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Effects of intermittent hypoxic training on amino and fatty acid oxidative combustion in human permeabilized muscle fibers.

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Abstract

The effects of concurrent hypoxic/endurance training on mitochondrial respiration in permeabilized fibers in trained athletes were investigated. Eighteen endurance athletes were divided into two training groups: normoxic (Nor, n = 8) and hypoxic (H, n = 10). Three weeks of endurance training (5 sessions of 1h-1h30 week^{-1}) were completed. All training sessions were performed under normoxic (160 mmHg P_IO_2) or hypoxic conditions (≈ 100 mmHg P_IO_2, ≈ 3000 m) for Nor and H group respectively at the same relative intensity. Prior to and after the training period, an incremental test to exhaustion in normoxia was performed and muscle biopsy samples were taken from the *vastus lateralis* and mitochondrial respiration in permeabilized fibers was measured. Peak power output (PPO) increased by 7.2% and 6.6% ($P < 0.05$) for Nor and H respectively, whereas $\dot{V}O_{2max}$ remained unchanged: 58.1 ± 0.8 *vs.* 61.0 ± 1.2 ml·kg^{-1}·min^{-1} and 58.5 ± 0.7 *vs.* 58.3 ± 0.6 ml·kg^{-1}·min^{-1} for Nor and H respectively between W0 and W4. Maximal ADP-stimulated mitochondrial respiration (Vmax) significantly increased for glutamate + malate (6.27 ± 0.37 *vs.* 8.51 ± 0.33 nmolO_2·min^{-1}·mg^{-1}dry weight), and significantly decreased for palmitate + malate (3.88 ± 0.23 *vs.* 2.77 ± 0.08 nmolO_2·min^{-1}·mg^{-1}dry weight) in H group. In contrast, no significant differences were found for Nor group. The findings demonstrate that: 1) a three-week training period increased the PPO at sea-level without any changes in $\dot{V}O_{2max}$ and 2) a three-week hypoxic exercise training seems to alter the intrinsic properties of mitochondrial function, *i.e.* substrate preference.

Key words: endurance exercise, substrate preference, hypoxic stress, aerobic adaptation, muscle biopsy

Introduction

It is common for endurance training to be supplemented with periods of exposure to hypoxia either by living at altitude or performing acute bouts of exercise in simulated altitude. Despite this, the effects of hypoxic training on sea-level performance are equivocal (48). Indeed, Roels *et al.* (35) concluded that intermittent hypoxic training (IHT) of two high-intensity cycling sessions per week (90-100% relative peak power output) during seven weeks at a simulated altitude of 3000 m in well-trained cyclists and triathletes did not improved performance (peak power output and 10-min cycle time trial) nor $\dot{V}O_{2max}$ to a greater extent than a similar sea level training. In contrast, Hendriksen and Meeuwsen (15) concluded that nine days after a 10-days IHT ($2h \cdot day^{-1}$ of cycling at 60-70% of heart rate reserve in a hypobaric hypoxia at a simulated altitude of 2500 m) significantly improved performance in terms of maximal power output and mean and peak anaerobic power compared to similar normoxic training.

Potential mechanisms underlying the observed performance enhancement include changes in a number of central (enhanced O_2 transport) and peripheral (enhanced muscle oxidative capacity) responses (11, 19, 45, 48). However there is little information available concerning the adaptations that occur in skeletal muscle when endurance training is performed entirely in hypoxia as compared to normoxic conditions alone.

When skeletal muscle contracts aerobically, nearly all O_2 consumption and most ATP resynthesis occur in the mitochondria (37). Indeed, muscular activity demands energy which will be supply by the selection of the preferred metabolic pathways (energy metabolism) and thus mitochondrial substrate utilization (37). This mitochondrial substrate utilization varies among muscle types and is dependant on exercise type and duration (17). It has been claimed often that mitochondrial function is impaired during exercise (12, 49). This contention is not supported by results from continuous moderate intensity, *i.e.* endurance exercise in which

mitochondrial function is well maintained in both animal (2, 39) and human skeletal muscle (26, 42). However one of the main adaptations of skeletal muscle in response to endurance training is improved muscle oxidative capacity (3, 4) which results from changes in mitochondrial substrate utilisation and in mitochondrial enzyme activities (18). Walsh *et al*. (47) observed that six weeks of endurance training in healthy subjects increased the maximal ADP-stimulated mitochondrial respiration (Vmax) by 38%. The reduced availability of oxygen under hypoxic conditions might also alter the mitochondrial functions, as it is known that hypoxia induces several physiological and cellular adaptations to maintain O_2 transport to the muscles (9). To date it is not yet been shown how mitochondrial substrate utilization, respiration and enzyme activities respond to exercise performed under hypoxic exposure. Therefore, it is possible that mitochondria are affected to a higher extent during endurance exercise performed in hypoxia compared with exercise in normoxia. Given this hypothesis, the present study was interested in comparing the effects of exercise performed in hypoxia and normoxia on the mitochondrial respiration. In particular, the present study investigated different specific metabolic pathways by using glutamate + malate and palmitate + malate as substrates. In addition, these substrates are being used as standard substrates in several other studies (28, 41, 52).

The hypothesis of this experiment was that the intrinsic properties of mitochondrial function, such as substrate preferences, are altered by exercise performed in hypoxia as compared to the same relative exercise stress performed in normoxia.

Methods

Subjects

Nineteen healthy male endurance-trained athletes gave written informed consent to participate in this study, which was approved by the institutional ethics committee (Nîmes, France). All

subjects were sea-level residents, familiarized with the testing protocols and equipment used in the experiment and none of them exhibited signs or symptoms of any pathology. In addition, none had a history of recent travel to altitude*, i.e.* three weeks prior to the training period. The subjects were initially randomized into two groups. The two groups comprised a Hypoxic group (H; n = 10), and a Normoxic group (Nor, n = 9). The groups were of comparable training status as evidence by similar maximal oxygen uptake ($\dot{V}O_{2max}$) measured during an incremental exercise test prior to the experimental training period. The experiment took place in the pre-season when the athletes had not reached a peak in endurance performance. Subject characteristics for each group are presented in Table 1. One athlete assigned to the Nor group did not complete the training due to illness.

Experimental design

During three weeks (W1-W3), the subjects performed two interval and three continuous endurance training sessions per week. Each training session began and ended with 15 min warm-up and 15 min cool-down. The continuous training session consisted of 60 min at 60% of $\dot{V}O_{2max}$. The interval-training required the subjects to perform two sets of three repetitions of two min duration at an intensity of 100% peak power output (PPO). Two min of rest was allowed between each repetition with six min rest between each set. The subjects trained on their own bicycle fixed on an electromagnetic resistant home-trainer (Elite Travel, Milan, Italy). During each laboratory training session, the target power output, *i.e.* 100% PPO or the power output corresponding to 60% of $\dot{V}O_{2max}$, and the heart rate corresponding to the target power output were continuously monitored and meticulously controlled by the same researcher. Moreover, subjects were continuously encouraged to perform at the target power output level.

Each training session was performed in either a normoxic (P_IO_2 of 160 mmHg) or hypoxic (P_IO_2 of 100 mmHg, simulated altitude of ~3000 m) environment for the Nor and the H group, respectively. The average duration of the hypoxic stimulus per week during the training period was 382 min. The subjects did not perform supplementary interval-training outside the two supervised interval-training sessions.

Prior to (pre-training = W0) and at the end (post-training = W4) of the training period a medical examination and determination of physical characteristics were completed (Table 1). The subjects performed an incremental exercise test to exhaustion in normoxic conditions, and an identical incremental test to exhaustion under hypoxic conditions (P_IO_2 ~100 mmHg) on a separate visit to the laboratory was also performed by the H group. These tests were randomized for the H group. Two days after the last test, muscle biopsies were taken at rest prior the training protocol. After the training protocol (W4), the muscle biopsies were taken at rest two to three days after the last training session. All tests and biopsies were performed at the end of W4, *i.e.* maximal five days after the last training session.

A physician was in attendance at all times and was responsible for the safety of the subjects during the study.

The weekly training regime that took place outside the experimental trials was controlled and documented. Lastly, the dietary intake of the subjects was monitored and controlled; the subjects were required to maintain consistency from week to week in their dietary intake. Both groups consumed a similar diet, *i.e.* the same relative proportion of fat (~30 %) and carbohydrates (CHO ~60 %).

Environmental stimulus

The hypoxic gas mixture was delivered continuously by a system which changes the inspired air by modifying nitrogen (N_2) content (Altitrainer 200®, SMTEC, Geneva, Switzerland) (35).

This device allows the production of large quantities of a hypoxic gas mixture (up to 200 liters per minute), with an easily adjustable O_2 fraction over a large range and with a short response time. The O_2 content of the mixture is continuously displayed and can be expressed either by the equivalent altitude (~3000 m) or the O_2 partial pressure (P_IO_2 ~100 mmHg) taking into account the barometric pressure. Air inhaled from outside the machine is controlled with a fixed quantity of N_2 coming from a bottle and then mechanically mixed prior to being stocked in a buffer tank of 30 liters. The first safety check is ensured by the mixer's mechanical limit that cannot exceed a certain N_2 fraction (F_IO_2 = 9.7%). The user inhales the mixture contained in the tank through a Hans Rudolph two-way respiratory valve. An O_2 probe, a second safety check, plunged in the buffer tank, measures the PO_2. This probe, with aid of a microprocessor, allows the PO_2 of the inhaled mixture, or the equivalent altitude to be displayed and cannot decrease less than 66 mmHg (5500 m). If necessary, the user can constantly modify the composition of the air which they breathe and thus change the simulated altitude. The respiratory mask could at all times be removed and so the subjects found themselves immediately in normoxic conditions. A breath-by-breath analyzer can be easily attached to the system in order to measure respiratory exchange.

Performance Tests

Prior to and following the training period, the subjects performed an incremental test to exhaustion to determine the maximal oxygen uptake ($\dot{V}O_{2max}$; $ml \cdot kg^{-1} \cdot min^{-1}$), the peak power output (PPO; W), maximal ventilation ($\dot{V}E_{max}$; $l \cdot min^{-1}$) and maximal heart rate (HR_{max}; bpm). The test began at an initial power output of 60 W for 3 min and then the workload was increased by 30 W every minute until exhaustion. Exhaustion was reached when two out of three of the following criteria were obtained: 1) heart rate (HR) approaching an age predicted maximum value (220 – age); 2) a plateau in $\dot{V}O_2$ despite an increase in exercise intensity; and

3) a RER > 1.1. Respiratory exchange was measured breath-by-breath then reduced to 30 s averages. $\dot{V}O_{2max}$ was determined as the highest 30 s $\dot{V}O_2$ average. PPO was defined as the highest mechanical power maintained during one min.

In addition, only the H group performed the same test also under hypoxic conditions on a separated laboratory visit and prior the start of the training protocol and biopsy (data not presented in this paper). This test was necessary to provide data to determine the workloads (100% PPO and 60% of $\dot{V}O_{2max}$) under hypoxic conditions for the training sessions for the H group, so that the same relative intensity between N and H groups could be established to ensure an equivalent training stimulus.

The test was performed on a bicycle equipped with a 'SRM® road professional' powermeter (Schoberer Rad Messtechnik, Jülich, Welldorf, Germany). The saddle height on the cycle ergometer measured during the first test was kept identical. Power output and the pedaling cadence were recorded with an acquisition frequency of one s then averaged over 30 s. The calibration procedure and technical aspects concerning SRM crank system have been described in detail by Jones and Passfield (23). The 'SRM® road professional' powermeter that has four strain gauges, was shown to have a high accuracy in power measurement. The 95% limits of agreement is 2.1 W which is equivalent to 1.8% (23).

Training outside experimental design

The training completed outside the experimental design was recorded daily using a computerized training diary during the three weeks of training period. The type of activity, *i.e.* cycling, swimming, running, etc, and the intensity was recorded with this training diary. The training intensity was divided into five intensity levels (30). All training sessions outside the protocol were individually timed and each exercise categorized according to the five intensity levels. The performed training duration was multiplied by its corresponding multiplying

factor, *i.e.* 2, 4, 6, 10, and 16, respectively and the sum was then divided by the overall durationof the session to calculate the average intensity of each training session. The recorded parameters were the number and average intensity of the sessions, calculated according to Mujika *et al.* (30) as it was not sensible to compare hourly volume as the subjects performed exercise training in different modes (swimming, running).

Physiological measurements

Pre (W0) and post training (W4), gas exchange was measured by a $K4^{b2}$ (Cosmed, Rome, Italy). The $K4^{b2}$ configuration was modified in order to calculate the ventilatory variables with the accurate F_IO_2 rather than with the default version. The aforementioned physiological variables were measured, breath-by-breath, and averaged every 30 s. Before each test, the system was calibrated using ambient air, whose partial O_2 composition was assumed to be 20.93% and a gas of known CO_2 (5%) and O_2 (16%) concentration. The calibration of the turbine flowmeter of the $K4^{b2}$ was performed with a 3-L syringe (Quinton Instruments, Seattle, WA).

During the different tests and training sessions, the HR was constantly recorded by the means of a HR monitor (S810, Polar, Kempele, Finland) integrated to the Cosmed system.

Muscle biopsy.

Muscle samples were obtained from the *vastus lateralis* using the percutaneous needle biopsy technique after administration of local anesthesia (xylocaine) as previously described and perfomed in our laboratory (41). The biopsies were taken by the same researcher from the same site, *i.e.* in the middle of the line between *spina iliaca* anterior superior and the upper outer corner of the *patella* at a depth of 1.5 – 2.0 cm from the fascia in all the subjects.

The muscle samples were divided into two portions with one portion immediately frozen in liquid nitrogen and stored at –80°C until enzymatic analysis. The second portion was used for *in situ* respiration studies and was immediately placed in an ice-cold relaxing solution [at ionic strength 160 (potassium methanesulfonate), pH 7.1] containing (mM): EGTA-calcium buffer 10 (free Ca^{2+} concentration 100 nmol·l^{-1}), Imidazole 20, KH_2PO_4 3, $MgCl_2$ 1, Taurine 20, DTT 0.5, MgATP 5 and PCr 15.

The muscle fiber bundles were separated with sharp-ended needles and were incubated in one ml of the relaxing solution (4°C) containing 50 µg ml^{-1} saponin for 30 min with continuous agitation. In order to completely remove the saponin, the incubated muscle fibers were washed with continuous stirring in relaxing solution for 10 min (4°C); in order to remove free ATP, they were then washed with oxygraph solution for 2 x 5 min (4°C), which was of the same composition as the relaxing solution except that MgATP and PCr were replaced (mM) by malate 2, phosphate 3, and fatty acid-free bovine serum albumin 2 [pH 7.1]. After washing, the fibers were stored in oxygraph solution for immediat determination of mitochondrial respiration activity.

The skinned fiber preparations met the necessary criteria defined by Saks *et al.* (36). All fiber preparations and respiratory measurements were performed under identical conditions and by the same researcher for the Nor and H group.

Unfortunately, due to technical reasons, the post biopsy in two subjects of the H group was not possible; therefore only eight subjects are included in the biopsy-derived measurements.

Mitochondrial respiration studies

The respiratory parameters of the total mitochondrial population were studied *in situ* as previously described (28, 44) using a Clark electrode (Strathkelvin Instruments, Glasgow, Scotland). These measurements are extensively being done in our laboratory (41).

Measurements were carried out at 30°C with continuous agitation in 3 ml of oxygraph solution with either glutamate 5 mM + malate, or palmitate 40 mM + malate as respiratory substrates. This continuous agitation was necessary to ensure stability of the preparation and reliable comparison of the data (36). Moreover, the O_2 consumption values were corrected for instrumental and chemical background O_2 consumption. Maximal ADP-stimulated respiration (Vmax) above basal oxygen consumption (V_0), *i.e.* oxygen consumption in the absence of nucleotides, was measured by addition of ADP (2000 µM). At the end of measurement, we used the cytochrome c test to investigate the state of the outer mitochondrial membrane (36). After the respiratory measurements, the muscle fiber bundles were removed, dried overnight, and weighed the next day. Respiration rates were expressed in micromoles of O_2 per minute per gram of dry weight.

Mean V_0 was determined and Vmax for each substrate was calculated using a non-linear mono-exponential fitting of the Michaelis-Menten equation with DataFit 6.0 software (Oakdale Engineering, USA). The acceptor control ratio (ACR) was calculated as $Vmax/V_0$, representing the degree of coupling between oxidation and phosphorylation. The ratio of maximal palmitate and glutamate oxidation (Vmax pal/Vmax glut) is calculated and expressed in $\mu molO_2 \cdot min^{-1} \cdot g\ dry\ weight^{-1}$.

The palmitate measures for the H and Nor groups were determined in two independent series of experiments.

Citrate synthase (CS) and hydroxyacyl-CoA-dehydrogenase (HADH) activity.

Homogenates for CS and HADH activity were prepared in buffer (mM): sucrose 210, EGTA 2, NaCl 40, HEPES 30, EDTA 5, and phenylmethylsulfonyl fluoride 2 [pH 7.4], and stored at –80°C. CS and HADH activity were assayed by a spectrophotometric method. Changes in

absorbance were recorded over 3 min at 412 nm, at 25°C for CS (38) and over 10 min at 340 nm, at 30°C for HADH analyses (50).

'Real Time' RT-PCR for CS and HADH mRNA

Total RNA was isolated using commercially available reagents (FastRNA Kit-Green (BIO 101, Vista CA) then quantified spectrophotometrically at 260 nm. First strand cDNA was generated from 1μg RNA using AMV RT (Promega, Madison, WI). The cDNA was then stored at –20 ^{o}C until further analysis. cDNA primers and probes were designed using Primer Express software package version 1.0 (Applied Biosystems, CA, USA) from gene sequences obtained from GenBank (CS, HADH). The cDNA samples were prepared for analysis using Brilliant^{TM} QPCR kit (Stratagene) with SYBR$^{®}$ Green I dye and forward/reverse primers (3 μM). The forward primer (5-3) for CS and HADH is GTGCCCATACCAGCCACTTG and TGGCTTCCCGCCTTGTC respectively and the reverse primer (5-3) is CTGCCAGCCCGTTCATG and TTGAGCCGGTCCACTATCTTC for CS and HADH respectively. Samples were run for 40 cycles in a total volume of 20 μl. The linearity of the primers was also confirmed with a serial of dilution of cDNA. Real-time PCR was performed using the ABI PRISM 5700 sequence detection system (Applied Biosystems, CA, USA). Samples for each gene were run in duplicate on each plate to control for amplification efficiency. Fluorescent emission data were captured and mRNA levels were quantified for each gene using the critical threshold (C_T) value. The relative expression of the gene of interest is calculated using the expression $2^{-\Delta CT}$ and normalized to baseline values and standardized to an unchanged internal control, then expressed as fold change.

Statistical analysis

All values are reported as mean ± standard error (SE). After analysis of normality and homogeneity of variance, the effects of the two training conditions on each variable were compared using a two-way (training group x time) variance (ANOVA) with repeated measures on the second factor. The reliability of the CS and HADH mRNA measurements and the inter-assay variability of Vmax Palmitate and Vmax Glutamate were also assessed as the typical (standard) error of measurement expressed as a coefficient of variation (CV; percent of the mean) between analyses. Significant effects were subsequently analyzed using the Student Newman-Keuls post-hoc test. All analyses were completed using SigmaStat 2.3 (Jandel Corporation, San Rafael, CA) and the statistical power was calculated for each analysis. Statistical significance was accepted at $P < 0.05$.

Results

Training in- and outside the training sessions

Table 2 presents the workloads of each group averaged over the three weeks for the interval training and continuous workload sessions.

Table 3 presents the average intensity of additional training outside the experimental design of each training week for both groups.

No significant differences in (relative) training workloads in- and outside the training sessions were observed between the groups.

Incremental test to exhaustion

There were no significant differences in the initial PPO and $\dot{V}O_{2max}$ between the groups. The physiological variables obtained from the incremental test are presented in Table 4. The PPO increased with training ($P < 0.001$, power = 0.969) by 7.2% and 6.6% for Nor and H respectively.

Mitochondrial respiration

Figure 1 represents the mean Vmax measured with (A) glutamate + malate, and (B) palmitate + malate, with an increase ($P < 0.05$, power = 0.851 in H, W0 vs. W4) in glutamate + malate Vmax, and a decrease ($P < 0.05$, power = 0.875 in H, W0 vs. W4) in palmitate + malate Vmax. CV of Vmax Palmitate was 2.01 and 4.39% at W0 for Nor and H group respectively and Vmax Glutamate at W0 was 4.87 and 0.44 % for Nor and H group, respectively.

Table 5 shows the V_0 and ACR of the skeletal muscle mitochondria for each of the substrates. There was no significant difference ($P > 0.05$) between V_0 and ACR of the two groups for the two substrates.

There was no change in Vmax after cytochrome c addition.

CS and HADH analyses

There were no significant differences between or within both groups for CS and HADH measurements. The CS values were 18.9 ± 0.6 *vs.* 19.9 ± 0.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ at W0 and 20.4 ± 0.6 *vs.* 19.2 ± 0.4 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ at W4 for the Nor *vs.* H group respectively. The HADH values were 1.27 ± 0.06 *vs.* 1.10 ± 0.09 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ at W0 and 0.88 ± 0.07 *vs.* 1.29 ± 0.05 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ at W4 for the Nor *vs.* H group respectively. There was no significant difference in the fold change in CS mRNA between the H and Nor groups (1.6 ± 0.3 *vs.* 1.4 ± 0.1). There was also no significant fold difference in HADH mRNA before and after the training between the two groups (4.2 ± 0.9 *vs*. 2.8 ± 0.3). CV was 1.31% and 1.65% for the duplicated analyses for CS and HADH respectively.

Discussion

The findings from the present study demonstrate that:

1) a hypoxic and normoxic exercise training period of three weeks increased the PPO at sea-level without any changes in $\dot{V}O_{2max}$.
2) a hypoxic exercise training seems to induce qualitative changes of skeletal muscle mitochondrial respiration without a change in enzymatic activity at protein and gene level.

Mitochondrial respiration

To our knowledge, the effect of a hypoxic exercise-training period on mitochondrial respiration in humans had not yet been studied. In the present study, the maximal muscle oxidative capacity was measured *in situ* with different types of substrates. Several studies used glutamate + malate as substrate to investigate maximal oxidative capacity (28, 41, 52). In addition, palmitate + malate was also tested to investigate the different specific metabolic pathways. The biopsies were taken from the *vastus lateralis* because this muscle is highly activated during cycling. Muscle oxidative capacity has been shown to be significantly improved by endurance training (3, 4, 46). However, the present study showed no alteration in mitochondrial respiration, measured *in situ,* after a normal, normoxic exercise-training period. This result is in agreement with the study of Ponsot *et al*. (32) who observed no changes in mitochondrial function in male distance runners after six weeks of two training sessions a week of treadmill running at the second ventilatory threshold (VT2) at sea level. In contrast, Walsh *et al.* (46) found an increase in maximal mitochondrial respiration after six weeks of endurance cycle training. However, the study was done in untrained subjects. Zoll *et al*. (51) found that eight weeks of voluntary wheel running increased the mitochondrial oxidative capacity; however this study was done on female Wistar rats. The present study is to our knowledge the first study to investigate the effect of endurance training in trained athletes.

The absent of changes in the Nor group in the present study might be explained by the fact that the subjects are trained endurance athletes and thus have already a high initial level of muscle oxidative capacity. Another explanation could be that the training stimulus was not high enough to evoke functional mitochondrial adaptations.

Meanwhile the hypoxic exercise-training period, to the contrary, seems to alter the mitochondrial respiration. These results are similar to those of the IHT group in the study of Ponsot *et al.* (32), who observed that IHT group, who performed six week treadmill running at VT2 at F_IO_2 of 14.5 %, improved mitochondrial function of the *vastus lateralis*, whereas as mentioned above no differences were observed for the normoxic control group.

The changes in mitochondrial respiration occur only in the H group, therefore, they can be attributed to the hypoxic exposure. This finding may be explained by a decreased mechanical efficiency, *i.e.* an altered recruitment pattern of fibers and muscles during hypoxic exercise. Unfortunately, the present study was not designed to investigate this. Because of the lack of oxygen during hypoxic exercise, the intrinsic properties of mitochondrial function, *i.e.* the substrate preferences, might be altered. Indeed, IHT increased carbohydrate utilization and seems to decrease fat oxidation. These findings are in accordance with Roberts *et al.* (33), who observed, by using indirect blood parameters measurements to investigate substrate preference, a decreased reliance on free fatty acids and a increase in glucose dependence after a chronic (21 days) altitude exposure (4300 m) in healthy men. However, no significant effect was found after an acute (4h) exposure to altitude (4300 m) (33). Moreover, the same authors (34) concluded that altitude exposure (3 weeks at 4300 m) increased glucose utilization both during rest and at exercise. The observed shift in substrate preferences, *i.e.* an increased glucose and decreased lipid utilization under intermittent hypoxic conditions may be more efficient as glucose is the most oxygen-efficient substrate for energy metabolism (33). Therefore, the hypoxic stimulus seems to overwrite the exercise training stimulus.

Indeed, it is well known that endurance training increases the subject's capacity to oxidize fat during exercise (16). Dyck *et al.* (5) found that palmitate oxidation increased in both untrained and trained rat *soleus* muscle after an eight-week endurance training protocol. It is generally accepted that a hypoxic training environment induces several physiological and cellular adaptations in order to maintain O_2 transport to satisfy tissue ATP demand. Moreover, the composition of skeletal muscle isoforms is modified by hypoxia in the sense of an increase in the fast type isoforms. Indeed, several studies found that rats, exposed to hypoxia, presented a shift in favor of glycolytic-oxidative fibers in comparison with normoxic control rats (20, 21). Tonkonogi *et al*. (42) showed that fiber-type-specific control of mitochondrial respiration is also present in human skeletal muscle mitochondria. Hypoxia alone affects the structural and biochemical properties of skeletal muscle by inducing a change in the profile of type I (oxidative) to type II (glycolytic) fibers (20, 21), which will affect the changes in substrate preference, *i.e.* an increase in glutamate and a decrease in palmitate utilization (33, 34) which is in agreement with the suggested shift in muscle fiber type. Unfortunately, we were not able to determine changes in muscle fiber type due to the quantity of muscle collected from that used for the mitochondrial respiration studies. However, fiber type does not change with acute training when quantified by standard histochemistry.

The fact that the ACR values for glutamate and palmitate did not change in both groups over time, might suggest that the electron transport to phosphorylation coupling did not increase (52). Therefore, this electron transport may have reached already their maximal level in these trained endurance athletes.

However, the pre-protocol difference in Vmax for palmitate + malate does limit the present conclusion on the effects of hypoxia on substrate preferences. Therefore, some caution has to be taken when discussing the observed decrease in palmitate utilization. As can be noticed, the initial level of palmitate oxidation is significantly higher in the H group compared to the

Nor group. All measurements were done under identical conditions by the same researcher and technical bias or equipment failure can be exclude. A possible explanation could be differences in dietary intake of the subjects. However, the diet was shown to be similar between subjects, *i.e.* high CHO. Another explanation can be muscle fiber differentiation as it is known that isolated mitochondria from fibers of type I are different in enzymes and substrate utilization compared to fibers of type II (22). Indeed, several studies observed that substrates differ in their concentration between different types of muscle fibers, with higher glycogen concentration in type II and higher triglyceride concentration for type I fibers (8). However, Erzen *et al*. (7) found no significant differences in terms of percentage and surface area in muscle fiber types, type I, IIa, IIb and IIc, from distinct symmetrical sites of the left and right of the *vastus lateralis* in 10 young health male subjects. The same authors (31) analyzed a total of 106 fascicles at six predetermined areas of the *vastus lateralis* biopsy samples in healthy men (18-40 yrs) and concluded that a consistent arrangement of fiber types within the fascicles was obtained, regardless of fascicles size, fiber type proportion, biopsy site and subject. The main characteristic of fiber distribution was a uniform distribution of IIa fibers in all layers in a *vastus lateralis* fascicle.

On the other hand, Elder et al.(6), using a more extensive sampling technique, demonstrated, that multiple samples need to be collected from quadriceps muscle to decrease the between-site standard deviation. Indeed, McGuigan et al. (27) suggested that sampling of 150-200 type I and IIA fibers from random blocks is required to provide an accurate reflection of fiber cross-sectional area. Therefore, fiber type differences in the sampling for the fascicles for the respiration measured may still present a source of bias for the pre-differences between H and Nor group.

Unfortunately, the present study did not analyze the fiber type distribution and therefore cannot totally exclude the possibility of muscle fiber differentiation. Therefore caution has to be taken when interpreting the results.

Mitochondrial enzyme activity

One of the main adaptations of skeletal muscle in response to endurance training is improved muscle oxidative capacity (3, 4), which results from changes in mitochondrial substrate utilization, but also from changes in mitochondrial enzyme activities (18). However, the present study did not observe any changes in CS and HADH nor in CS mRNA and HADH mRNA. According to the literature, several studies (14, 19) suggest that hypoxia *per se* is not a stimulus for increased mitochondrial oxidative enzyme activity (14, 25). Mizuno *et al.* (29) demonstrated a significant decrease in CS activity and in HADH in the gastrocnemius muscle however, in the *triceps brachii* muscle the enzyme activities were maintained on return to sea-level after a two week training period at 2100 to 2700 m. The performed training consisted mainly of cross-country skiing, which involved more arm and less leg muscles. In contrast, Terrados *et al.* (40) found a significantly greater increase in CS activity in leg musculature trained in hypobaric hypoxia (3 to 4 sessions of 30 min at 2300 m a week during 4 weeks) compared to normoxic training. Also a more pronounced increase, however not significantly different, was observed in HADH (40). Thus there are equivocal findings on the effect of hypoxia *per se* or combined with exercise on mitochondrial enzyme activity. It seems that the hypoxic stimulus of 382 $min \cdot week^{-1}$ at 3000 m during three weeks in the present study was not enough to evoke any changes in the mitochondrial oxidative enzyme activities while this stimulus is enough to evoke changes in the overall mitochondrial substrate utilization. Moreover, changes in muscle enzyme activities can be an indication of training- an/or hypoxic-induced changes in mitochondrial volume and density (41); however the fact that no

changes in enzyme activities were observed, suggest that there were no changes in mitochondrial volume and density in both groups. Moreover, the *vastus lateralis* of the subjects could have reached the maximal level in mitochondria. This result is in contrast with the studies of Geiser *et al*. (11) and Vogt *et al*. (45), where an increase in mitochondrial content after hypoxic training and exposure was found. Under hypoxic conditions the protein synthesis seems to be compromised (13).

Endurance performance

The present study showed that five sessions per week during three weeks of exercise training significantly improved normoxic PPO. However, performing such training under hypoxic or normoxic conditions did not further enhance these improvements. Therefore, the primary goal of hypoxic training, *i.e.* to enhance sea level performance more than normoxic training, is not validated in the present study.

The observed results are in accordance with Truijens *et al.* (43), who found that a five week training program with two high-intensity training sessions in a flume per week in well-trained swimmers did improve sea level performance, *i.e.* 100- and 400-m freestyle swim and even $\dot{V}O_{2max}$, however no additional improvement was induced by performing this training under hypoxia (F_IO_2 = 15.3 %). Similarly, Roels *et al.* (35) concluded that IHT of two high-intensity cycling sessions per week (100-90% relative peak power output, identical to the present high-intensity sessions) during seven weeks at a simulated altitude of 3000 m in well-trained cyclists and triathletes did not improve performance, *i.e.* PPO and 10-min cycle time trial nor $\dot{V}O_{2max}$ to a greater extent than a similar sea level training. In contrast, Julian *et al.* (24) observed that four weeks of 5:5 min hypoxia-to-normoxia ratio for 70 min at 10 % F_IO_2 of for five days a week in well-trained runners did not alter the 3km time-trial performance nor $\dot{V}O_{2max}$. Also, Hendriksen and Meeuwsen (15) concluded that nine days after a 10-days IHT

($2h \cdot day^{-1}$ of cycling at 60-70% of heart rate reserve in a hypobaric hypoxia at a simulated altitude of 2500 m) significantly improved performance in terms of maximal power output and mean and peak anaerobic power compared to similar normoxic training. Thus, the efficacy of IHT in terms of performance improvement remains controversial.

This study suggests that three weeks with two sessions of interval training and three steady work-training sessions are sufficient to obtain significant improvements in sea-level endurance performance, however, no improvements were found in $\dot{V}O_{2max}$. Meanwhile, it is well known that small, statistically non-significant improvements can result in a significant increase in endurance performance (10) and that for trained athletes endurance performance may be independent of $\dot{V}O_{2max}$ and that other submaximal variables may influence performance to a greater extent (1).

In terms of practical application of this IHT protocol for endurance athletes, the observed differences in mitochondrial oxidation could have an effect on submaximal parameters of endurance performance, which may influence performance (1). The subjects in this study were trained athletes, who have already a high initial level of oxidative capacity; therefore it might be that the IHT stimulus needs to be stronger, *i.e.* a higher level of simulated altitude or longer duration, to induce an observable impact of the differences in mitochondrial oxidation on maximal parameters of endurance performance. Further investigation is necessary to address this issue.

In addition, the fact that mitochondrial oxidative capacity increased in the H group without any improvement in whole-body $\dot{V}O_{2max}$ is in accordance with the consensus that $\dot{V}O_{2max}$ is limited more by cardiac output and not by peripheral changes in skeletal muscle.

The present results suggest that qualitative rather than quantitative changes in mitochondrial function of trained athletes with an already well developed mitochondrial oxidative capacity can be obtained after a three-week intermittent hypoxic training program. These qualitative

changes might increase the aerobic performance by ameliorating the integration of energy demand to utilization (32).

In summary, the present study has shown that three weeks of endurance training performed in hypoxia seems to alter substrate preference as measured by mitochondrial respiration compared to endurance training performed in normal environmental conditions, but does not alter normoxic $\dot{V}O_{2max}$ or PPO more than identical training in normoxia.

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Table 1: Subjects characteristics of Normoxic (Nor; n = 8) and Hypoxic (H; n = 10) groups.

	W0		W4	
	Nor	H	Nor	H
Age (years)	24.2 ± 0.4	24.4 ± 0.3		
Height (cm)	181.6 ± 0.03	180.1 ± 0.5		
Weight (kg)	71.3 ± 0.8	73.2 ± 0.8	72.0 ± 0.8	72.6 ± 0.8

Mean ± SE; W0: pre-training; W4: post-training.

Table 2: The averaged percentage of target power output (target PO), *i.e.* 100% PPO for interval sessions and 60% of VO_{2max} and the averaged absolute power output ($W_{absolute}$; W) for continuous training sessions, of Normoxic (Nor, n = 8) and Hypoxic (H; n = 10) groups.

	Continuous		**Interval**	
	% of target PO	**$W_{absolute}$**	**% of target PO**	**$W_{absolute}$**
Nor	91.7 ± 0.6	185.0 ± 7.5	91.0 ± 0.7	310.9 ± 4.5
H	91.8 ± 1.6	130.0 ± 0.3	91.8 ± 1.0	259.4 ± 0.3

Mean ± SE.

Table 3: The average intensity of additional training outside the experimental design of each training week for Normoxic (Nor; n = 8) and Hypoxic (H; n = 10) groups.

	W1	**W2**	**W3**
Nor	3.1 ± 0.05	3.1 ± 0.06	3.2 ± 0.1
H	2.9 ± 0.09	3.0 ± 0.05	3.0 ± 0.07

Mean ± SE; W1: first week of training protocol; W2: second week of training protocol; W3: third week of training protocol.

Table 4: Incremental test to exhaustion of Normoxic (Nor; n = 8) and Hypoxic (H; n = 10) groups.

	W0		**W4**	
	Nor	**H**	**Nor**	**H**
VO_{2max}($ml \cdot kg^{-1} \cdot min^{-1}$)	58.1 ± 0.8	58.5 ± 0.7	61.0 ± 1.2	58.3 ± 0.6
PPO (W)	341.7 ± 3.5	339.0 ± 0.5	366.3 ± 3.2 *	361.5 ± 4.4 *
HR_{max} (bpm)	190.1 ± 1.1	189.7 ± 1.1	188.3 ± 1.5	189.4 ± 1.0
VE_{max} ($L \cdot min^{-1}$)	159.9 ± 2.6	154.2 ± 1.4	174.3 ± 2.5	155.0 ± 1.7
RPE_{max}	17.9 ± 0.2	16.4 ± 0.2 §	17.9 ± 0.1	16.9 ± 0.2

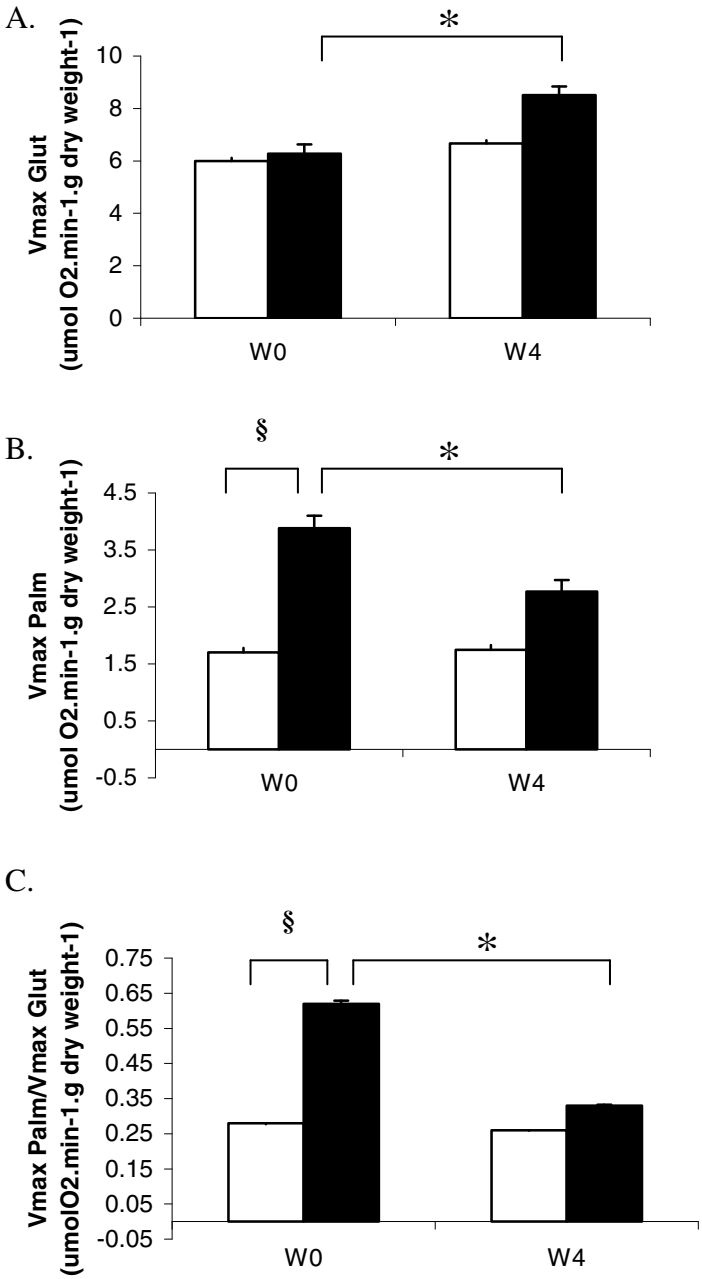
Mean ± SE; W0: pre-training; W4: post-training; VO_{2max}: highest value of the oxygen consumption averaged over 30 s; PPO: peak power output; VE_{max}: highest value of ventilation averaged over 30 s; RPE: rate of perceived exertion; *: $P < 0.05$ for the differences within a group versus W0;§: $P < 0.05$ for the differences between groups at a matched time point.

Table 5: Basal oxygen consumption (V_0) and acceptor control ratio (ACR) in saponin-permeabilised muscle fibers of Normoxic (Nor; n = 8) and Hypoxic (H; n = 8) groups.

	W0		**W4**	
	Nor	**H**	**Nor**	**H**
V_0 glutamate ($\mu molO_2 \cdot min^{-1} \cdot g$ dry weight^{-1})	1.70 ± 0.00006	1.94 ± 0.00007	1.79 ± 0.00005	1.93 ± 0.00005
V_0 palmitate ($\mu molO_2 \cdot min^{-1} \cdot g$ dry weight^{-1})	1.28 ± 0.0001	1.59 ± 0.00002	1.26 ± 0.00006	1.67 ± 0.00009
ACR glutamate	3.34 ± 0.16	2.44 ± 0.09	3.76 ± 0.21	4.15 ± 0.06
ACR palmitate	1.73 ± 0.10	2.21 ± 0.04	1.43 ± 0.05	2.08 ± 0.21

Mean ± SE. W0: pre-training; W4: post-training; V_0: basal oxygen consumption expressed in $\mu molO_2 \cdot min^{-1} \cdot g$ dry weight^{-1}; ACR: Acceptor Control Ratio.

Figure 1: Maximal respiratory rate with A. glutamate + malate (Vmax Glut), B. palmitate + malate (Vmax Palm), and C. the ratio of maximal palmitate and glutamate oxidation (Vmax Palm/Vmax Glut), expressed in $\mu molO_2 \cdot min^{-1} \cdot g$ dry $weight^{-1}$, in saponin-permeabilised muscle fibers of Normoxic (Nor; n = 8) and Hypoxic (H; n = 8) groups.



Mean ± SE; W0: pre-training; W4: post-training *: $P < 0.05$ for the differences within a group versus W0; §: $P < 0.05$ for the differences between groups at a matched time point.