Control of mitochondrial biogenesis, ROS level, and cytosolic Ca\(^{2+}\) concentration during the cell cycle and the onset of differentiation in L6E9 myoblasts

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Jahnke VE, Sabido O, Freyssenet D. Control of mitochondrial biogenesis, ROS level, and cytosolic Ca\(^{2+}\) concentration during the cell cycle and the onset of differentiation in L6E9 myoblasts. Am J Physiol Cell Physiol 296:C1185–C1194, 2009. First published March 18, 2009; doi:10.1152/ajpcell.00377.2008—Mitochondria can sense signals linked to changes in energy demand to affect nuclear gene expression. This retrograde signaling pathway is presumed to be involved in the regulation of myoblast proliferation and differentiation. We have investigated the regulation of mitochondrial biogenesis and production of putative retrograde signaling agents [hydrogen peroxide (H\(_2\)O\(_2\)) and Ca\(^{2+}\)] during the cell cycle and the onset of differentiation in L6E9 muscle cells. The biosynthesis of cardiolipin and mitochondrial proteins was mainly achieved in S phase, whereas the expression of mitochondrial biogenesis factors [peroxisome proliferator-activated receptor (PPAR)-α, PPAR-β, and neuronal nitric oxide synthase 1] was regularly increased from G\(_1\) to G2M phase. In agreement with the increase in mitochondrial membrane potential, mitochondria in S and G2M phases have a significantly higher H\(_2\)O\(_2\) level when compared with G\(_1\) phase. By contrast, the onset of differentiation was characterized by a marked reduction in mitochondrial protein expression and mitochondrial H\(_2\)O\(_2\) level. The capacity of mitochondria to release Ca\(^{2+}\) in response to a metabolic challenge was significantly decreased at the onset of differentiation. Finally, an increase in calmodulin expression in S and G2M phases and a transitory increase in phosphorylated nuclear factor of activated T cells (NFAT) c3 in S phase was observed. NFATc3 phosphorylation was markedly decreased at the onset of differentiation. Our data point to functional links between the control of mitochondrial biogenesis and the regulation of the level of retrograde signaling agents during the cell cycle and the onset of differentiation in L6E9 muscle cells.

mitochondria; myogenesis; reactive oxygen species; skeletal muscle

ADULT SKELETAL MUSCLE is a postmitotic tissue that retains the ability to repair and regenerate. In response to injury, satellite cells, the myogenic precursor cells, are rapidly activated. Myoblasts, the descendants of activated satellite cells, then proliferate by multiple rounds of cell division, fuse together and with pre-existing muscle fibers to form differentiated myofibers. Myoblasts can also return to quiescence and contribute to the self-renewal of satellite cell population (reviewed in Ref. 8). If the molecular mechanisms involved in the process of myoblast proliferation and differentiation are now well described with respect to the temporal expression of cell cycle and myogenic regulatory factors (14, 45), the activation of proteinases (13), as well as the regulation of critical signaling pathways (45), the energy dependence of this process is still poorly understood.

Recapitulation of the myogenic program requires energy production for the execution of a number of regulatory biosynthesis events, including DNA synthesis, mitosis but also muscle protein synthesis. As the major energy source in most cells, mitochondrial oxidative phosphorylation may therefore potentially play important regulatory roles during myogenesis. Earlier studies showed that inhibition of mitochondrial protein synthesis with chloramphenicol prevents the differentiation of myoblasts into myotubes (21, 29) and respiration-deficient myoblasts devoided of mitochondrial DNA fail to differentiate (23). More recently, the stimulation of mitochondrial oxidative metabolism by pyruvate has been shown to block the proliferation of L6E9 muscle cells (15), and the stimulation of mitochondrial biogenesis by p43, a triiodothyronine-dependent mitochondrial transcription factor, also exerts a myogenic influence, notably through the induction of myogenin and muscle-specific gene expression (46, 49). Variations in mitochondrial content and metabolism thus appear to actively contribute to regulate the proliferation and the onset of differentiation of myoblasts.

The events originating from the mitochondria that ultimately contribute to the regulation of cellular function have been defined as retrograde signaling. These events are still largely unknown, but molecules and/or ions whose cytosolic concentration is modulated by variations in mitochondrial metabolism logically constitute potential signaling agents (reviewed in Ref. 17). Reactive oxygen species (ROS) and ionized calcium (Ca\(^{2+}\)) are thus emerging as critical players. For instance, disruption of mitochondrial membrane potential in C2C12 myoblasts triggers a two- to three-fold increase in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)), which is accompanied by enhanced expression of several Ca\(^{2+}\)-responsive and Ca\(^{2+}\)-handling proteins [calcineurin, nuclear factor of activated T cells (NFAT)], as well as genes of NF-kB pathways (2, 4). These data, and other data obtained in different cell types (1, 2, 35), illustrate that mitochondria can be viewed as energy-sensing organelles that contribute to the regulation of nuclear gene expression under conditions where mitochondrial metabolism is challenged.

In the present study we hypothesized that mitochondrial biogenesis and mitochondrial metabolism are differentially regulated in myoblasts during progression through the cell cycle and at the onset of differentiation. Corollary, we also hypothesized that these variations may impact mitochondrial

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ROS level and the regulation of Ca2+ metabolism. To test this hypothesis, we determined the changes in protein levels of several markers of mitochondrial biogenesis as a function of myoblast position in the cell cycle and at the onset of differentiation (36 h of differentiation). We also examined the regulation of mitochondrial hydrogen peroxide (H2O2) level and [Ca2+]j, as well as the expression and phosphorylation level of Ca2+-dependent signaling proteins.

MATERIALS AND METHODS

Cell Culture

L6E9 muscle cells (gift from Dr. D. A. Hood, York University, Toronto, Ontario, Canada) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum and 1% penicillin (126.6 U/ml) and 1% streptomycin (0.126 mg/ml) at 37°C and 5% CO2 in air on 100-mm plastic dishes. At 80% confluence, cells were either trypsinized or allowed to differentiate for 36 h in DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin.

Flow Cytometry Analyses

Cells were trypsinized for 3 min at 37°C, harvested, and then resuspended with 10 ml Dulbecco’s phosphate-buffered saline (DPBS). Cells were centrifuged at 800 g × 3 min, resuspended in serum-free DMEM, and counted. Cells were analyzed on a FACSDiva (BD Biosciences, San Jose, CA) equipped with an Enterprise II argon ion laser (Coherent, Palo Alto, CA). Cells were first gated to perform measurements on morphologically normal single living cells (forward scatter vs. side scatter, doublet exclusion) and on morphologically normal single fixed cells (forward scatter vs. side scatter, doublet exclusion). With the exception of cell cycle Hoechst 33342 staining and mitochondrial membrane potential (DiOC6, Invitrogen) were used to determine mitochondrial content (16, 41), mitochondrial H2O2 level (H2DCFDA, Invitrogen) and mitochondrial membrane depolarization. H2O2 (16 mM, 30 min) was used as a positive control.

**Table 1. Biological parameters, fluorescent probes, channels, characteristics of electronic signals, and band-pass filters used in flow cytometry analyses**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Probe</th>
<th>Excitation Line, nm</th>
<th>Channel</th>
<th>Amplification</th>
<th>Emission, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>Hoechst 33342</td>
<td>351</td>
<td>FL5-A; FL5-W</td>
<td>Linear</td>
<td>424/44</td>
</tr>
<tr>
<td>Cell viability</td>
<td>PI</td>
<td>488</td>
<td>FL3-H</td>
<td>Logarithm</td>
<td>695/40</td>
</tr>
<tr>
<td>Cardiolipin content</td>
<td>NAO</td>
<td>488</td>
<td>FL1-H</td>
<td>Logarithm</td>
<td>530/30</td>
</tr>
<tr>
<td>Mitochondrial elongation</td>
<td>NAO</td>
<td>488</td>
<td>FL1-W</td>
<td>Linear</td>
<td>530/30</td>
</tr>
<tr>
<td>Mitochondrial membrane potential</td>
<td>DiOC6</td>
<td>488</td>
<td>FL1-H</td>
<td>Logarithm</td>
<td>530/30</td>
</tr>
<tr>
<td>H2O2 level</td>
<td>H2DCFDA</td>
<td>488</td>
<td>FL1-H</td>
<td>Logarithm</td>
<td>530/30</td>
</tr>
<tr>
<td>Cytosolic Ca2+ concentration</td>
<td>Fluo-4AM</td>
<td>488</td>
<td>FL1-H</td>
<td>Logarithm</td>
<td>530/30</td>
</tr>
<tr>
<td>Immunolabeling</td>
<td>FITC</td>
<td>488</td>
<td>FL1-H</td>
<td>Logarithm</td>
<td>530/30</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>488</td>
<td>FL2-H</td>
<td>Logarithm</td>
<td>585/40</td>
</tr>
</tbody>
</table>

H2DCFDA, 3,3′-dihexyloxacarbocyanine iodide; FITC, fluorescein-5-isothiocyanate; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; NAO, nonyl acridine orange; PE, phycoerythrin; PI, propidium iodide.
stained with Hoechst 33342. Fluorescence was visualized by microscopy (Leica confocal TCS-SP2 or Olympus inverse microscope IX81 system).

**Statistical Analyses**

Data are means ± SE from six independent culture dishes. Mean difference between myoblasts in G1, S, and G2M was determined by using a one-way analysis of variance. Scheffé post hoc test was used to identify specific mean differences. Unpaired t-test was used to determine specific mean difference between myoblasts in G1 and at the onset of differentiation. The α-level of significance was set at 0.05.

**RESULTS**

**Cell Cycle Distribution of L6E9 Muscle Cells**

L6E9 myoblasts were maintained in growth medium (Fig. 1A) or allowed to differentiate for 36 h in differentiation medium. Under this condition, myoblasts adopted an elongated shape and aligned with each other. Beyond this time point, myoblasts then started to fuse and form multinucleated myotubes. Quantitative analysis of Hoechst 33342 staining indicated that 69.5 ± 0.8%, 13.7 ± 0.9%, and 16.8 ± 0.8% (n = 24) of myoblasts were positioned in G1, S, and G2M phases of the cell cycle, respectively (Fig. 1B). By contrast, myoblasts allowed to differentiate for 36 h in differentiation medium were almost all positioned in G1 phase (Fig. 1C). Only 4% of cells were in S and G2M phases, clearly illustrating withdrawal from the cell cycle. In agreement with these observations, expression of PCNA, a cofactor of DNA polymerase-δ, was markedly decreased at the onset of differentiation (Fig. 1, D and E). Similarly, expression of Myf-5, a muscle regulatory factor whose expression decreases at the onset of differentiation (28, 34, 48), was also significantly reduced (Fig. 1, D and E).

**Regulation of Mitochondrial Biogenesis**

Cardiolipin content was first determined by using NAO, a metachromatic dye, which binds to the mitochondrial-specific phospholipid, cardiolipin (16, 41). NAO fluorescence intensity signal also correlates well with the mitochondrial content in skeletal muscle (37). When observed with fluorescence microscopy, NAO-stained mitochondria were distributed in the entire cell with a greater density at the vicinity of nucleus (Fig. 2A). The punctuated staining pattern suggests that mitochondria were definite entities. Cytometric analysis shows that mitochondrial content increased in a nonlinear fashion during the cell cycle, with most of the increase occurring during the transition from G1 to S phase (Fig. 2B). Myoblasts allowed to differentiate for 36 h had the same mitochondrial content than myoblasts in G1 phase. Analysis of NAO width signal fluorescence, which is indicative of mitochondria elongation (27), suggested that mitochondria became larger during the cell cycle (P < 0.001) (Fig. 2C). However, they did not differ in differentiating myoblasts comparatively to cells in G1 phase.

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![Fig. 1](http://www.ajpcell.org)
chondrial biogenesis in skeletal muscle (52), mainly occurred during the transition from G1 to S phase (Table 3). By contrast, the expression of PPAR-α, PPAR-δ, and NOS1 was regularly and markedly increased as myoblasts progressed through the cell cycle (Table 3). Expression of PPAR-α and PPAR-δ, two transcription factors that contribute to mitochondrial biogenesis by activating expression of genes involved in fatty acid oxidation pathway (19), were increased by about threefold from G1 to G2M phase (P < 0.001) (Table 3). Expression of NOS1, an important regulator of mitochondrial biogenesis in skeletal muscle (40), was also regularly increased to reach a level that was twofold higher in G2M phases compared with G1 phase (P < 0.001) (Table 3). Finally, the protein levels of PGC-1α, PPAR-α, PPAR-δ, and NOS1 were all strongly decreased after 36 h of differentiation to represent only 10–25% of the values obtained in G1 phase.

Mitochondrial H$_2$O$_2$ Level and Mitochondrial Membrane Potential

Mitochondria were identified as a major source of ROS as illustrated by a marked reduction in H$_2$DCFDA fluorescence, a marker of mitochondrial H$_2$O$_2$ level (5), after the addition of the mitochondrial uncoupler CCCP (Fig. 3A). A twofold increase in fluorescence was observed during the transition from G1 to S phase (P < 0.05) (Fig. 3B). Mitochondrial H$_2$O$_2$ level then remained elevated until completion of the cell cycle. By

Table 3. Expression of mitochondrial biogenesis factors

<table>
<thead>
<tr>
<th>Cell Cycle Phase</th>
<th>G1</th>
<th>S</th>
<th>G2M</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC-1α</td>
<td>100.0±4.2</td>
<td>126.7±6.5*</td>
<td>141.5±7.5†</td>
<td>23.3±1.5*</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>100.0±10.1</td>
<td>203.2±17.4*</td>
<td>329.7±30.3†</td>
<td>8.7±0.4*</td>
</tr>
<tr>
<td>PPAR-δ/δ</td>
<td>100.0±9.7</td>
<td>211.7±14.5*</td>
<td>314.4±16.3*</td>
<td>18.0±1.1*</td>
</tr>
<tr>
<td>NOS1</td>
<td>100.0±5.7</td>
<td>149.3±7.2*</td>
<td>206.1±10.2†</td>
<td>10.7±0.8*</td>
</tr>
</tbody>
</table>

Data are means ± SE from 6 culture dishes and are expressed as percentage of values obtained in G1 phase. Analyses were performed on fixed/permeabilized myoblasts cultured in growth medium (G1, S, and G2M) and after 36 h of differentiation. Hoechst 33342 was used to perform cell cycle analysis after doublet exclusion. Geometric means of the fluorescence intensity peaks were used to determine the expression of respiratory chain proteins. *P < 0.001, significantly different from G1 phase; †P < 0.001, significantly different from S phase.

Variations in mitochondrial content as a function of myoblast position in the cell cycle were also accompanied by an increase in the expression of mitochondrial respiratory chain protein. Expressions of three nuclear-encoded mitochondrial proteins of complex III (core II, 13.4) and V (F1-ATPase-α) were all increased by about 40–50% in S phase (P < 0.001) and 60–80% in G2M phases (P < 0.001) when compared with G1 cells (Table 2). As observed for mitochondrial content, the increase in mitochondrial protein expression mainly occurred during G1 to S transition. Induction of differentiation elicited a marked reduction in core II, 13.4, and F1-ATPase-α protein levels, which were decreased by 30%, 55%, and 40%, respectively (P < 0.001).

In agreement with the expression pattern of mitochondrial proteins, expression of PGC-1α, a master regulator of mitochondrial biogenesis in skeletal muscle (52), was strongly increased from G1 to S phase (Table 3). By contrast, the expression of PPAR-α, PPAR-δ, and NOS1 was regularly and markedly increased as myoblasts progressed through the cell cycle (Table 3). Expression of PPAR-α and PPAR-δ, two transcription factors that contribute to mitochondrial biogenesis by activating expression of genes involved in fatty acid oxidation pathway (19), were increased by about threefold from G1 to G2M phase (P < 0.001) (Table 3). Expression of NOS1, an important regulator of mitochondrial biogenesis in skeletal muscle (40), was also regularly increased to reach a level that was twofold higher in G2M phases compared with G1 phase (P < 0.001) (Table 3). Finally, the protein levels of PGC-1α, PPAR-α, PPAR-δ, and NOS1 were all strongly decreased after 36 h of differentiation to represent only 10–25% of the values obtained in G1 phase.

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Table 2. Expression of nuclear-encoded respiratory chain proteins of complex III (Core II, 13.4) and complex V (20D6)

<table>
<thead>
<tr>
<th>Cell Cycle Phase</th>
<th>G1</th>
<th>S</th>
<th>G2M</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core II</td>
<td>100.0±5.6</td>
<td>151.3±9.5*</td>
<td>171.5±10.4†</td>
<td>70.9±2.3*</td>
</tr>
<tr>
<td>13.4</td>
<td>100.0±5.3</td>
<td>151.3±8.3*</td>
<td>183.5±10.1†</td>
<td>45.4±3.7*</td>
</tr>
<tr>
<td>20D6</td>
<td>100.0±3.2</td>
<td>138.7±4.9*</td>
<td>161.4±5.6†</td>
<td>60.5±2.3*</td>
</tr>
</tbody>
</table>

Data are means ± SE from 6 culture dishes. Analyses were performed on fixed/permeabilized myoblasts cultured in growth medium (G1, S, and G2M) and after 36 h of differentiation. Hoechst 33342 was used to perform cell cycle analysis after doublet exclusion. Geometric means of the fluorescence intensity peaks were used to determine the expression of respiratory chain proteins. *P < 0.001, significantly different from G1 phase; †P < 0.001, significantly different from S phase.

Fig. 2. Mitochondrial cardiolipin content and mitochondrial elongation. A: representative image of mitochondrial staining by nonyl acridine orange (NAO) in L6E9 myoblasts cultured in growth medium. Nuclei were stained with Hoechst 33342. Cells were visualized by confocal microscopy (Leica TCS-SP2). B: mitochondrial cardiolipin content was determined from the geometric means of height NAO (NAO-H) fluorescence signal. C: mitochondrial elongation was estimated from the geometric means of width NAO (NAO-W) fluorescence. Flow cytometry analyses were performed on living cells as a function of myoblast position in the cell cycle (G1, S, and G2M) and after 36 h of differentiation (D). Data are means ± SE from 6 culture dishes. ***P < 0.001, significantly different from G1 phase; !!!!P < 0.001, significantly different from S phase.

Variations in mitochondrial content as a function of myoblast position in the cell cycle were also accompanied by an increase in the expression of mitochondrial respiratory chain protein. Expressions of three nuclear-encoded mitochondrial proteins of complex III (core II, 13.4) and V (F1-ATPase-α) were all increased by about 40–50% in S phase (P < 0.001) and 60–80% in G2M phases (P < 0.001) when compared with G1 cells (Table 2). As observed for mitochondrial content, the increase in mitochondrial protein expression mainly occurred during G1 to S transition. Induction of differentiation elicited a marked reduction in core II, 13.4, and F1-ATPase-α protein levels, which were decreased by 30%, 55%, and 40%, respectively (P < 0.001).

In agreement with the expression pattern of mitochondrial proteins, expression of PGC-1α, a master regulator of mito-
contrast, mitochondrial H$_2$O$_2$ level was decreased by about 50% when cells were allowed to differentiate in low-serum medium for 36 h ($P < 0.001$).

Because the mitochondrial membrane potential is a critical bioenergetic parameter controlling mitochondrial ROS production (30, 39), we examined the mitochondrial membrane potential signal by using the cationic dye DiOC$_6$ (47). DiOC$_6$ was targeted to the mitochondria (compare Fig. 4A with Fig. 2A), and DiOC$_6$ fluorescence was abolished by a mitochondrial uncoupler (Fig. 4B). DiOC$_6$ fluorescence was increased by about twofold during the transition from G1 to S phase ($P < 0.001$) and then remained elevated (Fig. 4C). Fluorescence was still above G1 values after 36 h of differentiation ($P < 0.001$).

Cytosolic Ca$^{2+}$ Concentration

Ca$^{2+}$ is a critical regulator of both mitochondrial respiration and mitochondrial ROS production (6). The capacity of mitochondria to release Ca$^{2+}$ in response to a metabolic challenge was therefore analyzed by using Fluo-4AM as a cytosolic Ca$^{2+}$ probe (18). Fluo-4AM fluorescence was calibrated as a function of Ca$^{2+}$-free concentration (Fig. 5A), and the Ca$^{2+}$ ionophore, ionomycin, was used as a positive control (Fig. 5B). Analysis of baseline fluorescence showed that [Ca$^{2+}$]$_{cyt}$ regularly increased from G1 to G2M phase to reach levels that were 300% above G1 phase values ($P < 0.001$) (Fig. 5C). [Ca$^{2+}$]$_{cyt}$ remained similar to G1 values in differentiating myoblasts. Fluo4-AM fluorescence was rapidly increased upon addition of CCCP (Fig. 5, C and D). Maximum increase in [Ca$^{2+}$]$_{cyt}$ was reached about 25 s after CCCP addition and then remained stable for at least 7 min. This increase in [Ca$^{2+}$]$_{cyt}$ was similar whatever the position of myoblasts in the cell cycle. However, mitochondria from differentiating myoblasts released less Ca$^{2+}$ in response to the CCCP challenge (Fig. 5D).

Calmodulin Protein Level, NFATc3 Phosphorylation, and NFATc3 Protein Level

We then asked whether the observed variations in [Ca$^{2+}$]$_{cyt}$ were associated with concomitant variations in the expression of Ca$^{2+}$-dependent signaling proteins. Calmodulin (CaM) is a Ca$^{2+}$ sensor protein that mediates the Ca$^{2+}$-dependent regulation of enzymes and downstream signaling pathways (9). CaM protein level was sharply increased during the transition from G1 to S phase and remained elevated throughout the remainder of the cell cycle ($P < 0.001$). Calmodulin protein level was sharply increased during the transition from G1 to S phase and remained elevated throughout the remainder of the cell cycle ($P < 0.001$). Calmodulin protein level was sharply increased during the transition from G1 to S phase and remained elevated throughout the remainder of the cell cycle ($P < 0.001$). Calmodulin protein level was sharply increased during the transition from G1 to S phase and remained elevated throughout the remainder of the cell cycle ($P < 0.001$). Calmodulin protein level was sharply increased during the transition from G1 to S phase and remained elevated throughout the remainder of the cell cycle ($P < 0.001$). Calmodulin protein level was sharply increased during the transition from G1 to S phase and remained elevated throughout the remainder of the cell cycle ($P < 0.001$). Calmodulin protein level was sharply increased during the transition from G1 to S phase and remained elevated throughout the remainder of the cell cycle ($P < 0.001$).

Fig. 3. Mitochondrial H$_2$O$_2$ level. A: oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; FL1-H) was determined in L6E9 myoblasts cultured in growth medium. Positive and negative controls were obtained by incubating cells with 100 µM H$_2$O$_2$ (right displacement) and 1 mM CCCP (left displacement), respectively. B: H$_2$DCFDA oxidation was determined from the geometric means of fluorescence. Analyses were performed on living cells cultured in growth medium (G1, S, and G2M) and after 36 h of differentiation. Data are means ± SE from 6 culture dishes. *$P < 0.05$ and ***$P < 0.001$, significantly different from G1 phase.

Fig. 4. Mitochondrial membrane potential. A: representative image of 3,3'-dihexyloxacarbocyanine iodide (DiOC$_6$) fluorescence of L6E9 myoblasts cultured in growth medium (Olympus inverse microscope IX81 system). Nuclei were stained with Hoechst 33342. B: representative frequency histogram of DiOC$_6$ fluorescence (FL1-H) obtained in myoblasts cultured in growth medium (G1, S, and G2M) and after 36 h of differentiation. Data are means ± SE from 6 culture dishes. *$P < 0.05$ and ***$P < 0.001$, significantly different from G1 phase.

Fig. 5. Calmodulin protein level. A: representative image of calmodulin (CaM) fluorescence of L6E9 myoblasts cultured in growth medium (Olympus inverse microscope IX81 system). Nuclei were stained with Hoechst 33342. B: representative frequency histogram of CaM fluorescence (FL1-H) obtained in myoblasts cultured in growth medium (G1, S, and G2M) and after 36 h of differentiation. Data are means ± SE from 6 culture dishes. **$P < 0.01$, ***$P < 0.001$, significantly different from G1 phase.
G1 to S phase ($P < 0.001$) and then modestly but significantly increased (Fig. 6A). By contrast, CaM protein level was decreased at the onset of differentiation ($P < 0.001$). NFAT translocation into the nucleus and transcriptional activity are regulated by calcineurin, a Ca$^{2+}$/CaM-activated phosphatase. In the present study, the phosphorylated inactive form of NFATc3 was mainly increased during the transition from G1 to
S phase ($P < 0.001$), whereas it was decreased by 40% at the onset of differentiation ($P < 0.001$) (Fig. 6B). NFATc3 total protein level (phosphorylated plus unphosphorylated forms) was regularly increased from G1 to G2M phase to reach a level that was 1.8-fold higher in G2M when compared with G1 (Fig. 6C). When cells were allowed to differentiate for 36 h, NFATc3 expression decreased by 20%. Finally, the phosphorylated-to-total NFATc3 ratio slightly but significantly increased between G1 and S phases and markedly decreased at the onset of differentiation ($P < 0.001$) (Fig. 6D).

**DISCUSSION**

In the present study, we have demonstrated that mitochondrial biogenesis and metabolism are tightly regulated during myoblast proliferation and upon the induction of differentiation. We further show that this may contribute to regulate the level of signaling agents.

The marked stimulation of the biosynthesis of the phospholipid cardiolipin during the transition from G1 to S phase is in agreement with previous observations on murine splenocytes (44) and liver C9 cells (36) and therefore indicates an increase in mitochondrial content and membrane synthesis (37). This enlargement is probably necessary to supply the amount of functional mitochondrial inner membrane necessary to anchor respiratory chain proteins required for ATP synthesis during DNA replication in S phase. The concomitant increase in the expression of respiratory chain proteins and mitochondrial biogenesis factors (PGC-1α, PPAR-α, PPAR-δ, and NOS1) observed in S phase further supports this hypothesis. However, expression of PPAR-α, PPAR-δ, and NOS1 was still markedly increased between S and G2M phases, whereas cardiolipin synthesis and mitochondrial protein expression were modestly stimulated, suggesting that PPAR-α, PPAR-δ, and NOS1 may be also involved in other biological processes. Furthermore, as major regulators of the expression of genes involved in fatty acid transport and oxidation (19), the regular increase in PPAR-α and PPAR-δ expression from G1 to G2M phase also suggests a progressive maturation of mitochondrial lipid metabolism as cells progress through the cell cycle. In agreement with a previous report in liver C9 cells (36), myoblasts had doubled their mitochondrial content after completion of the cell cycle. Mitochondria are therefore equally divided among daughter cells after cytokinesis, suggesting that the mitochondrial content is maintained through successive cell division. The difference between cells in cytokinesis and cells in G1 phase is therefore the result of the repartition of existing material in daughter cells. Our analysis indicating that mitochondria are modestly but regularly elongated during cell cycle progression suggests that mitochondria first undergo fusion and that fission then allows equitable distribution of mitochondria to daughter cells. Altogether, these data indicate a strong stimulation of mitochondrial biogenesis. They also show that mitochondria are quite labile and subject to an intense remodeling/reshaping during the cell cycle. Expression of mitochondrial fusion and fission proteins may be therefore tightly regulated during cell cycle progression.

Whereas the mitochondrial content of myoblasts in G1 and at the onset of differentiation was fairly similar, mitochondrial protein expression was strongly reduced upon the induction of differentiation, illustrating a decrease in protein density inside the organelle. This observation is also supported by the fact that the expression of PGC-1α, PPAR-α, PPAR-δ, and NOS1 was concomitantly decreased. Mitochondrial membrane biogenesis and mitochondrial protein expression can be therefore differentially regulated. It was previously reported that exposure of quiescent 3T3 fibroblasts to 0.5–20% fetal bovine serum led to a marked stimulation of mitochondrial respiratory capacity together with an increase in cytochrome c expression and nuclear respiratory factor (NRF) 1 and cAMP response element binding protein phosphorylation (24). In the present study, it is conceivable that the induction of differentiation by lowering serum level from 20% fetal bovine serum to 2% horse serum may conversely repress the expression of genes involved in mitochondrial respiration. The marked decrease in the expression of mitochondrial biogenesis factors, observed at the onset of differentiation, could be also necessary for the completion of the subsequent steps of the myogenic program. In support of this assumption, PPAR-δ has been described as an inhibitor of myogenesis through the negative regulation of myogenic protein expression, such as MyoD (25). Furthermore, induction of NOS1 expression 48–60 h after the onset of differentiation has been shown to be necessary for myoblast fusion (33). In the present study, the weak expression of NOS1 after 36 h of differentiation is in agreement with our observation that myoblasts have not fused yet at the time of analysis.

We and others previously reported that a marked stimulation of mitochondrial biogenesis occurs in vitro 3–4 days after the induction of differentiation (31, 38) and in vivo after 7–10 days of regeneration (14). These data combined with our observations therefore indicate that this stimulation of mitochondrial biogenesis occurs later during cell fusion and the subsequent hierarchical organization of muscle fibers.

We next investigated the impact of these variations in mitochondrial biogenesis on the regulation of H2O2 level and [Ca$^{2+}$]$_{cyt}$. It has been shown that the probability of electron leakage to form ROS increases at high mitochondrial membrane potential (26, 39). In agreement with these studies, our results show that progression of myoblasts through the cell cycle is accompanied by an increase in both mitochondrial H2O2 level and mitochondrial membrane potential. Importantly, ROS have been previously reported to promote the transition from G1 to S phase. Indeed, retardation of G1 to S transition in NSF/N1.H7 cells can be overridden by directly adding H2O2 to the cells in a mechanism that involves the downregulation of p27 and activation of Cdk2 (12). Conversely, antioxidant treatment inhibits G1 to S phase transition in different cell types (12, 22, 51). Although the mitochondrial origin of ROS was not investigated in these studies, these data suggest a potential role for mitochondrial ROS in the regulation of G1 to S phase transition. In the present study, the marked increase in mitochondrial H2O2 level during the transition from G1 to S phase supports this assumption.

Upon the induction of differentiation, mitochondria have a lower H2O2 level, despite the maintenance of an elevated mitochondrial membrane potential, suggesting that these mitochondria have a very efficient electron transport so that electron leakage toward ROS production is decreased. Furthermore, the lower density of respiratory chain proteins (see above) may also contribute to decrease ROS production. In rat hepatoma cells, ROS have been shown to activate the binding of NRF-1 to the promoter of mitochondrial transcription factor
A, a transcription factor required for mitochondrial DNA transcription and replication (42). In the present study, the decrease in H$_2$O$_2$ level at the onset of differentiation may thus decrease calcineurin activity, ultimately contributing to decrease the expression of proteins encoded by the mitochondrial genome. Conversely, the increase in H$_2$O$_2$ level observed during the transition from G$_1$ to S phase may stimulate the replication and transcription of mitochondrial DNA, therefore contributing to the stimulation of mitochondrial biogenesis. The decrease in mitochondrial H$_2$O$_2$ level may also have profound influence on the process of myogenic differentiation. Exposure of differentiating myoblasts to H$_2$O$_2$ has been shown to almost totally inhibit myogenic differentiation. Exposure of differentiating myoblasts released less Ca$^{2+}$ compared with cells in G$_1$. This also indicates that one of the main systems of Ca$^{2+}$ transport, the plasma membrane Ca$^{2+}$-ATPase, is sensitive to redox conditions (53). In the present study, the increase in mitochondrial H$_2$O$_2$ level in S and G$_2$M phases may therefore contribute to the reported increase in [Ca$^{2+}$]$_{cys}$. However, mitochondria can also couple their metabolic activity with the regulation of Ca$^{2+}$ transport processes, thereby controlling not only their own Ca$^{2+}$ concentration but also influencing the entire network of cellular Ca$^{2+}$ signaling. Our observation that CCCP led to a significant increase in [Ca$^{2+}$]$_{cys}$ further illustrates that mitochondria can function as a Ca$^{2+}$ sink that may be mobilized in response to variations in mitochondrial membrane potential (20). The similar response to chemical uncoupling between cells in G$_1$, S, or G$_2$M, together with the increase in mitochondrial content observed in S or G$_2$M phase, suggests that myoblasts in S and G$_2$M have more mitochondria with lower Ca$^{2+}$ contents compared with cells in G$_1$. This also means that the intense remodeling/reshaping of mitochondria that occurs in S and G$_2$M phases does not seem to disturb mitochondrial capacity to release Ca$^{2+}$. The dependence of [Ca$^{2+}$]$_{cys}$ on mitochondrial membrane potential also points to a potential mean of interfering with Ca$^{2+}$ signaling under physiological conditions where mitochondrial membrane potential is modulated by substrate availability. Mitochondria from differentiating myoblasts released less Ca$^{2+}$ upon a metabolic challenge. Taking into account the decrease in mitochondrial protein expression upon the induction of differentiation, the lower response of mitochondria in the face of the CCCP challenge may be due to an immature system of mitochondrial Ca$^{2+}$ transport. Corollary, this also suggests that the capacity of mitochondria to transduce a metabolic signal into a Ca$^{2+}$-dependent response is weakened at the onset of differentiation. It is also important to note that numerous Ca$^{2+}$-handling proteins, whose expression can be modified during myoblast proliferation and the onset of differentiation, may also contribute to the regulation of [Ca$^{2+}$]$_{cys}$.

The regulation of [Ca$^{2+}$]$_{cys}$ could contribute to a modulation of Ca$^{2+}$-dependent signaling pathways. We therefore asked whether the observed variations in [Ca$^{2+}$]$_{cys}$ were associated with concomitant variations in the expression and activity of Ca$^{2+}$-dependent signaling proteins. The reported increase in the expression of CaM during the progression from G$_1$ to S phase apparently contrasts with the increased phosphorylated-to-total NFATc3 ratio, which may rather indicate a decrease in calcineurin activity. This suggests that the increase in CaM protein level can be partly dissociated from the regulation of NFATc3 phosphorylation. This is further supported by the observation that ectopic expression of CaM accelerates the transition from G$_1$ to S phase in mouse mammary cells and that CaM inhibition triggers a cell cycle arrest in G$_1$ (43). This effect could be explained by the capacity of CaM to bind and activate cyclin E (10), which is essential for the transition from G$_1$ to S phase. Furthermore, NFAT has been described to repress the expression of cyclin A2 (7), which is involved in the transition from G$_1$ to S phase. This observation is therefore in agreement with the increase in NFATc3 phosphorylation reported in the present study. Although [Ca$^{2+}$]$_{cys}$ was unchanged and CaM expression was repressed in differentiating cells, phosphorylated-to-total NFATc3 ratio was decreased, suggesting an increase in the nuclear translocation of NFATc3 in the nucleus of differentiating cells. This is in agreement with the positive regulation of calcineurin and NFATc3 on the initiation of differentiation (3, 11, 50). It remains to be determined whether the relation between mitochondrial metabolism and [Ca$^{2+}$]$_{cys}$ observed in the present study may contribute to the regulation of Ca$^{2+}$-dependent signaling.

In summary, we have shown that mitochondrial content and metabolism are tightly regulated during the proliferation and at the onset of differentiation of myoblasts. Furthermore, we show that these variations were accompanied by variations in the regulation of mitochondrial H$_2$O$_2$ level and [Ca$^{2+}$]$_{cys}$, suggesting the existence of a functional link between mitochondrial biogenesis/metabolism and the regulation of cellular signaling. Our data therefore set the fundamentals for further studies aimed at identifying the targets of mitochondrial-dependent signaling pathways and their biological relevance in the context of myoblast proliferation and differentiation.

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