Mitochondrial biogenesis during skeletal muscle regeneration

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Received 27 July 2001; accepted in final form 9 November 2001

Mitochondrial biogenesis during skeletal muscle regeneration. Am J Physiol Endocrinol Metab 282: E802–E809, 2002; 10.1152/ajpendo.00343.2001.—Myogenesis requires energy production for the execution of a number of regulatory and biosynthesis events. We hypothesized that mitochondrial biogenesis would be stimulated during skeletal muscle regeneration. Tibialis anterior muscles of male Sprague-Dawley rats were injected with 0.75% bupivacaine and removed at 3, 5, 7, 10, 14, 21, or 35 days after injection (n = 5–7/group). Two main periods emerged from the histochemical analyses of muscle sections and the expression of proliferating cell nuclear antigen, desmin, and creatine phosphokinase: 1) activation/proliferation of satellite cells (days 3–14) and 2) differentiation into muscle fibers (days 5–35). The onset of muscle differentiation was accompanied by a marked stimulation of mitochondrial biogenesis, as indicated by a nearly fivefold increase in citrate synthase activity and state 3 rate of respiration, which was accompanied by a marked stimulation of mitochondrial respiration; muscle precursor cells; myogenesis; peroxisome proliferator-activated receptor-γ coactivator-1; mitochondrial transcription factor A.

Mitochondrial biogenesis is one of the striking responses observed in skeletal muscle exposed to a variety of physiological conditions (see Refs. 12 and 26 for reviews). The synthesis of mitochondrial proteins involves the expression of genes originating from both nuclear and mitochondrial genomes. This dual-genomic organization is coordinated by a set of transcription factors, including peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1), nuclear respiratory factor-1 (NRF-1), and NRF-2, among others. PGC-1 stimulates the expression of NRF-1 and NRF-2 (49), whose regulatory elements are shared by many genes encoding mitochondrial proteins (46).

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Recapitulation of the myogenic program requires energy production for the execution of a number of regulatory and biosynthesis events. Accordingly, mitochondrial biogenesis accompanies the in vitro differentiation of myoblasts into myotubes (30, 36). In addition, muscle cell differentiation appears to depend on mitochondrial function. Indeed, respiration-deficient myoblasts devoid of mitochondrial DNA fail to differentiate (23). Similarly, inhibition of mitochondrial protein synthesis with chloramphenicol prevents the differentiation of myoblasts into myotubes (22, 28). These studies illustrate the importance of the biosynthesis of functional mitochondria during in vitro myogenesis. However, much less is known about mitochondrial biogenesis during in vivo skeletal muscle regeneration.

The aim of this study was to analyze the relationship among mitochondrial content, mitochondrial function, and skeletal muscle regeneration. For this purpose, the...
bupivacaine-induced muscle degeneration model was employed to examine the expression of mitochondrial proteins and transcription factors in relation to muscle cell proliferation and differentiation.

**METHODS**

**Animal care and bupivacaine injection.** The animal protocol was approved by the Ministère de l'Agriculture et de la Forêt. Male Sprague-Dawley rats (278 ± 2 g; n = 44) were housed individually in a temperature-controlled room (21°C) with a 12:12-h light-dark cycle and allowed food and water ad libitum. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt). Injections of bupivacaine (4 × 150 μL of 0.75% bupivacaine in 0.9% NaCl) were done into the tibialis anterior (TA) muscle with a 30-gauge needle. The injection procedure used in the present study has been shown to be highly effective in eliciting a large and complete muscle degeneration (19). The contralateral TA muscle was injected with a saline solution.

**Tissue sampling.** After 3, 5, 7, 10, 14, 21, or 35 days of injection (n = 5–7/group), rats were anesthetized, and the entire TA muscle was removed from each limb. A portion of bupivacaine (4–7 μL of 10 μM bupivacaine) was mixed with a loading buffer [62.5 mM Tris, pH 6.8, 5% glycerol (vol/vol), 1% SDS (vol/vol), 2.5% β-mercaptoethanol (vol/vol), boiled for 3 min, applied to a 10% polyacrylamide gel, and electrophoresed at 115 V for 4 h at 4°C. Separated proteins were electrotransferred for 1 h at 4°C onto nitrocellulose membranes. The membranes were then blocked in TBS-5% milk solution (PCNA and desmin) or in TBS-Tween 20-5% milk solution (mtTFA) at room temperature for 1 h. The following primary antibodies were used for immunoblotting: PCNA, 1:500 dilution (PC10 NeoMarkers); desmin, 1:500 dilution (D33, DAKO); and mtTFA, 1:500 dilution [a generous gift of D. A. Hood and H. Inagaki, National Industrial Research Institute, Nagoya, Japan (17)]. Primary antibodies were incubated overnight at 4°C. Rabbit anti-mouse IgG [1:2,000 (vol/vol); P0161, DAKO] or goat anti-rabbit IgG [1:2,000 (vol/vol); P0448, DAKO] conjugated to horseradish peroxidase was used for chemiluminescent detection of proteins (ECL, Amersham). The films were scanned and quantified using NIH Image 1.61.

**Enzyme activities.** Enzyme activities were fluorometrically measured (exc = 340 nm and em = 450 nm) according to Lowry and Passonneau (32) with some modifications. For creatine phosphokinase activity (CPK, EC 2.7.3.2.), 10 μL of protein extract (1:100 dilution, vol/vol) were added to 960 μL of assay buffer [50 mM Tris, pH 8.0, 1 mM ADP, 2 mM glucose, 100 μM NADPH, 10 mM MgCl2, 5 mM dithiothreitol, 0.02% BSA (wt/vol), 3 U/ml hexokinase (Roche Diagnostics), and 0.35 U/ml glucose-6-phosphate dehydrogenase (Roche Diagnostics)]. Production of NADPH, H+ was recorded after addition of creatine phosphate (24 mM). For citrate synthase activity (CS, EC 4.1.3.7), 10 μL of protein extract (1:30 dilution, vol/vol) were added to 980 μL of assay buffer [100 mM Tris, pH 8.0, 2 mM EDTA, 1.25 mM l-malate, 0.25 mM NAD+, 0.01% Triton X-100 (vol/vol), 6 U/ml malate dehydrogenase (Sigma)]. Production of NADH, H+ was recorded after addition of acetyl-CoA (50 μM). For 3-hydroxyacyl-CoA dehydrogenase activity (HAD, EC 1.1.1.35), 3 μL of protein extract were added to 982 μL of assay buffer [50 mM Tris, pH 7.6, 2 mM EDTA, 50 μM NADH, H+, 0.01% Triton X-100 (vol/vol)]. Oxidation of NADH was measured after addition of acetoacetyl-CoA (75 μM). For phosphofructokinase activity (PFK, EC 2.7.1.1), 10 μL of protein extract (1:100 dilution, vol/vol) were added to 980 μL of assay buffer [50 mM Tris, pH 8.0, 50 μM NADH, H+, 1.25 mM ATP, 5 mM MgCl2, 2 mM β-mercaptoethanol, 0.05% BSA (wt/vol), 0.45 U/ml aldolase (Roche Diagnostics)], 50 U/ml triose phosphate isomerase (Sigma Chemical), and 0.85 U/ml glycerol-3-phosphate dehydrogenase (Roche Diagnostics). Oxidation of NADH+ was recorded after addition of fructose 6-phosphate (1 mM). For lactate dehydrogenase activity (LDH, EC 1.1.1.27), 5 μL of protein extract (1:30 dilution, vol/vol) were added to 985 μL of assay buffer [50 mM Tris, pH 7.6, 2 mM EDTA, and 50 μM NADH, H+]. Oxidation of NADH+ was recorded after addition of pyruvate (1 mM).

**Isolation of total RNA and RT-PCR conditions.** Total RNA from frozen powdered muscles (30–40 mg) was isolated as previously described (11). One microgram of total RNA was used in reverse transcription reactions performed with a Superscript II kit (GIBCO-BRL) by using 10 μL of the reverse transcription reaction products diluted 1:20 (vol/vol) and the LightCycler-DNA Master SYBR Green I kit. The reaction mixture was then inactivated for 5 min at 95°C. Primers homologous to nucleotides 2182 to 2203 (reverse: 5′-GTTTCATCTCTAGTTTAC-3′) and 2441 to 2460 (sense: 3′-CTCCTACTGGTCTCCTACCG-5′) of PGC-1 were used. PCR amplification was performed with the LightCycler System (Roche Diagnostics) using 10 μL of the reverse transcription reaction products diluted 1:20 (vol/vol) and the LightCycler-DNA Master SYBR Green 1 kit. The reaction mixture was first denatured for 2 min at 95°C and then cycled 40 times with a 55°C annealing for 10 s, a 72°C extension for 10 s, and a progressive denaturation to 95°C. Fluorescence was acquired during the extension period, and data were analyzed using the LightCycler analysis software.
No signs of necrosis were evident in control muscles. The final mitochondrial pellet was diluted (1:1, wt/vol) in 10 mM Tris, pH 7.4, 1 mM EDTA, 700 mM mannitol, and 220 mM saccharose solution. Protein content was measured as described above. Oxygen consumption (natom O\(\text{min}^{-1}\cdot g^{-1}\) of muscle) was recorded at 37°C under constant magnetic stirring with a Clarke oxygen electrode (Hansatech, UK). State 4 rate of respiration was measured in the presence of 100 µM of protein, 2.5 mM L-malate, and 5 mM pyruvate in 1 ml of respiration medium (20 mM Tris, pH 7.4, 225 mM mannitol, 5 mM MgCl\(_2\), 500 µM EDTA, 10 mM KH\(_2\)PO\(_4\)) saturated with air. State 3 rate of respiration was measured after the addition of ADP (350 µM). To assess intactness of mitochondria, CS activity was measured in the absence and presence of 0.1% Triton X-100 (vol/vol) as previously described (13).

**Statistical analysis.** All values are presented as means ± SE. Two-way ANOVA was used to evaluate the effects of bupivacaine as a function of time on desmin protein level, state 3 rate of respiration, and CK, CS, HAD, PFK, and LDH enzyme activities. Post hoc comparisons were performed with the Fisher's protected least significance difference test. One-way ANOVA was used to evaluate the time course changes in necrosis, number of fibers with central myonuclei, PCNA and mtTFA protein levels, and PGC-1 mRNA level. Individual means were compared with a paired t-test. Differences were considered to be statistically significant at the 0.05 level of confidence.

**RESULTS**

**Body mass, muscle weight, and total protein concentration.** Bupivacaine injection had no effect on the body mass, TA muscle weight, and total protein concentration during skeletal muscle regeneration (Table 1). At the end of the experimental period, control muscle weight was 868.1 ± 54.8 mg (\(n = 5\)). Muscle weight was significantly decreased (40%) by 5–10 days after bupivacaine injection and had returned to control levels by 21 days postinjection. The growth of animals after bupivacaine injection and had returned to control levels by 35 days after bupivacaine injection. The growth of animals after bupivacaine injection and had returned to control levels by 35 days after bupivacaine injection.

**Histochemical analyses of necrosis and regeneration.** No signs of necrosis were evident in control muscles. Normal fibers were characterized by regular outlines and peripherally located nuclei. Three days after bupivacaine injection, 99 ± 1% of the whole muscle cross-sectional area was affected by the drug (Fig. 1, Ab and B). At that time, most of the muscle fibers were disjoined or no longer discernable. Intense myophagy with interstitial edema and infiltrating cells was also observed. Proliferation and fusion of myoblasts occurred concomitantly with the phagocytosis of muscle debris, since the presence of many small colonies of regenerating fibers with central myonuclei was observed as soon as 7 days postinjection (Fig. 1C). Massive reduction in myonecrosis was then observed after 10 days of regeneration (\(P < 0.001;\) Fig. 1, Ac and B). At that time, the regenerating myofibers had grown larger, and 52.2 ± 9.9% of the muscle fibers had central myonuclei (Fig. 1, Ac and C). On day 35, despite the presence of numerous fibers with central myonuclei, muscle regeneration appeared to be stable (Fig. 1Ad). The normal polyhedric aspect of muscle fibers was restored, and the number of muscle fibers was virtually the same in controls (8,228 ± 807 fibers; \(n = 4\)) and bupivacaine-injected TA muscles (7,640 ± 1,372 fibers; \(n = 4\)).

**PCNA, desmin, and CPK protein levels.** To more closely delineate the period corresponding to myoblast proliferation, PCNA, a protein that functions as a co-factor for DNA polymerase-δ in S phase, was quantified by immunoblotting (Fig. 2, A and C). Whereas PCNA was not detected in control muscles, regenerating muscles strongly expressed PCNA. Expression peaked on day 3 and then decreased to represent only 7% of day 3 values by 14 days postinjection (14.5-fold decrease; \(P < 0.001\)). To assess the onset of muscle-specific protein expression, desmin protein level was determined (Fig. 2, B and C). Precocious expression of desmin is characteristic of proliferating myoblasts (41, 45). A nonsignificant, 2.3-fold decrease in desmin protein level (\(P = 0.11\)) was observed at 3 days postinjection. Expression then increased, attaining levels that were 2.3-fold above those observed in control muscles on day 10 (\(P < 0.05\)). Desmin protein level had returned to control levels at 35 days after bupivacaine injection. CPK is a myogenic marker expressed after the fusion of myoblasts into myotubes (9). Total CPK activity, whose 95% is due to the muscle isoform (1, 2), exhibited a dramatic 80% reduction at 3 days of regeneration (\(P < 0.001;\) Fig. 2C) and then gradually recovered, returning to control values 35 days after bupivacaine injection.

**CS and HAD enzyme activities.** Matrix mitochondrial enzymes (CS and HAD) were assayed to establish a possible relationship between mitochondrial content and skeletal muscle regeneration (Fig. 3). These mitochondrial enzymes correlate closely with mitochondrial volume fraction (8). CS activity decreased significantly

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<th>Parameter</th>
<th>Days of Regeneration</th>
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<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>284±10</td>
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<tr>
<td>Muscle weight, %</td>
<td>81.4±3.8$^*$</td>
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<tr>
<td>Total protein, %</td>
<td>76.2±4.7$^*$</td>
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Values are means ± SE; \(n = 5–7\)/group. Tibialis anterior (TA) muscle weight (g of muscle) and total protein concentration (mg protein/g muscle) are expressed as the percentage of contralateral muscle values. *\(P < 0.05\), †\(P < 0.01\), and ‡\(P < 0.001\), bupivacaine-injected vs. contralateral muscles.

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by 80% at 3 days after bupivacaine injection. Enzyme activity then increased sharply between days 5 and 10. On day 14, CS activity had returned to control values. The pattern of changes in HAD activity was similar to that observed in CS activity, exhibiting the same strong rise between days 5 and 10.

Mitochondrial respiration. To further study the effect of skeletal muscle regeneration on mitochondrial content and function, we isolated mitochondria from control and regenerating muscles and measured the rates of respiration. Protein yield of mitochondria isolated from control muscles did not change over the 35-day time period (5.59 ± 0.41 μmol·min⁻¹·g muscle⁻¹ in control muscles; n = 41). By 3 days postinjection, mitochondrial protein yield was significantly decreased fourfold in regenerating muscles compared with control muscles (Table 2). After 14 days of regeneration, mitochondrial yield had nearly returned to control values. Intactness of mitochondria in the suspension was virtually the same in control (88.3 ± 1.0%; n = 41) and bupivacaine-injected (88.6 ± 0.9%; n = 41) muscles over the experimental period. Addition of cytochrome c and NADH, H⁺ to the respiration medium did not increase state 3 rate of respiration, further indicating the intactness of mitochondrial membranes (data not
The efficiency of the mitochondrial isolation, expressed as the percentage of CS activity in the mitochondrial suspension over total muscle CS activity (3), was unchanged in control (32.4 ± 2.4%; n = 42) and bupivacaine-injected (33.1 ± 2.8%; n = 43) muscles. With the use of CS activity as a reference, mitochondrial respiration was therefore expressed per muscle mass (3, 13). Respiratory control ratios (RCR) indicated that mitochondria isolated from control muscles were well coupled (Table 2). In contrast, RCRs were dramatically decreased at 3 days postinjection (7-fold relative to control; P < 0.001). Control values were reached between days 14 and 21 of the experimental period. State 4 rates of respiration were similar in control (0.65 ± 0.05 natom O·min⁻¹·g muscle⁻¹; n = 41) and bupivacaine-injected (0.52 ± 0.05 natom O·min⁻¹·g muscle⁻¹; n = 41) muscles over the course of the regeneration process. By contrast, state 3 rates of respiration were only 12% of their respective contratateral values by 3 days postinjection (P < 0.01; Fig. 3). From 5 to 10 days, mitochondrial respiration sharply increased to reach control levels at 21 days postinjection. This pattern closely resembled the pattern obtained with CS and HAD activities.

**mtTFA protein level and PGC-1 mRNA level.** In view of the effects of muscle regeneration on mitochondrial content and function, mtTFA protein level was quantified (Fig. 4). Surprisingly, the changes in mtTFA protein level relative to contratateral values were moderate. Only day 7 values were significantly different from those of contratateral muscles. However, relative mtTFA protein levels were different over time, from being lower between days 3 and 7 to being nearly the same to control values between days 10 and 35. mtTFA expression is activated by PGC-1 (49). PGC-1 mRNA level exhibited a strong decrease 3 days after bupivacaine injection and then increased to peak on day 10 (5-fold increase; Fig. 4, inset). On day 21, PGC-1 mRNA level had almost returned to control levels.

**PFK and LDH enzyme activities.** PFK and LDH activities were decreased by ~50% at 3 days postinjection (P < 0.01; Fig. 5). No significant changes in enzyme activities were evident until 21 days of regeneration. Activities had returned to control levels by day 35. This pattern of expression was significantly different from the one observed for mitochondrial enzymes (two-way ANOVA; data not shown).

**DISCUSSION**

The ability to alter mitochondrial content and function is an important adaptive response of skeletal muscle. We hypothesized that skeletal muscle regeneration, which recapitulates embryonic myogenesis, could stimulate mitochondrial biogenesis. To address this question, we chose the bupivacaine-induced muscle degeneration model. The results indicate that skeletal muscle regeneration is accompanied by a dramatic stimulation of mitochondrial biogenesis concomitant with the onset of muscle differentiation.

Histochemical and biochemical analyses were performed to delineate some of the cellular events occurring over the course of the regeneration process. In agreement with other studies (20, 37, 39), bupivacaine was highly effective in eliciting muscle degeneration. The appearance of myogenic mitosis, as demonstrated by the expression of PCNA, was regarded as the first sign of the initiation of regeneration (38). Progression of myogenesis was also associated with the onset of muscle-specific protein expression, as indicated by the expression of desmin, one of the earliest known muscle-

**Table 2. Protein yield and RCR of isolated mitochondria**

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<th>Parameter</th>
<th>Days of Regeneration</th>
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<tr>
<td></td>
<td>3</td>
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<tr>
<td>Protein yield, %</td>
<td>26.3 ± 6.5‡</td>
</tr>
<tr>
<td>RCR</td>
<td>Con</td>
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<td></td>
<td>12.62 ± 0.77</td>
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Values are means ± SE; n = 5–7/group. RCR, respiratory control ratios; Con, contratateral muscle; Bup, bupivacaine-injected muscle. Protein yields are expressed as the percentage of contratateral muscle values. *P < 0.05, †P < 0.01, and ‡P < 0.001, bupivacaine-injected vs. contratateral muscles.
specific structural proteins (6, 16). The appearance of myotubes with central myonuclei concomitant with the increase in total CPK activity clearly indicates that myoblast fusion was initiated as early as 7 days of regeneration. The subsequent growth of regenerating myofibers (size and number) revealed that terminal differentiation was almost complete at the end of the experimental period, despite the persistence of fibers with central myonuclei. All together, two main periods emerged from the histochemical analyses of necrosis and regeneration and the temporal expressions of PCNA, desmin and CPK: 1) activation of satellite cells and proliferation of myoblasts (days 3–14) and 2) differentiation of myoblasts into muscle fibers (days 5–35).

The novel finding in this study was that in vivo skeletal muscle regeneration was accompanied by a marked stimulation of mitochondrial biogenesis. The most prominent changes in the yield of mitochondrial proteins, CS activity, and state 3 rate of respiration occurred between days 5 and 10 of regeneration, indicating a nearly fivefold increase in mitochondrial content (8, 42) during the onset of muscle differentiation. Gene expression of mitochondrial proteins depends on the existence of a set of specialized transcription factors (see Ref. 25 for review). Expression of mitochondrial genome as well as mtDNA replication is regulated by mtTFA, a nuclear-encoded protein imported inside the mitochondrion (35). Binding sites for NRF-1 and -2 are present in the human mtTFA promoter and are important for mtTFA gene expression (48). Furthermore, PGC-1 also coactivates NRF-1 transcriptional activity on mtTFA gene expression (49). One surprising finding in the present study was the moderate changes in mtTFA protein level compared with control values. This contrasts with the large decrease in CS enzyme activity and mitochondrial respiration observed on days 3 and 5. Therefore, one may hypothe-

![Graph](image-url)
cytokines by in
be determined, but they most likely result from the
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tween
synthesized and imported in mature lipid bilayers be-
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ci
c gene expression and CS activity through a
pression. Interestingly, calcium modulates muscle-spe-
tion could also contribute to mitochondrial gene ex-
during skeletal muscle regeneration (40) taken into
and functional characteristics of calcium homeostasis
(5) in muscle cells. With the modi
Connor, M. DiCarlo, and D.A. Hood, unpublished ob-
(24). Calcium is also known to modulate mitochondrial
modulation of cAMP response element-binding protein
muscle cell proliferation and differentiation.
This suggests that ATP production by mitochondria
depend on the ef
regenerating capability of rat skeletal muscle could
depend on the efficiency of oxidative phosphorylation.
Indeed, the onset of muscle differentiation was accom-
panied by the recovery of state 3 rate of respiration.
This suggests that ATP production by mitochondria
could regulate the execution of a number of regulatory
and biosynthetic events involved in myogenesis. Fur-
thermore, a recent report (43) demonstrates that mito-
chondria can also regulate in vitro muscle differentia-
tion through the regulation of myogenic transcription
factor expression. Together, these data corroborate
previous studies showing that inhibition of mitochon-
drial protein synthesis inhibits in vitro muscle cell
differentiation (22, 23, 28). Experiments are in
progress to further explore the function of mitochond-
dria during myogenesis.

In summary, we have shown that skeletal muscle
regeneration is accompanied by a marked stimulation
of mitochondrial biogenesis concomitant with the onset
of muscle cell differentiation. This work sets the stage
for in vivo studies designed to evaluate the physiolog-
ical function of mitochondria in regulating skeletal
muscle cell proliferation and differentiation.

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