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Exercise training in normobaric hypoxia in endurance runners. II. Improvement of mitochondrial properties in skeletal muscle

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Ponsot, Elodie, Stéphane P. Dufour, Joffrey Zoll, Stéphane Doutrelau, Benoit N'Guessan, Bernard Geny, Hans Hoppeler, Eliane Lampert, Bertrand Mettauer, Renée Ventura-Clapier, and Ruddy Richard. Exercise training in normobaric hypoxia in endurance runners. II. Improvement of mitochondrial properties in skeletal muscle. *J Appl Physiol* 100: 1249–1257, 2006. First published December 8, 2005; doi:10.1152/japplphysiol.00361.2005.—This study investigates whether adaptations of mitochondrial function accompany the improvement of endurance performance capacity observed in well-trained athletes after an intermittent hypoxic training program. Fifteen endurance-trained athletes performed two weekly training sessions on treadmill at the velocity associated with the second ventilatory threshold (VT_2) with inspired O_2 fraction = 14.5% [hypoxic group (Hyp), n = 8] or with inspired O_2 fraction = 21% [normoxic group (Nor), n = 7], integrated into their usual training, for 6 wk. Before and after training, oxygen uptake ($\dot{V}O_2$) and speed at VT_2, maximal $\dot{V}O_2$ ($\dot{V}O_{2\,max}$), and time to exhaustion at velocity of $\dot{V}O_{2\,max}$ (minimal speed associated with $\dot{V}O_{2\,max}$) were measured, and muscle biopsies of vastus lateralis were harvested. Muscle oxidative capacities and sensitivity of mitochondrial respiration to ADP (K_m) were evaluated on permeabilized muscle fibers. Time to exhaustion, $\dot{V}O_2$ at VT_2, and $\dot{V}O_{2\,max}$ were significantly improved in Hyp (+42, +8, and +5%, respectively) but not in Nor. No increase in muscle oxidative capacity was obtained with either training protocol. However, mitochondrial regulation shifted to a more oxidative profile in Hyp only as shown by the increased K_m for ADP (Nor: before 476 ± 63, after 524 ± 62 μM, not significant; Hyp: before 441 ± 59, after 694 ± 51 μM, $P < 0.05$). Thus including hypoxia sessions into the usual training of athletes qualitatively ameliorates mitochondrial function by increasing the respiratory control by creatine, providing a tighter integration between ATP demand and supply.

intermittent hypoxia training; skeletal muscle; mitochondria; time to exhaustion; endurance athletes

HYPOXIA AND PHYSICAL EXERCISE are two independent potent metabolic stressors (1) that induce adaptations of the O_2 supply and utilization at the whole body tissue as well as molecular levels. For this reason, to cumulate benefits of both stimuli, training under hypoxic conditions is widely used to improve athlete aerobic performance linked to peripheral adaptations. The "living low-training high" (LLTH) method, which consists of performing only the training sessions under hypoxia, has provided significant improvement in maximal O_2 uptake ($\dot{V}O_{2\,max}$) (23, 29). In addition, increased mitochondrial densities, capillary-to-fiber ratios, fiber cross-sectional areas, activities of oxidative enzymes like citrate synthase (CS), capillary density, and higher myoglobin content have been reported in muscle of sedentary humans subjected to LLTH protocols (7, 17, 23, 37, 45). In athletes, however, the loss of efficiency mainly due to lower training intensities and the lack of convincing effects in competitive performance are often pointed out (13, 21, 30). Moreover, improvements in performance seem to be obtained with LLTH only when hypoxic training sessions are of sufficient duration and intensity [typically above second ventilatory threshold (VT_2)] (38), but not for lower work rates (43).

To take into account these inconveniences, an intermittent hypoxic training (IHT) program has been proposed, whose specificity is the combination of hypoxic and normoxic training sessions performed by trained athletes, run at velocity associated with VT_2 (vVT_2) for at least 2 × 12 min per session twice a week (43) [see part I of this study (10)]. Although this new training protocol was without effect on maximal power output and hypoxic maximal work capacity in a previous study (43), the combination of hypoxic stimulation during exercise with the preservation of high workloads during the normoxic sessions could be expected to induce beneficial effects on aerobic performance, especially when the ability to sustain longer the minimal velocity associated with $\dot{V}O_{2\,max}$ [time to exhaustion (Tlim)] is considered. Indeed, we show in the accompanying paper (10) that introducing hypoxic training sessions in the usual training schedule of trained athletes greatly improved Tlim, thus opening the question of the muscular mechanisms accompanying the observed improvement in endurance performance capacity.

Although muscle oxidative capacity is a major component of endurance performance, after several years of endurance training, it seems that athletes reach the limit of their adaptive potential in terms of quantitative aspects of muscular oxidative capacities (27). At the cellular level, it is now clearly established that low sensitivity of mitochondrial respiration to cytosolic ADP and the control of respiration by the creatine kinase (CK) system with mitochondrial CK (mi-CK) as an ultimate element is a hallmark of fatigue-resistant oxidative

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muscle (19, 41). This tissue specificity of the control of mitochondrial respiration permits the highly oxidative muscle to respond to ATP utilization by modifying energy fluxes through the CK shuttle on a "pay as you go" basis, with mitochondria being less sensitive to cytosolic ADP levels but more sensitive to the local phosphocreatine (PCr)-to-Cr ratios (44). This allows close control of mitochondrial regulation by energy consumption at the sites of excitation-contraction coupling. It has recently been established in animal models and human subjects that increasing aerobic performance is associated with reorganization of muscle fiber cytoarchitecture and with quantitative and qualitative mitochondrial adaptations (39, 50, 52). These qualitative modulations of mitochondrial function involve decreased sensitivity of mitochondrial respiration to cytosolic ADP and increased coupling to phosphotransfer kinases, contributing to a better integration between ATP demand and supply. Such changes are expected to limit perturbations of homeostasis like the decrease of the ATP-to-ADP ratio (ATP/ADP), to improve oxidative ATP supply, and to delay the participation of anaerobic glycolysis to energy supply.

We hypothesized that qualitative changes of mitochondrial function could be a critical mechanism of muscular metabolic adaptation induced by a training protocol capable of increasing the maximal endurance capacity (Tlim) of athletes.

The goals of the present study were thus: *1*) to investigate whether the increased aerobic performance capacity of already trained athletes, following a training program, including moderate hypoxia stimulation, is accompanied by changes in the sensitivity to ADP and creatine of skeletal muscle mitochondria; and *2*) to verify whether alterations of one or more of the parameters of mitochondrial function are linked to the improvement in aerobic performance.

METHODS

Subjects. Fifteen highly trained male distance runners participated in the study. Biopsies were performed before and after training in the 15 subjects. Among them, eight trained with two weekly hypoxic training sessions to constitute the hypoxic group (Hyp, $n = 8$), and seven trained without hypoxic sessions to constitute the normoxic group (Nor, $n = 7$). Both groups had similar anthropometrical characteristics (Table 1), including the percentage of body fat mass (12). All subjects gave their written consent to the study and were fully informed about its potential risks. All experiments were approved by our institution's ethics committee.

Experimental design. Before and following the training period, all subjects performed: *1*) an incremental treadmill test to exhaustion at sea level [inspired O_2 fraction ($F_{I_{O_2}}$) = 21%] and at the simulated 3,000-m training altitude ($F_{I_{O_2}}$ = 14.5%), to assess oxygen consumption ($\dot{V}O_2$) at VT_2 and vVT_2 and $\dot{V}O_{2\,max}$ and the minimal velocity that elicited $\dot{V}O_{2\,max}$ ($v\dot{V}O_{2\,max}$); and *2*) a constant-load test at $v\dot{V}O_{2\,max}$ to determine the Tlim. For further details, see part I of this study (10). During incremental and all-out tests, athletes breathed normoxic or hypoxic air through a facial Hans-Rudolph mask, and $\dot{V}O_2$ was assessed by measuring both $F_{I_{O_2}}$ and expired O_2 fraction. For further details, see part I of this study (10).

Table 1. *Group characteristics*

	Groups	
	Hyp	Nor
Age, yr	29.9±2.3	31.3±2.3
Weight, kg	71.1±2.4	71.0±2.9
Height, cm	181±4	180±2
Body fat, %	11.6±1.0	11.9±1.5

Values are means ± SE. Hyp, group of subjects training under hypoxia ($n = 8$); Nor, group of subjects training under normoxia ($n = 7$).

Subjects were randomly assigned to one of the two groups for 6 wk and performed within their usual training program two weekly training sessions on a treadmill at vVT_2 calibrated by the previous incremental tests (10). The Hyp group ran the two laboratory sessions under simulated normobaric hypoxia ($F_{I_{O_2}}$ = 14.5%) by breathing through a face mask providing the hypoxic gas mixture, whereas the Nor group breathed room air. Identical sessions were performed by the control group (Nor) under normoxia ($F_{I_{O_2}}$ = 21%). Exercise duration of the sessions at vVT_2 was increased each week (from 2 × 12 min to 2 × 20 min), and exercise intensity was readjusted at the fourth week to elicit the same heart rate as at the first laboratory VT_2 session. For further details, see part I of this study (10).

Skeletal muscle biopsy. Biopsy samples were taken using the percutaneous Bergström technique after local anesthesia (lidocaine-lignocaine), as previously described (26). Subjects were asked to refrain from sporting activities 48 h before the biopsy, which always occurred before any other evaluation test. No complications occurred following biopsies in any subject. The muscle tissue retrieved was rinsed in ice-cold saline, one part was immediately frozen in liquid nitrogen for enzymatic activities, and another part served for in situ respiration studies. Muscles were kept at 4°C in *solution S* (see below) until fiber separation.

In situ study of mitochondrial respiration. Mitochondrial respiration was studied in situ in saponin skinned fibers, as previously described (32, 42). Briefly, fibers were gently separated under binocular microscope in *solution S* at 4°C (see below) and permeabilized in *solution S* with 50 μg/ml saponin for 30 min. After being rinsed for 10 min in *solution R* (see below) to wash out cytosolic adenine nucleotides and PCr, skinned fibers were transferred in a water-jacketed oxygraphic cell (Strathkelvin Instruments, Glasgow, UK) equipped with a Clark electrode containing 3 ml of *solution R*, as previously described (26), and basal respiration rate ($\dot{V}_0$) was measured at 22°C under continuous stirring. ADP-stimulated respiration ($\dot{V}_{ADP}$) above $\dot{V}_0$ was measured by stepwise addition of ADP as phosphate acceptor (from 10 to 2,000 μM), with or without creatine (20 mM). The apparent K_m values for ADP were calculated by using a nonlinear monoexponential fitting of the Michaelis-Menten equation. Nonlinear fitting for K_m assessment in skinned muscle fibers is an already established fitting method, giving consistent results and yielding correlation coefficients ≥0.99 for each measurement. Moreover, it gives an equal weight to each experimental measurement, avoiding the disadvantages of linear fitting that overweigh the extreme points compared with the others. Maximal respiration rate ($\dot{V}_{max}$) was calculated as ($\dot{V}_{ADP} + \dot{V}_0$). The acceptor control ratio (ACR) was calculated as ratio of $\dot{V}_{max}$ to $\dot{V}_0$. Examples of the data obtained by the skinned-fiber respiration experiment with increasing amounts of ADP and the corresponding Michaelis-Menten fit are presented in Fig. 1.

Following these ADP additions, functioning of various complexes of the electron transport chain (ETC) function was also assessed. Addition of 2 mM amytal, a specific inhibitor of complex I, followed by 25 mM succinate, allowed estimation of the maximal respiration from complexes II, III, and IV ($\dot{V}_{succ}$). Thereafter, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD; 0.5 mM) and ascorbate (0.5 mM) were added to estimate only maximal respiration from complex IV [$\dot{V}_{TMPD\text{-}Asc}$, cytochrome oxidase complex (COX)]. The $\dot{V}_{TMPD\text{-}Asc}$-to-$\dot{V}_{max}$ ratio, which represents the amount of excess respiration, is an expression of the COX excess (15).

Both *solutions R* and *S* contained 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA (100 nM free Ca^{2+}), 6.56 mM $MgCl_2$ (1 mM free Mg^{2+}), 20 mM taurine, 0.5 mM dithiothreitol, 50 mM potassium-methane sulfonate (160 mM ionic strength), and 20 mM imidazole (pH 7.1). *Solution S* also contained 5.7 mM Na_2ATP and 15 mM creatine

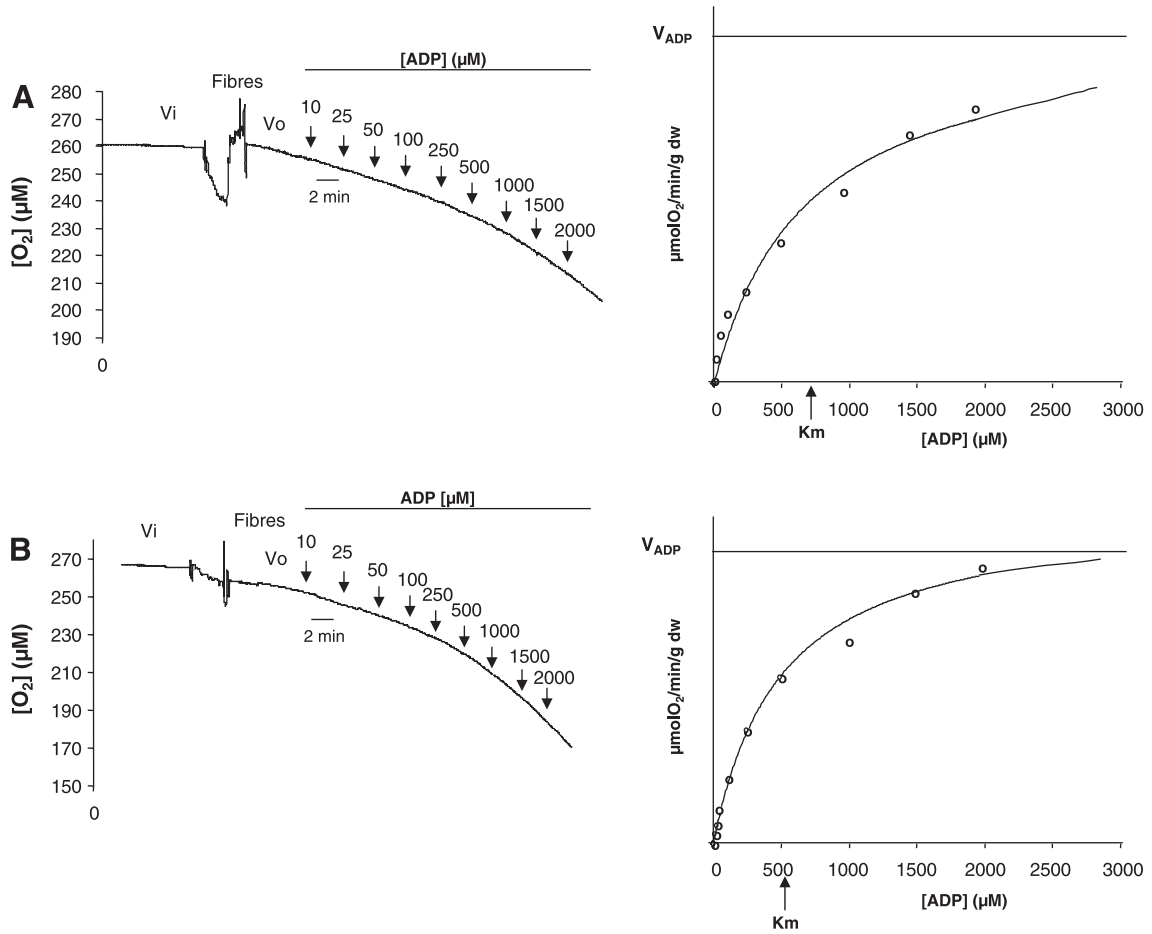

Fig. 1. Representative respiration experiment in vastus lateralis skinned fibers of one endurance runner who trained under hypoxia (*A*) and one who trained in normoxia (*B*). *Left*: decrease in O_2 concentration ($[O_2]$) within the oxygraphic chamber with increasing amounts of the phosphate acceptor ADP. $\dot{V}_i$, initial respiration rate; $\dot{V}_0$, basal respiration rate of the fibers. *Right*: Michaelis-Menten fit of respiration as a function of ADP concentration ([ADP]). $\dot{V}_{ADP}$, maximal oxygen consumption extrapolated for the Michaelis-Menten fit; $\dot{V}_{max}$ ($\dot{V}_0 + \dot{V}_{ADP}$), maximal oxidative capacity. *A*: $\dot{V}_{max}$ and K_m for ADP were 9.32 μmol·min^{-1}·g dry wt^{-1} (dw) and 693.9 μM, respectively, after training. Correlation coefficient of the fit was 0.9916. *B*: $\dot{V}_{max}$ and K_m for ADP were 9.94 μmol·min^{-1}·g^{-1} and 506.3 μM, respectively, after training. Correlation coefficient of the fit was 0.9981.

phosphate. *Solution R* contained 3 mM phosphate, 2 mg/ml fatty acid-free bovine serum albumin, 2 mM malate, and 5 mM glutamate. After the experiments, fibers were harvested, dried, and weighted to express respiration rates as micromoles O_2 per minute per gram dry weight.

Enzyme analysis. Part of the frozen muscle samples were weighted, homogenized into ice-cold buffer (30 mg/ml) containing 5 mM HEPES, 1 mM EGTA, 5 mM $MgCl_2$, and 0.1 Triton X-100 (pH 8.7), and incubated for 60 min at 0°C to ensure complete enzyme extraction. CS activity was determined according to Srere (35), and COX activity was assayed according to Wharton and Tzagoloff (49) at 30°C and pH 7.5 by spectrophotometric analysis.

Statistical analysis. Statistical analysis was performed by using the Sigmastat 3.0 software. To test for both treatment (hypoxia vs. normoxia) and time (before vs. after) effects on each of the measurements during the training period, we used a two-way ANOVA for repeated measures followed by a Student-Newman-Keuls post hoc procedure. Data are means ± SE. Differences were considered to be significant for $P < 0.05$.

RESULTS

Exercise tests. Both $\dot{V}_{O_2\,max}$ and $\dot{V}_{O_2}$ at the VT_2 were significantly improved by training in the Hyp group only, as shown in Table 2. Moreover, the time the subjects were able to sustain $v\dot{V}_{O_2\,max}$ until exhaustion (Tlim) was markedly longer after training in the Hyp group only (+41.7%; $P = 0.001$), as depicted in Fig. 2.

Mitochondrial function. Mean $\dot{V}_0$ in the absence of the phosphate acceptor ADP and mean $\dot{V}_0$ at saturating ADP concentration ($\dot{V}_{max}$), which characterize the muscle oxidative capacities, were similar in both groups before training (Fig. 3). None of these quantitative parameters were improved after 6 wk, whatever the training modality. The ACR ($\dot{V}_{max}/\dot{V}_0$), representing the coupling between oxidation and phosphorylation, was similar in the two groups before training (Nor: 5.4 ± 0.6; Hyp: 5.2 ± 0.7) and remained unchanged after training (Nor: 6.4 ± 1.1; Hyp: 5.1 ± 0.7).

Mean K_m values for ADP are presented in Fig. 4 in the absence or presence of creatine. Before training, both groups presented high and similar K_m values without creatine (inversely proportional to ADP sensitivity). In both groups, addition of creatine to stimulate mi-CK significantly decreased the K_m (K_{m+Cr}) values to a similar level. As expected, the fivefold increase in ADP sensitivity with creatine indicates an efficient mi-CK coupling with oxidative phosphorylation in the muscle of these highly trained subjects (52).

Table 2. *Effects of the two training modalities on $\dot{V}O_{2\ VT_2}$ and $\dot{V}O_{2max}$*

	Groups			
	Hyp		Nor	
	Before training	After training	Before training	After training
$\dot{V}O_{2\ VT_2}$, % $\dot{V}O_{2max}$	88.6±0.9	91.3±0.7*	88.8±1.4	89.1±1.3
$\dot{V}O_{2\ VT_2}$, ml·min^{-1}·kg^{-1}	56.4±1.3	61.1±0.8*	55.0±1.4	55.9±1.0
$\dot{V}O_{2max}$, ml·min^{-1}·kg^{-1}	63.6±1.1	67.0±1.2*	62.0±1.4	62.8±1.2

Values are means ± SE. Hyp, group of subjects training under hypoxia at the second ventilatory threshold (VT_2) ($n = 8$); Nor, group of subjects training under normoxia at VT_2 ($n = 7$); $\dot{V}O_{2\ VT_2}$, O_2 consumption at VT_2; $\dot{V}O_{2max}$, maximal O_2 consumption. *Significant difference after vs. before training ($P < 0.05$).

After training, the K_m values significantly increased in the Hyp group only, reaching values higher than in the Nor group (+57%; $P = 0.001$). Interestingly, in the Hyp group, pre- and posttraining K_m and Tlim individual values disclosed a consistent pattern of simultaneous increase after hypoxia training (Fig. 5*B*) that was not found after normoxia training (Fig. 5*A*). However, the correlation between changes in K_m and changes in Tlim did not reach significance ($P = 0.286$). After training, the ratio of K_m to K_{m+Cr} (K_m/K_{m+Cr}), which reflects the efficiency of mi-CK and oxidative phosphorylation coupling, increased two times more in Hyp (+124%; $P = 0.005$) than in Nor (+66%; $P = 0.04$).

Assessment of the different complexes of the mitochondrial ETC is shown in Table 3. The ADP-stimulated maximal $\dot{V}_{succ}$ and the ratio of $\dot{V}_{succ}$ to $\dot{V}_{max}$ remained unchanged after training in both groups. $\dot{V}_{TMPD\text{-}Asc}$ as well as $\dot{V}_{TMPD\text{-}Asc}$-to-$\dot{V}_{max}$ ratio were similar in both groups and did not change, whatever the training modality, showing neither specific adaptation nor deterioration at the ETC level after both training modalities.

Enzyme activities. Enzyme activities are presented in Table 3. The Krebs cycle enzyme CS as well as the complex of the respiratory chain COX activities were similar in both groups and remained unchanged by the 6-wk training.

DISCUSSION

Major findings. This study shows that skeletal muscle mitochondrial adaptations accompany exercise performance improvements in already trained athletes after the present IHT program. While oxidative enzyme activities (CS and COX), as well as muscle oxidative capacity ($\dot{V}_{max}$) remained unchanged, the control of mitochondrial respiration by cytosolic ADP (higher K_m) was depressed after IHT only.

Taken together, these results suggest that, in already trained athletes with high muscular oxidative capacities, qualitative rather than quantitative adaptations of skeletal muscle metabolism are still to be obtained after an IHT. These qualitative adaptations could participate in the increase of the endurance performance by improving integration of energy demand to utilization.

Normoxic and hypoxic training and performance. As presented in part I of this study (10) and reported in Table 1, $\dot{V}O_{2\ max}$ and $\dot{V}O_2$ at the ventilatory threshold were significantly improved by training in the Hyp group only. Moreover, improved endurance performance capacity was also clearly observed as a prolonged Tlim at the pretraining minimal velocity, eliciting $\dot{V}O_{2\ max}$ in the Hyp group only. Thus, despite moderate hypoxic exposure, this IHT program demonstrated a significant training effect in competitive runners (see Ref. 10 for further discussion).

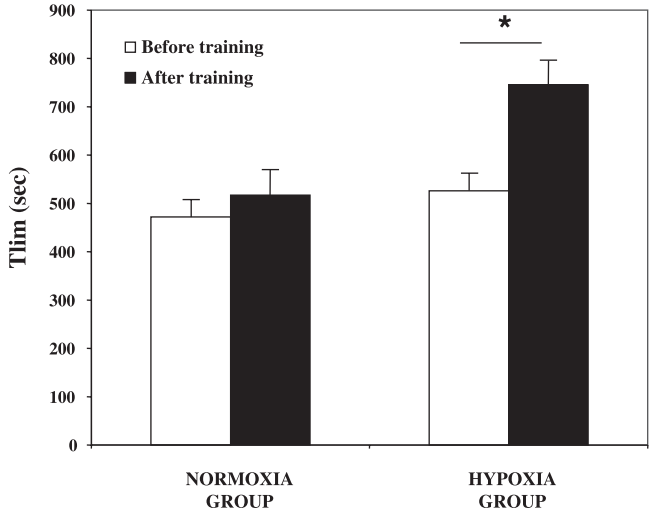

Fig. 2. Time to exhaustion (Tlim) at the minimal speed eliciting maximum O_2 uptake ($\dot{V}O_{2\ max}$) before and after 6 wk of training in hypoxia or normoxia. Values are means ± SE. *Significant difference after vs. before training ($P < 0.05$).

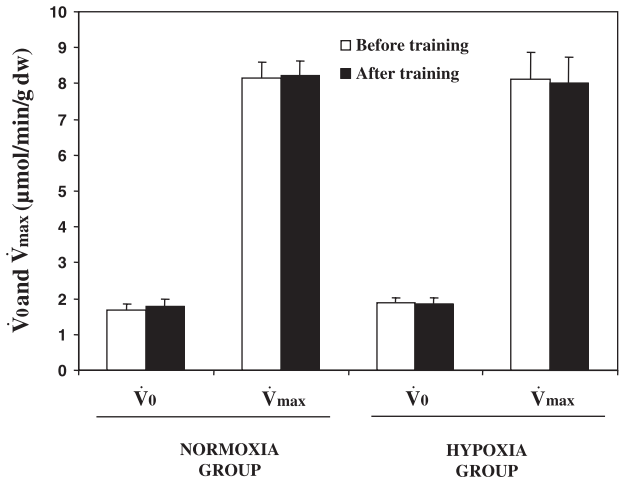

Fig. 3. $\dot{V}_0$ and $\dot{V}_{max}$ mitochondrial respiratory rates in saponin-treated fibers before and after 6 wk of training in hypoxia and normoxia. Values are means ± SE. No differences were observed between groups, either before or after training.

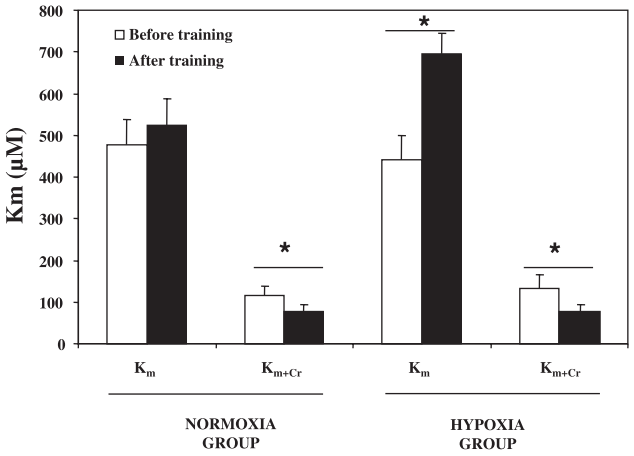

Fig. 4. Apparent K_m for ADP (μM), with or without creatine (Cr), before and after 6 wk of training in hypoxia and normoxia. Values are means ± SE. *Significant difference after vs. before training ($P < 0.05$).

Physiological consequences of the normoxic training programs on muscle oxidative capacity. Although it is well known that endurance training results in improvement in exercise capacity and muscle oxidative capacities when either ultrastructural (6, 40), biochemical (16), or functional parameters (47, 52) are examined, the present normoxic training protocol increased neither $\dot{V}O_{2\,max}$ and Tlim at $v\dot{V}O_{2\,max}$, nor any biochemical or functional markers of mitochondrial content. The origin of this difference may arise from one main reason: the training protocol, corresponding to the usual training of the athletes, neither increased the duration of the training sessions nor augmented the metabolic and mechanical components of the training load compared with the normal activity of the athletes. Effects obtained in the Hyp group could thus be mainly attributed to the added hypoxic stimulus.

Lack of quantitative changes of mitochondrial function after the IHT program. The measure of mitochondrial respiration in situ ($\dot{V}_{max}$), as well as COX and CS activities, showed that replacing two normoxic sessions of the usual training by two moderately hypoxic sessions at the same relative intensity did not change the muscle maximal oxidative capacity (quantitative changes) of endurance athletes. In the past, ultrastructural mitochondrial density and CS as a flux-generating enzyme in the Krebs cycle have been used, together with the COX activity, as markers of maximal oxidative capacity (25). Therefore, our results suggest that no further increase in mitochondrial density occurs after hypoxic training in already well-trained subjects, despite improvement in their endurance capacity (i.e., Tlim). These results contrast with previous studies showing an increase in mitochondrial content following hypoxic training (14, 45). The most likely explanation for these apparent discrepancies is that the latter were carried out on initially untrained animals or human subjects (14, 17, 23, 47) and reflect both training and hypoxia effects.

Together with the results obtained in the Nor group, this suggests that quantitative adaptations of the mitochondrial network to endurance training may progressively level off as oxidative capacity increases. This may appear, despite further exposition of muscles to the mechanical and metabolic stimuli induced by training and/or hypoxia. Therefore, oxidative capacity in vastus lateralis muscles of these trained athletes may have reached levels close to the maximal ones possibly induced by training. Actually, oxidative capacity of less trained subjects (7.9 ± 0.5 μmol $O_2 \cdot min^{-1} \cdot g$ dry wt^{-1}) (52) was similar to the values observed in the present study (Nor: 8.2 ± 0.7; Hyp: 8.0 ± 0.7 μmol $O_2 \cdot min^{-1} \cdot g$ dry wt^{-1}), despite a $\dot{V}O_{2\,max}$ 12 $ml \cdot min^{-1} \cdot kg^{-1}$ lower than in the present groups, strongly arguing for an upper limitation of mitochondrial content following intense training (27). The ACR also remained unchanged in both groups, suggesting that the electron transport to phosphorylation coupling was not further improved compared with less trained subjects (52). These results are in accordance with the observation that hypoxia hardly affects mitochondrial function. Hypoxic stress stabilizes and activates the hypoxia-inducible transcription factor-1. This transcription factor mainly activates the transcription of genes coding for glycolytic enzymes and angiogenic factors but hardly modify mitochondrial proteins (18). Nevertheless, the possibility that longer training duration may further modify the quantitative parameters of mitochondrial respiration needs further investigations. Altogether, this suggests that, for already trained subjects, either the skeletal muscle plasticity allows quantitative adaptations of mitochondrial oxidative capacity up to a

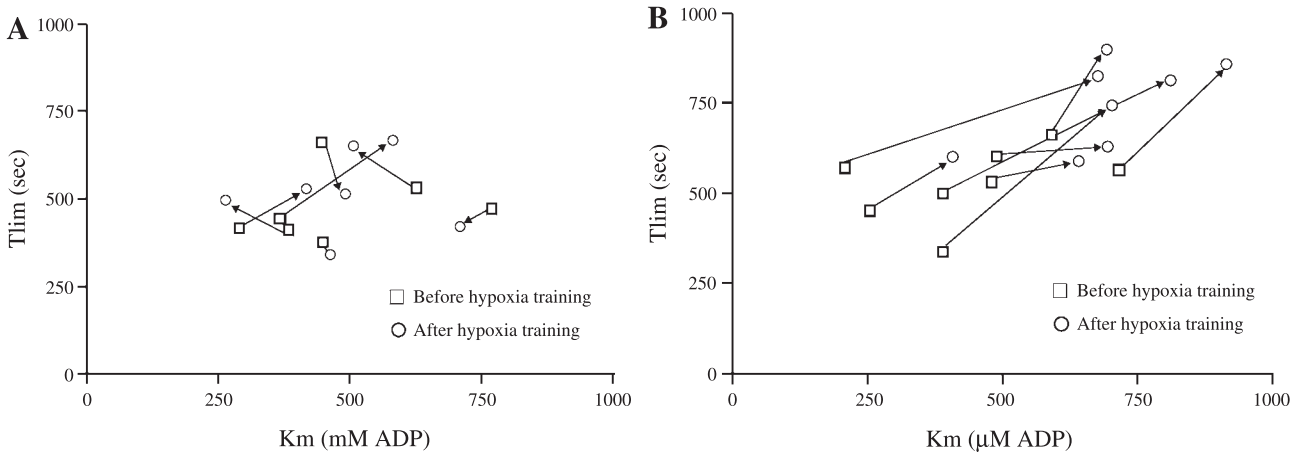

Fig. 5. Individual values of K_m for ADP (in absence of Cr) and Tlim before (□) and after (○) training in the normoxic group (*A*), which trained in normoxia only, and in the hypoxic group (*B*), which trained under hypoxia for two sessions per week.

Table 3. *Mitochondrial function*

	Groups			
	Hyp		Nor	
	Before training	After training	Before training	After training
Complexes of electron transport chain				
$\dot{V}_{succ}$, μmol O_2·min^{-1}·g dry wt^{-1}	9.2±0.8	11.7±1.0	10.5±0.6	11.7±0.7
$\dot{V}_{TMPD\text{-}Asc}$, μmol O_2·min^{-1}·g dry wt^{-1}	23.7±1.7	22.1±1.1	22.9±2.6	19.2±1.4
$\dot{V}_{succ}/\dot{V}_{max}$	1.3±0.2	1.7±0.1	1.3±0.1	1.4±0.1
$\dot{V}_{TMPD\text{-}Asc}/\dot{V}_{max}$	3.4±0.6	3.3±0.2	2.9±0.4	2.4±0.4
Biochemical data				
Citrate synthase, IU/g wet wt	19.4±0.4	19.8±0.9	18.3±1.6	16.9±1.7
Cytochrome oxidase, IU/g wet wt	5.6±1.4	5.5±1.1	4.2±0.6	3.6±0.4

Values are means ± SE; $n = 8$ for Hyp group and $n = 7$ for Nor group. $\dot{V}_{succ}$, respiration under succinate; $\dot{V}_{TMPD\text{-}Asc}$, respiration under *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine + ascorbate; $\dot{V}_{max}$, maximal respiration rate; $\dot{V}_{succ}/\dot{V}_{max}$, ratio of $\dot{V}_{succ}$ to $\dot{V}_{max}$; $\dot{V}_{TMPD\text{-}Asc}/\dot{V}_{max}$, ratio of $\dot{V}_{TMPD\text{-}Asc}$ to $\dot{V}_{max}$.

plateau and levels off thereafter, or hypoxic stimulus does not improve mitochondrial content.

Qualitative changes of mitochondrial function after the IHT program. Although improved muscle performances rely in part on increased mitochondrial content and oxidative capacity, we and others have recently shown that organization of intracellular energy fluxes is an integral part of the muscle phenotype and of the adaptation to endurance training (44, 47, 50, 52). The main observation of this study is a critical modification of the regulation of mitochondrial respiration by ADP and creatine after IHT. It is noteworthy that the apparent K_m for ADP (inversely proportional to the affinity of mitochondria for ADP) was already high in the two groups, in accordance with previous results obtained for highly trained athletes compared with untrained subjects (48, 52). In addition, it was more than 50% higher after integration of hypoxic sessions into the usual training program of athletes. The fact that quantitative and qualitative characteristics of mitochondrial respiration are not always coregulated was already suspected from our laboratory's previous study (52), where only mitochondrial quantitative adaptations were observed, together with increasing training status (comparison of sedentary vs. active subjects), whereas supplemental qualitative adaptations appeared with regular endurance training (comparison between active and athletic subjects). The apparent K_m for ADP has been shown to be related to the metabolic profile of the muscle, being higher in muscle with higher oxidative capacity (4, 19, 32, 41, 44, 50). In such oxidative muscles, addition of creatine decreases the K_m for ADP, indicating that ATP production is then coupled to PCr resynthesis within the intermembrane space. Thus the decrease in sensitivity to external ADP, together with the ability of creatine to increase the respiratory effects of ADP as a phosphate acceptor, is a hallmark of oxidative muscle fibers. In these fibers, cytosolic ADP is no longer the main stimulus of mitochondrial respiration that is then driven by the local Cr-to-PCr ratio, with mi-CK being coupled to ATP production and translocation. In the mixed human vastus lateralis muscle, a decrease in mitochondrial sensitivity to cytoplasmic ADP appears either with training or with increasing activity levels (39, 47, 52). Moreover, consistent with the higher K_m/K_{m+Cr} values observed in trained subjects (52), the dramatic increase of the K_m/K_{m+Cr} in Hyp (+124%) underlines the critical role of mi-CK coupled to ATP production. This increase in mi-CK coupling to oxidative phosphorylation enhances the transfer of the phosphate moiety to PCr, and ADP is recycled to oxidative phosphorylation. This allows amplification of the ADP signal for stimulation of mitochondrial respiration, so that a smaller cellular ADP signal is necessary to stimulate respiration in intact muscle when CK is active. Mathematical modeling has shown that, in cells where mi-CK is coupled to adenyl nucleotide translocase and where there is a restricted access of ADP to the mitochondrial intermembrane space, the sensitivity of cellular respiration to the PCr-to-ATP ratio is increased (31). Therefore, O_2 uptake in these cells is also driven by lower local changes in ATP/ADP. This explains the long-held observation that the sensitivity of intact muscle cell respiration to global changes in ATP/ADP, and therefore to ADP, increases with training (9). In oxidative muscles, PCr is then shuttled by cytosolic CK and ultimately transferred by the bound MM-CK isoenzyme to ADP produced locally by the ATPases, thus ensuring a better coupling between energy production and utilization (33, 46). The Cr/PCr system thus functions as a low-threshold ADP sensor, functionally coupling energy production to energy utilization. Such an increase in energy channeling within the cell may have happened after hypoxic training in the vastus lateralis of endurance runners.

As suggested by the concomitant increase of both K_m and Tlim in the Hyp group, this may further improve energetic performance, without necessarily increasing mitochondrial mass. However, lack of significant correlation between the changes in K_m and Tlim suggests that other factors may be involved in the improvement of Tlim, as suggested by the results presented in part III of this study (51). Indeed, the vastus lateralis muscle of already trained athletes may be quite optimally stocked with mitochondria, and further increase in mitochondrial mass would develop at the expense of sarcomeres and other organelles. Thus a better coupling of energy production to energy utilization by the CK system may provide an increase in energetic efficiency and an improvement of muscle performance without changes in mitochondrial content. This seems to be the case after hypoxia in our subjects. The hypoxic training sessions may be the source of lower intracellular P_{O_2} and reduced O_2 diffusion gradients to the mitochondria, as discussed previously (10). Adaptation of mitochondrial function toward a more efficient coupling between energy utilization and the local energy production units may help to

maintain longer the cellular homeostasis with high local ATP/ADP and may delay the use of anaerobic energy production and accumulation of protons.

The signals producing these cellular changes remain unclear at present. It is already known that training at low P_{O_2} results in a higher production of oxidants, generated by both the ETC and the NADPH oxidase in rats (3). Bailey et al. (1) showed, in already trained athletes ($\dot{V}_{O_2\,max} > 50$ ml·min^{-1}·kg^{-1}), that similar IHT, in terms of simulated altitude and exercise intensity, increased $\dot{V}_{O_2\,max}$ and lipid peroxidation with free-radical generation possibly induced by lower mitochondrial P_{O_2}. They proposed that oxidative stress might be considered as a biological prerequisite for performance adaptation. Reactive oxygen species (ROS) are generated by each complex of the ETC under hypoxia, and moderate ROS fluctuations may play a role as regulatory mediators of cell signaling processes (8, 18, 24, 36). In this case, ROS production may be involved in modifying mitochondrial function and cytoarchitecture (31).

Complexes of the ETC. We found no change in the intrinsic functional properties of individual respiratory chain complexes in skeletal muscle of already well-trained athletes after both normoxic and hypoxic training. This original observation suggests that IHT did not result in permanent deleterious effects on complexes of the ETC. According to previous studies, several potentially deleterious effects on complexes I and IV could be suspected to have occurred: for example, low arterial O_2 saturation increases the proportion of the inactive isoform of complex I with a resulting higher mitochondrial NADH concentration (2), in turn reducing the complex II inhibition by oxaloacetate (22). According to our observations, this is unlikely to have occurred in our subjects. Additionally, $\dot{V}_{TMPD\text{-}ASC}$ is shown to be >50% reduced under hypoxic conditions (5, 11). However, persistent changes in COX enzymatic activity are unlikely, as we did not observe changes in $\dot{V}_{TMPD\text{-}ASC}$ and COX activity, whatever the training modality. Thus we assume that our training protocols did not impair the mitochondrial complexes of the ETC.

Limitation of the study. One possible limitation of the study is that only the Hyp group used a mask during the hypoxic training sessions. By inducing a specific work of the respiratory muscles, the mask might be responsible for a potential effect on performance improvement. Considering a healthy subject, with anthropometrical characteristics comparable to our subjects, 15% (mostly 30%) of the theoretical maximal inspiratory pressure (16.23 cmH_2O in this case) is reported to be the minimal resistance required to induced a significant respiratory muscle work, resulting in endurance performance improvement (for review see Ref. 34). In the practical conditions of the present study, the resistance does not exceed 1.8 cmH_2O when the ventilation is equal to 200 l/min (Hans-Rudolph, 1999). Therefore, according to both the data available for the Hans-Rudolf valve combined to the mask and the previous studies on specific respiratory muscle training (34), the athletes of the Hyp group only experienced a negligible fraction of the theoretical minimal resistance needed to expect a respiratory training-induced performance improvement (1.8 vs. 16.23 cmH_2O). In these conditions, we assumed that the influence of mask breathing on our observed performance improvement is likely to have been limited. Another limitation could be the use of vastus lateralis muscle for biopsies. In elite endurance runners, gastrocnemius and vastus lateralis are, respectively, the first and the second most recruited leg muscles during the entire running cycle (20). For both of them, the fiber distribution, the capillary-to-fiber ratio, as well as the CS and lactate dehydrogenase activity values are included in the same range (28). We choose to study the vastus lateralis muscle for two main reasons. *1*) This muscle is far more safe to biopsy in humans compared with the gastrocnemius, because the former has an underlying bony layer, which helps homeostasis and does not contain major arteries. Conversely, the latter contains the twin arteries increasing the risk for intramuscular hemorrhagic complications after biopsy. *2*) The vastus lateralis muscle is known to be sensitive to the training status in terms of both quantitative and qualitative parameters of mitochondrial function (47, 50). Interestingly, even if this muscle contributes less than the gastrocnemius to the metabolic and mechanical work of running, the changes that occurred in the qualitative aspects of the mitochondrial function after IHT might even be more dramatic in the gastrocnemius. We believed that this point highlights the potential beneficial effect of IHT on the skeletal muscle mitochondrial function.

Important changes of K_m and Tlim occurred simultaneously and only after IHT, which, at first sight, suggests a potential contribution of a higher coupling between energy utilization and production sites to the Tlim improvement. However, because of our too small number of subjects per group and also because of the likely multifactorial nature of the mechanisms that influence changes in both K_m and Tlim, the respective variations of the K_m and Tlim values were not significantly correlated. Thus our hypothesis of an influence of qualitative mitochondrial changes within the myocyte on endurance performance as expressed by Tlim warrants further studies on larger cohorts of subjects.

In conclusion, inclusion of two weekly moderate hypoxic training sessions at VT_2 (never exceeding 80 min/wk) into the usual training of endurance runners induces skeletal muscle mitochondrial adaptations that may contribute to the improvement of endurance performance (Tlim). Our results suggest that adaptations of the athlete's skeletal muscle to the added hypoxic stress involves a better coupling between the energy utilization and production sites to promote more efficient oxidative pathways and to decrease intracellular energetic perturbations.

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