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ORIGINAL CONTRIBUTION

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Skeletal muscle expression of LDH and monocarboxylate transporters in growing rats submitted to protein malnutrition

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Abbreviations CTRL: Control, EDL: Extensor digitorum longus, L32: Ribosomal L32 protein, LDH: Lactate dehydrogenase, MALN: Malnourished, MCT1: Monocarboxylate transporter 1, MCT4: Monocarboxylate transporter 4, REx: Relative expression

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Abstract *Background* In different circumstances such as infant malnutrition, old age or chronic disease, decline of muscular strength, particularly anaerobic power, is shown. In this context, our laboratory, has demonstrated a decrease in anaerobic glycolytic power in pre-pubertal Bolivian children living at low and high altitude and suffering from marginal protein malnutrition. *Aim of the study* To bring molecular support to the relationship between protein malnutrition and anaerobic glycolytic metabolism, we studied the impact of prolonged protein malnutrition on lactate metabolism in different muscles of growing rats. Lactate dehydrogenase (LDH), monocarboxylate transporters (MCT1, MCT4) and membrane protein CD147 were chosen as specific markers of anaerobic glycolytic metabolism. *Methods* Two groups of 10 weaning male rats were fed for 10 weeks either ad libitum with a well-balanced diet containing 18% protein or an isocaloric-diet containing 8% protein. LDH activity and mRNA amounts of LDH isoforms, MCT, CD147 were measured. *Results* Protein deprivation during rat growth induced a decrease of LDH specific activity in skeletal muscles (mean value of −41%), accompanied by isoform distribution modifications in soleus, but not in glycolytic muscles (extensor digitorum longus (EDL) or plantaris). A reduction in mRNA amounts encoding the LDH A and B subunits was observed in EDL. A decrease in LDH B mRNA amounts was monitored in plantaris, whereas no modification in both LDH isoform mRNA quantities was observed in soleus. MCT1 mRNA quantities were decreased in EDL but MCT4 mRNA quantities remained stable. CD147 mRNA amounts were unchanged except for EDL with a 42% increase. *Conclusions* The global decreases of LDH activity, LDH and MCT gene expressions in growing rat skeletal muscles support the observed alterations of lactate metabolism associated with lowered muscular anaerobic performances in protein malnutrition.

Key words anaerobic metabolism – muscle – rat – LDH – MCT

EJN 606

Introduction

In animal models of hypocaloric diet, alterations of muscle glucose oxidation [1] and glycolysis [2, 3] were demonstrated. Reduction of glycolysis was associated with low glycolytic enzyme activities, mainly hexokinase and phosphofructokinase [1, 4]. Aerobic glucose degradation was also reduced in chronically protein-deprived rats with low succinic dehydrogenase and citrate synthase activities [5]. In chronic protein malnutrition, the effects on anaerobic glycolysis are more controversial. In one study, LDH activity was unchanged in rat fast and slow muscles [6], whereas another study indicated a glycolytic activity decrease through a reduced lactate production in rat muscles [7]. Likewise, a decrease in LDH activity was observed in heart after malnutrition [8] and an increase in LDH activity was reported in skeletal muscles of rats re-fed after a long period of protein deprivation [3]. In animals, these muscular alterations under malnutrition induced a loss of muscle strength [1, 2, 4] and a decrease of muscle relaxation [3, 9].

In humans, chronic protein malnutrition, not as severe as in animal models, causes muscle disturbances. Muscular fatigue has been observed in children suffering from malnutrition [10–12]. Specific decrease in anaerobic power in relation with marginal malnutrition on pre-pubertal Bolivian children was also reported by our laboratory [13, 14]. Similarly, a decline of muscular alactic anaerobic capacity was found in men living at high altitude with protein malnutrition brought on by a loss of appetite [15]. These modifications of muscle metabolism have also been observed in chronic diseases due to or leading to protein malnutrition. For instance, relationships between the nutritional status and the anaerobic force in respiratory [16, 17] and skeletal muscles [16] were found in cystic fibrosis. Similar respiratory and skeletal muscle alterations were also shown in chronic obstructive pulmonary disease [18, 19].

To understand these losses of muscle force in chronic malnutrition under physiological conditions, or in chronic diseases, and to bring a molecular support for our work in Bolivia, we searched for the impact of prolonged protein malnutrition on lactate metabolism in growing rat muscles. It was decided that protein malnutrition should be over a long time period and should not be too severe, in order to emulate the previously described physiological situations and also to avoid further pathologies in growing rats.

We chose lactate dehydrogenase (LDH; EC 1.1.1.27) as a specific marker of anaerobic metabolism. LDH catalyses the reversible conversion of pyruvate to lactate at the final step of the Embden–Meyerhof pathway, allowing cytosolic NADH + H^+ oxidation when the mitochondrial respiratory chain cannot carry out this function because of oxygen shortage. This enzyme is a tetramer made up of two types of subunits (A and B), forming a set of five isozymes that follow a binomial distribution suggesting a random combination of A (muscle) and B (heart) subunits: LDH1(B4), LDH2(B3A1), LDH3(B2A2), LDH4(B1A3), LDH5(A4). LDH A and B subunits are encoded by two different genes. In mammalian muscles, B subunits have been shown to be predominant in slow twitch fibers (type 1) characterized by an oxidative metabolism, whereas A subunits are principally expressed in fast twitch fibers (type 2) characterized by a more glycolytic metabolism [20].

We also studied muscle expression of monocarboxylate transporters (MCT) 1 and 4, and the membrane glycoprotein CD147. Monocarboxylates such as lactate and pyruvate play a central role in cellular metabolism. Lactate crosses the plasma membrane of many tissues, including heart and skeletal muscles, via a family of transporters. Muscles are able to produce and release large amounts of lactate and, at the same time, to take up lactate and use it as a respiratory fuel. This transport is performed by MCT1 and MCT4 proteins [21]. Brooks proposed an intra and extra-cellular lactate shuttle theory to explain exchanges of lactate in cells and between cells [22]. MCT1 expression is high in sarcolemma of type 1 fibers, low in type 2a fibers and almost undetectable in type 2b fibers. In contrast, MCT4 expression is low in membranes of most type 1 fibers but high in type 2 fibers [23]. Therefore, MCT1 expression is highly correlated with muscle oxidative fiber composition, whereas MCT4 content is correlated with anaerobic glycolytic metabolism [24]. This suggests that MCT4 is responsible for the efflux of lactate produced by glycolysis, whereas MCT1 allows lactate re-uptake [24, 25]. Both transporters require a protein partner for their correct plasma membrane expression and function. This protein, named CD147, is a broadly expressed plasma membrane glycoprotein containing two immunoglobulin-like domains and a single transmembrane helix [26].

We studied LDH and MCT expression in skeletal muscles of growing rats submitted to a regimen of chronic protein malnutrition between the ages of 3 and 13 weeks. We hypothesized that the expression of the enzyme and transporters would be reduced by malnutrition.

Methods

Animals

Twenty weaning male Sprague-Dawley rats (IFFA Credo, France) were individually housed in a controlled environment with an ambient temperature of

+22°C and a 12-h light–dark cycle. The rats were randomly assigned into two groups (2 × 10 animals): a control group (CRTL) was fed ad libitum with a well-balanced diet containing 18% protein (U.A.R, France) and a protein-depleted group (MALN) was fed on a diet containing 8% protein. In the 8% protein diet, starch replaced casein to make rations isocaloric. Rats from both groups had similar food intakes and free access to drinking water. These diets were given for 10 weeks. The animals were weighed twice a week.

Tissue sampling

At the age of 13 weeks, the animals were sacrificed according to the national Animal Welfare Committee recommendations. The animals were killed by cerebral dislocation followed by decapitation. We chose to study soleus a slow muscle type, EDL a postural fast muscle and plantaris, a fast muscle more involved in animal locomotion. The selected tissues were quickly removed, weighed, sampled in sterile cryotubes, frozen in liquid nitrogen and kept at −80°C prior to use.

LDH specific activity

About 50–100 mg of tissue sample was homogenized for 30 s with an Ultra Turrax homogenizer at +4°C in 19 volumes (1 vol/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 at +4°C. Supernatants were diluted in extraction buffer and assayed for LDH activity at +25°C by a spectrophotometric method using a LDH-P UV system (Roche, France). The measured LDH activities were reported to the total amount of protein in the supernatants determined using a bicinchoninic acid kit (Pierce, France) with bovine serum albumin as 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 with a Performance scanner (Sebia, France). The percentage of LDH A subunits was calculated as follows

$$\text{LDH A}(\%) = (4 \times \text{LDH5} + 3 \times \text{LDH4} + 2 \times \text{LDH3} + \text{LDH2})/4.$$

All tissue treatments, activity measurements and isoform determinations for one tissue were carried out on the same day for both groups of animals.

RNA preparations and reverse transcriptions

Tissues were ground in liquid nitrogen, and the powder was homogenised in Tri-InstaPure reagent (Eurogentec, France). Total RNA was extracted according to the manufacturer's recommendations. Once purified, total RNA was measured spectrophotometrically at 260 nm for concentration and at a 260/280 nm ratio for purity. Integrity and good quality of the purified RNAs were checked using formaldehyde–agarose gels containing ethidium bromide. RNAs were kept at −80°C until used.

Reverse transcription was performed as previously described [27]. A semi-quantitative RT-PCR assay was used to assess LDH, MCT and CD147 transcript levels in tissues. The method allows the estimation of the relative expression of these mRNAs (target genes) and rat ribosomal L32 protein mRNA, a house-keeping gene used as an internal standard [28]. PCR experiments were performed as reported earlier [27]. The PCR products were analyzed on 2% agarose gel containing ethidium bromide. Gel DNA picture analyses were performed using Kodak DC120 software. Results were expressed as the ratio of target gene over L32 expression (relative expression: REx). Primers used and RT-PCR characteristics are listed in Table 1.

Table 1 Oligonucleotide sequences, cDNA size, annealing temperature and number of PCR cycles amplification

Sequence			cDNA size	Annealing *T* (°C)	Number of cycles
LDHA	Sense	ggTgACACTgACTCCTgACgA	544 bp	61	28
	Antisense	gTTggTTCCATCATCCATATgCAgATC			
LDHB	Sense	TgACCTCATCgAATCCATgC	419 bp	62	28
	Antisense	TgCAATTgCTAAACTTTATTTgTTCAg			
MCT1	Sense	CCTAAgATgCCACCTgCg	698 bp	60	30
	Antisense	gggACTTCCTCCAATgAg			
MCT4	Sense	agCTgCTgCAggACCACTAT	597 bp	64	29
	Antisense	CTAACAgCAgCACgAgACCA			
CD147	Sense	AgAAATCggAACACgCCAgT	660 bp	62	28
	Antisense	TgCTTCTCCCAgATgggTTT			
L32	Sense	gTgAAgCCCAAgATCgTCAA	257 bp	59	29
	Antisense	TTgTTgCACATCAgCAgCAC			

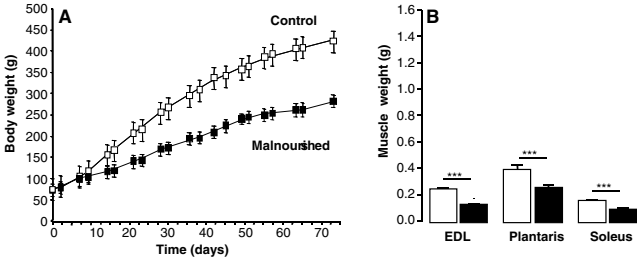

Fig. 1 Muscle and body weight growth measurements. Panel **A**: body weight growth curves of male Sprague-Dawley rats fed ad libitum with a well-balanced diet containing 18% protein (empty squares, $n = 10$) and an isocaloric protein-depleted diet containing 8% protein (filled squares, $n = 10$). Panel **B**: skeletal muscle weights ($n = 20$) of rats from the two groups (empty and filled bars are used for normal and protein-deprived diets, respectively). Data are presented as mean ± SEM. *** Significantly different $P < 0.001$

Statistics

Results are presented as mean ± SEM. Statistical differences between values were assessed using the χ^2 test. Comparisons between groups were carried out using a Mann–Whitney test for mRNA measures ($n = 4$) and ANOVA for LDH activities and isoform ($n = 10$) (Statview software, Abacus Concept Inc., Berkeley, CA, USA). Statistical significance was set at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

Results

Animal characteristics

After the 10-week protocol, rats from the MALN group exhibited a 6-week growth delay: they exhibited a smaller size and a lower body weight (−35%, $P < 0.001$) than CRTL group (Fig. 1A). Their muscle weights were also decreased: −45, −33 and −38% for EDL, plantaris and soleus, respectively (Fig. 1B).

LDH regulation

Specific activity and isoforms

In the four different tissues from the CTRL group, LDH activity was similar to that measured in a previous study from our laboratory [27]. As expected, EDL muscles exhibited the highest LDH specific activity whereas soleus, a slow muscle, had the lowest (Fig. 2). In the MALN group, LDH specific activity was reduced in all the tissues studied compared to the CRTL group (−33.6% in EDL, −48.3% in plantaris and −42.5% in soleus) (Fig. 2).

In both groups, the percentage of LDH A subunits was higher than 50% in the fast glycolytic muscles

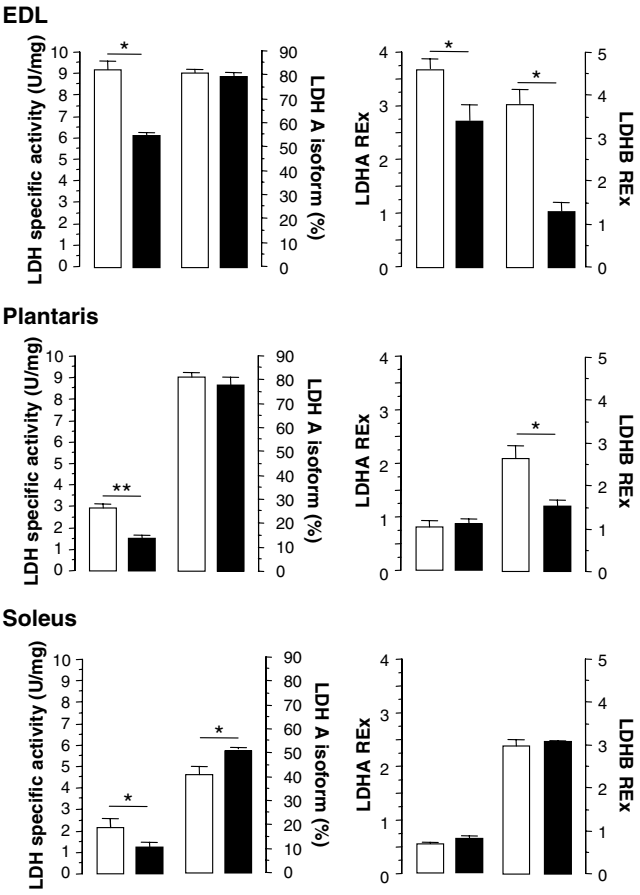

Fig. 2 LDH changes in EDL, plantaris, and soleus of male rats fed for 10 weeks with diets containing 18% (empty bars) and 8% protein (filled bars). Lactate dehydrogenase specific activity was determined in cytosolic extracts of skeletal muscles using a spectrophotometric method at +25°C and reported to the mg of total soluble proteins ($n = 6$ for each group). The percentage of LDH A subunits was measured using non-denaturing agarose gel electrophoresis ($n = 6$ for each group). LDH A and B REx were determined by semi-quantitative RT-PCR ($n = 4$ for each group). Data are presented as mean ± SEM. * Significantly different $P < 0.05$

(EDL and plantaris). These percentages were not subjected to significant modifications due to the low protein diet. However, LDH A percentage isoform was increased in the most oxidative tissues (+24%, $P < 0.05$ in soleus).

Distributions of the different LDH isozymes were therefore changed in the oxidative muscle only (soleus). In MALN rats there were significant decreases in LDH1 and LDH2 isoform percentages and increases in LDH4 isoform percentages (Table 2).

mRNA amounts of LDH isoforms

LDH A mRNA amounts remained steady in plantaris and were significantly decreased in EDL of MALN rats without affecting global LDH A isoform percentage.

Table 2 LDH isozyme composition (% total) in soluble fractions of skeletal muscles of rats from the two different groups. * Significantly different $P < 0.05$, ** $P < 0.01$

		EDL	Plantaris	Soleus
LDH5	CRTL	58.6 ± 3.1	57.9 ± 3.1	12.3 ± 4.2
	MALN	55.3 ± 2.4	54.7 ± 5.2	17 ± 0.8
LDH4	CRTL	21.9 ± 1.3	23 ± 1.7	18.5 ± 1.7
	MALN	25 ± 0.8	22.1 ± 1.6	28.2 ± 0.7**
LDH3	CRTL	8.9 ± 0.7	8.8 ± 1	15.9 ± 2
	MALN	9.5 ± 0.7	9.1 ± 1.2	16.8 ± 0.4
LDH2	CRTL	5.8 ± 0.5	6.5 ± 0.7	29.1 ± 3
	MALN	5.5 ± 0.7	7.1 ± 1.3	19.2 ± 0.1*
LDH1	CRTL	4.6 ± 0.6	3.6 ± 0.6	24.1 ± 1.8
	MALN	4.4 ± 0.4	6.8 ± 1.3	18.7 ± 0.4*

In soleus, a slight increase in LDH A mRNA levels (+18.9%, $P = 0.24$) was measured in MALN animals (Fig. 2). LDH B mRNA levels were reduced by the low protein diet in plantaris and EDL but not in soleus that exhibited no change (Fig. 2).

Monocarboxylate transporters 1 and 4, CD147 mRNA amounts

A significant decreased of MCT1 was observed only in EDL (−51.6%, $P = 0.043$; Fig. 3). MCT4 mRNA levels remained unchanged in the three skeletal muscles. In EDL, CD147 mRNA was increased (+42.8%, $P = 0.03$) whereas no significant change was measured in soleus and plantaris (Fig. 3).

Discussion

Ten weeks of protein deprivation in growing rats decreased their body weights by 35% as compared to the control group. According to past studies, the decline varied from 35 to 60% [5, 6], depending on the length and/or the extent of the protein deprivation protocol. The low body weight was associated with low muscle masses (a mean decrease of −38%) as previously reported in different protocols [5, 29]. Such fiber size reduction was observed in muscles of rhesus monkeys and rats [30, 31]. Fast muscles seemed to be more sensitive to malnutrition with a preferential atrophy of type 2 fibers, especially type 2b [5, 30, 32]. However, no fiber switch was observed in any of these previous studies [5, 30, 32]. Similar results were reported in humans with a marked reduction of fiber size in muscles of children suffering from protein deprivation [33].

As expected, the fast muscle (EDL) exhibited the highest LDH specific activity whereas soleus, a slow muscle had the lowest. The 10-week isocaloric low protein diet produced a significant reduction in LDH

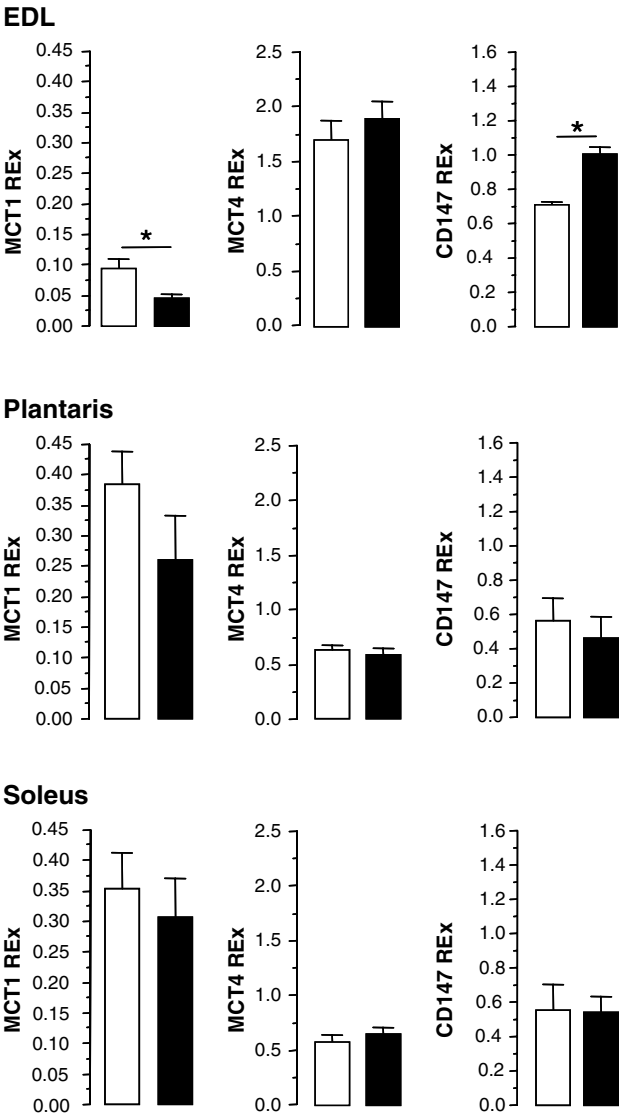

Fig. 3 MCT1, MCT4 and CD147 mRNA amount changes in EDL, plantaris, and soleus of male rats fed for 10 weeks with diets containing 18% (empty bars) and 8% protein (filled bars). MCT and CD147 REx were determined by semi-quantitative RT-PCR ($n = 4$ for each group). Results are presented as mean ± SEM. * Significantly different $P < 0.05$, ** $P < 0.01$

specific activity in both oxidative (−42%) and glycolytic (mean value of 41%) muscles of growing rats although protein deficiency was moderate (8% protein instead of 18%). The impact of these low LDH activities on anaerobic glycolytic metabolism may be of great importance: according to flux control theory, even small LDH activity variations could generate strong modifications of total glycolytic pathway potential in anaerobic conditions [34]. Similar results were suggested from a low lactate/pyruvate ratio in muscles of rats under protein malnutrition procedures [7]. Furthermore, in rat gastrocnemius muscle, an increase in LDH activity and a decrease of muscle

pyruvate concentration were observed after 10 weeks of 6% protein diet followed by re-feeding a control diet [3]. However, a decline of activity of both glycolysis and tricarboxylic acid cycle enzymes was found without alteration of LDH activity after various protocols of caloric restriction [1, 4, 6].

In humans, only one study in children indirectly supports that protein malnutrition was associated with a decrease of LDH activity. These children exhibited a reduced plasma lactate concentration and a rise of plasma alanine concentration explained by an increased pyruvate/alanine transamination due to a low LDH activity [34].

It is well-accepted that Insulin-like Growth Factor 1 (IGF-1) plays a major role in muscle growth regulation [35] and also induces an increase of muscle LDH activity [36]. Although IGF-1 was not measured, it is suggested that this parameter may be involved in our results. In fact, it has been demonstrated that protein malnutrition decreased plasma IGF-1 concentration and IGF-1 mRNA amounts in liver [37–39]. An identical pattern was obtained in rodents submitted to protein deprivation or prolonged malnutrition for IGF-1 protein and mRNA amounts in muscle [40]. Furthermore, in children with chronic malnutrition our laboratory has previously reported a decrease of plasma IGF-1 concentration correlated with a reduction of glycolytic anaerobic performances [41]. Therefore, all these observations suggest that a low IGF-1 synthesis was associated with, and perhaps was responsible for, the decrease in LDH activity observed under malnutrition.

As expected, the percentage of LDH A subunits was higher than 50% in the glycolytic muscle (EDL and plantaris). Ten weeks of protein deprivation had no effect on this subunits distribution in fast muscle but increased LDH A isoform percentage in the most oxidative tissue (soleus). The variations of amounts of LDH A and B mRNA were not correlated with the variations of LDH A and LDH B subunits, in concordance with a past study which did not report any correlation between the two LDH subunit quantities and their respective mRNA amounts in muscles of growing rabbits [42]. This discrepancy is probably due to the intervention of other control levels of protein production like gene transcription rate and mRNA degradation speed.

A major finding in our study is that the diminution of LDH activity in different muscles, particularly glycolytic muscles, is associated with a reduction of LDH A and B mRNA amounts. These results explain at a molecular level the loss of glycolytic anaerobic performance due to malnutrition.

In anaerobic muscular power, anaerobic glycolysis depends on LDH activity that sustains the presence of lactate. Variations in lactate production in muscle cells may be accompanied by differences in expression of monocarboxylate transporters. These transmembranar proteins play an essential role in energetic utilization of lactate and pyruvate in muscles [21]. They allow sarcolemmal membrane crossing, but are also suspected of participating in an intracellular lactate shuttle with a mitochondrial membranar location [22, 24, 43, 44]. Considering these elements, the second main result of our study is an important decrease of MCT1 mRNA amounts (−51%) in glycolytic muscle (EDL), suggesting a reduced lactate re-uptake in these muscles due to malnutrition [25, 45]. At the same time CD147 mRNA amounts increased by 42% in EDL. This reverse variation of MCT1 and CD147 mRNA quantities could be partly explained by previous data showing that increased pyruvate concentration was able to augment CD147 mRNA quantities in muscle cells [26, 46]. During protein deprivation, similar variations in pyruvate concentration could occur since lactate production is reduced [47].

In oxidative muscle (soleus), no significant changes were found in MCT1 and CD147 mRNA amounts between both groups of rats. In two previous studies, MCT1 protein expression was shown to depend on MCT1 mRNA levels indicating a transcriptional and/or a post-transcriptional regulation of the transporter [24, 43]. Additionally, the amount of MCT1 protein appeared not to change significantly in oxidative muscles of growing rats submitted to 6 weeks of food restriction [47]. Although MCT protein was not estimated in our study, concordance between our results and these previous studies is in favor of stable MCT1 mRNA and protein amounts in oxidative muscle of animals submitted to malnutrition.

MCT4 mRNA amounts were not modified in the three different skeletal muscles. This observation was in agreement with a previous study of food restriction in which no modification of MCT4 protein was found in sarcolemmal membranes of red gastrocnemius muscle [47].

In conclusion, diminution of LDH activity in all muscles of MALN rats associated with reductions of LDH mRNA and MCT1 mRNA amounts, specifically in glycolytic muscle (EDL), brings new insights to explain increased muscle fatigability of animals submitted to different malnutrition protocols [1, 2, 4]. These results bring a better understanding to our previous studies, which found a reduction of anaerobic glycolytic performances in malnourished Bolivian teenagers [13, 14]. They underline the importance of nutritional criteria in muscular performance in sportsmen submitted to a loss of appetite associated with stay at high altitude [2, 15]. Moreover, these results may contribute to better understand the loss of muscle strength in chronic pathology with malnutri-

tion: diminution of respiratory muscle forces in chronic respiratory insufficiency or cystic fibrosis [17, 18], or loss of skeletal muscle strength in patients with chronic heart disease or in elderly people [48, 49]. Our data could also support clinical studies which described a functional improvement of muscles after a protein supplementation in chronic pathologies associated with malnutrition [50]. Nevertheless, taking into consideration the ethical limitations, additional studies in human muscle would be performed to support our results in animals.

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