Influence of Tension Reduction and Peripheral Dissection on Histologic, Biochemical and Bioenergetic Profiles, and Kinetics of Skeletal Muscle Fast-to-Slow Transformation

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ABSTRACT **Seven goat latissimus dorsi muscles were submitted to a progressive electrostimulation program through intramuscular electrodes (Medtronic SP5528). Group 1 (n = 3) consisted of muscles stimulated in situ, and group 2 (n = 4), of muscles dissected distally and reinserted on the chest wall with a reduced tension. In group 1, complete fiber switch from type II to I occurred within 60–100 days after the beginning of stimulation, as demonstrated by myosin isoforms and lactate dehydrogenase (LDH) isozymes pattern. Respiratory chain oxidases first increased within 30–70 days after stimulation, then progressively decreased to stabilized values, higher than the basal ones. Total LDH activity showed progressive decrease to one-fifth of the initial value. Morphological analysis confirmed the structural integrity and physical reinforcement of the muscles. In group 2, respiratory chain oxidases showed initial increase followed first by a fast reduction to values less than the starting ones, and then by a slow secondary increase between day 40 and 90. LDH activity displayed a sharp decrease between day 15 and 36. Myosin as well as LDH isoforms showed progressive conversion. This kinetic study suggests a three-phase adaptative evolution of the goat latissimus dorsi submitted to increased workload (group 1): a fast increase (phase I) in oxidative capacities is followed by the development of an efficient contractile machinery (phase II), with subsequent adaptation (phase III) of the terminal chemosmotic enzymes involved in energy production. Transformation of the contractile apparatus occurred in group 2, but the process was noted to be slower. Furthermore, within 90 days the energetic capacities did not approach the peak capacities observed in group 1. This data suggests that preconditioning in situ may be a superior technique, almost as if muscles are expected to work under energy consuming conditions when providing assist to a falling heart.**

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muscle to adapt to increased work. This process is mediated through a sequence of changes involving modifications of sarcoplasmic reticulum, amplification of oxidative capacities with reduction of glycolytic ones, increase in capillar and mitochondrial density, and a shift of myosin isoforms. These modifications induce fatigue resistance and allow transformed muscle to be used for cardiac assistance or to substitute for the myocardium. In that sense, some have felt that muscle preconditioning is a prerequisite prior to definitive utilization. Following the work of Chachques et al.,[3] it was, however, shown that complete transformation can be achieved when training the muscle after it had been wrapped around the heart, and that long-term hemodynamic support can be expected. This one-stage operation has the advantage of avoiding successive surgical interventions in sick patients.

Since transformed skeletal muscles are used primarily for their expected capacity to produce useful work, determination of the parameters and factors that influence transformation is mandatory. This knowledge will contribute to optimize the procedure.

The present study was designed to investigate the role of tension and peripheral dissection on muscle behavior following chronic stimulation. Muscles stimulated in situ or after complete peripheral dissection and reinsertion on the thorax with modified tension were studied. In both situations, muscles were available for regular biopsies.

Our monitoring for transformation included the usual histochemistry, myosin- and lactate dehydrogenase (LDH) isoform electrophoresis, and, in order to appreciate the evolution of oxidative metabolism capacities, the measurement of succinate-cytochrome c oxidoreductase (succ-cyt c OR), NADH-cytochrome c oxidoreductase (NADH-cyt c OR), and cytochrome c oxidase (cyt c ox) activities. Indeed, the first two represent the converging points of all reducing equivalents produced by cellular dehydrogenases: the chemosmotic enzymes complex I and complex III.

used. The animals were cared for in a humane fashion according to guidelines published by the National Institute of Health (guides for care and use of laboratory animals, NIH Publications, 85–23, revised 1985). Sedation was obtained by intramuscular (IM) administration of xylasine, and anesthesia was induced by ketamin IM. The animals were intubated and placed under mechanical ventilation. The intervention consisted of a vertical skin incision at the level of the axilla and dissection of the proximal third of the latissimus dorsi (LD) to expose the thoracodorsalis neurovascular pedicle. Two electrodes (SP5528, Medtronic, Inc., Minneapolis, MN) were sewn transversely in the muscle at the level where the neurovascular pedicle divides, following the technique reported by Grandjean et al.[4] Impedance and threshold were checked. Both LD on the same animal were stimulated in parallel, using a unipolar Itrel pacemaker (Medtronic, Inc.). During implantation of the stimulating device, muscle threshold and the global circuit impedance were measured and controlled to fit calculated values for the parallel circuit. After a 15-day rest period, the muscles were stimulated by 2 pulses (35/min), then after 2 weeks, 3 pulses, and finally burst pulses (6 pulses, 30 Hz, 185 msec, 35/min). Voltage (2.5–5 volts[V]) was chosen to elicit palpable and visible contraction with movement of the forelimb on the anesthetized animal. Biopsies were regularly performed from distal to proximal, in order to avoid artifacts due to neurovascular damage. The site of sampling was carefully repaired. Samples were immediately processed for histochemistry and biochemical analysis.

The specimens for histochemistry were sectioned, 12-μm thick in a cryostat, and then routine ATPase staining at pH 4.35 and 10.4, as well as hematoxylin-eosin and NADH tetrazolium reductase stains were done. Determination of the percentage of type I and II fibers was made, as well as a general microscopic examination. LDH isozymes from the sarcoplasmic fraction were separated and revealed on polyacrylamide gel electrophoresis (PAGE), according to Leberer and Pette,[5] and myosin heavy

late polyacrylamide gel electrophoresis (SDS-PAGE)[6,7] the proportions of LDH isozymes, myosin MHC I and II, myosin MLC2-S (myosin light chain two-slow) and MLC2-F (myosin light chain two-fast) were measured by densitometry of gels. Mitochondrial oxidase activities were measured by spectrophotometry at 30°C (Amin-co Chance DW 2UV/VIS spectrophotometer (FLM, Inc., Urbana, IL]) according to Schneider et al.[8] for NADH-cyt c OR and for succ-cyt c OR, and according to Möller and Palmer[9] for cyt c ox. The LDH activity was determined according to Bergmeyer and Bernt[10] at a pyruvate concentration that does not inhibit H-isozyme (0.5 mM).

RESULTS

Control values prior to stimulation clearly showed that individual differences for the LD may be important. For a given muscle, and for right-to-left comparison, heterogeneity due to sampling of the muscle. The results in both groups 1 and 2 are presented as kinetic profiles.

Results in group 1

Although the three muscles involved in that group achieved complete fast-to-slow transformation, two extreme records of the observed changes are presented.

Muscle A (goat 1, left muscle) showed (Fig. 1) sharp increase (3.4 times) of NADH-cyt c OR activity within 7 days, followed by a plateau until day 21, and then a persistent increase up to a maximum value 10 times the initial activity (that was 0.4 nmol/min^{-1}/mg ww^{-1}) (ww = wet weight) after 70 days. A regular decrease followed the peak. The succ-cyt c OR activity also displayed a sharp increase (2.7 times) within 7 days, and reached its maximum activity after 21 days, and remained nearly constant until day 112 (3.8 times the initial activity, that was 0.66 nmol/ min-

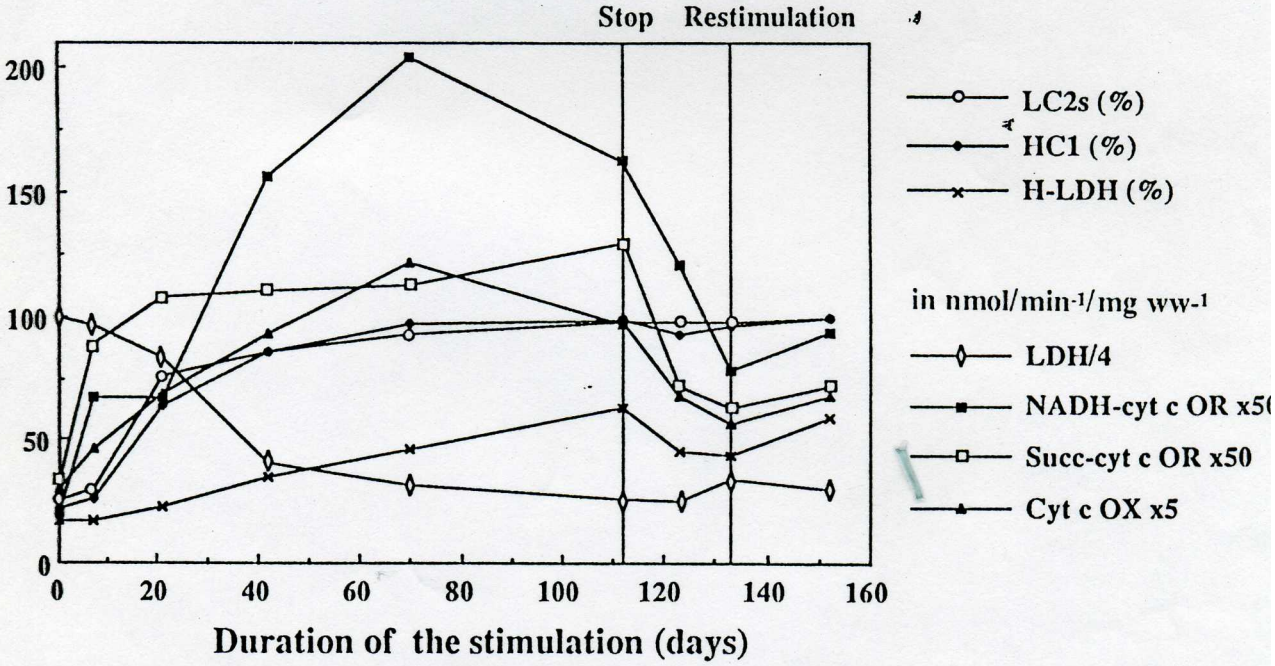

Figure 1. *Muscle A (group 1): synoptic time course of type I fibers (H-LDH) and slow myosin (LC2-S and HC1) markers expressed as percentage of the respective isoforms or subunits total content. Simultaneous recording of glycolytic anaerobic metabolism marker (LDH) and mitochondrial terminal oxidases expressed as maximal enzymatic activities (in nmol/min^{-1} w w^{-1}). Muscle stimulation was stopped on day 112 and reinstituted on day 133.*

reached after 70 days. Thereafter, a slow decrease was observed until day 112. LDH activity showed a slow decrease from its initial activity (400 nmol/ min^{-1} mg ww^{-1}) first, then a sharper one until day 42, and reached its minimal activity at day 112 (4 times less). In parallel, we observed a regular increase of the H-LDH isoform from 17%–70%.

Conversion of fast-to-slow myosin monitored by heavy chain one (HC1) and light chain two-slow (LC2-S) showed sigmoidal profiles with 75% and 100% slow types at 20 and 70 days. Histochemistry disclosed a normally structured muscle, with nearly 100% of fibers type I at day 70. Electron microscopy showed, after full transformation, mitochondria larger in size and number as well as widening of a Z line, indicating a physical reinforcement of the muscle. With cessation of stimulation at day 112, the mitochondrial oxidases underwent a significant fall after 10 and 21 days (29% and 53% for NADH-

28% reduction after 21 days. LDH activity increased 20% 21 days after arrest of stimulation. Histologically, the samples at day 10 showed persistent transformation, while the fibers heterogeneity was obvious on day 21, with 90% of type I fibers. Stimulating again (4 V 35/min) induced after 20 days a reverse phenomenon, characterized by increase of oxidase activities, weak decrease of LDH activity, and the percentage of H-isoform recovery. Histologic shift to a homogeneous population of type I fibers was observed.

Muscle B (goat 2, left muscle), behaved differently. The starting situation differed from muscle A mainly at the level of HC1 (12% instead of 22%), H-LDH isoform (12% instead of 17%), LDH activity (725 nmol/min^{-1}/mg ww^{-1} instead of 400), and cyt c ox (3.9 nmol/min^{-1}/mg ww^{-1} instead of 6). Soon after the onset of stimulation (day 4), a fivefold increase in NADH-cyt c OR activity was observed, contrary to succ-cyt c OR

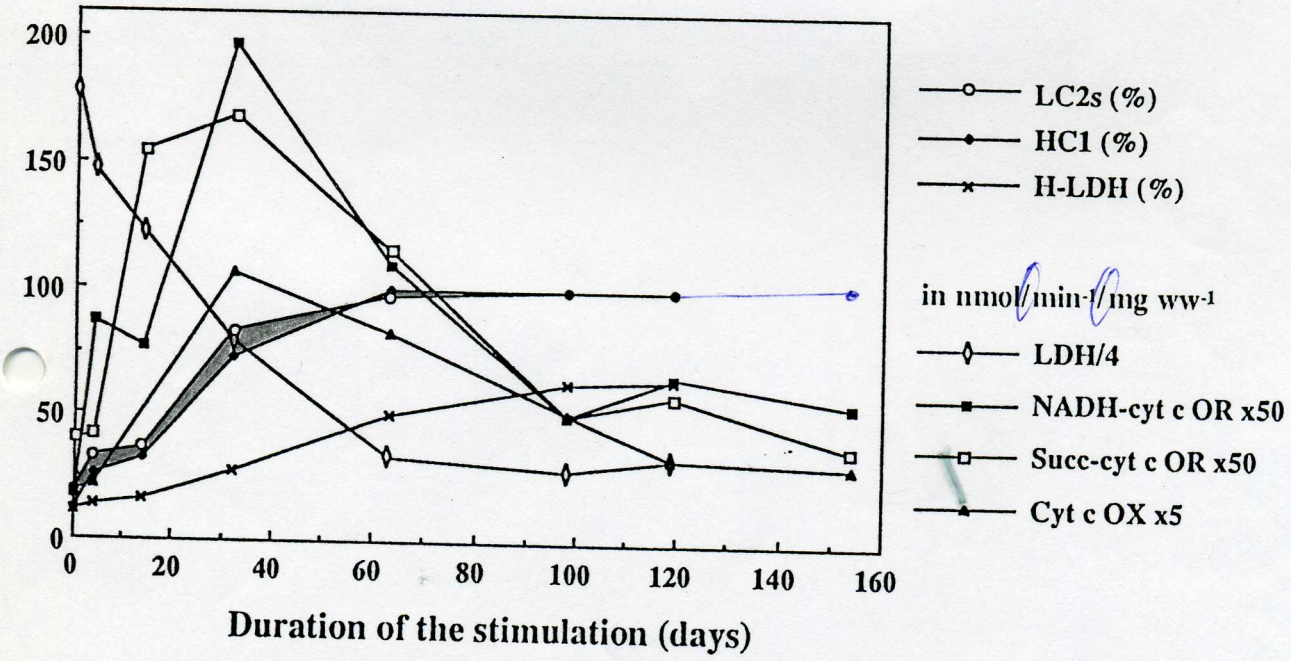

Figure 2. *Muscle B (group 1): similar recording as in muscle A. An evolution profile in three phases can be emphasized: initial major increase in oxidative capacities (phase I), followed by complete change at the myosin level (phase II), and readjustment of the metabolism to supply the energetic requirements of the new contractile machinery (phase III).*

direct regulation of the enzyme activity to face energy demand, was followed by a stagnation phase until day 15 when the number of pulses was increased. The peak activity was reached after 32 days, like in muscle A (4 nmol/min^{-1}/mg ww^{-1}). A regular decrease was observed from day 32–98, where a rather stable activity was measured around 1.1 nmol/min^{-1}/mg ww^{1} (3.2-fold the initial activity). The profiles of succ-cyt c OR and cyt c ox were similar after 4 days lag; a maximal value was noted at day 32 (3.4 and 21 nmol/min^{-1}/mg ww^{-1}, respectively), close to the values reached in muscle A. Thereafter, a regular decrease to quite stable values reached around 0.95 nmol/mm^{-1}/mg ww^{-1} at day 98 and 6.4 nmol/mm^{-1}/mg ww^{1} at day 119, that is 1.2 and 1.6 times the initial values, was observed. At the same time, a sharp decrease of total LDH activity was noted until day 63 (5.4 times less), followed by a stagnation period until day 119. In parallel, H-LDH isoform increased from 12%–64% after 98 days, and then plateaued. LC2-S and HCl isoforms of myosin exhibited nearly the same evolution compared to muscle A, with 75% and 100% slow types at, respectively, 32 and 63 days.

A complete homogeneous histoenzymological transformation (II to I switch) occurred by day 62, and muscle organization was intact, with preservation of interfascicular and interfibrillar space without signs of atrophy or anomaly in muscle density, compared to control. A new steady state between the contractile machine and energy metabolism occurred as a result of the electrical stimulation.

Results in group 2

Muscle C (goat 4, right muscle) was restrained at its equilibrium length (nearly 0 tension, providing the animal forelimb is in a neutral position). Although appropriately stimulated, it progressively deteriorated.

Interstitial infiltrates of histiocytic cells and fat substitution of actomyosin were prominent features. There were no signs of ischemic or neurologic injury. Simultaneously, LDH showed after a small peak, a marked decrease, and all muscle degeneration (not shown).

Muscle D (goat 4, left muscle) was dissected and reinserted on the chest wall with a tension equivalent to 75% of its rest length. After a rest of 15 days, the stimulation protocol was applied. Figure 3 shows that after 15 days of stimulation, the muscle underwent a moderate increase in succ-cyt c OR (1.3 times), in NADH-cyt c OR (1.6 times), and in cyt c ox (2.6 times) as compared with values obtained in the undissected muscle. Subsequent evolution showed a sharp decrease up to a minimum, below their initial activity, at day 36 for cyt c ox and succ-cyt c OR (1.6 and 2 times, respectively). The minimum was reached after 64 days for NADH-cyt c OR (1.4 times less than the initial value). After these falls, regular increase was observed for the three oxidases. LDH activity decreased to 0.2 times the starting value and reincreased slowly after 64 days. In parallel, H-LDH isozyme increased regularly from day 15–92. Myosin isoform conversion followed a similar evolution to muscle A and B, with 75% slow types after 30 days and 100% after 92 days. Histoenzymology showed conservation of muscle structure, disclosed perifascicular atrophy, and complete transformation after 60 days. It must be pointed out that the LD of goat 4 was "aerobic and slow," compared to those of goats 1 and 2; namely 45% of LC2-S and HC1, 1.2 and 1.7 nmol/min^{-1}/mg ww^{1} for NADH-cyt c and succ-cyt c OR, respectively.

Muscle E (goat 3, right muscle) (not shown) was studied in the conditions of muscle D. It presented a less aerobic initial metabolic profile and more isoforms of fast type than goat 4, left muscle. After 15 days of rest and 11 days of stimulation, a rather important adaptation of the contractile machine was observed (28%–53% for LC2-S, and 17%–29% for HCl). A major decrease of LDH activity (from 570–360 nmol/min^{-1}/mg ww^{-1}, and a modest increase in H-LDH isozyme (from 6%–8%) were noticed. Concomitantly, cyt c ox increased from 6.1–8.6 nmol/ min^{-1}/mg ww^{1}, while NADH-cyt c OR increased twofold, and succ-cyt c OR remained unchanged. Accidental arrest in electrostimulation occurred during the 4 days following the first biopsy; modification of the evolution pattern en-

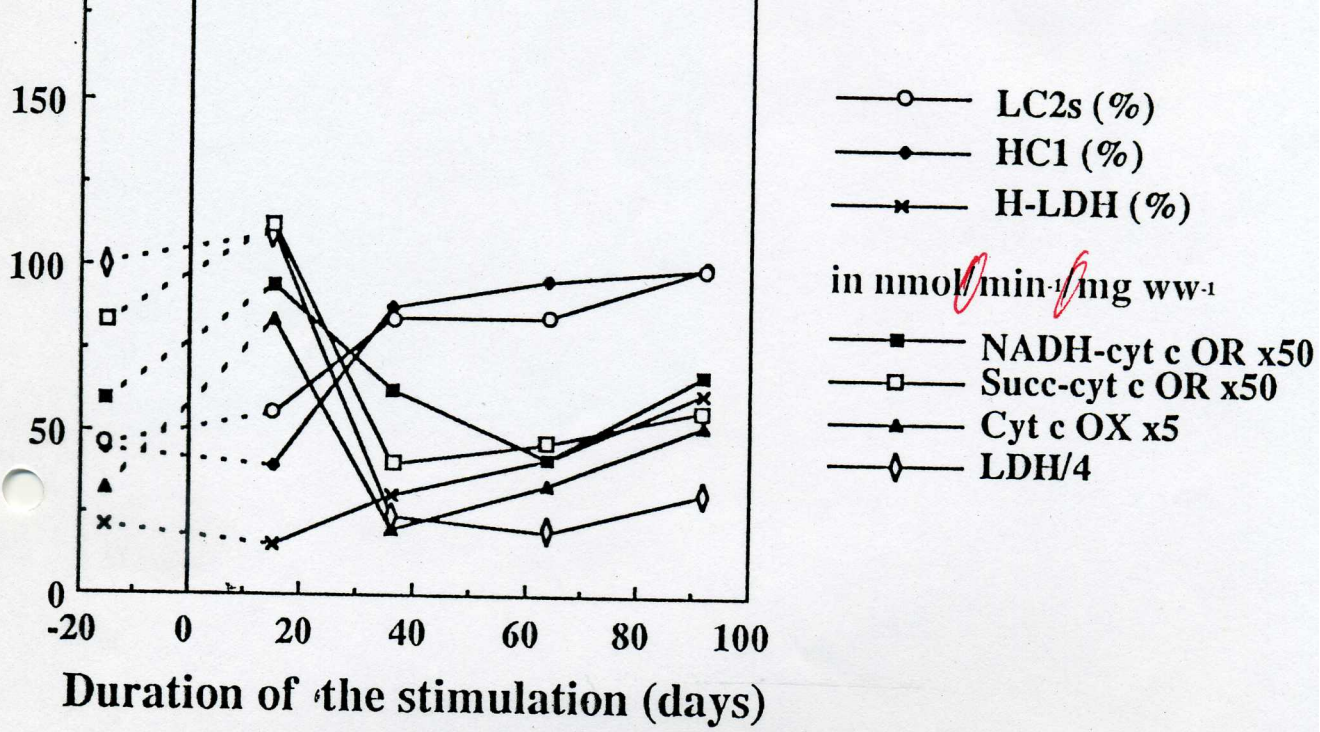

Figure 3: *Muscle D (group 2): time course recording of the same parameters as in Figures 1 and 2. Dotted lines point that data at the onset of stimulation (day 0) were not available. A multiple phases evolution is displayed but time dependence differs from muscles of group 1.*

sued. A decrease in myosin isoform transformation, and an increase in LDH activity (day 51 and 87) were noticed. Nevertheless, the stimulation protocol was resumed, in order to observe long-term events. After 116 days (Fig. 3), LDH activity was one-third the initial value, oxidase activities, probably after an intermediate fall, increased slowly, as well as myosin isoforms and LDH enzymes. The final features were assessed by biopsies. Microscopic observations were rigorously normal, and showed slowly evolving transformation.

Muscle F (goat 3, left muscle) showed a comparable evolution to muscle E.

DISCUSSION

The observations made in this study involve the simultaneous recording of histologic, biochemical, and energetic aspects in two groups of LD undergoing intermittent chronic electrostimulation (intramuscular leads) by regularly sampling the muscles to plot the time course of transformation parameters. Assessment of aerobic potentials were made by screening the mitochondrial respiratory chain complex operating room capacities correlated to other usual measurements. This study emphasized the evolution profiles of these enzymes during fast-to-slow transformation.[11]

In group 1, the time course profiles of our parameters were grossly similar in the studied muscles, but differences in both starting values and time dependence during transformation must be highlighted.

The dispersion of initial values illustrates in-

forms percentage was between 10% and 45% at the initial time). The speed of changes observed (Figs. 1 and 2), providing the workload is similar, and might be determined by the amount of type I fibers. This accounts for the extent of imbalance between demand and supply in muscle energetics, and modulates the stimulus strength inducing the fast-to-slow switch at the metabolic level. This consideration must, however, be tempered by a possible bias at the electrical level (electrode positioning, electric field intensity between anode and cathode).

The time course in both illustrated records of group 1 showed a mitochondrial oxidase increase together with a fall in anaerobic capacities appreciated by LDH activity, as well as an induction of the slow myosin isoforms and H-LDH isozymes. In the two time courses, the increase in oxidative capacity preceded the transformation of the contractile machinery. Peak values were reached when 95% of myosin fast-to-slow transformation (muscle A) and 80% (muscle B) had occurred. These timing differences may be attributed to different starting points, the muscle of goat 2 being more anaerobic. It is interesting to note that full transformation of the contractile machinery is reached after the same period (10 weeks). The increase in aerobic potentials was followed by a fall concomitant with or close to the completion of myosin fast-to-slow switch. This fall yielded to a new steady state between chemosmotic enzymes activities and the contractile machinery.

This phase must not be considered as a maladaptation, but rather as a readjustment of the metabolism to comply with a new contractile machinery endowed with a lower rate of energy consumption per unit force. This multiphase evolution that was evoked by Henriksson et al.[12] is strongly emphasized by our longitudinal observation of LD submitted to increased workload through chronic stimulation.

In muscle A, the fall of oxidase activity is a combination of a normal readjustment phase (phase III), together with the effects of a cessation of stimulation. Evidence of "de-transformation," with increase in anaerobic capacities and al.[13] Herein, it was shown that during this 21-day period, the extent of de-transformation remained modest, and that reinstitution of stimulation quickly reversed the phenomenon.

The fast and unique enhancement of NADH-cyt c OR activity (fivefold) within 4 days, as observed in Figure 2, might be explained by a direct regulation of complex I activity, that is known to be the less active oxidoreductase of the respiratory chain. This may indicate an important control strength of this enzyme in the overall oxidative metabolism in vivo, especially during high energy demand.

Muscle C illustrates the major role of tension[14] on the protein balance at the fibrillar level. Indeed, near complete tension suppression led to muscle degeneration. On the other hand, stretched muscles are known to undergo hypertrophy and fast-to-slow transformation.[15]

The metabolic adaptation observed in the group of less restrained muscles showed a different profile. The increase in oxidase activities was less impressive and, surprisingly, yielded to values smaller than initial ones. Nevertheless, a multiphasic evolution could also be discerned: (1) weak increase of oxidase activities with no modification of the contractile machinery, (2) important fall of aerobic capacity concomitant with fast-to-slow isoforms transformation, and (3) slow progressive increase of oxidases activity by the time the contractile machinery is completely switched.

The profiles of the three phases described in group 1 and 2 are, however, different as far as time dependence and synchronization between energy metabolism and the contractile machinery evolution are concerned. The differences lie in three points: (1) higher oxidases peak activities are reached before or in synchrony with the near complete transformation at the myosin level in group 1; (2) the subsequent decrease of oxidase activities started approximately after transformation of the contractile machinery in group 1, and during this process in group 2; (3) this decrease led to values higher than the initial ones in group 1, and lower than initial values in group 2, but with a slow subsequent increase. A

ered in group 1.

The global muscle behaviour described in Figure 3 is believed to approximate the conditions of a muscle transposed without preconditioning (one-step cardiomyoplasty procedure). Initial conditions are favorable because the muscle contains 45% of slow fibers. Nevertheless, after 36 days, even though the myosin is 80% of the slow type, the aerobic capacity is smaller than the initial one; 90 days are needed to recover the metabolic loss and to complete the contractile machinery transformation (despite the favorable initial features of the muscle). The behavior described in Figure 2 may be analyzed in the context of a two-stage procedure. The muscle initial situation is unfavorable with only 12% of slow fibers. However, after only 32 days of preconditioning, nearly 80% of the myosin is of the slow type, and an important aerobic capacity is already available. Since we have shown (Fig. 1) that the rest period (15-21 days) needed for vascular adaptation and adhesions between the muscle and the heart will not lead to extensive de-transformation, these data suggest an advantage to use pretransformed muscle. Indeed, they display both myosin fast-to-slow shift and high bioenergetic capacities 45 days after the onset of stimulation (Fig. 1). In such conditions they might be able to ensure maximal assistance to a failing heart. In contrast, the muscles of group 2 (Fig. 3), because of discrepancy between energetic potentials and myosin transformation, are in a less optimal situation.

We have observed in goats (unpublished data), as well as in humans,[16] that anastomoses between the thoracodorsal blood supply and the peripheral pedicles coming from intercostal and lumbar vessels exist. This rich vascular anastomotic network between the principle pedicles allow for suppression of the latter during peripheral dissection without ischemia. Data obtained in canines (a species in which the peripheral blood supply is preponderant) demonstrated that the distal blood flow increase observed in response to exercise after collateral vessel ligation was only evident in preconditioned muscles. Together with fatigue resistance, Recently, Chiu et al.[18] showed that after 30 minutes of fatigue test, the force of nontransformed muscle decreased to approximately 40% of the initial level, and remained steady thereafter. These authors postulated there is a progressive decline in activity of type II fibers with residual force only delivered by the fatigue-resistant (type I) fibers, thus a sufficient amount of force is still available for cardiac assistance. The long-term fate of exhausted type II fibers is not known, and in particular, whether they will transform remains unanswered. Chachques et al.,[3] who introduced the concept of one-stage cardiomyoplasty, showed evidence of muscle transformation and excellent long-term functional status when the stimulation protocol was begun on the muscle already applied to the heart.

The analysis of our results for quality and extent of transformation in muscle stimulated in group 1 (normal tension) or in group 2 (reduced tension, condition that simulates, at least in part, the muscle constrained in cardiomyoplasty) showed that similar long-term values for mitochondrial oxidases might well be reached, and that complete histologic transformation occurred. Muscle D (in group 2) showed, however, moderate signs of perifascicular atrophy. Because oxidative capacities were higher in group 1 during acquisition of the contractile machinery, and because signs of moderate atrophy were never observed in that group, preconditioning in situ may be preferable.

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