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Vitamin E prevents hypobaric hypoxia-induced mitochondrial dysfunction in skeletal muscle

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Abstract

The effect of vitamin E on mice skeletal muscle mitochondrial dysfunction and oxidative damage induced by an *in vivo* acute and severe hypobaric hypoxic insult (48h at a barometric pressure equivalent to 8500m) was analyzed.

Twenty-four male mice were randomly divided into the following 4 groups (n=6): control (C), hypoxia (H), vitamin E (VE; 60mg.kg⁻¹ i.p., 3 times/wk for 3 wks) and hypoxia+VE (HVE).

Significant increase in mitochondrial protein carbonyls groups (CGs) was found in H compared to C. Confirming previous observations from our group, hypoxia induced mitochondrial dysfunction, as identified by altered respiratory parameters. Hypoxia exposure increased Bax content and decreased Bcl-2/Bax ratio, although Bcl-2 content remained unchanged. Inner and outer mitochondrial membrane integrity were significantly affected by hypoxia exposure. However, vitamin E treatment attenuated the effect of hypoxia on mitochondrial oxidative phosphorylation and on the levels of CGs. Vitamin E supplementation also re-established the Bax and Bcl-2/Bax ratio impairments caused by hypoxia, as well as the decreased inner and outer mitochondrial membrane integrity.

In conclusion, data suggest that vitamin E prevents the loss in mitochondrial integrity and function, as well as the increase in Bax content that suggests that mitochondria are involved on increased cell death induced by severe hypobaric hypoxia in mice skeletal muscle.

Keywords: high altitude, antioxidants, α -tocopherol, membrane integrity, mitochondrial respiration, apoptotic signaling.

Introduction

It has been known for a long time that high-altitude exposure is an aggressive physiological stress which induces several cellular deleterious effects. Recent data evidenced that severe high-altitude hypoxia triggers increased oxidative stress with consequent damage inflicted to lipids, proteins and DNA [1-5]. Increased cellular oxidative stress is a favorable condition for the activation of apoptotic cellular death pathways [6]. Apoptosis is a systematic and programmed dismantling of the cell into discrete packages, being a phenomenon that can involve signal transduction pathways originating from mitochondria. In fact, mitochondria are intimately involved in apoptosis as important pro-apoptotic proteins are located in the inter-membrane space; furthermore, mitochondria are an active source of reactive oxygen and nitrogen species (RONS), which can also modulate the induction of cell death. Some mitochondrial proteins with an active role in the apoptotic process are cytochrome *c* and apoptosis inducing factor (AIF), which can be both released into the cytosol upon death stimuli, leading to cell death through distinct pathways [7, 8]. The release of either AIF or cytochrome *c* from mitochondria is thought to be partially dependent on the formation of a specific channel within the inner mitochondrial membrane, usually known as permeability transition pore (PTP), leading to outer membrane rupture [8]. Although some controversy surrounds the role of PTP in the control of mitochondria-mediated apoptosis, consistent data indicates that the formation of PTP complexes (formed by the adenine nucleotide translocator, the voltage-dependent anion channel and cyclophilin-D, among other proteins) on the inner mitochondrial membrane is involved in the release of pro-apoptotic proteins during apoptosis [see 8, 9]. It is also known that the conformational status of mitochondrial PTP is regulated by the content of Bcl-2 family proteins, which comprises both pro (i.e. Bax, Bad, tBid, Bak, Bok) and anti (Bcl-2, Bcl-XL, Bcl-w) apoptotic elements; the relative levels of each group (*viz* Bax/Bcl-2 ratio) determines the response to death stimuli [10]. Moreover, it is known that the enhanced production of RONS and the consequent oxidation of protein thiol groups, can contribute to PTP opening and determine the pore conductance and reversibility [11-13].

In a recent study from our laboratory [14], we reported that 48 hours of severe hypobaric hypoxia at a barometric pressure equivalent to 8500m induced mice skeletal muscle mitochondrial oxidative stress and damage, as well as respiratory deficit using substrates for both complex I and complex II. We also observed that vitamin E, a lipophilic antioxidant, was able to attenuate mitochondrial dysfunction induced by

hypobaric hypoxia. However, to the best of our knowledge, the impact of severe hypobaric hypoxia at a barometric pressure equivalent to 8500m on the activation of the intrinsic, mitochondrial-dependent, pathway of apoptosis, in skeletal muscle was not described so far. Most particularly, one of the aims of the present work was to investigate if hypobaric hypoxia damages mitochondrial membranes. Based on previous studies in which mitochondrial oxidative stress and dysfunction elicited by other stimuli resulted in the increased susceptibility to apoptosis [15, 16], it seems reasonable to hypothesize that skeletal muscle mitochondrial oxidative damage and respiratory dysfunction induced by hypobaric hypoxia plays a key role in tissue damage and is correlated with the activation of apoptosis. Given that vitamin E is one of the most important fat-soluble chain-breaking antioxidant in the body, another purpose of the present study was to analyze whether *in vivo* vitamin E treatment prevents or attenuates the impairment of mitochondrial respiration, membrane integrity and apoptosis induced by severe high-altitude hypobaric hypoxia in mice skeletal muscle.

Methods

Experimental Design

Twenty-four male Charles River CD 1, 8 wk aged mice were randomly divided into four groups (n=6) as follows: control (C), hypoxia (H), vitamin E and hypoxia+VE (HVE).

Two groups (VE and HVE) were intra-peritoneally (i.p.) treated with cumulative doses (three times a week during a course of three weeks, 60mg/Kg) of α -tocopherol acetate (vitamin E). The other two groups (C and H) received the correspondent vehicle solution (sterilized soybean oil) during the same period [17].

Immediately after the last injection, the animals of the C and VE groups were maintained at an atmospheric pressure of 101.3 kPa (760 mmHg) equivalent to sea level. Again immediately after the last injection and one at a time, the animals of the hypoxic groups (H and HVE) were acutely exposed during 48 hours inside a hypobaric chamber to a simulated atmospheric pressure of 35.2 kPa (265 mmHg) equivalent to an altitude of 8500m. The depressurization period to reach the simulated altitude of 8500m and the pressurization period back to sea level conditions lasted for 30 minutes each. All animals were kept at constant temperature (21-25°C) on a daily light schedule of 12 hours of light vs. dark with normal activity. Mice were provided with 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 the preparation of isolated mitochondria. Skeletal muscle mitochondria were prepared by conventional methods involving differential centrifugation, as previously described by Tonkonogi and Sahlin [18]. Briefly, muscles 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. 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) and stirred for 2 minutes. The sample was then 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 700xg for 10 minutes. The resulting pellet was removed and the supernatant suspension centrifuged at 10,000xg 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 7,000xg for 3 minutes. The supernatant was discarded and the final pellet, containing the mitochondrial fraction, was gently resuspended ($0.4\mu\text{l}\cdot\text{mg}^{-1}$ initial tissue) in a medium containing 225mM mannitol, 75mM sucrose, 10mM Tris and 0.1mM EDTA, pH 7.4. All mitochondrial isolation procedures were performed at 0-4°C. Mitochondrial protein concentration was spectrophotometrically estimated with the biuret method 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 (0-4°C) throughout this period. There was no significant alteration of the mitochondrial RCR between the first and the last measurements of the same animal.

Aliquots of the mitochondrial suspension were separated and rapidly frozen at -80°C for later biochemical analysis of vitamin E, protein carbonyl derivatives (CGs), Bax and Bcl-2.

Measurement of mitochondrial respiratory activity

Mitochondrial respiratory function was polarographically measured using a Clark-type oxygen electrode (Hansatech DW 1, Norfolk, UK). Reactions were conducted in a 0.75ml closed thermostated (25 °C) and magnetically stirred glass chamber containing 0.5mg of mitochondrial protein in a reaction buffer containing 225mM mannitol 75mM sucrose, 10mM Tris, 10mM KCl, 10mM K₂HPO₄ and 0.1mM EDTA, pH 7.5 in accordance to Tonkonogi *et al* [19]. After 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5mM) plus malate (2mM) or succinate (10mM) plus rotenone (4μM). State 3 respiration was determined after adding ADP to a final concentration of 200μM; state 4 respiration was measured as the rate of oxygen consumption which follows total ADP phosphorylation. The respiratory control ratio (RCR, the ratio between state 3 and state 4) and the ratio between the amount of ADP phosphorylated per oxygen consumed (ADP/O) were calculated according to Estabrook [20] and by using 235 nmolO₂/ml as the value for the solubility of oxygen at 25°C. The lag phase, determined as the time in seconds reflecting the capacity of mitochondrial to depolarize and repolarize after ADP addition, was also measured for both malate+pyruvate (M-P) and succinate+rotenone (S-R) as substrates.

Oligomycin (final concentration 1.5μg.ml⁻¹) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 2μM) were added during state 3 respiration with saturated amounts of ADP (final concentration 1mM).

Inner and outer mitochondrial membrane integrity

An estimate of the level of inner membrane integrity was made by measuring the ratio of uncoupled respiration induced by CCCP addition versus the rate in state oligomycin (CCCP/oligomycin) using M-P as substrates, according to Benamar *et al.* [21] with adaptations. It has been described that the ratio increases as the inner membrane impermeability to protons increases [21].

Outer-membrane integrity was also assayed according to Benamar *et al.* [21] (see Fig. 4). Briefly, 30 μM cytochrome *c* reduced by 10 mM ascorbate originated an initial oxygen consumption corresponding to the activity of a fraction of mitochondria with damaged outer membranes. The complete rupture of the outer membrane in mitochondria was achieved by the addition of 0.05 % (v/v) Triton X100. The final addition of 0.5 mM

cyanide made possible the measurement of the non-enzymatic oxidation of cytochrome c. The percentage of mitochondrial outer membrane integrity was calculated according to the following formula:

Outer membrane integrity = 100% – % of outer membrane rupture, where:

100% integrity = (respiration after cytochrome c + triton) – (respiration after cyanide)

% of outer membrane rupture = respiration after cytochrome c - respiration after cyanide

Biochemical assays in mitochondria extracted fraction

Vitamin E concentration

α -tocopherol was determined by high performance liquid chromatography (HPLC) according to Vatassery *et al.* [22]. Briefly, 2ml ethanol containing 0.025% (w/v) butylated hydroxytoluene (BHT) and 0.1ml of 30% (w/v) ascorbic acid was added to an aliquot of 100 μ l mitochondrial extract. Tubes were cooled and 2ml of water were 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 40°C. The residue was re-dissolved in the 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. α -tocopherol content was fluorimetrically detected ($\lambda_{\text{emission}}$: 290nm, $\lambda_{\text{absorption}}$: 330nm).

Analysis of Bax, Bcl-2 and protein carbonylation,

Equivalent amounts of proteins were separated by electrophoresis 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 TBS-T [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) 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 assay, a certain isolated mitochondrial volume (V) containing 20 μ g 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 β -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 0.001 μ g/ μ L, 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 immunoblots with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshire, UK), according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software (Bio Rad). Optical density results are expressed as percentage variation of control values.

Statistical analysis

Mean and standard deviation 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

Skeletal muscle mitochondria from VE group showed a significant higher content of vitamin E than the C group (1.24 \pm 0.21 vs. 0.65 \pm 0.13 nmol.mg⁻¹ of mitochondrial protein). Mitochondria from both hypoxic groups (H and HVE) had a significant decrease in vitamin E levels when compared to their non-hypoxic counterparts (C and VE) (0.12 \pm 0.06 vs. 0.65 \pm 0.13 and 0.46 \pm 0.12 vs. 1.24 \pm 0.21 nmol.mg⁻¹ of mitochondrial

protein). However, the vitamin E content of the H group was significantly lower than the HVE group.

Mitochondrial protein oxidative damage

Hypoxia exposure inflicted significant protein oxidation, as observed by the oxidation of protein carbonyls (Fig 1). A significant increase in mitochondrial CGs was found in the H group when compared to the C group. Vitamin E protected mitochondria from CGs formation induced by hypobaric hypoxia, although the values found on the HVE group were still higher than on the VE group.

**** INSERT FIG. 1 ****

Standard mitochondrial respiratory parameters

Mitochondrial respiration was significantly impaired in H as demonstrated by decreased state 3, respiratory control ratio (RCR) and ADP/O, as well as by the enhanced state 4 using both complex I and II-linked substrates. However, vitamin E treatment attenuated the effect of hypoxia on state 3, RCR, ADP/O and abolished hypoxia-induced changes in state 4 with M-P as substrates (data not shown).

Hypoxia significantly increased the phosphorylation lag-phase with both M-P (87.0 ± 11.03 seconds) and S-R (70.20 ± 10.22 seconds) when compared to control (60.24 ± 8.01 and 45.80 ± 6.76 seconds, respectively). Vitamin E treatment significantly reduced the augmented phosphorylation lag phase caused by hypoxia (to 73.20 ± 8.21 with M-P and 51.0 ± 6.01 seconds with S-R).

Mitochondrial inner and outer membrane integrity

As can be depicted from figure 2, mitochondria energized with M-P from the H group showed a significantly lower CCCP/oligomycin respiratory ratio when compared with all other groups (H vs. C, VE and HVE groups) indicating a higher inner membrane permeability to protons. However, vitamin E treatment attenuated the increased inner membrane permeability induced by hypoxia (H vs. HVE groups).

The intactness of the mitochondrial outer membrane was quantified by measuring the cytochrome *c*-dependent oxygen consumption (Fig 3). If mitochondria have intact outer membranes, exogenously added reduced cytochrome *c* cannot be oxidized by cytochrome *c* oxidase on the inner membrane as it cannot cross the outer membrane.

However, when the outer membrane is damaged, the oxidation can occur with an increased rate of oxygen consumption by mitochondria. Thus, the higher rate of oxygen consumption upon cytochrome *c* addition to mitochondrial suspension in H compared to other groups is indicative of the elevated permeability induced by severe hypobaric hypoxia on skeletal muscle mitochondria. In agreement with protection observed for other parameters, vitamin E attenuated the increased outer membrane permeability caused by hypobaric hypoxia (H vs. HVE).

**** INSERT FIG. 2 ****

**** INSERT FIG. 3 ****

Bax and Bcl-2 mitochondrial content

In what concerns to the expression of proapoptotic protein Bax (Fig. 4), the results showed a significant increase in animals submitted to acute and severe hypoxia when compared with both the C (+32.02%) and the HVE group (+22.54%). There was no significant difference in the levels of Bax between C, VE and HVE groups. On the other hand, the levels of the antiapoptotic protein Bcl-2 did not differ significantly within all groups independently of the conditions. Therefore, the Bcl-2/Bax ratio (Fig. 4) revealed a significant decrease in the H compared with the C group. Vitamin E supplementation was able to counteract the triggering of the apoptotic pathway induced by acute and severe hypobaric hypoxia.

**** INSERT FIG. 4 ****

Discussion

The results of the present study provided additional insights into the effects of vitamin E treatment against the impact of an *in vivo* acute and severe hypobaric hypoxic insult on mice skeletal muscle mitochondria. Data confirms the mitochondrial respiratory derangements and increased oxidative damage which was previously described by our own group [14]. Nevertheless, the present study is the first to show the relationship between impaired skeletal muscle mitochondrial function, increased inner and outer mitochondrial membrane permeability and also increased Bax expression caused by 48 hours of *in vivo* severe hypobaric hypoxia exposure. Data showed that the hypoxic insult

induced depletion on mitochondrial vitamin E content, increased protein oxidation, impaired mitochondrial respiratory function and decreased inner and outer mitochondrial membrane integrity, while decreased Bcl-2/Bax ratio due to increased Bax expression. Additionally, our study also demonstrated that vitamin E treatment was able to attenuate the impact of this hypoxic oxidant insult on mitochondrial function and on apoptotic signalling of mice skeletal muscle. Nevertheless, considering that mitochondria were isolated from different hindlimb muscles (soleus, gastrocnemius, tibialis anterior, and quadriceps) characterized by distinct biochemical features, data should not be extrapolated to each muscle individually. It is also important to note that *in vitro* studies performed after isolation of mitochondria from animal models have the ability to identify pre-existent defects on mitochondrial bioenergetics that can provide valuable information on the effects observed in the living organism. The experimental protocol, that can involve the assessment of mitochondrial respiratory parameters, calcium loading capacity or alterations in protein expression, represents a powerful tool for generating convincing hypotheses regarding how mitochondria are affected by xenobiotics, pathologies or environmental alterations to which the intact system is exposed to. Nevertheless, further complementary *in vivo* studies (*viz.* NMR) of mitochondrial metabolism would allow to take the next step of linking *in vitro* results with metabolic function in the intact organism [23, 24].

Regarding respiratory measurements, the decreased state 3 respiration and the increased phosphorylative lag phase obtained with energized mitochondria using both complex I and II substrates, as well as the increased rate of oxygen consumption during state 4 are consistent with data previously published by our group [14], and confirms that hypobaric hypoxic conditions induces mitochondrial dysfunction. Accordingly, the results also showed a decrease in RCR and ADP/O values, which are indicative of decreased coupling between respiration and ATP synthesis and decreased phosphorylation efficiency, respectively. The results from this and our previous work [14] indicate that hypoxia jeopardizes the ability of mitochondria to synthesize ATP. Moreover, both inner and outer membrane integrity assays revealed that the severe hypoxic exposure caused a decrease in the impermeability of these mitochondrial membranes. Altogether, these data suggest that severe hypobaric hypoxia may have inflicted mitochondrial functional and structural impairments through several distinct mechanisms. For example, the hypoxia-related reduced oxygen availability as electron acceptor seems to result in the accumulation of reducing equivalents throughout the components of the mitochondria

electron transport chain. The end result elicits the impairment of the respiratory chain activity, the increase in the so-called electron leakage to oxygen, and the consequent enhanced production of RONS [25, 26] resulting in increased mitochondrial oxidative damage to lipids and proteins, as demonstrated in ours and in other studies [14], which ultimately might compromise mitochondrial function. On the other hand, hypoxia may also increase calcium content in mitochondria, which accompanied with RONS and phosphate (Pi) accumulation may predispose to mitochondrial swelling and eventual PTP opening, thereby inducing membrane permeabilization, collapse of the transmembrane osmotic gradient and mitochondrial dysfunction [27]. Moreover, as suggested above, Pi tends to increase in hypoxic cells due to phosphocreatine cleavage, which may also had contributed to swelling and consequent increased permeability of both mitochondrial membranes. Nevertheless, another possibility that can not be excluded in the present study is that, due to swelling, hypoxic mitochondria might have become more prone to damage during the isolation procedure, which would also compromise mitochondrial integrity and function.

Clearly confirming that, at least in part, there is an oxidant-based aetiology for the referred mitochondrial dysfunction and membranes permeabilization, Vitamin E was considerably depleted in hypoxic mitochondria, and its supplementation was able to attenuate the mitochondrial impairments caused by hypoxia exposure, probably through an antioxidant-mediated effect. These data are in accordance with our previous observations [14].

Because inner and outer membrane permeation with or without physiological dependence of PTP is also suggested to be regulated by the pro and the anti-apoptotic members of Bcl-2 family [9, 28], and mitochondrial dysfunction has been considered an earliest step of the intrinsic pathway of apoptosis [29], we also studied that effect of the hypoxic insult on the expression of 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 [8, 30, 31]. Data from our study demonstrated a significant increase in Bax content from non-supplemented hypoxic mice suggesting that, in addition to the mechanisms referred above, severe hypoxic insult can also increase outer membrane permeability due to Bax translocation from cytosol to mitochondrial membranes and subsequent homodimerization into high molecular weight complexes which can cause cytochrome *c* release [31-33] through the formation of pores or

channels [34]. Considering the formation of Bcl-2/Bax heterodimers as a mechanism to inhibit apoptosis [31], 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 severe hypobaric hypoxia can predispose cells to apoptosis. In clear contrast with our data, and probably reflecting an hypoxia severity-dependent phenomenon and/or the protective effect of the acclimatization process against skeletal muscle oxidative stress already demonstrated in our lab [35], Riva and coworkers [36] showed an over-expression of Bax and Bcl-2 in skeletal muscle mitochondria of young rats growing under moderate chronic hypoxia conditions equivalent to ~6000m. In this case, the moderate and less severe level of hypoxia exposure was translated into an increase of the Bcl-2/Bax ratio allowing a better protection against apoptosis. In the present study, the expression of Bax was significantly depressed in vitamin E supplemented animals, when compared with non-supplemented mice, which corroborates the data from mitochondrial respiratory function.

In conclusion, it was demonstrated for the first time that severe hypoxic conditions resulted in decreased inner and outer mitochondrial membrane integrity with a presumable consequent triggering of the intrinsic pathway to apoptotic cell death. The fact that hypoxia increases the Bax/Bcl-2 ratio is worth of attention as it corroborates that hypoxia is able to trigger several phenomena conducting to cell death. Moreover, vitamin E attenuates the deleterious impact of this pro oxidant and pro apoptotic hypoxic insult in skeletal muscle mitochondria.

Considering the extreme severity of the hypoxic insult, the present results may have many implications in distinct areas of biology and medicine, particularly in chronic heart and lung disease, as this level of hypoxia may represent an insult causing systemic and tissue impairments characteristic of the referred pathophysiological conditions [37]. Studies regarding the impact of less severe hypobaric hypoxic conditions equivalent to those that most of the humans face on mitochondrial function are still lacking, although data regarding the impact of such hypoxic conditions on whole muscle tissue, blood or plasma oxidative stress and damage markers suggest that less intense alterations would probably occur at the mitochondrial level.

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FIGURE LEGENDS

Figure 1. Hypoxia and vitamin E treatment effects on mice skeletal muscle mitochondrial protein carbonyl groups. Control plus placebo solution of sterile soybean oil (C); hypobaric hypoxia plus placebo solution of sterile soybean oil (H); control plus vitamin E treatment (VE) and hypoxia plus vitamin E treatment (HVE). Data represent mean \pm SD and are expressed percentage of the control value. * $p < 0.05$, H vs. both C and HVE; ** $p < 0.05$, HVE vs. VE.

Figure 2. Hypoxia and vitamin E treatment effects on mice skeletal muscle outer (black bars) and inner (white bars) mitochondrial membrane integrity. Control plus placebo solution of sterile soybean oil (C); hypobaric hypoxia plus placebo solution of sterile soybean oil (H); control plus vitamin E treatment (VE) and hypoxia plus vitamin E treatment (HVE). Data represent mean \pm SD. * $p < 0.05$, H vs C, VE and HVE; ** $p < 0.05$, HVE vs H.

Figure 3. Oxygraphic analysis of outer membrane integrity (cytochrome c permeation assay) of mitochondria from control plus placebo solution of sterile soybean oil (C); hypobaric hypoxia plus placebo solution of sterile soybean oil (H); control plus vitamin E treatment (VE) and hypoxia plus vitamin E treatment (HVE). The arrows show the addition of compounds with their final reaction medium concentrations. Numbers under the traces indicate the rates of oxygen consumption in $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

Figure 4. Hypoxia and vitamin E treatment effects on mice skeletal muscle mitochondrial Bax (white bars), Bcl-2 (black bars) and Bcl-2/Bax ratio (gray bars). Control plus placebo solution of sterile soybean oil (C); hypobaric hypoxia plus placebo solution of sterile soybean oil (H); control plus vitamin E treatment (VE) and hypoxia plus vitamin E treatment (HVE). Data represent mean \pm SD and are expressed percentage of the control value. A scan of a representative Bax and Bcl-2 western blotting for each group ($n=4$) is immediately below the histogram. * $p < 0.05$, H vs. both C and HVE.







