Coordinated modulations of markers of energy metabolism and contractile apparatus in rat hind limb muscles following physiological activity changes

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Abstract

Although plasticity is a well-known property of skeletal muscle, we still have little understanding of the underlying mechanisms. To characterise at molecular level the coordinated modulations occurring in adapting striated muscles, we have examined two types of rat models resulting in opposite physiological neuromuscular changes: disuse and physical training. We observed that increased muscle activity induces modifications towards an increased oxidative phenotype visible only in slow muscles. Conversely, following muscle disuse, modifications occur towards an increased glycolytic phenotype of slow-oxidative muscles. Our novel observation of muscle-specific enolase increased expression, in the soleus muscle following three-week hind limb suspension, contributes to the demonstration that a global activation of glycolysis, in slow-twitch muscle, accompanies muscle disuse. Our results also indicate that the fast-glycolytic muscle undergoes a transition towards a less oxidative phenotype. In this muscle disuse model, micro methods allowed to obtain data on changes occurring in expression of transcripts and when possible proteins, for twelve chosen markers from one single muscle sample. Among them were two transcription factors implicated in the maintenance of the cellular metabolic balance, HIF-1α and PGC-1α. In conclusion, we have analysed changes in markers of gene modules including enzymes of energy metabolism and isoforms of contractile proteins. In every case, despite the complexity of generated results, our observations imply, but far from prove, the occurrence of coordinated changes in gene expressions of metabolic enzymes and contractile proteins. Furthermore, our data point to two sensitive indicators of complementary metabolic changes: β enolase (glycolysis) and HAD (lipid oxidation).

Key words: striated muscle, enolase, glycolysis, transcriptional regulation, myosin.

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Introduction

Plasticity is a fundamental property of skeletal muscle tissue. Thus, during the post-natal period, skeletal muscles rapidly adapt to environmental stimuli such as changes in neuromuscular activity (muscular exercise) and injury (denervation, contusion, etc…). This well documented plasticity is largely based on analyses of myofibre heterogeneity varying from slow oxidative to fast-glycolytic phenotypes [31]. In the past, most studies have analysed changes occurring at the level either of contractile proteins and/or of some metabolic enzymes [3], [16], [29]. Because of the numerous parameters involved in the observed phenotypic changes, researchers face difficulties that have been summarised in a recent review article by Spangenburg & Booth [34]. These authors have introduced the modular concept, recommending to analyse the molecular regulations of functional gene groupings or modules (contractile proteins, mitochondria, ion transport, fatty acid, glucose transport, etc.) rather than molecular regulations of muscle MHC classified fibre types (or of metabolically classified fibre types). This is in keeping with the

approach we have developed for the past few years [6], [41].

In order to progress in understanding the coordinated modulations occurring in adapting striated muscles, we are analysing changes in markers of gene modules including both enzymes of energy metabolism and isoforms of contractile proteins, in animal models of modified neuromuscular status (inactivity, physical training). We have developed micro methods allowing the acquisition of data on expression changes of different markers, from a single muscle. This should allow a more precise analysis of coordinated modulations because of the often-observed individual variability among animals. Another originality of this study is the choice, based on our previous studies, of the enzyme enolase as a marker of glycolytic metabolism [13], [20], [22].

We have analysed changes occurring in protein and enzymatic activity levels with two opposite models of physiological neuromuscular changes: training and disuse. In order to have better insights on the underlying molecular mechanisms, in the muscle disuse model, we have analysed variations occurring at transcript levels for markers analysed at protein levels. As only few data are available concerning transcription factors involved in striated muscle phenotypic changes we decided to analyse HIF-1α and PGC-1α transcription factors known to be involved respectively in control of glycolysis [17] and of oxidative metabolism, [15], [23].

Hind limb suspension (HS) is one of the most studied animal models of musculoskeletal disuse. The postural *soleus* muscle is particularly susceptible to HS-induced atrophy. In the present study, we chose to associate to the analysis of the slow-twitch *soleus* muscle, the *plantaris* of the same animals because this mixed fast-twitch muscle [1] is part of the same group of flexor muscles. Although *soleus* muscle is mainly an antigravity muscle, both *soleus* and *plantaris* muscles are likely involved in plantar flexion. In this way, it is possible to compare changes occurring in two muscles submitted to at least partly similar stimuli but exhibiting very different fibre type compositions.

In every case, despite the complexity of results generated here, changes in gene expressions of metabolic enzymes and contractile proteins appeared to be coordinated: in the increased muscle activity model, modifications occur, towards an increased oxidative phenotype visible only in slow oxidative muscles; conversely, in the muscle disuse model, the slow-oxidative muscle undergoes a transition towards a fast-glycolytic phenotype, whereas the fast-glycolytic muscle undergoes a transition towards a less oxidative phenotype. Furthermore, we demonstrate that the analysis of the glycolytic isozyme β enolase and of the enzyme of lipid β oxidation, β Hydroxy AcylCoA Dehydrogenase (HAD) should be recommended, as they are very sensitive indicators of complementary metabolic changes.

Materials and methods

Animals

All animals used in these studies were female Wistar rats purchased from IFFA Credo (L'Arbresle, France). Animals were housed four per cage with a 12-12 h light-dark cycle at an ambient temperature of 22 +/- 2°C. They had free access to food and water. After 4 days of acclimatisation, they were randomly assigned to control or experimental groups. This investigation was conducted in accordance with French legislation and the Helsinki Accords for Humane Treatment of Animals.

Modifications have been examined in one model of muscle disuse, and in two models of physical exercise; one model corresponds to bouts of intense exercises of short duration (voluntary physical exercise), the other is continuous and less intense (endurance training).

Model of chronic voluntary physical exercise

The experimental paradigm was previously described [43]. Briefly, after 4 days of acclimatisation, female adult rats (weighing 180 g) were randomly assigned to one of two groups designated as spontaneously active (n=8) or control sedentary (n=8). Exercising animals were placed in individual plastic cages with access to running-wheels. The wheels were attached to electronic devices allowing the number of running bouts, the duration and speed of individual bouts and the total distance run per hour and daily to be recorded in a computer. Limb muscles were collected at the end of the 8-weeks training period.

Model of endurance training

Study of adults: 8-weeks training. Sixteen adult female rats (~230 g) were randomly divided into two groups (8 controls, 8 trained animals). All animals were given food *ad libidum*. Training was as previously described [4]. Briefly, rats were running 30m/min on a 7% grade treadmill for 2 hours/day. At the end of the 8-weeks training period, limb muscles were collected.

Model of hind limb suspension

Female rats (weighing 100-120 g) were randomly assigned to one of two groups designated as hind limb suspension (HS group, n=8) or control (C group, n=8). The hind limb of HS animals were elevated for 21 days using a previously described tail-suspension model, in order to maintain head down suspension of approximately 45° [5]. Two series of such experiments were carried out for the present study. At the end of the experiments, *soleus* and *plantaris* muscles were collected.

Biochemical studies of muscles

At the end of the experimentation period, animals were weighed and anesthetised with sodium pentobarbital

(70 mg/kg) administered intra peritoneally. Muscles were isolated, weighed and freed of tendons and connective tissues under a binocular microscope. They were transversally cut in half and rapidly frozen in liquid nitrogen until further analyses. Muscles obtained from controls were simultaneously treated according to a similar procedure.

Micro methods of extractions

In the first series of HS animals, classical methods of protein extraction were used for protein and enzymatic measurements as well as mRNA extraction for Northern blot analyses [21],[24]. In the second series, procedures have been miniaturised in order to analyse a larger number of parameters on a single muscle sample. The middle larger parts of the transversally cut muscles were used for these studies. For enzymatic assays, about 10 cryostat sections (20 μm) were collected in Eppendorf tubes in the cold cryostat chamber (- 20°C) and then stored at –80°C until further use.

Enzyme activities

Frozen cryostat sections were dropped in 100 μl ice-cold extraction buffer: 15 mM sodium phosphate buffer pH 7.2, containing 4 mM magnesium acetate and a proteinase inhibitor, aprotinin, as indicated by the manufacturer (Boehringer Mannheim, Meylan, France). Following 1 hour centrifugation in the cold at 1500g, the supernatants were collected, one half for enolase enzymatic activity and one half for myokinase (MK) assay. Pellets were recovered for citrate synthase (CS) enzymatic activity measurements with 100 μl CS buffer (5 mM HEPES pH 8.7, 1 mM EGTA, 1mM dithiothreitol, 5 mM $MgCl_2$, triton X-100 (0.1%) followed by incubation for 60 min at 4°C to ensure complete enzyme extraction from mitochondria. All assays were performed in 96 wells plates, with a final volume of 200 μl. Protein concentrations were determined using a commercial kit (protein assay system kit 600-0005, Bio-Rad). Enolase activity was determined using a coupled enzyme assay [26]. MK assay was as previously described [38], [39]. Determination of CS activity was assayed according to Srere [35]. Each measurement was carried out at least in duplicate. All enzyme activities were reported as International Units per mg protein in the extract (IU / mg).

Quantification of MHC protein isoforms

Pellets obtained for CS enzyme activity measurements, were also utilised for MHC analysis by polyacrylamide gel electrophoresis as previously described [27].

Analyses of transcript levels

Expression of transcripts encoding all 4 MHC isoforms, and metabolic enzymes were determined by semi-quantitative RT-PCR using transcription factor IID (TFIID) as an internal control and as previously described [41]. New primers designed for this study are described in Table 1. Total RNA was prepared using the RNA Instapure kit according to the manufacturer's instructions (Eurogentec, Seraing, Belgium). The number of cycles used in the PCR and the annealing temperature were determined empirically for each primer pair to be within the linear range of amplification. In addition, the amount of RNA used for the RT reaction (1 μg) was determined to be in the linear response range when comparing RNA input to PCR product abundance. Accordingly, we usually used 30 cycles except for a few cases (α enolase, MHC1: 35 cycles). Each cycle consisted of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. For MK and MHC2x/d, 50°C was used instead of 55°C. PCR products were loaded on a 2% agarose gel (Tris-acetate-EDTA-buffer) containing ethidium bromide. Signal intensity of the bands under ultraviolet light was quantified using densitometry (ChemiGenius, Syngene Cambridge, Ozyme, Montigny le Bretonneux). Intensity of amplified transcript bands were expressed after normalisation with TFIID band intensity. Linearity of signal intensity was always checked. Only fragments of expected sizes were obtained ([41]; Table 1).

Table 1. Oligonucleotide primers used for PCR amplification reactions

Transcript	Sense primer	Antisense primer	Expected size (bp)
α enolase	5'CGTCATCAAAGAGAAGTACGGGAA3'	5'CAGTCTCCTCAGATCGATGGG3'	547
HAD	5'CCTGGATTTATCGTGAACCGTCT3'	5'TGGAAGAACCCCATTACCGTAACTA3'	456
MK	5'TAAACAGGGAGAAGAGTTTGAACGG3'	5'TCAGGGAGTCAAGATAGGTCGAAA3'	284
HIF-1α	5'GCAAGACATTTCTCAGTCGACACA3'	5'TGTTGAAGGGAGAAAATCAAGTCG3'	351
PGC-1α	5'CACGCAGTCCTATTCATTGTTCG3'	5'TCTCAGTTCTGTCCGCGTTGT3'	501

Statistical analysis

The data were expressed as means +/- SEM. In all experiments, data were submitted to one-way analysis of variance to determine the main statistical effects resulting from hind limb suspension, chronic voluntary physical exercise or endurance training of adult animals. Linear regression analysis was used to establish a relation between transcript levels and/or between enzyme activities. Statistical significance was accepted for $p \leq 0.05$. All these analyses were performed using standard software (Statistica 5.5, Stat Soft, Paris, France).

Results

I. Results obtained by the micro method are in keeping with those obtained by classical macro methods.

In order to verify the validity of our new experimental procedures (micro method), transcript levels for all the chosen markers were evaluated in 5 different muscles of a control rat. The relative abundance of transcripts in the different muscles was as expected (Table 2): β enolase expression was highest in the fast-twitch muscles, EDL and *plantaris* [21], [22]. MHC1 highest expression was in heart and was not observable in the EDL, whereas MHC2b was expressed at high levels in EDL and *plantaris*, and not detected in *soleus* and heart [31]. The reference sample (REF) used in all experiments was a mixture of total RNA extracted from diaphragm and *plantaris* muscles in equal amounts. Further data obtained by the micro procedures in the course of this study were also in keeping with previous results generated by us and by others, concerning enzyme activities, MHC isoform composition of muscles as well as changes in transcript levels (see below).

Table 2: Relative levels (a.u.) of specific transcripts in 5 muscles of a control animal

muscle	**beta enolase**	**MHC1**	**MHC2a**	**MHC2x/d**	**MHC2b**
EDL	1.71	0.00	0.67	0.70	1.28
plantaris	1.50	1.03	0.86	0.90	1.47
heart	1.08	1.67	0.00	0.00	0.00
soleus	0.56	0.97	0.47	0.00	0.00
diaphragm	0.49	1.00	1.14	1.09	0.53
REF	1.00	1.00	1.00	1.00	1.00

Transcript levels in each muscle are expressed in arbitrary units (a.u.) by comparison to a reference sample (REF) with a level arbitrary defined as 1. Data were standardized relative to TFIID (see materials and methods). Quantitative evaluations were repeated at least three times and variability was never above 10%.

II. Coordinated changes in metabolic enzymes and contractile proteins following chronic physical exercise.

Changes in the levels of β enolase, the muscle specific isoform of enolase, had never been examined following modifications in muscle physical activities. In order to check whether some intrinsic differences in the use of energy metabolism could parallel differences in performance, in a first experiment, we examined whether changes were induced in animals permitted voluntary activity. We did not observe any change in specific activities of enolase and MK, and in MHC isoform composition, neither in fast-twitch glycolytic *plantaris* nor in slow-twitch oxidative *soleus* (data not shown). Furthermore, no correlation could be detected between the amount of voluntary physical activity (running distance per day, ranging from 1.9 km to 7.7 km) and enzymatic levels. We then examined whether, in contrast to voluntary activity, endurance training would induce some changes. Eight weeks of such treatment did not induce any modification of enolase and MK specific activities as compared to the sedentary control animals, in either slow oxidative *soleus* (Table 3) or fast glycolytic muscle EDL. However, in contrast to the lack of change observed in the previous experimental group (voluntary activity), a 60% significant increase in CS activity occurred in the *soleus* muscle (Table 3).

Table 3: Changes in selected metabolic enzymes of soleus *muscle following eight weeks endurance training*

	Sedentary	Trained
Enolase	1.33 (0.18)	1.30 (0.14)
MK	5.38 (1.13)	4.62 (0.60)
CS	0.25 (0.02)	0.40* (0.04)

*Enzymatic activities are expressed in international units per mg protein (IU/mg), with SEM indicated in parenthesis. Number of animals per group: n=7-9; * $p \leq 0.05$*

III. Coordinated changes in metabolic enzymes and contractile proteins of fast-glycolytic and slow-oxidative muscles, in HS rats.

Parallel increases in total enolase activity and in β enolase transcript levels occur in soleus muscles following three-week hind limb suspension (HS).

A first set of animals had been studied using standard methods of extraction and analysis. *Soleus* muscles from HS rats exhibited atrophy, with a 50% reduction in absolute weight as compared to control muscles. These changes were accompanied by a large increase (+ 91%) in total enolase activity in this muscle, and a parallel increase in β enolase transcript levels (+86%) as measured by Northern Blot. Transcript levels of the ubiquitous α enolase isoform remained unchanged.

Similar changes were observed in the second set of animals analysed by micro methods, concerning atrophy of *soleus* muscle. A 114% increase in total enolase activity occurred in the *soleus* (Fig. 1A), but no significant change was observed in the *plantaris* (Fig. 2A). As in the previous experimental group, the increased enolase activity reported in *soleus* muscles was accompanied by a 23.5% significant increase in β

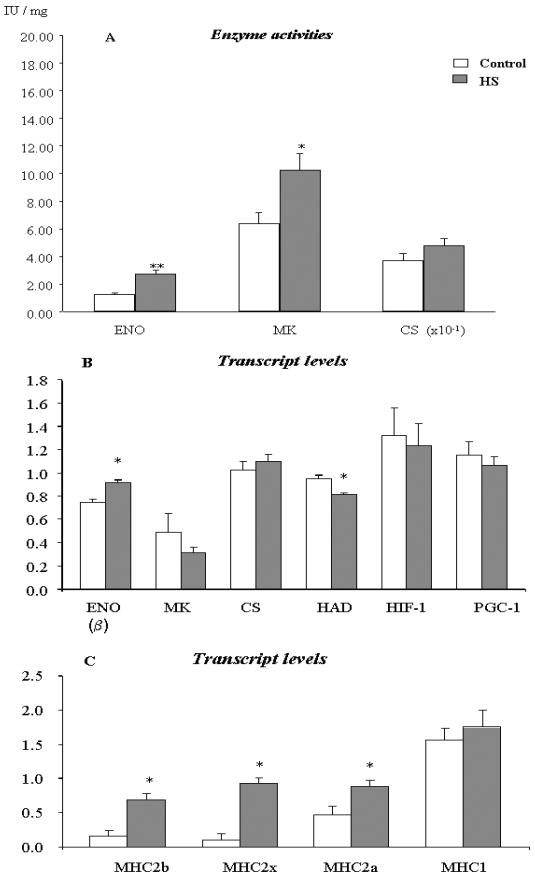

Figure 1. Changes in selected metabolic enzymes and MHC isoforms, in soleus muscle following three-weeks hind limb suspension.
*A: Enzymatic activities are expressed in international units per mg protein (IU/mg). B: Transcript levels are expressed in arbitrary units (a. u). C: MHC (myosin heavy chain) transcript levels. Number of animals per group: n=7-8; * p ≤ 0.05, ** p ≤ 0.0 1.*

enolase transcripts levels (Fig. 1B), without significant change in α enolase transcript abundance (data not shown). The micro methods applied to this experimental group allowed for the analysis of an expanded number of markers for each individual muscle as reported below.

Differential changes in enzymes of energy metabolism in soleus and plantaris muscles

We chose to analyse the enzyme activity of MK, the muscle specific isoform of adenylate kinase which synthesizes ATP and AMP from the ADP produced in excess during exercises [40], because it is expressed at higher levels in fast-glycolytic than in slow-oxidative muscles [25]. We also measured activities of the mitochondrial enzyme CS, a key-enzyme of the Krebs cycle commonly used as a marker of slow-oxidative fibres [25], [39].

Following three weeks suspension, a significant 61% increase in MK activity was observed in the *soleus* of HS animals (Fig. 1A) but no change was detected in the *plantaris* muscle (Fig. 2A). Conversely, HS led to a significant concomitant 30% decrease in CS activity in the *plantaris* (Fig. 2A), but not in the *soleus* muscle of the same animals (Fig. 1A). No significant change in the abundance of MK and CS transcripts was observed in these muscles (Fig. 1B, Fig. 2B). Interestingly, a significant 14% decrease in transcripts of β Hydroxy AcylCoADehydrogenase (HAD) was observed in the *soleus* (Fig. 1B) but not in the *plantaris* (Fig. 2B).

Changes in expressions of myosin heavy chain (MHC) isoforms

Following suspension, the amount of MHC2 fast isoforms was 32 % of the total MHC protein content in *soleus* of HS animals whereas it never exceeded 15 % in controls (data not shown). No significant change was observed in the level of MHC proteins in the *plantaris* muscle (data not shown). The only fast MHC isoform expressed in control *soleus* muscle is MHC2a, associated to the fast glycolytic-oxidative IIA fibre type. It is noteworthy that low amounts of transcripts encoding the two other fast MHC isoforms, MHC2x/d and MHC2b, are detected in control *soleus* muscles that do not express the corresponding proteins [36]. We demonstrate here a large increase in transcript levels in *soleus* of HS animals by about 800% for MHC2x/d, 350% for MHC2b and 85% for MHC2a (Fig. 1C). In this muscle, no significant change in MHC1 transcript expression was observed. Concomitantly, in the adjacent fast-twitch *plantaris*, a significant 60% decrease in MHC1 transcript level occurred (Fig. 2C), without any significant change in transcript amounts of each fast MHC isoform. Some (but not all) individual control *plantaris* muscles expressed low levels of MHC1 transcripts, in keeping with the previously described low content in MHC1 protein, in this muscle [43].

Differential expressions of the transcription factors HIF-1α and PGC-1α in various muscles of control and HS animals

In an effort to uncover mechanisms underlying the coordinated changes occurring in this experimental model of muscle disuse, we chose to analyse at transcript levels, two transcription factors which have been recently evidenced as playing key roles in cellular transitions of energy metabolic pathways, HIF-1α and PGC-1α. Only a few data are available concerning the expression of these transcription factors in different striated muscle types. We therefore determined the levels of these transcripts in 5 muscles of control rats.

We observed significant differences in expression levels as a function of muscles, although it was blurred by an important variability among individuals for both transcripts (Fig. 3). PGC-1α highest expression is in diaphragm, nearly 4 times as high as in the fast *plantaris* muscle that exhibits the lowest level of these transcripts. It is also high in heart where it is expressed at more than twice the level in *plantaris* (Fig. 3A). Furthermore, expression of PGC-1α is significantly lower in the fast plantaris muscle (-29.9%, $p < 0.05$) than in the slow soleus muscle. Differences are less marked for the HIF-1α transcripts. Similarly to PGC-1α, HIF-1α is expressed the least in the fast *plantaris* muscle; it is expressed the most in heart, where its level is close to twice that in *plantaris* (Fig. 3B). No significant difference in levels of the transcription factor HIF-1α is detectable when comparing fast (plantaris, EDL) and slow (soleus) skeletal muscles.

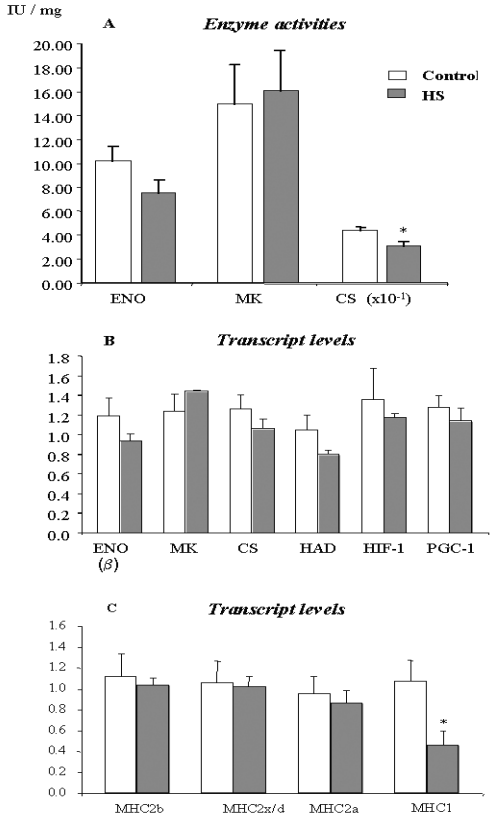

Figure 2. Changes in selected metabolic enzymes and MHC isoforms, in plantaris muscles following three weeks suspension.
*A: Enzymatic activities are expressed in international units per mg protein (IU/mg). Observe the decrease in citrate synthase activity (CS) in plantaris muscles following three weeks suspension. B: Transcript levels are expressed in arbitrary units (a. u.). No change in transcript levels in the same muscle samples. C: Decrease in slow MHC transcript levels in the same plantaris muscle samples. Number of animals per group: n=8; * $p \leq 0.05$. Abbreviations as in legend to Fig. 1.*

After three-week hind limb suspension, comparison to controls did not reveal any significant change in expression of the transcription factors HIF-1α and PGC-1α, whether in *soleus* (Fig. 1B) or in *plantaris* (Fig. 2B) muscles.

Discussion

In both physical training and disuse models, changes at protein levels are clearly correlated to functional adaptations of hind limb muscles.

The results obtained in the present study demonstrate that increased muscle activity induces molecular modifications visible only in the slow muscles. Following endurance training of adult rats, we observe an increase in the oxidative marker CS of about 60%, supporting data reported by others [33]. However, no change in markers of fast-twitch fibres (MK, β enolase) was observed.

In the other model of increased activity, no correlation could be observed between the amount of voluntary physical exercise and metabolic enzyme levels of muscles. We had expected that some intrinsic differences in the use of energy metabolism could

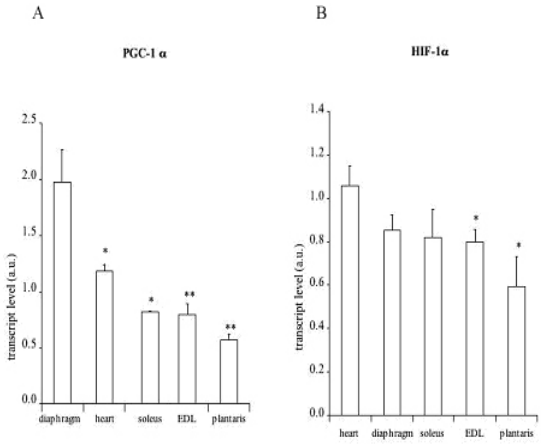

Figure 3. Differential expression of transcription factors in 5 control rat muscles.
*All transcript levels are expressed by comparison to a standard sample (REF) as explained in table 2. Significant differences between muscles are estimated by comparison to the highest values obtained: diaphragm for PGC-1α (A); heart for HIF-1α (B). * $p \leq 0.05$, ** $p \leq 0.0$ 1. Number of analysed animals: n = 5.*

parallel differences in physical activity, in the case of the voluntary physical exercise. Our observations did not support this hypothesis. Changes following exercise are visible only during forced endurance training. Most probably, a selection of animals would be necessary to observe such correlations, in order to overcome problems arising from individual variability [18].

In the opposite model – muscle disuse – the observed modifications favour a less oxidative phenotype. We

demonstrate that the changes occurring in two muscles belonging to the same anatomical and functional grouping, but of opposite phenotypes (one slow-oxidative, the other fast-glycolytic), converge to support similar physiological consequences: the modifications visible in the fast-twitch *plantaris* muscle underline a transition towards a less oxidative phenotype, with a diminution of the oxidative enzyme CS, whereas in the slow-oxidative *soleus* muscle, metabolic and contractile parameters indicate an incomplete transition towards a fast-glycolytic phenotype.

Modifications in transcript abundance of adjacent slow- and fast-twitch hind limb muscles indicate a less oxidative phenotype in the disuse model

With the aim of getting information on the molecular regulations of different functional modules (glycolysis, oxidative metabolism, transcription factors, contractile proteins) within muscles of each animal, we have analysed the expression of 12 transcripts in *soleus* and *plantaris* muscles of HS animals. This has allowed us to uncover, in the slow-twitch muscle, the diminished expression of a key-enzyme of lipid β oxidation (HAD) that accompanies the increased expression of the muscle-specific glycolytic isozyme, β enolase. Several researchers had already described, in similar experimental conditions, the increased expression of other glycolytic enzymes, such as hexokinase [25], GAPDH [10], PGAM-M [19], pyruvate kinase [5], [25], and less oxidative LDH isozymes [5]. For many of these enzymes, the changes affected only their muscle-specific isoform, analysed either as enzyme activity and /or transcript levels. Our novel observations with the β muscle-specific isoform of enolase, brings support to the idea that energy production is modified by full activation of the glycolytic pathway in the slow-oxidative *soleus* muscle following HS. The mitochondrial energy production is regulated concomitantly, as underlined by our observation of decreased HAD transcript expression, supporting similar observations by others of a decreased HAD enzyme activity [8], [28]. In the same individual samples, increased transcriptions of the fast MHC isoforms occurred, in agreement with observations at the protein levels (this study, [5], [7], [19], [36], [37].

Results obtained with the *plantaris*, the fast muscle adjacent to the *soleus*, demonstrate changes in the activity of the oxidative enzyme CS that is significantly decreased, in keeping with the decrease previously observed for this same enzyme in another fast-twitch limb muscle submitted to microgravity, the *tibialis anterior* [25]. Our simultaneous measurements in the same *plantaris* samples demonstrate, associated to this decrease in oxidative enzyme, the diminished expression of the MHC1 isoform, usually associated to the slow-twitch oxidative phenotype. Our data support the idea that changes occurring in disused hind limb muscles are more important in the postural slow-twitch *soleus* than in the fast-witch muscles, whether in rats or in humans [3], [12].

Despite important individual variations, strong correlations do exist between enolase and other metabolic and contractile markers

The individual variability, observed among animals of all the analysed groups, increased the difficulty to uncover statistically significant differences. However interesting correlations appeared from analysing grouped data gathered for one muscle type in one experimental model. Grouping the 15 analysed *soleus* muscles from control and HS animals, demonstrates a positive correlation between specific activities of the glycolytic enzyme enolase and the MK, an important muscle isoenzyme of ATP transfer (Fig. 4). Both enzymes are good markers of the fast-twitch glycolytic fibres [22], [25], and exhibit increased activities in slow muscles from suspended animals, as shown in this study (Fig. 1A).

In order to simplify the presentation, only a selection

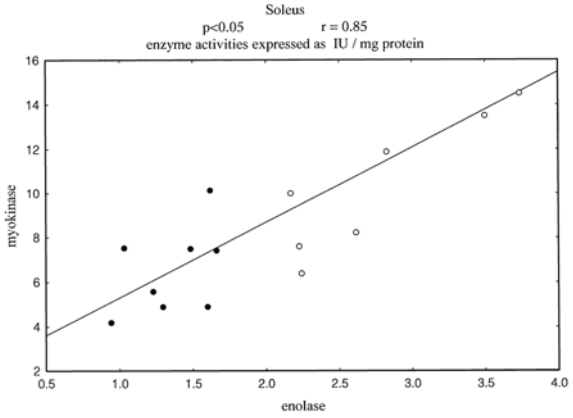

Figure 4. Correlation analyses
Linear regression analysis shows positive correlations between enolase and MK enzyme activities of soleus muscles. Pooled data from control (filled circle) and suspended (open circle) animals. n=15.

of pertinent results obtained from correlation analyses, at both protein and transcript levels are discussed and shown in Table 4.

In *soleus*, a negative correlation exists between specific activities of enolase and HAD transcript levels (Table 4). A similar negative correlation is observed between MK activities and HAD transcript levels (r = -72, $p < 0.05$). These data suggest that, in this slow muscle, energy originating from glycolysis and ATP transfer would replace that fuelled by β oxidation.

In both *soleus* and *plantaris* muscles, a positive correlation is apparent between β enolase and MHC2x/d transcript levels, illustrating the parallel activation of both genes contributing to the fast-glycolytic phenotype of muscle fibres (Table 4). Similarly, positive

Table 4: Summary of correlation analyzes

Markers	MK Protein	CS Protein	CS Transcripts	HAD Transcripts	MHC2b Transcripts	MHC2x/d Transcripts	MHC2a Transcripts
ENO protein	0.87(s)	--	--	-0.77 (s)	0.71 (s)	0.74 (s)	0.61 (s)
ENO β transcripts	--	--	--	0.95 (pl)	0.71 (pl)	0.60 (s) 0.65 (pl)	0.61 (pl)
HIF-1α transcripts	--	--	0.91 (s) 0.84 (pl)	--	--	--	--
PGC-1α transcripts	--	--	0.80 (s) 0.88 (pl)	--	--	--	--

Correlation analyses were performed with levels of markers listed in headers of lines and columns by reporting correlation constants r. *Analyzed muscles are indicated in parenthesis: s for* soleus *(16 independent samples); pl for* plantaris *(16 independent samples). All values presented here are significant with p≤ 0.05, except for --: non significant. Enzymes activities are measured as IU /mg (see legend to Table 3), transcript levels as a.u. (arbitrary units, see legend to Table 2). Abbreviations as in legend to Fig. 1.*

correlations are visible between enolase expression (specific activity or transcript levels) and transcript levels of the three fast MHC isoforms (Table 4). Data at MHC protein levels were not produced in this study, as samples were scarce (micro methods) and it did not seem necessary to repeat already published results [5]. However, the strong correlations calculated here underlines that mechanisms do exist for coordinated regulation of energy producing pathways and contractile ATP consuming proteins, such as the MHC isoforms.

Differences are observed in correlation constants (*r*) obtained in correlation analyses with enolase protein *versus* β enolase transcripts: they most probably reflect the delay in time of expression of proteins as compared to the corresponding transcripts. Indeed, different time-courses at transcript and protein levels have been reported in this model of muscular disuse [10], [19], [36].

Search for transcription factors implicated in the metabolic plasticity of muscle

In recent years, two transcription factors have emerged as master regulators of cellular metabolism, HIF-1 α for the genes of the glycolytic pathway [17] and PGC-1α for the genes of mitochondrial and oxidative metabolism, [15], [23]. HIF-1α is a bHLH-PAS protein that, in hypoxic conditions, interacts with promoters of many genes and thus activates them. Among those are several glycolytic enzymes, including enolase [11], [32]. PGC-1α**,** a transcriptional co-activator known to activate mitochondrial biogenesis, has been implicated in the switch from fast glycolytic to slow oxidative fibre type [2], [14], [23].

We had hypothesized that an increased expression of HIF-1α and a decreased expression of PGC-1α would be associated to the transition towards a more glycolytic (less oxidative) phenotype observed in hind limb suspended animals. No such observation could be made. We did not observe any significant modifications of expression of these two transcription factors in the analysed hind limb muscles following HS. These results are not conclusive however, because if such changes should take place, they most probably would occur at early times of the muscle disuse experiment, as observed by others for PGC-1α following physical activity modifications [2].

Our observation (Table 4) of a strong correlation between CS and HIF-1α transcript levels in both fast- and slow-twitch muscle was unexpected. It should be related to our data on HIF-1α in various striated muscles, demonstrating a less important expression in fast muscle, in agreement with a recent study [30]. These last authors underline the complexity of HIF-1α mode of action: cellular adaptation to low oxygen conditions is at least partly mediated by increased HIF-1α protein stability. These authors investigated the yet poorly understood HIF-1α transcriptional regulation and demonstrated that it involves natural anti-sense RNA. Clearly, more work is necessary to clarify the role of this transcription factor in physiological metabolism regulations. In future studies, both transcript and protein levels should be analysed as a function of time following physiological modifications.

Concluding remarks

In all types of models studied here, it is possible to observe coordinated changes in gene expressions of both metabolic enzymes and contractile proteins. The results imply, but far from prove, convergent modifications occurring in the two adjacent muscles examined in the disuse muscle model. We observe that the slow-oxidative muscle undergoes a transition towards a fast-glycolytic phenotype, whereas the fast-glycolytic muscle undergoes a transition towards a less oxidative phenotype. In the opposite model of increased muscle activity, opposite modifications occur towards

an increased oxidative phenotype, visible only in the slow oxidative muscles. However, coordination does not always appear complete, perhaps because its visibility depends on both the complexity of the involved regulatory loops and the analysed experimental models. Fig. 5 is a schematic representation of our working hypotheses, showing how striated muscle plasticity has been studied with selected metabolic and contractile markers. The equilibrium between energy (ATP) consumption (especially by contractile proteins) and production (by glycolytic and oxidative metabolisms) is usually maintained. Still unknown mechanisms underlie muscle plasticity and ensure that coordinated changes occur, governing a balanced expression of metabolic enzymes and contractile proteins (MHC). We have demonstrated that the analysis of the glycolytic isozyme β enolase and of the enzyme of lipid β oxidation, β Hydroxy AcylCoA Dehydrogenase (HAD) are very sensitive indicators of complementary metabolic changes. The phenotypic transitions we have observed correspond to adaptations that can be compromised in cases of many myopathies including that induced by mutations in the β enolase gene [9]. Anyhow, correlation analyses strongly imply the existence of factors (HIF-1α? PGC-1α?others?) involved in subtle molecular mechanisms responsible for the co-regulation of energy producing pathways and the ATP consuming contractile proteins, such as MHC isoforms. One of these newly emerging transcription factors of interest is PPARδ [42]. The roles of these factors remain to be unambiguously demonstrated. We are currently developing in *vitro* models that should help analyse regulatory mechanisms that remain to be discovered.

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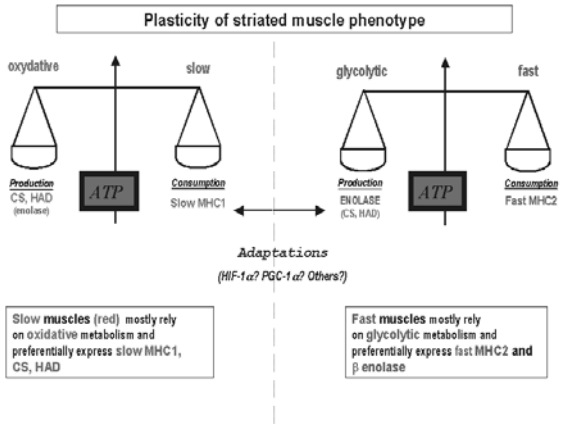

Figure 5. In striated muscle cells, the equilibrium between energy (ATP) consumption (by contractile proteins) and production (by glycolytic and oxidative metabolisms) is usually maintained. Mechanisms underlying muscle plasticity ensure that coordinated changes occur, governing a balanced expression of metabolic enzymes and contractile proteins (MHC), as depicted here. This equilibrium is disturbed in cases of metabolic myopathies, as in glycogenose XIII, corresponding to mutations in the β enolase gene [9].

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List of abbreviations

a.u.: arbitrary units
bHLH : basic Helix Loop Helix
CS: citrate synthase
EDL: extensor digitorun longus
ENO: enolase
GADPH: GlycerAldehyde Phosphate Dehydrogenase
HAD: β Hydroxy AcylCoA Dehydrogenase
HIF-1α: hypoxia inducible factor 1α
IU: international units
MHC: myosine heavy chain
MK: myokinase
PAS domain: PAS (per-ARNT-Sim motif) domains are newly recognized signaling domains that are widely distributed in proteins from bacteria to vertebrates.
PGAM-M : phosphoglyserate mutase, M form.
PGC-1α: PPAR gamma coactivator 1α
PPAR δ : peroxisome proliferator receptor δ.
LDH: Lactate deshydrogenase
HS: hind limb suspension