

Effects of Long-Term Stimulation on Skeletal Muscle Phenotype Expression and Collagen/Fibrillin Distribution

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Abstract

The effects of chronic electrical stimulation on muscle fiber phenotype and metabolism are well known, but its impact on the extracellular matrix is poorly understood. Material properties of skeletal muscle are largely influenced by the viscoelastic properties of connective tissues which occupy the interstitium. Changes in collagen and fibrillin content may therefore play a key role in muscular adaptation processes. Latissimus dorsi (LD) of eight rabbits were used to study muscular adaptation to long-term electrical conditioning. Muscles were conditioned using burst stimuli delivered over 6 or 12 weeks. Contralateral LD were used as control. Stimulation produced marked reductions in maximum isometric force, but improved endurance capacity due to increased percent cross sectional area (CSA) occupied by slow-twitch oxidative muscle fibers. Stimulation also increased percent CSA occupied by type I collagen and fibrillin. In contrast, the amount of type III collagen and fast-twitch glycolytic fibers decreased in stimulated muscle. These data suggest that muscular adaptation to long-term stimulation includes both alterations in fiber type expression and remodeling of the extracellular matrix.

Key words: collagen, extracellular matrix, fibrillin, skeletal muscle, stimulation.

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Skeletal muscle is highly adaptable in that its metabolic and contractile characteristics are largely regulated by its pattern of use. Muscles used intermittently against large loads contain mostly thick, fast-twitch glycolytic (FG) fibers which are powerful but prone to fatigue. Muscles used against lighter loads over prolonged periods retain thinner, slow-twitch oxidative (SO) fibers which are less powerful but more fatigue-resistant. Several studies have shown that muscle phenotype can be manipulated via chronic electrical stimulation to enhance fatigue resistance at the expense of contractile power [14, 17, 23]. This property (termed "muscle plasticity") has prompted many researchers to investigate the possibility of using conditioned skeletal muscle as an endogenous power source for cardiac assist [1, 16, 24].

Although many aspects of muscular adaptation to long-term stimulation are well-known, several facets remain unexplored. Changes in fiber-type, metabolic activity, muscle mass, contractile strength, and speed have been documented in numerous reports [6, 22, 25], but little attention has been paid to alterations within the extracellular matrix. This is an important aspect of the adaptation process since the mechanical properties of muscle tissue are

heavily influenced by the interstitium which binds individual muscle cells into a cohesive unit.

Collagen types I and III are the major forms of interstitial collagen found in both skeletal muscle and heart tissue [8, 18] and have been shown to strongly influence tissue extensibility [2]. Type III collagen is also the main component of connective tissue in non-capillary blood vessels [7] and hence, collagen staining techniques can be used to identify small vessels in the muscle interstitium [4]. A network of elastic fibers in the extracellular matrix provides muscle tissue with the resilience required to recoil after a transient stretch. These flexible fibers comprise an amorphous core of elastin surrounded by microfibrils, of which fibrillin is the primary element [10]. The amount and relative proportion of these interstitial components has been shown to change with exercise training [9]. Hence, it is reasonable to hypothesize that chronic electrical stimulation might also alter collagen and fibrillin content in skeletal muscle.

This study is designed to test this hypothesis using electrically-stimulated rabbit latissimus dorsi (LD) muscle. The objective is to quantify changes in LD morphology (both intracellular and extracellular) brought

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about by the conditioning process and to determine how these structural changes relate to muscle function and long-term work capacity.

Materials and methods

Animal model and surgical procedure

Experiments were performed on eight female New Zealand white rabbits (2.5-3.5 kg) in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institution of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication 85-23, revised 1985). Rabbits were randomly assigned to one of two groups of four: 1) 6-week stimulation (6-wk), and 2) 12-week stimulation (12-wk). Sterile techniques were used for all operative procedures. Medtronic Irel neuromuscular stimulators (Model 7420) were used with custom paraneural leads (U.S. Pat. No. 5,158,097) to condition the muscles. Animals were pre-anesthetized with xylazine and ketamine, intubated, and placed on a ventilator. Anesthesia was maintained with a mixture of 50% oxygen, 48-49% nitrous oxide, and 1-2% isoflurane. Acepromazine and atropine were given to control secretions. EKG monitoring was performed in each case. A lateral incision was made in the skin and subcutaneous muscle along the left hemithorax. Hemostasis was achieved without the use of electrocautery. The proximal medial border of the LD muscle was dissected free from adjacent structures and elevated from the chest wall by gentle retraction. The thoracodorsal nerve branch entering the insertion of the LD muscle was identified and the cathode of the paraneural electrode sutured to the muscle fibers adjacent to the neural bundle. The LD muscle was secured in its native location in preparation for *in situ* stimulation. The stimulator was tunneled into the abdominal subcutaneous tissue and the incision closed in the standard fashion. Antibiotics were given preoperatively and for three days postoperatively.

Stimulation parameters

The left LD from each animal was stimulated for either 6 or 12 weeks while the right LD served as an unstimulated, paired control. Muscles were conditioned using burst stimuli delivered at a rate of 53 pulse trains per minute for 24 hours/day. Burst frequencies were increased gradually from 10 to 25 Hz over the first four weeks to prevent stimulation-induced muscle damage. Other stimulator settings remained constant throughout the training period and were set as follows: pulse amplitude = 2.0 volts; pulse width = 210 μ sec; burst duration = 250 msec; inter-burst interval = 880 msec.

Muscle mechanics testing

Upon completion of the stimulation protocol, the animals received premedication similar to that used for surgery, without endotracheal intubation. Muscle peak isometric force was tested over five contractions with the same stimulation pattern used for muscle conditioning (each burst comprising 7 pulses separated by an interval of 40 msec). Muscle contractile energetics and fatigue resistance were determined under the same stimulation conditions for 40 minutes. Each study employed a custom skeletal muscle ergometer designed to impose isometric or near isotonic loading conditions on the muscle as described previously [5].

This apparatus enables the measurement of linear motion under conditions of active shortening and passive extension. The muscle load comprised a stack of weights attached to the LD by a thin cord traversing a stationary pulley. Motion was measured with a pair of sonomicrometry crystals mounted beneath the rack on telescoping, fluid-filled pipettes. Forces were measured through a strain gauge mounted between the load and the muscle. The load level was chosen to approximate the peak tension generated by the normal human myocardium (which ranges from about 1.0-2.0 N/cm²). Because the cross-sectional area of the muscles under investigation average 1.5-2.0 cm², the afterload applied during these tests was 1.5 N/cm² x 1.75 cm², or 2.6 Newtons (roughly 265 gf). To ensure that this load was transmitted directly to the LD, the lead cable of the ergometer was secured to the LD origin via Teflon felt sutured to the cable. Each animal was held in position to minimize body displacement in response to muscle contraction against the load.

Tissue preparation

Muscles harvested from both sides of all animals underwent the same set of studies. Two biopsies (0.5-1.0 cm²) were taken from the distal, proximal, anterolateral, and posterolateral areas of LD muscle. These samples were used for histochemistry, immunofluorescence and biochemistry determinations.

Histochemical analysis for muscle fiber types

Samples were frozen in 2-methylbutane cooled with liquid nitrogen. Serial 8-mm cross sections were cut on a cryostat microtome at 21°C, mounted on a coverslip, and air dried. Cross sections were subsequently stained for ATPase activity (preincubation at pH 4.3, 4.55, and 10.4) for fiber type identification and nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) activity as an indicator of oxidative potential [3, 11]. Fibers were identified as fast-twitch glycolytic (FG), fast-twitch oxidative glycolytic (FOG), and slow-twitch oxidative (SO). Identification of 300-500 fibers from each sample were made and presented as percentages. Fiber cross sectional area was measured using myosin

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ATPase stained with preincubation pH 4.55. Muscle cross sections were divided into 4-5 evenly spaced regions, depending on the size of the sample. Representative fascicles with fibers cut perpendicular to their long axes were measured with the use of an OPTIMAS 6 image processing system. This system consists of a microscope with an attached camera coupled to a Compaq personal computer, optical mouse, and an image processor.

Immunofluorescent analysis for collagen and fibrillin

Fresh frozen sections were cut on a cryostat, placed on slides and put into -20°C acetone fixative for 10 minutes. The fixative was removed with a 10 minute wash in (1X) PBS. The slides were then placed into a humidity chamber and the tissue blocked with (1X) PBS containing 5% normal goat serum at room temperature with gentle rotation. The block was tapped-off by (1X) PBS washing. Three kinds of antibodies were used: monoclonal anti-type I collagen (Sigma C-2456), monoclonal anti-type III collagen (Calbiochem CP19), and polyclonal anti-fibrillin (EPC PR210). The tissues were incubated with antibody (or 4% BSA/PBS alone for negative control) for three hours at room temperature in a humidity chamber. Unbound primary antibodies were removed with 3-fold (1X) PBS washing. Tissues were then incubated with goat anti-mouse secondary antibody (Sigma F-4143) conjugated to FITC for 30 minutes at room temperature. Following 8-fold washing and fixing with mounting medium, tissues were examined by fluorescent microscopy using 450-490 nm filter [21]. The OPTIMAS 6 image processing system was used to determine the location of these proteins and measure the percent of muscle CSA made fluorescent via this technique. The relative proportion of the muscle cross section made fluorescent via antibody binding was used as an indicator of change in collagen/fibrillin content. Because this technique has not been validated for measuring absolute amounts of these proteins present in skeletal muscle, changes in fluorescence were taken only as gross indicators of alterations in collagen and fibrillin concentrations.

Biochemical analysis for metabolic enzymes

Samples were frozen and pulverized in liquid nitrogen for enzyme analysis. Enzymes involved in glycolysis (phosphofructokinase [PFK] and lactate dehydrogenase [LDH]), terminal oxidation (citrate synthase [CS] and malate dehydrogenase [MDH]) and β -oxidation (3-hydroxyacyl CoA dehydrogenase [HADH]) were determined spectrophotometrically and in duplicate. Enzyme activities were expressed as mmol/g-muscle/min [12, 13, 15, 20].

Statistical analysis

Results are expressed as means $\pm$ SE unless otherwise stated. Data were analyzed using one-tailed ANOVA with non-repeating measures and Duncan's multiple

range test. The use of one-tailed analyses is justified since the direction of change for those parameters tested are well documented from prior studies of electrically conditioned skeletal muscle [3, 6, 14, 23]. Differences were considered significant at the $p < 0.05$ level.

Results

Isometric contraction and fatigue resistance

Muscle stimulated for either 6 or 12 weeks produced a marked reduction in maximum isometric force (Figure 1). Comparison of maximum isometric force generation with control muscles shown a 44.3% overall decrease in the 6-week group and a 48.8% decrease in the 12-week group. Strength differences were not significant between the two stimulation groups. Stimulated muscle groups displayed significantly improved endurance capacity relative to control, as illustrated in Figure 2. Stroke work was reduced by 67% after the first five minutes of testing and then dropped 94% by the ten-minute mark in the control group. At the conclusion of the 40 minutes test, control muscles retained only 2% of their initial work capacity. Conditioned muscles were fatigued by 8% in the 6-week group and 18% in the 12-week group after the first five minutes of testing and retained 66% (6-wk) and 57% (12-wk) of their initial stroke work at 40 minutes.

Muscle fiber types

Muscle fiber composition and cross-sectional area data are shown in Table 1. The percentage of SO fibers in both 6-wk and 12-wk groups was higher than control. Conversely, the percentage of FG fibers in 6-wk and 12-wk groups was lower than control. Differences in FOG fibers were not significant between stimulated and con-

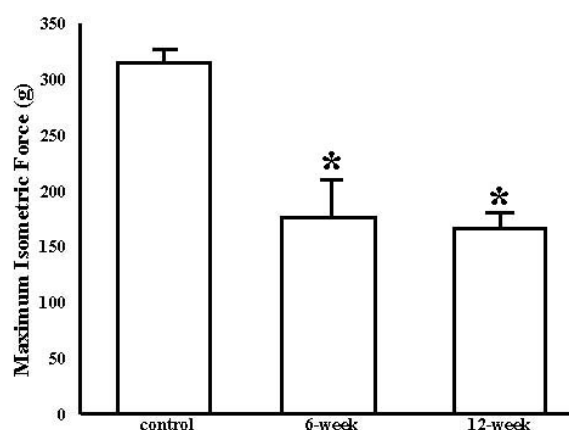


Figure 1. Peak isometric force generated by control and stimulated muscles. Long-term stimulation decreased force generation by 44.3% in the 6-wk group and by 48.8% in the 12-wk group relative to control. Strength differences were not significant between the two stimulation groups. *Statistically significant change relative to control ($P < 0.05$).

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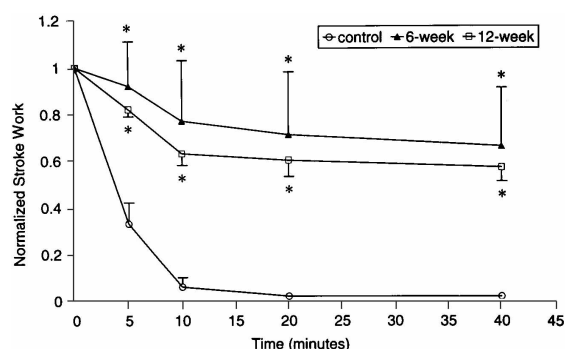


Figure 2. Relative endurance characteristics of control and conditioned LD muscles. Stroke work is normalized to initial work levels and is charted over 40-minutes of continuous activation at 53 contractions per minute. The two conditioned muscle groups displayed significantly improved endurance capacities, retaining 66% (6-wk) and 57% (12-wk) of their initial stroke work compared to 2% for control. *Statistically significant change relative to control ($P < 0.05$).

control groups. Typical fiber-type distributions for control and conditioned groups are shown in Figure 3. There were no differences in the CSA of SO fibers among the three groups. However, the CSA of FOG fibers was decreased in the 6-wk group and the CSA of FG fibers was reduced in both 6-wk and 12-wk groups.

Metabolic enzyme analysis

Muscle enzyme activities measured in LD muscle are presented in Table 2. PFK and LDH activities were reduced by both 6-wk and 12-wk stimulation. Conversely, MDH was enhanced in both stimulation groups. Significant increases in CS and HADH activities were seen only in the 12-wk group.

Table 1. Muscle fiber type and cross-sectional area of rabbit LD muscle in control, 6-wk, and 12-wk burst stimulation.

	Percent Muscle CSA Occupied			Mean CSA of Fibers (mm ²)		
	control	6-week	12-week	control	6-week	12-week
SO	17.9±2.4	59.0±5.8*	62.3±6.7*	2724±241	2233±140	2321±193
FOG	19.9±2.2	28.3±2.4	28.5±7.0	3068±140	2376±138*	2465±57
FG	62.3±4.3	12.8±4.4*	9.3±2.2*	3904±102	2526±179*	2294±150*

Data are mean ± SD. * Statistically significant change relative to control ($p < 0.05$).

Table 2. Muscle enzyme activities of rabbit LD muscle in control, 6-wk, and 12-wk burst stimulation.

	PFK	LDH	MDH	HADH	CS
control	31.8±5.9	474.8±68.4	114.0±10.8	4.0±0.6	6.6±0.7
6-week	9.0±2.4*	139.0±22.8*	157.0±11.7*	3.9±0.3	10.4±1.2
12-week	11.6±13.3*	162.5±26.5*	234.5±13.0*= ^a	6.0±0.6*= ^a	24.1±2.1*= ^a

Data are mean ± SD. Muscle enzyme activities are expressed as $\mu\text{mol/g/min}$. * Statistically significant change relative to control ($p < 0.05$). = Statistically significant change relative to 6-wk stimulation ($p < 0.05$).

Collagen and fibrillin analysis

The percentage of muscle CSA seen to fluoresce due to antibodies bound to type I collagen was lower in control LD than in both 6-wk and 12-wk groups, indicating an increase in type I collagen content with stimulation (Figure 4). Fibrillin concentrations also appeared to increase in response to LD stimulation in both 6-wk and 12-wk stimulated groups (Figure 5). Conversely, the proportion of type III collagen decreased with stimulation, total antibody fluorescence appearing lowest in the 6-wk group. Some segments of type III collagen formed circles in the extracellular matrix, (indicative of small blood vessels), which were seen to both proliferate and expand after 6 weeks of stimulation (Figure 6). The number and size of these circles were seen to increase further after 12 weeks of burst stimulation.

Discussion

The purpose of this study was to determine adaptations in muscle function, fiber type, metabolic characteristics, and collagen and fibrillin content due to long-term stimulation in rabbit LD muscle. Control LD muscles were composed mainly of FG fibers (62.3%, Table 1). FG fibers occupied an even greater proportion of muscle fiber CSA in control muscles (68.9%) due to their larger cross sectional area. Any changes in FG fibers should translate directly to functional and structural changes in the LD muscle. Our results support this hypothesis, since decreases in FG fibers corresponded with reductions of isometric force, improvements in fatigue resistance, increases in type I collagen and fibrillin, and decreases in glycolytic enzyme activities.

Observed changes in muscle mechanics, myofiber distribution, collagen volume, and fibrillin content have

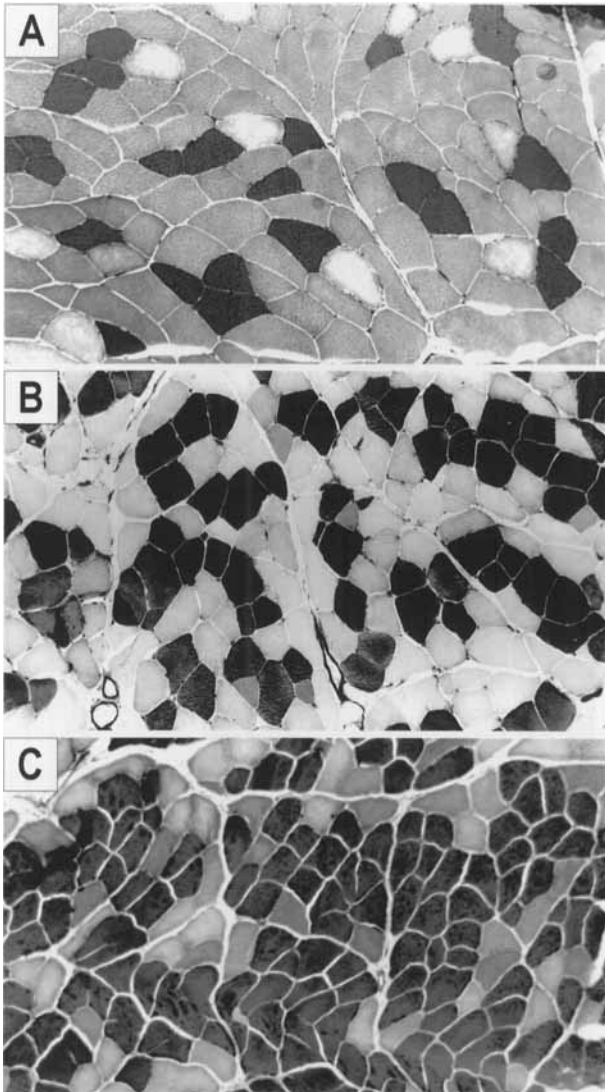


Figure 3. Fiber typing of rabbit latissimus dorsi muscle in control (A), 6 week (B), and 12 week (C) stimulated according to histochemical staining for myosin ATPase (pH 4.45 preincubation). Three populations of fiber types are indicated by dark (SO), medium (FG) and light (FOG) staining.

longed activation on muscle morphology and function. In this study, stimulation-induced reductions in muscle strength accompanied FG fiber transformation and atrophy. Improvements in fatigue resistance occurred with the conversion from thicker FG fibers to thinner SO fibers. Losses in total muscle fiber CSA (34.5% in 6-wk, 33.1% in 12-wk) were accompanied by increases in type I collagen and fibrillin content. In light of these data, we hypothesize that atrophy is an intermediate stage of muscle fiber transformation and connective tissue accumulation.

Again, long-term stimulation was seen to increase fatigue resistance and oxidative enzyme activity secondary to the proliferation of SO muscle fibers, type I collagen, and fibrillin. Increases in oxidative metabolic capacity

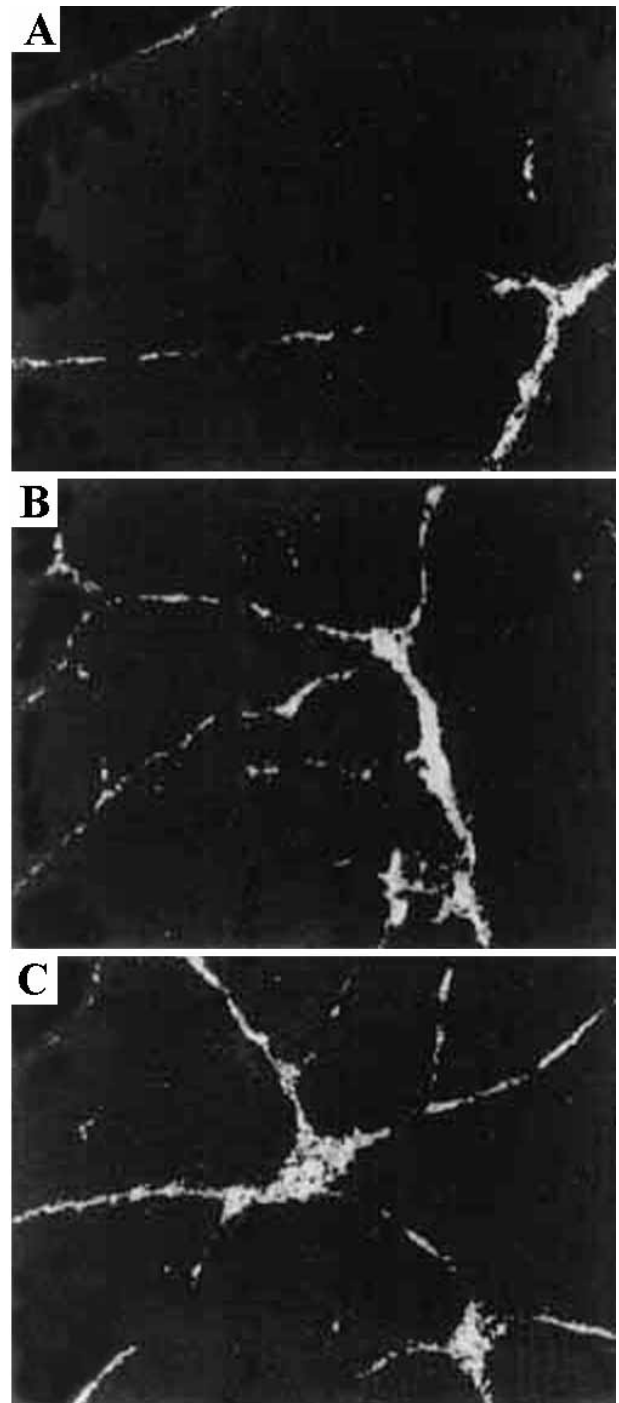


Figure 4. Latissimus dorsi muscle in rabbit of control (A), 6 week (B), and 12 week (C) stimulated groups. Light sections indicate the presence of antibodies bound to type I collagen. Magnification: 250X.

were sustained throughout the 12-week stimulation period. Levels of MDH, CS, and HADH were all higher at 12 weeks than at 6 weeks. However, significant changes in fiber type, type I collagen and fibrillin did not continue beyond 6 weeks. These results suggest that mitochondrial

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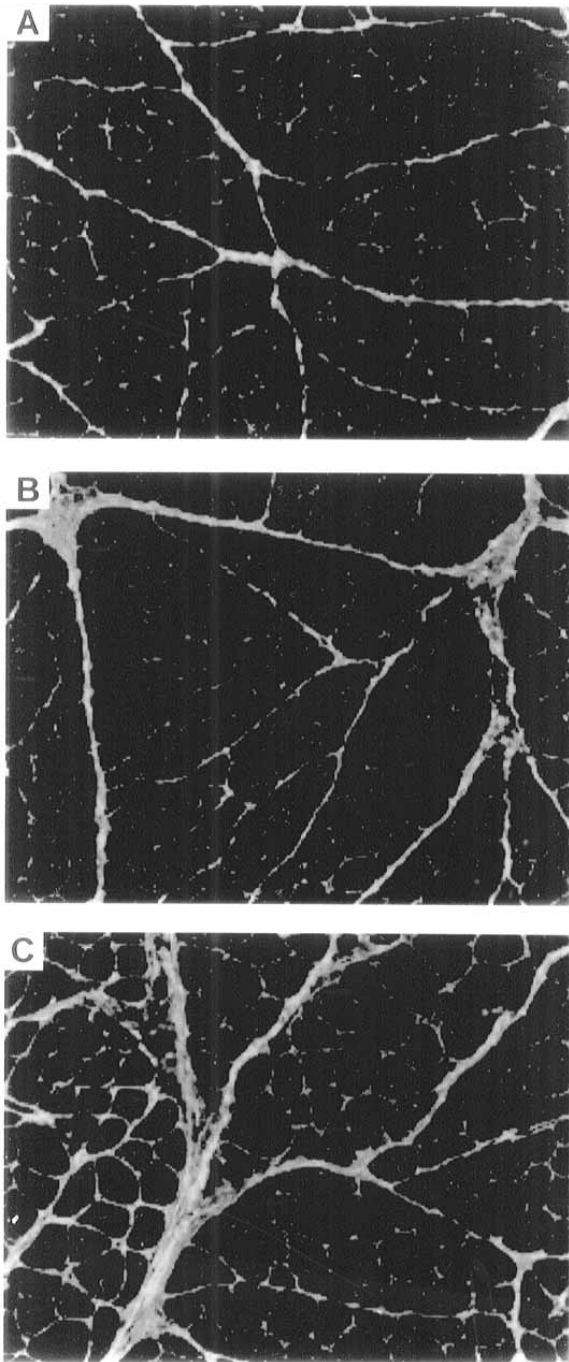


Figure 5. Latissimus dorsi muscle in rabbit of control (A), 6 week (B), and 12 week (C) stimulated groups. Light sections indicate the presence of antibodies bound to fibrillin. Magnification: 250X.

function may continue to adapt to chronic muscular activation even after the process of fiber transformation and collagen deposition is largely complete.

Stimulation-induced increases in type I collagen content of LD muscle coincided with reductions in collagen type III concentrations. As a result, the CSA ratio of

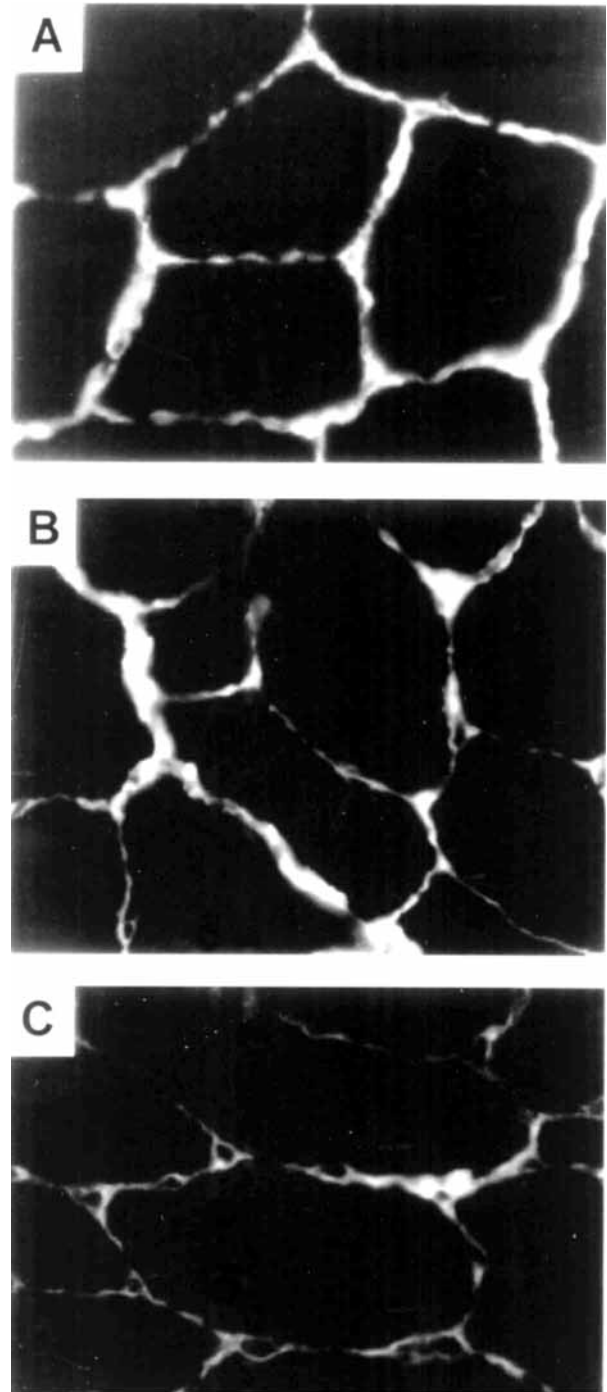


Figure 6. Latissimus dorsi muscle in rabbit of control (A), 6 week (B) and 12 week (C) stimulated. Light sections indicate the presence of antibodies bound to type III collagen. Circles in the muscle extracellular matrix (blood vessels) were most prevalent in the 12-wk stimulation group (panel C). Magnification: 1375X.

collagen types I and III was substantially increased during stimulation. Since type III collagen is known to enhance tissue extensibility [2], reductions due to chronic

stimulation may contribute to decreased muscle elasticity noted in prior work [19, 23].

Muscular blood supply is a critical factor in maintaining contractile function over prolonged periods. Serial sections taken from trained and control muscles immediately following 40-min fatigue testing suggest that long-term stimulation leads to angiogenesis and the expansion of pre-existing blood vessels (Figure 6). The relatively sparse distribution and modest size of blood vessels seen in control LD may partially explain why these muscles are susceptible to fatigue. It should be noted however, that our method of collagen staining measures the outside diameter of the vessel only (since the collagen III layer is located outside the smooth muscle and endothelial layers). Hence, the true size of the vessel lumen cannot be measured directly via this technique.

Conclusion

In summary, the present study suggests that stimulation-related transformation of muscle fibers from fast to slow is associated with increases in fatigue resistance, oxidative capacity, type I collagen content, and fibrillin concentration - as well as decreases in muscle strength, type III collagen content, and glycolytic metabolism. Sustained changes in metabolic enzyme activities indicate that mitochondrial function may continue to adapt to chronic muscular activation even after the process of fiber transformation and collagen deposition is largely complete. Moreover, detected changes in intercellular collagen and fibrillin levels suggest that muscular adaptation to long-term stimulation involves both alterations in fiber type expression and remodeling of the extracellular matrix.

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Abbreviations

CSA: cross-sectional area
FG: fast-twitch glycolytic
FOG: fast-twitch oxidative glycolytic
LD: latissimus dorsi (muscle)
SO: slow-twitch oxidative.

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