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Intermittent increases in cytosolic Ca^{2+} stimulate mitochondrial biogenesis in muscle cells

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Ojuka, Edward O., Terry E. Jones, Dong-Ho Han, May Chen, Brian R. Wamhoff, Michael Sturek, and John O. Holloszy. Intermittent increases in cytosolic Ca^{2+} stimulate mitochondrial biogenesis in muscle cells. *Am J Physiol Endocrinol Metab* 283: E1040–E1045, 2002. First published July 24, 2002; 10.1152/ajpendo.00242.2002.—Muscle contractions cause numerous disturbances in intracellular homeostasis. This makes it impossible to use contracting muscle to identify which of the many signals generated by contractions are responsible for stimulating mitochondrial biogenesis. One purpose of this study was to evaluate the usefulness of L6 myotubes, which do not contract, for studying mitochondrial biogenesis. A second purpose was to evaluate further the possibility that increases in cytosolic Ca^{2+} can stimulate mitochondrial biogenesis. Continuous exposure to 1 μM ionomycin, a Ca^{2+} ionophore, for 5 days induced an increase in mitochondrial enzymes but also caused a loss of myotubes, as reflected in an ~40% decrease in protein per dish. However, intermittent (5 h/day) exposure to ionomycin, or to caffeine or W7, which release Ca^{2+} from the sarcoplasmic reticulum, did not cause a decrease in protein per dish. Raising cytosolic Ca^{2+} intermittently with these agents induced significant increases in mitochondrial enzymes. EGTA blocked most of this effect of ionomycin, whereas dantrolene, which blocks Ca^{2+} release from the sarcoplasmic reticulum, largely prevented the increases in mitochondrial enzymes induced by W7 and caffeine. These findings provide evidence that intermittently raising cytosolic Ca^{2+} stimulates mitochondrial biogenesis in muscle cells.

caffeine; exercise; gene expression; ionomycin; L6 myotubes

THIRTY-FIVE YEARS HAVE ELAPSED since the discovery that endurance exercise induces an increase in muscle respiratory capacity, mediated by an increase in mitochondrial enzymes (11). Although progress has been made in explaining how the increase in muscle mitochondria contributes to enhancement of exercise capacity and endurance, relatively little is known regarding the mechanisms responsible for this adaptation (2, 4, 5). One of the reasons for this slow progress is that muscle contractions cause numerous disturbances in intracellular homeostasis. This makes it impossible to use contracting muscle for elucidating which of the many signals generated by exercise is/are responsible for stimulation of mitochondrial biogenesis. It is, therefore, necessary to use another model in which it is possible to study these potential signaling mechanisms individually. In this context, one purpose of this study was to evaluate the usefulness of L6 myotubes in culture for studying the regulation of mitochondrial biogenesis.

One of the signals by which contractile activity stimulates mitochondrial biogenesis could be the increase in cytosolic Ca^{2+} that is triggered by each wave of sarcolemmal depolarization. In support of this possibility, Lawrence and Salsgiver (16) reported that exposure of primary cultures of fetal rat muscle cells to 3 μM A-23187, a Ca^{2+} ionophore, induced increases in mitochondrial enzymes. Similarly, Freyssenet et al. (6) found that continuous exposure of L6E9 myoblasts to A-23187 induced an increase in cytochrome *c* mRNA. In a preliminary experiment in our laboratory, using primary cultures of fetal rat skeletal muscle, we confirmed that continuous exposure to 3 μM A-23187 induces an increase in mitochondrial enzymes; however, we also found that continuous exposure to 3 μM A-23187 for 4 days has a deleterious effect on cell viability, with a decrease in total protein content, a decrease in number of cells, and atrophy of some cells (E. A. Gulve and J. O. Holloszy, unpublished findings). Enzyme activities vary markedly between individual myotubes in primary cultures of rat skeletal muscle (21). So, this finding of a decrease in myotubes raised the possibility that the sustained increase in cytosolic Ca^{2+} may have selectively killed myotubes with a low mitochondrial content rather than stimulated mitochondrial biogenesis. Therefore, a second aim of this study was to evaluate further the possibility that intermittent increases in cytosolic Ca^{2+} can stimulate mitochondrial biogenesis in muscle cells. L6 myotubes do not contract in response to an increase in cytosolic Ca^{2+}, thus avoiding the generation of other potential signals induced by breakdown of high-energy phos-

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phates (~P), increases in glycolytic intermediates, changes in redox state and pH, and so forth, in contracting muscle.

MATERIALS AND METHODS

Materials. Reagents for SDS-PAGE were purchased from Bio-Rad. Reagents for enhanced chemiluminescence (ECL) were obtained from Amersham Pharmacia Biotech. L6 myocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA). [1-^{14}C]oleic acid was from American Radiolabeled Chemicals (St. Louis, MO). Rabbit polyclonal antibodies (directed) against the 19 COOH-terminal amino acids of δ-aminolevulinate synthase (ALAS) and the 20 COOH-terminal amino acids of citrate synthase were generated by Alpha Diagnostic International (San Antonio, TX). A mouse anti-human cytochrome oxidase (COX) subunit I monoclonal antibody was purchased from Molecular Probes (Eugene, OR). A mouse anti-cytochrome *c* monoclonal antibody was purchased from Pharmingen International (San Diego, CA). A rabbit polyclonal phospho-AMP kinase-α antibody (Thr^{172}) was obtained from Cell Signaling Technology. Horseradish peroxidase-conjugated secondary antibodies were from the Jackson Laboratory. 5-Aminoimidazole-4-carboxamide-β-D-ribofuranoside (AICAR) was obtained from Toronto Research Chemicals (North York, Ontario, Canada). All other reagents were purchased from Sigma.

Cell culture. L6 myocytes were maintained at 37°C on 100-mm collagen-coated plastic dishes in 5% CO_2-95% humidified air. The culture medium consisted of low glucose (5 mM) DMEM supplemented with 0.5 mM oleic acid, 1% BSA, 1 mM L-carnitine, 100 μU/ml penicillin, 100 μU/ml streptomycin, 0.25 μg/ml fungizone, 5% horse serum, and 10% FBS. The oleate was solubilized in 1 mM fatty acid-free albumin. Media were sterilized by filtration through a 0.2-μm filter. Cells were maintained in continuous passage by trypsinization of subconfluent cultures with the use of 0.25% trypsin. Differentiation was induced by switching to medium containing 2% heat-inactivated horse serum when the myoblasts were ~80% confluent. The experimental treatments were started after 7–9 days, by which time nearly all of the myoblasts had fused to form myotubes. At this time, we switched back to the medium containing 5% horse serum and 10% FBS. Treatment of the myotubes with 5 mM caffeine, 1 μM ionomycin, or 50 μM W7 with or without 10 μM dantrolene or 1 mM EGTA was for 5 h/day for 5 days. To remove these agents, the myotubes were washed twice with PBS.

Measurement of cytosolic Ca^{2+} levels. Cytosolic Ca^{2+} was determined with the use of fura-2 epifluorescence digital microscopy as described previously (10, 23).

Western blotting. Myotubes were homogenized in 250 mM sucrose containing 10 mM HEPES and 1 mM EDTA, pH 7.4. Homogenate protein concentration was measured, and the homogenate volumes were adjusted to give the same protein concentration in homogenates of cells from the different culture dishes. Aliquots of homogenate were solubilized in Laemmli sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C with 5% nonfat dry milk in PBS containing 0.1% Tween. The blots were probed with the following primary antibodies: a rabbit polyclonal antibody against the COOH-terminus of ALAS, a rabbit polyclonal antibody against the COOH-terminus of citrate synthase, a monoclonal antibody against COX-I, a monoclonal antibody against cytochrome *c*, or a rabbit polyclonal antibody against a phosphopeptide corresponding to residues surrounding Thr^{172} of AMP kinase-α. The blots were then incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody. Antibody-bound protein was detected using ECL.

Oleate oxidation. Myotubes were scraped from the culture plates and homogenized in 175 mM KCl containing 0.1 mM EDTA, using a glass Potter-Elvehjem homogenizer immersed in ice water. The capacity of whole homogenates of myotubes to oxidize [^{14}C]oleate was assessed by measuring the rate of $^{14}CO_2$ production as described previously (1, 20). The reaction mixture, contained in a final volume of 2 ml, consisted of homogenate equivalent to 2 mg protein, 5 mM $MgCl_2$, 87.5 mM KCl, 40 mM potassium phosphate buffer, 2 mM EDTA, 2 mM ADP, 10 mM Tris·HCl, 0.078 mM cytochrome *c*, 0.15 mM fatty acid-free albumin, and 0.75 mM oleate containing 0.25 μCi [1-^{14}C]oleate (per flask). The oleate was solubilized in 1 mM fatty acid-free albumin. The reaction mixtures were placed in flasks fitted with serum caps and hanging center wells containing 0.4 ml hyamine hydrozide. The flasks were incubated in Dubnoff shaking incubators. The $^{14}CO_2$ produced was trapped, and radioactivity was measured using a scintillation counter. Results are expressed per milligram protein.

ATP measurement. Cells were scraped from plates and homogenized in 0.6 M perchloric acid (PCA) at −10°C. PCA was extracted from the homogenates by use of 1:4 (vol/vol) trioctylamine:Freon. ATP concentration was determined with the use of high-pressure liquid chromatography as described by Scott et al. (27).

Statistics. Values are expressed as means ± SE. Statistically significant differences were determined using unpaired Student's *t*-tests or ANOVA as appropriate. When ANOVA showed significant differences, post hoc analysis was performed using Fisher's least significant differences post hoc test.

RESULTS

AICAR induces increases in ALAS and cytochrome c in L6 myotubes. AICAR is converted to the AMP analog 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (ZMP) after it is transported into cells and thus results in activation of AMP kinase (AMPK). It has been shown that administration of AICAR to rats induces an increase in mitochondrial enzymes in skeletal muscle (32). To validate L6 myocytes as an appropriate model for studying mitochondrial biogenesis, we therefore examined the effect of AICAR. As shown in Fig. 1, exposure of myotubes to 1 mM AICAR for 5 days resulted in significant increases in ALAS and cytochrome *c*, which were used as mitochondrial marker proteins. Exposure of L6 myotubes to AICAR also resulted in a significant increase in AMPK phosphorylation (Fig. 2), providing evidence for activation of AMPK kinase and AMPK.

Continuous vs. intermittent exposure of L6 myotubes to ionomycin. We used ionomycin instead of A-23187, because it is a more effective Ca^{2+} ionophore (18). Continuous exposure to 1 μM ionomycin for 5 days induced large increases in the protein concentrations of COX-I and ALAS, which were used as mitochondrial marker enzymes (Fig. 3). On microscopic examination, there were fewer myotubes in the culture dishes treated with ionomycin than in the untreated controls. This was reflected in a significant decrease in total protein per dish: 3.5 ± 0.3 mg/plate for ionomycin

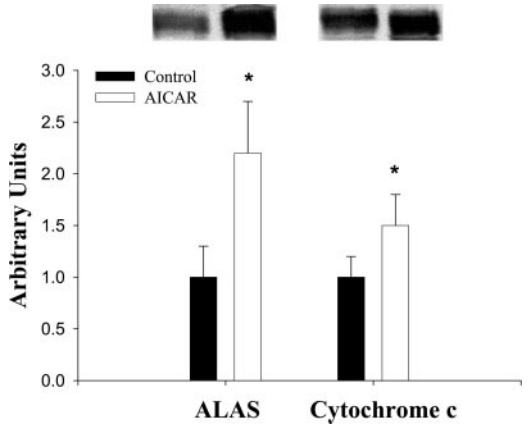

Fig. 1. 5-Aminoimidazole-4-carboxamide-β-D-ribofuranoside (AICAR) induces increases in δ-aminolevulinate synthase (ALAS) and cytochrome *c*. L6 myotubes were incubated with 1.0 mM AICAR for 5 days. Enzyme protein concentrations were determined by Western blot analysis. Values are means ± SE for 7–8 dishes/group. $*P < 0.01$, AICAR vs. control.

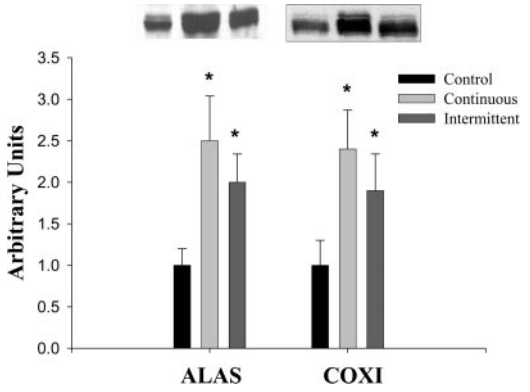

Fig. 3. Continuous vs. intermittent exposure of L6 myotubes to 1 μM ionomycin. L6 myotubes were exposed to 1 μM ionomycin either continuously or for 5 h/day for 5 days. ALAS and cytochrome oxidase subunit I (COX-I) proteins were determined by Western blot analysis. Values are means ± SE for 6 dishes/group. $*P < 0.01$, control vs. continuous or intermittent.

treatment vs. 5.8 ± 0.4 mg/plate for control, $P < 0.05$. This finding, which confirmed our pilot study findings in primary muscle cell cultures, led us to investigate the effect of intermittent exposure to ionomycin. Exposure of myotubes to 1 μM ionomycin for 5 h/day for 5 days resulted in slightly smaller, but still highly significant increases in COX-I and ALAS (Fig. 3) but no significant decrease in protein: 5.8 ± 0.4 mg/plate for controls and 5.5 ± 0.4 mg/plate for myotubes treated for 5 h with ionomycin.

Effects of ionomycin, W7, and caffeine on cytosolic Ca^{2+} concentrations. Before conducting further studies of the effect of raising cytosolic Ca^{2+}, we measured the effects of ionomycin, which acts as a Ca^{2+} ionophore and facilitates Ca^{2+} entry from the medium (18), and of two agents, W7 and caffeine, that release Ca^{2+} from the sarcoplasmic reticulum (SR) (25) on cytosolic Ca^{2+} concentration. As for ionomycin, the intermittent exposure of myotubes to caffeine or W7 did not result in a decrease in protein. As shown in Fig. 4, all three agents caused increases in cytosolic Ca^{2+} in L6 myotubes. [Fig. 4*C*, showing the effect of caffeine, has been published previously (23) and is included here for comparison with W7 and ionomycin.]

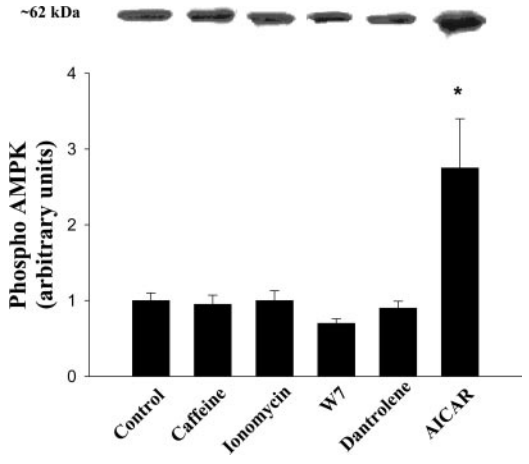

Fig. 2. AMP kinase (AMPK) phosphorylation. L6 myotubes were exposed to either AICAR, caffeine, ionomycin, W7, or dantrolene for 5 h. AMPK phosphorylation was assessed by Western blot analysis with the use of an anti-phospho-AMPK-α (Thr172) antibody. Values are means for 4 dishes/group. $*P < 0.01$ vs. control.

ATP concentrations in L6 myotubes exposed to ionomycin or W7. Although L6 myotubes do not contract in response to an increase in cytosolic Ca^{2+}, it still seemed possible that the increase in Ca^{2+} might result in a decrease in ATP. However, exposure of myotubes to 1 μM ionomycin or 50 μM W7 for 5 h had no significant effect on ATP concentrations, which averaged 5.01 ± 0.23 μmol/100 mg protein for untreated control myotubes, 4.92 ± 0.29 μmol/100 mg protein for myotubes exposed to ionomycin, and 4.80 ± 0.24 μmol/100 mg protein for myotubes exposed to W7. L6 myotubes contain negligible amounts of phosphocreatine, which were too low to measure accurately.

Increases in mitochondrial enzymes induced by agents that raise cytosolic Ca^{2+}. As shown in Fig. 5, exposure of L6 myotubes to either 50 μM W7, 1 μM ionomycin, or 5 mM caffeine for 5 h/day for 5 days induced significant increases in ALAS, COX-I, and cytochrome *c* or citrate synthase, which were used as mitochondrial marker proteins. The increases in these enzymes induced by W7 and caffeine were, like the increase in cytosolic Ca^{2+}, partially blocked by inclusion of 10 μM dantrolene in the medium containing these agents. Dantrolene is an inhibitor of Ca^{2+} release from the SR (29). Similarly, the Ca^{2+} chelator EGTA partially inhibited the increases in enzyme protein induced by ionomycin. As shown in Fig. 2, neither caffeine, ionomycin, W7, nor dantrolene treatment resulted in an increase in AMPK phosphorylation.

Rates of oxidation of oleate. To determine whether the increases in mitochondrial marker enzymes reflect an increase in functional mitochondria, we measured the capacity of myotube homogenates to oxidize [^{14}C]oleate to $^{14}CO_2$ under conditions in which availability of ADP and P_i are not limiting. As shown in Fig.

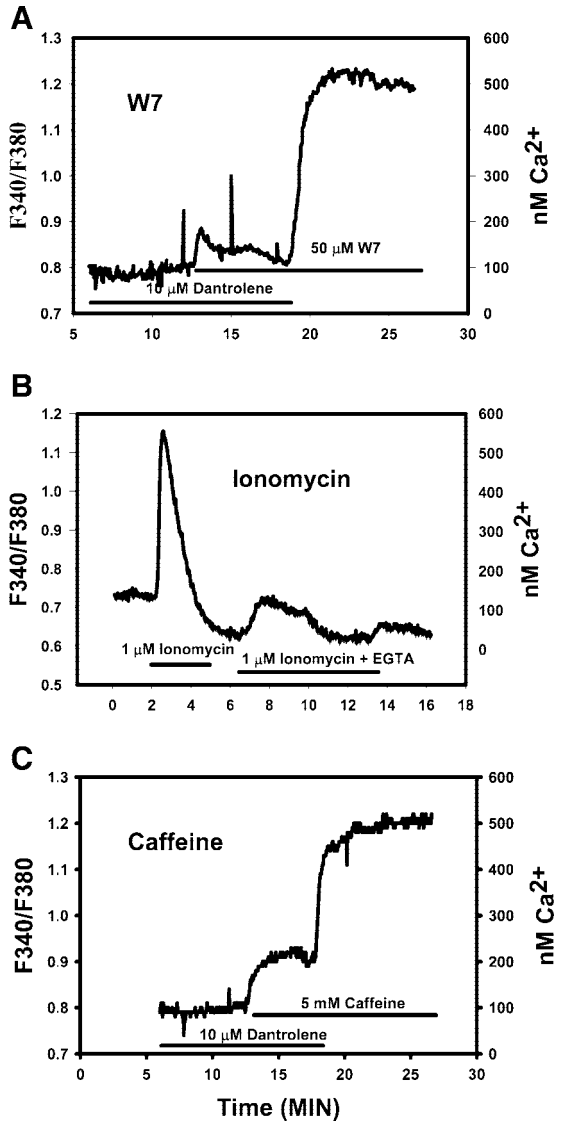

Fig. 4. W7 (*A*), ionomycin (*B*), and caffeine (*C*) increase cytosolic Ca^{2+}. Levels of Ca^{2+} were determined by use of digital epifluorescence microscopy as described in MATERIALS AND METHODS. The ratios of emitted light at 340 and 380 nm (F340/F380) represent the cytosolic Ca^{2+} levels. Estimated Ca^{2+} concentrations, based on numerous calibration curves, are also shown. Caffeine (5 mM) and W7 (50 μM) induce small increases in cytosolic Ca^{2+} in the presence of 10 μM dantrolene [which inhibits Ca^{2+} release from the sarcoplasmic reticulum (SR)] and an ~4-fold increase in the absence of dantrolene. Ionomycin (1 μM) caused an ~4-fold increase in cytosolic Ca^{2+}, most of which was prevented by 1 mM EGTA. The tracings represent average Ca^{2+} responses of 10 myotubes for each agent.

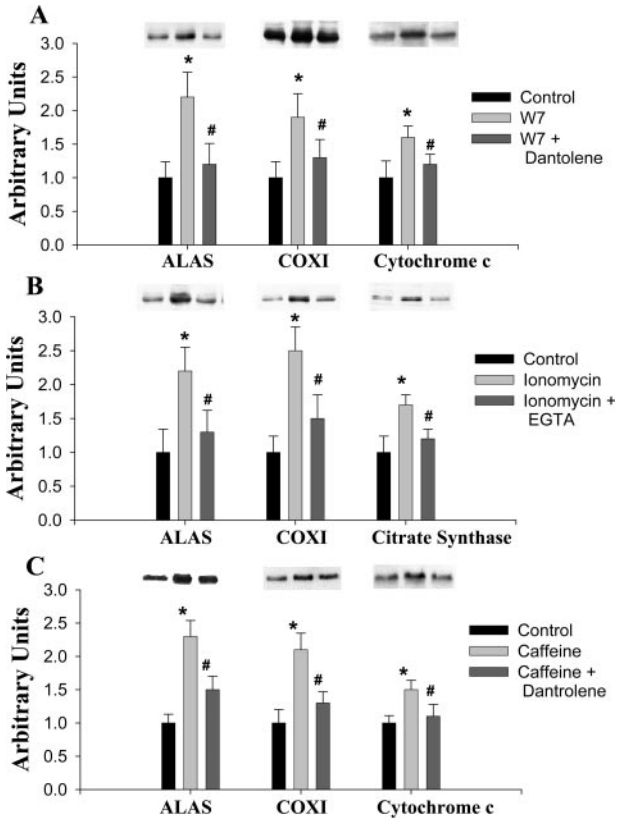

Fig. 5. Effects of intermittent exposure of L6 myotubes to W7, ionomycin, or caffeine on mitochondrial enzyme expression. L6 myotubes were exposed to 50 μM W7 with or without 10 μM dantrolene, 1 μM ionomycin with or without 1 mM EGTA, or 5 mM caffeine with or without 10 μM dantrolene for 5 h/day for 5 days. ALAS, COX-I, cytochrome *c*, and citrate synthase proteins were measured by Western blot analysis. Values are means for 7–10 dishes. *W7, ionomycin, or caffeine vs. control: $P < 0.01$ for ALAS and COX-I, and $P < 0.05$ for cytochrome *c* and citrate synthase. #W7 + dantrolene, ionomycin + EGTA, or caffeine + dantrolene vs. W7, ionomycin, or caffeine: $P < 0.05$.

6, oleate oxidation was increased significantly in response to 5 days of caffeine treatment.

DISCUSSION

One aim of this study was to evaluate the usefulness of L6 myotubes as a model for studying the regulation of mitochondrial biogenesis. One reason for choosing L6 myotubes instead of primary cultures of rat skeletal muscle is that L6 myotubes have the advantage that they do not contract. Our results show that L6 myo-

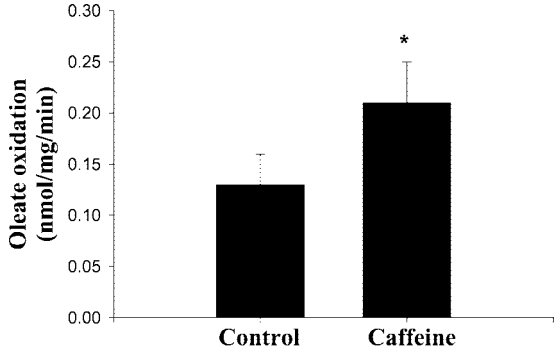

Fig. 6. Rate of oxidation of oleate by control L6 myocytes and L6 myocytes exposed to caffeine. L6 myocytes were exposed to 5 mM caffeine for 5 h/day for 5 days. Rates of oleate oxidation by homogenates of L6 myocytes were determined by measurement of production of $^{14}CO_2$ from [^{14}C]oleate as described in MATERIALS AND METHODS. Results are expressed as nmol substrate oxidized·mg protein^{-1}·min^{-1}. Values are means ± SE for 8 dishes/group. *$P < 0.05$, caffeine treatment vs. control.

tubes can respond to adaptive stimuli with rapid increases in the concentrations of mitochondrial enzymes and in the capacity to oxidize fat and carbohydrate. The latter finding provides good evidence for an increase in functional mitochondria. From these findings, we conclude that L6 myotubes are a suitable model for studying mitochondrial biogenesis.

The second purpose of this study was to reevaluate the effect of raising cytosolic Ca^{2+} on mitochondrial biogenesis in muscle cells. The evidence that Ca^{2+} stimulates mitochondrial biogenesis has come from two studies in which continuous exposure of primary cultures of rat myotubes (16) or L6E9 myotubes (6) to A-23187 induced increases in a number of mitochondrial enzymes. One reason for reevaluating the role of Ca^{2+} was the evidence that activation of AMPK stimulates mitochondrial biogenesis (32) and the possibility that the effect of A-23187 on mitochondrial biogenesis was mediated by a decrease in ATP, with an increase in AMP, resulting in activation of AMPK, rather than by the increase in Ca^{2+} per se. Another reason was our finding in a preliminary experiment that continuous exposure of primary cultures of rat myotubes to 3 μM A-23187 had a deleterious effect on cell viability, with an ~50% decrease in total protein because of atrophy and loss of cells (E. A. Gulve and J. O. Holloszy, unpublished results). Because enzyme activities can vary up to eightfold between individual myotubes in primary cultures of rat myotubes (21), this finding raised the possibility that the increases in mitochondrial enzymes might be due to selective killing by A-23187 of myotubes with the lowest mitochondrial content. A third reason was that during contractile activity, Ca^{2+} is released from and increases in the region of the SR, whereas A-23187 mediates Ca^{2+} entry through the sarcolemma. We therefore also wanted to evaluate the effects of agents that release Ca^{2+} from the SR.

Our results provide strong evidence that increases in cytosolic Ca^{2+}, mediated either by intermittent release of Ca^{2+} from the SR or Ca^{2+} entry through the sarcolemma, stimulate mitochondrial biogenesis. They also show that this effect is mediated by Ca^{2+}, not by a decrease in ~P. In support of the role of Ca^{2+}, we found that dantrolene, at a concentration that inhibited most of the increase in cytosolic Ca^{2+} induced by caffeine or W7, also largely inhibited the increases in mitochondrial proteins. Similarly, removal of Ca^{2+} from the medium with EGTA attenuated both the increase in cytosolic Ca^{2+} and the stimulation of mitochondrial biogenesis by ionomycin.

These findings, which implicate increases in cytosolic Ca^{2+} in stimulating mitochondrial biogenesis, raise a question regarding the role of decreases in phosphocreatine and ATP (~P) in mediating the increase in mitochondria induced by endurance training. The evidence that a decrease in ~P stimulates mitochondrial biogenesis, although not conclusive, is also strong. This evidence includes the finding that a number of other stimuli that lower ~P also induce increases in mitochondrial proteins. These include thyrotoxicosis (30), cold exposure (14), severe iron deficiency (22), feeding of β-guanidinopropionate (β-GPA) (26, 28, 33), and mitochondrial uncoupling (17, 19). Although severe iron deficiency results in a decrease in the iron-containing mitochondrial constituents, it can result in large increases in non-iron-containing mitochondrial enzymes (22). A mutation that results in partial mitochondrial uncoupling is associated with a remarkable increase in muscle mitochondria (19). In HeLa cells containing plasmids allowing doxycycline-inducible expression of uncoupling protein 1 (UCP-1), induction of UCP-1 expression resulted in a decrease in phosphorylation potential, as evidenced by an increase in oxygen consumption and rapid increases in nuclear respiratory factor (NRF)-1 and ALAS expression (17). Feeding rats the creatine analog β-GPA, which competitively inhibits creatine uptake and markedly lowers ~P concentrations, induces increases in mitochondrial enzymes (26, 28, 33) and in GLUT4 (26), the expression of which is regulated in parallel with mitochondrial biogenesis (3, 7, 12, 26, 32).

Recent studies have shown that many of the metabolic effects induced by a decrease in ~P are mediated by activation of AMPK (31). Inhibition of this enzyme is released by decreases in phosphocreatine and ATP, and activation is induced by increases in AMP (31). Much of the information regarding the roles of AMPK has come from the use of AICAR, which is taken up by muscle and converted to the AMP analog ZMP, which activates AMPK (31). Activation of AMPK appears to be the mechanism by which muscle contractions stimulate glucose transport (8, 9, 15). AICAR also induces increases in mitochondrial enzymes and GLUT4 in skeletal muscle (13, 24, 32, 34) and L6 myotubes (23, Fig. 1). These findings suggest that activation of AMPK may mediate the effects of a decrease in ~P not only on glucose transport but also on mitochondrial biogenesis and GLUT4 expression.

In view of this evidence, it is our tentative conclusion that both increases in cytosolic Ca^{2+} and decreases in ~P stimulate signaling pathways that lead to increases in mitochondrial biogenesis and GLUT4 expression. Mitochondria are necessary for survival, so the existence of more than one mechanism for the regulation of mitochondrial biogenesis is not surprising. Although there is now evidence for only two independent signaling pathways, one activated by increases in Ca^{2+}, the other by decreases in ~P, for stimulating mitochondrial biogenesis, there may well be additional mechanisms still to be discovered.

In conclusion, the present results show that L6 myocytes are a useful model for investigation of mitochondrial biogenesis. Using this model, we have shown that intermittently raising cytosolic Ca^{2+}, either by release from the SR or entry across the sarcolemma, stimulates mitochondrial biogenesis in muscle cells, with a rapid increase in mitochondria.

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