severe hypobaric hypoxia exposure on mice skeletal muscle mitochondria. Data showed that the hypoxic insult induced mitochondrial increased superoxide radical production, protein oxidation, impaired mitochondrial respiratory function, and ultimately the triggering of the intrinsic pathway to apoptotic cell death. Furthermore, our study also suggests that Vitamin E is able to attenuate the impact of this hypoxic oxidant insult on mitochondrial function and on apoptotic cell fate of mice skeletal muscle. Considering that mitochondria were isolated from an overall sample of muscle specimens, characterized by different biochemical features, it is possible that the sensitivity to hypobaric hypoxia and to Vitamin E might be distinct in each muscle.

Mitochondrial oxidative stress and damage after acute and severe hypobaric hypoxia

In addition to the well-described (Singh *et al.*, 2001; Magalhaes *et al.*, 2004a) and at the present confirmed paradoxical phenomenon of hypoxia-induced oxidative stress and damage in whole

Table IV. Mitochondrial oxidative damage markers in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil. Values are mean and SEM.

	SH nmol/mg	CGs OD
C+P	0,028 ± 0,002	34,62 ± 1,70
H+P	0,013 ± 0,001 *	60,33 ± 1,44 *
C+V	0,029 ± 0,002	39,56 ± 1,97
H+V	0,018 ± 0,002 **	49,37 ± 0,87 **

Values represent mean ± SEM; Sulfhydryl protein groups (SH), Carbonyl derivative groups (CGs), Optical density arbitrary units (OD); *p<0.05, H+P vs C+P and H+V; **p<0.05, H+V vs C+V.

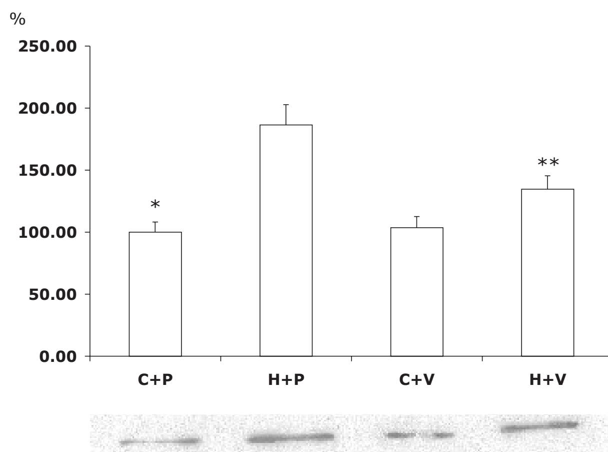

Figure 4. Mitochondrial HSP60 expression in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil. Values represent mean ± SEM and are expressed as percentage of the Vitamin E non-supplemented control (C+P). A scan of a representative western blotting for each group (n=6) is immediately below the histogram. *p<0.05, H+P vs C+P and H+V; **p<0.05, H+V vs C+V.

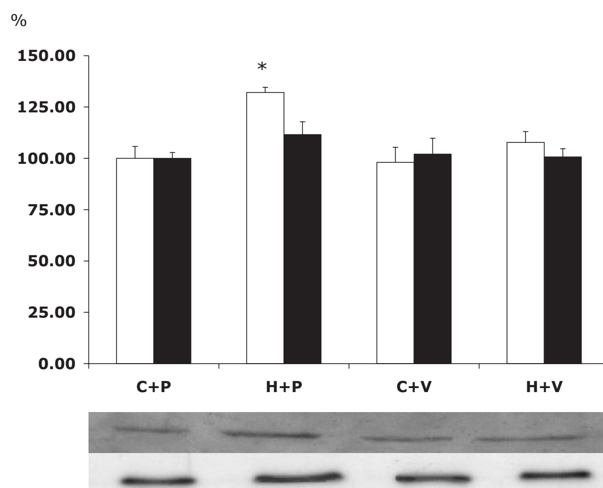

Figure 5. Mitochondrial expression of the Bcl2 family proapoptotic protein Bax (open bars) and Bcl-2 (close bars) in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil. Values represent mean ± SEM and are expressed as percentage of the Vitamin E non-supplemented control (C+P). A scan of a representative Bax and Bcl-2 western blotting for each group (n=6) is immediately below the histogram. *p<0.05, H+P vs C+P and H+V

Table V. Bcl2 / Bax ratio in mice exposed either to hypobaric hypoxia equivalent to 8500m or sea level conditions and treated either by Vitamin E or a placebo solution of sterile soyabean oil.

	C+P	H+P	C+V	H+V
Bcl2/Bax ratio	1,00±0,03	0,84±0,01*	1,04±0,00	0,95±0,01

Values represent mean ± SEM; *p<0.05, H+P vs C+P and H+V.

skeletal muscle (table III), our study supports a role for mitochondria as a potential ROS source and also as a specific oxidative target under severe hypoxic conditions. It is currently stated that hypoxia-mediated partial inhibition of mitochondrial electron transport results in *redox* changes in the electron carriers with consequent enhanced generation of ROS (Dada *et al.*, 2003). In accordance, our data regarding mitochondrial aconitase activity impairment in hypoxic mice (see Fig. 3) constitute an indirect index of increased mitochondrial $O_2^{-\cdot}$ production (Melov *et al.*, 1999). In fact, the loss of mitochondrial aconitase activity in aged SOD2 knockout mice, but not cytosolic aconitase, previously demonstrated by Williams *et al*. (1998) suggests that the Fe-S center of aconitases can be reversely inactivated by $O_2^{-\cdot}$ and related species (Castro *et al.*, 1994) and its inactivation is a selective measure of enhanced *in-vivo* mitochondrial $O_2^{-\cdot}$ production. Accordingly, clear evidences of protein oxidative

damage (SH and carbonyl-derivative groups) were found in mitochondria isolated from hypoxic mice (table IV), which suggest that under these severe hypoxic conditions the mitochondrial antioxidant defense system was overwhelmed by the increased ROS production. This assumption was confirmed in our study, since the markers of ROS production and oxidative damage in Vitamin E supplemented mice were substantially attenuated compared to the non-supplemented hypoxic mitochondria. Moreover, and in accordance with data reported by Ilavazhagan (2001), the above results suggest that Vitamin E is a valuable antioxidant able to counteract the oxidative stress and damage inflicted by a hypobaric hypoxia pro-oxidant insult at distinct levels of cell organization.

Accordingly, in our study, the enhanced expression of HSP60 seems to correlate well with the magnitude of the physiological stress and damage inflicted to mitochondria under the distinct experimental conditions (Fig. 4). In fact, the highest levels of HSP60 exhibited by the hypoxic mice, particularly the Vitamin E non-supplemented group, seem to confirm the mitochondrial aggression perpetrated by hypobaric hypoxia as well as the protective role of Vitamin E against these deleterious cellular conditions, which reinforces the suspicion of a predominant oxidative etiology for the damage inflicted by hypoxia. Complementary to anti-oxidants scavengers, the HSP60 overexpression might be seen as a second line of mitochondrial defense against oxidative damage, i.e., counteracting the overwhelmed antioxidant capacity by increased ROS production (Sammut *et al.*, 2001). This protective complementary effect assumes particular importance since when O_2 is limited, the levels of some mitochondrial and nuclear genome encoding proteins of ETC are repressed compromising mitochondrial regenerative processes (Webster *et al.*, 1990; Vijayasarathy *et al.*, 2003) and consequently mitochondrial respiratory features.

Impairment of skeletal muscle mitochondria functionality upon an acute and severe in-vivo hypobaric hypoxic insult

Given that mitochondrial protein and phospholipid damage induced by oxidative stress is widely suggested as being implicated in the onset and/or development of several mitochondria pathophysiological states either by impairing integral enzyme functioning or by destroying mitochondrial bilayer packing order (Megli and Sabatini, 2003) with influence on membrane fluidity (Zini *et al.*, 2002), we looked for the effects of hypoxia induced-oxidative stress on *in-vitro* mitochondrial respiratory function.

To the best of our knowledge, this is the first study dealing with skeletal muscle mitochondria functionality after an acute and severe *in-vivo* hypobaric hypoxic insult. State 3 respiratory rates of energized mitochondria with both NADH- and $FADH_2$-linked substrates were significantly diminished in the hypoxic groups (tables I and II), which indicate that the maximal skeletal muscle oxidative power was severely affected and that complex I-mediated respiration was more affected than succinate-dependent respiratory rate. Since the maximal rates of respiration in ADP stimulated mitochondria reflect the availability of NADH and $FADH_2$ for electron supply, one possible explanation, among others, may be related to the scarcity of ETC-linked substrates. In fact, Nulton-Persson and Szweda (2001) reported that the reversible impairment in some mitochondrial Krebs cycle enzyme activities, namely succinate dehydrogenase and α-ketoglutarate dehydrogenase after *in-vitro* pro-oxidant stimulation, resulted in diminished NADH levels that closely paralleled the changes in the rate of state 3 respiration. Moreover, our findings concerning the substantial mitochondrial aconitase inactivation followed by hypoxia-induced oxidative stress (Fig. 3) also suggest an impairment of the Krebs cycle metabolism that might had compromised the regular and adequate supply of substrates to ETC.

Nevertheless, the scarcity of ETC-linked substrates might probably affect both complexes-linked

respiration but does not seem to be sufficiently powerful to explain the relative differences between the respiratory rates with NADH- and $FADH_2$-linked substrate. Thus, the decreased activity of some of the ETC enzymatic complexes through free radical-mediated protein oxidation (Yen *et al.*, 1996) or by the ROS-induced inner membrane cardiolipin peroxidation (Paradies *et al.*, 2000, 2002), might be an important reason to explain the diminished electron transport capacity through the ETC and to justify the decreased state 3 respiration observed in the hypoxic groups. Because mitochondrial enzyme complexes consist of polypeptides encompassing iron-sulfur clusters, they can become subject to oxidative modification and deactivation by free radical-mediated interactions, resulting in accumulating products of protein oxidation such as carbonyl groups as well as in the depressed content of SH- protein groups (table IV)**,** thereby reducing ETC complexes enzyme activities and compromising state 3 respiratory rates with both NADH and $FADH_2$-related substrates (Sammut *et al.*, 2001). Although the activity of complex I and II enzymes was not measured in our study, it is possible that [4Fe-4S] clusters in those complexes were oxidatively targeted with distinct magnitudes by the enhanced mitochondrial superoxide production. Indeed, in hypoxic Vitamin E supplemented and non-supplemented mice, NADH-related state 3 respiration was more affected than with $FADH_2$-linked succinate, which suggests that, probably because complex I contains more [4Fe-4S] clusters than complex II (Yen *et al.*, 1996), it was more sensitive to hypoxia-induced free radical-mediated inactivation than complex II.

In what concerns to state 4 respiration, our data showed that in mitochondria isolated from both Vitamin E supplemented and non-supplemented hypoxia-submitted animals, respiratory rates did not return to control state 4 values after ADP stimulation. Since the stimulation of state 4 respiration reflects an increased back-leakage of protons through the mitochondrial inner membrane to compensate the decreased transmembrane potential (Cardoso *et al.*, 2001), these data suggest a mitochondrial-uncoupled state through non-identified inner membrane sites and an unexpected inefficacy of Vitamin E to counteract the oxidative attack inflicted against the mitochondrial membrane of hypoxic mice. In fact, being Vitamin E a lipophilic substance present in the hydrophobic domain of cellular membranes (Traber, 2000), it would be expected that this antioxidant had prevented or, at least, had attenuated mitochondria inner membrane lipid peroxidation by scavenging lipid peroxyl radicals contributing for the increased stability of the lipid and protein membrane constituents (Sumien *et al.*, 2003) with consequent benefits for coupled mitochondrial respiration. However, the simple analysis of state 4 respiration rate *per se* seems to be insufficient to conclude about the effectiveness of Vitamin E regarding inner membrane protection. Accordingly, to further clarify the above-referred findings concerning depressed state 3 and enhanced state 4 respiration, oligomycin and CCCP were used in mitochondria previously energized with M-P and stimulated with ADP. This trial avoids the possible interference of the permeability to protons through the Fo-F1 ATP synthase on state 4 (state oligomycin) and, on the other hand, ensures that the variations in membrane permeability do not interfere with the inhibition of the respiratory chain, since the permeability in the presence of CCCP is always maximal. Since state CCCP was also severely affected in mitochondria isolated from hypoxic mice, one can argue that the hypoxic insult clearly affects the respiratory function, hindering the normal electron flux. The study of the effect of this hypoxic insult on the respiratory rate after the addition of olygomycin conveniently checked out the increased state 4. Data showed that state oligomycin was in accordance with state 4 in hypoxic non-supplemented mice, which confirms the increased permeability of mitochondrial inner membrane through other components rather than Fo-F1 ATP synthase. Nevertheless, since in Vitamin E supplemented animals state 4 increased but state oligomycin did not differed from control, it is reasonable to assume that mitochondrial inner membrane permeability was preserved by a Vitamin E antioxidant-mediated effect, but the intrinsic H^+

stoechiometry of the ATP synthase was impaired (intrinsic uncoupling of ATP synthase).
The phosphorylation efficiency of mitochondria from non-supplemented mice previously submitted to hypobaric hypoxia was clearly affected as inferred from either the decrease in RCR and ADP/O ratios. In fact, as a consequence of the state 3 and state 4 significant alterations, the RCR value suffered a substantial decrease, which suggests that the substrate utilization was uncoupled concerning the demands of ATP synthesis. Since RCR is a standard respiratory parameter that represents the functional and structural integrity of respiring mitochondria, i.e., a measure of dependence of the respiratory rate on ADP, these findings suggest that hypobaric hypoxia act as a mitochondrial-uncoupling stimulus but also as an inhibitor of respiration and oxidative phosphorylation. Furthermore, the ADP/O ratio was also impaired, which corroborates the less efficient utilization of oxygen *per* molecule of ATP synthesized in mitochondria isolated from hypoxic mice. It is worth to note that Vitamin E, probably through an antioxidant-mediated effect (table IV), attenuated the dysfunction of mitochondria phosphorylation parameters induced by the severe hypoxic insult. Taken together, the overall abovementioned findings suggest that, presumably by an oxidative stress-mediated phenomenon, acute and severe hypoxia *per se* induced increased mitochondria inner membrane permeability through distinct membrane constituents, and that Vitamin E modulated the mitochondrial membrane integrity only on specific membrane sites resulting in the partial inhibition of the mitochondrial uncoupling.

Impact of skeletal muscle mitochondrial malfunction on cell apoptotic fate

Since mitochondrial dysfunction has been considered an earliest step of the intrinsic pathway of apoptosis (Skulachev, 1997), we also looked for the influence of the hypoxic insult on the expression of the mitochondrial pro- and anti-apoptotic Bcl-2 family proteins (Bax and Bcl-2, respectively). In fact, the relative expression of these proteins in the mitochondrial outer membrane is thought to decide the apoptotic fate of the cell by regulating membrane integrity (Reed *et al.*, 1998; Crompton, 1999; Sreedhar and Csermely, 2004). Data from our study demonstrated a significant increase in Bax content from non-supplemented hypoxic mice, suggesting that this severe hypoxic insult activates the intrinsic apoptotic pathway. Bax translocation from cytosol to mitochondrial outer membrane and subsequent homodimeryzation into high molecular weight complexes constitutes a requisite gateway to mitochondrial outer membrane permeabilization and subsequent apoptotic fate through cytochrome *c* release (Reed *et al.*, 1998; Wei *et al.*, 2001; Mikhailov *et al.*, 2003). Considering the formation of Bcl-2/Bax heterodimers as a mechanism to inhibit apoptosis (Reed *et al.*, 1998), the imbalance between the expression of Bax and Bcl-2 in non-supplemented hypoxic animals, expressed by the reduced Bcl-2/Bax ratio, suggests that hypobaric hypoxia is a prone stimulus to trigger cellular apoptosis. In clear contrast with our data, and probably reflecting the protective effect of the acclimatization process against skeletal muscle oxidative stress already demonstrated in our lab (Magalhaes *et al.*), Riva and coworkers (Riva *et al.*, 2001) showed that in young rats growing under moderate chronic hypoxia conditions, skeletal muscle mitochondria overexpressed Bax and Bcl-2 proteins but the hypoxic global effect on Bcl-2/Bax ratio increased, allowing a better protection against apoptosis. As expected, the expression of the mitochondrial intrinsic apoptotic pathway markers was significantly depressed in Vitamin E supplemented animals, compared to the one's non-supplemented, which corroborates the data from mitochondrial respiratory function.

In conclusion, our data suggest that acute and severe hypobaric hypoxia exposure induces mitochondrial oxidative damage, impairs mitochondrial respiratory function, and triggers the intrinsic pathway to apoptotic cell death, and that Vitamin E attenuates the impact of this hypoxic oxidant insult.

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PAPER V

Skeletal muscle ultrastructural and plasma biochemical signs of endothelium dysfunction induced by a high-altitude expedition (Pumori, 7161m)

José Magalhães[1], António Ascensão[1], Franklim Marques[2], José M.C. Soares[1], Maria J. Neuparth[1,3], Rita Ferreira[1], Francisco Amado[3], José A. Duarte[1]

[1] Department of Sport Biology
Faculty of Sport Sciences and
Physical Education
University of Porto
Portugal

[2] Departament of Clinical Analysis
Faculty of Pharmacy
University of Porto
Portugal

[3] Department of Chemistry
University of Aveiro
Portugal

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Abstract

The aim of this study was to analyze whether or not a high-altitude expedition to a Himalayan peak (Pumori, 7161m) induces skeletal muscle ultrastructural and plasma biochemical changes suggestive of microvascular dysfunction. To achieve this purpose 6 mountaineers spent 3 weeks at an altitude range between 5250–7161m after 1 week in an acclimatization trek (2800-5250m). Muscle biopsies from *vastus lateralis* and blood drawn from antecubital vein were collected at sea level 1 day before and after the expedition to analyze qualitative and quantitative (capillary and fiber basement membrane thickness) ultrastructural muscle alterations as well as the plasma activity of tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1). In contrast with a regular skeletal muscle pattern observed before the expedition, the post-expedition muscle samples revealed profound structure alterations in the tissue organization. Severe and chronic high altitude exposure also induced significant capillary basement membrane thickness as well as a significant increase in plasma tPA, PAI-1 and PAI-1/tPA ratio. From the present data it could be concluded that sustained and severe hypobaric-hypoxia exposure constitutes an insult to skeletal muscle with deleterious microvasculature consequences even in acclimatized climbers.

Keywords
Hypobaric hypoxia, humans, microvascular damage, basement membrane, fibrinolysis, morphology, oxidative stress

Introduction

Chronic exposure to extreme high-altitude has been widely considered as a stressful stimuli even to acclimatized dwellers (West, 1996). In response to this hypobaric hypoxic condition, several systemic and peripheral physiological adaptive responses are acutely and chronically triggered-out to counteract the body overall hypoxic status imposed by environmental oxygen scarceness (West, 1996). Nevertheless, despite the contribution of these orchestrated mechanisms to diminish the arterial hypoxemia and related tissue hypoxia during the course of an acclimatization process, cell oxygen levels at high-altitude seem to be far below the normal sea-level values (West, 2003) and thus, even the acclimatized body remains hypoxic compromising tissue *redox* status homeostasis.

In fact, several evidences from chronic high-altitude biological research report cellular disturbances in a variety of organs and tissues related, at least in part, to impaired cellular oxygen tension (West, 1996). Among others, and despite of it extensive plasticity, skeletal muscle may also suffer serious disturbances from a hypoxic environmental condition, such as the one experienced during high-altitude exposure, which lead to impaired homeostasis and disruption in a wide range of cellular functions (Fluck and Hoppeler, 2003). Indeed, several studies described cumulative evidences of biochemical down-regulation and morphological detrimental effects in skeletal muscle fibers after chronic hypoxia, like enzymes involved in oxidative metabolic pathways, decrease of muscle fibers cross sectional area, decrease mitochondrial volume density, and accumulation of degradation products such as lipofuscin-like substances (Hoppeler and Vogt, 2001). However, concerning skeletal muscle microvascular bed, despite the importance of the capillary supply to muscle metabolism and the effect of chronic hypoxia on muscle capillary to fiber structure are well established, there is still a lack of data regarding the influence of chronic high altitude hypoxia on the functional and morphological phenotype of skeletal muscle endothelial cells (Mathieu-Costello, 2001). Taking into account the impairment of physiological mechanisms to cope with severe environmental oxygenless, even in acclimatized subjects, it is likely that skeletal muscle endothelial cells could also present signs of hypoxia-mediated homeostatic disturbances, as previously reported in other tissues (Gonzalez and Wood, 2001).

In this sense, the purpose of the present study was to analyze whether or not a 4 weeks high-altitude expedition to a Himalayan peak (Pumori, 7161m) induces skeletal muscle microvascular ultrastructural changes in response to sustained hypobaric hypoxia. Moreover, since vascular bed stress could modify the expression of several plasmatic substances (Pearson, 1993), the activity of tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1) in plasma was measured as general markers of endothelial dysfunction.

Material and methods

Experimental Design

Six (6) male non-smokers high-altitude skilled and fit recreational climbers (age: 31.0±2.1 yrs; height: 172±6.8 cm; weight: 64.3±3.9 kg; % fat mass: 13.4±1.3; VO_{2max}: 63.0±0.7 $ml.kg^{-1}.min^{-1}$) who didn't experience high-altitude conditions within a period of, at least, 6 months, participated in this study. All the experimental procedures and the possible risks involved in this study were explained to the subjects whose written consent was obtained. The study protocol was approved in advance by the Ethics Committee of the Scientific Board of Faculty of Sport Sciences, University of Porto and was designed in accordance to the recommendations of the Declaration of Helsinki. Nepalese Sherpa packers and base-camp personnel supported the expedition, however no data was collected from these Nepalese citizens.

After the flight from Portugal to Nepal (2800m; day 1), the climbers hiked over one week in an acclimatization trek until Mount Pumori base-camp

(day 7). Additionally, all the dwellers spent three weeks in high-altitude expedition routines at an altitude range between base-camp (5250m) and the summit of Mount Pumori (7161m). All the subjects successfully achieved the top of Pumori on days 24/25. After two subsequent days, the climbers returned to Lukla and flew back to Portugal at day 29. The detailed altitude profile of the expedition is shown in figure 1. Symptoms of acute mountain sickness were unimportant and decreased after a few days, thus none of the climbers needed any medication. During all the experimental protocol food and fluid ingestion was allowed *ad libitum,* however, antioxidant supplementation were not allowed 4 weeks prior to protocol or during the expedition period.

Blood sampling and biochemical assays

Blood samples were collected twice during the experimental protocol from the antecubital vein. The first one was taken in Portugal at sea level condition, one day before the flight to Nepal, and the other one was collected in the day after the subjects arrived to Portugal. All the venous blood samples were taken by conventional clinical procedures using EDTA as anticoagulant. Plasma was separated by centrifugation (3,000*g* during 10 min at 4ºC) into several aliquots and rapidly frozen at –80ºC for later analysis of tPA and PAI-1 activities. These parameters were measured by enzyme immunoassay (ELISA) using TintElize tPA and TintElize PAI-1 Biopool International commercial kits, respectively.

Muscle Biopsies, preparations and microscopic analyzes

A biopsy from the *vastus lateralis* muscle was obtained in each subject one day before the departure to Himalayans, and another one was collected in the day after the climbers returned to Portugal. After infiltration of the skin with 1% lidocaine, a 3mm incision through the skin and fascia was made in the midlateral thigh, and a muscle sample was achieved using a 5-mm diameter side-cutting Bergström needle with suction system (Evans *et al.*, 1982) that deep penetrated 3 cm in muscle. The

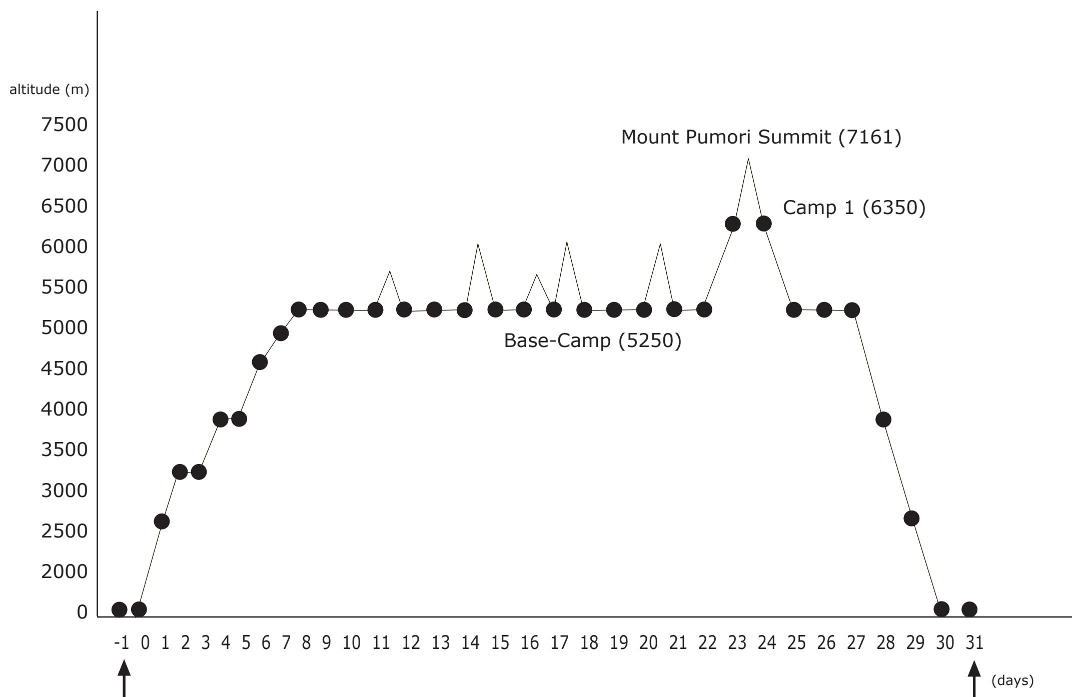

Figure 1. Altitude profile of the expedition during the four weeks. Circles represent the altitude at which the climbers slept. Arrows represent the days of blood drawn and muscular biopsies before and after the expedition

sample was cleansed of excess blood, connective tissue and fat, and immediately transferred to 2.5% glutaraldehyde for two hours to ensure rapid fixation. Using routine methods, the samples were further fixated with 1% osmiumtetroxide, dehydrated in graded alcohol, and embedded in Epon. Ultrathin sections were contrasted with 0.5% uranyl acetate and lead citrate and examined in a Zeiss EM 10A electron microscope. Electron micrographs from cross-sections, were digitized to a computer and used to estimate the thickness of the vascular and fiber basement membrane. The morphometric analysis was made using the software ImageJ 1.30V (National Institutes of Health, USA). In addition, a qualitative analysis of muscle fibers ultrastructure was also performed.

Statistical procedures

Mean and standard error mean were used as descriptive statistics. To test differences between pre and post high-altitude exposure a *t*-test for repeated measures was used. The significant level was set at 5%.

Results

From a qualitative point of view, the overall picture of biopsies obtained from pre-expedition muscle samples revealed a regular skeletal muscle pattern without any notorious ultrastructural disturbances (Fig 2a). Nevertheless, an abundant subsarcolemmal and intermyofibrillar mitochondrial volume density and glycogen content (Fig 3a) were evident. Conversely, the ultrastructural analysis of post-expedition muscle samples (Fig 2b, 3b) revealed a marked increase in the intermyofibrillar space with loss of myofilaments and organelles in the subsarcolemmal area. Additionally, a decrease in subsarcolemmal and intermyofibrillar mitochondria volume density and a presumable concomitant increase in lipid inclusions in mitochondria vicinity, suggesting

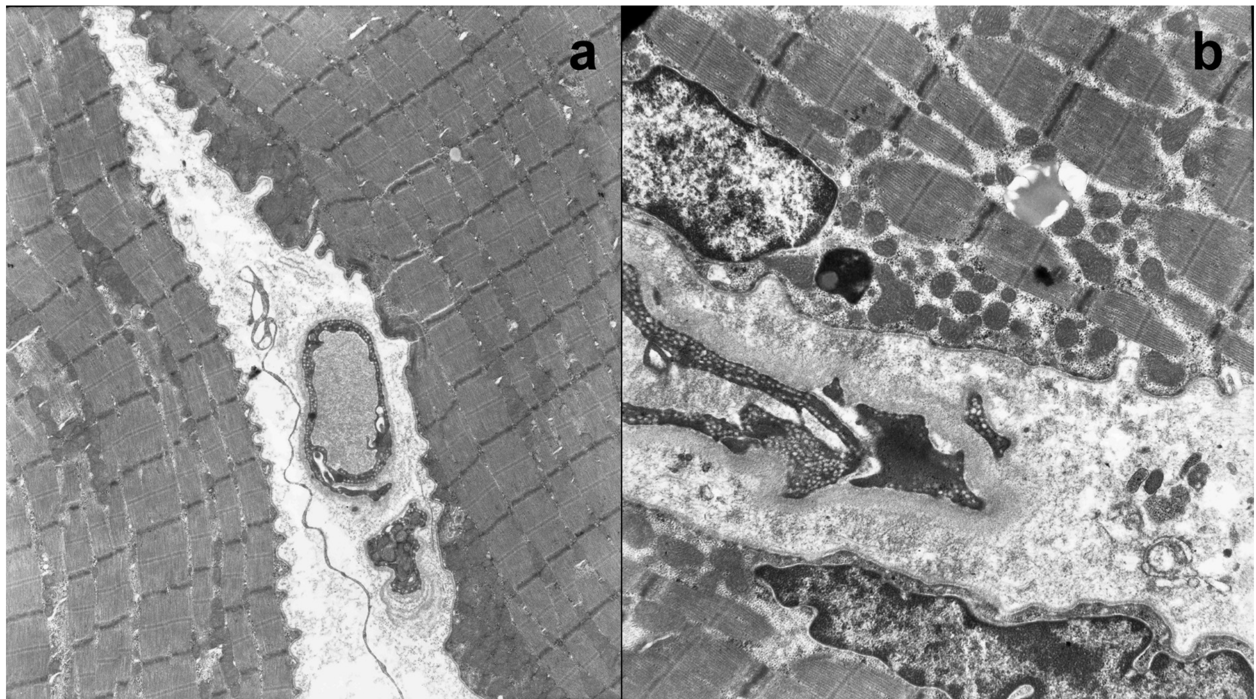

Figure 2 – Electron micrographs showing the general ultrastructural appearance of skeletal muscle before (a) and after the expedition (b); in contrast with the regular skeletal muscle pattern exhibit in section a, the high-altitude exposure induced capillary basement membrane thickness, notorious decrease of mitochondrial volume density with inter-myofibrilar space enlargement, accumulation of lipofuscin-like pigments, and an apparent reduction in glycogen content (original magnification a - x5,000; b - x10,000).

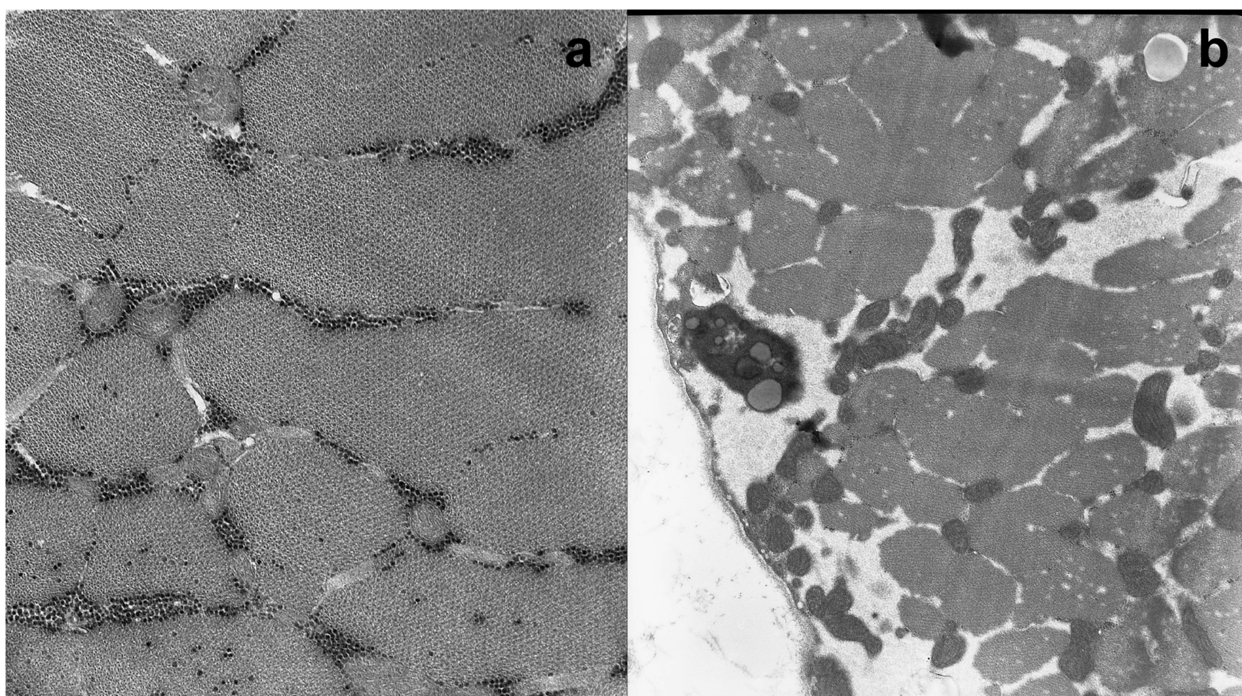

Figure 3 – Cross-section skeletal muscle electron micrographs showing the general tissue ultrastructural appearance before (a) and after the expedition (b); in section a it appears evident a notorious accumulation of glycogen granules within a normal intermyofibrillar space. In a clear contrast, the high-altitude exposure (section b) increased intermyofibrillar and subsarcolemmal space with a lack of organelles and glycogen content; it could also be oberved a lipid-droplet and a lipofuscin-like pigment as well as a wide myofibrillar ultrastructure vacuolization (original magnification a – x24,000; b - x12,500).

lipofuscin-like substances accumulation, were noted after the expedition. The glycogen granules concentration suffered a noticeable decrease in both subsarcolemmal and intermyofibrillar locations in the post-expedition biopsies when compared to pre-expedition (Fig. 3b). Furthermore, a wide and spread vacuolization was observed in endothelial cells in electron micrographs obtained from post-expedition muscle samples (Fig 4b).

Concerning the skeletal muscle ultrastructure morphometrical analysis, and as can be observed in table 1, the capillary basement membrane was significantly enlarged in the post-expedition muscle samples. Conversely, no significant morphological changes were found in fibers basement membrane after the hypoxic insult.

Plasma tissue-type plasminogen activator and plasminogen activator inhibitor type 1 activities can be depicted in table 2. Regarding these fibrinolytic markers, a significant increase was observed in both tPA and PAI-1 after the expedition period. However, as a consequence of a disproportionate increase in PAI-1 activity, a significant increase in PAI-1/tPA ratio was also found after the hypoxic insult.

Discussion

The overall picture of the qualitative morphological observations confirmed previous data from several other studies dealing with the long-established findings of profound structure alterations in the muscle organization so far demonstrated in humans (Hoppeler and Vogt, 2001) and animals (Amicarelli *et al.*, 1999) after hypoxia exposure.

The main finding of our study suggests that significant modifications on skeletal muscle microvasculature biochemical and ultrastructural variables also occurred during the high-altitude expedition. In fact, from an ultrastrutural point of view, a noticeable morphometric feature observed

at capillary level after the high-altitude expedition was the significant thickness of the capillaries basement membrane. This enlargement might be interpreted as a defense mechanism against the sympathetic-mediated increase in systemic pressure involving both systolic and diastolic pressures, and also systemic resistance described in mountaineers during high-altitude sojourns (for refs see Hultgren, 1997). In fact, in several known pathologies-induced gradually increase in capillary pressure over long periods of time, such as mitral stenosis or pulmonary venoocclusive disease (for refs see West, 2000), capillaries show thickening of the endothelial cell basement membrane. The importance of this basement membrane remodeling process in providing additional strength to the capillary wall is also supported by the fact that the systemic capillaries basement membrane thickness increases as the hydrostatic pressures within these capillaries increases down the body (West and Mathieu-Costello, 1995). Additionally, it might be looked as the morphological correlate of functionally puffed constituents of the basement membrane due to extravasal water accumulation as suggested in ischemia-reperfusion (IR) models (Appell *et al.*, 1999).

Table 1. Skeletal muscle capillaries (CBMT) and fibers (FBMT) basement membrane thickness before and after the high-altitude expedition

	Before expedition	After expedition
CBMT	43.00 ± 0.93 *	75.84 ± 1.47
FBMT	23.91± 0.51	20.80 ± 0.40

*p<0,05; Before vs after the high-altitude expedition

Nevertheless, since IR-induced endothelium disturbances have been related at least in part to oxidative stress-mediated mechanisms (Walker, 1991), another hypothetical explanation for this morphometric feature would be based on enhanced endothelium pro-oxidant redox conditions. In fact, according to current metabolic concepts, blood capillaries under hypoxia or IR are among the first structures affected from enhanced reactive oxygen species (ROS) production that, at least in part, might

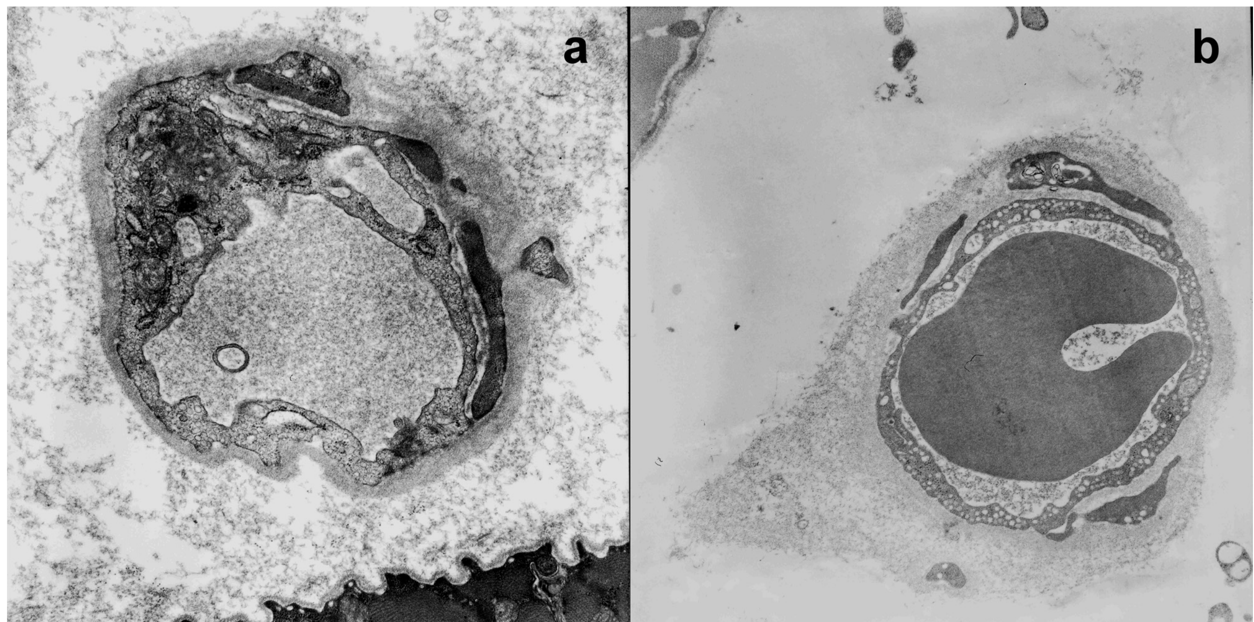

Figure 4 – Skeletal muscle electron micrographs showing the ultrastructural appearance of capillaries before (a) and after the expedition (b); note the enlargement of the basement membrane and the intense cytoplasmic vacuolization resulting from high-altitude exposure (original magnification of a & b x12,500).

be derived from endothelial xanthine oxidase (XO) increased activity (Duarte *et al.*, 1993; Erdogan *et al.*, 1999). Moreover, animal studies showed that systemic hypoxia modify endothelial cells physiology inducing a generalized and rapid microvascular inflammatory response characterized by increased ROS levels, leukocyte-endothelial adherence and emigration, and increased vascular permeability (reviewed in Gonzalez and Wood, 2001). In humans, systemic enhanced oxidative stress induced by several pathologies, such as diabetes (Jakus, 2000) or cardiovascular diseases (reviewed in Brown and Hu, 2001) have been described as having aggressive and deleterious effects in capillary walls. Thus, and as expected, due to their intrinsic hypoxic status-mediated XO activation (Hoshikawa *et al.*, 2001) and to a close vicinity relationship with such a pro-oxidant circulatory pool (Frei *et al.*, 1988; Houston *et al.*, 1999; Wood *et al.*, 1999), endothelial cells seem to be simultaneously oxidative source and target during sustained high-altitude insult. However, to our knowledge, despite the reported occurrence of oxidative stress in skeletal muscle induced by hypobaric hypoxia (Singh *et al.*, 2001; Sarada *et al.*, 2002; Magalhaes *et al.*, 2004a; Magalhaes *et al.*, 2004b), there is no strong scientific rationale to associate the morphometric microvascular modulation found in our study with the presumable enhanced oxidative stress in hypoxic endothelial cells (Houston *et al.*, 1999; Kayyali *et al.*, 2001; Steiner *et al.*, 2002; Paddenberg *et al.*, 2003). In fact, only a moderate increase in retinal capillary basement membrane was found in □-tocopherol deficiency-induced oxidative stress rats (Robison *et al.*, 2000). Moreover, in another recent study conducted with rats, a long-term administration of a vitamin antioxidant mixture did not affect the diabetes-induced thickness of capillary basement membranes (Kowluru *et al.*, 2001). Since no changes in endothelial basement membrane thickness were reported during moderate hypobaric hypoxia in cerebral microvasculature (Stewart *et al.*, 1997), the hypoxic vasculature remodelling observed in our study could be a tissue and/or altitude-dependent phenomenon.

Table 2. Plasma plasminogen activator inhibitor (PAI-1), tissue-type plasminogen activator (tPA) activities and PAI-1/tPA ratio before and after the high-altitude expedition

	Before expedition	After expedition
PAI-1 (U/mL)	20,93 ± 4,49 *	39,62 ± 5,3
tPA (U/mL)	4,19 ± 0,56 *	5,98 ± 0,70
PAI-1 / tPA	4,88 ± 0,47 *	6,64 ± 0,53

*p<0,05; Before vs after the high-altitude expedition

The significant increase in both plasma *t*-PA and PAI-1 activities was found after the expedition, confirming that severe and chronic high-altitude exposure led to the development of noticeable endothelial cell stress-related disturbances. In fact, the plasma concentration of these and other substances related to coagulation and fibrinolytic systems are directly dependent from the level of endothelial cell function (Pearson, 1993). After chemical or mechanical stress, endothelial cells alter their functional pattern, characterized, among other events, by the exposition of cell adhesion molecules, by the reduction of prostacyclin and nitric oxide production and by the release of tPA and PAI-1 to blood circulation (Pearson, 1993) with impact in leukocyte-endothelium interaction and in the regulation of coagulation and fibrinolytic systems. In fact, Wood and coworkers (1999) showed that systemic hypoxia prompt an enhanced leukocyte-endothelial interaction involving decreased nitric oxide levels, a phenomenon mediated by increased ROS production that resulted in microvascular damage and endothelial cell activation. Accordingly, some recent studies supported the notion of a hypoxia-mediated pathway for PAI-1 up-regulation. Indeed, it has been suggested that hypoxia-inducible factor (HIF-1) binds to the promoter of human PAI-1 gene through tightly regulated hypoxia-response elements (Kietzmann *et al.*, 1999; Fink *et al.*, 2002). Moreover, alterations in the vascular fibrinolytic pathways associated to hypoxia towards fibrinolytic impairment may be, at least in part, related to *redox*-sensitive mechanisms, since ROS seem to play an important role in PAI-1 activation. This could explain

the increase in PAI-1/*t*PA ratio observed in our study and the concomitant tendency to a prothrombotic state during sustained high-altitude exposure. Accordingly, in a study conducted by Antoniades et al (2003), antioxidant supplementation of Vit C and E decreased PAI-1/*t*PA ratio enhancing fibrinolytic activity and decreased coagulability.

In accordance with other authors observations (reviewed in Fluck and Hoppeler, 2003), the ultrastructural data showed evidence of lipofuscin particles accumulation in muscle subsarcolemmal area (figure 2b). The quantity of lipofuscin has been described as increasing by over two-threefold after returning from a high-altitude expedition (Martinelli *et al.*, 1990; Howald and Hoppeler, 2003). This lipofuscin accumulation within the cells of capillary walls is consistent with current information (Robison *et al.*, 2000) on the effects of enhanced cellular autoxidation and consequent accumulation of highly peroxidized membrane remnants as lipofuscin in various tissues. In fact, lipofuscin might be considered as a degradation product probably formed by mitochondria lipid peroxidation that characterizes cytological damage incurred by enhanced free radical formation in muscle cells, which also corroborates a condition of oxidative stress associated to high-altitude exposure (Fluck and Hoppeler, 2003). Moreover, another related mechanism favoring this characteristic lipofuscin accumulation could be the loss of muscle mitochondria volume density observed in the present study and elsewhere after the prolonged hypoxic exposure (Hoppeler *et al.*, 1990). In fact, observations regarding several pathological conditions suggest that during cellular enhanced reparative process, mitochondria lysosomic autophagocytation is dependent on the organelle size, i.e., while larger mitochondria are less prone to autophagocitose causing damaged mitochondria accumulation, small mitochondria are preferentially autophagocitosed (reviewed in Brunk and Terman, 2002), which could favor lipofuscin accumulation in hypoxic muscle.

In conclusion, our main findings seem to support that a sustained and severe hypobaric-hypoxia exposure within a field-based Himalayan expedition constitutes an insult to skeletal muscle with deleterious microvasculature consequences even in acclimatized climbers. The hypothesis that the referred morphological and biochemical findings were, at least in part, associated to a previously reported hypoxia-induced oxidative stress phenomenon, should not be excluded.

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PAPER VI

Effect of a high-altitude expedition to a Himalayan peak (Pumori, 7161m) on plasma and erythrocyte antioxidant profile

José Magalhães[1], António Ascensão[1], Franklim Marques[3], José M.C. Soares[1], Rita Ferreira[1], Maria J. Neuparth[1,2], José Oliveira[1], José A. Duarte[1]

[1] Department of Sport Biology
Faculty of Sport Sciences and
Physical Education
University of Porto
Portugal

[2] Department of Chemistry,
University of Aveiro
Portugal

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Abstract

The effects of a high-altitude exposure were studied in six mountaineers who spent 3 weeks at an altitude range between 5250–7161m after 1 week in an acclimatization trek (2800-5250m). Blood drawn from the antecubital vein was collected at sea level 1 day before and after the expedition to analyze some hematological variables (hemoglobin, hematocrit and red blood cells count), erythrocyte antioxidant enzyme activity (superoxide dismutase - SOD, glutathione peroxidase - GPx and glutathione reductase - Gr) and membrane fatty acid profile (monounsaturated fatty acids - MUFA, polyunsaturated fatty acids - PUFA, saturated fatty acids - SFA, trans fatty acids - TRANS). Moreover, total antioxidant status (TAS), thiobarbituric acid reactive substances (TBARS), thiol protein groups (SH), SOD, GPx and Gr were measured in plasma. High-altitude exposure induced polycythaemia, with significant increase in RBC count (5.26%), Hb concentration (4.83%) and Htc (6.26%). Furthermore, a significant increase in plasma TBARS, SOD and Gr was observed after the expedition, whereas SH, TAS and GPx decreased. Erythrocyte glutathione cycle-related antioxidant enzymes activity was up-regulated, whereas SOD activity was maintained after the expedition. In addition, despite the unchanged (MUFA+PUFA)/SFA ratio, the membrane erythrocyte fatty acid content showed a significant increase in PUFAs and a decrease in TRANS, suggesting enhanced membrane fluidity. In conclusion, it seems that high-altitude exposure, besides quantitative variations in RBC expression, induced plasma oxidative stress and damage, and significant changes in erythrocyte components, namely in antioxidant enzyme activity and membrane fatty acid profile that might modify RBC functionality.

Keywords:
Hypobaric hypoxia, humans, blood, oxidative stress, membrane fatty acid

Introduction

Exposure to extreme hypobaric-hypoxia has been widely considered as a severe and potentially life-threatening physiological stimulus, not only for non-acclimatized, but also, for high-altitude acclimatized humans (West, 1996). In response to this oxygen deficient environmental condition, several systemic and tissue physiological adaptive responses are acutely and chronically triggered to counteract the body's overall hypoxic status imposed by the scarcetty of environmental oxygen (West, 1996).

Paradoxically, this "oxygenless" condition seems to be a triggering stimulus for increased production of reactive oxygen species (ROS) and hence, to a condition of hypoxia-induced oxidative stress and enhanced oxidative damage to lipids, proteins and DNA (Bailey *et al.*, 2000; Joanny *et al.*, 2001; Moller *et al.*, 2001) that might contribute, at least in part, to the organic deleterious effects perpetrated by a high-altitude hypoxic insult. Recently, and in accordance with several other authors (Bailey *et al.*, 2000; Singh *et al.*, 2001), we showed in animal studies conducted in our lab that acute and severe hypoxia induces oxidative stress and damage in skeletal muscle (Magalhaes *et al.*, 2004b). Moreover, despite the protective effect of acclimatization**,** a trend to a gradual increased oxidative deleterious effect in mice skeletal muscle seems to occur during persistent severe hypoxia exposure even after a previous acclimatization period (Magalhaes *et al.*, 2004a). These results supported previous data from Joanny and co-workers (2001) in Operation Everest III, yet some of the underlying mechanisms remain insufficiently clear regarding acclimatized climbers engaged in high-altitude expeditions.

As a hallmark of the orchestrated adaptive response to chronic hypoxia referred to previously, bone marrow is severely stressed through erythropoietin-mediated regulation to increase erythropoiesis in an attempt to minimize the physiological impairment related to diminished tissue oxygen tension (Samaja, 2001). Nevertheless, during enhanced sustained conditions of systemic ROS production, such as those induced by chronic high-altitude exposure, red blood cells (RBC) might also be considered as potential oxidative stress targets. They transport high concentrations of a potentially pro-oxidant haem protein (haemoglobin) and oxygen inside a membrane rich in polyunsaturated fatty acid side-chains (Halliwell and Gutteridge, 1999). Moreover, ROS are able to diffuse through the RBC membrane (Halliwell and Gutteridge, 1999) and RBC have limited biomolecular repair and regenerating mechanisms due to the absence of DNA. Thus, it is reasonable that some RBCs may not overwhelm the enhanced oxidative stress developed within sustained stress conditions and thus, some RBC components such as membranes, could be oxidatively affected. One of the few studies dealing with human erythrocyte membrane changes in acute hypoxia reported damage in erythrocyte membrane protein and modifications on membrane lipid dynamics probably due to hypoxia-mediated enhanced oxidative stress (Celedon *et al.*, 1998). In such pro-oxidant conditions, peroxidation of erythrocyte membranes, among other possible oxidant deleterious effects, could lead to the loss of their ability to change shape and squeeze through the smallest capillaries (Halliwell and Gutteridge, 1999) and alter the oxygen transport to peripheral tissues, which might be particularly critical in the frozen, dry and hypoxic conditions of a long-term high-altitude exposure.

Despite the limited adaptive response of mature erythrocytes, the sustained and systemic pro-oxidant *redox* status induced by hypoxia can also directly affect bone narrow erythrogenic cells, probably contributing to the modulation of the erythrocytes. Being true, this condition would possibly allow the genesis of more resistant and functional RBCs in order to cope with this aggressive systemic environment. However, despite a remarkable amount of scientific information regarding the quantitative hypoxia-mediated phenomenon of high-altitude polycytaemia (West, 1996), there is a lack of data concerning the qualitative changes induced by this hypoxia-mediated sustained and enhanced systemic oxidative stress in the newly formed red

blood cells (RBC). In this sense, the purpose of the present study was to analyse whether or not a 4 weeks high-altitude expedition to a Himalayan peak (Pumori, 7161m) induces plasma antioxidant defence changes as well as qualitative erythrocyte alterations (antioxidant enzyme activity, membrane fatty acid profile) in addition to the commonly used quantitative haematological variables.

Material and methods

Experimental Design

The study was performed in six male non-smokers high-altitude skilled and fit recreational climbers (age: 31.0±2.1 yrs; height: 172±6.8 cm; weight: 64.3±3.9 kg; % fat mass: 13.4±1.3; VO_{2max}: 63.0±0.7 $ml.kg^{-1}.min^{-1}$) who had not experienced high-altitude conditions within a period of, at least, 6 months prior to the study.

After the flight from Lisbon, Portugal to Lukla, Nepal (2800m; day 1), the six subjects hiked for 1 week in an acclimatization trek until they reached Mount Pumori base-camp (day 7). Additionally, all the climbers spent 3 weeks in high-altitude expedition routines, such as setting an altitude camp (6350m), at an altitude range between base-camp (5250m) and the summit of Mount Pumori (7161m). All the subjects successfully achieved the summit without oxygen supplementation on days 24/25. After a subsequent 2 days of rest and dismantling base-camp, the climbers returned to Lukla and flew to Portugal on day 29. The exact altitude profile of the expedition is shown in figure 1. Symptoms of acute mountain sickness were unimportant and decreased after a few days, thus none of the climbers needed any medication. During all the experimental protocol food and fluid ingestion was allowed *ad libitum,* however, antioxidant vitamins were not allowed 4 weeks prior to protocol or during the expedition period.

All the experimental procedures and the possible risks involved in this study were explained to the subjects whose written consent was obtained. The study protocol was approved in advance by the

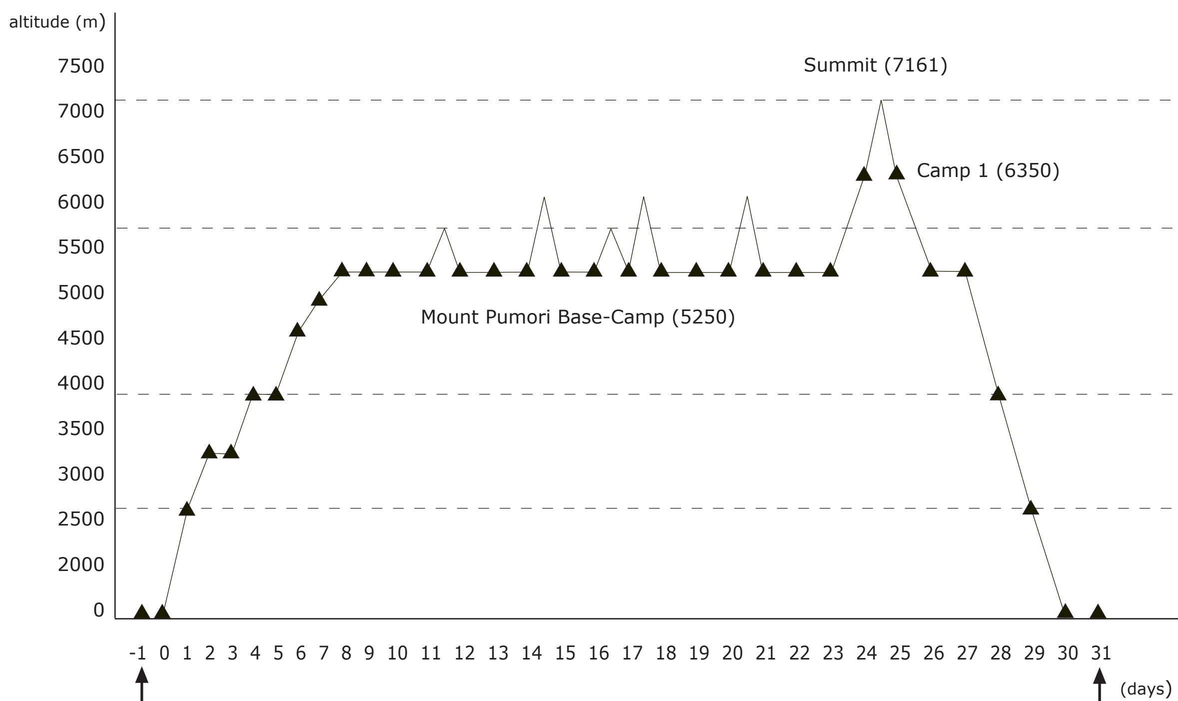

Figure 1. Altitude profile of the expedition during the four weeks. Triangles represent the altitude at which the climbers slept. Arrows represent the days blood was drawn before and after the expedition

Ethics Committee of the Scientific Board of Faculty of Sport Sciences, University of Porto and was designed in accordance to the recommendations of the Declaration of Helsinki. Nepalese Sherpa packers and base-camp personnel supported the expedition; however, no data was collected from these Nepalese citizens.

Blood sampling and preparations

Blood samples were withdrawn twice during the experimental protocol from the antecubital vein. The first blood drawn was taken in Portugal at sea level condition, one day before the flight to Katmandu-Lukla (Nepal) and the other was collected on the day after the subjects arrived back in Portugal. All the venous blood samples were taken by conventional clinical procedures using EDTA as anticoagulant. Nevertheless, in accordance to Bailey et al. (2001), no tourniquet constriction was used in order to minimize potentially enhanced oxidative stress induced by an ischaemia-reperfusion maneuver.

An aliquot of the whole blood was used to analyze routine hematological parameters [red blood cells count (RBC count), hemoglobin (Hb) and hematocrit (Htc)]. The remaining freshly withdrawn blood was immediately centrifuged at 2500 rpm for 15 minutes. After careful removal of the plasma, erythrocytes were washed three times with triple-volume of 0.9% NaCl solution and centrifuged at 900 *g* for 15 min after each washing.

Plasma was separated into several aliquots and rapidly frozen at –80ºC for later biochemical analysis of antioxidant enzyme activity (total superoxide dismutase - SOD, total glutathione peroxidase - GPx and total glutathione reductase - Gr), total antioxidant status (TAS), thiobarbituric acid reactive substances (TBARS) and protein SH content (SH). Likewise, isolated erythrocytes were also stored at –80ºC for biochemical assays of antioxidant enzyme activities (SOD, GPx and Gr).

To obtain isolated RBC membranes, washed RBC were lysed by thermo shock with ice-cold distilled water and centrifuged at 3500 *g* during 15 min. The erythrocyte membrane pellet was then ressuspended for extraction of total lipids using methanol and chloroform according to the method proposed by Bligh and Dyer (1959), and used for the determination of membrane fatty acids profile (monounsaturated fatty acids - MUFA, polyunsaturated fatty acids - PUFA, saturated fatty acids - SFA, trans fatty acids - TRANS).

Biochemical Assays

Regarding enzyme activities in plasma and erythrocyte hemolysates, SOD activity was measured spectrophotometrically at 550 nm using a commercial RANSOD kit (catalogue no. SD 125, Crumlin, United Kingdom). The activity of GPx was assayed by a spectrophotometric technique at 340 nm using a commercial RANSEL kit (catalogue no RS 505, Crumlin, United Kingdom). The activity of Gr was measured with a spectrophotometric procedure at 340 nm using a commercial Gr RANDOX kit (catalogue no. GR 2368, Crumlin, United Kingdom). Plasma TAS was measured spectrophotometrically at 600 nm using a RANDOX commercial kit (catalogue no NX 2332, Crumlin, United Kingdom).

Plasma lipid peroxidation was assayed according to the method described by Bertholf et al. (1987) and spectrophotometrically measured by the formation of TBARS at 540 nm. Oxidative modification of protein SH groups in plasma was quantified by spectrophotometric measurement according to the method proposed by Hu (1990) at 414nm.

The erythrocyte membrane fatty acids were determined by gas chromatography with flame-ionization detector (GC-FID)/capillary column based on the method described by Amaral (2003). Fatty acids methyl esters (FAMEs) were prepared by hydrolysis with an 11g/L methanolic potassium hydroxide solution, methyl esterification with BF_3/MeOH, and extraction with *n*-heptane. Fatty acids (MUFA, PUFA, SFA and TRANS) were identified by comparing their retention times on the column to appropriate standards.

Statistical procedures

Mean and standard error mean were used as descriptive statistics. To test differences between pre and post high-altitude exposure a *t*-test for repeated measures was used. The significant level was set at 5%.

Results

As can be depicted from figure 2, high-altitude exposure induced polycythaemia with significant increase in blood relative standard quantitative parameters such as RBC count, hemoglobin concentration and hematocrit.

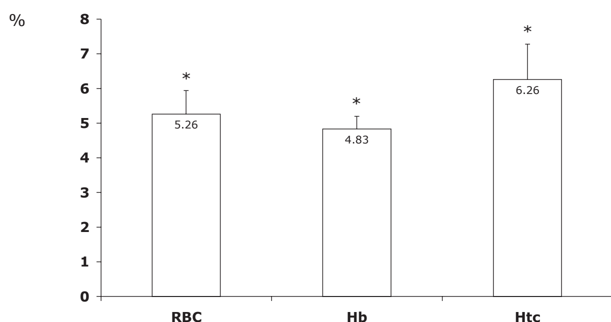

Figure 2. Increment (%) of RBC count (RBC), hemoglobin concentration (Hb) and hematocrit (Hct) induced by the high-altitude expedition. * p<0,05; After vs before the high-altitude expedition.

Plasma lipid peroxidation levels measured by the formation of TBARS and SH content as an indicator of protein oxidation are given in table 1. As can be observed, TBARS enhanced significantly after the expedition, whereas a significant decrease in SH protein content was found.

Regarding plasma antioxidant enzymes (Table 2), our data suggested that the high-altitude exposure period induced a distinct modulation pattern of their activities. In fact, SOD and GR activities were clearly up-regulated, though GPx activity was severely depressed by this long-term *in-vivo* hypoxic insult. As a consequence, the SOD/GPx ratio increased significantly in the end of the expedition. Likewise, our data showed a significant reduction in plasma TAS after the expedition, compared to pre-expedition baseline conditions.

Table 1. Plasma thiobarbituric reactive substances (TBARS) and protein SH content before and after the high-altitude expedition.

	Before expedition	After expedition
TBARS (M*10^{6})	6.98 ± 0.14	10.32 ± 0.32 *
SH (mol/g prot)	10.67 ± 0.21	8.24 ± 0.31 *

* p<0,05; After vs before the high-altitude expedition

Table 2. Plasma antioxidant enzyme activity of total superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (Gr), SOD/GPx ratio and total antioxidant status (TAS) concentration before and after the high-altitude expedition.

	Before expedition	After expedition
SOD (U/mL)	53,80 ± 5,32	69,80 ± 6,13 *
GPx (U/mL)	540,92 ± 22,20	268,74 ± 27,11 *
Gr (U/L)	49,60 ± 2,30	62,50 ± 3,62 *
SOD/GPx	0,10 ± 0,01	0,26 ± 0,01 *
TAS (mmol/L)	1,27 ± 0,06	1,11 ± 0,02 *

* p<0,05; After vs before the high-altitude expedition

Table 3. Erythrocyte antioxidant enzyme activity of total superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (Gr) and SOD/GPx ratio before and after the high-altitude expedition.

	Before expedition	After expedition
SOD (U/gHb)	1236,0 ± 60,4	1099,2 ± 110,4
GPx (U/gHb)	41,66 ± 7,3	53,82 ± 7,9 *
Gr (U/gHb)	8,06 ± 0,48	10,6 ± 0,86 *
SOD/GPx	33,42 ± 5,39	21,84 ± 3,10 *

* p<0,05; After vs before the high-altitude expedition

Concerning erythrocyte antioxidant enzymes (Table 3), significant increases in the activity of the glutathione-cycle related enzymes (GPx and GR) were observed after the expedition, whereas no change was observed in SOD activity. Therefore, the SOD/GPx ratio decreased significantly after the high-altitude exposure.

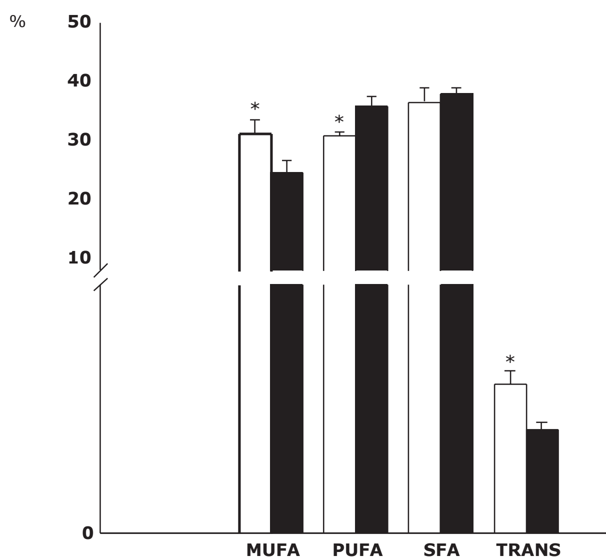

Figure 3. Erythrocyte membrane fatty acid profile expressed as percentage of total fatty acids in RBC membrane phospholipids. Levels of monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA) and trans fatty acids (TRANS) before (open bars) and after (solid bars) the high-altitude expedition. * p<0,05; After vs before the high-altitude expedition.

The erythrocyte membrane fatty acid composition expressed as a percentage of the total fatty acids in the erythrocyte membrane phospholipids is shown in figure 3. Regarding unsaturated fatty acids, our data showed that MUFA decreased significantly after the expedition, whereas the PUFA content was significantly enhanced in post-expedition erythrocytes. On the other hand, no changes in SFA content were observed after the expedition when compared to baseline conditions. TRANS isomers decreased significantly in erythrocyte membranes after the expedition.

Discussion

The overall picture of our results suggests that a significant up-regulation of the plasma and erythrocyte enzymatic antioxidant defence system occurred during the high-altitude expedition.

Concerning plasma antioxidant enzymes activity, our results could be interpreted as the cumulative effect of, at least, two distinct mechanisms. First, as suggested by others (Childs *et al.*, 2001), the increase in plasma SOD and Gr activities could have been directly due to oxidative damage inflicted to tissue membranes, resulting in increased enzyme leakage to plasma. This hypothesis corroborates our assumption regarding an enhanced condition of systemic stress and it seems to be supported by our data concerning increased concentration of lipid peroxidation markers (Table 1). On the other hand, since the method we used to measure total SOD enzyme activity does not differentiate between the different isoforms, our results could also be influenced by an increase in extracellular SOD (eSOD) secretion by endothelial cells in order to cope with an enhanced plasma pro-oxidant status (Spranger *et al.*, 1997; Horiuchi *et al.*, 2004).

In accordance with the kinetic founded for plasma SOD and Gr, a similar increase in plasma GPx activity after the high-altitude expedition would be expected. In fact, besides the contribution derived from the presumable tissue leakage, some studies (Tham *et al.*, 2002) suggest that, at least in animals, renal production of extracellular GPx (eGPx) may be sensitive to distal insults, such as inflammation or oxidative stress occurring in distinct organs or tissues, and that the kidney may respond to these distal insults by an increase in eGPx mRNA expression with a resultant increase in eGPx in the plasma. However, our data failed to show any GPx up-regulation, instead, a dramatic decrease was found after the expedition. The renal pathophysiological changes and blood flow disturbances described in humans submitted to hypobaric hypoxia (Singh *et al.*, 2003) could, eventually, justify the decrease in GPx activity observed in plasma after the expedition.

Nevertheless, future studies on plasma GPx kinetics are needed to highlight the true meaning of these results for overall regulation of antioxidant enzyme under sustained hypoxic-mediated oxidative stress conditions.

In the context of these systemic condition of enhanced oxidative stress, the antioxidant activity of plasma may be an important factor providing endothelial protection against oxidative damage perpetrated by several pro-oxidant compounds, such as ROS or oxidative metabolites such as phospholipid hydroperoxides (Maddipati and Marnett, 1987; Avissar *et al.*, 1996). However, taking into a account the critical physiological role of the balance between the first (SOD) and second step (GPx and/or catalase) antioxidant enzymes (de Haan *et al.*, 1996), the significant increase in plasma SOD/GPx ratio observed after the expedition, mainly due to a drastic decreased in GPx activity as referred above, seems to favor H_2O_2 accumulation and thus, could be indicative of a low plasma scavenging efficiency contributing to enhanced oxidative stress (Gaeta *et al.*, 2002). In fact, under this enzymatic disequilibrium, enhanced oxidative damage may occur since H_2O_2 could give rise to the highly reactive and deleterious hydroxyl radical ($OH^{\cdot}$) through a Fenton reaction (Halliwell and Gutteridge, 1999). These enzymatic data are consistent with the results provided by TAS analysis after the hypoxic period. TAS is an integrated parameter that considers the cumulative status of all the different antioxidants present in plasma and it provides an insight into the delicate *in vivo* balance between oxidants and antioxidants (Ghiselli *et al.*, 2000). Thus, the decrease in TAS observed after the expedition supported the hypothesis of an exacerbated systemic oxidative stress during this severe and prolonged hypoxic insult, which is confirmed by the increase in plasma lipid peroxidation and protein oxidation markers. In fact, the increased oxidative stress caused by high-altitude exposure seems to lead to peroxidative modification of membrane lipids measured as TBARS probably altering normal cell function, since polyunsaturated fatty acids are usually considered highly susceptible to ROS attack. These results are in accordance with other reports, in which real (Bailey *et al.*, 2000) or simulated (Joanny *et al.*, 2001) high-altitude conditions induced increase in serum and plasma lipid peroxidation by-products, respectively. Likewise, this prolonged hypoxic insult also induced a significant reduction in sulfhydryl residues (-SH), indicating increased disulfide linkages (-S-S-) from oxidativelly modified proteins and in a lesser extent from GSH oxidation, which probably contributed to loss of catalytic function and structural integrity of proteins (Stadtman and Levine, 2000). These data seem to support our previous assumption concerning the deleterious effects of persistent severe hypoxia even after a period of acclimatization (Magalhaes *et al.*, 2004a).

Regarding the erythrocyte antioxidant enzymatic modulation, our results showed that the activity of the glutathione cycle-related enzymes (GPx and Gr) seems to be up-regulated after the high-altitude expedition, increasing the intracellular capacity for reduced glutathione (GSH) utilization and re-synthesis. Because erythrocyte GSH accounts for most of the blood GSH (Halliwell and Gutteridge, 1999), these changes could be a sign of enhanced reactive oxygen species during hypobaric hypoxia and of a concomitant satisfactory response to elevate the erythrocyte antioxidant protection via the glutathione system. Moreover, since SOD activity was maintained and GPx increased significantly, the decrease in SOD/GPx ratio might be interpreted as a positive enzymatic coordinated induction to cope with enhanced free radical production. Thus, these adaptive enzymatic modulations suggest an increased antioxidant defense in erythrocytes under hypoxic environmental stress, and support the role of these blood cells as potential free radical buffers against high altitude-induced oxidative stress.

Due to the genetic inability of mature erythrocytes, the above results could only be explained by the appearance of newly formed erythrocytes in the blood circulation with a modified phenotype regarding the enzymatic antioxidant defense system. In fact, since the pro-oxidant *redox* status induced by hypoxia seems to be a systemic phenomenon affecting different tissues (Gonzalez and Wood, 2001; Joanny

et al., 2001; Magalhaes *et al.*, 2004a), it is possible that oxidative stress could also affect the bone marrow. This enhanced free radical production disturbing erythrocyte progenitor nucleated cells could activate signaling/transcription pathways and promote, among others, the synthesis of antioxidant enzymes. In this sense, after losing the nucleus, these newly formed erythrocytes will be equipped with strong and suitable antioxidant machinery to cope with enhanced oxidative stress.

In accordance with animals studies regarding chronic high-altitude exposure (Grandjean *et al.*, 1998), the present data on erythrocyte membrane fatty acid profile does not support a decrease in PUFAs. In fact, despite the unchanged (MUFA+PUFA)/SFA ratio, the membrane erythrocyte fatty acids content showed a significant increase in PUFAs, which could probably result in enhanced membrane fluidity (Halliwell and Gutteridge, 1999) and therefore in the facilitation of oxygen transport through the smallest capillaries to peripheral tissues. In addition, because of the steric differences between the two configurations of the unsaturated fatty acids, the increase in *cis*-double bound fatty acids observed in our study after the expedition, could also contribute to the erythrocyte ability to change shape and to squeeze through the smallest capillaries. In fact, the presence of a *cis* double bond introduces a bend that would create some non-conformability between chains and increase chain disorder, which seems to be a condition that enhances membrane fluidity (Subczynski and Wisniewska, 2000).

Considering that PUFAs are the fatty acids most susceptible to oxidative damage, and that their relative concentration could be considered as an indirect marker of membrane lipid peroxidation (Frei *et al.*, 1988; Grandjean *et al.*, 1998; Suarez *et al.*, 1999; Solans *et al.*, 2000; Griffiths *et al.*, 2001), an increase in PUFA's suggests that despite a presumable increased in ROS production, peroxidative erythrocyte damage was prevented, probably due to enhanced antioxidant capacity of RBCs. However, since in our study the climbers' nutritional regimen was not controlled, it can not be established whether or not these results were also due to significant changes in dietary habits favoring the increased consumption of lipid rich macronutrients, which could compensate an eventual increase in lipid peroxidation. In fact, since dietary fatty acids are exchanged with membrane fatty acids, dietary fat composition is reflected in membrane lipid composition and could influence cell function through effects on membrane properties. Accordingly, daily dietary fish oil supplementations seem to induce significant changes in membrane erythrocyte composition of humans (Guezennec *et al.*, 1989) and dogs (Grandjean *et al.*, 1998) submitted to moderate hypobaric hypoxia suppressing the decrease in red cell deformability found in control groups. Nevertheless, despite the strong scientific rationale supporting the influence of dietetic factors on membrane fatty acid profile throughout the half-life of the erythrocytes, the hypothesis that those membrane adaptations could be genetically induced in bone marrow during erythropoiesis must also be considered.

In conclusion, our main findings seem to support that a period of severe high-altitude exposure within a Himalayan expedition constitutes a systemic pro-oxidant challenge with deleterious consequences even to acclimatized climbers. Moreover, besides quantitative changes in RBC expression, significant alterations in erythrocyte antioxidant enzyme activity and membrane fatty acid profile seem to contribute to their preserved functionality under oxidative stress, which might be derived from their vital and irreplaceable role in oxygen transport.

Acknowledgments

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General Discussion

General Discussion

The overall picture of the studies comprised in this dissertation sustains the role of severe hypobaric hypoxia as an extreme harmful environmental stress for animals and humans submitted to acute or chronic exposure under simulated or high-altitude field-based conditions. In fact, complementing data collected in several other studies conducted in hypobaric hypoxia (reviewed in Cerretelli and Hoppeler 1996; Ward *et al*. 2000; Hoppeler and Vogt 2001), significant signs of cellular metabolic and morphological disturbances at different levels of cellular organization were found in all the experiments that constitute this dissertation. Moreover, despite the fact that many of the undesirable effects of the high altitude exposure could be attribute to several hazards of the environment, such as extreme cold (Kanstrup *et al*. 1999), low absolute humidity (Piccoli *et al*. 1996) and intense solar radiations (Ambach *et al*. 1993), the experiments developed under controlled simulated conditions both with animals (I, III and IV) and humans (II) confirmed that hypobaric hypoxia *per se* plays a major role in some of the deleterious physiological constrains associated to high altitude exposure.

Additionally, our data (I, II, III, IV, VI) clearly showed that a phenomenon of systemic and local (tissue) oxidative stress and damage, resulting from a broadly imbalance between oxidant production and the antioxidant capacity, takes place during hypobaric hypoxia exposure. In this sense, and like other pro-oxidant environmental stimuli (Askew 1995; Meacher and Menzel 2000; Weber 2000; Benedetti *et al*. 2004), hypobaric hypoxia seems to be a prone condition to exacerbate the production of RONS. Thus, in accordance with other studies dealing with systemic (reviewed in MacNee 2000; Dekhuijzen 2004) or local (Vanden Hoek *et al*. 1997; Powell *et al*. 2001; Monteiro *et al*. 2003) hypoxic or anoxic pathophysiological states, one might argue that hypobaric hypoxia truly engenders a biological paradox, i.e., too less molecules of stable oxygen seem to generate too much molecules of unstable and reactive oxygen with systemic and tissue deleterious consequences to organism. Therefore, although the use of O_2 as metabolic fuel allows a vital and attractive harvest of energy-rich phosphates *per* molecule of glucose, aminoacids or fatty acids, it seems that in oxygen depressed environments, such as high altitude, a significant fraction of the O_2 utilized by the body undergoes a univalent reduction, resulting in the formation of reactive oxygen and nitrogen species and their derivatives, and thus it is associated to mechanisms of cellular toxicity (Askew 2002; Bakonyi and Radak 2004).

Under several distinct experimental hypoxic conditions conducted with humans and animals and in accordance with others (Ilavazhagan *et al*. 2001; Joanny *et al*. 2001; Singh *et al*. 2001; Sarada *et al*. 2002), our data revealed both in plasma (II) and skeletal muscle (I, III and IV), significant disturbances in the glutathione system. Since glutathione (GSH) contains a sulfhydryl (-SH) residue, it plays an important role (i) maintaining the reduction-oxidation status by forming a disulfide bond, oxidized glutathione (GSSG), in the reaction catalyzed by glutathione peroxidase (GPx), and (ii) restoring the sulfhydryl bond in the reaction catalyzed by glutathione reductase (Gr) (Ji and Leichtweis 1997). In this way, the increase in the ratio GSSG/Total glutathione consistently found in our studies clearly suggests that under hypobaric hypoxia conditions an enhanced generation of pro-oxidant molecules overwhelmed the antioxidant capacity.

In accordance to data suggesting increased oxidative stress, we also found a regular pattern of systemic and tissue signs of oxidative damage confirming the incapacity of the antioxidant system to cope with the increased production of RONS under hypobaric hypoxia. In fact, lipid peroxidation evaluated through the increased levels of thiobarbituric acid reactive substances (TBARS) or malondialdehyde (MDA) and protein oxidation estimated by augmented content of carbonyl derivatives groups (CGs) or decreased sulfhydryl groups were found in human plasma (II and VI) and in mice skeletal muscle (I, III, IV) at distinct levels of cell organization. These data are consistent with other studies reporting oxidative damage in animals and humans submitted to hypobaric hypoxia under simulated or real high altitude conditions (Radak *et al*. 1994; Chao *et al*. 1999; Bailey *et al*. 2000; Ilavazhagan *et al*. 2001; Moller *et al*. 2001; Sarada *et al*. 2002; Lundby *et al*. 2003), and reflect the high susceptibility of different cellular constituents, namely lipids (Kappus 1985) and proteins (Stadtman and Levine 2000) to an exacerbated oxidative attack. Some *in-vivo* pharmacological treatments (I and IV, respectively) using L-buthionine–[*SL*]-sulfoximine (BSO), an overall cellular GSH deplector (Uhlig and Wendel 1992), and Vitamin E, the most powerful lipophilic chain-breaking antioxidant (Traber 2000), allow us to substantiate the oxidative aetiology of some of the cellular stress and damage imposed by the hypobaric hypoxic insults. In fact, in accordance with other studies (Wagner *et al*. 1996; Venditti *et al*. 1999; Mohamed *et al*. 2000; Leichtweis and Ji 2001) using the same pharmacological drugs, BSO administration exacerbated the stress and damage inflicted by hypobaric hypoxia, while Vitamin E supplementation attenuated the effects of the oxidant hypoxic insult.

Besides all the evidences showing that systemic physiological hypoxia induced by real or simulated high-altitude exposure exacerbate RONS production and oxidative stress, our results (II) also demonstrated that, in opposition to the model of ischemia/reperfusion (I/R) (reviewed in Ferrari *et al*. 2004), the levels of oxidative stress and damage in plasma, did not increased further after the reoxygenation induced by post-systemic hypoxia pressurization, at least, at the end of the reoxygenation period. These data rejected the hypothesis that, in many simulated or high-altitude field-based conditions of hypobaric hypoxia, like those tested in our studies (I, II, III, IV, V and VI), oxidative stress and damage mostly derived from the reoxygenation period and not from the period of hypoxia *per se*. Moreover, these results also reflect fundamental differences in the mechanisms responsible' for *redox* status homeostasis during conditions of physiological hypoxia-reoxygenation vs. the classical model of I/R. In fact, in clear contrast with the usual data from I/R model (reviewed in Walker 1991; Ferrari 1995; Rubin *et al*. 1996) reporting that oxidative stress and cellular injury are dramatically aggravated during the reperfusion period, our study (II) showed that, comparatively to the hypoxic phase, in the reoxygenation period the levels of oxidative stress and damage markers, at least in plasma, did not rise. In accordance, in a study conducted with rats submitted to an *in-vivo* protocol of normobaric hypoxia (10% O_2) followed by an normoxic (21% O_2) recovery period, Wood *et al* (1999a) showed through dihydrorhodamine 123 fluorescence signals that RONS generation increased significantly during hypoxia, when a deficit in physiological O_2 delivery to the tissues was imposed, although did not increased further, it even decreased, during the recovery reoxigenation period. Taking into account that in skeletal muscle I/R injury the conversion of xanthine dehydrogenase (XD) into the pro-oxidant oxidase form (xanthine oxidase - XO) is dependent from the severity of the ischaemic period (Kadambi and Skalak 2000), perhaps systemic hypoxia is not a sufficiently

severe hypoxic stress condition to induce an XD/XO conversion and, consequently, an exacerbated response of XO activity during the post-hypoxic reoxigenation period. In this sense, a pro-oxidant imbalance between the hypoxia mechanisms of RONS production, particularly mitochondria reductive stress as reported in study IV and elsewhere (Duranteau *et al*. 1998), and those related to reoxygenation would favor the former resulting in the lack of additional RONS production during the reoxygenation period. However, since Hoshikawa (2001) found an increased activation of XO during hypobaric hypoxia and our own data from study V revealed noticeable endothelial cell stress-related disturbances suggesting, at least in part, an oxidative aetiology that might have been mediated by XO, other possible explanations should be addressed in future studies.

In accordance with current knowledge regarding the protective role of acclimatization in several hypoxia-related organic disturbances (reviewed in West 1993; Green 2000), data from study III revealed that this gradual and staged process of hypobaric hypoxia adaptation attenuates substantially the impact of an acute hypoxic insult on skeletal muscle oxidative stress and damage markers. This protective role of acclimatization against oxidative stress assumes particular importance since some investigators (Bailey and Davies 2001; Bailey *et al*. 2001; Gelfi *et al*. 2004) had linked the development of some pathophysiological states associated to high altitude, at least in part, to a condition of increased RONS formation, i.e., to an oxidative aetiology. For example, justifying hypothetically some data obtained in study II, a field study with trekkers in Nepal at altitudes up to 5400m (Roncin *et al*. 1996) reported a marked effectiveness of Gingko Biloba (Egb761), a well documented oxygen-derived free radical-quenching (for refs see Roach and Hackett 2001), preventing acute mountain sickness (AMS). The authors reported that none of the Egb761 treated subjects developed AMS compared with 41% in the placebo-treated group, which could be partially explained by the possible role of its antioxidant properties preventing endothelial damage that could cause blood-brain barrier opening in AMS pathophysiology (Roach and Hackett 2001).

Being hypoxia *per se*, a triggering stimulus to increase cellular RONS generation, one possible explanation to the protective role of acclimatization is that the modification of several cardiovascular (reviewed in Mirrakhimov and Winslow 1966), ventilatory (reviewed in Bisgard and Forster 1996) and skeletal muscle (reviewed in Cerretelli and Hoppeler 1996; Hoppeler and Vogt 2001) parameters might attenuate the decrease in intracellular oxygen tension and contribute to diminish RONS formation. Additionally, Wood *et al*. (1999b) showed in mesenteric that, in clear contrast with non-acclimatized animals, no leukocyte rolling, endothelial adherence, migration into the perivascular space or increased vascular permeability occurred in acclimatized rats submitted to hypobaric hypoxia. Since inflammatory processes have been associated to RONS production in acute hypoxia (Moller *et al*. 2001), this might be another possible reason that contribute for the attenuation of oxidative stress and damage after a period of acclimatization. Furthermore, other mechanisms related to the increasingly complex behavior of RONS within biological systems might also contribute to justify the protective role of acclimatization against the stress imposed by hypobaric hypoxia. In fact, high concentrations of RONS are frequently accompanied by harmful processes to cell survival, such as lipid peroxidation and oxidative modification of proteins and nucleic acids. However, at low concentrations, RONS might act as secondary messengers responsible' for a signal transduction from extracellular

signaling molecules and their membrane receptors to the intracellular regulatory systems which control gene expression (reviewed in Thannickal and Fanburg 2000; Droge 2002; Turpaev 2002). Indeed, several molecular adaptations induced by chronic hypoxia, through the activation of the hypoxia-inducible factor 1 (HIF-1), such as the increased expression of vascular endothelial growth factor (VEGF), erythropoietin (EPO), glucose transporters (glut1 and 3) and some glycolytic enzymes (Hoppeler *et al*. 2003) seem to be, partially, related to moderate disturbances in cellular *redox* status (Clanton and Klawitter 2001; Droge 2002; Turpaev 2002). Moreover, the exposure of organisms to graduated mild oxidative stress often causes a prompt increased expression of antioxidants and other defense systems that help to protect the cell against the insult and sometimes against oxidative insults applied subsequently (Halliwell and Gutteridge 1999). Accordingly, the total activity of the antioxidant enzyme superoxide dismutase (tSOD) in mice skeletal muscle (III) and of some glutathione-cycle related enzymes, like glutathione peroxidase and glutathione reductase in human erythrocytes (VI), increased significantly after chronic hypobaric hypoxia exposure. This antioxidant enzymatic modulation correlated well, respectively, with the decreased levels of lipid peroxidation and protein oxidation in mice skeletal muscle (III), and with the preservation of the relative content of polyunsaturated fatty acids in human erythrocytes (VI). Concerning erythrocytes, this up-regulation of the enzymatic machinery assumes particular importance. Indeed, since mature erythrocytes lack DNA, i.e., have genetic inability to synthesize new proteins and to trigger biomolecular repair and regenerating mechanisms, our data suggest that (i) the pro-oxidant *redox* status induced by hypoxia exposure affect several tissues, including the bone narrow and that (ii) newly formed erythrocytes are "equipped" with strong and suitable antioxidant machinery to cope with enhanced oxidative stress. In accordance with a modifiable phenotype of the enzymatic antioxidant defense system, Wood *et al*. (1999b) found, in the above referred acclimatized mice submitted to hypobaric hypoxia, a clear relationship between the decreased levels of leukocyte adherence and migration through the endothelial cells of the mesenteric and the increased levels of nitric oxide due to an up-regulation of the inducible nitric oxide synthase (iNOS) (Wood *et al*. 1999b). Since increased leukocyte adherence and migration in non-acclimatized mice seems to be related to nitric oxide depletion via mechanisms involving peroxynitrite formation (Arteel *et al*. 1999), the up-regulation of iNOS had probably reduced the magnitude of the inflammatory response contributing for the attenuation of RONS production. Nevertheless, conflicting data regarding a hypoxia-mediated antioxidant enzymatic modulation are present in literature. Indeed, data from Radak (1994) and Nakanishi (1995) revealed a decreased activity of some antioxidant enzymes in several tissues of rats chronically exposed to simulated altitudes of 4000m and 5500m, respectively. However, differences related to selective tissue-sensitivity to oxidative stress and, particularly, to an altitude-dependent effect, might probably explain some discrepancies between these and our results.

Nevertheless, in accordance with data obtained in non-acclimatized humans submitted to acute hypoxia (II) and with results reported by Joanny (2001) in Operation Everest III, a trend to a gradually increased oxidative deleterious effect was found in animal skeletal muscle (III) during persistent severe hypoxia even after a previous period of acclimatization. Moreover, this condition of increased oxidative stress and damage under prolonged and severe high altitude exposure was also found in the acclimatized climbers involved in study VI, and suggests a cumulative time-dependent increased of pro-oxidant

stress and damage throughout the time of hypoxia exposure both in non-acclimatized and acclimatized conditions. That is, the severity of the oxidative stress and consequent damage inflicted to some cell constituents follows an incremental and degenerative course, probably related to the increasing incapacity of the antioxidant system to cope with a continuous and exacerbated production of RONS. In accordance, recent data (Jefferson *et al*. 2004) obtained in the Andean plateau showed that high altitude natives living in Peru at 4300m, compared to seal level residents, presented elevated levels of urinary F(2)-isoprostane, 8-iso PGF(2 alpha), plasma TBARS and plasma total glutathione, denoting a persistent metabolic condition of increased oxidative stress and oxidative peroxidation. Nevertheless, corroborating the distinct regional pattern of altitude adaptation found in Andean populations and Tibetans from the Himalayan plateau (reviewed in Moore *et al*. 1998; Beall 2000), a new proteomic approach in Tibetans high altitude natives (Gelfi *et al*. 2004) brought new insights concerning the relationship between prolonged high altitude exposure and oxidative stress. Hence, Tibetans born and living between 3500 and 4500m, compared to Nepali control subjects resident a 1300m, demontrated an increased expression (+80%) of the enzyme glutathione-S-transferase in skeletal muscle, particularly of the P1-1 isoform, and had decreased levels of 4-hydroxynonenal (4-HNE) protein adducts. Given that this enzyme plays an important role in cellular detoxication by conjugating GSH with a range of electrophilic acceptors, the authors argue for a close association between this phylogenetic antioxidant enzymatic modulation and the levels of aldehydes (4-HNE) generated by RONS attack to lipid membranes. Moreover, these results were also compatible with the observation of a reduced accumulation of lipofuscin pigments in Tibetans skeletal muscle, which suggest that, in contrast to their Andean counterparts and with acclimatized climbers (as supported in study V), Tibetans might had had activated a RONS detoxifying system throughout the centuries that prevents and/or limits tissue damage of an oxidative aetiology, and thus preserve muscle function allowing them to live and be better succeed in such hostile environments.

Amongst other reasons, one possible explanation to the gradual increased oxidative deleterious effect during persistent severe hypoxia exposure is that, despite the contribution of the orchestrated mechanisms to diminish the arterial hypoxemia and related tissue hypoxia during the course of an acclimatization process, cellular oxygen levels at high-altitude seem to be far below the normal sea-level values (West 2003) and thus, even the acclimatized body remains hypoxic compromising tissue *redox* status homeostasis. In such cellular hypoxic conditions, not only the mechanisms responsible for RONS production are triggered, but also the antioxidant defense system could be compromised. For instance, increased utilization of glucose-6-phosphate through the glycolytic pathway (Jones 1985; LeGrand and Aw 1998) might result in decreased substrate availability for pentose phosphate pathway limiting NADPH supply for glutathione reductase, thereby, increasing the sensitivity of hypoxic tissues to oxidative stress and injury by impairing GSSG reduction and consequently compromising hydroperoxide metabolism (Jones 1985; LeGrand and Aw 1998). Moreover, the loss of appetite and the consequent reduced food intake clearly observed in our study III and described elsewhere (Westerterp-Plantenga *et al*. 1999; Ward *et al*. 2000), besides resulting in deficient dietary ingestion of antioxidant compounds, might also limits glucose availability and, indirectly, affects GSH turnover and glutathione *redox* status (Leeuwenburgh and Ji 1996) exacerbating oxidative stress and damage.

Above and beyond the modulation of some antioxidant enzymatic mechanisms under

chronic hypoxia (III and VI), data obtained in acute and chronic hypoxic conditions, both at tissue (I and III) and organelle levels (IV), showed an up-regulation of the expression of heat shock proteins of 60 and 70 kDa (HSP60, 70). The expression of HSPs correlated well with the magnitude of the physiological stress and damage in all the studies (I, III and IV) confirming the aggression perpetrated by hypobaric hypoxia at distinct levels of skeletal muscle organization. Moreover, HSPs content corroborated the impact of the glutathione depletion induced by BSO on tissue antioxidant capacity (I), as well as, the protective role of Vitamin E against these deleterious cellular conditions (IV), which reinforces the suspicion of a predominant oxidative aetiology for the damage inflicted by hypoxia. On the other hand, their overexpression also suggests that these molecular chaperones are, collectively or independently, involved with other defense mechanisms in the protection against the oxidative damage mediated by hypobaric hypoxia (I, III and IV). In fact, taking into account that in those studies the levels of HSPs paralleled the severity of the oxidative damage but were inversely proportional to the antioxidant and scavenging capacity in distinct levels of cellular organization, skeletal muscle HSP60 and HSP70 seem to act as a second line of defense against hypoxia-induced oxidative stress and damage. In accordance, intermittent (Zhong *et al*. 2000) and continuous (Wen *et al*. 2002) chronic altitude-hypoxia exposure had respectively showed to increase resistance and survival of rats from cardiac ischaemic injury by protective mechanisms related to the overexpression of HSP70. Moreover, Zhong (2000) demonstrated that the overexpression of HSP70 and the increased tolerance to subsequent acute ischemia/reperfusion injury could last for 2 weeks after the rats returned to normoxia. These findings might have particular interest in the current high altitude-field practice used by experienced climbers at high altitude. Actually, since a prolonged stay at altitudes above 5000m results in organic deterioration rather than continued improvement through acclimatization, climbers whom are setting up routes and camps up to 8000m usually return to base camp for several days before moving up rapidly for the summit climbs (West 1993). In such conditions of intermittent high altitude exposure, the hypothetical overexpression of HSPs during the permanence at moderate altitude, similarly to a preconditioning ischemic strategy (Lepore *et al*. 2001; Snoeckx *et al*. 2001), might contribute as an endogenous defensive mechanism to counteract, at least partially, the exacerbated oxidative stress and damage inflicted by the exposure to extreme high altitude. Thereby, the overexpression of HSPs induced by hypobaric hypoxia during the acclimatization, as reported in study III, might be considered an additional hypothetical mechanism to counteract the exacerbated physiological stress suffered by climbers, especially in extreme high altitude expeditions. Although the biochemical and molecular mechanisms responsible' for HSP60 and HSP70 induction were not determined in our studies (I, III and IV), the increased oxidative stress and damage, among other possible hypoxia-inducible signaling mechanisms, could explain, at least in part, their overexpression (Locke *et al*. 1995; Noble 2002). In fact, the enhanced production of RONS has been described as a signal for the up-regulation of HSPs (Hamilton and Powers 2002) due to their intrinsic protection against oxidative deleterious stimuli, interacting with other proteins and minimizing the probability of these other proteins to interact inappropriately with others, i.e., facilitating protein synthesis, folding and assembly, and ultimately minimizing the extent of the cellular decay (Feder and Hofmann 1999; Sammut *et al*. 2001). Concerning the impact of the hypoxic insult on mitochondrial functionality (IV), this hypothetical protective complementary effect assumes particular importance since,

when O_2 is limited, the levels of some mitochondrial and nuclear genome encoding proteins of ETC are repressed, which compromise mitochondrial regenerative processes (Webster *et al*. 1990; Vijayasarathy *et al*. 2003) and consequently mitochondrial respiratory features.

Taking into account the current hypothesis concerning the mechanisms involved in the phenomenon of hypoxia-mediated oxidative stress (reviewed in Askew 2002) and also reported in studies I, II, III and VI, data obtained in study IV support that skeletal muscle mitochondria is able to increase significantly the rate of superoxide radical production leading to the overwhelming of the antioxidant capacity and, consequently, to oxidative stress and damage. These findings were consistent with several data obtained elsewhere (Lemasters and Nieminen 1997; Vanden Hoek *et al*. 1998; Kulisz *et al*. 2002) and confirm the role of mitochondria as an important source of reactive oxygen species under hypobaric hypoxic conditions. Moreover, according to others (Yen *et al*. 1999; Paradies *et al*. 2002), our data showed that under these pro-oxidant conditions, mitochondria become oxidatively targeted, which results in increased lipid peroxidation and protein oxidation of their constituents. Consequently, hypoxia-induced oxidative stress (IV) acted as a mitochondrial-uncoupling stimulus and, as an inhibitor of the mitochondrial respiration and oxidative phosphorylation, contributing to decreased mitochondrial phosphorylation efficiency, i.e., to a less efficient utilization of oxygen *per* molecule of ATP synthesized. This impairment of the mitochondrial functionality, expressed by the loss of oxidative power and the increase of inner and outer membrane permeability seems to justify several metabolic disturbances that culminate in mitochondrial failure and, at a cellular dimension, in the triggering of cellular intrinsic pathway of apoptosis, as supported by study IV in skeletal muscle.

Being mitochondria the powerhouse cellular organelles, it is reasonable to argue that in such oxygen-limited environments, the oxidative stress-mediated mitochondria malfunction and ATP impaired production, might be dramatic and will probably be involved in the aetiology of many of the high altitude deleterious constrains. In fact, this mitochondrial dysfunction probably explains several skeletal muscle morphological evidences previously demonstrated by others (reviewed in Cerretelli and Hoppeler 1996; Hoppeler *et al*. 2003) and also confirmed in study V, disclosing signs of notorious mitochondrial swelling, cristae degeneration and significant accumulation of lipofuscin-like pigments probably resulting from oxidant-mediated mitochondrial degradation products. Indeed, this lipofuscin accumulation within the cells of capillary walls is consistent with current information (Robison *et al*. 2000) about the effects of enhanced cellular autoxidation and consequent accumulation of highly peroxidized membrane remnants as lipofuscin in several tissues. Since lipofuscin might be considered as a degradation product possibly formed by mitochondria lipid peroxidation that characterizes cytological damage incurred by enhanced free radical formation in muscle cells, it also corroborates a condition of oxidative stress associated to high-altitude exposure (Fluck and Hoppeler 2003). In accordance, the morphological and biochemical findings obtained from the humans engaged in the Himalayan high-altitude expedition (V), denoting profound structure alterations in skeletal muscle organization and evident deleterious morphological and biochemical microvasculature modifications, correlated well with the systemic redox disequilibrium (VI) and thus, might had had, in some cases, an oxidative aetiology (Hoppeler *et al*. 2003; Gelfi *et al*. 2004). Taken together, these cytological disturbances as well as the above referred mitochondrial dysfunction with increased permeability and consequent leak of pro-apoptotic inductors, might be involved in the development of several skeletal

muscle physiopathological states, including atrophy that characterizes the phenotype of both lowlanders acclimatized climbers engaged in prolonged sojourns at high altitude and highlanders natives (Fluck and Hoppeler 2003; Gelfi *et al*. 2004).

Conclusions

Conclusions

Based in the general conclusions of each of the different studies presented in this dissertation, it seems possible to highlight the following major conclusions:

a) The exposure of animals and humans to acute or chronic hypobaric hypoxia, under simulated or real high altitude conditions, engenders an increased oxidative stress and damage in blood and in skeletal muscle, with biochemical, functional and histological deleterious consequences at distinct levels of cellular organization, that increases throughout the time spent in hypobaric hypoxia, both in acclimatized and non-acclimatized animals and humans;
b) The reoxygenation period after a severe hypobaric hypoxia insult does not increase further the levels of oxidative stress and damage induced by the previous phase of simulated altitude exposure, at least, at the end of the reoxygenation period;
c) The levels of oxidative stress and damage induced by severe hypobaric hypoxia do not increase further after the reoxigenation period
d) A gradually staged process of acclimatization to real or simulated high-altitude hypobaric hypoxia attenuates the levels of oxidative stress and damage induced by acute hypoxia;
e) An antioxidant treatment with Vitamin E protects skeletal muscle mitochondria against hypobaric hypoxia-mediated enhanced oxidative stress and damage, mitochondrial dysfunction and the triggering of the intrinsic pathway of cellular apoptosis;
f) In consequence of a chronic hypobaric hypoxia exposure, besides quantitative changes in red blood cells expression, significant alterations in erythrocytes antioxidant enzyme activity and membrane fatty acid profile seem to contribute to erythrocyte preserved functionality under oxidative stress conditions.

In summary, this dissertation presents an interesting scenario where RONS may actually play an important role during hypobaric hypoxia exposure by modulating cell *redox* status, homeostasis and function in a hypoxic cell, tissue and organism.

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Appendix