Mitochondrial biogenesis during skeletal muscle regeneration

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Duguez, Stéphanie, Léonard Féasson, Christian Denis, and Damien Freyssenet. 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 between days 5 and 10. Peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) mRNA level and mitochondrial transcription factor A (mtTFA) protein level peaked on day 10 concurrently with the state 3 rate of respiration. Therefore, transcriptional activation by PGC-1 and mtTFA may be one of the mechanisms regulating mitochondrial biogenesis in regenerating skeletal muscle. Taken together, our results suggest that mitochondrial biogenesis may be an important regulatory event during muscle regeneration.

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). PGC-1 and NRF-1 also coactivate the expression of mitochondrial transcription factor A (mtTFA) (49), the only known regulator of mitochondrial replication and transcription. Thus the transcription factors involved in mitochondrial biogenesis are key contributors to the nuclear control of mitochondrial phenotypic alteration and energy production in skeletal muscle.

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

SKELETAL MUSCLE HAS A REMARKABLE CAPACITY to regenerate after injury. Different events must occur to achieve complete regeneration of the muscle: phagocytosis of muscle debris, revascularization, activation, proliferation, and differentiation of muscle precursor cells, and reinnervation (10). Experimentally, bupivacaine is frequently used to study skeletal muscle regeneration. This myotoxic agent causes severe perturbations of calcium homeostasis (31, 47) associated with hypercontractions of muscle fibers and disruptions of plasma-lemma membranes (20, 44), which lead ultimately to rapid muscle fiber necrosis (18, 20). However, muscle satellite cells are resistant to the action of bupivacaine, and are thus available for the subsequent recapitulation of the myogenic program (21).
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 mg/kg body wt) 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 first portion of muscle was embedded in cryopreservative (Cryomount, Electrophoresis). The second portion of muscle was embedded in paraffin and sectioned (7 µm) with a microtome at −20°C. Sections were placed in 0.75% bupivacaine in 0.9% NaCl and counted the number of infiltrating cells, interstitial edema, necrotic fibers, and regenerating cells with central myonuclei divided by the total number of fibers. Analyses were performed using a light microscope connected to a computerized image analysis system (National Institutes of Health (NIH) Image 1.61).

**Protein extraction for immunoblotting and enzyme assays.** Total proteins were extracted from powdered muscles as previously described (14) and stored at −80°C. Concentrations were spectrophotometrically measured at 750 nm using the Bio-Rad protein assay.

**Enzyme activities.** Enzyme activities were fluorometrically measured (λex = 340 nm and λem = 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 NADP+, 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 3-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, 0.01% Triton X-100 (vol/vol), 0.01% Triton X-100 (vol/vol), 0.85 U/ml lactate dehydrogenase (Roche Diagnostics), and 0.45 U/ml aldolase (Roche Diagnostics), 50 U/ml triose phosphate isomerase (Sigma Chemical), and 0.85 U/ml glyceraldehyde-3-phosphate dehydrogenase (Roche Diagnostics). Oxidation of NADH, H+ 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, H+ 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) with the use of random hexamer primers at 42°C for 50 min. The reverse transcriptase was then inactivated for 5 min at 95°C. Primers homologous to nucleotides 2182 to 2203 (reverse: 5′-TACCTACCCCTATATACCG-3′) of PGC-1 were used. PCR amplification was performed with the LightCycler System (Roche Diagnostics) with 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.
and had recovered to control levels at pivacaine injection, 99 and peripherally located nuclei. Three days after bupivacaine was affected by the drug (Fig. 1, A 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, A 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 cofactor 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 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).

### RESULTS

**Body mass, muscle weight, and total protein concentration.** Bupivacaine injection had no effect on the weight of control muscles over the 35-day time period (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 did not affect total protein concentration of control muscles, which averaged 168.4 ± 4.3 mg protein/g of muscle (n = 44). Total protein concentration was moderately decreased by 3–5 days postinjection (P < 0.05) and had recovered to control levels at day 7 (Table 1).

**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 disjointed or no longer discernable. Intense myophagy

![Table 1. Body mass, TA muscle weight, and total protein concentration during skeletal muscle regeneration](http://ajpendo.physiology.org)

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<th>Days of Regeneration</th>
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<td></td>
<td>3</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>284 ± 10</td>
</tr>
<tr>
<td>Muscle weight, %</td>
<td>81.4 ± 3.8†</td>
</tr>
<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.
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 mg protein/mg of muscle; 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 shown).

Fig. 1. Histochemical analysis of skeletal muscle regeneration. A: representative transverse muscle sections stained with hemalum-eosin-safran in contralateral (a) and bupivacaine-injected muscles at 3 (b), 10 (c), and 35 (d) days after bupivacaine injection. See RESULTS for complete description of muscle sections. B: area affected by the action of bupivacaine. C: percentage of fibers with central myonuclei in bupivacaine-injected tibialis anterior (TA) muscles. Contralateral muscles exhibited 2.7 ± 0.2% (n = 25) of fibers with central myonuclei. All values are means ± SE; n = 4–5/group. ††P < 0.01 and †††P < 0.001, significantly different from day 3 values.

Fig. 2. Proliferating cell nuclear antigen (PCNA), desmin, and creatine phosphokinase (CPK) protein levels during skeletal muscle regeneration. Representative immunoblots of PCNA (A) and desmin (B) protein levels. Contralateral TA muscles did not express PCNA. C: PCNA protein level is expressed as the percentage of day 3 values. Desmin level and CPK activity are expressed as the percentage of contralateral muscle values. Absolute CPK activity averaged 1.11 ± 0.03 μmol·min⁻¹·g muscle⁻¹ in contralateral muscles (n = 44). Values are means ± SE; n = 5–7/group. †P < 0.05, ††P < 0.01, significantly different from day 3 values. *P < 0.05, **P < 0.01, and ***P < 0.001, bupivacaine-injected vs. contralateral muscles.
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 levels was quantified (Fig. 4). Surprisingly, the changes in mtTFA protein levels relative to contralateral values were moderate. Only day 7 values were significantly different from those of contralateral 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>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></td>
</tr>
<tr>
<td>Con</td>
<td>12.62 ± 0.77</td>
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<tr>
<td>Bup</td>
<td>1.98 ± 0.39‡</td>
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Values are means ± SE; n = 5–7/group. RCR, respiratory control ratios; Con, contralateral muscle; Bup, bupivacaine-injected muscle. Protein yields are expressed as the percentage of contralateral muscle values. *P < 0.05, †P < 0.01, and ‡P < 0.001, bupivacaine-injected vs. contralateral 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 hypothesize that mtTFA protein levels observed on days 3, 5, and 7 of regeneration would be necessary mainly for the replication of mtDNA. However, mtDNA replication also depends on factors other than mtTFA, such as DNA polymerase-γ, mitochondrial single-strand DNA-binding protein, or RNA mitochondrial processing enzyme (35). Protein level measurements of these factors would therefore be necessary to confirm the physiological significance of elevated mtTFA protein level during the onset of regeneration. Later during the regeneration process, PGC-1 mRNA and mtTFA protein levels peaked on day 10, concurrently with the state 3 rate of respiration, suggesting that the transcriptional activation by PGC-1 and mtTFA may be one of the mechanisms regulating mitochondrial biogenesis in regenerating skeletal muscle. Other aspects may also be important in the overall regulation of mitochondrial biogenesis, like the transcriptional activation by other transcription factors, the regulation of mRNA stability, and/or the regulation of the import process (25). Overall, these different regulatory influences may determine the expression pattern of mitochondrial proteins observed during in vivo skeletal muscle regeneration.

Biogenesis of functional mitochondria also requires intact mitochondrial membranes to house the newly synthesized proteins and maintain an electrochemical proton gradient during respiration. In skeletal muscle, the resting oxygen consumption measured in the absence of ADP (state 4 respiratory rate) is mainly due to passive flux of protons through the inner mitochondrial membrane (29). Therefore, the state 4 respiratory rates reported in the present study indicate that the proton permeability of the inner mitochondrial membrane was unaffected by bupivacaine injection, supporting the concept that the gross lipid composition of mitochondrial membranes was fairly similar in control and bupivacaine-injected muscles (7). Taken together, these results suggest that mitochondrial proteins were
synthesized and imported in mature lipid bilayers between days 5 and 14 to give functional, well-coupled mitochondria.

The signals involved in the stimulation of mitochondrial biogenesis during muscle regeneration remain to be determined, but they most likely result from the combinatorial interplay of different molecular events. For example, the production of growth factors and cytokines by infiltrating cells and muscle cells (27, 33, 34) could modulate mitochondrial gene expression during skeletal muscle regeneration. Indeed, evidence has been recently presented for the role of growth factors in inducing cytochrome c gene expression through the modulation of cAMP response element-binding protein and NRF-1 transcriptional activity in BALB/C3T3 cells (24). Calcium is also known to modulate mitochondrial gene expression (15) (D. Freyssenet, I. Ircher, M. K. Connor, M. DiCarlo, and D.A. Hood, unpublished observations) as well as mitochondrial-nuclear cross talk (5) in muscle cells. With the modification of structural and functional characteristics of calcium homeostasis during skeletal muscle regeneration (40) taken into account, perturbations in cytosolic calcium concentration could also contribute to mitochondrial gene expression. Interestingly, calcium modulates muscle-specific gene expression and CS activity through a calcineurin-dependent pathway (4), therefore opening the possibility that a shared signal could regulate the expression of both mitochondrial and muscle-specific genes during skeletal muscle regeneration.

A new and potentially important observation has emerged from this study, an observation that establishes possible relationships between mitochondrial biogenesis and muscle cell differentiation. The different response of glycolytic and mitochondrial metabolisms observed in the present study suggests that the regenerating capability of rat skeletal muscle could depend on the efficiency of oxidative phosphorylation. Indeed, the onset of muscle differentiation was accompanied 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. Furthermore, a recent report (43) demonstrates that mitochondria can also regulate in vitro muscle differentiation through the regulation of myogenic transcription factor expression. Together, these data corroborate previous studies showing that inhibition of mitochondrial protein synthesis inhibits in vitro muscle cell differentiation (22, 23, 28). Experiments are in progress to further explore the function of mitochondria 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 physiological function of mitochondria in regulating skeletal muscle cell proliferation and differentiation.

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