Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle

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Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. J Appl Physiol 99: 1247–1253, 2005. First published May 19, 2005; doi:10.1152/japplphysiol.01324.2004.—Severe high-altitude hypoxia exposure is considered a triggering stimulus for redox disturbances at distinct levels of cellular organization. The effect of an in vivo acute and severe hypoxic insult (48 h at a pressure equivalent to 8,500 m) on oxidative damage and respiratory function was analyzed in isolated skeletal muscle mitochondria isolated from vitamin E-supplemented (60 mg/kg ip, 3 times/wk for 3 wk) and nonsupplemented mice. Forty male mice were randomly divided into four groups: control + placebo, hypoxia + placebo (H + P), control + vitamin E, and hypoxia + vitamin E. Significant increases in mitochondrial heat shock protein 60 expression and protein carbonyls group levels and mitochondrial respiration was significantly impaired in animals from the H + P group when compared with the control + placebo group. Mitochondrial respiration was significantly impaired in animals from the H + P group, as demonstrated by decreased state 3 respiratory control ratio and ADP-to-oxygen ratio and by increased state 4 with both complex I- and II-linked substrates. Using malate + pyruvate as substrates, hypoxia decreased the respiratory rate in the presence of carbonyl cyanide m-chlorophenylhydrazone and also stimulated oligomycin-inhibited respiration. However, vitamin E treatment attenuated the effect of hypoxia on the mitochondrial levels of heat shock protein 60 and markers of oxidative stress. Vitamin E was also able to prevent most mitochondrial alterations induced by hypobaric hypoxia. In conclusion, hypobaric hypoxia increases mitochondrial oxidative stress while decreasing mitochondrial capacity for oxidative phosphorylation. Vitamin E was an effective preventive agent, which further supports the oxidative character of mitochondrial dysfunction induced by hypoxia.

high altitude; oxidative damage; antioxidants; heat shock proteins; vitamin E

IT HAS BEEN CONSIDERED that high-altitude exposure is an aggressive physiological stress inducing wide cellular deleterious effects. Recently, increasing evidence (4, 29, 30, 34, 46) has also pointed out that severe high-altitude hypoxia can cause increased cellular oxidative stress with consequent damage to lipids, proteins, and DNA. Mitochondria reductive stress (13) has been suggested as one possible source of free radicals involved in this apparently paradoxical phenomenon of hypoxia-induced oxidative stress. Based on previous studies with hepatocytes (11) and cardiomyocytes (13), the reduction in oxygen availability to act as an electron sink that is normally associated with hypoxia may result in the accumulation of reducing equivalents in the mitochondrial electron transport chain, which can lead to enhanced production of reactive oxygen species (ROS) (8, 11, 13, 19, 33, 45). Mitochondria themselves may become oxidative targets, leading to peroxidation of membrane lipids (37), protein oxidation, DNA cleavage, and consequently impaired ATP production (1, 3, 39–41). In fact, ultrastructural data obtained from rat (1) and human (16, 28) skeletal muscle exposed to hypobaric hypoxia revealed significant mitochondrial morphological changes, namely considerable swelling and cristae degeneration, which also have been described in several other tissues (20, 43, 44).

In vitro measurements of oxygen consumption by isolated mitochondria have been used to assess mitochondrial functionality in several normal and pathological conditions (48). Early data from Kramer and Pearlstein (21) showed that isolated mitochondria submitted to in vitro severe hypoxic oxygen suffered from low phosphorylation efficiency [ADP-to-oxygen ratio (ADP/O)], presumably reflecting an increased electron leakage from complex III to oxygen. However, in vivo studies revealed conflicting data regarding oxygen consumption rates of mitochondria isolated from cerebral cortex (9), liver (10), and heart (10, 27). Furthermore, despite the fact that previous studies from our laboratory (29) reported signs of skeletal muscle oxidative stress and damage after acute hypobaric hypoxia, it is not sufficiently clear whether mitochondria contribute to such tissue redox disturbances. Additionally, to our knowledge, no information has been published concerning the effect of in vivo acute and severe simulated high-altitude hypoxia exposure on rodent skeletal muscle mitochondrial function. In this context, and based on previous studies in which oxidative stress was elicited by several other stimuli (23, 37, 43, 49), it seems reasonable to hypothesize that hypoxia-induced oxidative stress and damage to skeletal muscle correlates with disturbances of mitochondrial functionality.

Therefore, the aim of this study was to investigate whether acute and severe hypobaric hypoxia induces mitochondrial oxidative damage and dysfunction. Moreover, and considering the effectiveness of vitamin E supplementation in preventing tissue oxidative damage inflicted by high-altitude stress (18), we complementarily intended to test whether vitamin E could be effective against the severe hypoxia-induced, free radical-mediated, mitochondrial dysfunction.

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HYPOBARIC HYPOXIA AND MITOCHONDRIAL FUNCTIONALITY

METHODS

Experimental Design

Forty male Charles River CD1 10-wk-old mice were randomly divided into four groups (n = 10) according to the following independent variables: nonhypoxic control (C), hypoxia (H), vitamin E (V), and placebo (P). Two groups (C + V and H + V) were intraperitoneally supplemented with cumulative doses (3 times/wk during 3 wk, 60 mg/kg) of α-tocopherol acetate (V), and the other two groups (C + P and H + P) received the correspondent vehicle placebo solution (sterilized soybean oil) during the same period (2).

Immediately after the last injection, the animals of the C + P and C + V groups were maintained at an atmospheric pressure of 101.3 kPa (760 mmHg), which was equivalent to sea level. Also immediately after the last injection and one at a time, the animals of the hypoxic groups (H + P and H + V) were acutely exposed during 48 h inside a hypobaric chamber to a simulated atmospheric pressure of 35.2 kPa (265 mmHg), which was equivalent to an altitude of 8,500 m. The depressurization period to reach the simulated altitude of 8,500 m and the pressurization period back to sea-level conditions took 30 min each. All animals were kept at constant temperature (21–25°C) on a daily light schedule of 12 h of light vs. dark with normal activity. Mice were provided with food and water ad libitum during the experimental protocol and were killed 49 h 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 killed 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 (48). Briefly, muscles were immediately excised and minced in ice-cold isolation medium containing (in mM) 100 sucrose, 0.1 EDTA, 50 Tris-HCl, 100 KCl, 1 KH₂PO₄, 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, 25°C) and a magnetically stirred glass chamber containing 0.5 mg of mitochondrial protein in a reaction buffer of (in mM) 225 mannitol, 75 sucrose, 10 Tris, 10 KCl, 10 K₂HPO₄, and 0.1 EDTA, pH 7.5 in accordance to Tonkonogi et al. (49). After a 1-min equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) or succinate (10 mM) plus rotenone (4 μM). State 3 respiration was determined after adding ADP to a final concentration of 200 μM; state 4 respiration was measured as the rate of oxygen consumption in the absence of ADP phosphorylation. The RCR, i.e., the ratio between state 3 and state 4 respiration, and ADP/O were calculated according to Estabrook (14), using 235 nmol O₂/ml as the value for the solubility of oxygen at 25°C.

To quantify mitochondrial inner membrane permeability and the maximal rate of uncoupled oxidative phosphorylation, oligomycin (final concentration of 1.5 μg/ml) and carbonyl cyanide m-chlorophenylhydrazone (CCCP, 2 μM), respectively, were added during state 3 respiration with saturated amounts of ADP (final concentration of 1 mM).

Biochemical Assays in Mitochondria Extracted Fraction

Vitamin E concentration. α-Tocopherol was determined by high-performance liquid chromatography according to Vatassery et al. (51). Briefly, 2 ml of ethanol containing 0.025% (wt/vol) butylated hydroxytoluene and 0.1 ml of 30% (wt/vol) ascorbic acid were added to an aliquot of 100 μl of mitochondrial extract. Tubes were cooled, and 2 ml of water were added followed by 2 ml of hexane containing 0.025% (wt/vol) butylated hydroxytoluene. The hexane phase was separated out and evaporated down under a stream of nitrogen at 40°C. The residue was re-suspended in the mobile phase and analyzed by high-performance liquid chromatography using the following conditions: column = Inertsil, 5 Si, 250 × 3 mm (Varian); mobile phase = n-hexane with 3% dioxane; flow rate = 0.7 ml/min. α-Tocopherol was fluorimetrically detected (emission wavelength: 290 nm; absorbance wavelength: 330 nm).

Aconitase enzyme activity. The activity of aconitase was assayed in isolated mitochondria as an index for superoxide radical generation (24). Immediately before aconitase activity measurements, the mitochondrial fraction was diluted in 0.5 ml of buffer containing 50 mM Tris-HCl and 0.6 mM MnCl₂ (pH 7.4) and sonicated for 2 s. Aconitase activity was immediately measured spectrophotometrically by monitoring at 240 nm the formation of cis-aconitate after the addition of 20 mM isocitrate at 25°C, according to Krebs and Holzach (22). One unit was defined as the amount of enzyme necessary to produce 1 μM cis-aconitate/min [molar extinction coefficient (ε) at 240 nm (ε₂₄₀) = 3.6 mM⁻¹·cm⁻¹].

Analysis of HSP60 and Protein Carbonylation

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 TBS with Tween 20 and the membrane was incubated with HSP60 (1:2,000; 386028 mouse monoclonal IgG; Calbiochem) antibodies for 2 h at room temperature, washed, and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:1,000; Amersham Pharmacia Biotech) for 2 h.

For protein carbonyl derivatives assay, a certain isolated mitochondrial volume (vol) containing 20 μg of protein was derivatized with dinitrophenylhydrazine. Briefly, the sample was mixed with 1 vol of 12% SDS plus 2 vol of 20 mM dinitrophenylhydrazine 10% trifluoroacetic acid, followed by 30 min of dark incubation, after
which 1.5 vol of 2 M Tris/18.3% β-mercaptoethanol was added. A negative control was simultaneously prepared for each sample. After the derivatized proteins were diluted in TBS to obtain a final concentration of 0.001 μg/μl, a 100-ml volume was spotted into a Hybond-polyvinylidene difluoride membrane. Immunodetection of carboxyls was then performed using rabbit polyclonal anti-dinitrophenyl (1:2,000; V0401 DakoCyntomation) as the primary antibody and anti-rabbit IgG-peroxidase (Amersham Pharmacia) as the secondary antibody (1:2,000 dilution).

For both referred assays, the bands were visualized by the immunoblots with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshire, UK), according to the supplier’s instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, MO). The films were analyzed with QuantityOne Software version 4.3.1 (Bio-Rad). Optical density results were expressed as percentage variation of control values.

Analysis of Total Mitochondrial SH Groups

The mitochondrial content of oxidative modified SH groups, including GSH and other SH-containing proteins, were quantified by spectrophotometric measurement according to the method proposed by Hu (17). Briefly, mitochondria were sonicated for 2 s, and after 1 min centrifugation at 1,000 g, 50 μl of supernatant was added to a medium containing 150 μl of Tris (0.25 M), 790 μl of methanol, and 10 μl of 5,5′-dithio-bis (2-nitrobenzoic acid) (10 mM). The colorimetric assay was performed at 414 nm against a blank test. Total SH content was expressed in nanomoles per milligrams of mitochondrial protein (ε414 = 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 −80°C 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 (47). The aliquots for glutathione assay were previously extracted for a medium containing perchloric acid at 5% (wt/vol) to precipitate proteins. Regarding TGSH, after neutralization with potassium hydrogen carbonate (0.76 M), samples were centrifuged for 1 min at 13,000 g. Then, a supernatant aliquot was incubated for 15 min at 30°C in a microtiter plate with a reagent solution containing NADPH (1.68 mM) and 5,5′-dithio-bis (2-nitrobenzoic acid) (0.7 mM). Immediately after the addition of glutathione reductase (20 U/ml), a kinetic analysis was performed at 412 nm. For GSSG content evaluation, addition of 2-vinylpyridine, to a final concentration of 5% (vol/vol), was made before the neutralization step to inactivate the SH groups. The remained assay was analogous to the total glutathione measurement. TGSH and GSSG concentrations (nmol/mg protein) were established based on calibration curves made with commercial standards. Protein content was spectrophotometrically assayed using bovine serum albumin as standard according to Lowry et al. (26).

Statistical Analysis

Means and 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 version 10.0) was used for all analyses. 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 (+85.8%) of vitamin E when compared with skeletal muscle mitochondria from C + P mice (Fig. 1). Moreover, skeletal muscle mitochondria isolated from mice from both hypoxic groups (H + P and H + V) showed a significant decrease in vitamin E levels (81.4 and 61.8%, respectively), compared with their control counterparts (C + P and C + V). Vitamin E content in skeletal muscle mitochondria from the H + P group was significantly lower than in the H + V group.

Mitochondrial Respiratory Rates

The state 3 and the state 4 respiration rates as well as the RCR obtained in this study were comparable to other data reported elsewhere with mitochondria isolated from skeletal muscle (5).

As illustrated in Tables 1 and 2, the acute and severe hypoxic insult induced a significant impairment in the respiratory rates of isolated skeletal muscle mitochondria energized with malate + pyruvate (M-P) and succinate + rotenone (S-R). The acute and severe hypoxic insult significantly decreased state 3 respiration by 40.5% with M-P and by 27.7% with S-R compared with control mitochondria from nonhypoxic animals. On the other hand, hypoxic mice (H + P) showed a 26.8 and 35.8% significant increase in state 4 respiration with M-P and S-R as substrates, respectively, when compared with the C + P group. The RCR was also significantly affected in hypoxic mice. In fact, the RCR decreased 53.6% using M-P and 46.5% with S-R as respiratory substrates when compared with control animals. With both M-P and S-R, ADP/O was also significantly impaired. Indeed, the ratio of the ADP to oxygen consumption significantly decreased 28.2% with M-P and 25.8% using S-R as substrates.

Vitamin E supplementation induced a significant protective effect in state 3 respiration both with M-P and S-R as substrates (Tables 1 and 2). Indeed, vitamin E was able to prevent the decrease in state 3 respiration induced by hypobaric hypoxia (H + P vs. H + V groups). However, regarding state 4 respiratory rates, our data showed that vitamin E-supplemented animals did not differ from their nonsupplemented counterparts after the hypoxic insult with both substrates. In what concerns to the RCR and ADP/O obtained with both substrates, our data

![Fig. 1. Effect of hypobaric hypoxia equivalent to 8,500 m and vitamin E treatment on mice skeletal muscle mitochondrial α-tocopherol (vitamin E) content obtained from 10 independent experiments. C + P, control plus placebo solution of sterile soybean oil; H + P, hypoxic hypoxia plus placebo solution of sterile soybean oil; C + V, control plus vitamin E treatment; H + V, hypoxia plus vitamin E treatment. Data are means ± SE. *P < 0.05, H + P and C + V vs. C + P. #P < 0.05, H + V vs. C + V and H + P.](http://jap.physiology.org/10.1152/japplphysiol.02284.2005)
showed that vitamin E supplementation was helpful against the deleterious effect of acute and severe hypoxia. Moreover, concerning RCR and taking into account the above-referred effect of vitamin E supplementation in state 3 and state 4 respiratory rates with both substrates, data revealed that the effective protection of this antioxidant derived essentially from preventing the decrease in state 3.

After the hypoxic insult, mitochondria energized with malate-pyruvate and stimulated with excess ADP showed a respiratory rate in the presence of oligomycin (Table 3) significantly increased (28.1%) when compared with the group C + P. On the other hand, vitamin E supplementation did not prevent the increased inner membrane permeability induced by hypobaric hypoxia compared with C + V. Uncoupled mitochondrial respiration with CCCP (Table 3) was reduced in the H + P group compared with the C + P group (43.8%). Vitamin E supplementation attenuated the inhibition of uncoupled respiration (18.3%) after acute and severe hypoxia (H + V vs. H + P). Nevertheless, the uncoupled respiration in the presence of CCCP in the H + V group was still significantly distinct from the one obtained in vitamin E-supplemented 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 4. There was a significant reduction in TGSH and GSH in the H + P group compared with the C + P group. Also, a remarkable increase in GSSG concentration and %GSSG was found in these animals compared with nonhypoxic controls. When H + P and H + V groups were compared, vitamin E significantly attenuated the impact of the hypoxic insult on TGS, GSH, GSSG, and %GSSG. Nevertheless, for all the above-referred parameters, vitamin E-supplemented animals presented values that were substantially distinct from controls (H + V vs. C + V) after the acute and severe hypoxic insult.

As presented in Fig. 2, aconitase activity was severely affected by the experimental hypoxic protocol. In fact, in animals of the H + P group, the activity of this superoxide-sensitive enzyme significantly decreased (34.9%) when compared with the C + P group. Vitamin E significantly attenuated the inactivation of aconitase (H + P vs. H + V) induced by the hypoxic insult; however, mitochondrial aconitase activity in hypoxic supplemented mice was still significantly affected compared with control (17.9%).

Regarding markers for mitochondrial oxidative damage (Table 5), our data showed that hypoxia exposure inflicted substantial deleterious effects through protein oxidation. With respect to protein SH content, a significant decrease (53.6%) was found in animals from the H + P group compared with animals from the C + P group. Despite the significantly antioxidant protective effect of vitamin E (H + P vs H + V), hypoxic mice (H + V) supplemented with vitamin E also showed significant diminished levels of SH protein groups compared with animals from the C + V group. Concerning...
carbonyl groups, a significant increase was found in animals from the H + P group when compared with their controls (74.3%). Vitamin E protected mitochondria from the generation of carbonyl groups induced by hypobaric hypoxia; however, supplemented mice (H + V) still differed from their control counterparts (C + V).

Regarding HSP60 expression (Fig. 3), our data showed that acute and severe hypoxia exposure induced a significant mitochondrial HSP60 overexpression (86.4%) when compared with nonhypoxic conditions (H + P vs. C + P). Hypoxic animals supplemented with vitamin E also showed significant overexpression (34.6%) of HSP60 compared with C + P animals; however, the result obtained was still significantly distinct from the values obtained from their nonsupplemented counterparts (H + P vs. H + 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 acute and severe hypobaric hypoxia on skeletal muscle mitochondria of exposed mice. Data showed that the hypoxic insult induced increased mitochondrial superoxide radical production, protein oxidation, and impaired respiratory function. Furthermore, our study also suggests that vitamin E is able to attenuate the impact of hypoxia on mitochondrial function of mice skeletal muscle. Nevertheless, considering that mitochondria were isolated from different hind-limb muscles (soleus, gastrocnemius, tibialis anterior, and quadriceps) characterized by different biochemical features, data should not be extrapolated to each muscle individually.

**Mitochondrial Oxidative Stress and Damage after Acute and Severe Hypobaric Hypoxia**

In accordance with several other reports (29, 46), the present study confirmed the paradoxical phenomenon of hypoxia-induced oxidative stress and damage in whole skeletal muscle (Table 4). In fact, the data support the role of mitochondria as a potential ROS source and also as a specific oxidative target under severe hypoxic conditions.

As demonstrated in isolated hepatic (11) and cardiac cells (13), hypoxia-mediated partial inhibition of mitochondrial electron transport results in redox changes in the electron carriers with consequent enhanced generation of ROS. Our data regarding mitochondrial aconitase activity impairment in hypoxic mice (see Fig. 2) constitute an indirect index of increased mitochondrial superoxide production (32). In fact, the loss of mitochondrial, but not cytosolic, aconitase activity in aged SOD2 knockout mice, previously demonstrated by Williams et al. (55), suggests that the Fe-S center of aconitase can be reversely inactivated by superoxide and related species (7). Accordingly, clear evidence of protein oxidative damage (SH and carbonyl-derivative groups) were found in mitochondria isolated from hypoxic mice (Table 5), which suggests 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 markers for ROS production and oxidative damage in vitamin E-supplemented mice were substantially decreased compared with the nonsupplemented hypoxic mitochondria. Moreover, and in accordance with data reported by Ilavazhagan et al. (18), the results presented above suggest that vitamin E is a valuable antioxidant to counteract the oxidative damage inflicted by hypobaric hypoxia at distinct levels of cell organization.

Primarily considered a mitochondrial-abundant heat shock protein, HSP60 appears to have an important assignment in maintaining mitochondrial function and integrity (25). In fact,
besides stabilizing mitochondrial proteins under stressful conditions, this chaperone also assists in the folding and assembly of proteins as they enter the mitochondria (36). In our study, the overexpression of HSP60 can be undoubtedly interpreted as an acute sign of oxidative mitochondrial stress (42). The increase in HSP60 levels seems to correlate well with the magnitude of the physiological stress and damage inflicted to mitochondria under the distinct experimental conditions (Fig. 3). The levels of HSP60 exhibited by both hypoxic animals supplemented with vitamin E or placebo (H + P vs. H + V) confirmed the levels of stress imposed to mitochondria as well as the protective role of vitamin E against the oxidative stress induced by hypoxia. This protective effect of HSP60 overexpression assumes particular importance as the levels of some mitochondrial and nuclear genome encoding proteins of electron transport chain are repressed on reduced oxygen levels compromising mitochondrial regenerative processes (52, 53) and consequently mitochondrial respiration.

Impairment of Skeletal Muscle Mitochondria Functionality on an Acute and Severe In Vivo Hypobaric Hypoxic Insult

Mitochondrial protein and phospholipid damage induced by oxidative stress is widely suggested as being implicated in the onset and/or development of several mitochondrial pathophysiological states, either by impairing integral enzyme function or by destroying mitochondrial bilayer packing order (31). To the best of our knowledge, this is the first study dealing with skeletal muscle mitochondria functionality after acute and severe in vivo hypobaric hypoxic exposure. State 3 respiratory rates of energized mitochondria with both NADH- and FADH2-linked substrates were significantly diminished in the hypoxic groups. We hypothesize that the decrease in state 3 respiration observed in hypoxia-exposed animals can be due to one or more of the following causes: 1) decreased availability of reduced substrates for the electron transport chain (35); 2) oxidative inactivation of enzymes involved in the Krebs cycle [like aconitase (15)] or in oxidative phosphorylation (12, 54, 56); or J) peroxidative damage to mitochondrial membranes (38, 39).

With regard to state 4 respiration, our data showed that mitochondria isolated from both vitamin E-supplemented and nonsupplemented animals submitted to hypoxia possessed higher rates of state 4 respiration. Since the increase in state 4 respiration may reflect an increased inner membrane leakage of protons (6), the data suggest that hypoxia can indeed cause an increased membrane permeability in a vitamin E-insensitive manner. However, the simple analysis of state 4 respiration rate per se is insufficient to conclude about the mechanisms of mitochondrial damage and about the effectiveness of vitamin E regarding inner membrane protection. To further clarify the above-referred findings concerning depressed state 3 and increased state 4 respiration, oligomycin and CCCP were used in mitochondria that were previously energized with M-P in the presence of ADP. Since respiratory rates in the presence of CCCP were also severely affected in mitochondria isolated from hypoxic mice, we confirm that hypoxia clearly affects the maximal rate of electron transfer through one or more of the mechanisms previously proposed.

Data obtained in the presence of oligomycin confirmed our initial hypothesis that hypoxia increased the permeability of the mitochondrial inner membrane through other components rather than the Fo-F1 ATP synthase, again in a vitamin E-independent manner. Because vitamin E is a lipophilic substance present in cellular membranes (50), it would be expected to offer some protection against increases in membrane permeability due to peroxidation of membrane lipids (41). Further work is necessary to clarify this subject.

The coupled respiratory control and the phosphorylation efficiency of mitochondria from nonsupplemented mice previously submitted to hypobaric hypoxia were clearly affected, as inferred from both RCR and ADP/O decreases, respectively. These findings suggest that hypobaric hypoxia affects both the mitochondrial phosphorylation efficiency and the coupling between respiration and ATP synthesis. Vitamin E, probably through an antioxidant-mediated effect (Tables 1 and 2), attenuated the mitochondrial dysfunction, as evaluated by the decreases in RCR and ADP/O.

In conclusion, our data demonstrate for the first time that acute and severe hypobaric hypoxia exposure induces mitochondrial oxidative damage with impairment of mitochondrial function. The oxidative nature of the mitochondrial damage induced by hypobaric hypoxia was demonstrated by the protective effect of vitamin E against the appearance of several markers of cell and mitochondrial stress. The results demonstrate that, physiologically, the administration of antioxidants before exposure to hypoxia may be beneficial to counteract mitochondrial alterations resulting from oxidative stress.

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