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Expression of Lactate Dehydrogenase A and B Genes in Different Tissues of Rats Adapted to Chronic Hypobaric Hypoxia

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Abstract Lactate dehydrogenase (LDH) is a tetramer made up of two different subunits A and B. In cellular models, severe hypoxia increases LDH A gene expression whereas LDH B gene does not exhibit any regulation. The aim of our work was to characterise LDH expression in different tissues of rats bred at high altitude. For this purpose, we chose a Sprague–Dawley rat strain adapted to chronic hypoxia in La Paz (3700 m), Bolivia. Two normoxic control groups were bred at low altitude in Clermont-Ferrand (350 m), France, one group was ad libitum with free access to food and water as was the hypoxic one, and the second normoxic group was nourished with the food intakes measured for the animals from La Paz. We measured total LDH specific activity, isoform distribution and LDH A and B mRNA amounts in three skeletal muscles (soleus, extensor digitorum longus (EDL), plantaris), heart and brain. Our study demonstrates that, unlike what has been shown in cellular models under severe hypoxia, LDH A gene is not systematically up-regulated in tissues of rats living at high altitude. Furthermore, chronic hypoxia limits LDH B gene transcription or its mRNA stability in both soleus and EDL. These regulations occur at various molecular levels like gene transcription, mRNA stabilisation or translation and protein stability, depending on the tissue studied, and are partly attributed to caloric restriction provoked by high altitude. These data provide insight into LDH gene expression underlying the diverse and complex tissue-specific response to chronic hypoxia. J. Cell. Biochem. 89: 67–79, 2003.

Key words: high altitude; anaerobic metabolism; caloric restriction; tissue-specific

In acute hypoxic conditions, data clearly demonstrate an overexpression of genes encoding proteins involved in homeostatic response to hypoxia, i.e., erythropoesis, angiogenesis, anaerobic metabolism [Semenza, 2000]. These gene overexpressions are essentially due to the hypoxic stabilisation of the hypoxia inducible factor 1 (HIF-1) [Wang and Semenza, 1993]. Among these genes, some encoding glycolytic isozymes are up-regulated at a transcriptional level, as enolase 1, aldolase A, glyceraldehyde-3-phosphate dehydrogenase or lactate dehydrogenase A [Firth et al., 1994, 1995; Ebert et al., 1996; Semenza et al., 1996]. It seems logical that in low oxygen conditions, glycolysis rate should be enhanced in order to compensate oxidative energetic metabolism decay. Since in the absence of oxygen, glycolysis produces only two molecules of ATP per glucose oxidised, thus glucose transport has to be improved to maintain a constant energy supply [Loike et al., 1992] and pyruvate to lactate transformation must be efficient in order to keep a high $NAD^+/NADH + H^+$ ratio and therefore to allow continued glycolytic flux. In cells, this pyruvate reduction is catalysed by lactate dehydrogenase (LDH; E.C. 1.1.1.27).

LDH is a tetrameric protein made up of two different types of 35 kDa subunits, A and B, encoded by two different genes. The LDH A gene encodes the former called M-subunit which exhibits a higher K_m for pyruvate and a higher V_{max} for pyruvate reduction than the LDH B subunit [Markert et al., 1975; Ji et al., 1986].

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Besides HIF-1 mediated up-regulation of LDH A gene during hypoxia [Marti et al., 1994], it has been shown that in rat retina, LDH B gene expression was down-regulated by low oxygen availability [Buono and Lang, 1999]. In that particular case, HIF-1 involvement has not been evoked. Moreover, it seems that tissue-specific gene regulations have to be taken into account. Indeed, LDH protein expression could depend on the type of tissue considered [McClelland and Brooks, 2002] but as well on the oxygen supply level.

Hypoxic up-regulation of LDH A gene in cellular models exposed to severe O_2 deprivation is a well-described fact [Firth et al., 1994, 1995]. What is the situation in different tissues of animals adapted to chronic low oxygen conditions? Is there a systematic increase of LDH A expression? Is there a tissue-specific regulation of both LDH isoforms making cellular models too simplistic? To answer these questions, we measured total LDH specific activity, isoform distribution, and LDH A and B mRNA relative amounts in three different skeletal muscles, heart and brain of rats adapted to chronic hypoxia and compared these data to the same parameters measured in animals bred in normoxic conditions.

Our model is a Sprague–Dawley rat strain adapted to chronic hypoxia in La Paz, Bolivia. As a reference, a normoxic control group of this rat strain was bred at low altitude in Clermont-Ferrand, France. These two groups of rats were either allowed free access to food and water (ad libitum). During the study, we observed that the body weight growth and food intake curves of the hypoxic animals were particularly impaired. This observation has already been made with humans and animals maintained at high altitude [Tschop and Morrison, 2001]. Then, another control group of low altitude-living Sprague–Dawley rats was used. These normoxic pair-fed rats were nourished with the food intakes measured for the hypoxic group in La Paz in order to discriminate caloric restriction effects from hypoxia consequences.

MATERIALS AND METHODS

Animals

Since 1992, a Sprague–Dawley rat strain has been adapted to hypobaric chronical hypoxia in La Paz (Bolivia, 3700 m, PB = 490 mmHg). Ten young male rats (3 weeks of age) were assigned to a hypoxic group (H). In Clermont-Ferrand (France, 350 m, PB = 750 mmHg), a group of ten Sprague–Dawley male rats (IFFA Credo, France) was received at 3 weeks of age (40–45 g) and housed in a temperature-controlled room ($21 \pm 1^{\circ}C$) with a dark light cycle of 12:12 h. These animals formed the normoxic ad libitum group (N_{AL}) with free access to both food and water. According to the low food intake measurements observed in La Paz with the hypoxic group of animals, a second group of 10 Sprague–Dawley male rats was assigned to a normoxic pair-fed group (N_{PF}) with free access to water but with food restriction according to the measurements made in Bolivia.

Analysis of food composition (sugars, lipids, proteins, vitamins and minerals) did not show any significant difference between the food used in La Paz and the one used in Clermont-Ferrand. During their growth, the animals from the three groups were weighted once a week and their food intakes were estimated daily.

Tissue Sampling

At the age of 90 days the animals were sacrificed according to the local Animal Welfare Committee recommendations. The animals were killed by cerebral dislocation followed by decapitation. The selected tissues, soleus, plantaris, extensor digitorum longus (EDL), brain, and heart, were removed, weighted, sampled in sterile cryotubes, then frozen in liquid nitrogen and kept at $-80^{\circ}C$ prior to use.

RNA Preparations and Reverse Transcriptions

Tissues were ground in liquid nitrogen, and then the frozen powder was incubated in TRI InstaPure reagent (Eurogentech, France). The total RNA was extracted according to the manufacturer's recommendations. The amounts of total RNA were measured by estimating their absorbance at 260 nm. Integrity and good quality of the purified RNAs were checked using formaldehyde-agarose gels containing ethidium bromide.

Four micrograms of total RNA was reverse-transcribed at $42^{\circ}C$ for 50 min in a volume of 100 μl with 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 800 μM dNTPs, 10 U RNasin ribonuclease inhibitor, 0.5 μg of oligod$(T)_{18}$ primer and 200 U of M-MLV reverse transcriptase RNase H minus (Promega, France). The enzyme was inactivated at $94^{\circ}C$ for 5 min.

mRNA Quantification

Two different methods were used giving rather identical results: the first one was a semi-quantitative RT-PCR assay used to assess LDH A and B transcript levels in tissues. This method allows the estimation of the relative expression of these RNAs (target genes) and rat ribosomal L32 protein mRNA, a house-keeping gene used as internal standard in order to normalise these amplifications to the cellular transcription yield and to the RT amount present into each PCR tubes [Thellin et al., 1999]. In a PCR tube, in a final volume of 25 μl, 0.5 μl of the cDNA (RT) was mixed with 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 μM dNTPs, 1.5 mM $MgCl_2$, 12.5 pmoles of sense and antisense primers and 0.5 U of Taq polymerase (Promega). The PCR was carried out as follows using a Hybaid MBS0.5 gradient thermo-cycler: 94°C (1 min), annealing temperature (1 min), 72°C (2 min) for *n* cycles and 72°C (8 min) for the last cycle. The PCR products were analysed on 2% agarose gel containing ethidium bromide (final concentration 0.5 μg/ml). Gel DNA picture analyses were performed using a Kodak DC120 software. Results were expressed as the ratio of target gene over L32 expression (ratio expression rates: RER).

The second method was a competitive RT-PCR technique. We used internal controls of amplification (ICA) as competitors, constructed by PCR, and being amplified with the primers used to amplify the target cDNA (LDH A or LDH B). A constant amount of reverse transcription mixture was added to PCR tubes in which various numbers of copies of the corresponding competitor DNA were added (Fig. 1). PCR reactions were performed in conditions identical to those described in the above paragraph. Here again the number of cDNA copies was normalised using the rat ribosomal L32 protein as a housekeeping gene. With this procedure, the LDH A and B copy number ranges were comparable to those previously described with a slot blot technique [Marieze et al., 1994]. Primer sequences and RT-PCR characteristics are detailed in Table I.

LDH Specific Activities

Tissue samples (50–100 mg) were homogenised for 30 s with an Ultra Turrax homogenizer at 4°C in 19 volumes (1 volume/100 mg of tissue) of extraction buffer (50 mM Tris-acetate, pH 7.5, 250 mM sucrose, 1 mM EDTA) containing an anti-protease mixture (Roche, France). The homogenates were then centrifuged at 10,000*g* for 5 min. Supernatants were diluted in the extraction buffer and assayed for LDH activity at 31 ± 1°C by a spectrophotometric method using an LDH-P UV system (Roche, France). Measured LDH activities were reported to the total amount of proteins in the supernatants determined using a bicinchoninic acid kit (Pierce, France) with bovine serum albumin as

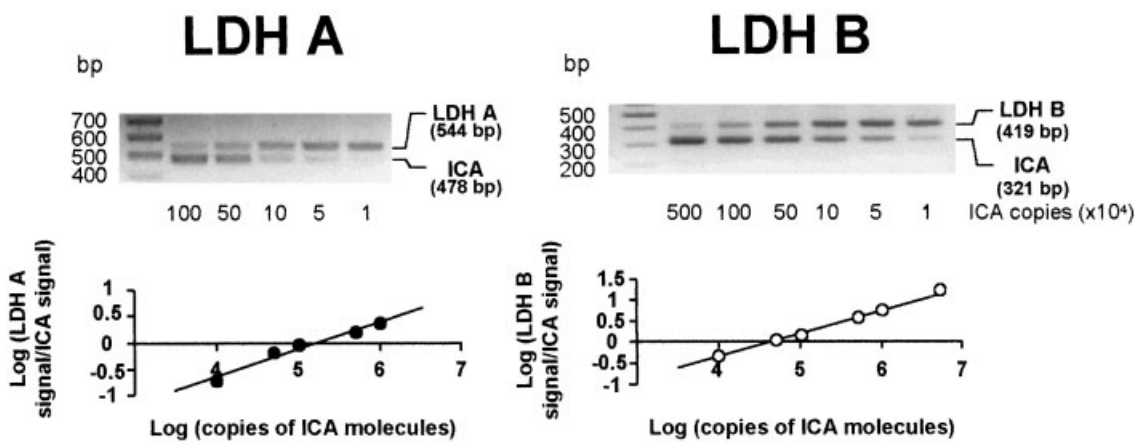

Fig. 1. Analysis of pictures of competitive RT-PCR, loaded onto 2% TAE-agarose gel containing ethidium bromide. Various copy numbers of internal controls of amplification (ICA) were added to each PCR reaction tube as indicated under each lane. The left lane on the gels represents DNA markers. The logarithm of template/ICA ratio versus logarithm of number of ICA molecules present in each PCR tube is represented. When the number of ICA copies is equal to the number of template, the straight line crosses the abscissa axis. The obtained values are then normalised using the ribosomal protein L32 signal observed in identical RT-PCR conditions.

TABLE I. Oligonucleotide Sequences, cDNA Size, Annealing Temperature and Number of PCR Cycles Giving a Linear Amplification

	Sequence	cDNA size	Annealing T (°C)	Linearity (cycle)
Lactate dehydrogenase A		(F/R)		
F sense	ggTgACACTgACTCCTgACgA	544 bp		
R antisense	gTTggTTCCATCATCCATATgCAgATC	(F-F′/R)	69 (F/R)	27
F-F′ sense ICA	**ggTgACACTgACTCCTgACgA**CTTCC-CAgTgTCCTAgCACT	478 bp		
Lactate dehydrogenase B		(F/R)		
F sense	TgACCTCATCgAATCCATgC	419 bp		
R antisense	TgCAATTgCTAAACTTTATTTgTTCAg	(F-F′/R)	60 (F/R)	27
F-F′ sense ICA	**TgACCTCATCgAATCCATgC**gCTCggg-gACTgACCAgCg	321 bp		
Ribosomal L32 protein		(F/R)		
F sense	gTgAAgCCCAAgATCgTCAA	257 bp		
R antisense	TtgTTgCACATCAgCAgCAC		60	27

LDH A and B ICA molecules were both obtained using RT-PCR and an F-F′/R combination of primers. The ICA RT-PCR products were then loaded on an agarose gel, DNA bands were cut off from the gel and were purified using a PCR Product Purification Kit (Promega). The amount of the two ICA molecules (LDH A and B) was then determined using a 260 nm spectrophotometric method. The common parts between F-F′ and F primers are shown in bold.

a standard. LDH isoform determinations were performed by native agarose gel electrophoresis using a Hydragel Iso-LDH kit (Sebia, France). The electrophoregrams were automatically scanned using a Performance scanner (Sebia, France). All tissue treatments, activity measurements and isoform determinations for one tissue were performed the same day.

Statistics

Results are presented as means ± SEM. Group means were compared using a simple one-way ANOVA. When appropriate, differences between groups were tested with a Student's *t*-test for unequal variances. Using Bonferroni's values for simultaneous test, statistical significance was set at $P < 0.017$ (*), $P < 0.0034$ (**), $P < 0.00034$ (***).

RESULTS

Animal Observations

We first carried out both H and N_{AL} groups in order to measure and compare their daily food intakes. High altitude has already been described as having an anorexic effect on humans and animals [Tschop and Morrison, 2001]. As expected, the rats from La Paz had lower food intakes than those bred in Clermont-Ferrand (Fig. 2A). In order to estimate this under-nutrition effect, we decided to study a second normoxic group of male rats, with animals having restrained food access (N_{PF}). In these conditions, the body weight curve of these normoxic animals after 8 weeks of growth after weaning is entirely superimposable to the one obtained with the H group. Animals from

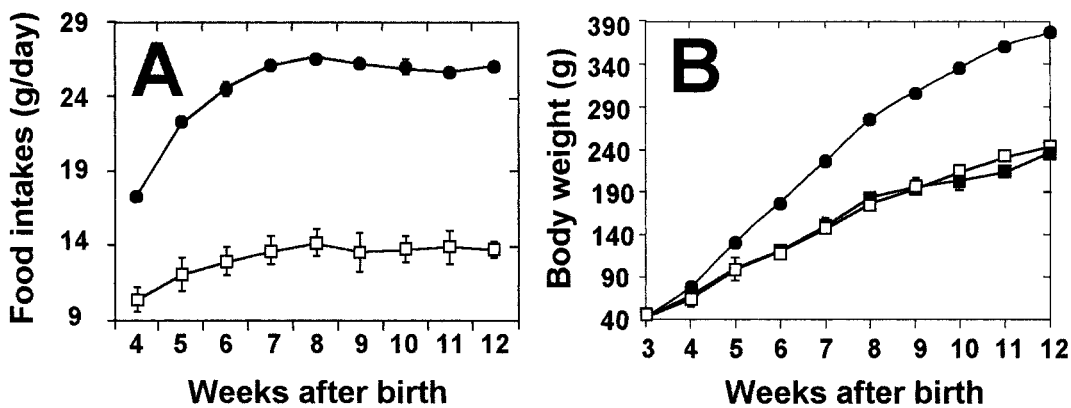

Fig. 2. Food intake and body weight growth measurements. **Panel A**: Time course evolution of the daily food intakes (n = 6) of the N_{AL} (filled circles) and H animals (empty squares). The food intakes measured for the hypoxic group of rats have been used to perform the normoxic pair-fed group (N_{PF}: filled squares). **Panel B**: Body weight growth curves of the animals from the three groups (n = 7). Results are presented as means ± SEM.

the N_{AL} group exhibit higher body weights (+35%) than those from H and N_{PF} groups (Fig. 2B).

We weighted the three different skeletal muscles removed from the animals of the three groups. There is no significant change in the mass of these tissues between the three groups of rats (Table II).

For all groups, we measured LDH specific activities, percentage of the five different LDH isoforms and then mRNA amounts of both LDH genes in various tissues. We chose soleus a low muscle type, EDL a postural fast muscle and plantaris, which is also a fast muscle but which is more involved in animal moving. We also studied heart, which is an oxidative tissue previously described to be an organ very sensitive to altitude at least at a morphological level [Pissarek et al., 1997; Rumsey et al., 1999]. Lastly, we selected brain as an organ in which changes are generally minimal.

LDH Expression in soleus

In soleus, no significant change in LDH specific activity can be measured between the three different groups of animals (Fig. 3A) despite an increase in the percentage of the LDH A subunit in rats submitted to chronic hypoxia compared to rats from the two other groups (Fig. 3B). The distribution of the different LDH isozymes, consisting of combinations of the two LDH A and LDH B subunits, were estimated using gel electrophoresis. LDH being a tetramer, there are five LDH isozymes: LDH1 (B_4), LDH2 (A_1B_3), LDH3 (A_2B_2), LDH4 (A_3B_1) and LDH5 (A_4). soleus from rats submitted to chronic hypoxia exhibits lower proportions of LDH1 and 2 isoforms but higher amounts of LDH3 and 4 than N_{PF} or N_{AL} animals (Table III). At a transcriptional level, LDH A gene expression seems to be constant even in hypoxic conditions even though a slight increase of this gene expression can be observed (Fig. 3C). Conversely, LDH B mRNA amounts are significantly decreased in soleus of rats exposed to chronic hypoxia (Fig. 3D). LDH A mRNA relative percentage (Fig. 3E) is then notably augmented in hypoxia and apparently no effect of food intake restriction can be noted.

LDH Expression in EDL

In this fast postural muscle, one can observe that food restriction decreases total LDH specific activity. However, high altitude counterbalances in part this effect (Fig. 4A). The percentage of LDH A subunit in rats from the N_{PF} group is higher than either H or N_{AL} groups of rats (Fig. 4B). If N_{PF} animals have the highest LDH5 content (Table III), EDL from animals submitted to chronic hypoxia display a global increase of the isoforms containing B subunits. At an mRNA level, the amounts of LDH A transcripts are higher in EDL of animals from the N_{PF} group compared to that observed in animals from the N_{AL} group. In EDL of rats raised in chronic hypoxia, there is an increase of LDH A mRNA quantity (60%) compared to EDL of animals from the N_{AL} group (Fig. 4C). LDH B mRNA amounts in EDL are reduced by food intake decrease and even more when chronic hypoxia is present (Fig. 4D). LDH A mRNA relative percentage (Fig. 4E) is then notably increased in the H and N_{PF} groups compared to the N_{AL} one.

LDH Expression in plantaris

In this glycolytic muscle, LDH specific activity is slightly decreased in the N_{PF} group compared to the N_{AL} one. In the H group, an LDH specific activity decrease is measured although it is not statistically significant (Fig. 5A). In spite of variations in total LDH activity no significant change can be monitored in the percentage of LDH A subunit between the three different groups of rats (Fig. 5B). At a transcriptional level, LDH A and B gene expression or mRNA stability remains unchanged regardless of the conditions the rats were exposed to (Fig. 5C–E).

LDH Expression in Heart

We first studied separately left and right hearts since it is well known that hypoxia

TABLE II. Muscle Weights Measured in the Three Different Groups of Animals

	N_{AL} (n = 20)	N_{PF} (n = 5)	H (n = 10)
Soleus weight (mg)	101.5 ± 12.3	98.2 ± 11.3	105.0 ± 14.0
Plantaris weight (mg)	260.5 ± 36.3	267.0 ± 34.1	256.4 ± 20.0
EDL weight (mg)	131.6 ± 18.5	121.8 ± 16.0	117.2 ± 8.8

TABLE III. LDH Isozyme Composition (% Total) in Soluble Fractions of Skeletal Muscles, Left and Right Heart and Brain of Rats From the Three Different Groups

		soleus	plantaris	EDL	Left heart	Right heart	Brain
LDH5 A_4	H	11.02 ± 0.93	51.35 ± 5.44	47.15 ± 3.35	7.40 ± 0.38	8.62 ± 1.00	10.83 ± 0.96
	N_{PF}	11.50 ± 1.01	64.52 ± 5.56	68.33 ± 1.40	5.48 ± 1.32	6.28 ± 1.43	10.30 ± 0.91
	N_{AL}	12.30 ± 4.24	57.98 ± 3.17	58.60 ± 3.14	6.60 ± 0.80	13.67 ± 3.53	15.73 ± 3.99
LDH4 A_3B_1	H	28.20 ± 1.06	27.28 ± 2.96	31.70 ± 1.67	13.18 ± 0.35	11.72 ± 1.38	20.88 ± 0.49
	N_{PF}	23.37 ± 0.66	17.13 ± 1.33	18.13 ± 0.98	8.88 ± 0.76	10.57 ± 0.89	19.70 ± 1.27
	N_{AL}	18.50 ± 1.72	23.00 ± 1.79	21.90 ± 1.39	11.70 ± 0.10	12.53 ± 2.58	25.83 ± 3.27
LDH3 A_2B_2	H	22.00 ± 0.57	9.57 ± 1.37	10.25 ± 0.93	28.45 ± 0.85	28.16 ± 1.15	27.53 ± 1.19
	N_{PF}	13.00 ± 0.68	8.12 ± 1.65	5.97 ± 0.23	24.40 ± 0.78	27.45 ± 3.08	25.53 ± 1.34
	N_{AL}	15.90 ± 2.10	8.80 ± 1.03	8.98 ± 0.76	26.80 ± 0.30	25.33 ± 0.87	18.97 ± 0.27
LDH2 A_1B_3	H	21.62 ± 0.39	6.27 ± 0.91	6.02 ± 0.69	28.90 ± 0.75	29.52 ± 0.32	21.35 ± 0.91
	N_{PF}	22.97 ± 0.57	5.05 ± 1.25	3.90 ± 0.23	31.03 ± 0.35	28.60 ± 1.69	22.50 ± 0.78
	N_{AL}	29.12 ± 3.00	6.54 ± 0.75	5.88 ± 0.60	26.75 ± 1.05	26.13 ± 0.85	17.47 ± 2.28
LDH1 B_4	H	17.17 ± 0.43	5.32 ± 0.51	4.88 ± 0.22	22.08 ± 0.49	21.98 ± 1.12	19.43 ± 0.80
	N_{PF}	29.17 ± 1.27	5.18 ± 1.67	3.67 ± 0.38	30.20 ± 1.01	27.10 ± 1.38	21.97 ± 3.16
	N_{AL}	24.18 ± 1.84	3.68 ± 0.61	4.62 ± 0.63	28.15 ± 0.65	22.33 ± 0.98	22.00 ± 5.76

In all experiments, tissues of three to seven different animals were used.

induces hypertrophy of right ventricle [Pissarek et al., 1997; Rumsey et al., 1999]. For both heart ventricles, food restriction greatly reduces total LDH specific activity (Fig. 6A,C). However, high altitude totally corrects this effect as previously observed in EDL. The percentage of the LDH A subunit in left heart of rats belonging to the N_{PF} group is lower than the one measured in the H group of rats but is unchanged if it is compared to the one observed in the N_{AL} group (Fig. 6B).

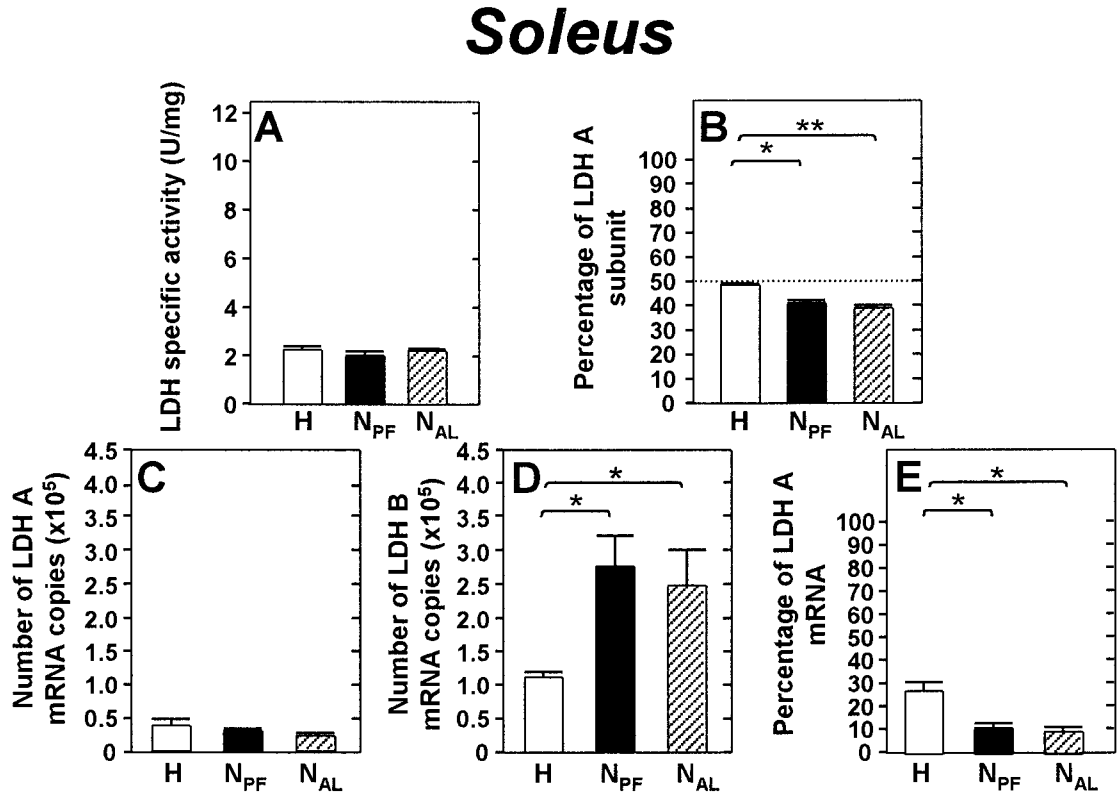

Fig. 3. LDH changes in soleus of male rats raised in normoxia and chronic hypoxia. **Panel A**: LDH specific activity measurements. Lactate dehydrogenase activities were determined using a spectrophotometric method at 31 ± 1°C and reported to the mgs of total soluble proteins (H: n = 10; N_{PF}: n = 8; N_{AL}: n = 6). **Panel B**: Percentage of LDH A subunits determined by non-denaturing agarose gel electrophoresis (H: n = 10; PF: n = 8; AL: n = 6). **Panel C** & **D**: Number of LDH A and B (respectively) mRNA copies for 20 ng of total RNA determined by competitive RT-PCR and normalised using ribosomal protein L32 signal. For all these experiments, the number of animals was four to five. **Panel E**: Percentage of LDH A mRNA copies. Results are presented as means ± SEM. *Significantly different $P < 0.017$, **$P < 0.0034$, ***$P < 0.00034$.

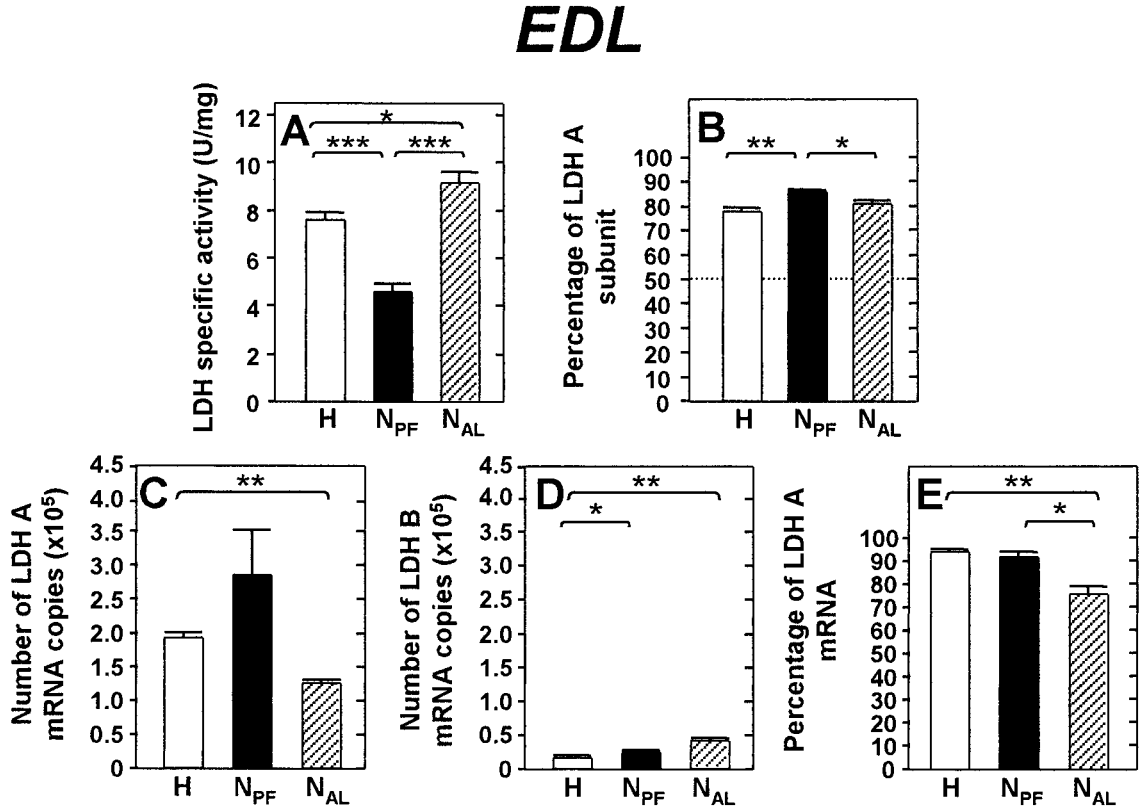

Fig. 4. LDH changes in EDL of male rats raised in normoxia and chronic hypoxia. **Panel A**: LDH specific activity measurements. **Panel B**: Percentage of LDH A subunits determined by non-denaturing agarose gel electrophoresis. **Panel C** & **D**: Number of LDH A and B (respectively) mRNA copies for 20 ng of total RNA determined by competitive RT-PCR and normalised using ribosomal protein L32 signal. **Panel E**: Percentage of LDH A mRNA copies. Results are presented as means ± SEM. *Significantly different $P < 0.017$, **$P < 0.0034$, ***$P < 0.00034$.

In right heart, LDH A subunit percentage is slightly decreased in both H and N_{PF} groups of rats compared to the N_{AL} one (Fig. 6D) indicating that food restriction could be implicated in this down-regulation. If we look carefully at the isoform distribution data, we can see that hypoxia seems to decrease the relative amounts of the LDH1 isoform in left heart compared to both normoxic groups, whereas the LDH4 isoform relative amount seems to augment. In right heart, chronic hypoxia decreases LDH1 isoform amount compared to the value found with the N_{PF} group. Nevertheless, the magnitude of these changes is not very important.

As the global changes in LDH activity seem very comparable between left and right hearts, we used right heart to measure LDH mRNA amount variations. Food intake reduction augments LDH A mRNA amounts in heart as does hypoxia (Fig. 6E). LDH B RER does not seem to be changed by rat growth conditions (Fig. 6F). If we analyse LDH A/LDH B mRNA ratios then we can note that food intake, alone or associated with hypoxia, augments LDH A mRNA proportion in rat heart (Fig. 6G).

LDH Expression in Brain

The whole body tries to maintain a constant brain metabolism whatever be the living conditions. In spite of that, LDH specific activity is significantly decreased in chronic hypoxia group compared to that observed in both N_{PF} and N_{AL} groups (Fig. 7A). Despite variations in total LDH activity of hypoxic rats no significant change can be monitored in the percentage of LDH A subunit between H and N_{AL} groups (Fig. 7B). However there is a small but significant decrease in the percentage of LDH A subunit between N_{AL} and N_{PF} groups, indicating that food restriction could be involved in that decline. If we closely examine LDH isoform distribution, we can note that a small increase of the LDH3 isoform in both hypoxic and pair-fed groups compared to the N_{AL} one is measured but no other significant change can be monitored (Table III).

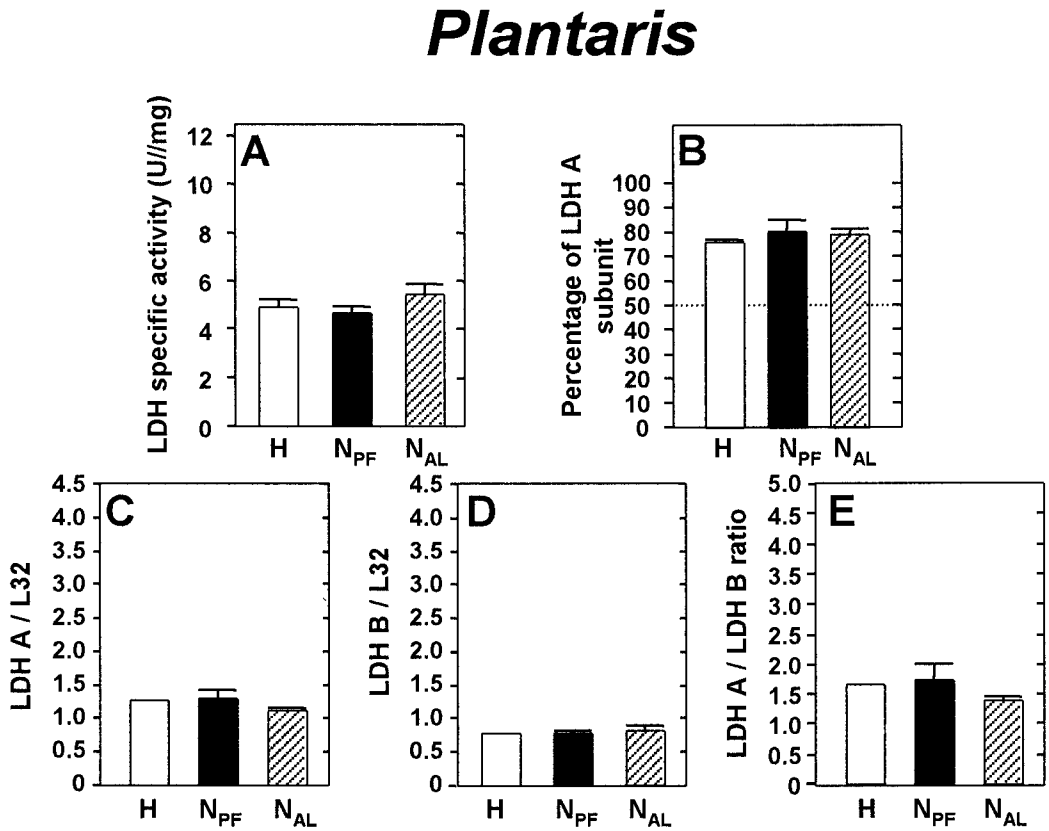

Fig. 5. LDH changes in plantaris of male rats raised in normoxia and chronic hypoxia. **Panel A**: LDH specific activity measurements. **Panel B**: Percentage of LDH A subunits determined by non-denaturing agarose gel electrophoresis. **Panel C** & **D**: LDH A and B (respectively) RER determined by semi-quantitative RT-PCR. For all these experiments, the number of animals used was four to five. **Panel E**: LDH A mRNA/LDH B mRNA ratio. Results are presented as means ± SEM. *Significantly different $P < 0.017$, **$P < 0.0034$, ***$P < 0.00034$.

At a transcriptional level, LDH A RER is significantly decreased in brain of N_{PF} rats compared to both N_{AL} and H rats (Fig. 7C). However, LDH B gene expression or mRNA stability in brain remains remarkably unaffected in the three groups of rats we studied (Fig. 7D). LDH A/LDH B RER ratios do not differ significantly between the three groups of animals (Fig. 7E).

DISCUSSION

Lactate dehydrogenase is the enzyme catalysing the final step of the anaerobic metabolic pathway, glycolysis. This tetrameric protein is made up of two different subunits A and B encoded by two different genes. If this protein has been considered as a strict cytosolic marker, a mitochondrial location is evoked [Brandt et al., 1987; Szczesna-Kaczmarek, 1990] to support the hypothesis of an intracellular lactate shuttle model [Brooks, 1998; Brooks et al., 1999; McClelland and Brooks, 2002], although conflicting reports have been recently published [Rasmussen et al., 2002; Sahlin et al., 2002].

In living organisms, low oxygen amounts provoke a large number of molecular adaptations mainly via the activation of a transcription factor named HIF-1 [Semenza, 2001]. Lactate dehydrogenase A gene is among the genes controlled by HIF-1 [Firth et al., 1994] whereas lactate dehydrogenase B gene does not seem to be regulated by low oxygen availability [Ebert et al., 1996].

The aim of our work was to explore regulation of LDH expression in different tissues of rats adapted to chronic hypoxia. A non-native Sprague–Dawley rat strain adapted to altitude and low oxygen availability (La Paz, 3700 m, PB = 490 mmHg) has been used. These animals exhibit a lower body weight than those bred in normoxic and normobaric conditions after 12 weeks of growth. Their daily food intakes were lower than the animals from the normoxic

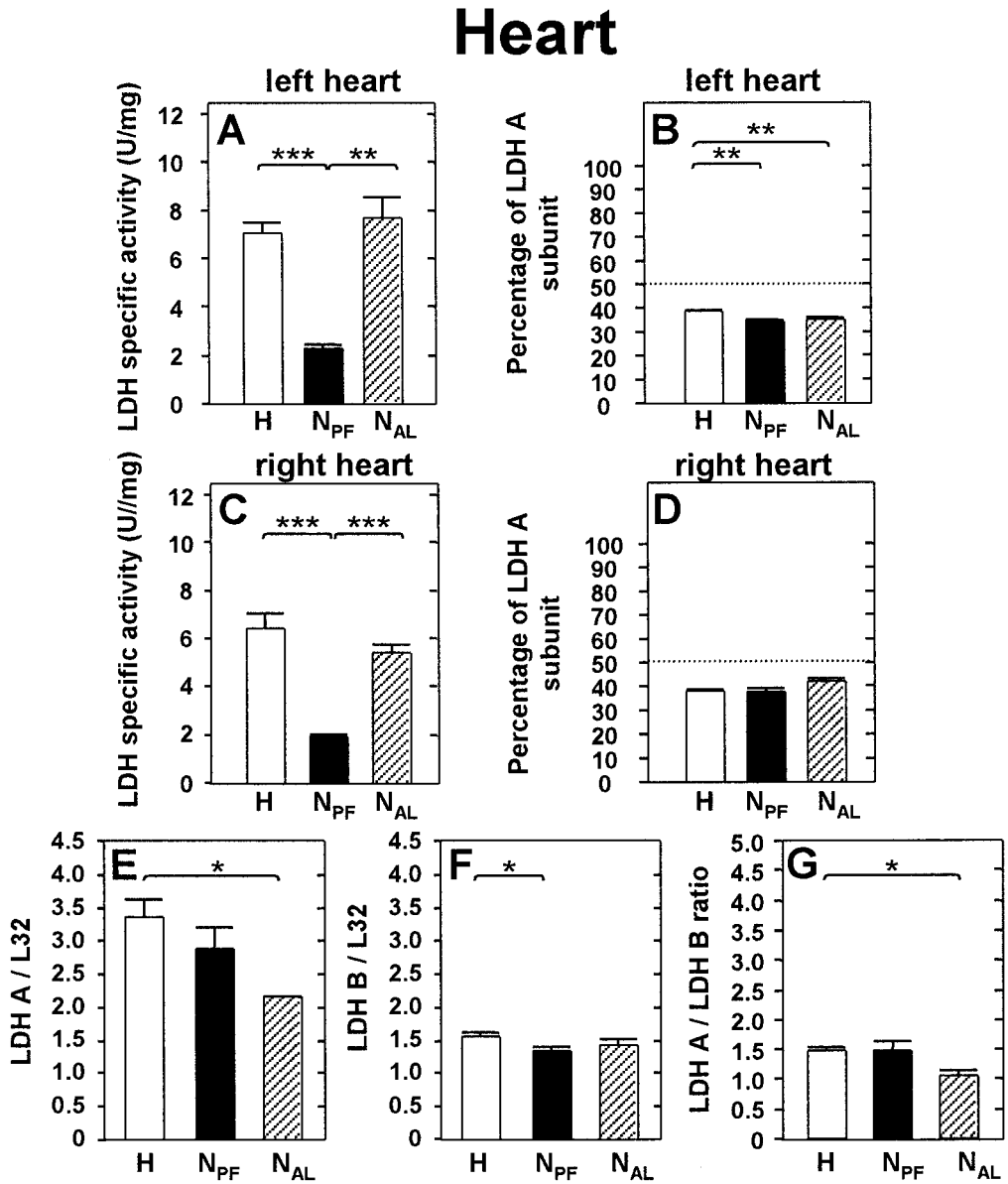

Fig. 6. LDH changes in heart of male rats raised in normoxia and chronic hypoxia. **Panel A** & **C**: LDH specific activity measurements in left and right heart, respectively. **Panel B** & **D**: Percentage of LDH A subunits determined by non-denaturing agarose gel electrophoresis in left and right heart. **Panel E** & **F**: LDH A and B (respectively) RER determined by semi-quantitative RT-PCR. **Panel G**: LDH A/LDH B mRNA ratio. Results are presented as means ± SEM. *Significantly different $P < 0.017$, **$P < 0.0034$, ***$P < 0.00034$.

ad libitum group. It is well known that high altitude has an anorexic effect on mammals [Raff et al., 1999]. Consequently, we used these food intake values to perform a normoxic pair-fed group (N_{PF}) as control. The animals from this third group exhibit a similar growth curve to the rats bred in chronic hypoxia (Fig. 2A). Skeletal muscle weights of animals from the three groups are not significantly different (Table II). This observation indicates that the overweight measured in the N_{AL} group is probably due to a superior mass of fat and that low oxygen supply is hardly involved in the lower body weights measured in the H group.

For each group of rats, total LDH specific activity, LDH isoform percentages and LDH A and B mRNA amounts were measured in three different skeletal muscles (soleus, EDL, plantaris), heart and brain.

In soleus muscles, chronic hypoxia does not change LDH specific activity. LDH A subunit percentage increases in muscles of hypoxic ani-

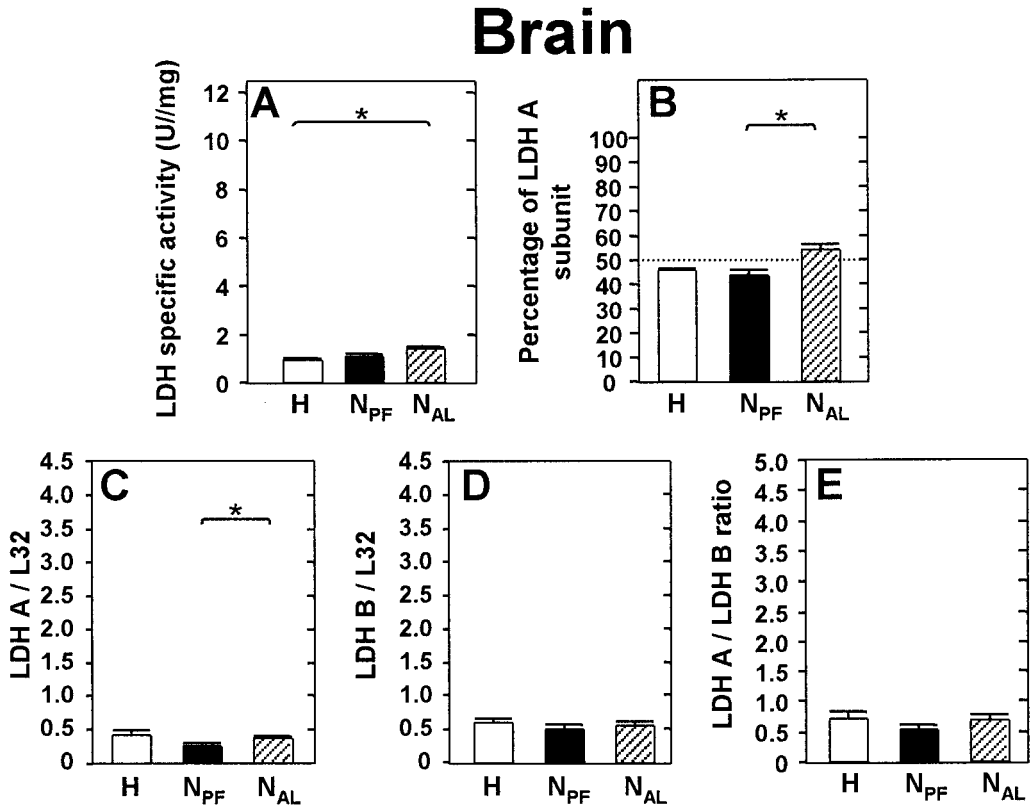

Fig. 7. LDH changes in brain of male rats raised in normoxia and chronic hypoxia. **Panel A**: LDH specific activity measurements. **Panel B**: Percentage of LDH A subunits determined by non-denaturing agarose gel electrophoresis. **Panel C** & **D**: LDH A and B (respectively) RER determined by semi-quantitative RT-PCR. **Panel E**: LDH A mRNA/LDH B mRNA ratio. Results are presented as means ± SEM. *Significantly different $P < 0.017$, **$P < 0.0034$, ***$P < 0.00034$.

mals. This increase is due to a down-regulation of LDH B gene transcription or a lower stability of LDH B mRNA transcripts, but not to a higher LDH A mRNA amount.

In EDL, a fast postural muscle, caloric restriction (N_{PF} vs. N_{AL}) decreases LDH specific activity. LDH A and B gene expressions are regulated in opposite ways that leads to a higher percentage of LDH A subunits. If we just compare the two H and N_{PF} groups in order to minimise nutrition effects, we can see that chronic hypoxia partially compensates diet restriction consequences on LDH global activity. LDH A mRNA copy number remains unchanged whereas LDH B mRNA copy number is lower in EDL of hypoxic animals than in EDL of N_{PF} rats.

In the other fast muscle plantaris, no significant change can be noted between the three groups of rats (Fig. 5), indicating that a low oxygen supply does not induce any adaptation at LDH level.

In left and right heart, a global LDH activity pattern, similar to the one observed in EDL is found, with a much lower activity in heart of N_{PF} rats and no significant change between LDH activities in heart of H and N_{AL} groups contrary to what had been previously observed with adult female rats exposed to transient caloric restriction [Daneshrad et al., 2000]. However, our experimental conditions are very different from the ones used in this previous study. LDH expression has been shown to depend on the age of the animals [Mager et al., 1968].

No significant difference on LDH specific activity pattern can be noted between the two heart ventricles. Caloric restriction induces in both cases a strong decrease in total enzyme activity without affecting subunit percentage distribution. Hypoxia seems to counterbalance food restriction effects. mRNA levels of both LDH genes cannot explain by themselves the measured enzymatic activity differences (Fig. 6). Indeed mRNA levels in heart of H and N_{PF} rats are equal or higher than that of N_{AL} animals. One can suspect a low efficiency in the translation of these LDH A and B mRNA in the

H and N_{PF} groups. Here again, lower protein stability cannot be ruled out.

In brain of animals raised under chronic hypoxia, a slight decrease in total LDH activity is measured. This LDH activity reduction cannot be directly explained by mRNA level changes. A possible decrease of mRNA translation could give an explanation to this observation (Fig. 7). The physiological significance of such an activity decrease in brain of animals bred in chronic hypoxia could be an optimisation of pyruvate use, this molecule being directed to mitochondria oxidation instead of being reduced into lactate. This probably shows that brain cells of these high altitude-living animals probably have sufficient oxygen supply.

It emerges from these observations that LDH regulation depends on the tissue considered and occurs at various molecular levels. In all the tissues studied except in plantaris, there is a poor association between mRNA amounts and LDH specific activity. The abundance of LDH mRNA not being correlated to enzyme activity has already been described in several mammal [Sass et al., 1989; Skidmore and Beebee, 1990; Marieze et al., 1994] and fish tissues [Yang and Somero, 1996]. This lack of correlation could be due to LDH A mRNA stability variations. Indeed, rat LDH A mRNA possesses a 3′ untranslated region known to contain an AU rich region, making it very instable in various conditions [Tian et al., 1998]. It also exhibits a protein kinase A-regulated instability element [Huang et al., 1995] as well as structural determinants for post-transcriptional stabilisation by the protein kinase C signalling pathway [Short et al., 2000].

LDH A gene regulation has been shown to be complex [Jungmann et al., 1998]. In many cellular models submitted to very low oxygen concentrations (1% O_2), HIF-1 is known to upregulate LDH A gene transcription, LDH A gene promoter exhibiting an HRE sequence [Semenza et al., 1996]. However, this transcription factor alone is unable to augment LDH A gene transcription. Indeed it requires the presence of other nuclear proteins like CREB and p300 [Ebert and Bunn, 1998]. LDH A promoter can be regulated via epidermal growth factor [Matrisian et al., 1986], phorbol ester [Huang and Jungmann, 1995], catecholamines [Derda et al., 1980] or growth hormone [Kaaja and Are, 1996]. All these regulators operate via common or different transcription factors. Moreover, there is a negative regulatory element (NRE) that binds a still unknown regulatory protein that represses LDH A gene transcription [Chung et al., 1995]. One can imagine that all these different transcription factor combinations could explain the apparent inconsistent regulation of LDH A gene transcription during chronic hypoxia. Indeed, on the opposite of what is observed in cellular models of hypoxia, in our study, LDH A gene does not appear to be systematically up-regulated as expected in tissues of animals living under low oxygen supply. Therefore, one can doubt about HIF-1 control of LDH A gene in tissues of rats adapted to chronic hypoxia. This may signify that HIF-1 expression levels could be also tissue-specifically regulated.

Another not exclusive hypothesis could be a different regulatory mechanism of the protein half-life, which could be decreased by the caloric restriction as seen in heart and EDL.

If LDH B gene is generally considered not to respond to low oxygen supply [Ebert et al., 1996], some studies have demonstrated that its transcription can be down-regulated by hypoxia in retina [Buono and Lang, 1999]. On the opposite, earlier physiological studies using rats as a model have shown that LDH B activity in skeletal muscle and heart [Barrie and Harris, 1976; Vergnes and Moret, 1976] could be upregulated during chronic hypoxia. Rat LDH B gene promoter structure is poorly documented but in fish, it exhibits a putative hypoxia response element [Rees et al., 2001]. In our model, long-term adaptation to chronic hypoxia seems to limit LDH B gene transcription or LDH B mRNA stability in at least two of the three skeletal muscles studied.

In conclusion, long-term hypobaric hypoxia in rats does not produce identical effects in all the tissues we considered. In these physiological conditions, there is not a systematic increase of LDH A expression due to low oxygen supply on the opposite of what is seen in cellular models submitted to severe hypoxia.

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