Type II to Type I Transformation of Chronically Stimulated Goat Latissimus dorsi Muscle: A Histoenzymological, Biochemical, Bioenergetic, and Functional Study

Abstract

Five goat latissimus dorsi muscles (LDM) were submitted to a progressive chronic electrostimulation program to reach an integrated understanding of the fast-to-slow transformation process in large mammals. LDM were regularly sampled and followed during a period of 8 months. Each sample was simultaneously assessed for histoenzymological study, myosin and LDH isoforms and bioenergetic capacities [NADH dehydrogenase cytochrome c oxidoreductase (NADH Cyt c OR), succinate dehydrogenase cytochrome c oxidoreductase (Succ Cyt c OR), cytochrome c oxidase (Cyt c Ox) and LDH]. Such muscles were also tested with and without completion of II to I transformation for their mechanical properties in isometric and isotonic strain gauge testing. The conversion of fast-to-slow myosin monitored by heavy chain (HC I) and light chain slow component (LC5s) began a few days after stimulation and was almost 100% after 100 days. The H-LDH isoforms evolved similarly but did not reach 100% conversion after 200 days. The activity of respiratory chain oxidases increased within 36 h but to a variable extent and peaked after 32 days, corresponding to a 75% transformation of myosin compared to initial levels. NADH Cyt c OR, Succ Cyt c OR, and Cyt c Ox, respectively increased 10-, 5- and 5-fold. These activities then significantly decreased before the completion of the myofibrillar transformation and reached a plateau with stable activities that remained 2- to 3-fold higher than the unstimulated LDM. LDH activity sharply decreased until day 62 (5-fold) and then plateaued. Functionally, muscle showed a reduced speed of contraction and moderate reduction in power output but had become fatigue-resistant. This study documents the transformation process in large mammals and suggests the dynamic relation between workload, aerobic-anaerobic metabolism and the contractile myofibrillar system.

Key Words
Latissimus dorsi
Muscle transformation
Mitochondrial oxidases
Myosine
LDH
Chronic stimulation

Introduction

The distinction between fast-twitch and slow-twitch skeletal muscle is basically histological, biochemical, and mechanical. Fast-twitch muscles (mostly populated with type II fibers) are involved in brief phase activity and are characterized by a pale aspect. Fatigue-prone type II fibers have an energetic supply mainly from anaerobic glycolytic metabolism.
involved in postural tonus and are capable of sustaining tonic contraction without fatigue. They have a red aspect due to their cytochrome and myoglobin contents and their higher blood supply, and are constituted of type I fatigue-resistant slow-twitch fibers. They are characterized by a high vascular and mitochondrial content. Such muscles rely on aerobic oxidative metabolism for their energy supply. These contractile properties are strongly related to the myosin isoforms present in the fibers and to their ATPase activity [1].

Most muscles, however, such as the LDM, exhibit an intermediate profile and are composed of a mixed population of type II (predominant) and type I fibers. Following the experimental work of Buller et al. [2], it was progressively realized that the phenotypic aspect of muscles and their fiber content may be susceptible to deep modifications as a consequence of modified neural influx or chronic stimulation. This was interpreted as an extraordinary adaptive potential of muscles to cope with increased functional demand. It was shown that a fast-twitch fatigue-prone muscle may acquire the features of a slow-twitch fatigue-resistant one through a complex transformation of type II to type I fibers [3]. These new physiological properties are obtained through deep changes in metabolic pathways, vascular supply and myofibrillar protein isoforms. This remarkable plasticity, together with progress in cardiac stimulation, led to utilizing transformed muscles as power generators in different sorts of cardiac assist configurations [3–6]. Although the plasticity exhibited by striated skeletal muscles and the fast to slow transformation process have been extensively studied [3], little information is available for larger mammals (apart from dogs and sheep) [4, 7–

**Material and Methods**

**Material**

Five adult female goats (weight 30–50 kg) were used. The animals were cared for according to guidelines published by the National Institutes of Health. Sedation was obtained by intramuscular (IM) administration of xylazine, and anesthesia was induced by ketamine IM. The animals were intubated and placed under mechanical ventilation [12]. 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 neuro-
Histological Methods

The specimens for histochemistry were sectioned, 12 μm thick in a cryostat, and then routine hematoxylin-eosin stain, ATPase stain at pH 4.1 and 9.4 [14], and NADH tetrazolium reductase stain were done. The percentage of type I and II fibers was determined, as well as a routine microscopic examination. Electron microscopic examination was done in fully transformed muscles.

Biochemical Methods

For the biochemical analyses, muscle samples of approximately 0.05 g were immediately weighed and finely cut in 10 vol of a solution containing KCl 0.05 M, Tris 0.01 M, dithiothreitol (DTT) 0.001 M, NaF 0.005%, glycerol 50% (v/v), pH 7.5, kept 24 h at 4°C with occasional mixing and stored at −18°C for at least 8 days before use. After centrifugation (20 min, 39,000 g), the supernatant containing crude sarcoplasm-0.01 M, NaHCO₃ 0.04 M, MgCl₂ 0.001 M, NaCl 50/0.01 M, β-mercaptoethanol 0.002 M, pH 8.8 for 5 min in a mortar with sand and precipitated by 10 times dilution at pH 6.3. The pellet of acryomysin was disassociated in a sodium dodecyl sulfate (SDS) solution (Tris 0.0625 M, SDS 0.69 M, glycerol 10%, β-mercaptoethanol 5%; pH 6.8) and heated at 100°C for 2 min.

Polyacrylamide gel electrophoresis (PAGE) was performed on vertical slab gels (18 × 8 × 0.2 cm). LDH isoenzymes were separated on continuous PAGE and enzymatically revealed according to Leberer and Pette [15]. Myosin heavy chain subunit (HC) separation was carried out on high porosity gels according to Danieli-Betto et al. [16]. To improve the resolution of fast-type myosin heavy chains HC Ila and HC Iib, the concentration in acrylamide of the separation gel was adjusted to 8% and the concentration in glycerol to 45%(v/v). Electrophoresis was run at 10 mA (constant current) for 16 h. Myosin light chain subunits (LC) were resolved on 20% acrylamide gels at pH 8.4 according to Laemmli [17].

Gels were stained with Coomassie brilliant blue R-250 (Serva). Densitometric tracings were obtained with a Helena Quick-Scan densitometer (Beaumont, Tex.). The relative amounts of each LDH isofrom or myosin subunit were calculated from the integrated values of the peak areas. The amount of H monomer of LDH isoenzymes was computed with the method of Thorling and Jensen [18].

Bioenergetic Methods

Mitochondrial oxidative activities were measured with spectrophotometry at 30°C (Aminco Chance DW 2UV/VIS spectrophotometer, FLM, Urbana, III) according to Schneider et al. [19] for NADH dehydrogenase, cytochrome c oxidoreductase (NADH Cyt c OR), and for succinate dehydrogenase, cytochrome c oxidoreductase (SuCe Cyt c OR), and according to Möller and Palmer [20] for Cyt c Ox. The LDH activity was determined according to Bergmeyer and Bernt [21] at a pyruvate concentration that does not inhibit H isoenzymes (0.5 mm).

Electron Microscopy

Fresh biopsy samples before and after LDM transformation were immersed in a fixative solution (2.5% glutaraldehyde (LADD) in 0.1 M phosphate buffer pH 7.4) for 90 min, changed 3 times at 4°C and postfixed in 2% osmium tetroxide in the same buffer. They were rinsed, dehydrated and embedded in an Epon mixture.
Strain Gauge Test
In anesthetized goats, the left LDM was dissected with preservation of the neurovascular bundle. Stimulating IM electrodes were implanted. Muscles were maintained in a warm atmosphere (± 30°C). For isometric testing, the muscle tendon and its distal part were grasped in a mechanical device permitting a progressive augmentation of the length of the muscle thus modifying the starting length or preload. Passive and total tension were obtained via an FT10 gauge (Grass Instrumental Company, Quincy, Mass.). For isotonic measurements, the muscle was fixed at the tendon and its distal part was attached via the FT10 force displacement transducer to selected loads. The muscle was allowed to slide in a horizontal plane on a smooth silastic surface to minimize friction effects. For each given load, the muscle distension (starting length) was established and the shortening during contraction as well as the speed of shortening were recorded (Tektronix Amplifier AM502 and Gould TA200 recorder). Muscles were stimulated with 6 pulses (30 Hz, 185 ms) and a voltage corresponding to twice the full recruitment threshold leading to a supramaximal contraction. The fatigue test consisted of muscle stimulation in isometric conditions at 6 pulses, 5 V, 90/min. With isotonic measurement, the working capacity (W, expressed in J) was computed for each preload via the formula:

\[ W = F \cdot d = m \cdot g \cdot d, \]

where m = mass, g = gravity and d = shortening.

The power (P, expressed in W) was derived from the formula:

\[ P = \frac{W}{t} = \frac{m \cdot g \cdot d}{t} = m \cdot g \cdot v, \]

where m = mass, g = gravity and \( v = \frac{\Delta x}{\Delta t} \) = instantaneous velocity.

Results
Histoenzymeology
The normal goat LD muscle (LDM) is composed to a variable extent of fast type II fibers (± 75%) and slow type I fibers (± 25%).

Biochemical and Bioenergetic Results
The electrophoretic distribution of myosin heavy chains and light chains in the goat LDM is shown in figure 2a and was compared to the corresponding subunits from the cardiac muscle. The low-percentage PACE, in the presence of glycerol, made possible a good separation between the different myosin heavy chains. In the unstimulated LDM three bands were visible: the faint ahead-migrating band was the slow-fiber myosin HC I while the two slower ones corresponded to fast-fiber myosin HC IIb and IIa. After 4 months of stimulation, the LDM myosin exhibited only HC I clearly pointing out the complete replacement of fast fibers by their slow counterparts. This latter electrophoretic pattern was identical to that of ventricular muscle. The atrial muscle HC isoform seemed to migrate a
little slower although the phenomenon was indiscernible in comigration experiments. The different isoforms of myosin light chains were discriminated by high-percentage PAGE of the goat actomyosin (fig. 2b). The unstimulated LDM showed a large content of fast light chains LC1f and LC2f and a trace of LC3f. Both slow light chains (LC3s and LC5s) were also well separated. Our light chain identification was confirmed by previous work on sheep myosin [7]. Low amounts of TN-I contaminating the actomyosin preparations comigrated with LC1 (TN-I with LC1f and TN-II with LC3s); therefore, no precise evaluation of these latter light chains was possible. Because LC3f and LC3s were electrophoretically better discriminated and free from contaminant, the relative percentages of these two light chains were used in the computation of the fast- and slow-fiber proportions during the time course of muscle transformation. In the transformed LDM, the fast myosin chain LC3f had disappeared as did the fast myosin heavy chain, whereas some LC1f was still visible. The faint band that remained at the level of LC3f did not exactly comigrate with LC2f and could have been a contaminant protein. The comparative analysis of ventricular and atrial myosin light chain pattern revealed the presence of a similar LC2 (LC2v and LC2a) corresponding to the skeletal muscle LC2s isoform. By contrast, the ventricular and atrial muscle LC1 were distinct from each other but also when compared with both skeletal LC1f and
confirmed by PAGE where proteins are discriminated according to their electrical charge [data not shown]. The results indicated that the myosin extracted from transformed LDM muscle were similar to the myosin present in the slow fibers from the unstimulated muscle but clearly differed from ventricular or atrial myosin at the level of the LC\textsubscript{1}f light chain.

The time course of the transformation of myosin heavy and light subunits in five muscles is illustrated in figure 3a and b. In the five muscles studied, the transformation of the contractile proteins was initiated a few days after stimulation and regularly progressed to reach a 90–100% conversion after 60–100 days. The myosin light chain LC\textsubscript{2}S followed the time course of HC I. The increase of HC I was the mirror image of HC IIa which almost disappeared after 60 days. The amount of H monomer in LDH tetrameric isoenzymes increased steadily but never reached more than 70–80% after 7 months (fig. 3c). The electrophoretogram [data not shown] displayed the sequential development of the transformation of myosin heavy chains: the progressive replacement of HC IIa by HC I and disappearance of HC IIb (HC IIb → HC IIa → HC I).

Cessation of the stimulation after complete transformation (100% of HC I and LC\textsubscript{2}S) induced little if any regression towards the fast-type myosin. The reversibility in LDH conversion looked more pronounced. In any case, the prearrest aspect was quickly restored when the stimulation was continued.

The enhancement of mitochondrial oxidases was already observed 36 h after onset of stimulation. However, each complex had a characteristic time course. NADH-Cyt c OR, Succ Cyt c OR, and Cyt c Ox peaked between 30 and 100 days, and declined thereafter. This progressive decrease ultimately led to stabilized values 2–3 times higher than initial levels (fig. 4). When the terminal mitochondrial activities were expressed as ratio of LDH activity, to emphasize the relative capacities of oxidative and glycolytic anaerobic metabolism, three distinct profiles could be discerned. The NADH Cyt c OR/LDH evolved in a multiphasic pattern characterized by a fast enhancement (3–4 times) within 3 days after stimulation, then a plateau phase followed after 14–21 days by a subsequent increase (fig. 5a). The Succ Cyt c OR/LDH showed a linear increase with individual dif-
**Fig. 3.** Time course of the synthesis of the slow myosin heavy chain HC I (a) and light chain LC2-s (b) subunits and LDH-H monomer (c) expressed as percentages of the respective subunit or monomer total content. The black arrows represent cessation of the stimulation and the white arrows continued stimulation.

**Fig. 4.** Time course of the myosin subunits (HC I, LC2-s), LDH total activity, H-LDH isoform content (all %) and mitochondrial oxidases activities (all nmol·min⁻¹·mg WW⁻¹) during stimulation, and its cessation after 216 days in 1 goat.

**Fig. 5.** Kinetic aspects of, NADH Cyt c OR/LDH (a), Succ Cyt c OR/LDH (b) and Cyt c OX/LDH during the early phase of progressive chronic stimulation. Pulses refer to the number of aggregated impulses delivered to the muscle at each stimulation. (c). d Evolution of LDH activities.
Fig. 6. Histograms illustrating the relative percentages of myosin heavy and light chains as well as H-LDH monomer contents of the whole LDM samples. **a** Mean values of 3 unstimulated LDM (3 goats). **b** Values of 1 transformed L.D. **c** Schematic representation of the whole left LDM localizing the analyzed samples (1–13) and the area of biopsies used in the time course of the muscular transformation study (hatched area).

Differences of the slopes (fig. 5b). The Cyt c OX/LDH ratio followed a sigmoidal course (fig. 5c). At 75% conversion of HC I, mitochondrial oxidases peaked and decreased soon after. When the stimulation was suspended during this phase, the decrease of maximal activities was accelerated, but easily reversed by reinstitution of stimulation. A similar behavior was observed when cessation occurred during the plateau phase [data not
The close examination of the fiber compositions in the native LDM used in this study obviously showed quite a broad fluctuation of the type II fiber population. Thirteen uniformly distributed muscle samples (approximately 1 g, from outer to inner muscle surface; fig. 6c) from three unstimulated and one transformed muscles (4 goats) were analyzed for the initial fiber composition and the homogeneity of the fiber repartition following complete transformation.

Before stimulation, the mean values (n = 3) of myosin subunits (HC IIb: 15.2%, SD: 2.9; HC IIa: 65.7%, SD: 9.3; HC I: 19.1%, SD: 6.9; LC2s: 29.5%, SD: 5.8) and H-LDH monomers (11.6%, SD: 2.7) indicated a fairly homogeneous fast aerobic muscle type rich in type IIA fibers (fig. 6a). The amount of slow myosin subunit LC2s (29.5), greater than that of HC I subunit could imply the presence of IIA fibers with hybrid myosin. The comparatively low amount of HC IIb (below 20%) was relatively constant within the muscle mass, while an inverse relation was observed between the percentage of subunits HC IIa and HC I. The HC IIa was the most represented subunit within the goat LD. Only the ventral and dorsal edges differed from the general pattern. The upper border contained a greater amount of slow-type fibers rich in HC I, LC2s and H-LDH monomers, while the lower border contained HC IIb fibers.

Functional Assessment

Control LDM behaved in isometric conditions as predicted by the length-tension curve (Frank-Starling relationship). The isometric tension was a function of prestretch until a threshold was achieved. Beyond the threshold, any increase in starting length resulted in a fall of the global tension. The global isomet-
Fig. 7. Longitudinal sections of goat LDM as demonstrated by electron microscopy before (a) and after (b) II to I transformation (× 10,600).

In isotonic conditions, the muscle power output was calculated taking into account the speed of contraction, displacement and mobilized mass. The results showed that the power output depends on the preloading conditions (fig. 8b) and the stimulating pattern [data not shown]. Transformed muscle displayed similar gross behavior both in isometric and isotonic conditions. However, their contraction velocity was decreased (± 50%) and they were fatigue-resistant. Preliminary
Fig. 8. a Plot of total force (curve 1), passive force (curve 3) and active force (curve 2, obtained by subtraction of curve 3 from curve 1). Graphic representation of velocity, work and power at different preloads for muscles tested in isotonic conditions and stimulated with 6 impulses (30 Hz, 185 ms) at V. b Native LDM. c Chronically stimulated and transformed LDM.

data suggest that the power output was moderately reduced by the fast to slow transformation initiated by our stimulation protocol (≤ 50%), apparently owing to a reduction in muscle velocity. Residual power was approximately 120 mW for a 180-gram muscle (fig. 8c).

Discussion

The fast to slow transformation process has not been studied in detail in large mammals (apart from dogs). Goats are considered exemplary models for the study of both basic questions and surgical techniques [10, 12]. This multidisciplinary time course study was
Following electrostimulation, muscles displayed an orderly sequence of changes involving structural, functional and molecular aspects of the fibers. Metabolic adaptations appeared to precede changes in myosin isoforms (thick filament), agreeing with a previously reported sequence of events [28]. Electron microscopy demonstrated a marked augmentation of mitochondrial volume and density, illustrating the important increase in aerobic oxidative capacities (fig. 7). Chronic stimulation also modified the isoenzyme pattern of the LDH. The normally predominant M-type isoenzyme was progressively replaced by the H-type isoenzyme with a lower enzymatic activity. This transformation however was incomplete (80%). The replacement of fast heavy and light chain myosin subunits by their slow counterparts, monitored by the appearance of slow HC I and LC₂s, evolved simultaneously within 60–100 days of stimulation. As first shown by Brown et al. [29] in stimulated rabbit muscles, the replacement of the LC₁f by the LC₁₅ did not occur synchronously with that of LC₂ but appeared later. The heavy chain transformation followed the transition sequence described by Maier et al. [30] and Pette [31]: HC IIb → HC IIA → HC I. The heavy chain HC IId, usually found in fast oxidative muscles such as the diaphragm, was not detected in goat muscles. The myosin slow isoform found in transformed goat LDM was similar to that present in the unstimulated slow-twitch fibers. This was consistent for both heavy and light chains. These observations contradict the report of Chachques et al. [4]. The slow myosin isoform from transformed LDM differs from the ventricular isoform with respect to molecular weight and the isoelectric point of LC₁f. Moreover, unlike

Eur Surg Res 1996;28:80–95
suggests in agreement with Brown et al. [39] that the changes in myosin synthesis do not recapitulate the developmental sequence.

The time course of the aerobic/anaerobic relative activities (fig. 5a) is characterized by the early enhancement (2–4 days) of NADH Cyt c OR. This can be explained by a direct activation of complex I that may indicate an important control strength of this complex in the overall oxidative metabolism in vivo, especially during high energy demand. Indeed, this enzyme must turn over the reducing equivalent NADH produced in the cytosol and entering the mitochondria via the malate-aspartate shuttle. In contrast, the lag period observed in the Cyt c Ox profile might indicate that the activity of this enzyme correlates closely with the neoformation of mitochondria (fig. 5c). The lack of a strict correlation with the number of impulses suggests that these patterns of evolution are part of a metabolic response to the increased workload as a result of chronic stimulation rather than a response to the three steps of the stimulation protocol.

The integrated analysis of the markers of oxidative metabolism (mitochondrial oxidases), glycolytic-anaerobic metabolism (LDH isozymes and LDH activity), and myosin isoforms (HC I and LC3s) suggests a development in three phases of muscle experiencing an increased workload. (1) The initial response (1–2 weeks) after initiation of chronic electrostimulation consists of an immediate enhancement of oxidative capacities with a modest transformation of the contractile proteins (phase 1). (2) This is followed by the development of a slow contractile apparatus (containing myosin HC I and LC3s) while the oxidative capacities still increase to reach their peak values between days 20 and 35.
which had been completely transformed at the myofibrillar level, the effect of cessation of stimulation was studied. Muscle oxidase activity was either in the plateau (phase III) or decreasing phase (phase II). In each case, a reversal was observed after 3 weeks when the LDH isoforms and the markers of aerobic metabolism moved towards prestimulation values. The LDH activity increased, the activity of the mitochondrial oxidases decreased, whilst the myosin isoforms showed a very limited return to the pattern of untransformed LDM. Histoenzymology also confirmed this reversal with the reappearance of type II fibers in a muscle previously populated by 100% type I fibers. This was promptly reversed by reinstitution of stimulation. These data illustrate the reversibility of the transformation phenomenon and demonstrate the linkage between metabolic-structural adaptation and functional demand.

The mechanical and functional evaluation of muscles submitted to similar protocols was performed to validate the teleological hypothesis related to fatigue resistance. One main

Acknowledgements

This work was supported by the research grants No. 3.4542.91 and No. 3.4514.93 from the Belgian Fonds de la Recherche Scientifique Médicale (FRSM) and research grants FRFC No. 2.4503.91 and Loterie Nationale No. 9.4578.91. B.F. is Research Associate of the Belgian FNRS.

We are grateful to Professor G. Goessens for the ultrastructural analysis of LD samples and to Professor D. Serteyn from the Department of Veterinary Surgery for his collaboration. Special thanks go to Miss Delouse for the typing of the manuscript and to Miss Delycur for valuable laboratory assistance.

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Eur J Surg 1996;28:90-95