

**Joffrey Zoll, Elodie Ponsot, Stéphane Dufour, Stéphane Doutreleau, Renée Ventura-Clapier, Michael Vogt, Hans Hoppeler, Ruddy Richard and Martin Flück**

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E. Ponsot, S. P. Dufour, J. Zoll, S. Doutreleau, B. N'Guessan, B. Geny, H. Hoppeler, E. Lampert, B. Mettauer, R. Ventura-Clapier and R. Richard  
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## Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts

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**Zoll, Joffrey, Elodie Ponsot, Stéphane Dufour, Stéphane Doutreleau, Renée Ventura-Clapier, Michael Vogt, Hans Hoppeler, Ruddy Richard, and Martin Flück.** Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts. *J Appl Physiol* 100: 1258–1266, 2006; doi:10.1152/jappphysiol.00359.2005.—We hypothesized that specific muscular transcript level adaptations participate in the improvement of endurance performances following intermittent hypoxia training in endurance-trained subjects. Fifteen male high-level, long-distance runners integrated a modified living low-training high program comprising two weekly controlled training sessions performed at the second ventilatory threshold for 6 wk into their normal training schedule. The athletes were randomly assigned to either a normoxic (Nor) (inspired O<sub>2</sub> fraction = 20.9%, *n* = 6) or a hypoxic group exercising under normobaric hypoxia (Hyp) (inspired O<sub>2</sub> fraction = 14.5%, *n* = 9). Oxygen uptake and speed at second ventilatory threshold, maximal oxygen uptake ( $\dot{V}O_{2\max}$ ), and time to exhaustion (T<sub>lim</sub>) at constant load at  $\dot{V}O_{2\max}$  velocity in normoxia and muscular levels of selected mRNAs in biopsies were determined before and after training.  $\dot{V}O_{2\max}$  (+5%) and T<sub>lim</sub> (+35%) increased specifically in the Hyp group. At the molecular level, mRNA concentrations of the hypoxia-inducible factor 1 $\alpha$  (+104%), glucose transporter-4 (+32%), phosphofructokinase (+32%), peroxisome proliferator-activated receptor gamma coactivator 1 $\alpha$  (+60%), citrate synthase (+28%), cytochrome oxidase 1 (+74%) and 4 (+36%), carbonic anhydrase-3 (+74%), and manganese superoxide dismutase (+44%) were significantly augmented in muscle after exercise training in Hyp only. Significant correlations were noted between muscular mRNA levels of monocarboxylate transporter-1, carbonic anhydrase-3, glucose transporter-4, and T<sub>lim</sub> only in the group of athletes who trained in hypoxia (*P* < 0.05). Accordingly, the addition of short hypoxic stress to the regular endurance training protocol induces transcriptional adaptations in skeletal muscle of athletic subjects. Expressional adaptations involving redox regulation and glucose uptake are being recognized as a potential molecular pathway, resulting in improved endurance performance in hypoxia-trained subjects.

athletes; messenger ribonucleic acid; redox regulation; glucose uptake

HYPOXIA IS AN IMPORTANT STIMULUS related to exercise in muscle tissue (29). For 15 years, the “living low-training high” (LLTH) concept, which consists of sleeping and living in normoxia but training in moderate hypoxia (~2,500 m above sea level), demonstrated training-induced improvement in sea level endurance capacity (23, 37, 39, 40, 54, 55). In untrained or recreationally trained subjects, certain muscular adaptations

involving increased citrate synthase (CS) activity, mitochondrial density, capillary-to-fiber ratio, and fiber cross-sectional area have been evoked to participate in the increased endurance performance (12, 23, 54).

Nevertheless, a persisting detrimental aspect of the LLTH method is that all training sessions are performed in hypoxia. As a consequence, training velocities must be lowered to account for the hypoxia-induced reduction in aerobic power and not to exceed the athlete's tolerance capacity (37). These deficits may lower any performance advantage gained through altitude-induced improvements in O<sub>2</sub> delivery or utilization. To conciliate hypoxia exposure during training sessions with a maintained training volume, our laboratory recently proposed to include controlled high-intensity training sessions in hypoxia into a regular normoxic training program performed in normoxia (57). In the first article of our trilogy, we demonstrated that a 6-wk intermittent hypoxia training (IHT) protocol leads to better improvements in endurance capacity than normoxic endurance training in well-trained runners (13a). In addition to the usual running training program of athletes, this IHT paradigm comprised two weekly hypoxic training sessions consisting of two repeated hypoxic bouts performed at the second ventilatory threshold (VT<sub>2</sub>). Such an intermittent, high-intensity approach thereby seems to avoid the detraining effects associated with training under permanent hypoxia.

This functional improvement suggested that an adapted IHT realized at VT<sub>2</sub> would enable athletes to optimize the stimuli necessary to achieve central and/or peripheral changes that enhance O<sub>2</sub> delivery and/or utilization. To date, the critical links between improved running performance and the hypoxic stimulus are not identified. Particularly, it remains to be demonstrated to which extent muscular adjustments are involved in the improvement of endurance capacities following modified hypoxic training in an endurance-trained population.

Adaptations of biological systems related to myocellular functioning were implicated in the superior altitude performance after hypoxia training at reasonable altitude, i.e., 2,500 m above sea level, in humans. An enhancement of buffer capacity has been implicated in improved performance of endurance athletes after hypoxia training interventions (43). Other hypoxia-specific muscular responses to endurance training, such as improved oxygen delivery and increased oxidative capacity, are known to occur in previously untrained individ-

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uals (12, 28, 40, 54, 58). High-altitude exposure and acclimatization also have been noted to increase utilization of blood glucose and improve metabolic coupling, whereas increased protection against the reactive oxygen species (ROS) was observed in native Tibetans (9, 24, 25). These adjustments may contribute to the metabolite flux adaptations due to reduced oxygen availability in hypoxia (29, 34).

Increased gene expression during recovery from consecutive bouts of exercise has been recognized as a major muscular strategy for muscular adaptations to training (7, 17, 27, 44, 45). In a training state, after repetition of the exercise stimuli, an accumulation of preexercise RNA levels of metabolic factors is matched to structural-functional equivalents of muscular phenotype (47, 51). Steady-state transcript level adaptations, therefore, provide a sensitive indication for training-specific muscular adjustments with endurance exercise.

We hypothesized that expressional adjustments of distinct biological systems (gene ontologies) can be implied in the muscular adaptations to hypoxia and would link to the observed functional ameliorations of endurance performance of athletes with a modified IHT (13a; first article of the trilogy). Specifically, we tested whether preexercise levels of selected transcripts of those gene ontologies involved in the functional and regulatory aspects of acid-base control [monocarboxylate transporter 1 (MCT-1) and carbonic anhydrase (CA) 3], the oxidative stress defense [manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), glutathione *S*-transferase pi], the glucose metabolism [glucose transporter 4 (GLUT-4), 6-phosphofructokinase, muscle type (PFK<sub>m</sub>)], the mitochondrial biogenesis and metabolism [peroxisome proliferator activated receptor gamma coactivator 1 $\alpha$  (PGC-1 $\alpha$ ), mitochondrial transcription factor A, CS, mitochondrial-encoded cytochrome oxidase (COX) subunit 1 (COX-1), nuclear-encoded COX subunit 4 (COX-4)], the oxygen transport [myoglobin (Mb), VEGF], as well as the oxygen signaling [hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ )] would be increased after modified IHT training. Finally, the coregulation and the correspondence of altered mRNA levels with improved time to exhaustion (T<sub>lim</sub>) and maximal oxygen uptake ( $\dot{V}O_{2\max}$ ) characteristics of hypoxia-trained athletes were tested with correlation analysis.

## METHODS

### Subjects

Fifteen highly trained male distance runners were recruited from local athletic teams and completed the study before the beginning of their competitive season. After all the potential risks were explained, the subjects gave a voluntary written and informed consent to participate to the protocol approved by the institution's ethics committee of the hospital of Strasbourg, France. In the weeks before and during the study, all of the subjects lived between 100 and 300 m above sea level, and all were engaged in a regular training schedule, including five training sessions per week. All were highly motivated to participate in the study, familiar with treadmill running, with current 10,000 m or equivalent personal-best times of less than 35:00 (min:s). The athletes were randomly assigned to either the normoxic (Nor) or the hypoxic (Hyp) group. The subjects constituted a subpopulation of the athletes studied in Dufour et al. (13a; first article of the trilogy). Hence, the functional values were recalculated from athletes engaged in this study.

### Experimental Design

**Basal medical examination.** All athletes received a basal medical examination, as already described in the first part of this trilogy (13a).

**Treadmill performance evaluation.** All procedures were explained in detail in the article by Dufour et al. (13a; first article of the trilogy). Briefly, normobaric hypoxic conditions, corresponding to an altitude of 3,000 m [inspired O<sub>2</sub> fraction (F<sub>I</sub>O<sub>2</sub>) = 14.5%], were simulated by diluting ambient air with nitrogen (Altı Trainer<sub>200</sub>, Sport and Medical Technology). In the week before and after the training intervention, all of the subjects performed three exercise tests on a motorized treadmill (13a; first article of the trilogy). The tests were separated by at least 24 h: 1) a treadmill incremental test to exhaustion (IET) in normoxia (IET<sub>N</sub>; F<sub>I</sub>O<sub>2</sub> = 20.9%) to determine the  $\dot{V}O_{2\max}$  ( $\dot{V}O_{2\max N}$ ), the first (VT<sub>1N</sub>) and second ventilatory thresholds (VT<sub>2N</sub>), as well as their associated velocities ( $v\dot{V}O_{2\max N}$ ,  $vVT_{1N}$ , and  $vVT_{2N}$ , respectively); 2) an IET in hypoxia (IET<sub>H</sub>; F<sub>I</sub>O<sub>2</sub> = 14.5%) to obtain the same parameters under hypoxic conditions ( $\dot{V}O_{2\max H}$ ,  $v\dot{V}O_{2\max H}$ , VT<sub>1H</sub>,  $vVT_{1H}$ , VT<sub>2H</sub>,  $vVT_{2H}$ ); and 3) an all-out test at  $v\dot{V}O_{2\max N}$  to determine the T<sub>lim</sub>. For a given subject, all tests were performed at the same time of day in a climate-controlled environment (21–23°C).

Ventilatory thresholds were assessed by using established criteria (3, 59). VT<sub>1</sub> corresponds to the breakpoint in the plot of CO<sub>2</sub> production ( $\dot{V}CO_2$ ) as a function of O<sub>2</sub> uptake ( $\dot{V}O_2$ ). At that point, the ventilatory equivalent for O<sub>2</sub> ( $\dot{V}E/\dot{V}CO_2$ ) increases without an increase in the ventilatory equivalent for CO<sub>2</sub> ( $\dot{V}E/\dot{V}CO_2$ ), where  $\dot{V}E$  is minute ventilation. VT<sub>2</sub> was located between VT<sub>1</sub> and  $\dot{V}O_{2\max}$ , when the  $\dot{V}E/\dot{V}CO_2$  starts to increase, while  $\dot{V}E/\dot{V}O_2$  continues to increase.

During the IET, the initial running speed was set at 10 km/h, and steps were increased every 2 min by 1 km/h until exhaustion occurred. Each subject was encouraged to give a maximum effort.

The all-out running test was performed at the same absolute running speed before and after training, corresponding to pretraining  $\dot{V}O_{2\max}$ . The test began with a 10-min warm-up at 60% of the subject's  $\dot{V}O_{2\max}$ . The subject was then connected to the test equipment during a 5-min period of rest and immediately asked to run at his corresponding  $\dot{V}O_{2\max}$  for as long as possible (T<sub>lim</sub>).

**Training program.** During the 6 wk of the study, both groups continued their usual running training program (5 sessions a week), including two weekly VT<sub>2</sub> sessions that were performed in the laboratory. A total of 12 laboratory-training sessions were performed during the 6 wk. At the beginning of the study and according to the training environment, the Hyp group trained at a significantly lower running speed (15.4 km/h in Hyp vs. 16.8 km/h in Nor,  $P < 0.05$ ). These different running speeds corresponded to the same exercise heart rate expressed in absolute (Hyp: 166  $\pm$  3 beats/min vs. Nor: 172  $\pm$  3 beats/min) or relative values (Hyp: 96  $\pm$  1% vs. Nor: 94  $\pm$  1%). Each VT<sub>2</sub> session began with a 10-min warm-up at 60%  $v\dot{V}O_{2\max}$ , followed by two periods at  $vVT_{2}$ , separated by 5-min recovery at 60%  $\dot{V}O_{2\max}$ . During the first 3 wk of training, exercise duration at  $vVT_{2}$  was increased each week: 2  $\times$  12 min for the first week (*week 1*), 2  $\times$  16 min for *week 2*, 2  $\times$  20 min for *week 3*. At the fourth week,  $vVT_{2}$  was increased to achieve the same heart rate as in *week 1* but was maintained for 2  $\times$  12 min. Thus training volume was subsequently again increased weekly at the new  $vVT_{2}$  for 2  $\times$  16 min for *week 5* and 2  $\times$  20 min for *week 6*.

For the group who trained in normoxia (Nor),  $vVT_{2N}$  was determined during the IET<sub>N</sub>, and, for the group who trained in hypoxia (Hyp),  $vVT_{2H}$  was determined during the IET<sub>H</sub>. For the Hyp group, subjects trained under hypoxic conditions only during the running periods at  $vVT_{2H}$ , by breathing through face masks connected to a mixing chamber via appropriate tubing. The Nor group performed their training at  $vVT_{2}$  without the face masks.

**Evaluation of usual training.** All of the runners were asked to report their individual training schedule into detailed training logs, including duration, distance, and intensity of each training session. Laboratory as well as field work bouts were taken into account to

provide both quantitative and qualitative characterization of the total training period. The duration and intensity of the training sessions performed out of the laboratory were assessed based on the running velocity and divided in four intensity zones: low ( $<vVT_1$ ), moderate ( $vVT_1$ - $vVT_2$ ), heavy ( $vVT_2$ - $v\dot{V}O_{2\max}$ ), and severe intensity ( $>v\dot{V}O_{2\max}$ ) [for details see Table 2 in Dufour et al. (13a), first article of the trilogy].

#### Blood Lactate

Blood samples were obtained from the earlobe at rest, as well as at the 1st and 3rd min of recovery after the treadmill incremental and constant-load tests to exhaustion. They were immediately analyzed by an enzymatic method (ABL 700 series, Radiometer).

#### Muscle Biopsy Samples

With the use of the Hultman and Bergström technique (31), biopsies were taken at midhigh level from vastus lateralis muscle, before and after the 6-wk training period. Before the biopsies, there were 48 h without any exercise activity. For mRNA analysis, the major part of the muscle tissue was immediately frozen in isopentane cooled by liquid nitrogen and then stored in the latter until required for analyses. The other part was processed for enzyme activities analysis and histochemistry.

#### Enzyme Analysis

Part of the frozen tissue samples were weighed, homogenized in ice-cold buffer (30 mg/ml) containing (in mM) 5 HEPES, 1 EGTA, 5  $MgCl_2$ , and Triton X-100 (0.1%), pH 8.7, and incubated for 60 min at 0°C to ensure complete enzyme extraction. CS and cytochrome-c oxidase were assessed by standard spectrophotometric methods.

#### Histochemistry

Cryostat cross sections (12- $\mu m$ ) were processed for myofibrillar ATPase (alkaline or acid preincubation at pH 10.4 and pH 4.5), as

described (6). Consistent with the proposition of Berchtold et al. (5), the muscle fibers were classified into type I, IIA, and IIB(IIx) fibers. The percentage of each fiber type was obtained from stained sections. One to three sections from different areas of each muscle biopsy were analyzed, depending on the size of the specimens. All of the fibers that appeared reasonably cross sectioned (minor to major fiber axis  $> 0.5$ ) were counted. A mean of  $124 \pm 11$  fibers was counted per muscle biopsy.

#### RNA Extraction and Reverse Transcription

Total RNA was extracted from the human vastus lateralis muscle samples by using the RNeasy minikit (Qiagen, Basel, Switzerland) and quantified with RiboGreen (molecular probes, Invitrogen, Basel, Switzerland), as described previously (16). Formaldehyde-agarose gel analysis demonstrated the integrity of all RNA samples. RNA aliquots (300 ng) of these reactions were reverse transcribed in 20  $\mu l$ , with four units of reverse transcriptase using random hexamer primers (1  $\mu M$ ) and 0.5 mM dNTPs, following the manufacturer's instructions (Omni-script Reverse Transcriptase kit, Qiagen).

Real-time PCR amplification reactions were carried out in triplicates on 30- $\mu l$  aliquots in a 96-well plate on an ABI Prism 5700 Sequence Detection System with cDNA signal detection via SYBRgreen (PE Biosystem, Rotkreuz, Switzerland). Primers were designed with the Primer Express software (PE Biosystems). Sequences of the primers used are given in Table 1. For the level estimation of single transcript, all samples from the two training series were assayed on the same 96-well plate. The amount of target mRNA relative to the reference (28S) was calculated using the comparative threshold cycle for target amplification method, as already described (16). The individual PCR efficiency was calculated from the mean slope of monitored SYBRgreen intensity within the linear phase of cDNA amplification. Specificity of amplified cDNA was verified from the dissociation curve, as determined on the ABI Prism 5700, and by

Table 1. PCR primer sequences for the measured gene transcripts

Gene	Gene Name	Genebank	Forward Primer	Reverse Primer
28S	ribosomal 28S RNA	M11167	5' ATATCCGCGAGCAGGTCTCCAA 3'	5' GAGCCAATCCTTATCCCGAAG 3'
CA3	carbonic anhydrase 3	BC004897	5' TTGACCCATCCTGCCTGTTC 3'	5' GGTGAATGAGCCCTGGTAGGT 3'
COX-1	cytochrome oxidase subunit 1	M10546	5' CTATACCTATTATTCGGCGCATGA 3'	5' CAGCTCGGCTCGAATAAGGA 3'
COX-4	cytochrome oxidase subunit 4	X54802	5' GCCATGTTCTTCATCGGTTTC 3'	5' GGCCGTACACATAGTGCTTCTG 3'
CS	citrate synthase	BTO07414	5' CTCAGGACGGGTTGTTCCTCA 3'	5' CAGGTATATCGCGGATCAGTCTTC 3'
Cu/ZnSOD	cytoplasmic copper/zinc superoxide dismutase	X02317	5' CAGGGCATCATCAATTTCTGA 3'	5' TGCTTCCCCACACCTTCCAC 3'
GLUT-4	glucose transporter 4	M20747	5' AGCCCCGCTACCTCTACA 3'	5' GTCAGGCGCTTCAGACTCTTTC 3'
GSTpi	glutathione S-transferase pi	X15480	5' CAGGAGGGCTCACTCAAAGC 3'	5' TGAGGTCTCCGTCTGGAAGT 3'
HIF-1 $\alpha$	hypoxia-inducible factor 1, alpha subunit	U22431	5' TAGTGAACAGAATGGAATGGAGCAA 3'	5' TTTTGGCACTGGTGGCTC 3'
Mb	myoglobin	BC018001	5' GCATGCCACCAAGCACAAG 3'	5' TGATGCATTCGGAGATGAAGTC 3'
MCT-1	monocarboxylate transporter 1	L31801	5' CCAAGGCGAGGAAAGATAAGTCT 3'	5' ATCTTTTTTTCACACCAGATTTTCCA 3'
MHC-I	myosin heavy chain type I	M21665	5' AAGGTCAAGGCCTACAAGC 3'	5' CGGAACTTGGACAGGTTGGT 3'
MHC-IIx	myosin heavy chain type IIx	AF111785	5' GGAGGAACAATCCAACGTCAA 3'	5' TGACCTGGGACTCAGCAATG 3'
MnSOD	manganese superoxide dismutase	M36693	5' CACGCGGCTACGTGAA 3'	5' GCCAACGCCTCCTGGTACT 3'
PFK $m$	6-phosphofructokinase, muscle type	J05533	5' TTGGCTCCACTGTGAGGATTG 3'	5' CCTGAAACCATCATGGACAA 3'
PGC-1 $\alpha$	peroxisome proliferator activated receptor gamma coactivator 1 $\alpha$	AF159714	5' GTAATCTGCGGGATGATGGA 3'	5' GCAGAAAAGCATCACAGGTAT 3'
Tfam	mitochondrial transcription factor A	NM003201	5' CCAAAAAGACCTCGTTTCAGCTTA 3'	5' TGCGGTGAATCACCTTAGC 3'
VEGF	vascular endothelial growth factor	M32977	5' CATGGCAGAAGGAGGAGGCAGAATCA 3'	5' ATCTTCAAGCCATCCTGTGTGCCCTG 3'

checking the amplified fragment for correct size after separation of the PCR reaction on a 1% agarose gel.

### Statistical Analysis

For each transcript and subject, 28S-related RNA levels were normalized to the corresponding pretraining mRNA value. Values are expressed as means  $\pm$  SE. Differences between groups before the training period were analyzed with one-way ANOVA. To test and compare the effects of training in hypoxia and normoxia, we used two-way repeated-measures ANOVA with factors "type of training" (i.e., for categories Hyp and Nor) and "measured variables" (i.e., for all transcripts or functional parameters) for the repeated performance and transcript measures pre- and posttraining. When significant modifications were found, Student Newman-Keuls post hoc test was performed to localize the effect. When a specific hypothesis could be formulated, a one-sided test statistic was adopted. Pearson linear regression analysis was used to determine any potential linear relationship between variables. Statistical analyses were performed with SigmaStat 3.0 software (SPSS, Chicago, IL) and Statistica software package 6.1 [StatSoft (Europe), Hamburg, Germany]. The level of significance was fixed at  $P < 0.05$ .

## RESULTS

The anthropometric characteristics, treadmill performance, and training conditions of the subjects are shown in Tables 2 and 3.  $\dot{V}O_{2\max}$  was significantly higher in the Hyp group before training ( $P < 0.05$ , Table 3).

### Total Training

The total training schedule (i.e., field and laboratory training sessions) of the athletes was very comparable. During the 6-wk training, Hyp and Nor groups performed, respectively,  $33.0 \pm 0.6$  and  $31.0 \pm 2.0$  training sessions, leading to no difference in total training time and total training distance (Hyp:  $2,013 \pm 114$  min and  $478 \pm 27$  km vs. Nor:  $2,085 \pm 266$  min and  $496 \pm 78$  km). No differences appeared either in total time or in total distance run by the subjects in the respective intensity zones [see Table 2 of Dufour et al. (13a), first article of the trilogy].

### Normoxic Performance

The effects of the experimental intervention in the studied population on the results of the IET<sub>N</sub> are shown in Table 3. The Hyp group significantly enhanced  $\dot{V}O_{2\max N}$ , as well as the speed at VT<sub>2N</sub>, by +5% and +4%, respectively ( $P < 0.05$ ). The Nor group did not demonstrated significant improvement in  $\dot{V}O_{2\max}$ , whereas athletes increased their speed at VT<sub>2N</sub> by 4% ( $P < 0.05$ ). There was a significant interaction ( $P = 0.008$ )

Table 2. Anthropometric data

	Group	
	Nor	Hyp
Number of subjects	6	9
Body weight, kg	75.7 $\pm$ 2.7	70.6 $\pm$ 2.2
Height, cm	180 $\pm$ 2	180 $\pm$ 1
Age, yr	31.3 $\pm$ 3.1	30.3 $\pm$ 2.1
%Body fat	13.4 $\pm$ 2.5	11.8 $\pm$ 0.8

Values are means  $\pm$  SE. Hyp and Nor are groups that included two training sessions at the velocity corresponding to the second ventilatory threshold under hypoxic or normoxic condition, respectively, in their usual weekly training schedule. %Body fat, percentage of body fat determined according to Ref. 2.

Table 3. Treadmill running performance indexes measured in normoxia before and after the 6-wk training period

	Nor		Hyp	
	Pre	Post	Pre	Post
Incremental test				
$vVT_{2N}$ , km/h	16.8 $\pm$ 0.7	17.5 $\pm$ 0.7*	18.1 $\pm$ 0.1	18.9 $\pm$ 0.1†
$\dot{V}O_{2\max N}$ , ml $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup>	59.4 $\pm$ 1.0	60.9 $\pm$ 1.4	64.3 $\pm$ 1.2‡	67.3 $\pm$ 1.3*
Time to exhaustion				
Tlim, s	500 $\pm$ 59	558 $\pm$ 55	540 $\pm$ 34	764 $\pm$ 55†
Lactate max, mM	7.1 $\pm$ 0.7	7.4 $\pm$ 1.0	6.9 $\pm$ 0.8	6.5 $\pm$ 0.8

Values are means  $\pm$  SE. Inspired O<sub>2</sub> fraction (FI<sub>O<sub>2</sub></sub>) in normoxia = 20.9%; Hyp FI<sub>O<sub>2</sub></sub> = 14.5%. Pre and Post, before and after the 6-wk training period, respectively;  $vVT_{2N}$ , running speed at second ventilatory threshold in the normoxic incremental test;  $\dot{V}O_{2\max N}$ , maximal oxygen uptake determined in the normoxic incremental test; Tlim, time to exhaustion at the end of the all-out exercise test; Lactate max, maximal blood lactate measured at the end of the all-out test. Significant difference to Pre values: \* $P < 0.05$ , † $P < 0.01$ . Significant difference to Nor: ‡ $P < 0.05$ .

between the training-induced increase in  $\dot{V}O_{2\max N}$  and type of training (Nor and Hyp). The all-out exercise tests were performed at the same absolute running velocity before and after training (Table 3). While it was precisely realized at  $v\dot{V}O_{2\max N}$  before training, this speed amounted to 96 and 97% of the posttraining  $v\dot{V}O_{2\max N}$  for the Hyp and Nor groups, respectively. The Tlim was significantly enhanced in the Hyp group (+43%,  $P < 0.05$ ) but was not changed in the Nor group. There was a significant interaction ( $P < 0.001$ ) between the training-induced increase in Tlim and type of training. Table 3 reported that the maximal blood lactate concentration was unchanged after both training protocols.

**Fiber-type composition and mitochondrial enzymes.** There were no significant alterations in fiber-type percentage with either training protocol (Table 4). As shown in Table 4, mitochondrial enzyme CS and COX activities were not altered after both training modalities.

### Skeletal Muscle mRNA Expression Analysis

Normalized values obtained for the mRNA level for each gene of interest, as determined by RT-PCR, before and after 6 wk of training in Nor or Hyp conditions, are given in Fig. 1. There was a significant interaction between the training-induced alterations in transcript levels and the type of training (Nor vs. Hyp,  $P = 0.017$ ). Additionally, a significant effect of transcript identity on training-induced transcript level changes was revealed ( $P < 0.001$ ).

**Oxygen sensing.** The preexercise HIF-1 $\alpha$  mRNA level was selectively increased by 104% in the Hyp group only.

Table 4. Fiber-type distribution and enzymes activities

	Nor		Hyp	
	Pre	Post	Pre	Post
CS, IU/g wet wt	19.2 $\pm$ 2.3	16.0 $\pm$ 1.8	19.4 $\pm$ 2.0	19.5 $\pm$ 2.2
COX, IU/g wet wt	5.5 $\pm$ 1.3	3.6 $\pm$ 0.5	6.0 $\pm$ 1.5	5.5 $\pm$ 1.1
%Type I fibers	59.0 $\pm$ 7.7	66.1 $\pm$ 6.2	70.6 $\pm$ 6.9	70.1 $\pm$ 6.2
%Type IIA fibers	37.6 $\pm$ 6.9	32.5 $\pm$ 5.9	28.7 $\pm$ 7.0	29.9 $\pm$ 6.2
%Type IIB(X) fibers	3.4 $\pm$ 1.2	1.4 $\pm$ 0.7	0.7 $\pm$ 0.4	0.0 $\pm$ 0.0

Values are means  $\pm$  SE. Nor FI<sub>O<sub>2</sub></sub> = 20.9%; Hyp FI<sub>O<sub>2</sub></sub> = 14.5%.

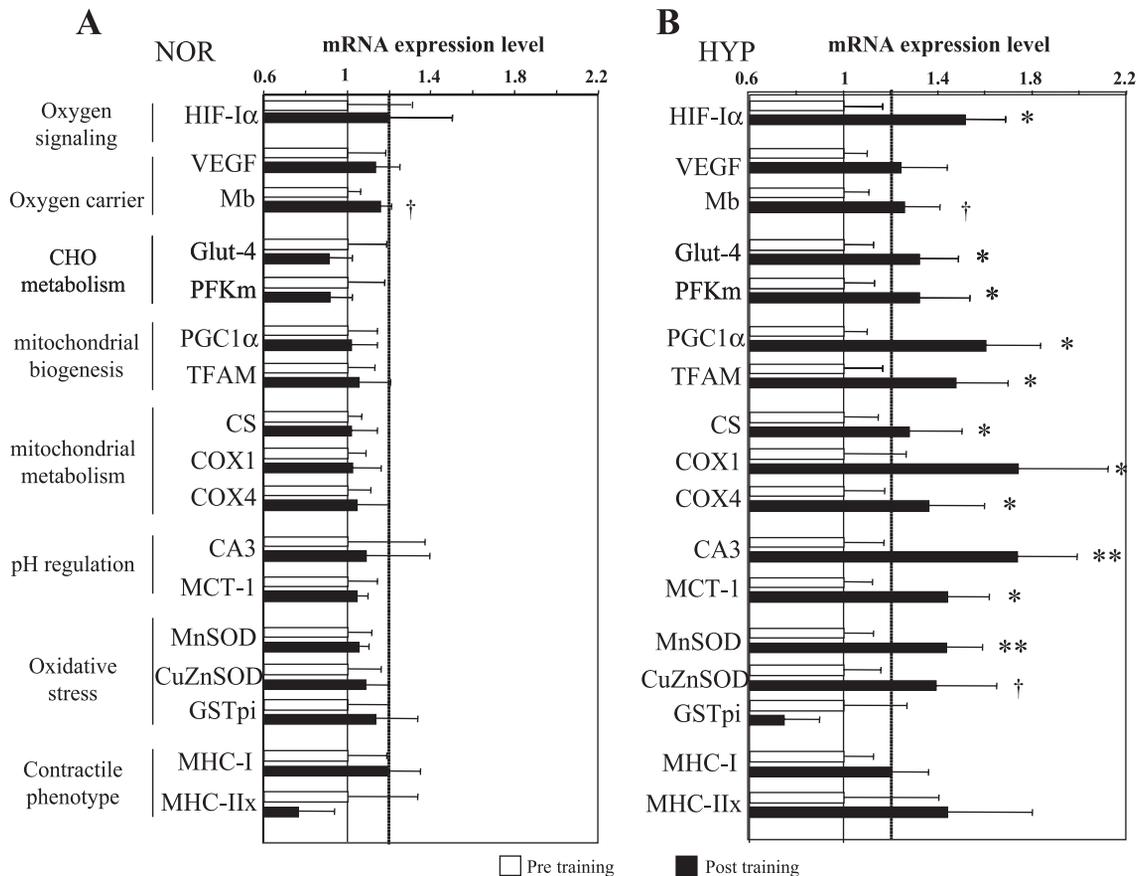


Fig. 1. Relative mRNA concentrations for gene products involved in muscular phenotype definition before and after 6 wk of training in normoxia (Nor; A) and hypoxia (Hyp; B). Open bars represent pretraining values, and solid bars are posttraining values of 28S-related mRNA concentrations in vastus lateralis muscle of endurance athletes. Posttraining values were related to the prevalues, which are set to 1. CHO, carbohydrate; HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; Mb, myoglobin; GLUT-4, glucose transporter 4; PFK<sub>m</sub>, 6-phosphofructokinase, muscle type; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1 $\alpha$ ; TFAM, mitochondrial transcription factor A; CS, citrate synthase; COX, cytochrome oxidase; CA3, carbonic anhydrase; MCT-1, monocarboxylate transporter 1; MnSOD, manganese superoxide dismutase; CuZnSOD, copper/zinc superoxide dismutase; GSTpi, glutathione S-transferase pi; MHC, myosin heavy chain. Values are means  $\pm$  SE. \*\* $P < 0.01$ , \* $P < 0.05$ , and † $P < 0.10$  vs. before training.

**Oxygen transport.** The mRNA level of Mb showed a trend toward a higher level after both training modalities (+16 and +26% after Nor and Hyp, respectively,  $P = 0.05$ ). The regulator of vascular growth, VEGF mRNA, was not significantly increased after either training modality.

**Metabolic phenotype.** After training in Hyp, the mRNA levels of GLUT-4 and glycolytic enzyme PFK<sub>m</sub> were both significantly increased by 32% ( $P < 0.05$ ).

Transcript levels of regulators of mitochondrial biogenesis PGC-1 $\alpha$  (+60%), transcription factor A (+48%), as well as mitochondrial metabolism, CS (+28%), COX-1 (+74%), and COX-4 (+36%), were significantly increased in biopsies of Hyp athletes.

**Oxidative stress.** PCR quantification revealed the level of MnSOD mRNA to be increased after training under hypoxic conditions (+44%,  $P < 0.01$ ), and Cu/ZnSOD mRNA level showed a tendency for an increase ( $P = 0.054$ ).

**pH regulation.** The hypothesis of a training-induced increase in the muscular mRNA concentration of CA3 (+74%,  $P < 0.01$ ) and MCT-1 (+44%,  $P < 0.05$ ) was confirmed for the athletes who trained in hypoxia.

**Contractile phenotype.** There was no significant alteration of myosin heavy chain-I and myosin heavy chain-IIx mRNA levels after both training modalities.

**Coregulation of RNA level changes.** Intergene comparisons revealed several significant correlations of genes implicated in oxidative metabolism and its metabolites (i.e., Mb vs. COX-4/COX-1, as well as Mb vs. MnSOD/Cu/ZnSOD,  $P < 0.0001$ ). Moreover, PFK<sub>m</sub> correlated with Mb ( $r = 0.76$ ), COX-4 ( $r = 0.86$ ), GLUT-4 ( $r = 0.7$ ), VEGF ( $r = 0.85$ ), and MnSOD ( $r = 0.76$ ), but only in postexercise biopsies of athletes who trained in hypoxia ( $P < 0.05$ , data not shown).

**Correlations between endurance performance and mRNA expression.** GLUT-4, CA3, and MCT-1 mRNAs specifically and significantly correlated with Tlim when pre- and posttraining biopsies of athletes who trained in hypoxia were pooled ( $r = 0.65$ ,  $P < 0.01$ ;  $r = 0.81$ ,  $P < 0.001$ ;  $r = 0.60$ ,  $P < 0.01$ , respectively, Fig. 2). There was no correlation between mRNA level and Tlim in pre- and posttraining biopsies of athletes who trained in normoxia.

## DISCUSSION

### Major Findings

To enhance the comprehension of the endurance capacity improvement following a modified IHT program, we assessed the expression levels of a set of selected mRNAs in skeletal muscle of athletes. Measurement of the transcripts encoding

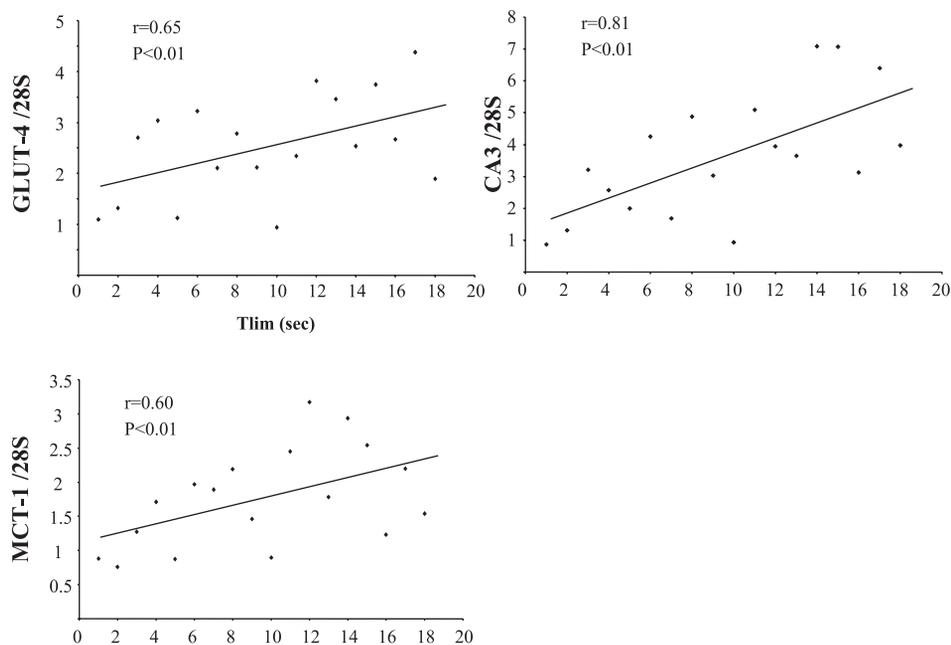


Fig. 2. Correlation analysis of mRNA levels with time to exhaustion at velocity of maximal oxygen uptake. Comparison is shown between the running time to exhaustion and GLUT-4, CA3, and MCT-1 in vastus lateralis muscle of athletes. The correlation coefficient ( $r$ ) and the statistical significance level  $P$  of linear regression analysis are shown.

enzymes involved in different metabolic, homeostatic, and regulatory pathways suggests that, in addition to the mechanical and contractile training stimuli, the added hypoxic stress during exercise seems to play a major role in the muscle phenotypic adaptations. In this context, we showed increased mRNA levels of factors implicated in the regulation of mitochondrial biogenesis, of enzymes implicated in carbohydrate and mitochondrial metabolism, oxidative stress defense, and pH regulation. As well, significant correlations were noted between muscular mRNAs levels of MCT-1, CA3, GLUT-4, and the endurance capacities (Tlim) of athletes only in the group who trained in hypoxia. Consequently, the addition of a hypoxic stress during two sessions per week realized at VT<sub>2</sub> during 6-wk induced specific gene expressional adaptations, which may contribute to the biological processes, which allow an increase in endurance performance.

#### *O<sub>2</sub> Sensing and Gene Regulation*

Investigations in trained human skeletal muscle on oxygen availability, in combination with variations in  $\dot{V}O_{2\max}$  during maximal exercise, have revealed that there is a very low cytoplasmic  $P_{O_2}$  at  $\dot{V}O_{2\max}$  and that variations in systemic  $O_2$  supply alter intracellular  $P_{O_2}$  (48). Consequently, intracellular oxygenation and then maximal muscular oxidative utilization are reduced, whereas muscle lactate efflux is accelerated in hypoxia. These changes are consistent with the concept that  $O_2$  supply limits  $\dot{V}O_{2\max}$  in trained human skeletal muscle (48, 49). This also suggests that intracellular  $P_{O_2}$  is an important modulator of muscle metabolism and ultimately muscle fatigue (50).

HIF-1 $\alpha$  protein is a major hypoxia sensor and transcriptional regulator of oxygen-dependent gene expression (52). Consequently, a HIF-1 $\alpha$  deficiency in HIF-1 $\alpha$  heterozygous mice blunts the hypoxia-induced expression of transcripts for glycolytic and respiratory factors in skeletal muscle (10). HIF-1 $\alpha$  protein is stabilized in hypoxia, thereby causing association with its partner, HIF-1 $\beta$ , and triggering the transcription of

multiple glycolytic and angiogenic genes (52). Under normoxic conditions, it has been demonstrated that HIF-1 $\alpha$  protein is degraded within several minutes by the ubiquitin-proteasome pathway (35). The observed increase of HIF-1 $\alpha$  mRNA in the Hyp group after training links to the previous observations of its enhanced levels after hypoxic training (58). This HIF-1 $\alpha$  mRNA elevation possibly reflects an augmented potential for the translation of HIF-1 $\alpha$  and downstream activation of HIF-1-dependent pathways. This supports the notion that the HIF-1 system is involved in the regulation of muscle adaptations, specifically when some training sessions are realized in hypoxia (58).

#### *Metabolic Phenotype*

It has been demonstrated that mitochondrial adaptation to endurance training in humans is associated with activation of PGC-1 $\alpha$ , as well as its downstream transcription factors (nuclear respiratory factor-1, mitochondrial transcription factor A), which induce coordinated expression of mitochondrial transcripts (20).

In our study, even if we found that the mRNA level encoding the coactivator PGC-1 $\alpha$  and the oxidative enzymes COX-1, COX-4, and CS increased after training in hypoxia, CS and COX enzyme activities did not show any alterations after both training modalities in skeletal muscle of athletes (Table 4). It can, therefore, be assumed that, after several years of endurance training, athletes reached the limit of their adaptive potential concerning quantitative aspects of the muscular oxidative capacities (47). We and others observed, in untrained subjects, a higher mitochondrial volume density as well as an upregulation of the nuclear- and mitochondrial-encoded COX subunit mRNAs after training in hypoxia (54, 58). On the other hand, in our population of athletes, we showed a lack of increase of muscular oxidative capacities as well as a lack of correlation between index of endurance capacity and the oxidative enzyme activities. This suggested that the endurance time at  $v\dot{V}O_{2\max}$  (Tlim) was not limited by the muscular

oxidative capacities in endurance athletes. Hence the increase of exercise capacities (i.e.,  $\dot{V}O_{2\max}$  and  $T_{lim}$ ) after training in hypoxia could not be explained by a simply quantitative adaptation of the muscular oxidative capacities. Rather, the increases of mRNA level of respiratory factors, therefore, possibly indicate increased mitochondrial turnover in the hypoxia-trained group.

Glucose transport has been shown to be the rate-limiting step in muscle glucose uptake under most conditions (36), and the importance of contraction-induced glucose transport for the maintenance of muscle ATP has been recognized for many years. The capacity of contraction-induced glucose uptake as well as the amount of GLUT-4 are higher in skeletal muscle of athletes than in untrained age-matched control subjects (1, 15, 19, 30). Correspondingly, physical exercise training has consistently been shown to increase GLUT-4 content (11, 13, 53). Acute hypoxia exposure is known to increase the dependence on blood glucose (9) and to induce glucose uptake by the same mechanism as contractile activity, in part via an AMP kinase pathway. Our data extend these results, showing that the GLUT-4 mRNA level was specifically increased after the 6-wk modified IHT regime. Moreover, it was significantly correlated with endurance capacities of athletes who trained in hypoxia. This suggests that hypoxia not only plays a functional role in glucose uptake, but could also lead to enhance GLUT-4 gene expression in response to hypoxia stimuli. This alteration may represent a specific hypoxia pathway (4), allowing a longer lasting increase in glucose uptake in muscle, which could participate in improving the endurance capacity of athletes.

#### *Oxidative Stress Defense*

Regular exercise training has been shown to increase endogenous production of antioxidants and to provide subsequent protections against further exercise-induced oxidative damage (32, 46). We showed in our study that the mRNA level of MnSOD was significantly higher after some training sessions in hypoxia when Cu/ZnSOD demonstrated a tendency to be increased. Because hypoxia seems to induce an additional increase of ROS generation (14), we hypothesize that the increase of the mRNA level is a transcriptional adaptation, allowing the increase of cellular antioxidant capacity and then the reduction of possible oxidative injury.

This increase of the mitochondrial superoxide dismutase isoform, i.e., MnSOD, is in line with a previous work in animals demonstrating that MnSOD is primarily responsible for the increased superoxide dismutase activity after a training program in normoxia (26). Our data support that increased MnSOD and Cu/ZnSOD transcript levels are an adaptive response to reduce the presumably higher level of muscular ROS during exercise in hypoxia. Alternatively, it may represent an adaptive response of turnover related to replenishment of this antioxidant enzyme during its enhanced use in hypoxia.

#### *pH-Regulating System*

pH-regulating transport systems are of major importance for endurance capacities of athletes. Effective pH regulation in muscle depends on the cotransport of lactate and  $H^+$  via the monocarboxylate transporters (33), as well as the CAs, which influence the rate of  $H^+$  and  $HCO_3^-$  transport (22). Recent data indicated that permanent hypoxia modifies skeletal muscle

acid-base control via increases in the capacity for lactate,  $HCO_3^-$ , and  $H^+$  fluxes from muscle to blood (34). In this regard, we found that modified hypoxic training induced increase in the muscular mRNA concentration of CA3 (+74%) and MCT-1 (+44%). This is in line with the enhanced buffering capacity in well-trained cross-country skiers after 2 wk of living and training at 2,100–2,700 m above sea level (43).

The importance of CA during exercise was previously demonstrated in experiments showing that the inhibition of CA enzyme with acetazolamide is associated with increased perception of leg fatigue during maximal exercise realized in hypoxia, which may be due to the effect of acidosis within muscle cells (21, 38). We can suggest that the increased expression of CA3 could accelerate the interstitial  $CO_2/HCO_3^-$  buffer system so that  $H^+$  ions can be rapidly delivered or buffered in the interstitial fluid (22, 60).

Because all subjects trained at  $VT_2$ , we can speculate that the net lactate efflux was identical during exercise sessions in both groups (56). On the other hand, a strong negative linear relationship between intracellular pH and muscle lactate efflux has previously been documented as well as a reduced intracellular pH in hypoxia compared with normoxia for a given muscle lactate efflux (50). Based on these observations, our present data suggest that, when training was realized in hypoxia at the same relative intensity as normoxia, the increase of the preexercise level of CA3 and MCT-1 mRNAs could represent a transcriptional adaptation in response to the reduced intracellular pH. The linear relation between the endurance capacity ( $T_{lim}$ ) and the muscular CA3, MCT-1, and GLUT-4 mRNA levels in hypoxia-trained athletes in our study relates to the significant correlation between hypoxia markers and glycolytic enzyme mRNAs after low-resistance/high-repetition strength training in hypoxia (18). This suggests that fine transcriptional tuning of enzymes implicated in pH regulation is part of the molecular program underlying improved endurance performance after training in hypoxia (34, 43). Indeed, lactate exchange and removal capacities have been shown to contribute to lengthening of  $T_{lim}$  (8, 41, 42). Then the increase in MCT-1 mRNA suggests a transcriptional mechanism, allowing the improvement of lactate exchange and removal, which could lead to the slow down of the progressive lowering of muscle pH at a given absolute running velocity, thereby allowing the athletes to run longer before fatigue occurs. In good agreement with this hypothesis is the fact that training did not change the maximal lactate values recorded at exhaustion during the all-out test, suggesting a lower rate of blood lactate accumulation after this modified IHT program.

In conclusion, the current investigation reveals that addition of intermittent, high-intensity hypoxic sessions to the normal training schedule of endurance athletes elicits a correlated increase in the transcripts that encode for factors implicated in myocellular homeostasis. Thus the increased mRNA expression of factors involved in glucose uptake, oxidative stress defense, and pH regulation is seen to reflect the potential for improved metabolism of carbohydrates with a maintained redox balance during exercise at the  $VT_2$ . The observed increase in endurance performance of athletes after training with a LLTH paradigm needs further studies to understand the coordinated metabolic regulations that allow for the functional improvements observed.

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