Respostes adaptatives sanguínies i musculars en condicions d'arribada limitada d'oxigen

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RESPOSTES ADAPTATIVES SANGUÍNIES I MUSCULARS EN CONDICIONS D'ARRIBADA LIMITADA D'OXIGEN.

Tesi Doctoral

Santiago Esteva i Gras

7. Publicacions

“Respostes adaptatives sanguínies i musculars en condicions d’arribada limitada d’oxigen”

Els doctors Ginés Viscor Carrasco i Teresa Pagès Costas, com a directors de la Tesi Doctoral presentada per Santiago Esteva i Gras, fem constar que el doctorand ha participat activament en els articles que formen aquesta memòria, tal com queda reflectit en l'ordre i composició de l'equip d'autors de cada un d'ells. El doctorand ha tingut un paper fonamental en el disseny experimental, en l'execució dels treballs experimentals, en els processos d'anàlisi i tractament de les dades. També ha tingut un important paper en el procés de difusió i publicació dels resultats i conclusions, és a dir, en la redacció dels manuscrits i en el procés de revisió per pars.

Els índexs de factor d'impacte (IF) de les publicacions en que s'han acceptat o s'han enviat els articles que conformen aquesta memòria són els següents:

1. **Títol de la publicació**: Capillary supply, fibre types and fibre morphometry in rat tibialis anterior and diaphragm muscles after intermittent exposure to hypobaric hypoxia.

Autors (p.o. de firma): Panisello, P.; Esteva, S.; Torrella, J.R.; Pagés, T.; Viscor, G.

Revista: European Journal of Applied Physiology **Volum:** 103 **Número:** 2 **Pàgines, inicial:** 203 **final:** 213 **Any:** 2008 **ISSN:** 1439-6319

Participació del doctorand: Participació en el protocol d'exposició intermitent a hipòxia hipobàrica dels animals. Realització de les tècniques descrites al manuscrit. Col·laboració en la redacció del manuscrit.

I.F. (2008): 1.931 **5 Years I.F.** (2008): 2.174

Eigenfactor Score (2008): 0.01543 *Article Influence* Score (2008): 0.587

Times Cited: 7 (a 22 de març de 2010)

2. **Títol de la publicació**: Morphofunctional responses to anaemia in rat skeletal muscle.

Autors (p.o. de firma): Esteva, S.; Panisello, P.; Casas, M.; Torrella, J.R.; Pagés, T.; Viscor, G.

Revista: Journal of Anatomy **Volum:** 212 **Número:** 6 **Pàgines, inicial:** 836 **final:** 844 **Any:** 2008 **ISSN:** 0021-8782

Participació del doctorand: Participació en el protocol d'exposició intermitent a hipòxia hipobàrica dels animals. Realització de part de les tècniques descrites al manuscrit. Col·laboració en la redacció del manuscrit i en el procés de revisió per pars.

I.F. (2008): 2.063 **5 Years I.F.** (2008): 2.625

Eigenfactor Score (2008): 0.01195 *Article Influence* Score (2008): 0.933

Times Cited: 1 (a 22 de març de 2010)

3. **Títol de la publicació**: Enzyme activity and myoglobin concentration in rat myocardium and skeletal muscles after passive intermittent simulated altitude exposure.

Autors (p.o. de firma): Esteva, S.; Panisello, P.; Torrella, J.R.; Pagés, T.; Viscor, G.

Revista: Jpurnal of Sports Sciences **Volum:** 27 **Número:** 6 **Pàgines, inicial:** 633 **final:** 640 **Any:** 2009 **ISSN:** 0264-0414

Participació del doctorand: Participació en el protocol d'exposició intermitent a hipòxia hipobàrica dels animals. Realització de les tècniques descrites al manuscrit. Col·laboració en la redacció del manuscrit i en el procés de revisió per pars.

I.F. (2008): 1.625 **5 Years I.F.** (2008): 2.296

Eigenfactor Score (2008): 0.00692 *Article Influence* Score (2008): 0.589

Times Cited: 0 (a 22 de març de 2010)

4. **Títol de la publicació**: Blood rheology adjustments in rats after a program of intermittent exposure to hypobaric hypoxia.

Autors (p.o. de firma): Esteva, S.; Panisello, P.; Torrella, J.R.; Pagés, T.; Viscor, G.

Revista: High Altitude Medicine & Biology **Volum:** 10 **Número:** 3 **Pàgines, inicial:** 275 **final:** 281 **Any:** 2009 **ISSN:** 1527-0297

Participació del doctorand: Participació en el protocol d'exposició intermitent a hipòxia hipobàrica dels animals. Realització de totes les tècniques descrites al manuscrit. Col·laboració en la redacció del manuscrit i en el procés de revisió per pars.

I.F. (2008): 1.667 **5 Years I.F.** (2008): 1.839

Eigenfactor Score (2008): 0.00185 *Article Influence* Score (2008): 0.540

Times Cited: 0 (a 22 de març de 2010)

5. **Títol de la publicació**: Oxidative stress markers status in rats after Intermittent Exposure to Hypobaric Hypoxia.

Autors (p.o. de firma): Esteva, S.; Pedret, R.; Torrella, J.R.; Pagés, T.; Viscor, G.

Revista: Wilderness & Environmental Medicine (en procés de revisió editorial)

Participació del doctorand: Participació en el protocol d'exposició intermitent a hipòxia hipobàrica dels animals. Realització de totes les tècniques descrites al manuscrit. Col·laboració en la redacció del manuscrit i en el procés de revisió per pars.

I.F. (2008): 0.518 **5 Years I.F.** (2008): 0.765

Eigenfactor Score (2008): 0.00075 *Article Influence* Score (2008): 0.188

Times Cited: No aplicable

Altres publicacions addicionals que no formen part de la tesi:

1. **Títol:** Data de naixement i èxit en el basquetbol professional.
   Autors: Esteva S, Drobnic F, Puigdellívol J, Serratosa L, Chamorro M.
   Revista: Apunts: Medicina de l'Esport. Volum: 41. Núm: 149. Pàg.: 25-30. Any: 2006.

2. **Títol:** Bithdate and Basketball Success.
   Autors: Esteva S, Drobnic F, Puigdellívol J, Serratosa L.
   Revista: FIBA Assist Magazine. Volum: 18. Pàg.: 64-66. Any: 2006.

3. **Títol:** Blood rheological behaviour in rats after intermittent hypobaric hypoxia exposure to 5000 m .
   Autors: Esteva S, Panisello R, Torrella R, et al.
   Revista: Comparative Biochemistry and Physiology. A-Molecular & Integrative Physiology. Volum: 146. Issue: 4. Pàg.: S164-S164. Suplement: Suppl. S Meeting Abstract: 16. Any: Abril 2007.

4. **Títol:** Intermittent hypobaric hypoxia induces changes at a different extent in biochemical parameters depending on muscle activity degree .
   Autors: Panisello P, Esteva S, Torrella R, et al.
   Revista: Comparative Biochemistry and Physiology. A-Molecular & Integrative Physiology. Volum: 146. Issue: 4. Pàg.: S184-S184. Supplement: Suppl. S Meeting Abstract: 40. Any publicació: Abril 2007.

5. **Títol:** Intermittent hypobaric hypoxia exposure enhances running economy in untrained rats .
   Autors: Pages T, Marin J, Esteva S, Torrella JR, Viscor G.
   Revista: Archivos de Medicina del Deporte. Volum: 25. Issue: 6. Pàg.: 453 Meeting Abstract. Any publicació: Nov-Des 2008.

6. **Títol:** Gender differences in the exercise response after sildenafil administration at simulated altitude .
   Autors: Pages T, Torrella JR, Fort N, Esteva S, Leal C, Ricart A, Viscor G.
   Revista: Archivos de Medicina del Deporte. Volum: 25. Issue: 6. Pàg.: 534 Meeting Abstract. Any publicació: Nov-Des 2008.

Resum article 1:

Subministrament capil·lar, tipus i morfometria de fibres en els músculs *tibialis anterior* i diafragma de rata després d'una exposició intermitent a hipòxia hipobàrica.

Tres grups de rates mascles sedentàries foren exposades a hipòxia hipobàrica intermitent (HHI) durant 22 dies (4h/dia, 5 dies/setmana) i a una altitud simulada de 5000m. Per aconseguir aquestes condicions, s'utilitzà una cambra hipobàrica dissenyada específicament per animals. S'extragueren els músculs *tibialis anterior* (TA) i el diafragma (DG) al finalitzar el programa (grup H), i als 20 i 40 dies després d'haver-lo finalitzat (grup P20 i grup P40). Un grup control (C) fou mantingut a pressió de nivell del mar i el seus músculs TA i DG foren comparats amb els dels animals H, P20 i P40. Es mesurà la morfometria de les fibres i capil·lars de cada un dels músculs. Els nostres resultats demostraren que l'HHI no produeix un canvi de la composició de tipus de fibres (pel que fa a les seves propietats contràctils i oxidatives) en la major part de les regions del músculs analitzats. Trobàrem algunes diferències significatives en el TA després del protocol HHI pel que fa a la morfometria de les fibres. Tanmateix, el protocol HHI va induir diversos canvis estadísticament significatius al DG: un augment de la densitat capil·lar en les rates H (736 capil·lars/mm^2) comparat amb els animals C (610 capil·lars/mm^2). Malgrat que el període d'exposició no produí un canvi en la capil.larització ni en els paràmetres morfomètrics de les fibres ràpides, s'observaren reduccions del 7% al 13% en l'àrea de la fibra, en el perímetre i en la distància de difusió entre C i H per a les fibres lentes. A més a més, aquests canvis morfomètrics representen valors entre el 10% i el 20% significativament més alts en la capil·larització, en l'àrea i el perímetre de les fibres. Aquest descobriment indica que les fibres SO són més sensibles a IHH que ambdós tipus de fibres ràpides.

Eur J Appl Physiol (2008) 103:203–213
DOI 10.1007/s00421-008-0691-0

ORIGINAL ARTICLE

Capillary supply, fibre types and fibre morphometry in rat tibialis anterior and diaphragm muscles after intermittent exposure to hypobaric hypoxia

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Accepted: 25 January 2008 / Published online: 13 February 2008

Abstract Three groups of sedentary male rats were exposed to intermittent hypobaric hypoxia (IHH) for 22 days (4 h/day, 5 days/week) in a hypobaric chamber at a simulated altitude of 5,000 m. Tibialis anterior (TA) and diaphragm (DG) were removed at the end of the programme (*H* group), and 20 or 40 days later (*P20* and *P40* groups). A control group (*C*) was maintained at sea-level pressure and their TA and DG were compared to those of the experimental rats at the end of the IHH programme, and also 20 and 40 days later. We measured the fibre morphometry and capillaries of each muscle. Our results demonstrate that IHH does not change the fibre type composition (with reference to either their contractile or oxidative properties) for most muscle regions of the muscles analysed analysed. We found few significant differences in muscle capillarity and fibre morphometry for TA after IHH. However, IHH did induce some statistically significant changes in DG: capillary density of the *H* rats (736 capillaries/mm^2) increased compared to *C* animals (610 capillaries/mm^2). Although IHH did not change the fibre capillarization or morphometric parameters of fast fibre types, we observed reductions ranging from 7 to 13% in fibre area, perimeter and diffusion distances between *C* and *H* for slow fibres. Moreover, these morphometric changes accounted for increases of 10–20% in capillarization, fibre unit area and fibre unit perimeter. This indicates that SO fibres are more sensitive to IHH than both fast fibre types.

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Keywords Intermittent hypoxia · Tibialis anterior · Diaphragm · Capillarization · Fibre types

Introduction

One of the most important functions of skeletal muscle vasculature is to ensure the metabolic activity required for the different functions of the muscle tissue fibres by distributing blood flow (see Weibel 1984). Skeletal and cardiac muscle capillarization alters in response to changes in muscle activity (Messonier et al. 2001). Thus, exercise increases capillarization in skeletal and cardiac muscle (see, for example, Bigard et al. 1991); and, conversely, a decrease in muscle activity (such as that observed in microgravity during space flight) induces skeletal muscle microvascular atrophy (Desplanches 1997). High-altitude hypoxia is a muscle acclimation factor that seriously affects human physiology. Under this condition, the low oxygen partial pressure of the inspired air induces adjustments to improve tissue oxygen availability. Among these adjustments, is an increase in active ventilation: greater respiratory muscle activity (West 1993). Inspiration is the active period of the respiratory cycle and depends on the pressure changes caused on the thoracic cavity by contraction of inspiratory muscles. Expiration at rest is passive. The main muscle responsible for the active process of inspiration is the diaphragm (DG). This preponderant role in ventilation has led to many studies of the structure and function of the mammalian DG (Sieck et al. 1987; De Troyer and Estenne 1988; Reid et al. 1992; Sexton and Poole 1995; Zobundzija et al. 1998; Deveci et al. 2002). In spite of its specific task, which means that it can never be at rest, the DG has the same structure and functionality as limb musculature and other skeletal muscles (Polla et al. 2004). The DG has a

great plasticity and capacity to acclimatize to different environmental situations such as hypoxia, microgravity or physical training by changing its capillarity and fibre type distributions (see Polla et al. 2004 for revision).

We designed the present study to investigate the effect of intermittent hypoxia on the capillarity and fibre types of rat DG and tibialis anterior (TA). Intermittent hypobaric hypoxia exposure (IHHE) consists of alternating hypoxia episodes with immediate recovery in normoxia and are usually performed in hypoxic chambers. These procedures can induce adaptive responses to hypoxia while preventing the negative effects of chronic hypoxia: weight loss, poor appetite, slow recovery from fatigue and lethargy and irritability (see Ward et al. 2000). Thus, IHHE has been evaluated as an efficient high-altitude acclimatization method (Wagner et al. 1987; Sutton et al. 1988; Richalet et al. 1992). Human IHHE induces some responses that enhance blood oxygen transport capacity (such as hyperventilation and acid–base changes and erythropoiesis) that in turn increases arterial oxygen saturation at altitude and maximal oxygen consumption at sea level, and also shifts the lactate threshold at sea level (Rodriguez et al. 1999, 2000; Casas et al. 2000a, b; Ricart et al. 2000).

The principle of symmorphosis (Taylor and Weibel 1981) postulates the optimal design of the mammalian respiratory system, with no structure that exceeds the requirements of maximal oxygen flux and maximal oxygen consumption. Although symmorphosis is not a general principle, it might apply in a variety of cases and conditions. The symmorphosis principle would mean that the central responses induced by IHHE must be accompanied by other adjustments in gas exchange and oxidative capacity at a peripheral level, especially in skeletal muscle and myocardium. With this approach in mind, we developed animal experiments using techniques and methods that cannot be used on humans for ethical or technical reasons. In a recent work we applied an IHHE protocol to laboratory rats in order to study myocardial morphofunctional parameters (Panisello et al. 2007). Our findings indicate that the programme induces an adaptive response in rat myocardium for more efficient O_2 delivery to the mitochondria of cardiac muscle cells. Capillarization and fibre morphometric changes show significant differences in capillary and fibre density, fibre size and shape, capillary to fibre ratios and maximal diffusion distances from surrounding capillaries to the fibre core after IHHE.

Here we study two skeletal muscles with different activation patterns: the DG and the TA. The former is responsible for inspiration (Sieck 1988), is continuously active and consists of three metabolically different fibre types (Powers et al. 1990). The latter is a fast-contracting locomotory muscle that dorsiflexes the ankle (Ariano et al. 1973) and also possesses a heterogeneous population of fibre types (Torrella et al. 2000). However, the TA has a different activation pattern from the DG, since it is only active during locomotion and hence not active during hypobaric chamber confinement. Preliminary results from this study were presented at the Annual Main Meeting of the Society for Experimental Biology (Barcelona, July 2005).

Methods

Animals

A total of 58 male Sprague-Dawley rats aged 6 weeks at the beginning of the experiment were randomly divided into four groups. The first experimental group (H, for *Hypoxic*) of 17 rats was subjected to a programme of intermittent hypobaric hypoxia (IHH), described in detail below. Muscle samples were removed at the end of this programme. A second experimental group (P20, for *Post-hypoxic* 20 days) of 16 rats was simultaneously subjected to the same programme, but muscle samples were obtained 20 days after the end of the programme. A third experimental group (P40, for *Post-hypoxic* 40 days) of six rats was also simultaneously subjected to the same exposure programme, but muscle samples were obtained 40 days after the end of the protocol. Finally, 19 rats were used as a triple-respective control group (*C*, for *Control*). All control animals were maintained under the same conditions as the three experimental groups. Samples from seven of the control animals (subgroup C1) were obtained at the same time as those from H; samples from another eight (subgroup C2) were obtained at the same time as those from P20 and, finally, samples from the remaining four (subgroup C3) were taken at the same time as those from P40. The change in body weight of the animals in all four groups during the complete experiment (70 days) is shown in Fig. 1.

The study was authorized by the University of Barcelona's Ethical Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the *Departament de Medi Ambient i Habitatge* (file No. 1899) of the regional government of Catalonia (*Generalitat de Catalunya*).

Hypobaric chamber and IHH programme

A hypobaric chamber was used to subject the rats to IHH. The total capacity of the hypobaric chamber was approximately 450 l, which allowed us to place three rat cages inside it. The chamber walls were made of Perspex, which enabled us to observe animal behaviour during the programme. A rotational vacuum pump (TRIVAC D5E; Leybold, Köln, Germany) created a partial vacuum by regulating the air inlet via a micrometric valve. Pressure

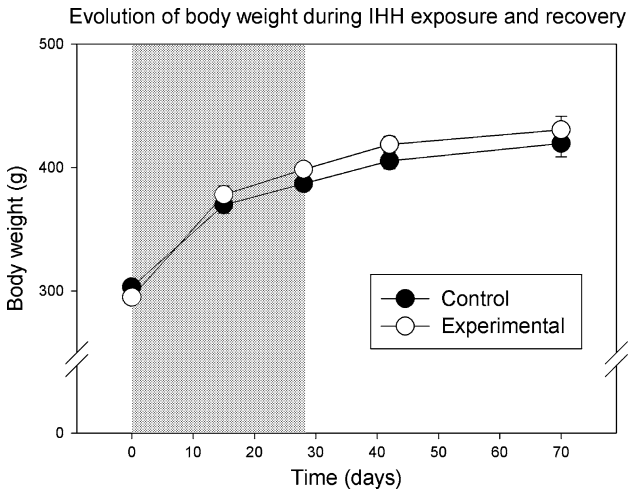

Fig. 1 Evolution of animal body weight during the experiment, for control (*black circles*) and experimental (*open circles*) groups. The *grey* area indicates the IHH exposure period

was regulated using two differential pressure sensors (ID 2000; Leybold, Köln, Germany) connected to a vacuum controller (Combivac IT23; Leybold, Köln, Germany) and a DG (MR16; Leybold, Köln, Germany). Depending on the altitude to be simulated, a fixed low-pressure point was set in the control system. Once the desired vacuum was reached, the internal barometric pressure of the chamber was regulated and maintained by the control system.

After a quarantine period of 2 weeks, the animals were moved into the conditioned room containing the hypobaric chamber. Habituation was completed during an initial period of 5 days, free from all disturbances. The IHH programme consisted of a single daily 4-hour session (0900–1300 h) repeated 5 days a week over four consecutive weeks and two additional days, thus completing 22 days of exposure to hypoxia (88 h in total). The altitude simulated during each session was 5,000 m (400 mmHg = 533 hPa), which is equivalent to 11% oxygen at normobaric hypoxia. Group C was subjected to the same procedure, but the hypobaric chamber was open at normal room pressure.

Muscles and histochemical procedures

The animals were anesthetized with urethane (1.5 g/kg BM). Following Greene (1959), the left TA and the left leaflet of the DG were excised from each rat. The muscles were immediately soaked in 3-methyl-butane pre-cooled to −160°C and stored in liquid nitrogen until subsequent sectioning (Dubowitz 1985). Serial cross sections were cut at a thickness of 14–20 µm in a cryostat (Frigocut, Reichart-Jung, Heidelberg, Germany) at −22°C. The sections were mounted on gelatinized slides and incubated for 5 min in a buffered fixative (Viscor et al. 1992) in order to prevent shrinkage or wrinkling. After rinsing the slides thoroughly, the following histochemical assays were performed: (1) succinate dehydrogenase, SDH (Nachlas et al. 1957), to identify the aerobic and anaerobic fibres; (2) myofibrillar adenosine triphosphatase, mATPase (Brooke and Kaiser 1970), following pre-incubation in alkaline (pH 10.7) and acid (pH 4.2 and 4.5) solutions, to differentiate between slow and fast fibres; and (3) the ATPase method developed by Fouces et al. (1993), in order to reveal muscle capillaries.

Morphofunctional measurements

Since rat TA (Torrella et al. 2000) and DG (Green et al. 1984) muscle fibre is heterogeneous, several *fields* or sample zones were selected from each muscle. The equatorial zone of the TA was analysed using five fields from the cross-sectional area following the protocol described in Torrella et al. (2000) as represented schematically in Fig. 2. The DG was analysed by taking three consecutive fields that covered the full section from the medial part of the muscle.

Images of the stained sections were obtained using a light microscope (Olympus, BX40, Japan) connected to a digital camera (Hitachi, KP-C550, Japan). To ensure accurate scaling, an image of a stage micrometer was obtained each time images of samples were taken. Capillary density

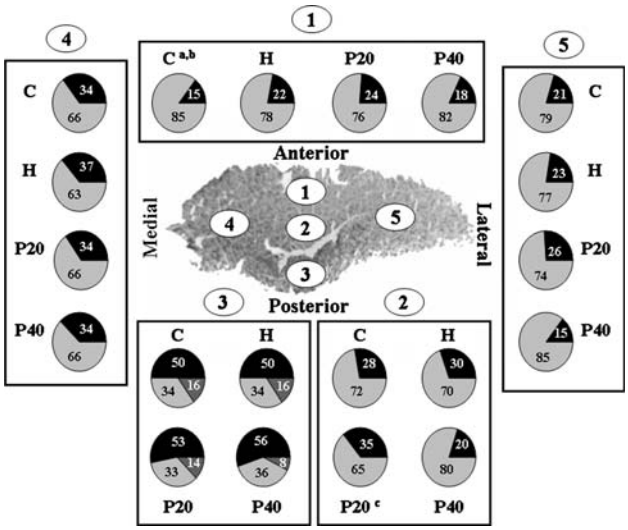

Fig. 2 Equatorial cross section of rat TA stained for succinate dehydrogenase (×9). Numbers (1–5) indicate the areas (fields) the fibre type frequencies, capillarization and morphometric measurements correspond to. Sector graphs show the percentage of fibre types for each field and experimental group. *C* all the control animals (C1 + C2 + C3); *H* hypoxic animals tested at the end of the exposure programme; P20 and P40, hypoxic animals from which samples were removed 20 or 40 days, respectively, after the hypoxic protocol ended; FG, fast glycolytic (*light grey*); FOG, fast oxidative glycolytic (*black*); SO, slow oxidative (*dark grey*). Significant differences ($P < 0.05$) between groups are indicated thus: **a** *C* versus H; **b** *C* versus P20; **c** *C* versus P40

(CD), fibre density (FD), the number of capillaries in contact with each fibre (NCF) and the percentage of each fibre type were then empirically determined from 2×10^5 μm^2 windows of tissue for each field. Capillary and fibre counts were expressed as capillaries and fibres per mm^2. Fibre cross-sectional area (FCSA) and fibre perimeter (FPER) were determined directly from digital images for each fibre type using a personal computer connected to a digitizer tablet and SigmaScan software (SPSS Science, USA). Two indices, CCA and CCP, expressing the relationship between NCF and the FCSA (CCA = NCF 10^3/FCSA) or FPER (CCP = NCF·10^2/FPER) were also calculated. These indices are taken to be the number of capillaries per 1,000 μm^2 of muscle area and the number of capillaries per 100 μm of muscle perimeter. The capillary to fibre ratio (*C*/*F*) was also calculated as the CD/CF quotient. In addition, Feret's diameter and shape factor (SF) were automatically determined for each fibre measured. Feret's diameter (the furthest distance between any two points along the selection boundary of the fibre; also known as the calliper length) can be used as an accurate estimate of the maximal diffusion distance (MDD) between a surrounding capillary and the central region of the fibre in contact with it. SF indicates the fit of the fibre cross section to a circular shape (SF = 1 for a perfect circle).

Statistics

Data for all the parameters are expressed as the sample mean ± standard error of the mean. For the percentage of fibre types, the arcsine transformation was applied as a prior step. To test data for normality, the Kolmogorov–Smirnov test (with Lilliefors' correction) was used. Comparisons between the experimental and control groups were analysed by a one-way ANOVA test. Afterwards, a multiple comparison test using the Holm-Sidak procedure was run to determine the differences between each pair of experimental and control conditions. All statistical tests were performed using a Sigma Stat software package (SYSTAT Software; Erkrath, Germany) with a significance level of $P < 0.05$.

Results

Normal growth was not affected by IHHE, as reflected by body weight evolution during the experiment (Fig. 1). Moreover, no statistically significant differences were found between C1, C2 and C3 for any of the parameters. Unless otherwise indicated, these three control subgroups were grouped together and named group C. Neither was any statistically significant differences found between the three DG fields for any of the parameters (measured or calculated). For this reason, DG data are grouped together in a single value for each parameter. This was not the case for TA, which presented considerable heterogeneity between fields. For this reason, the five fields sampled were studied separately as reflected in Fig. 2.

Fibre types

TA

Only two fibre types (FOG and FG) were found in most regions of this muscle (fields 1, 2, 4 and 5), although SO fibres were present in field 3 (the posterior muscle region nearest to the bone). The percentages of each fibre type in the different TA muscle regions for each experimental group are shown in Fig. 2. This figure shows that in most of the muscle fields analysed IHH does not induce statistically significant changes. Significant increases in the percentage of oxidative fibres (FOG) in hypoxic groups (H and P20) were only found in field 1 (anterior region). We also found a higher percentage of FOG in P20 than in P40 for field 2 (middle region).

DG

All three fibre types (FOG, FG and SO) were found in all the DG fields. Figure 3 shows the relative proportions of each fibre type in each experimental group for this muscle. Only the 10% increase in the proportion of FOG fibres between P20 and P40 was statistically significant; and,

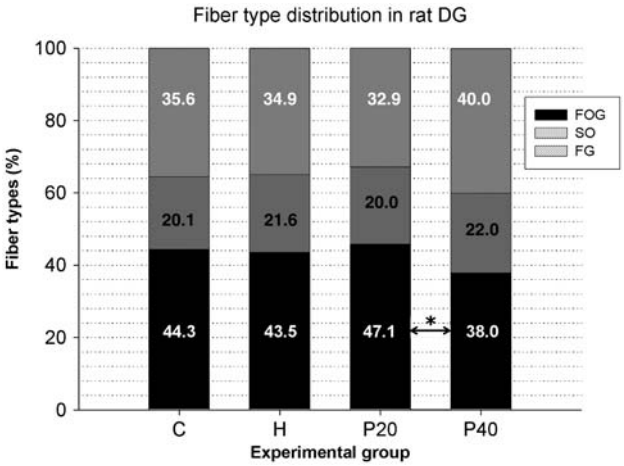

Fig. 3 Stacked bar diagrams showing the percentage of fibre types in rat DG. *C* control animals; *H* hypoxic animals tested at the end of the exposure programme; P20 and P40, hypoxic animals from which samples were removed 20 or 40 days, respectively, after the hypoxic protocol ended; FG, fast glycolytic (*light grey*); FOG, fast oxidative glycolytic (*black*); SO, slow oxidative (*dark grey*). *Asterisk* indicates significant differences ($P < 0.05$) between P20 and P40 in FOG fibres

Table 1 Capillary supply and morphometric fibre parameters in field 1 of the rat TA after IHHE

		C	*H*	P20	P40
CD		314 ± 18	375 ± 18[e]	370 ± 22[f]	285 ± 10
FD		158 ± 12	196 ± 20[e]	175 ± 14	120 ± 9
C/F		2.03 ± 0.12	2.02 ± 0.13	2.15 ± 0.10	2.42 ± 0.16
NCF	FOG	5.27 ± 0.19	5.42 ± 0.14	5.56 ± 0.10	5.31 ± 0.28
	FG	6.02 ± 0.18	6.15 ± 0.14	6.64 ± 0.35	6.63 ± 0.37
FCSA	FOG	3,459 ± 329	3,069 ± 197[e]	3,602 ± 176	4,363 ± 274
	FG	6,682 ± 600[c]	5,937 ± 357[e]	6,480 ± 1090[f]	8,829 ± 676
FPER	FOG	241 ± 11.3	230 ± 8.1	248 ± 4.8	272 ± 9.6
	FG	345 ± 15.1[c]	323 ± 9.5[e]	336 ± 9.7	390 ± 14.8
CCA	FOG	1.60 ± 0.12	1.83 ± 0.11[e]	1.56 ± 0.06	1.23 ± 0.07
	FG	0.94 ± 0.06	1.06 ± 0.05[e]	1.04 ± 0.06[f]	0.76 ± 0.03
CCP	FOG	2.21 ± 0.10	2.38 ± 0.09[e]	2.25 ± 0.05	1.96 ± 0.09
	FG	1.76 ± 0.07	1.91 ± 0.04[e]	1.98 ± 0.09	1.70 ± 0.05
MDD	FOG	65.6 ± 3.1	61.8 ± 2.0[e]	67.3 ± 1.7	73.9 ± 2.3
	FG	91.1 ± 3.9	86.1 ± 2.6[e]	90.0 ± 2.7	104.8 ± 4.1
SF	FOG	0.74 ± 0.02	0.72 ± 0.01	0.73 ± 0.01	0.74 ± 0.02
	FG	0.70 ± 0.02	0.71 ± 0.01	0.71 ± 0.01	0.72 ± 0.01

C control animals; *H* hypoxic animals tested at the end of the exposure programme; P20 and P40, hypoxic animals from which samples were removed 20 or 40 days, respectively, after the end of IHHE programme. *CD* capillary density; *FD* fibre density; *C/F* capillary-to-fibre ratio; *NCF* number of capillaries in contact with a fibre; *FCSA* fibre cross-sectional area; *FPER* fibre perimeter; *CCA* number of capillaries per 1,000 μm^2 of fibre area; *CCP* number of capillaries per 100 μm of fibre perimeter; *MDD* maximal diffusion distance between capillaries and the centre of the fibre. *SF* shape factor (the closer the value is to 1, the nearer a perfect circle the cross section of the fibres). Values are mean ± standard error of the mean. Significant differences ($P < 0.05$) between groups are indicated. [a]*C* versus *H*; [b]*C* versus P20; [c]*C* versus P40; [d]*H* versus P20; [e]*H* versus P40; [f]P20 versus P40

although not statistically significant, it is also interesting to note the 3% increase in FOG fibres from C to P20 animals.

Capillary supply and fibre morphometry

TA

Tables 1, 2, 3, 4, 5 show capillarization parameters (CD, *C/F*, NCF, CCA and CCP) and fibre morphometric measurements (FD, FCSA, FPER, MDD and SF) for fields 1–5, respectively. Capillary and fibre densities increased from C to H and again from H to P20 in all the muscle regions. In P40 both parameters tend to revert to their C values. However, these changes showed little statistical significance (Table 1). A combination of the two parameters showed increases in all hypoxic groups (even in P40) compared to group C. However, this only indicates a vague tendency since only a few fields yielded a statistically significant change (Tables 3,5). The NCF of both fast fibres (FOG and FG) is slightly higher (from 2 to 18%) in H and P20 than in C. Here also P40 values tend to revert to those of C. These findings are in sharp contrast to the values found in SO fibres (Table 3) where all hypoxic animals had lower NCF than the C animals. There are few statistical differences in NCF between experimental groups (Tables 4,5). In most fields (Tables 1,2,5) the fibre morphometric parameters (FCSA, FPER and MDD) of fast fibres tended to decline in *H* and P20 compared to *C* but there is a surprising increase in P40, which is statistically significant for the FG fibres of some fields (Tables 1,5). Only slow fibres of *H* animals showed a reduction, which was not significant, in fibre morphometric parameters with respect to *C* animals (Table 3). The capillarization indexes, CCA and CCP, behave in a similar way in the three fibre types and in all the muscle fields studied. In general, there is a mean increase of 15% in *H* animals as compared to *C* animals; P20 values tend to approximate to *C* values and P40 completely revert back to *C* values, or are even lower. Only some pairs of data in antero-lateral fields are statistically significant (Tables 1,5). Finally, fibre morphology showed no changes (in SF) after IHHE since no changes were found between any of the experimental groups (Tables 1–5).

DG

Table 6 shows capillarization parameters (CD, *C/F*, NCF, CCA and CCP) and fibre morphometric measurements (FD, FCSA, FPER, MDD and SF) for DG. Similar to TA, capillary and FD increased from C to H and P20 animals, whilst in P40 they tended to revert to *C* values. Only the difference

Table 2 Capillary supply and morphometric fibre parameters in field 2 of the rat TA after IHHE

		C	*H*	P20	P40
CD		303 ± 28	396 ± 30	436 ± 52	317 ± 38
FD		142 ± 16	186 ± 18	211 ± 38	145 ± 31
C/*F*		2.04 ± 0.07	2.06 ± 0.06	2.17 ± 0.15	2.25 ± 0.15
NCF	FOG	5.17 ± 0.11	5.57 ± 0.13	5.73 ± 0.28	5.45 ± 0.26
	FG	6.09 ± 0.17	6.49 ± 0.15	6.61 ± 0.30	6.42 ± 0.53
FCSA	FOG	3,877 ± 314	3,561 ± 410	3,416 ± 320	4,485 ± 964
	FG	7,344 ± 636	6,554 ± 571	6,363 ± 634	8,019 ± 1303
FPER	FOG	263 ± 11.6	251 ± 18.4	244 ± 10.2	287 ± 39.2
	FG	376 ± 16.0	351 ± 21.2	337 ± 15.6	392 ± 39.6
CCA	FOG	1.41 ± 0.16	1.82 ± 0.14	1.78 ± 0.23	1.37 ± 0.27
	FG	0.87 ± 0.08	1.09 ± 0.07	1.10 ± 0.13	0.87 ± 0.15
CCP	FOG	1.99 ± 0.11	2.40 ± 0.11	2.38 ± 0.16	2.02 ± 0.20
	FG	1.64 ± 0.07	1.96 ± 0.08	1.98 ± 0.11	1.69 ± 0.14
MDD	FOG	69.4 ± 3.0	65.8 ± 3.6	65.1 ± 3.3	74.1 ± 8.4
	FG	95.1 ± 4.3	89.8 ± 3.8	88.7 ± 4.8	99.0 ± 8.6
SF	FOG	0.70 ± 0.01	0.71 ± 0.02	0.71 ± 0.02	0.69 ± 0.03
	FG	0.65 ± 0.02	0.67 ± 0.02	0.69 ± 0.02	0.65 ± 0.03

See Table 1 for abbreviation key

Table 3 Capillary supply and morphometric fibre parameters in field 3 of the rat TA after IHHE

		C	*H*	P20	P40
CD		598 ± 25	658 ± 19	674 ± 46	570 ± 46
FD		276 ± 10	286 ± 12	295 ± 19	240 ± 20
C/*F*		2.17 ± 0.08[b,c]	2.31 ± 0.05	2.29 ± 0.09	2.54 ± 0.24
NCF	FOG	5.92 ± 0.14	6.32 ± 0.14	6.28 ± 0.23	6.14 ± 0.19
	FG	6.26 ± 0.38	7.39 ± 0.20	7.41 ± 0.35	7.46 ± 0.30
	SO	5.81 ± 0.39	5.26 ± 0.14	5.52 ± 0.27	5.06 ± 0.29
FCSA	FOG	2,667 ± 109	2,590 ± 112	2,692 ± 97	2,876 ± 169
	FG	4,492 ± 315	4,674 ± 208	4,763 ± 293	5,669 ± 507
	SO	2,158 ± 77	1,907 ± 84	2,146 ± 52	2,176 ± 187
FPER	FOG	210 ± 4.0	206 ± 4.7	210 ± 2.7	215 ± 6.4
	FG	281 ± 8.8	287 ± 5.8	292 ± 7.4	309 ± 12.8
	SO	185 ± 2.7	175 ± 3.2	186 ± 1.5	186 ± 8.0
CCA	FOG	2.25 ± 0.12	2.46 ± 0.06	2.38 ± 0.07	2.25 ± 0.15
	FG	1.58 ± 0.08	1.60 ± 0.04	1.61 ± 0.09	1.42 ± 0.13
	SO	2.39 ± 0.06	2.78 ± 0.07	2.62 ± 0.08	2.54 ± 0.21
CCP	FOG	2.83 ± 0.09	3.06 ± 0.03	3.04 ± 0.11	2.92 ± 0.13
	FG	2.47 ± 0.07	2.58 ± 0.04	2.57 ± 0.13	2.47 ± 0.13
	SO	2.78 ± 0.08	3.00 ± 0.06	2.98 ± 0.16	2.82 ± 0.16
MDD	FOG	57.8 ± 1.2	56.8 ± 1.3	58.0 ± 1.0	59.8 ± 1.8
	FG	74.9 ± 2.6	76.5 ± 1.7	77.2 ± 2.4	84.0 ± 3.7
	SO	52.0 ± 0.9	48.9 ± 1.1	52.0 ± 0.6	52.1 ± 2.2
SF	FOG	0.76 ± 0.01	0.76 ± 0.01	0.76 ± 0.01	0.77 ± 0.01
	FG	0.71 ± 0.02	0.71 ± 0.01	0.70 ± 0.02	0.74 ± 0.01
	SO	0.78 ± 0.01	0.78 ± 0.01	0.77 ± 0.01	0.78 ± 0.02

See Table 1 for abbreviation key

between *C* and *H* groups in CD was statistically significant (Table 6). Once more, the combination of both parameters resulted in non-significant increases in all hypoxic groups (even P40) with respect to C. However, if CD and FD are plotted (Fig. 4) a clear segregation of C from the hypoxic groups is evident. This plot also shows that P40 breaks the linearity of the plot due to the tendency for FD to revert and CD to remain high. When measuring the NCF of the three

Table 4 Capillary supply and morphometric fibre parameters in field 4 of the rat TA after IHHE

		C	*H*	P20	P40
CD		400 ± 22	458 ± 33	465 ± 28	442 ± 25
FD		207 ± 15	226 ± 9	210 ± 16	198 ± 18
C/*F*		1.95 ± 0.04	2.04 ± 0.13	2.24 ± 0.07	2.27 ± 0.08
NCF	FOG	5.13 ± 0.14[a,b]	5.59 ± 0.07	5.80 ± 0.21	5.44 ± 0.07
	FG	5.99 ± 0.15	6.75 ± 0.12	6.85 ± 0.37	6.80 ± 0.15
FCSA	FOG	2,673 ± 211	2,420 ± 72	2,782 ± 163	2,655 ± 166
	FG	5,607 ± 538	5,271 ± 206	6,088 ± 534	5,885 ± 473
FPER	FOG	209 ± 7.5	201 ± 3.7	215 ± 6.3	206 ± 6.7
	FG	312 ± 12.6	306 ± 5.9	322 ± 11.7	311 ± 12.5
CCA	FOG	1.98 ± 0.11	2.32 ± 0.06	2.11 ± 0.09	2.05 ± 0.17
	FG	1.13 ± 0.08	1.30 ± 0.05	1.17 ± 0.09	1.19 ± 0.09
CCP	FOG	2.47 ± 0.05[a]	2.81 ± 0.04	2.70 ± 0.08	2.60 ± 0.14
	FG	1.93 ± 0.04[a,c]	2.22 ± 0.04	2.13 ± 0.08	2.20 ± 0.09
MDD	FOG	57.7 ± 2.3	55.1 ± 0.8	59.0 ± 1.7	57.6 ± 1.8
	FG	82.9 ± 4.0	81.1 ± 1.6	86.7 ± 3.9	85.2 ± 3.4
SF	FOG	0.76 ± 0.01	0.75 ± 0.01	0.75 ± 0.02	0.78 ± 0.01
	FG	0.71 ± 0.01	0.70 ± 0.01	0.72 ± 0.02	0.75 ± 0.01

See Table 1 for abbreviation key

Table 5 Capillary supply and morphometric fibre parameters in field 5 of the rat TA after IHHE

		C	*H*	P20	P40
CD		323 ± 17	376 ± 22	369 ± 28	294 ± 13
FD		166 ± 8	184 ± 35	179 ± 23	126 ± 9
C/*F*		1.96 ± 0.07[c]	2.05 ± 0.04	2.17 ± 0.12	2.35 ± 0.09
NCF	FOG	5.22 ± 0.18	5.36 ± 0.15	5.84 ± 0.27	5.49 ± 0.35
	FG	5.85 ± 0.18[b,c]	6.34 ± 0.13	6.73 ± 0.20	6.96 ± 0.19
FCSA	FOG	3,288 ± 219	2,792 ± 215	3,262 ± 330	3,911 ± 239
	FG	6,429 ± 365	5,881 ± 383[e]	6,968 ± 593	8,412 ± 603
FPER	FOG	236 ± 8.9	214 ± 8.5	231 ± 10.7	254 ± 7.5
	FG	332 ± 9.3	317 ± 9.8[e]	347 ± 13.0	376 ± 13.5
CCA	FOG	1.63 ± 0.09	2.02 ± 0.18[e]	1.90 ± 0.14	1.39 ± 0.08
	FG	0.92 ± 0.04	1.10 ± 0.09	1.03 ± 0.08	0.84 ± 0.06
CCP	FOG	2.23 ± 0.08	2.59 ± 0.12[e]	2.55 ± 0.10	2.11 ± 0.10
	FG	1.77 ± 0.05	2.03 ± 0.08	1.95 ± 0.07	1.83 ± 0.07
MDD	FOG	64.1 ± 2.1	59.0 ± 2.3	63.4 ± 3.2	70.2 ± 2.1
	FG	89.7 ± 2.4	85.5 ± 2.7[e]	92.8 ± 4.0	102.5 ± 3.7
SF	FOG	0.74 ± 0.01	0.76 ± 0.01	0.75 ± 0.01	0.76 ± 0.01
	FG	0.73 ± 0.01	0.73 ± 0.01	0.71 ± 0.01	0.74 ± 0.01

See Table 1 for abbreviation key

fibre types slight non-significant increases from 4 to 7% in *H* and P20 with respect to *C* were observed. Also in this parameter a trend for P40 values to revert to those of C was detected. The fibre morphometric parameters (FCSA, FPER and MDD) and the capillarization indexes (CCA and CCP) tended to reduce in all fibre types for all hypoxic groups (*H*, P20 and P40) compared to C. However, these reductions were only statistically significant for SO fibres (Table 6). Similar to TA, fibre morphology showed no changes after IHHE in any fibre type, since no changes in SF were found for any of the experimental groups.

Discussion

Body weight

Chronic hypoxia has a deleterious effect on body mass (Boyer and Blume 1984; Rose et al. 1988). Seemingly, a recent experimental study of chronic IHH in rats with a 4 × 4 and 2 × 2 alternating daily schedule of sea level and simulated 4,600 m altitude demonstrated a severe body weight reduction and compromised survival rate (Siqués et al. 2006). However, possibly due to the lower hypoxia

Table 6 Capillary supply and morphometric fibre parameters in rat DG after IHHE

		C	*H*	P20	P40
CD		610 ± 36[a]	736 ± 29	712 ± 39	729 ± 59
FD		314 ± 15	368 ± 21	346 ± 24	335 ± 30
C/F		1.94 ± 0.06	2.02 ± 0.06	2.08 ± 0.08	2.19 ± 0.09
NCF	FOG	5.42 ± 0.13	5.72 ± 0.18	5.73 ± 0.10	5.53 ± 0.15
	FG	7.42 ± 0.20	7.49 ± 0.37	7.87 ± 0.25	7.62 ± 0.23
	SO	5.16 ± 0.12	5.53 ± 0.24	5.44 ± 0.12	5.25 ± 0.11
FCSA	FOG	2,230 ± 89	2,018 ± 69	2,058 ± 81	1,947 ± 156
	FG	6,980 ± 336	6,178 ± 375	6,261 ± 437	6,166 ± 521
	SO	1,911 ± 45[a]	1,668 ± 60	1,820 ± 83	1,670 ± 83
FPER	FOG	202 ± 4.7	190 ± 3.2	193 ± 3.9	184 ± 6.8
	FG	355 ± 8.1	327 ± 9.3	333 ± 9.3	325 ± 11.3
	SO	179 ± 2.4[a,c]	167 ± 2.8	174 ± 3.9	164 ± 4.1
CCA	FOG	2.46 ± 0.09	2.82 ± 0.12	2.81 ± 0.08	2.94 ± 0.27
	FG	1.09 ± 0.05	1.27 ± 0.07	1.29 ± 0.07	1.29 ± 0.13
	SO	2.70 ± 0.08[a]	3.23 ± 0.13	3.02 ± 0.10	3.18 ± 0.19
CCP	FOG	2.70 ± 0.07	3.06 ± 0.12	2.99 ± 0.06	3.04 ± 0.15
	FG	2.10 ± 0.07	2.36 ± 0.08	2.37 ± 0.09	2.36 ± 0.11
	SO	2.87 ± 0.07[a]	3.19 ± 0.09	3.14 ± 0.07	3.20 ± 0.10
MDD	FOG	52.4 ± 1.1	49.9 ± 0.9	50.4 ± 1.0	49.0 ± 2.0
	FG	93.1 ± 2.3	87.4 ± 2.6	87.9 ± 3.1	86.9 ± 3.7
	SO	48.7 ± 0.6[a,c]	45.6 ± 0.8	47.5 ± 1.1	45.5 ± 1.2
SF	FOG	0.68 ± 0.01	0.70 ± 0.01	0.69 ± 0.01	0.72 ± 0.01
	FG	0.69 ± 0.02	0.71 ± 0.02	0.69 ± 0.02	0.72 ± 0.01
	SO	0.74 ± 0.01	0.74 ± 0.01	0.75 ± 0.01	0.77 ± 0.01

See Table 1 for abbreviation key

exposure, we detected no negative effects on normal growth rate (Fig. 1).

Fibre types

With few exceptions, no statistical differences were detected in the percentage of fibre types either in TA or in DG between control and hypoxic animals. In general, there were slight increases in the percentages of FOG fibre types in H and P20 (Figs. 2,3). The few significant changes detected (especially in DG) suggest an increase in the oxidative character of the muscles. When fibres were grouped according to their oxidative (FOG and SO versus FG) or contractile (FOG and FG versus SO) character, or when a correction was applied to consider the proportional area occupied by each fibre type, once again no differences were found. These results indicate that this IHHE protocol does not induce significant changes in the contractile or in the oxidative properties of the muscle fibres. Similar results have been reported for other rat muscles subjected to chronic hypoxia. Muscle extensor digitorum longus, which has similar fibre type characteristics to TA, showed small, but non–significant, increases in FOG fibres (McGuire et al. 2003). However, contrasting results have been reported for the soleus muscle, which is a predominantly aerobic muscle. Many authors (Ishihara et al. 1995; Abdelmalki et al. 1996) also found no significant changes in fibre type proportions, but others (Sillau and Banchero 1977; Itoh et al. 1990) described a transformation from SO fibres to FOG under chronic hypoxic conditions.

Capillary supply and fibre morphometry

TA

Previous work reports the effects of chronic hypoxia on the capillarization of leg skeletal muscles in several animal species living in high-altitude adapted ecological niches. León-Velarde et al. (1993) obtained significant higher values for CD, NCF and *C/F* in coots living at 4,200 m of altitude as compared to coots at sea level. They also found significant decreases in FCSA in several leg muscles (including TA) of animals living at altitude. Eby and Banchero (1976) reported CD values three times higher and reductions of a half in MDD in leg muscles of Andean dogs living at 4,320 m compared to dogs living at sea level. However, when restrained rats were subjected to protocols of chronic hypoxia, hind limb skeletal muscle showed a

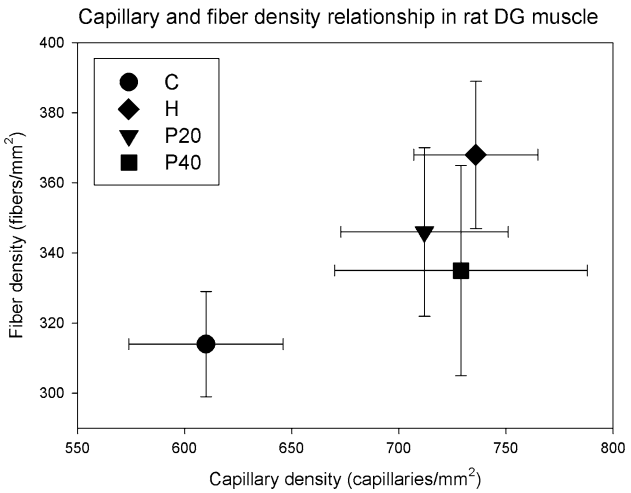

Fig. 4 Capillary and fibre density relationship in rat DG. *Bars* represent the standard error of the mean

non-significant increase in muscle capillarization and also a non-significant reduction in fibre size (Bender et al. 1984; Snyder et al. 1985; Yamashita et al. 1994). In agreement with these chronic hypoxia studies, our results show that intermittent hypoxia does not induce significant changes in the capillarization and fibre morphometry of rat TA, since we found significant differences only in some parameters (*C*/*F*, NCF and CCP) from some muscle regions (see Tables 3,4). It is interesting to note that if longer and higher simulated altitude chronic hypoxia protocols are applied, significant higher capillarization is observed in plantaris and gracilis muscles (Cassin et al. 1971). It remains to be seen whether the trends towards increasing capillarity or fibre muscle reduction we observed would become statistically significant in rats subjected to more intensive IHH protocols (higher duration and/or higher altitude). It also remains for future work to study the effects of a combination of intermittent hypoxia and exercise on leg muscles (see Clanton and Klawitter 2001) in order to compare this with chronic hypoxia situations. The combination of chronic hypoxia with exercise shows contrasting results in the literature: increases in CD and *C*/*F* with no changes in FCSA are observed in humans (Desplanches et al. 1996), no changes in CD but considerable reductions in FCSA were seen in soleus and extensor digitorum longus muscles in rats (Luedeke et al. 2004), increases in *C*/*F* as a result of high FCSA (Desplanches et al. 1993) and decreases in *C*/*F* were also found in similar situations (Abdelmalki et al. 1996).

DG

Several studies of DG subjected to chronic hypoxia report increases in capillarization. Thus, the DG of rats exposed to a simulated altitude of 6,000 m for 5 weeks showed significant increases in CD that were attributed to decreases in MDD, since no changes in *C*/*F* were observed (Snyder et al. 1985). Similar chronic hypoxia protocols also reported significant increases in CD accompanied by increases in *C*/*F*, which suggests that new capillaries should have formed since the increase in CD was not accompanied by a change in the fibre size (Deveci et al. 2001). The present study demonstrates that IHHE also induces significant increases in CD in the DG of the hypoxic rats. These changes are produced without changes in *C*/*F* since we also observed increases in FD (although non-significant) (Table 6). This is reinforced when CD and FD are plotted in a Cartesian representation: separation of control from hypoxic animals can be clearly seen because of the higher values in CD and FD in the hypoxic animals (Fig. 4). However, our results do not show significant increases in NCF after IHHE. Neither do they show a significant reduction in any morphometric parameters (FCSA, FPER, CCA, CCP and MDD) of either FOG or FG fibres. This is not the case for SO fibres where significant differences are clear between *C* and *H* and P20 rats (Table 6). This indicates that not only are the hypoxic load and the metabolic fibre type important, but the contractile fibre characteristics must also be considered in order to understand the effects of IHHE on DG. This is especially noteworthy when we consider that the more sustained motor behaviour of DG is achieved by recruiting slow motor units (SO fibres) whilst fast motor units are mainly required during forced inspirations (Mantilla and Sieck 2003).

Hypoxia and muscle workload

Previous work on myocardium muscle showed a progressive increase from C to H to P20 in capillary and fibre densities associated with significant reductions in fibre area, perimeter and diffusion distances (Panisello et al. 2007). Those results contrast with the results obtained in the present study due to the different oxidative demands of the three muscles (myocardium, TA and DG) considered. TA is an anaerobic leg muscle that did little work since the animals were confined. Hence, only postural muscles and SO fibres were active. In this situation, the predominant FOG and FG fibres found in this muscle are not affected by the lower oxygen pressure. This could explain why there were so few significant differences in muscle capillarity and fibre morphometry after IHHE. The metabolic characteristics of DG are predominantly oxidative with a mixture of slow and fast fibres that are differentially recruited. When comparing *C* to *H* rats, some significant differences have been found in the total muscle capillarization and in the morphometric parameters of SO; the active fibres in normal inspiratory work. However, compared to the results obtained for myocardium muscle, IHHE seems to induce few changes in

total DG capillarity and DG fibre morphometry. This is especially surprising considering the report that hypoxia increases respiratory frequency, and hence work per time unit, from 20 to 30% (Thomas and Marshall 1997). We hypothesize that, in order to respond to the higher ventilatory demand as a consequence of the reduced ambient oxygen pressure, other inspiratory muscles (such as the intercostals) could give support to DG activity to cope with the increased ventilatory requirements.

Further studies should be undertaken to clarify some apparent discrepancies between the effects of chronic and intermittent hypoxia. Factors such as the duration and intensity of the intermittent hypoxic stimulus may play a key role in the persistency of the morphofunctional changes in the skeletal muscles studied. In addition, individual variation in IHH responsiveness and differences among species are also important issues to be considered.

In conclusion, the present study shows that IHHE induces different changes in skeletal muscles according to their oxidative and contractile workloads. Thus, TA from hypoxic rats did not show significant changes either in total muscle capillarization or in fibre morphometry. This contrasted with the findings in DG where IHHE induced significant increases in total CD and reductions in morphometric parameters of SO fibres.

Acknowledgments This study was supported by grant BFI2003-03439 as part of the *Plan Nacional I+D+I* of the Spanish *Ministerio de Educación y Ciencia*. The authors wish to thank Robin Rycroft and Christopher Evans (UB Language Advisory Service) for their help in editing the manuscript.

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Resum article 2:

Respostes morfofuncionals a l'anèmia en múscul esquelètic de rata.

Rates adultes Sprague-Dawley foren assignades de forma aleatòria a dos grups: un grup control i un d'anèmic. L'anèmia va ser induïda mitjançant extraccions periòdiques de sang. Els músculs estudiats foren l'*extensor digitorum longus* i el *soleus*, els quals foren extrets i processats per ser analitzats en el Microscopi Electrònic de Transmissió (MET), histoquímicament i bioquímicament. Mitjançant el MET, es determinà el volum mitocondrial en tres regions diferents de cada fibra muscular: pericapil·lar, sarcolemal i sarcoplasmàtica. Seccions de mostres de cada múscul foren també tenyides utilitzant mètodes histoquímics (SDH i m-ATPasa) amb l'objectiu de revelar la capacitat oxidativa i la velocitat d'escurçament de cada una de les fibres musculars. Les determinacions de la densitat de fibres, la densitat capil·lar i la composició de fibres, foren realitzades a partir de micrografies de diferents camps fixats i seleccionats de la regió equatorial de cada múscul. La determinació de diversos metabòlits (ATP, fosfat inorgànic, creatinina, creatina fosfat i lactat) fou duta a terme utilitzant mètodes enzimàtics ja establerts i per detecció espectofotomètrica. Es trobaren diferències significatives en el volum mitocondrial entre les regions pericapil·lar, sarcolemal i sarcoplasmàtica quan els grups d'animals foren tractats independentment. A més a més, es verificà que les rates anèmiques presentaven valors significativament més baixos que els animals controls en totes les mostres de les regions d'ambdós músculs. Aquests canvis estaven associats a una proporció més elevada de fibres ràpides en el múscul *soleus* en les rates anèmiques (slow oxidative = 63.8%; fast glycolytic = 8.2%; fast oxidative glycolytic = 27.4%) que en les controls (slow oxidative = 79.0%; fast glycolytic = 3.9%; fast oxidative glycolytic = 17.1%). No es detectaren canvis significatius en el múscul *extensor digitorum longus.* Fou observat un increment significatiu en la concentració dels diversos metabòlits en els dos músculs de les rates anèmiques quan es compararen amb el grup control. Podem concloure que la hipòxia hipoxèmica causa una reducció del volum mitocondrial de les regions pericapil.lar, sarcolemal i sarcoplasmàtica. Tanmateix, fou mantingut en les fibres un patró comú en la

distribució zonal de les mitocòndries. Es trobà un increment significatiu en la concentració d'alguns dels metabòlits i en la proporció de fibres ràpides en el múscul *soleus* (de caràcter més oxidatiu), en contrast amb l'*extensor digitorum longus*, que és un múscul predominantment anaeròbic.

Journal of Anatomy

J. Anat. (2008) **212**, pp836–844 doi: 10.1111/j.1469-7580.2008.00908.x

Morphofunctional responses to anaemia in rat skeletal muscle

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Abstract

Adult male Sprague-Dawley rats were randomly assigned to two groups: control and anaemic. Anaemia was induced by periodical blood withdrawal. Extensor digitorum longus and soleus muscles were excised under pentobarbital sodium total anaesthesia and processed for transmission electron microscopy, histochemical and biochemical analyses. Mitochondrial volume was determined by transmission electron microscopy in three different regions of each muscle fibre: pericapillary, sarcolemmal and sarcoplasmatic. Muscle samples sections were also stained with histochemical methods (SDH and m-ATPase) to reveal the oxidative capacity and shortening velocity of each muscle fibre. Determinations of fibre and capillary densities and fibre type composition were made from micrographs of different fixed fields selected in the equatorial region of each rat muscle. Determination of metabolites (ATP, inorganic phosphate, creatine, creatine phosphate and lactate) was done using established enzymatic methods and spectrophotometric detection. Significant differences in mitochondrial volumes were found between pericapillary, sarcolemmal and sarcoplasmic regions when data from animal groups were tested independently. Moreover, it was verified that anaemic rats had significantly lower values than control animals in all the sampled regions of both muscles. These changes were associated with a significantly higher proportion of fast fibres in anaemic rat soleus muscles (slow oxidative group = 63.8%; fast glycolytic group = 8.2%; fast oxidative glycolytic group = 27.4%) than in the controls (slow oxidative group = 79.0%; fast glycolytic group = 3.9%; fast oxidative glycolytic group = 17.1%). No significant changes were detected in the extensor digitorum longus muscle. A significant increase was found in metabolite concentration in both the extensor digitorum longus and soleus muscles of the anaemic animals as compared to the control group. In conclusion, hypoxaemic hypoxia causes a reduction in mitochondrial volumes of pericapillary, sarcolemmal, and sarcoplasmic regions. However, a common proportional pattern of the zonal distribution of mitochondria was maintained within the fibres. A significant increment was found in the concentration of some metabolites and in the proportion of fast fibres in the more oxidative soleus muscle in contrast to the predominantly anaerobic extensor digitorum longus.

Key words anaemia; capillary density; fibre types; hypoxaemia; mitochondrial volume; oxidative metabolism; skeletal muscle.

Introduction

Like any kind of cell, muscle fibres need a continuous supply of oxygen and nutrients to be able to carry out their vital functions. The muscle tissue has a well developed microvascular system made up of blood capillaries with an average diameter of 3–5 μm, depending on the muscle (Wiedeman, 1984; Mathieu-Costello, 1991).

August S. Krogh proved in a pioneering work that the number of capillaries was clearly higher when muscle had oxidative characteristics (Krogh, 1919). Muscles with evident glycolytic metabolism had less capillarization. More variables, such the dimensions of the muscle vascularization and its functional implications, are required to quantify the capillary network accurately (Egginton & Ross, 1992).

Physiological, biochemical and mechanical factors that play an important role in muscle capillarization are now widely known (Mathieu-Costello, 2001; Christov et al. 2007). Although muscles formed mainly by oxidative fibres have a high capillary density, some researchers have not found a direct correlation between the oxidative capacity of muscle and capillarity (Gray & Renkin, 1978; Maxwell et al. 1980). Despite their low mitochondrial content, glycolytic fibres apparently have a high number of capillaries (Weibel, 1984). This may be because of the dual role of muscle capillaries, which not only participate in respiratory gases exchange, but also supply energy substrates and eliminate metabolic waste substances such as lactate

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Accepted for publication *8 February 2008*

(Weibel, 1984; Hudlicka et al. 1987). This suggests that mitochondria act as a possible modulatory factor, supplying a higher volume of oxygen to muscle fibres (Ingjer, 1979).

In skeletal muscles, mitochondrial densities and volumes are a reliable oxygen supply indicator associated with capillary density (Londraville & Sidell, 1990). Many studies have shown that there was a higher mitochondrial volume close to capillaries and just under the sarcolemma than in other deeper regions of muscle fibre (Hoppeler et al. 1981; Kayar et al. 1986; Kayar & Banchero, 1987). These studies speculate whether the mitochondrial density can also reflect the local PO_2. In other words, PO_2 would be higher where the mitochondrial volume is higher and *vice versa*.

An important characteristic of the capillary network in skeletal muscle is its plasticity and its capacity to adapt to metabolic changes which is induced by different factors: training, hypoxia, electrical stimulation of muscle, low temperatures, etc. (Butler & Turner, 1988; Hudlicka & Price, 1990; Leon-Velarde et al. 1993). Chronic hypoxia has been considered a stimulus that elicits capillary growth (Hudlicka, 1982).

Capillary tortuosity seems to increase in skeletal muscle after chronic exposition to hypobaric hypoxia (Apell, 1980; Mathieu-Costello, 1987; Poole & Mathieu-Costello, 1989). Structural adaptations of skeletal muscle resulting from prolonged exposure to hypobaric hypoxia seem to be conducted by vascular endothelial growth factor (VEGF) signalling, which ultimately depends on the expression balance of the hypoxia inducible factor (HIF) system (Pugh & Ratcliffe, 2003).

Using different enzyme activities from biochemical determinations, Peter et al. (1972) classified mammalian muscle fibres into three different groups: SO (slow oxidative), FOG (fast oxidative glycolytic) and FG (fast glycolytic). Despite being an 'academic' simplification of the muscle reality, this nomenclature has been widely accepted by researchers in the field of muscle physiology because it attributes some metabolic characteristics to each type of fibre. These characteristics were subsequently shown to be related to the physiological properties of the different motor units.

Differences at histochemical and morphometrical level in capillary supply between the different types of fibres present in mammalian skeletal musculature were evaluated. Soleus (SOL) muscle and the extensor digitorum longus (EDL) of rat were taken as a model. Soleus muscle is a postural and slow-contraction (slow-twitch) muscle with strong oxidative metabolism. Extensor digitorum longus is a fast-contraction (fast-twitch) muscle that takes part in rapid activities and high intensity processes. Therefore, its metabolism is essentially glycolytic (Close, 1972). There are also differences in these two muscles in contractile properties (Close, 1964), types of fibres (Armstrong & Phelps, 1984), blood flow and blood supply (Armstrong & Laughlin, 1985).

Nowadays, a great amount of information is available in the literature on the effects of altitude, cold, training and other factors on capillarization and skeletal muscle morphofunctional parameters (Leon-Velarde et al. 1993). In contrast, there are few data about the effect of anaemia (Celsing et al. 1988; Sakkas et al. 2004). The aim of the present work was to study the different adaptations of morphofunctional levels in physiological responses to anaemia in rat skeletal muscle. The main goal was to describe the effect of the presumable tissue hypoxia induced by limited oxygen transport capacity of the blood on fibre composition, capillary density, metabolic oxidative capacity, mitochondrial density, mitochondrial distribution and the muscle concentration of different metabolites.

Materials and methods

Laboratory animals

Sprague-Dawley male rats weighing 329.6 ± 22.9 g (mean $\pm$ SE) were used. These animals were randomly assigned to two different groups of seven animals each. One group was used as a control (C). The animals in the other group were submitted to hypoxaemic hypoxia by anaemization (A). The anaemization process took 3 weeks and consisted of whole blood withdrawal from each animal (approximately 4 mL in each extraction) on alternate days. Haematocrit values fell ($P < 0.0001$) to 31.7 ± 2.0, which was significantly lower than the normal levels (48.5 ± 2.4) found in the control group.

Muscle sampling procedure

After the hypoxaemic hypoxic period, extensor digitorum longus and soleus muscles were extracted from both legs. These skeletal muscles have different metabolic and ergonomic characteristics. Soleus is a postural muscle with oxidative properties. It therefore has a homogeneous composition. Extensor digitorum longus is a mixed muscle. The heterolateral muscles were destined for morphofunctional and biochemical studies, respectively. Muscle samples from one leg were processed for transmission electronic microscopy (TEM) and histochemistry. Muscles samples from the other hind limb were used for biochemical studies of some of the metabolites involved in muscle function: ATP, inorganic phosphate (Pi), creatine (Cr), creatine phosphate (CrP) and lactate (Lac). Muscle samples were taken by autopsy. They were then frozen and stored in 2-methylbutane cooled to –160 °C with liquid nitrogen until further study, according to the recommendations of Dubowitz (1985).

TEM study: distribution and mitochondrial volume

Sample preparation

The equatorial regions of soleus and extensor digitorum longus muscles were used to study mitochondrial volume. Small cylinders (4 mm long $\times$ 1 mm $\times$ 1 mm) of tissue were prepared from each equatorial muscle section. These muscle tissue samples were fixed by immersion in vials which contained 1 mL of mixed solution glutaraldehyde 1.25% and paraformaldehyde 1% in a phosphate-buffered saline (PBS) buffer solution, at 4 °C for 48 h. Samples were washed with PBS 0.14 M (pH = 7.4) for 45 min. This process was repeated

three times. Subsequent steps were carried out by the Serveis Cientifico-Tècnics (SCT) of the Universitat de Barcelona. In the SCT, samples were subjected to a post-fixation process in osmium tetraoxide (OsO_4) 1% in PBS for 1 h. Samples were then dehydrated in acetone. They were left in different solutions with ascending acetone concentrations for 10 min. Dehydrated samples were mounted in Spurr's epoxide resin blocks. The resin blocks were cut and semithin sections (1.5 μm) were processed. These sections were then observed with an optic microscope at 40× to choose the regions of interest for processing and analysing using TEM. The chosen regions were cut and put into square and eyelet grids. They were contrasted so that they could be studied with TEM.

Determination of mitochondrial volume

Mitochondrial volume was studied using TEM in three different regions of each muscle fibre: pericapillary (pc), the fibre region close to a capillary; sarcolemmal (sl), the fibre region close to the sarcolema; and sarcoplasmatic (sp), the innermost part of the muscle fibre. To study all of these regions, we obtained photomicrographs from different randomly chosen fibres. Different magnifications were used to cover all studied areas. The images were digitally processed and the mitochondrial volume and density calculated. Each photomicrograph (×6600) obtained by TEM (Philips model 600A) was scanned to be used with Sigma Scan image analysis software (Jandel Scientific, Erkrath, Germany). The stereological methods described by Weibel (1979) were used to determine the mitochondrial volume. These methods have been widely used in the study of different tissues. To ensure that no mitochondria were counted twice, we used the 'point-counting' method. A grid was drawn on the image to determine the number of intersection points that fell on mitochondria. The length of each square side of the grid was double the diameter of the mitochondria. Classic stereological techniques assume that mitochondria are randomly oriented and that the probability of finding mitochondria in a two-dimensional section of a tissue is related to the total mitochondria volume in a three-dimensional cell (Weibel, 1973). However, some authors (Eisenberg et al. 1974; Eisenberg, 1986) demonstrated that mitochondrial volume was not affected by its orientation in the fibre. Therefore, the geometrical probability theory can be used to calculate the average three-dimensional volumes from the study of two-dimensional samples without considering the orientation of mitochondria in the fibre. Mitochondrial volume measured by the 'point-counting' method was expressed as a percentage.

Histochemical procedures

Sample preparation

Histochemical studies were performed to determine the oxidative capacity, the contraction characteristics of each type of fibre, the capillary and fibre density, and the fibre composition. To carry out these studies, we used muscle samples that had not been processed for TEM. These samples were marked during the cryostat cuts to be able to identify the anatomic orientation of the muscle fragment and subsequent cuts. Greene's nomenclature for muscles was used (Greene, 1959). Serial transverse slices were obtained on the axis of the muscle fibres, with a thickness of 25 μm to SDH and mATPase stain, and 14 μm to the capillary mATPase stain. These cuts were done in a Frigocut Reichert-Jung (Heidelberg, Germany) cryostat between –22 °C and –20 °C. Samples were collected on gelatinized glass coverslips.

Sections were incubated for 5 min in a buffered fixative (Viscor et al. 1992) and stained for the following histochemical assays to identify the fibre types and capillaries: succinate dehydrogenase, SDH according to Nachlas et al. (1957) and myofibrillar adenosine triphosphatase, mATPase (Brooke & Kaiser, 1970). Endothelial ATPase was used to reveal muscle capillaries (Fouces et al. 1993; Torrella et al. 1993).

Capillarization and types of fibres in skeletal muscle

Once slices had been obtained in the cryostat, they were processed and mounted in a glycerine drop and photomicrographs were taken through an image capture system. This system enabled the images observed with the optical microscope to be digitalized directly. Sigma Scan Image software was used to count the capillaries and fibres and to measure the following fibre dimensions: area, perimeter, Feret's diameter and the shape factor. Feret's diameter is the longest distance possible between any two points along the boundary of a region of interest. Here it is used as an estimation of the maximum diffusion distance between surrounding capillaries and the centre of the muscle fibre. The shape factor measures the shape of a geometrical figure. This unit less the parameter is defined as $4 \times \pi \times$ the object's area divided by the perimeter squared (a perfect circle will have a shape factor of 1, whereas a line's shape factor will approach zero).

All photomicrographs were taken using 40× and 200× magnification in an optical microscope (Olympus, BX40, Japan) which incorporated a digital camera (Hitachi, KP-C550, Japan). Image calibration was done by taking a photo of a micrometric glass coverslip at each magnification at the beginning of each image capture session.

Capillary density (CD) and the percentage of each type of muscle fibre were counted in tissue fields of 2×10^5 μm^2. The obtained number was multiplied by 5 to express the parameter value in mm^2. The number of measurements oscillated between 20 and 100 fibres of each type in each field. However, all visible fibres were counted when the total number of fibres in the studied field was lower than this number.

In all cases, we used the equatorial region of each muscle to obtain the data. By means of the sample procedure designed by Torrella et al. (1996), equatorial transverse sections from each muscle were divided into a grid-like structure from which some muscle fields were selected for measurement. As a result of this protocol, five fields were sampled in this study. Due to the highly homogeneous distribution pattern of the fibre types in soleus muscle, only two fields per equatorial section were studied. In contrast, three fields were analysed in extensor digitorum longus (Fig. 1). Non-significant differences between fields in the same muscle and for the same condition were detected. As a consequence, and for clearer presentation, mean values for all the sampled fields of each muscle are presented in Tables 1–3 and Figs 2–4.

Biochemical determinations

Immediately after soleus and extensor digitorum longus muscles had been removed, metabolite extraction (ATP, PCr, Cr, Pi and lactate) was performed. Muscle tissue was crushed in a mortar which contained liquid nitrogen, and distributed to Eppendorf tubes containing 0.5 mL of PBS buffer. Each tube contained similar muscle tissue mass (between 15 and 20 mg). Samples were then homogenized using a Teflon tip on ice and low rotation velocity. Aliquots of 50 μL were obtained from the homogenate to determinate the different metabolites. In most cases, aliquots were diluted at 1/40. This entire process was carried out inside a cold chamber at 4 °C. Before proceeding to the assay for determining each different metabolite, aliquots were centrifuged and the

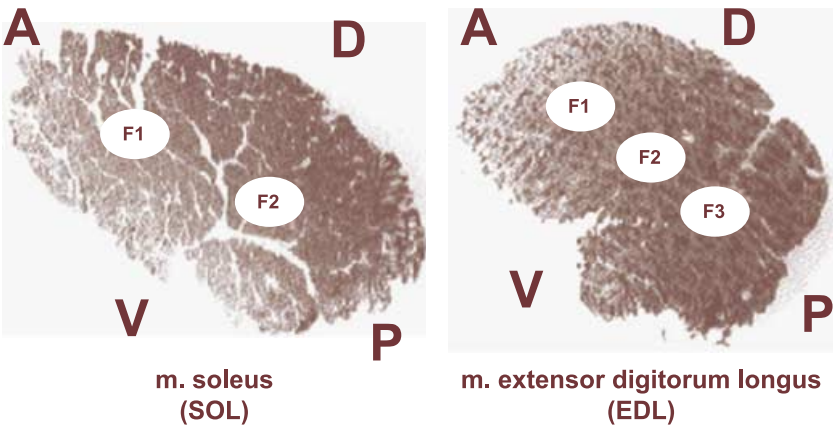

Fig. 1 Transverse sections in the equatorial region of soleus (SOL) and extensor digitorum longus (EDL) rat muscles. The images show the exact location of the studied fields (F1 to F3) used for fibre typing and capillarity measurements. Anatomical orientation: A, anterior (cranial); D, dorsal; P, posterior (caudal), V, ventral.

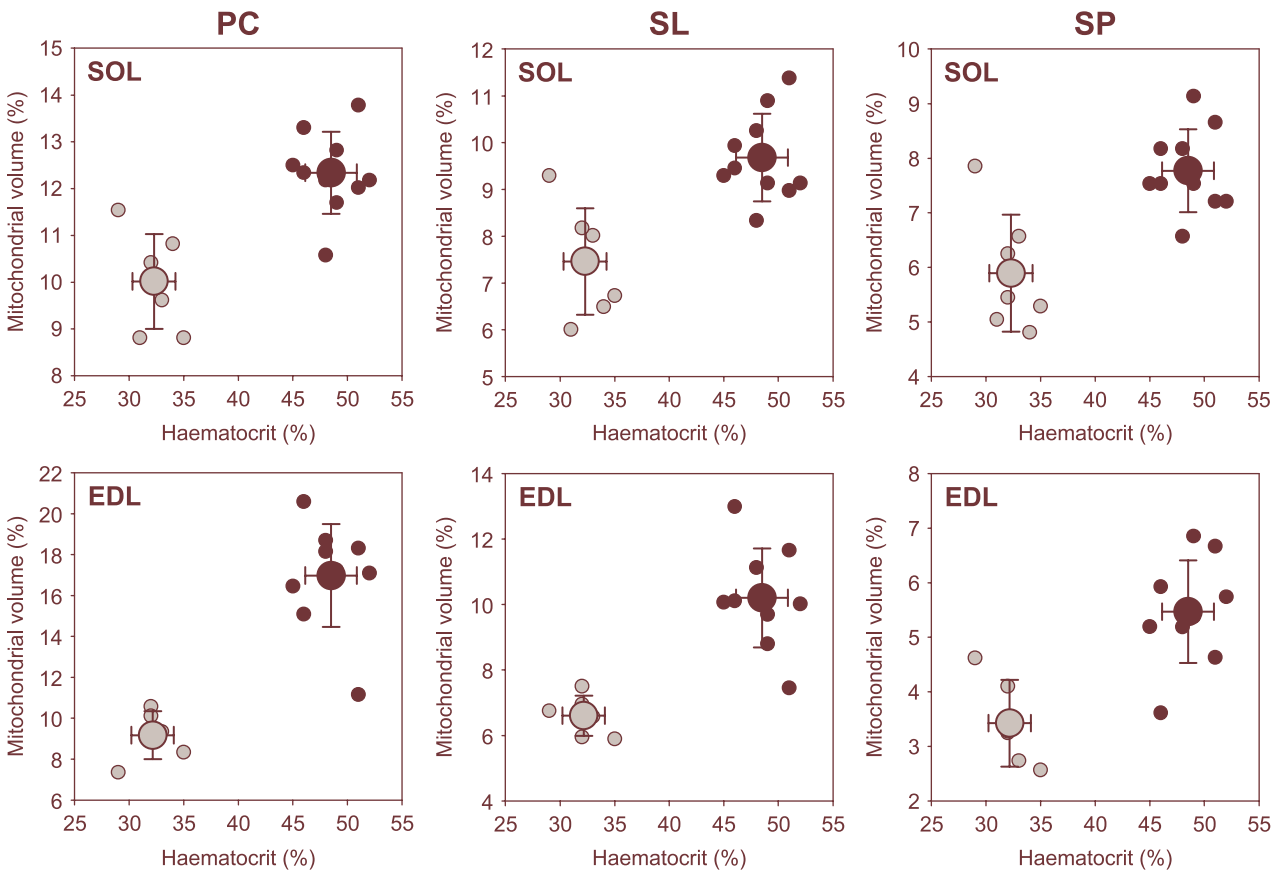

Fig. 2 Relationship between the mitochondrial volume of extensor digitorum longus (EDL) and soleus (SOL) muscles and haematocrit. Plots are presented for the three fibrillary studied regions: pericapillary (PC), sarcolemmal (SL) and sarcoplasmatic (SP). Marked differences between hypoxemic animals and controls are evidenced. Black circles: normoxia; grey circles: hypoxia. Mean values are indicated by bigger symbols. Crosshair indicates the standard deviation of the mean.

Table 1 Mitochondrial volumes (%) in the pericapillary, sarcolemmal and sarcoplasmatic regions of soleus (SOL) and extensor digitorum longus (EDL) rat muscles. Results are expressed as mean ± SE. Significant differences between anaemic and control condition are indicated when observed (***$P < 0.001$)

	SOL		EDL	
	Control	Hypoxaemic	Control	Hypoxaemic
Pericapillary	12.34 ± 1.26	9.98 ± 1.31***	16.98 ± 3.25	9.17 ± 1.64***
Sarcolemmal	9.68 ± 1.15	7.59 ± 1.39***	10.20 ± 2.06	6.60 ± 1.14***
Sarcoplasmatic	7.77 ± 0.85	6.00 ± 1.22***	5.47 ± 1.01	3.42 ± 1.06***

Table 2 Comparison of morphometric and capillarization parameters in extensor digitorum longus (EDL) and soleus (SOL) muscles between control and hypoxaemic animals. Results are expressed as mean ± SE. Significant differences between anaemic and control condition are indicated when observed (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

	SOL					
	Control			Anaemia		
Capillary density (cap mm^{-1})	1073 ± 103			888 ± 87***		
Fibre density (fib mm^{-1})	302 ± 27			273 ± 46		
Capillary to fibre ratio	3.6 ± 0.5			3.3 ± 0.3		
Fibre type	SO	FG	FOG	SO	FG	FOG
Area (μm)	3003 ± 503	3810 ± 659	3399 ± 1340	3632 ± 520**	4885 ± 931**	3363 ± 480
Perimeter (μm)	235 ± 26	271 ± 41	243 ± 43	250 ± 21	297 ± 32	244 ± 25
Feret diameter (μm)	61.8 ± 4.4	69.4 ± 6.1	64.3 ± 13.7	67.5 ± 4.8**	78.3 ± 7.4**	65.3 ± 4.7
Shape factor	0.70 ± 0.08	0.67 ± 0.14	0.69 ± 0.10	0.72 ± 0.04	0.71 ± 0.05	0.70 ± 0.06
	EDL					
	Control			Anaemia		
Capillary density (cap mm^{-1})	756 ± 71			670 ± 84**		
Fibre density (fib mm^{-1})	643 ± 86			575 ± 67*		
Capillary to fibre ratio	1.2 ± 0.1			1.2 ± 0.1		
Fibre type	SO	FG	FOG	SO	FG	FOG
Area (μm)	1519 ± 438	3659 ± 487	2239 ± 244	1577 ± 279	3761 ± 571	2463 ± 437
Perimeter (μm)	151 ± 14	244 ± 17	186 ± 14	155 ± 14	241 ± 18	195 ± 20
Feret diameter (μm)	42.2 ± 3.2	69.1 ± 2.5	53.6 ± 2.9	44.3 ± 4.0	68.6 ± 5.4	55.5 ± 5.1
Shape factor	0.79 ± 0.02	0.78 ± 0.02	0.78 ± 0.03	0.81 ± 0.02***	0.80 ± 0.02**	0.80 ± 0.03*

Table 3 Basal concentrations of some metabolites of the phosphate energy system in soleus (SOL) and extensor digitorum longus (EDL) muscles from control and hypoxemic animals. Metabolite concentrations are expressed as μmol per gram of tissue weight. Results are expressed as mean ± SE. Significant differences between control and hypoxemic muscles are indicated as *$P < 0.05$; **$P < 0.01$

	SOL		EDL	
	Control	Hypoxaemic	Control	Hypoxaemic
ATP	2.4 ± 0.5	7.7 ± 0.5**	2.84 ± 0.43	12.36 ± 7.3*
Creatine	23.9 ± 4.5	32.5 ± 6.3*	23.7 ± 3.13	49.3 ± 15.2*
Creatine phosphate	1.2 ± 0.9	7.1 ± 0.9**	2.37 ± 0.87	9.34 ± 5.8*
Inorganic phosphate	15.2 ± 4.8	34.4 ± 7.8*	15.6 ± 9.03	52.1 ± 15.9**
Lactate	9.7 ± 4.7	14.36 ± 2.6	12.9 ± 1.7	42.3 ± 18.5*

supernatant was kept for further analysis. Metabolite concentrations, expressed in μmol g^{-1} tissue weight, were determined using spectrophotometric methods.

Statistics

The normality and homoscedasticity of data were studied for each parameter to apply the correct statistical methods (parametric or non-parametric). The Kolmogorov-Smirnov test (Lilliefors table) was used to verify the normal distribution of the data. The arcsine function was used to study the percentages of fibre types. Student's *t*-test for paired data was used in normal distributions to evaluate whether there were differences in metabolite concentration. The Wilcoxon sign-rank test of paired data was used in non-normal distributions. One-way and two-way analyses of variance (ANOVA) were used to study the statistical differences between data groups. Morphometric differences between control and anaemic rats were calculated in each muscle. This method was also used to study the differences in distribution and mitochondrial volume between fibre regions (pericapillary, sarcolemmal and sarcoplasmatic) and the animals' conditions (hypoxaemic hypoxia and controls). Unless otherwise indicated, results are expressed as sample mean ± SE.

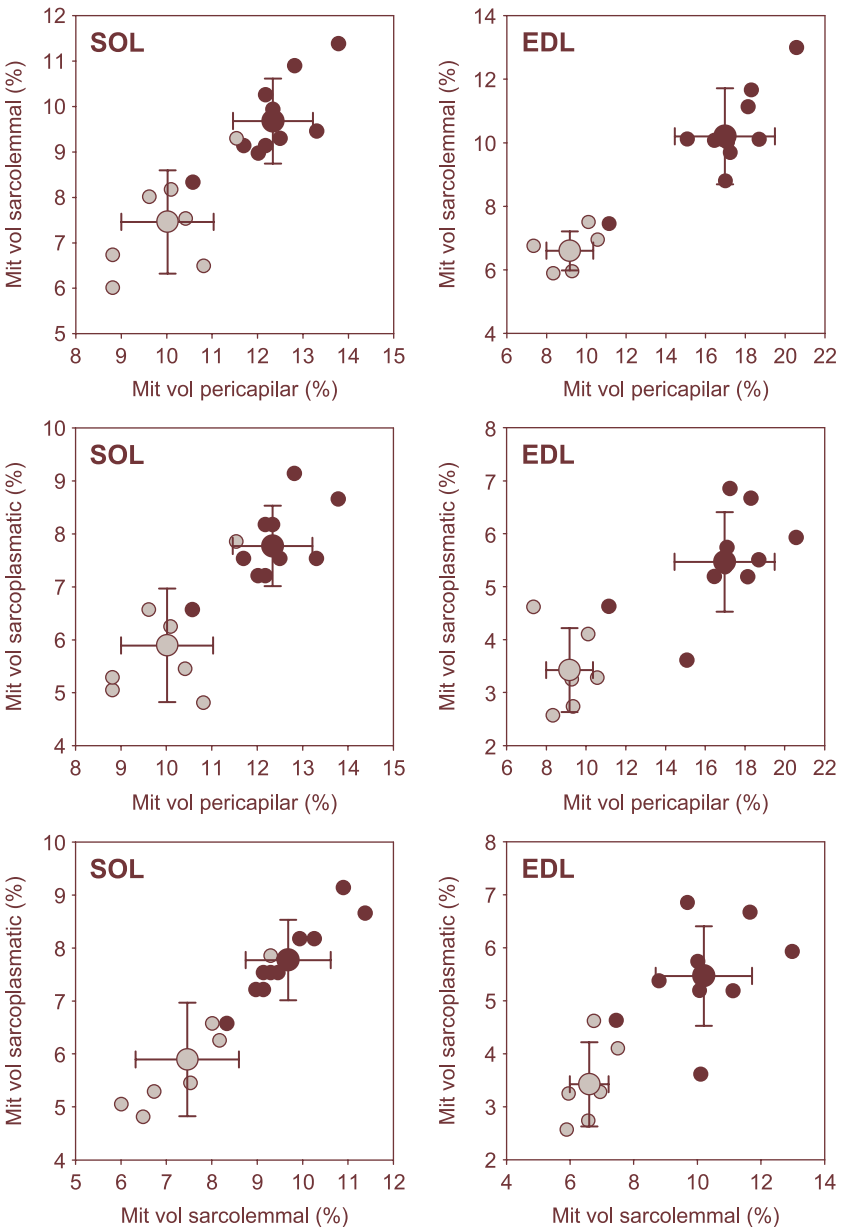

Fig. 3 Mitochondrial distribution pattern in the three muscle fibre regions (pericapillary, sarcolemmal and sarcoplasmatic) of soleus (SOL) and extensor digitorum longus (EDL) muscles. A similar trend is observed for hypoxemic (grey circles) and control animals (black circles).

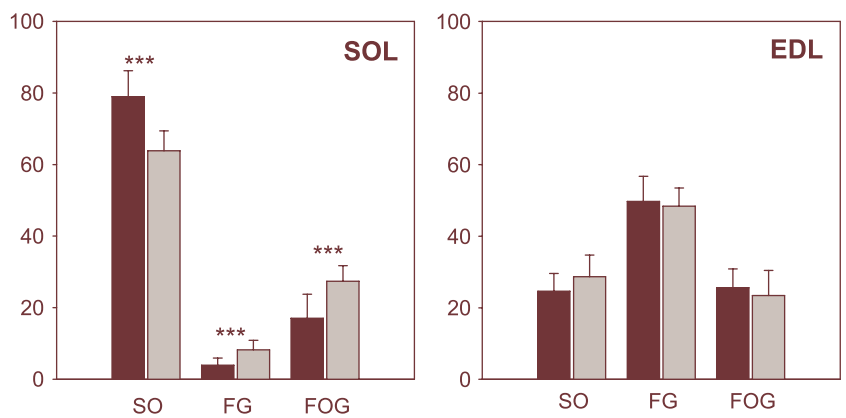

Fig. 4 Changes in fibre type composition in soleus (SOL) and in extensor digitorum longus (EDL) muscles of animals submitted to hypoxemic hypoxia (grey bars) in comparison to controls (black bars). Hairlines indicate the standard deviation of the mean. Significant differences (control versus hypoxemic) are indicated: ***$P < 0.001$.

Results

Mitochondrial volume and distribution

A statistically significant difference in the distribution ($P < 0.001$) of mitochondrial structures was found among the muscle fibres in the three studied regions: pericapillary, sarcolemmal and sarcoplasmatic (Table 1). When data from animal groups were tested independently, significant differences between control animals and those exposed to hypoxaemic hypoxia were detected for the same region. The anaemic rats had significantly lower values in all sampled regions than the control animals. Mitochondrial volume reduction was homogeneous in pericapillary, sarcolemmal and sarcoplasmatic regions. This decrease was directly related to the drop in haematocrit levels (Fig. 2).

A diminishing gradient in mitochondrial volume inside the fibre was clearly apparent when the mitochondrial volume of the three studied regions was compared, regardless of the hypoxaemic status (pericapillary > sarcolemmal > sarcoplasmatic). This was in accordance with the oxygen diffusion cascade inside the cell (Fig. 3).

Types of fibres in soleus and extensor digitorum longus muscles

It has been shown that there is a certain degree of heterogeneity in the fibre composition along the longitudinal axis of the skeletal muscles (Torrella et al. 2000). Therefore, the present paper only focused on the study and exhaustive description of the morphofunctional parameters in the equatorial region of each muscle. Figure 1 shows representative transverse sections of soleus muscle (SOL, on the left) and muscle extensor digitorum longus (EDL, on the right). Each section displays the anatomical orientation and the location of the fields used for the fibre typification and capillarization measurements. Figure 4 shows the significant increase ($P < 0.001$) in the proportion of fast fibre in the soleus of the hypoxaemic animals (SO = 63.8%; FG = 8.2%; FOG = 27.4%), as compared to control rats (SO = 79.0%; FG = 3.9%; FOG = 17.1%). No significant changes were found in extensor digitorum longus muscle, although a slight increase in slow fibres was observed in hypoxic animals (SO = 28.7%; FG = 48.4%; FOG = 23.5%) when compared with the animals used as controls (SO = 24.7%; FG = 49.7%; FOG = 25.6%).

Muscle morphometry and capillarization

In soleus muscle, a significant increase ($P < 0.01$) in the cross-sectional area and Feret diameter of SO and FG type fibres was observed in hypoxaemic animals. In addition, a significant decrease in capillary density was observed. A slight reduction in fibre density and C/F ratio was also observed in the soleus muscle of anaemic rats. In extensor digitorum longus muscle, a significant decrease in capillary and fibre densities was found in anaemic animals. There was also a marked alteration in cross-sectional morphology of all the fibre types, as indicated by noticeable changes in the shape factor (Table 2).

Biochemical data

In all the samples of the studied muscles, a significant increase ($P < 0.05$ or $P < 0.01$) in metabolite concentration ($\mu mol \cdot g^{-1}$ w/w) was observed in the anaemic animals as compared with the control group (Table 3).

Discussion and Conclusions

Hypoxaemic hypoxia is a stressful situation for most animals. To cope with the oxygen requirements, the organism is able to perform physiological and morphological adjustments to maintain a minimum level of aerobic metabolism under conditions of poor oxygen delivery to the tissues. The symmorphosis principle states that structural elements are formed to satisfy functional requirements without excess (Taylor & Weibel, 1981). Therefore, we would expect that when blood oxygen transport capacity is reduced, some adaptive responses must be elicited at muscle level to adjust the oxidative power of the muscle to this convective limitation.

In fact, in the present study we observed that the lower blood oxygen transport capacity associated with anaemia causes a change in the site of oxygen exchange at peripheral level. A clear diminution of soleus muscle capillarization was observed, accompanied by a similar, but less marked, trend in extensor digitorum longus muscle. This decrease in the number of capillaries per mm^2 in both muscles does not agree with the findings of most studies on hypoxia effects (Valdivia, 1958; Banchero, 1975; Banchero et al. 1976), including studies previously undertaken in our laboratory (Panisello et al. 2007). However, the present results are in partial agreement with the few studies on anaemia and capillary density (Celsing et al. 1988). This trend could be explained by a simultaneous increase in fibre cross-sectional area whilst maintaining capillary numbers. Capillary density was quite different in the two studied muscles. It has also been shown that the number of capillaries is higher in soleus than in extensor digitorum longus (Hoppeler et al. 1981; Leon-Velarde et al. 1993) after hypoxia conditions. Therefore, our data indicate that hypoxaemic hypoxia leads to a marked reduction in the oxidative character of soleus muscle (which is a postural and slow-twitch muscle), whereas changes are less noticeable in a muscle such as extensor digitorum longus, which has fast-contraction activity and a marked anaerobic character. This is because the metabolism of extensor digitorum longus is essentially glycolytic (Itoh et al. 1990; Faucher et al. 2005). It can be concluded that hypoxemic hypoxia transforms the soleus

muscle into a faster and less aerobic muscle, whereas extensor digitorum longus muscle is only modestly affected. The reduced response of extensor digitorum longus muscle to hypoxaemic hypoxia could result from its initial low capacity for O_2 uptake.

In agreement with previous reports (Hoppeler et al. 1990), we have observed that a long period of exposure to hypoxaemic hypoxia conditions reduces the muscle oxidative capacity and the mitochondrial volume in all regions of muscle fibres of rat soleus muscle. As can be observed in Fig. 3, the decrease in mitochondrial volume was homogeneous when the three fibre regions (pericapillary, sarcolemmal and sarcoplasmatic) were compared. Thus, in spite of the fall in oxygen diffusion gradient, hypoxaemic animals showed the same arrangement as control animals in the relationship between mitochondrial volume and fibre regions depth (pericapillary > sarcolemmal > sarcoplasmatic). Under hypoxaemic conditions, extensor digitorum longus muscle also suffers a reduction in mitochondrial volume that is concomitant with the decrease in the capillary and fibre densities. This indicates that hypoxaemia can also affect the morphofunctional organization of predominantly anaerobic muscles. This finding can be considered an efficient response against anaemia. This interpretation is supported by studies showing preservation of mitochondrial function in young anaemic patients with chronic renal failure (Miro et al. 2002).

The increase in metabolite concentration found in the muscles of animals exposed to hypoxaemic hypoxia could be interpreted as a compensatory response to decreased perfusion and oxygen supply. This interpretation is in agreement with a clinical study using ^{31}P magnetic resonance spectroscopy (MRS) and near-infrared spectroscopy (NIRS) in the calf muscle of patients with peripheral vascular disease. This study describes normal muscle cross-sectional area, ATP turnover and contractile efficiency, and higher phosphocreatine (PCr) changes during exercise (i.e. an increased shortfall of oxidative ATP synthesis). Slower PCr recovery and a decrease in functional capacity for oxidative ATP synthesis were also reported. These results indicate that a primary deficit in oxygen supply dominates muscle metabolism (Kemp et al. 2001). Thompson et al. (1993) described similar findings in chronically anaemic Wistar rats. The metabolic changes described were consistent with either reduction of the oxygen supply to the muscle or altered oxidative phosphorylation by mitochondria. Considering the possibility of mitochondrial function preservation, as proposed by Miro et al. (2002), reduced oxygen supply appears to be the main factor responsible for the increase in anaerobic character of skeletal muscle fibres manifested by anaemic animals in the present study. This interpretation is consistent with increased metabolite concentration in the muscles of the anaemic group.

In conclusion, under chronic oxygen delivery restrictions, skeletal muscle became more anaerobic and less dependent on blood flow. Considerable alterations in morphological organization are associated with these metabolic changes. Further studies are required to elucidate the functional significance of these changes and their possible limiting role in muscle work.

Acknowledgements

This study was supported in part by research grants PB96-0999 and BFI2003-03439 from Spain's Ministry of Science and from the High Sports Council of Spain 20/UNI21/97 and 09/UNI21/98. We acknowledge Robin Rycroft from the Language Advisory Service at the Universitat de Barcelona for his technical assistance in editing the manuscript.

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Resum article 3:

Activitat enzimàtica i concentració d'hemoglobina en miocardi i músculs esquelètics de rata després d'una exposició passiva intermitent a altitud simulada.

En aquest treball s'estudià l'efecte de l'exposició a hipòxia hipobàrica intermitent sobre l'activitat dels enzims lactat deshidrogenasa i citrat sintasa, juntament amb el contingut de mioglobina dels músculs cardíac, *tibialis anterior* i diafragma de rata. El programa d'exposició a hipòxia intermitent consistí en sessions de 4 hores en una cambra hipobàrica (5000m) durant un període de 22 dies. Les mostres s'extragueren al finalitzar el programa (grup H), i 20 (grup P20) i 40 (grup P40) dies després d'haver finalitzat el mateix. Els resultats foren comparats amb els obtinguts en els animals control (C). L'activitat de la lactat deshidrogenasa en el miocardi disminuí en els animals P20 (314.6 ± 15.3 $IU \cdot g^{-1}$) quan es comparà amb l'activitat de l'enzim en els animals control (400 ± 14.3 $IU \cdot g^{-1}$). Per altra banda, l'activitat de la citrat sintasa i la concentració d'hemoglobina mostraren un increment significatiu des dels animals C (88.2 ± 3.6 $IU \cdot g^{-1}$ i 4.38 ± 0.13 $\mu m \cdot mg^{-1}$), passant pels animals P20 (104.7 ± 3.7 $IU \cdot g^{-1}$ and 5.01 ± 0.17 $\mu m \cdot mg^{-1}$), i fins arribar als P40 (108.8 ± 6.5 $IU \cdot g^{-1}$ and 5.11 ± 0.22 $\mu m \cdot mg^{-1}$). Per contra, no s'observaren diferències en els músculs *tibialis anterior* i diafragma. Els nostres resultats mostren que una exposició a hipòxia hipobàrica intermitent permet l'increment del caràcter oxidatiu del miocardi, fins i tot vint dies després del cessament de l'estímul hipòxic. Aquest efecte es mantindrà durant més de 40 dies per a l'activitat citrat sintasa i per a la concentració d'hemoglobina. Aquestes troballes recolzen els nostres estudis previs en capil·larització del múscul cardíac i músculs esquelètics després d'una exposició intermitent passiva a altitud simulada, tot facilitant-nos evidències morfofuncionals i bioquímiques de l'increment de l'eficiència aeròbica cardíaca.

Journal of Sports Sciences, April 2009; 27(6): 633–640

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Enzyme activity and myoglobin concentration in rat myocardium and skeletal muscles after passive intermittent simulated altitude exposure

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(Accepted 27 December 2008)

Abstract
We studied the effect of intermittent hypobaric hypoxia exposure on lactate dehydrogenase and citrate synthase activities, together with myoglobin content, of rat myocardium, tibialis anterior, and diaphragm muscles. The intermittent hypoxia exposure programme consisted of daily 4-h sessions in a hypobaric chamber (5000 m) over a period of 22 days. Samples were taken at the end of the programme, and 20 and 40 days later, and compared with those of control animals. In myocardium, lactate dehydrogenase activity was significantly depressed in animals 20 days post-exposure (314.6 ± 15.3 IU $\cdot$ g^{-1}) compared with control animals (400 ± 14.3 IU $\cdot$ g^{-1}), while citrate synthase activity and myoglobin concentration showed a significant stepwise increase from control animals (88.2 ± 3.6 IU $\cdot$ g^{-1} and 4.38 ± 0.13 μm $\cdot$ mg^{-1}) to animals 20 days (104.7 ± 3.7 IU $\cdot$ g^{-1} and 5.01 ± 0.17 μm $\cdot$ mg^{-1}) and 40 days post-exposure (108.8 ± 6.5 IU $\cdot$ g^{-1} and 5.11 ± 0.22 μm $\cdot$ mg^{-1}). In contrast, no differences were found in diaphragm and tibialis anterior muscles. Our results show that intermittent hypobaric hypoxia exposure increased the oxidative character of myocardium even 20 days after the hypoxic stimulus has ceased, and that this effect lasts for more than 40 days for citrate synthase activity and myoglobin concentration. These findings support our previous results on skeletal and cardiac muscle capillarization after passive intermittent simulated altitude exposure, thus providing morphofunctional and biochemical evidence for increased cardiac aerobic efficiency.

Keywords: *Intermittent hypoxia, skeletal muscle enzymes, lactate dehydrogenase, citrate synthase, myoglobin*

Introduction

The reduced partial pressure of oxygen at altitude has several consequences for the oxygen economy of the body. Many acclimation responses are triggered to compensate for the reduced oxygen availability of the inspired air, among which those involving the cardiovascular system are the most noteworthy, since this system is responsible for supplying oxygen to tissues. Increases in haemoglobin concentration and haematocrit (Ferretti et al., 1990), a greater affinity of haemoglobin to oxygen (Cerretelli & Samaja, 2003), and elevated erythropoietin concentrations (Eckardt et al., 1989) have all been reported in blood from individuals exposed to chronic hypoxia. The oxygen supply to skeletal muscle tissue and myocardium under chronic hypoxic conditions has also been studied. Several studies have reported higher capillary density values and reductions in diffusion distances and fibre cross-sectional areas in muscle and myocardial fibres from various species exposed to chronic hypoxic conditions (Eby & Banchero, 1976; Kayar & Banchero, 1985; León-Velarde et al., 1993; Polla, D'Antona, Bottinelli, & Reggiani, 2004). These adaptive responses can be applied as a method to improve oxygen transport capacity in humans. However, prolonged or chronic exposure to hypobaric hypoxia also induces some degree of physical deterioration denoted by weight loss, fatigue, slowing of mental processes, and impaired cognitive function (Kayser, 1994; Milledge, 2003; Terrados, 1992). To avoid these negative effects of chronic hypoxia exposure, some programmes alternate short-term hypoxia exposure with recuperation in normoxia. These procedures can take place in hypobaric chambers and have been reported to be efficient methods for high altitude acclimatization (Richalet et al., 1992; Sutton et al., 1988; Wagner et al., 1987). In our laboratory, we have studied the effect of intermittent simulated altitude on some

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ISSN 0264-0414 print/ISSN 1466-447X online © 2009 Taylor & Francis
DOI: 10.1080/02640410802713480

functional parameters commonly used to quantify physical fitness in humans (H. Casas et al., 2000; M. Casas et al., 2000; Rodriguez et al., 1999, 2000). However, the efficiency of the different hypoxic exposure methods on well-trained humans remains controversial (Truijens et al., 2008).

According to the symmorphosis principle, the formation of structural elements in animal organisms is oriented to fit but not to exceed their functional requirements (Taylor & Weibel, 1981). Although this principle was first proposed in a study of the relationship between structure and function in the mammalian respiratory system, it has since been established as a general hypothesis of economic design (Weibel, 2000; Weibel, Taylor, & Bolis, 1998). Thus, it can be hypothesized that the adaptive responses elicited by hypoxia in humans at the haematological and central levels (respiratory and cardiac control) must be accompanied by corresponding adjustments at the peripheral level. However, non-erythropoietic adaptive responses to intermittent hypoxia in cardiac and muscular parameters cannot be evaluated in humans for ethical and technical reasons. Therefore, we developed an intermittent hypobaric hypoxia exposure protocol for laboratory rats to study morphofunctional and metabolic parameters in myocardial and skeletal muscle. Our findings in heart muscle indicate that intermittent hypobaric hypoxia elicits an adaptive response in cardiac muscle cells by increasing capillarization parameters and reducing fibre cross-sectional area, perimeter and diffusion distances, which facilitates pxygen diffusion from the capillaries to the mitochondria (Panisello, Torrella, Pagés, & Viscor, 2007). The same intermittent hypobaric hypoxia exposure protocol produces different changes in skeletal muscles according to both the oxidative and contractile workload. The tibialis anterior muscle from hypoxic rats did not show significant changes in either total muscle capillarization or fibre cross-sectional area, perimeter and diffusion distances, while in diaphragm muscle significant increases in total capillary density and reductions in these morphometric parameters of slow oxidative fibres were observed (Panisello, Torrella, Esteva, Pagés, & Viscor, 2008). In the present study, we wished to analyse further the basic mechanisms underlying the adaptive responses elicited by intermittent hypobaric hypoxia exposure. We studied myocardium and two skeletal muscles (tibialis anterior and diaphragm) to compare our previous morphofunctional findings with the following biochemical indicators: lactate dehydrogenase activity, as a marker of glycolytic anaerobic activity; citrate synthase, as a marker of aerobic activity; and myoglobin, a key oxygen release–storage protein. Preliminary results of this work were presented at the Annual Main Meeting of the Society for Experimental Biology (Panisello, Esteva, Torrella, Pagés, & Viscor, 2007).

Methods

Animals

Fifty-eight male Sprague-Dawley rats aged 6 weeks at the beginning of the experiment were randomly divided into three groups. A first experimental group of 17 rats with a mean body weight ($\pm s_x$) of 289 ± 6.9 g was subjected to a programme of intermittent hypobaric hypoxia exposure (described in detail below) and samples were drawn right at the end of this programme. A second experimental group of 16 rats with a mean body weight of 303 ± 5.8 g was simultaneously subjected to the same procedure, but samples were obtained 20 days after the hypoxic protocol ended. A third experimental group of six rats with a mean body weight of 290 ± 6.9 g was simultaneously subjected to the same exposure programme, but this time muscle samples were obtained 40 days after the end of the protocol. Finally, 19 rats with a mean body weight of 303 ± 5.1 g were used as a control group. Control animals were maintained in parallel under the same conditions as the three experimental groups. Samples from seven of the control animals (subgroup 1) were obtained at the end of the hypoxia exposure protocol, samples from eight more controls (subgroup 2) were obtained 20 days post-exposure and, finally, samples from the four remaining controls (subgroup 3) were taken 40 days post-exposure.

The present study was authorized by the University of Barcelona's Ethics Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the *Departament de Medi Ambient i Habitatge* (file #1899) of the Government of Catalonia (*Generalitat de Catalunya*).

Hypobaric chamber and intermittent hypobaric hypoxia programme

A hypobaric chamber was used to expose the rats to the intermittent hypobaric hypoxia programme. The total volume of the hypobaric chamber was approximately 430 litres, which allowed the housing of three rat cages. The chamber walls were made of Perspex, which enabled us to observe animal behaviour during the programme. Low pressure (simulated altitude) into the chamber was produced by a rotational vacuum pump (TRIVAC D5E; Leybold, Köln, Germany), the air flow rate at the inlet being regulated via a micrometric valve. Inner pressure was controlled by two differential pressure sensors (ID 2000; Leybold, Köln, Germany) connected to a

vacuum controller (Combivac IT23; Leybold, Köln, Germany) driving a diaphragm pressure regulator (MR16; Leybold, Köln, Germany). Depending on the simulated altitude to be reached, a low pressure set point was established in a control system. Once the desired level was reached, the internal barometric pressure of the chamber was regulated and maintained by the control system.

After a quarantine of 2 weeks, animals were moved into the conditioned room containing the hypobaric chamber. An initial period of 5 days, free of disturbance, was established for complete habituation. The intermittent hypobaric hypoxia exposure programme consisted of a single daily 4-h morning session (09.00–13.00 h), which was repeated 5 days a week over four consecutive weeks plus two additional days, thus completing 22 days of exposure to hypoxia (88 h in total). The simulated altitude reached during each session was 5000 m (400 mmHg = 533 hPa). The control group was subjected to the same procedure, although the hypobaric chamber was open to room pressure.

Sampling and biochemical procedures

Animals were anaesthetized with urethane ($1.5\ g \cdot kg^{-1}$ of body mass) and the right tibialis anterior muscle, the right leaflet of the diaphragm muscle, and the right ventricle of the heart were excised from each rat; these were then weighed, placed in a vial containing ice-cold saline, immediately frozen in liquid nitrogen and stored at $-80^{\circ}C$ until biochemical determinations were performed. Samples were homogenized in an ice-cold medium (1:100 w/v) containing $75\ mmol \cdot l^{-1}$ of Tris-HCl buffer. For each group, lactate dehydrogenase (LDH) activity (Beutler, 1984), citrate synthase (CS) activity (Srere, 1969), myoglobin content (Reynafarje, 1963), and total protein concentration (Bradford, 1976) were quantified. The quotient between LDH and CS activities (Hochachka, Stanley, Merkt, & Sumar-Kalinowsky, 1983) was also calculated and expressed adimensionally as the LDH/CS ratio.

Statistics

Data from all parameters are expressed as sample means ± standard errors. To test the data for normality, the Kolmogorov-Smirnov test (with Lilliefors' correction) was used. Comparisons between the two experimental groups and the control conditions were analysed using a one-way analysis of variance (ANOVA). A multiple comparison test using the Student-Newman-Keuls procedure was then performed to determine the differences between each pair of experimental and control conditions. All statistical tests were run using Sigma Stat software (SYSTAT Software GmbH; Erkrath, Germany) and statistical significance was set at $P < 0.01$.

Results

Since no significant differences were observed among control subgroups for any of the parameters, data for these three subgroups were averaged for all comparisons.

Table I shows mean LDH and CS activities, myoglobin concentration, and total protein content values of the control and hypoxic rats in the three

Table I. Lactate dehydrogenase (LDH) activity, citrate synthase (CS) activity, myoglobin concentration (Mb), and total protein content (TP) in rat myocardium (MC), diaphragm (DG), and tibialis anterior (TA) expressed as the mean ± s_x in three groups of experimental animals (see text for details) – H, hypoxic; P20, 20 days post-hypoxia; P40, 40 days post-hypoxia – and controls, C.

	C	H	P20	P40
LDH ($IU \cdot g^{-1}$)				
MC	400 ± 14.3[a]	352.2 ± 14.3	314.6 ± 15.3	367.4 ± 18.4
DG	258.6 ± 8.2	258.1 ± 10.1	258.8 ± 11.5	284.1 ± 22.9
TA	385.6 ± 14.9	383.2 ± 14.8	420.7 ± 17.5	438.4 ± 18.4
CS ($IU \cdot g^{-1}$)				
MC	88.2 ± 3.6[a,b]	91.7 ± 4.5	104.7 ± 3.7	108.8 ± 6.5
DG	23.6 ± 1.3	21.1 ± 1.7	25.1 ± 1.0	20.8 ± 1.5
TA	15.3 ± 0.8	12.9 ± 0.6	13.6 ± 0.6	13.8 ± 2.6
Mb ($\mu g \cdot mg^{-1}$)				
MC	4.38 ± 0.13[a,b]	4.81 ± 0.11	5.01 ± 0.17	5.11 ± 0.22
DG	3.62 ± 0.23	3.44 ± 0.22	3.57 ± 0.18	4.33 ± 0.44
TA	2.28 ± 0.09	2.26 ± 0.12	2.56 ± 0.17	2.83 ± 0.20
TP ($\mu g \cdot mg^{-1}$)				
MC	253.4 ± 13.2	282.4 ± 9.3	258.9 ± 11.4	393.5 ± 20.7
DG	297.9 ± 9.7	261.6 ± 8.2	284.3 ± 8.7	305.0 ± 26.1
TA	309.0 ± 14.4	270.1 ± 15.7	293.9 ± 19.0	280.9 ± 3.3

Note: Superscript letters indicate significant differences at $P < 0.01$ as follows: [a]C vs. P20; [b]C vs. P40.

muscles studied. Significant differences were noted in LDH activities of myocardium, with a mean decrease of 21% in animals 20 days post-exposure compared with the controls. In contrast, CS activities showed a significant stepwise increase from control animals to those 20 days and then 40 days post-exposure (increases of 19% and 23%, respectively). The differences observed in diaphragm and tibialis anterior muscles for both enzyme activities were non-significant. Myoglobin concentration showed similar behaviour to CS activity in myocardium, with significant increases from controls to animals 20 days and then 40 days post-exposure (increases of 14% and 17%, respectively), and non-significant differences in both diaphragm and tibialis anterior muscles. Table I shows that the changes reported in myocardium were achieved without changing the total protein concentration of the tissue.

Figure 1 shows the combined effect of the decrease in LDH activity and increase in CS activity by means of the LDH/CS ratio. This index showed a significant reduction in myocardium muscle from 4.58 ± 0.24 in control animals to 3.07 ± 0.12 at 20 days and 3.48 ± 0.34 at 40 days post-exposure. There was also a significant difference between the myocardium of animals at the end of the hypoxia protocol (3.89 ± 0.22) and 20 days post-exposure, but no significant difference between any pair of diaphragm and tibialis anterior muscles.

Thus, our results show a significant increase in indicators of aerobic capacity such as the LDH/CS ratio and myoglobin concentration in the myocardium of rats exposed to the intermittent hypobaric hypoxia exposure protocol after 20 and 40 days. In contrast, no significant differences were observed in any case for the two skeletal muscles studied.

Discussion

Lactate dehydrogenase

Lactate dehydrogenase catalyses the final step of the anaerobic pathway and its activity is one of the most frequently measured enzyme activities related to glycolysis. A common feature of most forms of hypoxic adaptation is the inhibition of lactic acid production, but it is possible that in different models the strategies used to accomplish this are different (Clanton & Klawitter, 2001). Lactate dehydrogenase catalyses the conversion of pyruvate to lactate or the reverse reaction, and two different isoforms of this enzyme appear to be involved in this key metabolic role (Daneshrad et al., 2003). This could explain the variety of results reported in relation to changes in LDH activity using several models of chronic, acute, and intermittent hypoxia. In most studies of humans exposed to chronic hypoxia, there is little or no appreciable shift in muscle LDH activity (Green, Sutton, Cymerman, Young, & Houston, 1989; Howald et al., 1990). In contrast, exposure of rats to hypobaric hypoxia increases their total heart LDH activity (Anderson & Bullard, 1971), while long cycles of intermittent hypoxia induce large LDH activity in skeletal (Clanton & Klawitter, 2001) and cardiac muscle (Ostadal et al., 1981). As regards experiments involving intermittent hypobaric hypoxia, the contradictory results reported in the literature may be due to experimental conditions such as the level of hypoxia, the duration of the hypoxic session, and the number of days of exposure. Our results clearly demonstrate that a moderate (400 torr) but sustained hypobaric hypoxia protocol (a long-cycle model maintained for 22 days) reduces LDH activity in rat myocardium (Table I). The reduction in LDH activity was only significant in samples obtained 20 days after the hypoxic protocol ended, which indicates that the response induced by the hypoxic stimulus takes some time to be completely developed.

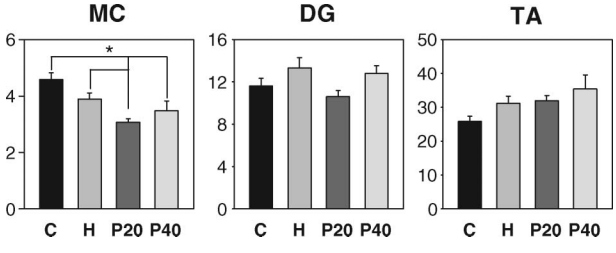

Figure 1. The LDH/CS ratio in rat myocardium (MC), diaphragm (DG), and tibialis anterior (TA) in three groups of experimental animals (see text for details) – H, hypoxic; P20, 20 days post-hypoxia; and P40, 40 days post-hypoxia – and controls, C. *Significant differences at $P < 0.01$.

Citrate synthase is often used as a key marker of oxidative metabolism, since it is the first enzyme of the Krebs cycle (Essen-Gustavsson & Henriksson, 1984). Interestingly, the values for CS activity in rat myocardium show the opposite trend from that observed in LDH activity (Table I). These results indicate that activation of the aerobic metabolic pathways is coupled with a reduction in LDH activity in hypoxic rat myocardium. This is especially evident when the LDH/CS ratio is considered, there being a significant reduction in this ratio from control to 20 days and then 40 days post-exposure (Figure 1). This index is generally used as an indicator of the relative adjustment between anaerobic and aerobic metabolism and expresses lower values when higher oxidative activities are present (Hochachka et al., 1983). The response induced by intermittent hypoxia on myocardial CS activity also needs time to become completely developed, since the increases were significant 20 days post-exposure but not immediately after the intermittent hypobaric hypoxia

protocol (Table I), while the LDH/CS ratio shows a significant decrease between recently hypoxic animals and animals 20 days post-exposure, but not between controls and recently hypoxic rats (Figure 1). However, a sharp contrast is seen between the behaviour of myocardial LDH and CS activities 40 days post-exposure. The effect of intermittent hypobaric hypoxia exposure on myocardial LDH activity was a tendency to revert towards basal values, while the increase in myocardial CS activity lasted for more than 40 days, with high values present 40 days post-exposure (Table I). Our results demonstrate that even passive intermittent simulated altitude exposure induces a significant increase in the oxidative capacity of myocardium, in a similar way to that reported in other studies that combined exercise and hypoxia (Abdelmalki, Fimbel, Mayet-Sornay, Sempore, & Favier, 1996; Messonnier et al., 2001) or which developed chronic hypoxia exposure (Hochachka et al., 1983; Sheafor, 2003). Thus, passive intermittent hypoxia, via morphofunctional and enzymatic changes, could play a role in improving heart function, in addition to routine training programmes.

However, our results show that intermittent hypobaric hypoxia exposure does not affect the LDH or CS activity of the two skeletal muscles studied, diaphragm and tibialis anterior (Figure 1 and Table I). In the case of diaphragm, this finding is in contrast to previous studies combining exercise and hypoxia (Bigard, Brunet, Guezennec, & Monod, 1991; Ogura et al., 2005; Powers & Criswell, 1996) and the work of Sheafor (2003), who found increases in diaphragm CS activity with altitude. Since diaphragm is the major muscle involved in respiration and is constantly active (Sieck, 1988), it is surprising that our results are not consistent with those found for myocardium. We believe that some of the inconsistencies between these results may be due to the fact that myocardium is an exclusively aerobic muscle, while diaphragm is a mixed muscle with 36% fast glycolytic fibres (Panisello et al., 2008). As regards locomotory muscles, many studies have reported increases in CS activity when hypoxia is combined with exercise (Melissa, MacDougall, Tarnopolsky, Cipriano, & Green, H., 1997; Perhonen, Takala & Kovanen, 1996; Terrados, Jansson, Sylven, & Kaijser, 1990), but no changes have been reported in either soleus or gastrocnemius when the exposure to hypoxia was under resting conditions (Pastoris, Foppa, Catapano, & Dossena, 1995). Similarly, our results indicate that passive intermittent hypoxia does not affect the CS activity of rat tibialis anterior (Table I). We attribute this finding to the fact that tibialis anterior is a predominantly anaerobic muscle, with zones that have up to 80% fast glycolytic fibres (Panisello et al., 2008; Torrella, Whitmore, Casas, Fouces, & Viscor, 2000), and hence it is not affected by low oxygen availability.

Myoglobin

Since Millikan's (1939) review of myoglobin function, it is generally agreed that myoglobin is produced in heart and skeletal oxidative muscles in response to the demand for oxygen. Subsequent studies and reviews have stated that myoglobin facilitates the diffusion of oxygen from the sarcolemma to the mitochondria of muscle cells (Wittenberg & Wittenberg, 1989; Takahashi & Doi, 1998). However, recent data obtained in isolated rat hearts (Chung, Huang, Glabe, & Jue, 2006) and also studies in knock-out mice lacking myoglobin (Grange et al., 2001; Meeson et al., 2001) have challenged this role of myoglobin. Myoglobin can also play a role as an intracellular scavenger of nitric oxide, which is an inhibitor of mitochondrial cytochrome-c oxidase, the terminal enzyme of the respiratory chain, thereby protecting respiration in the skeletal muscle and the heart (Brunori, 2001a, 2001b). Additional functional roles for myoglobin, such as limiting the toxic effects of reactive oxygen species in the heart, have also been proposed (Garry, Kanatous & Mammen, 2003). A notable review by Wittenberg and Wittenberg (2003) reassessed the role of myoglobin in heart and oxidative skeletal muscle.

Our results demonstrate that a sustained hypobaric hypoxia protocol markedly increases the myoglobin content of rat myocardium (Table I). The increase is higher in post-hypoxic than in hypoxic animals, indicating, once again, that adaptive changes to hypoxia are reached gradually and that they remain for more than 40 days after the regularly repeated hypoxic stimulus ceases. A reasonable explanation for the increased myoglobin content found in this study could be the need to improve the transport of oxygen to mitochondria in myocardium cells and to protect the heart from nitric oxide or the toxic effects of reactive oxygen species, which are generated as a consequence of the intermittent hypoxia conditions. Interestingly, the significant increase in myoglobin content in rat myocardium is also coupled with significant increases in CS activity and significant decreases in LDH activity; as discussed in the preceding section, this indicates an adjustment to relative anaerobic/aerobic potential of rat myocardium after intermittent hypoxia. This adjustment could explain the similar values obtained for total protein content in rat myocardium in the three experimental groups of animals (Table I). In contrast with the findings in myocardium, the myoglobin values of the two skeletal muscles studied (diaphragm and tibialis anterior) did not vary

significantly (Table I). In a recent study, Robach et al. (2007) reported a reduction in myoglobin concentration in human skeletal muscle after exposition at 4500 m. These authors proposed that myoglobin skeletal muscle is used to obtain iron to sustain the high iron erythopoietic demand in hypoxia. This possible role and also the short duration of the hypoxic stimulus could explain why in the present study myoglobin was not increased in skeletal muscle.

The biochemical findings reported here are in close agreement with rat myocardial capillarization after intermittent hypobaric hypoxia exposure (Panisello et al., 2007). We found a progressive increase from controls to hypoxic animals to animals 20 days post-exposure in capillary and fibre densities associated with significant reductions in fibre area, perimeter, diffusion distances, and increase in capillaries per fibre area and per fibre perimeter. Capillarization and fibre morphometric changes also showed marked differences over time, with rats 20 days post-exposure having higher capillarization parameters and fibre morphometry reductions than the hypoxic group. This supports the idea that there is a delay of about 20 days after the hypoxic stimulus ceases until the increase in myocardium oxidative capacity is reached. According to the Hill model of oxygen diffusion in cylindrical cells (Murray, 1974), the extracellular oxygen tension required to prevent an anoxic core depends on the oxygenated myoglobin concentration at the sarcolemma. As previously suggested (van Beek-Harmsen, Bekedam, Feenstra, Visser, & van der Laarse, 2004), when the workload of myocardium cells increases due to intermittent hypobaric hypoxia exposure, the increase in oxygenated myoglobin concentration at the sarcolemma is accomplished by increasing the total myoglobin concentration (as found in the present study) and by an upregulation of the capillarization parameters or a downregulation of fibre cross-sectional area (Panisello et al., 2008).

Furthermore, the biochemical results obtained here for diaphragm and tibialis anterior also correlate strongly with previous histochemical findings in our laboratory (Panisello et al., 2008). Intermittent hypobaric hypoxia exposure does not elicit changes in the fibre type proportion – in neither contractile nor oxidative properties – for most muscle regions from diaphragm and tibialis anterior. In addition, muscle capillarity and fibre morphometry were not altered after intermittent hypobaric hypoxia exposure in tibialis anterior muscle, and only significant increases in capillary density in the diaphragm muscle were found. This is in agreement with Lundby et al. (2004), who did not find changes in skeletal muscle capillarization in humans exposed to chronic hypoxia (4100 m during 8 weeks).

Our findings support the hypothesis that intermittent simulated altitude, such as that developed in this study, could involve a limitation of LDH gene transcription or LDH mRNA stability in the rat myocardium, which contrasts with the enhancement of CS and myoglobin formation, angiogenesis, and fibre reduction processes. Further research is required to understand better the mechanisms by which the hypoxia inducible factor (HIF-1) can modulate gene activity under the above-mentioned intermittent hypoxic conditions. A recent review by Lahiri et al. (2006) analyses the function of reactive oxygen speciea as signal transducers for specialized oxygen-sensing cells. These cells induce the transcription of specific genes involved in oxygen homeostasis after the mediation of HIF-1, which is strongly activated after intermittent hypoxia (Yuan, Nanduri, Bhasker, Semenza, & Prabhakar, 2005). Moreover, Vogt et al. (2001) have proposed low tissue oxygen pressure as a stimulus to initiate myoglobin formation, as deduced from increased mRNA contents of myoglobin found under hypoxic conditions. The observed differences in myoglobin response between myocardium, diaphragm, and tibialis anterior could be explained if differences in local PO_2 between heart and the skeletal muscles are developed during hypoxic exposure in resting animals. Oxygen delivery to inactive hind-limb muscles is not probably so challenged during simulated altitude exposure in resting animals as could be heart muscle, and to a lesser extent diaphragm muscle.

In conclusion, a 4-week programme of short intermittent hypobaric hypoxia exposure reduces the LDH activity of rat myocardium and enhances its oxidative character by means of increasing CS activity and myoglobin concentration. These changes are statistically significant 20 days after the hypoxic stimulus ceases and last for more than 40 days in the case of aerobic indicators (CS activity, myoglobin concentration, and the LDH/CS ratio), whereas they reverse after 20 days for LDH activity. However, no changes were observed in the studied skeletal muscles for any of the biochemical markers considered. This can be explained by passive exposure to hypoxia and the absence of exercise in the experimental animals. These findings support previous results obtained in our laboratory regarding skeletal and cardiac muscle capillarization after intermittent hypoxia and demonstrate that passive intermittent hypoxia induces enzymatic changes in the myocardium, leading to increased oxidative capacity (Panisello et al., 2007, 2008). This suggests that passive intermittent hypobaric hypoxia exposure could be useful to improve cardiac aerobic efficiency. In addition, intermittent hypobaric hypoxia protocols could make a valuable contribution to cardiac rehabilitation, especially in Central Asia and Andean regions, where intermittent exposure to altitude can

be easily accomplished and is low in cost. Further studies on the combined effect of intermittent hypoxia and training are needed to elucidate the complex nature of the functional adjustments elicited by both factors.

Acknowledgements

This study was supported by grant BFI 2003-03439 through the Plan Nacional I+D+I of Spain's Ministerio de Educación y Ciencia. The authors wish to thank Robin Rycroft (UB Language Advisory Service) for their help in editing the manuscript.

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Resum article 4:

Ajustaments reològics de la sang en rates després d'un programa d'exposició intermitent a hipòxia hipobàrica.

L'exposició a hipòxia hipobàrica intermitent (HHI) indueix un augment de la concentració d'hemoglobina i incrementa la massa eritrocitària tant en animals com en humans. Tot i que aquesta resposta incrementa la capacitat de transport d'oxigen de la sang, paradoxalment, afectaria també la fluïdesa de la sang i el bescanvi gasós degut a les alteracions de la viscositat sanguínia associades a l'augment de l'hematòcrit. En el present estudi, rates mascle foren sotmeses a un programa d'HHI consistent en sessions de 4 hores per dia durant 5 dies a la setmana fins completar 22 dies d'exposició a la hipòxia. S'utilitzà una cambra hipobàrica i s'aconseguí una altitud simulada de 5000m. Les mostres de sang foren extretes al final del període d'exposició (H), als 20 dies (P20) i als 40 dies (P40) després de finalitzar el mencionat programa, tot comparant-ho amb un grup control (C) que fou mantingut a pressió baromètrica de nivell del mar. Es mesurà la viscositat aparent de la sang (η_a) i la viscositat del plasma (η_p) en un microviscosímetre tipus con-placa. Malgrat que l'hematòcrit fou incrementat significativament en el grup H, la viscositat aparent de la sang no varià considerablement entre els quatre grups experimentals (els valors anaren des de 7.67 a 6.57 mPa·s a un gradient de velocitat de 90 s^{-1}). La viscositat relativa de la sang mostrà un clar increment (entorn d'un 27%) en les rates H, principalment degut al decreixement significatiu de la viscositat del plasma. Aquestes troballes podrien ser interpretades com una resposta compensatòria, la qual redueix l'efecte de l'increment del volum de la massa eritrocitària en la viscositat total de la sang. Els índexs indicadors de la capacitat de transport d'oxigen romangueren sense canvis en els quatre grups estudiats. Aquests resultats indiquen que el programa HHI te un efecte profund però transitori en els paràmetres eritrocítics i un efecte moderat en el comportament reològic de la sang.

HIGH ALTITUDE MEDICINE & BIOLOGY
Volume 10, Number 3, 2009

DOI: 10.1089/ham.2008.1086

Scientific Article

Blood Rheology Adjustments in Rats after a Program of Intermittent Exposure to Hypobaric Hypoxia

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Abstract

Esteva, Santiago, Pere Panisello, Joan Ramon Torrella, Teresa Pagés, and Ginés Viscor. Blood rheology adjustments in rats after a program of intermittent exposure to hypobaric hypoxia. *High Alt. Med. Biol.* 10:275–281, 2009.—Intermittent hypobaric hypoxia (IHH) exposure induces a rise in hemoglobin concentration and an increase in erythrocyte mass in both rats and humans. Although this response increases blood oxygen transport capacity, paradoxically, it could impair blood flow and gas exchange because of the blood viscosity alterations associated with the rising hematocrit. In the present study, male rats were subjected to an IHH program consisting of a daily 4-h session for 5 days/week until they had completed 22 days of hypoxia exposure in a hypobaric chamber at a simulated altitude of 5000 m. Blood samples were taken at the end of the exposure period (H) and at 20 (P20) and 40 (P40) days after the end of the program and were compared to control (C) maintained at sea- level pressure. Apparent blood viscosity (η_a) and plasma viscosity (η_p) were measured in a cone-plate microviscometer. Although the hematocrit significantly increased in the H group, blood apparent viscosity did not differ among groups, ranging from 7.67 to 6.57 mPa • sec at a shear rate of 90 sec^{-1}. Relative blood viscosity showed a clear increase (about 27%) in H rats, mainly due to the significant decrease in plasma viscosity. This finding could be interpreted as a compensatory response, which reduced the effect of increased erythrocyte mass volume on whole-blood viscosity. Oxygen delivery index and blood oxygen potential transport capacity remained unchanged in all groups. These data indicate that the IHH program has a deep but transitory effect on red cell parameters and a moderate effect on blood rheological behavior.

Key Words: intermittent hypoxia; hypobaric chamber; blood rheological behavior; blood viscosity; red blood cells

Introduction

Intermittent or continuous hypoxia has gained popularity as a tool for the enhancement of aerobic capacity, and an increasing number of elite athletes use these strategies in combination with training programs (Levine and Stray-Gundersen, 1997; Wilber, 2004; Robach et al., 2006). The most serious effects of high altitude on human physiology are due to the low oxygen partial pressure of the inspired air; consequently, several adjustments are needed to improve the tissue oxygen availability (Leon-Velarde et al., 2000). Hypobaric hypoxia increases ventilation (West, 1993), arteriovenous O_2 difference, hemoglobin concentration, and hematocrit (Ferretti et al., 1990a; Rodriguez et al., 1999). It also has profound effects on the structure and function of skeletal muscle tissue (Ferretti et al., 1990b; Hoppeler et al., 1990; Panisello et al., 2008), it induces acid–base alterations and affects the affinity of hemoglobin for oxygen (Cerretelli and Samaja, 2003), and it raises erythropoietin levels (Eckardt et al., 1989).

Moreover, prolonged exposure to hypobaric hypoxia also induces physical deterioration, which increases with altitude (Kayser, 1994). This deleterious effect is reflected in a marked decrease in body weight, due in part to a reduction in muscle mass (Terrados, 1992). Also, due to the increase in red cell mass, the viscosity of blood (apparent viscosity) can be increased with the possibility of a subsequent reduction in oxygen transport capacity. Other circulatory complications such as deep venous thrombosis may be caused by abnormally higher hematocrit, although compensatory mechanisms can reduce the effective hematocrit after intense exercise (Reinhart et al., 1983). Prolonged exposure to high altitude in nonnative residents or relatively recent high altitude populations frequently yields a complex syndrome: Monge's disease or chronic mountain sickness (Leon-Velarde et al., 2005;

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Rivera-Chira et al., 2007; Xing et al., 2008). To prevent the negative effects of chronic hypoxia exposure, several procedures alternating short hypoxia exposure with immediate recovery in normoxia have been proposed (Brugniaux et al., 2006; Robach et al., 2006). These intermittent hypoxia-exposure procedures are performed in hypoxic chambers and have led to relevant findings, such as the efficacy of a hypoxic stimulus to elicit an erythropoietic response and also other nonerythropoietic physiological adjustments affecting aerobic capacity. Thus, these exposure protocols have been considered as efficient methods for high altitude acclimatization (Wagner et al., 1987; Sutton et al., 1988; Richalet et al., 1992).

In the present study we analyze some of the parameters of the adaptive responses previously described in humans that are elicited by intermittent hypoxia. We applied an IHH exposure protocol to laboratory rats to study its effect on blood rheology and other rheological parameters during hypoxia exposure and recovery periods. Preliminary results from this study were presented at the Annual Main Meeting of the Society for Experimental Biology (Glasgow, April 2007).

Materials and Methods

Animals

A total of 70 male Sprague–Dawley rats, aged 6 weeks and with average body weight of 312.8 ± 4.6 g at the beginning of the experiment, were randomly divided into four groups. The first experimental group of 18 rats (H, for hypoxic) was submitted to a program of IHH (described in detail later), and blood was drawn at the end of this program. A second experimental group of 13 rats (P20, for posthypoxia 20 days) was simultaneously submitted to the same program, but blood samples were obtained 20 days after the end of the protocol. A third experimental group of 14 rats (P40, for posthypoxia 40 days) was also simultaneously submitted to the same exposure program, but samples were obtained 40 days after the end of the protocol. Finally, 22 rats were used as a triple control group (group C, for control). Control animals were maintained under the same conditions as the three experimental groups. Samples from 9 control animals (subgroup C1) were obtained at the same time as those from H; samples from another 9 controls (subgroup C2) were obtained at the same time as those from P20, and, finally, samples from the remaining 4 (subgroup C3) were taken at the same time as those from P40. No significant differences in body weight among control and experimental animals were detected in this study. Animal growth was normal, and the body mass of the last set of animals was 428.6 ± 9.4 g.

This study was part of a general procedure for studying peripheral gas exchange; thus all rats were killed and used to obtain other tissues samples.

The present study was authorized by the University of Barcelona's Ethical Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the Departament de Medi Ambient i Habitatge (file 1899) of the Catalan Government (Generalitat de Catalunya).

Hypobaric chamber

A hypobaric chamber was used to submit the rats to the IHH program. The total volume of the hypobaric chamber was approximately 450 L, which allowed the housing of three rat cages. The chamber walls were made of polymethyl methacrylate plastic, which facilitated observation of animal behavior during the protocol. Relative vacuum was developed by a rotational vacuum pump (TRIVAC D5E, Leybold, Köln, Germany) by regulating the airflow rate at the inlet with a micrometric valve. Inner pressure was controlled by two differential pressure sensors (ID 2000, Leybold, Köln, Germany) connected to a vacuum controller (Combivac IT23, Leybold, Köln, Germany) driving a diaphragm pressure regulator (MR16, Leybold, Köln, Germany). Depending on the simulated altitude required, a low-pressure set point was established in a control system. After the desired level was reached, the internal barometric pressure of the chamber was regulated and maintained by the control system.

IHH program

After a quarantine of 2 weeks, animals were moved into the conditioned room containing the hypobaric chamber. An initial period of 5 days, free of disturbance, was allowed for complete habituation. The IHH program consisted of 4-h sessions 5 days a week for 4 weeks and 2 additional days, thus making 22 days of exposure to hypoxia (88 h in total). The simulated altitude reached during each session was 5000 m (400 mmHg = 533 hPa). Group C was subjected to the same procedure, although the hypobaric chamber was open to room pressure. Animals had access to laboratory chow and tap water ad libitum. However, for technical reasons, water reservoirs were removed during hypoxia sessions from all animal groups.

Blood sampling procedure

Blood samples were collected by cardiac puncture. Prior to collection, animals were anesthetized with urethane (1.5 g/kg BM). Sodium heparin was used as an anticoagulant. A fraction of each blood sample was separated for immediate hematological analyses, which were always completed within 10 min of blood withdrawal. A second portion of the sample was simultaneously processed for blood rheology determinations. The cellular portion of a third part of the blood sample was removed by centrifugation, and the plasma obtained was separated without delay for the measurement of viscosity. Hematological and hemorheological values were determined immediately after collection.

Hematology and blood rheology

The following hematological parameters were measured using an electronic cell counter (Celltac α, Nihon Kohden Corp., Tokyo, Japan): red blood cells (RBC), hemoglobin (Hb), hematocrit (Hc), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Total plasma protein and fibrinogen concentrations were determined by spectrophotometric techniques according to Bradford (1976) and sulfite precipitation (Rampling and Gaffney, 1976) methods, respectively. The apparent viscosity (η_a) of blood and plasma was measured using a cone-plate microviscosimeter (Brookfield Digital Rheometer Model DV-III +, Middleboro, MA, USA) equipped with a CP40 spindle (0.8°) and connected to a thermostatic bath. Brookfield viscosity standard fluid 5 (4.9 mPa/sec at 25°C) was used for calibration just before each measuring session. A sample volume of 0.5 mL was tested at different shear rates ($\dot{\gamma}$), ranging from 2.25 to

$450\,sec^{-1}$. Measurements were done at 38.0°C. Due to the low viscosity values and to obtain the highest accuracy, plasma viscosity was measured only at $450\,sec^{-1}$, since the Newtonian behavior of plasma is well established.

Considering limitations due to the viscoelastic behavior of the whole blood and for easy understanding, the effect of plasma viscosity on whole-blood apparent viscosity was considered by studying the quotient of the apparent viscosities of whole blood and plasma, also known as blood relative viscosity (η_r). According to Chien and colleagues (1970), erythrocyte aggregability (RBC_a) and deformability (RBC_d) are the main factors affecting blood viscosity at low and high shear rates, respectively. As a consequence, the contribution of these microrheological characteristics to blood flow properties could be estimated from the variation of apparent blood viscosity values within low (when cell plasma protein interactions are strong) and high (with high probabilities of cell–cell interaction) ranges of shear rate, respectively. Thus, we applied the following formulas, also defined as the degree of shear dependence (Usami et al., 1969):

$$RBC_a = (\eta_{2.25} - \eta_{4.5})/\eta_{4.5}$$

$$RBC_d = (\eta_{225} - \eta_{450})/\eta_{450}$$

where η_x indicates the apparent blood viscosity at a shear rate level $\dot{\gamma} = x$.

Calculations were also performed to evaluate the effect of blood viscosity changes induced by hypobaric hypoxia on oxygen transport function. We calculated two different coefficients previously defined in the literatur: first, the oxygen delivery index (Hc/η) (Koch, 1995), which shows the relationship between the hematocrit (Hc) and blood viscosity (η) and, second, the blood oxygen potential transport capacity ($\beta[Hb]/\eta$) (Hedrick et al., 1986), which relates the hemoglobin oxygen capacity (β) and hemoglobin concentration [Hb] with blood viscosity (η). β values were assumed to be 1.34 mL $O_2 \bullet g^{-1}$ at 38°C for the four experimental groups. For the oxygen delivery index and oxygen potential transport capacity calculations, blood viscosity value at a shear rate of $90\,sec^{-1}$ was taken.

Statistics

Data for all parameters are expressed as the sample mean ± standard error of the mean. Differences between the experimental and control groups were analyzed by a one-way ANOVA test. Afterward, a multiple comparison test using the Scheffé procedure was run to determine the differences between each pair of experimental and control conditions. Descriptive statistics and analyses of normality were made with the SigmaStat software package, whereas one-way ANOVA and the Scheffé test were performed by the application of suitable subroutines from the SPPS/PC + package (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant for $p < 0.05$.

Results

Normal growth was not affected by IHH, as reflected by body weight changes during the protocol. Moreover, no statistically significant differences were found among C1, C2, and C3 for any of the parameters and, unless otherwise indicated, these three control groups were combined for all figures and tables and named group C.

Hematological parameters

Hematological parameters for the three species are given in Table 1. The one-way ANOVA and Scheffé tests showed significant differences among the four experimental groups in most of the hematological parameters. Hematological changes after exposure to hypobaric hypoxia were characterized by a significant increase in erythrocytes in the hypoxic group (H vs. C, P20, and P40; $p < 0.001$), hematocrit (H vs. C, P20, and P40; $p < 0.001$), and hemoglobin concentration (H vs. C, P20, and P40; $p < 0.001$). Also, some significant differences in the hematological indexes MCV and MCH were found; they

Table 1. Hematological Parameters, Plasma Viscosity, Microrheological Indexes, Blood Oxygen Potential Transport Capacity, and Oxygen Delivery Index for the Different Groups of Experimental Animals

		C (n = 17)	H (n = 10)	P20 (n = 13)	P40 (n = 14)
RBC ($cells \bullet 10^3 \bullet \mu L^{-1}$)	a	8.81 ± 0.10	9.98 ± 0.20[a]	8.91 ± 0.16	8.60 ± 0.14
Hb ($g \bullet dL^{-1}$)	a	15.66 ± 0.19	18.75 ± 0.37[a]	16.26 ± 0.28	15.56 ± 0.25
Hc (%)	a	47.03 ± 0.66	55.22 ± 1.02[a]	48.09 ± 0.79	47.03 ± 0.77
MCV (μm^3)	b	53.33 ± 0.41	55.25 ± 0.37[c]	53.86 ± 0.50	54.72 ± 0.44
MCH (pg)	c	17.77 ± 0.12	18.76 ± 0.15[b]	18.03 ± 0.85	17.92 ± 0.20
MCHC (%)		33.34 ± 0.17	33.96 ± 0.16	33.90 ± 0.21	33.09 ± 0.23
WBC ($cells \bullet 10^3 \bullet \mu L^{-1}$)	d	9.85 ± 0.83	13.50 ± 1.47[c]	12.61 ± 0.65	9.40 ± 0.78
Plasma viscosity (mPa • sec)	a	1.24 ± 0.02	1.14 ± 0.02[b]	1.25 ± 0.03	1.30 ± 0.03
Total plasma proteins ($g \bullet 100\,mL^{-1}$)	e	4.95 ± 0.08	4.69 ± 0.18	4.88 ± 0.08	5.34 ± 0.08[b]
Fibrinogen ($mg \bullet 100\,mL^{-1}$)		129.26 ± 8.42	96.01 ± 7.67	128.52 ± 9.76	136.64 ± 9.89
RBC_a	f	0.46 ± 0.03[b]	0.66 ± 0.03	0.61 ± 0.03	0.66 ± 0.03
RBC_d		0.13 ± 0.02	0.14 ± 0.07	0.16 ± 0.03	0.11 ± 0.01
Hc/η $(mPa \bullet sec)^{-1}$		7.34 ± 0.35	7.50 ± 0.37	7.25 ± 0.67	7.14 ± 0.23
$\beta[Hb]/\eta$ ($mLO_2 \bullet 100\,mL^{-1} \bullet mPa^{-1} \bullet sec^{-1}$)		3.30 ± 0.15	3.39 ± 0.37	3.26 ± 0.30	3.18 ± 0.10

Mean values and standard error are indicated. Significant differences between groups are indicated by the following code: (a) H vs. all other groups, (b) C vs. H, (c) H vs. C and P40, (d) H vs. P40, (e) P40 vs. all other groups, and (f) C vs. all other groups. Levels of significance are indicated as [a]$p < 0.001$, [b]$p < 0.01$, or [c]$p < 0.05$.

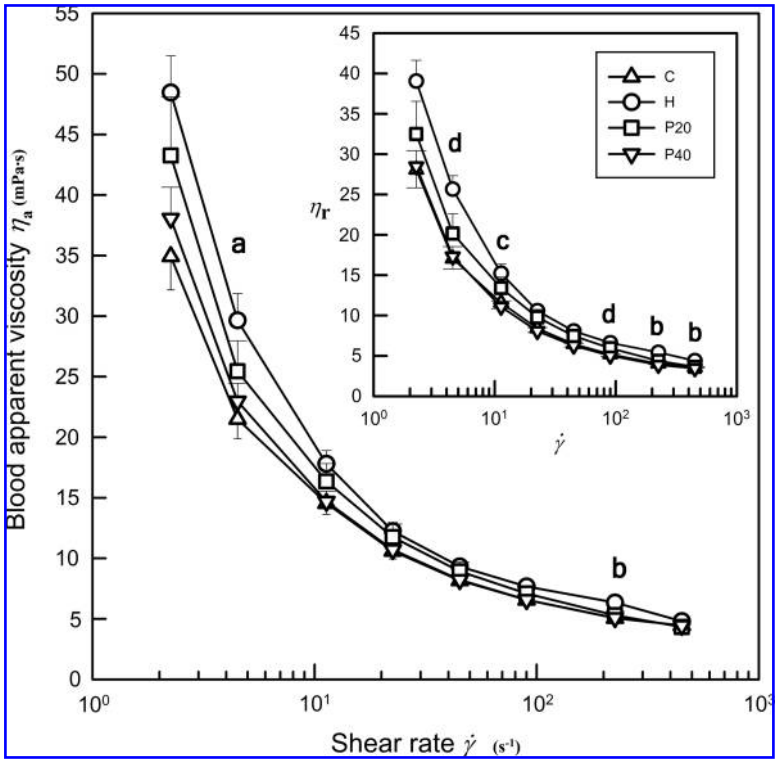

FIG. 1. Rheograms of apparent blood viscosity (η_a) and relative viscosity (η_r) for whole blood from the different experimental groups. Vertical bars represent standard error of the mean. Significant differences are indicated according to the following code: (a) $p < 0.05$ H vs. C; (b) $p < 0.01$ H vs. all other groups; (c) $p < 0.05$ H vs. P40; (d) $p < 0.05$ H vs. C and P40.

were slightly increased in the H group and later returned to basal values.

Blood rheology parameters

As expected, blood samples showed a non-Newtonian shear-thinning behavior manifested as a clear reduction of viscosity as the applied shear rate increased in each experimental condition. When the apparent viscosity of the native whole blood was compared by means of a one-way ANOVA, a significant effect of the shear rate on blood viscosity was found (Fig. 1).

Plasma viscosity values (Table 1) showed a significant decrease in the H group compared with the other three groups (H vs. C, P20, and P40; $p < 0.01$). Fibrinogen and plasma protein concentration showed a similar variation profile.

Table 1 also shows that, whereas RBC deformability indexes were very uniform, ranging from 0.11 to 0.16, the RBC aggregability indexes (Table 1) differed widely between C and the other experimental groups (C vs. H, P20, and P40; $p < 0.05$). However, these results must be considered with caution, because these indexes indicate the rheological behavior and red cell interactions in cone-plate conditions, which are distinct from those in vivo in microcirculatory vessels.

Blood oxygen potential transport capacity and the oxygen delivery indexes are listed in Table 1. In both indexes, the four experimental groups were similar, although group H showed a trend to be higher, thus indicating that oxygen supply to the tissues could be slightly improved in the hypoxia group.

The relationship between apparent and relative blood viscosities and the hematocrit is plotted in Fig. 2. A clear-cut trend was observed in animals according to their group. Both graphs show that group H is clearly apart from the others because of its high viscosity and hematocrit values.

Discussion

Many studies have reported that chronic hypoxia induces deleterious effects on body mass (Boyer and Blume, 1984; Rose et al., 1988). A recent experimental study of chronic IHH in rats with a 4 by 4 and 2 by 2 alternating daily schedule of sea level and simulated 4600-m altitude demonstrated a severe body weight reduction and compromised survival rate (Siques et al., 2006). However, possibly due to the lower degree of hypoxia exposure, we detected no negative effects on normal growth rate. This indicates that our hypoxia exposure regime offers good compatibility with the standard living conditions of these experimental animals.

Hematological parameters

The hematological parameters and oxygen transport indexes for the four experimental groups were within the range of the results previously found in rats (Siques et al., 2006) and humans (Casas et al., 2000a; Casas et al., 2000b). The most remarkable adaptations to the acclimatizing program are probably those observed in the hematological profile. The significant increases in red blood cells, hematocrit, and hemoglobin concentration values in group H are clearly associated with an enhancement of blood oxygen transport capacity. After the exposure period was over, raised values of RBC, Hb, and Hc had the tendency to return to the lower normal range. Consequently, it seems clear that intermittent exposure to hypoxia can also stimulate erythropoiesis in the

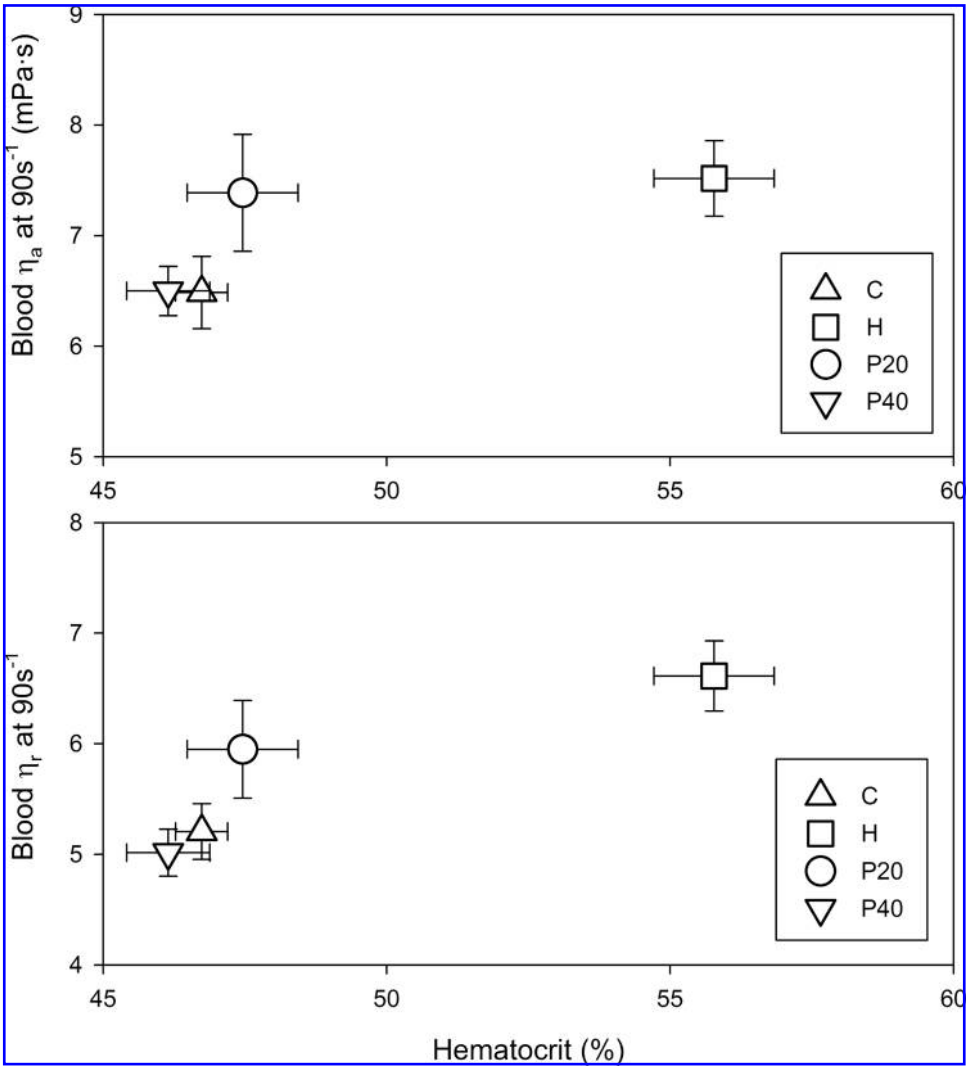

FIG. 2. Relationships between apparent blood viscosity (upper panel) and relative viscosity (lower panel) and mean hematocrit for each experimental group.

rat to the same extent as chronic exposure (Lamanna et al., 1992; Rivera et al., 1994; Biljanovic-Paunovic et al., 1996). Slight differences in hematimetric indexes may be due to the influence of a significantly higher percentage of reticulocytes in group H rats, thus causing a small increase in the volume of blood cells (Rodriguez et al., 1999; Savourey et al., 2004).

Blood rheology parameters

At low shear rates, the whole-blood viscosity values for all groups of rats were notably higher than those described in humans, even in previous studies in our laboratory (Rodriguez et al., 1999). As a consequence of the rise in the red cell packed volume induced by the intermittent hypoxia exposure program, one would expect a greater increase in blood viscosity; but the hemorheological characteristics did not significantly change in our observations after the exposure period, although a clear increasing trend was observed in the hypoxic group compared to the others, especially at low shear rates. Regrettably, for technical reasons, viscometer precision at very low rotational speeds is substantially reduced when low-viscosity biological fluids are measured. For this reason, data at low shear rates must be considered with caution. In spite of this uncertainty, a coherent trend toward increased values for whole-blood viscosity induced by hypoxia was observed (Fig. 1). The effect is more apparent at low shear rates than at higher ones, thus indicating different dynamics between erythrocyte deformability, the main factor influencing blood viscosity at high shear rates, and erythrocyte aggregability, the most important factor affecting blood viscosity at low shear rates. As reflected by the high viscosity values at low shear rate, our data indicate that, besides deformability, aggregation of rat erythrocytes may be an important factor limiting blood oxygen supply to the tissues.

The presence of some compensatory mechanisms could eventually prevent the negative effects evoked by an excessive increase in blood viscosity. The decrease in plasma viscosity and possible erythrocyte microrheological changes may be the main factors driving these compensatory responses. Plasma protein concentration showed a similar figure to plasma viscosity changes in this study and, of interest, fibrinogen concentration tightly followed these variations, maintaining in each case about 25% of total plasma protein mass. A similar response that regulates blood viscosity has been

described in transgenic mice overexpressing erythropoietin (Vogel et al., 2003). Changes in blood rheological behavior during hypoxia exposure can markedly affect microcirculation in peripheral tissues, resulting in more severe frostbite damage than in normoxic conditions (Zengren et al., 1999).

The oxygen carrying capacity and oxygen delivery indexes were similar in the four experimental groups (no significant differences were found), in spite of marked differences in hematocrit, blood viscosity, and hemoglobin concentration. Several factors, other than the well-known adjustment between hematocrit and blood viscosity for sustained oxygen delivery to tissues (optimal hematocrit theory), may explain this finding. Our data seem to indicate that the blood oxygen-carrying capacity and oxygen- delivery index are only slightly affected by increased hematocrit induced by hypoxia in rats of the H group. A similar finding was obtained in a previous comparative study in different species of vertebrates (Viscor et al., 2003).

The relationship between apparent and relative blood viscosities and hematocrit shows that a different time course for viscosity changes could exist when the four groups are compared (Fig. 2). This graph shows asymmetry in the effect on whole-blood viscosity between erythropoiesis activation and the recovery to normal conditions. Thus, 40 days after the end of exposure the rats had almost recovered from the effects of our IHH program. This time course does not differ from that described in humans (Rodriguez et al., 2000) and must be considered when planning exposure for preacclimatization expeditions or competition events.

We conclude that intermittent exposure to hypobaric hypoxia (simulated altitude) activates the erythropoietic response, but this does not necessarily imply a direct improvement in the aerobic performance capacity of rats. In fact, the oxygen delivery index and blood oxygen potential transport capacity remained unchanged in all groups due to a compensatory response that reduced the effect of increased red cell mass volume on whole-blood viscosity. All these data indicate that the IHH program applied has a deep but transitory effect on red cell parameters and a very moderate effect on blood rheological behavior.

Acknowledgments

This study was supported by grant BFI2003-03439 through the Spanish Plan Nacional I + D + I. The authors thank Rafael Pedret for his technical assistance and Robin Rycroft (Language Advisory Service–UB) for editing the manuscript.

Disclosures

Authors Esteva, Panisllo, Torrella, Pagés, and Viscor have no conflicts of interest or financial ties to disclose.

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Received November 4, 2008;
accepted in final form January 14, 2009.

Resum article 5:

Status de marcadors d'estrès oxidatiu en rates després d'una exposició intermitent a hipòxia hipobàrica.

Programes d'exposició a hipòxia hipobàrica intermitent (HHI) s'utilitzen per incrementar la concentració d'hemoglobina i la massa eritrocitària. Aquests han estat dissenyats amb l'objectiu de millorar el rendiment d'atletes però també es postula que poden contribuir al benestar de la gent. Malgrat que la resposta d'aclimatació afavoreix l'increment de la capacitat de transport d'oxigen facilitant l'increment de VO_{2max}, l'efecte de les espècies reactives de l'oxigen (ROS) podrien influenciar en el comportament dels eritròcits i del plasma, tot causant un empitjorament de la fluïdesa perifèrica de la sang. Rates mascle foren sotmeses a un programa d'HHI consistent en sessions de 4 hores per dia durant 5 dies a la setmana fins completar 22 dies d'exposició a la hipòxia. S'utilitzà una cambra hipobàrica i s'aconseguí una altitud simulada de 5000m. Les mostres de sang foren extretes al final del període d'exposició (H), als 20 dies (P20) i als 40 dies (P40) després de finalitzar el programa, tot comparant-ho amb un grup control (C) que fou mantingut a pressió de nivell del mar. Es mesuraren paràmetres hematològics juntament amb diversos indicadors d'estrès oxidatiu: els TBARS en plasma i els enzims CAT i SOD en eritròcits. El contingut de glòbuls vermells, la concentració d'hemoglobina i l'hematòcrit evolucionaren com era d'esperar ($p<0.001$, H *vs*. tots els altres grups). Tanmateix, no s'observaren diferències significatives en cap dels paràmetres oxidatius quan els 4 grups experimentals foren comparats entre sí. L'absència de diferències significatives entre els grups indicaria que el nostre programa HHI presenta un lleu impacte en l'estat redox general, fins i tot en les rates de laboratori, les quals són més sensibles a la hipòxia que els humans. Podem concloure que el programa d'HHI no incrementa l'estrès oxidatiu i, per tant, seria apropiat per a aplicacions biomèdiques.

Title

Oxidative stress markers status in rats after intermittent exposure to hypobaric hypoxia (IHH)

Authors

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Keywords

Intermittent hypoxia; hypobaric chamber; oxidative stress; free radicals; red blood cells.

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Running title

Oxidative stress after intermittent hypoxia

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Abstract

Programs of intermittent hypobaric hypoxia (IHH) exposure are used to raise hemoglobin concentration and erythrocyte mass. They were designed to improve the performance of athletes but it is postulated that can also contribute to the welfare of people. Although acclimation response increases blood oxygen transport capacity leading to a VO_{2max} increase, the effects of reactive oxygen species (ROS) might determine the behavior of erythrocytes and plasma, thus causing a worse peripheral blood flow. Male rats were subjected to an IHH program consisting of a daily 4-h session for 5 days/week until complete 22 days of hypoxia exposure in a hypobaric chamber at a simulated altitude of 5000 m. Blood samples were taken at the end of the exposure period (H) and at 20 (P20) and 40 (P40) days after the end of the program, and compared to control (C), maintained at sea-level pressure. Hematological parameters were measured together with several oxidative stress indicators: plasma TBARS and erythrocyte CAT and SOD. RBC count, hemoglobin concentration and hematocrit evolved as expected ($p<0.001$, H vs. all other groups). However, there were no significant differences between the four groups in any of the oxidative stress parameters. The absence of significant differences between groups indicates that our IHH program has little impact on the general redox status, even in the laboratory rat, which is more sensible to hypoxia than humans. We conclude that IHH does not increase oxidative stress and is thus suitable for use in biomedical applications.

Introduction

Intermittent or continuous hypoxia has gained popularity as a way to enhance aerobic capacity, and an increasing number of elite athletes use these strategies in combination with training programs.[1-3] The most serious effects of high altitude on human physiology are due to the low oxygen partial pressure of the inspired air; consequently, several adjustments are needed to improve the delivery of oxygen to tissues.[4] Hypobaric hypoxia causes an increase in ventilation[5] and arteriovenous O_2 difference, and also raises erythropoietin levels[6] and, as a consequence, hemoglobin and hematocrit concentrations.[7,8] Hypoxia exposure also alters the redox balance through increased ROS production.[9-12] It also modifies the structure and functioning of skeletal muscle,[13-15] by inducing acid–base alterations that affect the affinity of hemoglobin for oxygen.[16] Moreover, prolonged exposure to hypobaric hypoxia also induces physical deterioration, which increases with altitude.[17] This is reflected in a marked decrease in body weight, due in part to a reduction in muscle mass.[18] Also, due to the increase in red cell mass, the apparent viscosity of blood can be increased with the possibility of a subsequent reduction in oxygen transport capacity. Other circulatory complications such as deep venous thrombosis cannot be ruled out in the presence of abnormally high hematocrit, although compensatory mechanisms have been described that can reduce the effective hematocrit after intense exercise.[19] Prolonged exposure to high altitude in nonnative residents or recent altitude populations at a biological scale often induces a complex syndrome: chronic mountain sickness.[9,20,21] To prevent the negative effects of chronic hypoxia exposure, several procedures that alternate short hypoxia exposure with immediate recovery in normoxia have been proposed.[3,22,23] These intermittent hypoxia-exposure protocols have revealed that such hypoxic stimulus can elicit an erythropoietic and induce other non-erythropoietic physiological adjustments that also affect aerobic capacity. Thus, these exposure protocols have been considered as efficient methods for high altitude acclimatization.[8,24-26.]

Low levels of O_2 produce an increase in reactive oxygen species (ROS).[11,27-29] These molecules are crucial for the maintenance of oxygen homeostasis. ROS are abundantly formed during hypoxia and can serve as signaling molecules that help to

maintain homeostasis.[9] During hypoxic stress, the delivery of oxygen to working muscles may damage the polyunsaturated fatty acids in cell membranous structures.[30] High oxygen radical levels might increase lipid peroxidation, which is usually measured by quantification of plasma malondialdehyde (MDA) levels. As lipid peroxidation occurs, the cell membrane reduces its fluidity, permeability and excitability and alters the function of membrane-bound enzymes. In cells with decreased membrane fluidity, the membrane fails to maintain tonic gradients.[31, 32] An elaborate defense system providing various degrees of protection to cells against free radicals has evolved in all species. Various components of this protective system are increased in tissues or organs following exposure to extreme physiological conditions such as exercise training or altitude acclimation.[30,33,34] Two intraerythrocytary enzymes: superoxide dismutase (SOD) and catalase (CAT) provide a primary defense line against ROS generated during hypoxic exposure.

Here we analyze parameters of hematological and oxidative stress after applying an intermittent hypobaric hypoxia (IHH) exposure protocol to laboratory animals. These parameters were measured during hypoxia exposure and recovery. The goals of the study were to identify the hematological changes and to discern whether an IHH protocol (using hypobaric hypoxia, 5000 m) modifies the antioxidant/pro-oxidant balance in laboratory rats. This article completes a series of studies performed in our laboratory concerning peripheral oxygen delivery to muscle tissue and blood rheology after IHH.[15,35,36]

Materials and Methods

Animals

A total of 67 male Sprague-Dawley rats aged 6 weeks at the beginning of the experiment were randomly divided into four groups. The first experimental group of 18 rats (named H, for hypoxic), were submitted to a program of IHH (described in detail later) and blood was drawn at the end of this program. The second experimental

group of 13 rats (named group P20, for post hypoxia 20 days), were simultaneously submitted to the same program, but blood samples were obtained 20 days after the end of the protocol. The third experimental group of 14 rats (named group P40, for post hypoxia 40 days), were also simultaneously submitted to the same exposure program, but samples were obtained 40 days after the end of the protocol. Finally, 22 rats were used as a triple control group (named group C, for control). Control animals were maintained in parallel under the same conditions as the three experimental groups. Samples from 9 of the controls (group C1) were obtained at the same time as those from H, samples from 9 of the controls (group C2) were obtained at the same time as those from P20, and finally, samples from the 4 remaining controls (C3) were taken at the same times as those from P40.

This study was a part of a general procedure for studying peripheral gas exchange and blood rheology, thus all rats were killed and also used to provide other organ and tissues samples.

The present study was authorized by the University of Barcelona's Ethical Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the *Departament de Medi Ambient i Habitatge* (file #1899) of Catalonia Government (*Generalitat de Catalunya*).

Hypobaric Chamber and intermittent hypobaric hypoxia (IHH) program

A hypobaric chamber was used to submit the rats to the IHH program. The total volume of the hypobaric chamber was approximately 450 L which allowed the housing of three rat cages. The chamber walls were made of transparent polymethyl methacrylate acrylic plastic, thus facilitating the observation of animals during protocol application. A negative pressure gradient was produced inside the hypobaric chamber by means of a rotational vacuum pump (TRIVAC D5E, Leybold, Köln, Germany) by regulating the air flow rate at the inlet with a micrometric valve. Inner pressure was controlled by two differential pressure sensors (ID 2000, Leybold, Köln, Germany) connected to a vacuum controller (Combivac IT23, Leybold, Köln, Germany) driving a diaphragm pressure regulator (MR16, Leybold, Köln, Germany).

Depending on the simulated altitude to be reached, a low-pressure set point was established in a control system. After the desired level was reached, the internal barometric pressure of the chamber was regulated and maintained by the vaccum control element.

After 2 weeks' quarantine, the animals were moved into the conditioned room containing the hypobaric chamber. An initial period of 5 days, free of disturbance, was established for complete habituation. The IHH program consisted of 4-hour sessions 5 days a week for 4 weeks and 2 additional days, thus completing 22 days of exposure to hypoxia (88h in total). The simulated altitude reached during each session was 5000 m (400 mmHg = 533 hPa), which is equivalent to 11% oxygen at normobaric hypoxia. The control group was subjected to the same procedure, although the cages were installed above the hypobaric chamber rather than inside it. Water reservoirs were removed during hypoxia sessions from all rat cages, including the control group, in order to avoid the liquid ejection caused by the inevitable gas expansion into the drinking bottles inside the chamber.

Blood sampling procedure

Blood samples were collected by cardiac puncture. Before extraction, animals were anesthetized with urethane (1.5 g/Kg BM). Sodium heparin was used as anticoagulant. A fraction of each blood sample was separated for immediate hematological analysis, which was always completed within 10 minutes of blood withdrawal. A second portion of the sample was centrifuged at 1000*g* for 10 min at 4°C to separate the cells from plasma for analysis. These two blood parts were then processed for storage at -80ºC. The erythrocyte pellet was frozen in non-heparinized 5mL tubes, and 1µL of a butylated hydroxytoluene (BHT) solution at a concentration of 1mmol/L in methanol was added for each mL of cell homogenate to prevent peroxidation amplification during sample storage. Plasma was also frozen in heparinized 5mL tubes.

Before analysis of the oxidative stress parameters, both RBC (for SOD and CAT analysis) and plasma for thiobarbituric acid reactive substances (TBARs) were defrosted at room temperature.

Hematology and Oxidative Stress Markers

The following hematological parameters were measured using an electronic cell counter (Celltac-alpha, Nihon Kohden Corp., Tokyo, Japan): red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Hc), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

The parameters of oxidative stress measured were TBARS, CAT and SOD. TBARS is widely used to quantify the lipid peroxidation caused by high ROS levels. The concentration of TBARS was measured in plasma as a product of lipid peroxidation following Yagi.[37] Results are expressed as nmols TBARS/mL plasma compared to a standard obtained by acid hydrolysis of tetraethoxypropane. This technique is based on the quantification of malondialdehyde (MDA) produced by lipoperoxidation that reacts with the thiobarbituric acid.

Catalase (CAT) contributes to the detoxification of H_2O_2 and its conversion to other less hazardous products.[38,39] Its activity was determined in the erythrocyte hemolyzate following Aebi,[40] by monitoring the H_2O_2 consumption at 240 nm. Results are expressed as *k*/gHb.

Superoxide dismutase (Cu,Zn-SOD) is one of the main detoxification enzymes in the cell.[30] Superoxide (O_2^-), which is the product on the first step of the Haber-Weiss reaction, is one of the most dangerous ROS in the cell.[39] This enzyme was assayed in the hemolysate by the inhibition of pyrogallol autoxidation, due to the concentration of SOD in the cell.[41] The product concentration was measured at 414 nm and is expressed as arbitrary units of SOD/gHb.

Statistics

Data for all the parameters are expressed as the sample mean ± standard error of the mean. Comparisons between the experimental and control groups were analyzed by one-way ANOVA test. Afterwards, a multiple comparison test using the Scheffé procedure was run to determine the differences between each pair of experimental and control conditions. Descriptive statistics and analyses of normality were made with SigmaStat software package, whereas one-way ANOVA and Scheffé tests were performed by the application of suitable subroutines from SPPS/PC+ package (SPSS Inc). Differences were considered statistically significant for $p<0.05$.

Results

Normal growth was not affected by IHH, as reflected in body weight evolution during the protocol. Moreover, no significant differences were found between C1, C2, or C3 for any of the parameters and, unless otherwise indicated, these three control groups were combined for all figures and tables and named Group C (see Figure 1).

Hematological parameters

Hematological parameters for the three species are given in Table 1. The one-way ANOVA and Scheffé tests showed significant differences between the four experimental groups in most of the hematological parameters. Hematological changes after exposure to hypobaric hypoxia were characterized by a significant increase in erythrocytes in the hypoxic group (H vs. C, P20, and P40; $p<0.001$), hematocrit (H vs. C, P20, and P40; $p<0.001$), and hemoglobin concentration (H vs. C, P20, and P40; $p<0.001$). In addition, significant differences in the hematological indexes MCV and MCH were found; they were slightly increased in the H group and later returned to basal levels (see Table 1).

Oxidative Stress parameters

Data of oxidative stress markers are plotted in Fig. 2 (panels A, B and C). No significant differences were observed in the three parameters studied (TBARS, CAT and SOD) when the four experimental groups where compared, although a slight

trend is observed in TBARS results (increased values for H compared to the other groups).

Discussion

Many studies have reported that chronic hypoxia induces deleterious effects on body mass.[42,43] A recent experimental study of chronic IHH in rats with an alternating schedule of 4x4 and 2x2 days of sea level and simulated 4,600m altitude demonstrated a severe body weight reduction and compromised survival rate.[44] However, possibly due to the lower duration of daily hypoxia exposure, in the present study we detected no negative effects on normal growth rate. This indicates that our hypoxia exposure regime is compatible with the standard living conditions of these experimental animals.

Hematological parameters

The hematological parameters and oxygen transport indexes for the four experimental groups were within the range of the results previously found in rats[44] and humans.[45,46] The most remarkable adaptations to the acclimatizing program are those observed in the hematological profile. The significant increases in red blood cells, hematocrit, and hemoglobin concentration values in group H are clearly associated with an enhancement of blood oxygen transport capacity. After the exposure period was over, the elevated values of RBC, Hb, and Hc tended to return to the lower, normal range. Consequently, it seems clear that intermittent exposure to hypoxia can also stimulate erythropoiesis in the rat to the same extent as chronic exposure.[47,48,49] Slight differences in hematimetric indexes may be due to the influence of a significantly higher percentage of reticulocytes in group H rats, which would cause a small increase in mean erythrocyte volume.[8,50]

Oxidative Stress parameters

Results obtained for oxidative stress parameters in the four experimental groups after HHI can be considered positive for all possible future applications of this kind of protocol. Some authors report that oxidative stress parameters were increased after

acute hypoxia exposure. [11,51,52] Although our results do not show any significant differences in the three parameters studied (TBARS, CAT, SOD) we observed a trend in TBARS levels in the H groups.

One possible explanation for a rise in lipid peroxidation after the cessation of hypoxia is the increased susceptibility of unsaturated fatty acids. This is because hypoxia, as intense exercise, markedly increase the concentration and the degree of unsaturation of non-esterified fatty acids in blood.[30,53] Many studies have provided evidence for substantial increases in plasma lipid-peroxidation levels after aerobic exercise,[38,54,55] although others have reported no change.[56,57] Moreover, although there are some reservations about the validity of the TBARS assay in detecting lipid peroxidation *in vivo*, since it is not specific to malondialdehyde and lipid peroxidation is not the only source of malondialdehyde, it is still widely used for this purpose.

SOD provides the first line of defense against oxidative stress by catalyzing the dismutation of superoxide to hydrogen peroxide. CAT protects the cells from the toxic effects of hydrogen peroxide by catalyzing its decomposition to water without free radical production. CAT and SOD did not show significant differences between experimental groups, which indicates that HHI did not affect the activities of these enzymes either. Several studies on different conditions of hypoxia have demonstrated that an abnormal increase in the production of ROS has an important role in the defense against oxidative stress. This increase would facilitate antioxidant gene expression and so reduce the subsequent cell damage.[58,59] The amount, intensity, and type of exercise might be related to the extent of lipid peroxidation, the previous training state being more important than the type of exercise.[57]

Our study has some limitations. We studied oxidative stress markers from a general descriptive approach, because abundant information has been accumulated on the oxidative stress generated in response to acute hypoxia. However, a more detailed study of redox status during hypoxia exposure and in the hours following each acclimatization session might be much more informative. Further studies are required to clarify this point.

In conclusion, after a program of intermittent hypobaric hypoxia in rats, blood and plasma oxidative stress markers offered no evidence of a significant imbalance in redox status. This finding argues in favor of the use of intermittent hypobaric hypoxia protocols in several well established applications—such as altitude pre-acclimation

and athletic performance improvement—and for other potential future purposes in certain pathological states.

Acknowledgements

The authors thank Robin Rycroft (Language Advisory Service –U.B.) for his help in editing the manuscript.

Figures and Tables Legends.

Figure 1: Rat body mass comparison between control animals (solid circles) and those submitted to the IHH program (open circles). The grey area indicates the IHH exposure period. Mean values ± SE are indicated.

Figure 2: Oxidative Stress markers for the four groups of experimental animals: C (black), H (white), P20 (light grey) and P40 (dark grey). Mean values and standard errors are indicated. Panels A: TBARS, B: CAT, C: SOD.

Table 1: Hematological parameters for the different groups of experimental animals. Mean values and standard errors are indicated. Significant differences between groups are indicated by the following code: (a) H vs. all other groups, (b) C vs. H, (c) H vs. C and P40, (d) H vs. P40. Levels of significance are indicated as a ($p<0.001$), b ($p<0.01$), or c ($p<0.05$).

Figure 1

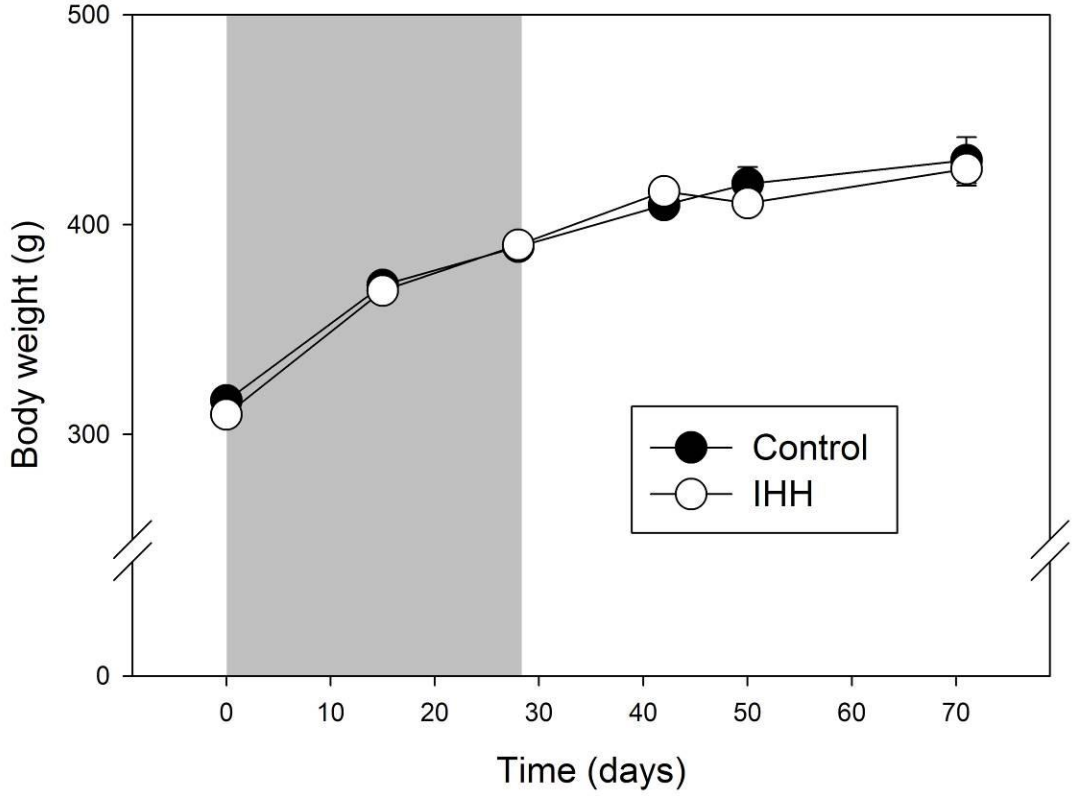
500
400
300
0
Body weight (g)
0
10
20
30
40
50
60
70
Time (days)
Control
IHH

Figure 2

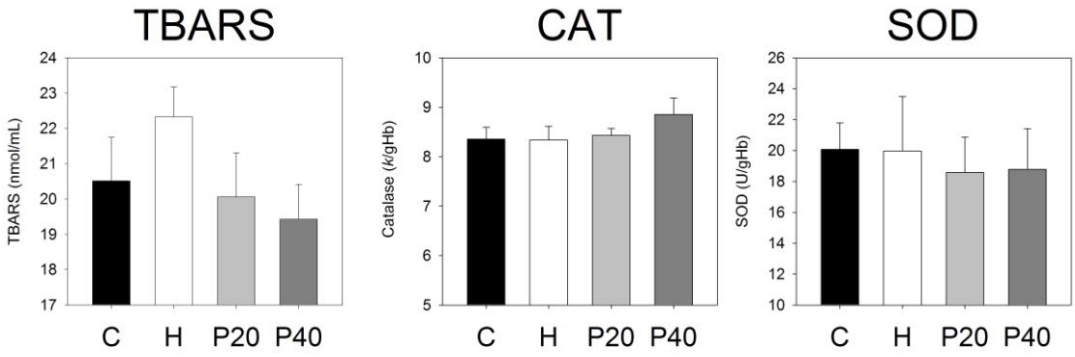
TBARS
TBARS (nmol/mL)
24
23
22
21
20
19
18
17
C
H
P20
P40
CAT
Catalase (k/gHb)
10
9
8
7
6
5
C
H
P20
P40
SOD
SOD (U/gHb)
26
24
22
20
18
16
14
12
10
C
H
P20
P40

Table 1

Table 1

		C (n=17)	H (n=10)	P20 (n=13)	P40 (n=14)
RBC (cells·10^6.µL^{-1})	a	8,81±0.10	9,98±0.20***	8,91±0.16	8,60±0.14
Hb (g·dL^{-1})	a	15,66±0.19	18,75±0.37***	16,26±0.28	15,56±0.25
Hc (%)	a	47,03±0.66	55,22±1.02***	48,09±0.79	47,03±0.77
MCV (µm^3)	b	53,33±0.41	55,25±0.37*	53,86±0.50	54,72±0.44
MCH (pg)	c	17,77±0.12	18,76±0.15**	18,03±0.85	17,92±0.20
MCHC (%)		33,34±0.17	33,96±0.16	33,90±0.21	33,09±0.23
WBC (cells·10^3.µL^{-1})	d	9,85±0.83	13,50±1.47*	12,61±0.65	9,40±0.78

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