CALCIUM INDUCES INCREASES IN PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ COACTIVATOR-1α AND MITOCHONDRIAL BIOGENESIS BY A PATHWAY LEADING TO p38 MITOGEN ACTIVATED PROTEIN KINASE ACTIVATION

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SUMMARY

Previous studies have shown that raising cytosolic calcium in myotubes induces increases in peroxisome proliferator-activated receptor γ coactivator-1α expression and mitochondrial biogenesis. This finding suggests that the increases in cytosolic calcium in skeletal muscle during exercise may mediate the exercise-induced increase in mitochondria. The initial aim of this study was to determine whether raising calcium in skeletal muscle induces the same adaptations as in myotubes. We found that treatment of rat epitrochlearis muscles with a concentration of caffeine that raises cytosolic calcium to a concentration too low to cause contraction induces increases in peroxisome proliferator-activated receptor γ coactivator-1α expression and mitochondrial biogenesis. Our second aim was to elucidate the pathway by which calcium induces these adaptations. Raising cytosolic calcium has been shown to activate calcium/calmodulin dependent protein kinase in muscle. In the present study, raising cytosolic calcium resulted in increases in phosphorylation of p38 mitogen activated protein kinase and activating transcription factor-2 which were blocked by the calcium/calmodulin dependent protein kinase inhibitor KN93 and by the p38 mitogen activated protein kinase inhibitor SB202190. The increases in peroxisome proliferator-activated receptor γ coactivator-1α expression and mitochondrial biogenesis were also prevented by inhibiting p38 activation. We interpret these findings as evidence that p38 mitogen activated protein kinase is downstream of calcium/calmodulin dependent protein kinase in a signaling pathway by which increases in cytosolic calcium lead to increases in peroxisome proliferator-activated receptor γ coactivator-1α expression and mitochondrial biogenesis in muscle.

INTRODUCTION

Exercise induces an increase in the mitochondrial content of skeletal muscle (1,2). This adaptation results in increases in the capacities to oxidize carbohydrate and fat, and generate ATP (3-6). The transcription coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) regulates the coordinated expression of the mitochondrial genome and of the nuclear genes encoding mitochondrial enzymes (7-9). Overexpression of PGC-1α in muscle cells and in skeletal muscle results in an increase in mitochondria similar to that induced in muscle by exercise (10-12). Exercise results in rapid increases in PGC-1α activation (13) and expression (14-17) in skeletal muscle that are thought to mediate the exercise-induced increase in mitochondria. Studies on myotubes in culture have provided evidence that raising cytosolic Ca²⁺ induces increases in PGC-1α expression (17,18) and mitochondrial biogenesis (17-21), and that this adaptation is prevented by inhibition of calcium calmodulin dependent protein kinase (CAMK) (18). These findings support the hypothesis that increases in cytosolic Ca²⁺ resulting from release of sarcoplasmic (SR) Ca²⁺ during excitation-contraction coupling is the signal or one of the signals generated in muscle during exercise that mediates the increase in mitochondrial biogenesis.

The initial purpose of this study was to determine whether raising cytosolic Ca²⁺ in a more biologically relevant model, skeletal muscle, induces an increase in mitochondrial biogenesis similar to that found in myotubes. After finding that raising cytosolic Ca²⁺ in our skeletal muscle preparation does result in increases in PGC-1α expression and mitochondrial biogenesis, we used this model to try to elucidate the pathway by which the Ca²⁺-induced increases in PGC-1α and mitochondrial...
biogenesis are mediated. Both the activity and expression of PGC-1α appear to be regulated by p38 MAPK (7,22-24), which is activated in muscle by contractile activity (24-26) and in myotubes by raising cytosolic Ca2+ (27). P38 activates the transcription factor ATF-2, which binds to the CRE binding site on the PGC-1α promoter and induces PGC-1α transcription (24,28). Our results provide evidence that Ca2+ induces increases in PGC-1α and mitochondrial biogenesis in muscle by activating a pathway that leads from CAMK to p38 MAPK.

**EXPERIMENTAL PROCEDURES**

**Materials** - Reagents for SDS-PAGE were purchased from Bio-Rad (Hercules, CA). Reverse Transcription (RT) kits were obtained from Promega (Madison, WI). Chemiluminescence reagents were a product of Amersham (Piscataway, NJ). SB202190 and KN93 were obtained from Calbiochem (La Jolla, CA). An antibody against cytochrome oxidase subunit I (COXI) was purchased from Molecular Probes (Eugene, OR). The anti-PGC-1 antibody was a product of Calbiochem (La Jolla, CA). δ-Aminolevulinate synthase (ALAS) and citrate synthase antibodies were generated by Alpha Diagnostics (San Antonio, TX) (16). Phosphospecific p38 MAPK, activating transcription factor 2 (ATF2) and AMP-activated protein kinase (AMPK) antibodies were purchased from Cell Signaling (Beverly, MA). Antibody against threonine phosphorylated protein kinase B (PKB) was purchased from Upstate Biotechnology (Lake Placid, NY). The horseradish peroxidase secondary antibodies were purchased from Jackson Immuno-Research Laboratories (West Grove, PA). All other chemicals were obtained from Sigma (St. Louis, MO).

**Animals** - This research was approved by the Animal Studies Committee of Washington University. Male Wistar rats weighing 51-75 grams were obtained from Charles River and maintained on a diet of Purina rat chow and water ad libitum. Food was removed at 5:00 PM the day before an experiment. Rats were anesthetized with pentobarbital sodium (5 mg/100 g body weight) given intraperitoneally, the epitrochlearis muscles were removed. These muscles weighed ~6-8 mg and are sufficiently thin so that oxygenation and substrate supply by diffusion are adequate, as evidenced by maintenance of normal ATP and phosphocreatine concentrations. The rat epitrochlearis contains approximately 76% type 2b fibers, 12% type 2a fibers and 12% type 1 fibers (29).

**Muscle Incubations** - Rat epitrochlearis were incubated in oxygenated (95% O2/5%CO2) culture medium containing Dulbecco’s modified eagle medium (alpha MEM), 10% fetal bovine serum, 50 μU/ml purified pork insulin, 100 μU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. The flasks containing the muscles were maintained at 35°C in a shaking water bath and gassed continuously with 95% O2/5% CO2. In one series of experiments, muscles were incubated in culture medium supplemented with 3.5 mM caffeine for 6 hours, to raise cytosolic Ca2+ concentration by releasing Ca2+ from the SR (20,30,31), and then allowed to recover for 18 hours in oxygenated culture medium in the absence of caffeine. Some muscles were preincubated in culture medium supplemented with 3.5 mM caffeine for 6 hours, to raise cytosolic Ca2+ concentration by releasing Ca2+ from the SR (20,30,31), and then allowed to recover for 18 hours in oxygenated culture medium in the absence of caffeine. Some muscles were preincubated in culture medium supplemented with 10 μM SB202190 or 10 μM KN93 for 1 hour prior to and during the 6-hour caffeine exposure. Muscles recovered for 18 hours in culture medium in the absence of KN93, SB202190 and caffeine. Because KN93, SB202190 are light sensitive, glass vials containing these
compounds were wrapped in foil. The medium was changed every 6 hours. At the end of the incubations, muscles were briefly washed in PBS and clamp frozen with tongs cooled to the temperature of liquid nitrogen. In another experiment, muscles were frozen after 2 hr, 4 hr or 6 hr of exposure to caffeine, or after 6 hr of exposure to caffeine and 2 hr of recovery. Muscles were subsequently processed for Western blotting or mRNA extraction.

**p38 MAPK Phosphorylation and Signaling** - Epitrochlearis muscles were dissected out and allowed to recover in culture medium for 1 hour in a shaking water bath maintained at 35°C with a gas phase of 95% O₂/5% CO₂. Muscles were then incubated in the absence or presence of 10 µM KN93 or 10 µM SB202190 (32) for 1 hour, followed by a 15-minute incubation with or without 3.5 mM caffeine in the continued absence or presence of inhibitors. Muscles were then clamp frozen with tongs cooled to the temperature of liquid nitrogen and kept at -70°C until they were processed for Western analysis.

**Western blotting** - Frozen epitrochlearis muscles were homogenized in modified RIPA buffer (33) and protein concentration was measured by the method of Lowry et al. (34). Proteins were separated by SDS-PAGE, using a 10% resolving gel and Western blotting was performed as described previously (33,35) to determine changes in the phosphorylation status of p38 MAPK, ATF-2, AMPK (thr172), PKB (thr308), and the levels of PGC-1α, COX1, ALAS and citrate synthase proteins. The blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Antibody bound protein was detected by ECL.

**Determination of mRNA** - Total RNA was isolated from epitrochlearis muscles using Trizol reagent (Invitrogen). Caffeine-induced changes in ALAS, citrate synthase (CS), PGC-1α and COX1 mRNAs were determined using semi-quantitative RT-PCR as described in detail previously (36). Universal 18S (Ambion, Austin, TX) was used as an internal control. PCR products were run on a 2% agarose gel and visualized with Sybr Green (Molecular Probes, Eugene, OR). Transcript intensity was expressed relative to 18S rRNA. The following primers were used:

- Δ ALAS, Forward: 5’GCTCTTTTCTCTCTGGCTTTCG3’
- Reverse: 5’CAAAGCTGTGGCAATGTATC3’
- Citrate Synthase Forward: 5’CACAGCCCTCAACAGTGAAAGC3’
- Reverse: 5’GTAGTTCTGTAATTGTCCCAGACG3’
- PGC-1α Forward: 5’TGACATAGAGTGTGGCTGACG3’
- Reverse: 5’TGTTTCTGAGTCTAAGACCGCTG3’
- COXI Forward: 5’GGACTGATTACCCATCAT3’
- Reverse: 5’CGGCAGAAGATGAGATGAAT5’

**Statistical Analysis** - Data are presented as means ± SE. Differences between two groups were analyzed using unpaired T-tests. Comparisons between the means of multiple groups were made using a one way analysis of variance (ANOVA) followed by a post hoc comparison using Fishers least significant difference method.

**RESULTS**

**Mitochondrial marker protein and beta actin levels in fresh and incubated muscles.** A potential problem in studies on incubated epitrochlearis muscles is that decreases in the levels of mitochondrial and other proteins might occur as the result of accelerated proteolysis or reduced protein synthesis during the in vitro incubation.
We, therefore, compared the levels of four mitochondrial marker proteins and beta actin in freshly dissected muscles and muscles incubated in vitro for 24h. As shown in Fig. 1, the levels of these five proteins were similar in the fresh and the incubated muscles. Apparently, the anabolic effects of the growth factors in the fetal calf serum, the amino acids, and the insulin in the incubation medium are sufficient to maintain the muscles in good condition for up to 24h.

Raising Cytosolic Ca\(^{2+}\) Induces an Increase in Mitochondrial Biogenesis in Skeletal Muscle. Previous studies have shown that raising cytosolic Ca\(^{2+}\) induces increases in mitochondrial proteins (17-20) and respiratory capacity (20) in L6 myotubes. Exposure of rat epitrochlearis muscles to 3.5 mM caffeine for 6h resulted in increases in the mRNA’s of PGC-1\(\alpha\), cytochrome oxidase subunit 1 (COX1), \(\delta\)-aminolevulinate synthase (ALAS) and citrate synthase measured 18h after caffeine exposure (Fig. 2A). This concentration of caffeine raises cytosolic Ca\(^{2+}\) to a concentration that is too low to cause muscle contraction or a decrease in high-energy phosphate levels (37,38). PGC-1\(\alpha\), COX1 and ALAS proteins, which have short half-lives, were also increased 18h after caffeine exposure (Fig. 2B). In contrast, citrate synthase protein, which appears to have a half-life of ~6-7 days (39) was not increased 18h after caffeine exposure (Fig. 2B). These findings provide evidence that, as in myotubes (17-21), raising cytosolic Ca\(^{2+}\) provides a signal that leads to an increase in mitochondrial biogenesis in skeletal muscle.

Inhibition of p38 MAPK Prevents the Ca\(^{2+}\) Induced Increase in Mitochondrial Biogenesis. Both the activity (7,22,23) and expression (24) of PGC-1\(\alpha\) are regulated by p38 MAP kinase, and p38 MAPK is activated in muscle by exercise and contractions (17,24,26) and by raising cytosolic Ca\(^{2+}\) in myotubes (27). As shown in Fig. 3, treatment of muscles with caffeine to raise cytosolic Ca\(^{2+}\) induced an increase in p38 phosphorylation, that was blocked by the inhibitor of p38 activation SB202190 (40). Raising cytosolic Ca\(^{2+}\) in muscle by means of contractile activity or exposure to caffeine also results in activation of calcium-calmodulin dependent kinase II (CAMKII) (38,41,42), which is the isoform of CAMK expressed in skeletal muscle (41-43). Inhibition of CAMKII activation using the CAMK inhibitor KN93 (32) prevents the increase in mitochondrial biogenesis induced by raising cytosolic Ca\(^{2+}\) (18). p38 MAPK lies downstream of CAMK in a signaling pathway by which increases in cytosolic Ca\(^{2+}\) lead to activation of p38 (44-46). As shown in Fig. 4, the CAMK inhibitors KN62 and KN93 prevented the phosphorylation of p38 induced by raising cytosolic Ca\(^{2+}\) while the inactive KN93 analog, KN92, had no effect. Prevention of p38 activation with SB 202190 completely blocked the Ca\(^{2+}\) induced increases in expression of PGC-1\(\alpha\), COX1 and ALAS proteins (Fig. 5). In contrast to the activation of p38, raising cytosolic Ca\(^{2+}\) using caffeine had no effect on AMP kinase or PKB/AKT phosphorylation (Fig. 6).

Phosphorylation of ATF-2. The mechanism by which activation of p38 brings about an increase in PGC-1\(\alpha\) expression appears to involve phosphorylation and activation of the transcription factor ATF-2, which binds to the CRE binding site on the PGC-1\(\alpha\) promoter and induces PGC-1\(\alpha\) transcription (24,28). As shown in Fig. 7, raising cytosolic Ca\(^{2+}\) resulted in an increase in ATF-2 phosphorylation that is blocked by either the CAMK inhibitor KN93 or by SB202190, an inhibitor that prevents p38 phosphorylation (32).

Time Course of Ca\(^{2+}\)-Induced Increase in PGC-1\(\alpha\) Protein. There was no
appreciable increase in PGC-1α protein level in muscles exposed to caffeine for 2, 4 or 6 hr (Fig. 8). However, PGC-1α protein level was significantly increased 2 hr after the caffeine treatment was completed (Fig. 8). An increase in COX1 mRNA preceded the increase in PGC-1α protein, with significant increases detectable in muscles treated with caffeine for either 4h or 6h (Fig. 9). This finding is compatible with the possibility that activation rather than increased expression of PGC-1α is involved in mediating the early phase of the adaptive response (13).

DISCUSSION

Our results provide evidence that p38 MAPK is downstream of CAMKII in a signaling pathway by which an increase in cytosolic Ca^{2+} leads to increases in PGC-1α expression and mitochondrial biogenesis in skeletal muscle. It has been known for many years that endurance exercise induces an adaptive increase in skeletal muscle mitochondria with increases in the capacity to oxidize carbohydrate and fat, and generate ATP (1-6). This adaptation plays a major role in the exercise-training induced increase in endurance by decreasing the disturbance in homeostasis induced by muscle contractions/strenuous exercise, (47,48). It has been our long-term goal to elucidate the mechanisms by which the exercise-induced increase in muscle mitochondria is mediated. However, for a long time there was no progress in this area because nothing was known regarding how expression of the mitochondrial genome and of the nuclear genes that encode mitochondrial proteins is regulated and coordinated. More recent research has provided much new information and insight regarding how this complex process is mediated. The initial breakthrough involved the discovery of the transcription factors NRF-1 and NRF-2, which regulate expression of various respiratory chain subunits, ALAS, and mitochondrial transcription factor A (Tfam) (49-51). This finding was followed by discovery of other transcription factors and nuclear receptors that control expression of a range of mitochondrial constituents (52-55). The key breakthrough that explained how the activity of these transcription factors is regulated and orchestrated was the discovery by Spiegelman’s group of the transcription coactivator PGC-1α, which binds to and coactivates the transcription factors involved in mitochondrial biogenesis (7,10,56-58).

Overexpression of PGC-1α in muscle cells or skeletal muscle increases mitochondrial biogenesis (10-12), and there is considerable evidence that PGC-1α provides the link between adaptive stimuli and increased mitochondrial biogenesis (23,56,58). These stimuli include exercise, such as running and swimming, which results in increases in PGC-1α expression, and mitochondrial biogenesis, (13,14,16,24,59). It is not feasible to use exercise as the stimulus for elucidating the signaling pathways by which exercise induces an adaptive increase in mitochondria, because muscle contractions result in numerous disturbances in cellular homeostasis, many of which are potential candidates as inducers of mitochondrial biogenesis. One of these is the increase in cytosolic Ca^{2+} due to release of Ca^{2+} from the sarcoplasmic reticulum (SR) during excitation-contraction coupling. Following-up on earlier studies that suggested that raising cytosolic Ca^{2+} in cultured myotubes can result in increases in mitochondrial proteins (19,21) we have conducted studies on the effect of raising cytosolic Ca^{2+} in L6 myotubes (18,20). This model has the advantage that L6 myotubes are viable for many days, making it possible to determine
whether raising cytosolic Ca\(^{2+}\) results in an increase in functional mitochondria.

In our first study we raised cytosolic Ca\(^{2+}\) by treating L6 myotubes with caffeine or W7, agents that release Ca\(^{2+}\) from the SR, or with the Ca\(^{2+}\) ionophore ionomycin, for 5h per day for 5 days (20). These treatments resulted in increased expression of a range of mitochondrial proteins, and in the capacity to oxidize oleate, which we used as a marker of an increase in functional mitochondria (20). This adaptation was blocked by preventing the caffeine or W-7 induced release of Ca\(^{2+}\) from the SR using dantrolene, and the effect of ionomycin by use of Ca\(^{2+}\) -free medium (20). In a subsequent study using the same approach, we found that raising cytosolic Ca\(^{2+}\) in L6 myotubes induces increases in the expression of PGC-1\(^{\alpha}\) and Tfam, as well as increased binding of NRF-1 to the ALAS promoter and of NRF-2 to the COXIV promoter (18). The increases in cytosolic Ca\(^{2+}\) induced by caffeine result in increased phosphorylation of CAMKII, which is blocked by the CAMK inhibitor KN93 (38). Inhibition of CAMKII activation by KN93 also prevents the increase in mitochondrial biogenesis induced by raising cytosolic Ca\(^{2+}\) (18). This finding provides evidence that the first step in the signaling pathway leading from an increase in cytosolic Ca\(^{2+}\) to an increase in PGC-1\(^{\alpha}\) is mediated by CAMKII, which is the CAMK isoform present in skeletal muscle (41-43). Exercise also results in activation of CAMKII in muscle (41,42), suggesting that our findings in muscle in vitro have relevance to the normal adaptive response to exercise.

There has been considerable interest in the role of CAMKIV in the regulation of PGC-1\(^{\alpha}\) expression and mitochondrial biogenesis in muscle (60-62). Overexpression of a constitutively active form of CAMKIV in skeletal muscle of mice induced an increase in mitochondria (60). It has also been reported that lowering muscle high-energy phosphate levels, which results in activation of AMPK and an increase in muscle mitochondria, results in increased CAMKIV expression and that this mediates the increase in mitochondria (61). In light of these findings, it has been proposed that activation of CAMKIV by increases in cytosolic Ca\(^{2+}\) mediates the adaptive increase in muscle mitochondria induced by exercise (60-62). However, CAMKIV is not expressed in skeletal muscle (41-43).

Activation of p38 MAPK results in both phosphorylation, with an increase in activity, of PGC-1\(^{\alpha}\) (7,22,23) and increased PGC-1\(^{\alpha}\) expression (24). The latter effect appears to be mediated through phosphorylation of activating transcription factor-2 (ATF-2), which binds to and activates the CRE binding site on the PGC-1\(^{\alpha}\) promoter, resulting in increased PGC-1\(^{\alpha}\) expression (24,28). Exercise activates p38 MAPK (17,24,26,63) in skeletal muscle, and there is evidence that p38 MAPK lies downstream of CAMK in a pathway activated by Ca\(^{2+}\) (44-46). In this context, we evaluated the possibility that p38 MAPK lies downstream of CAMKII in the pathway by which raising cytosolic Ca\(^{2+}\) leads to an increase in PGC-1\(^{\alpha}\) in skeletal muscle. In support of this possibility, we found that treatment of muscles with caffeine results in an increase p38 phosphorylation. We have shown that inhibition of CAMK activation using KN93 blocks the Ca\(^{2+}\)-induced increase in mitochondrial biogenesis (18). In the present study, we found that KN93 also blocks the phosphorylation of p38 that occurs in response to raising cytosolic Ca\(^{2+}\). Furthermore, the inhibitor of p38 activation, SB202190 (32,40) blocks the increases in PGC-1\(^{\alpha}\), COX1 and ALAS expression induced by raising cytosolic Ca\(^{2+}\).

The final step in the Ca\(^{2+}\)-activated pathway leading to increased PGC-1\(^{\alpha}\)
expression is likely the phosphorylation and activation of ATF-2 by p38 (24). ATF-2 binds to the CRE binding site on the PGC-1α promoter and appears to be involved in mediating the increase in PGC-1α expression (24,28). Inhibition of either CAMKII with KN93 or p38 with SB202190 prevented the increase in ATF-2 phosphorylation induced by raising cytosolic Ca\(^{2+}\) in our muscle preparation, providing further evidence for involvement of CAMKII and p38 MAPK in mediating the increases in mitochondrial biogenesis. In this context, we interpret our finding as evidence that raising cytosolic Ca\(^{2+}\) in muscle results in activation of CAMKII, that this is the first step in a pathway that leads to activation of p38 and ATF-2, and that activated ATF-2 then induces an increase in PGC-1α expression.

p38 MAPK has also been shown to phosphorylate and activate PGC-1α, resulting in its enhanced binding to, and activation of, transcription factors (7,22,23). We have found that exercise results in increased DNA binding of NRF-1 and NRF-2, as well as increased expression of a number genes encoding mitochondrial enzymes, prior to an increase in PGC-1α protein (13). These findings suggest that the initial phase of the increase in mitochondrial biogenesis induced by exercise or raising cytosolic Ca\(^{2+}\) is mediated by activation of PGC-1α by p38, and that this adaptation is maintained and enhanced by a subsequent increase in PGC-1α expression. Support for this interpretation is provided by the finding in the present study that COXI mRNA is increased in muscles treated for 4h with caffeine, while PGC-1α protein expression was not increased until 4h later.

In conclusion, we interpret the present results, together with previous findings (13,18,20,24) as evidence that raising cytosolic Ca\(^{2+}\) in muscle induces an increase in mitochondrial biogenesis by both activation and increased expression of PGC-1α by a pathway that leads from CAMKII to p38 MAPK.
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FOOTNOTES

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The abbreviations used are: PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; NRF-1, nuclear respiratory factor 1; NRF-2, nuclear respiratory factor 2; COXI, cytochrome oxidase subunit 1; ALAS, δ-aminolevulinate synthase; CS citrate synthase; p38 MAPK, p38 mitogen activated protein kinase; ATF-2; activating transcription factor 2; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; PKB, protein kinase B; AMPK; 5’ AMP activated protein kinase; CAMK, calcium/calmodulin dependent protein kinase
FIGURE LEGENDS

Figure 1. Protein levels in freshly dissected and 24h incubated epitrochlearis muscles. Mitochondrial marker protein and beta actin levels in freshly dissected muscles and muscles that were incubated in culture medium for 24h. Values are means ± SE for 6 muscles per group.

Figure 2. Caffeine induces increases in (A) the mRNA levels and (B) protein levels of a number of mitochondrial enzymes. Rat epitrochlearis muscles were exposed to 3.5 mM caffeine for 6h and measurements were made on muscle frozen 18h after caffeine treatment. Values are means ± SE for 4 pooled muscle samples with 3 muscles per sample for A, and for 5-7 muscles per group for B. * P<0.05 caffeine vs. control.

Figure 3. Treatment of muscles with 3.5 mM caffeine causes an increase in p38 MAPK phosphorylation that is blocked by SB202190, an inhibitor of p38 MAPK phosphorylation. Values are means ± SE for 7 muscles per group. *P<0.05 caffeine vs. basal and caffeine + SB202190.

Figure 4. CAMK inhibitors prevent the increase in p38 MAPK induced by raising cytosolic Ca$^{2+}$ in muscles using 3.5 mM caffeine. A. The CAMK inhibitor KN93, but not the inactive K93 analog KN92, blocks the increase in p38 MAPK phosphorylation induced by 3.5 mM caffeine. B. The CAMK inhibitor KN62 prevents the increase in p38 MAPK phosphorylation induced by 3.5 mM caffeine. Values are means ± SE for 6-12 muscles per group. A *P<0.05 caffeine + KN92 vs. control and caffeine + KN93; B *P<0.05 caffeine vs. control and caffeine + KN62.

Figure 5. The inhibitor of p38 MAPK activation, SB202190, blocks the increases in PGC-1α, COXI and ALAS protein levels induced by raising cytosolic Ca$^{2+}$ in muscles using 3.5 mM caffeine. Values are means ± SE for 5 to 10 muscles per group. * P<0.05, caffeine vs. control and caffeine + SB202190.

Figure 6. Raising cytosolic Ca$^{2+}$ with 3.5 mM caffeine has no effect on PKB or AMPK phosphorylation. Values are means ± SE for 6 muscles per group.

Figure 7. Treatment of muscles with caffeine induces an increase in ATF-2 phosphorylation that is blocked by SB202190, an inhibitor of p38 MAPK activation, and by KN93, an inhibitor of CAMKs. Values are means ± SE for 6 to 8 muscles per group. * P<0.05, caffeine vs. other three groups.

Figure 8. Time course of PGC-1α protein increase induced by raising Ca$^{2+}$ in muscles using caffeine. Epitrochlearis muscles were frozen after either 2h, 4h or 6h of exposure to caffeine or after 6h of caffeine treatment plus 2h of recovery. Values are means ± SE for 8 to 10 muscles per group. *P<0.05 vs. previous time points.

Figure 9. COX I mRNA levels are increased in epitrochlearis muscles treated with 3.5 mM caffeine for 4h or 6h. Values are means ± SE for 4 pooled muscle samples, with 3 muscles per sample. * P <0.05, caffeine vs. control.
Figure 2

A. mRNA (Arbitrary Units)

- PGC-1α
- COX I
- ALAS
- Citrate synthase

B. Protein (Arbitrary Units)

- PGC-1α
- COX I
- ALAS
- Citrate synthase

* Denotes significant difference between control and caffeine conditions.
Figure 3

[Graph showing phospho p38 levels under basal, caffeine, and caffeine + SB202190 conditions.]

Phospho p38 (arbitrary units)

- Basal
- Caffeine
- Caffeine + SB202190

* indicates a significant difference.
Figure 4

A. 

p-P38 MAPK

basal caffeine + KN92 caffeine + KN93

P38 MAPK

basal caffeine + KN92 caffeine + KN93

B. 

p-P38 MAPK

basal caffeine + KN62 caffeine + KN62

P38 MAPK

basal caffeine + KN62

Phospho p38 (Arbitrary Units)
Figure 5

Protein (Arbitrary Units)

- COX I
- ALAS
- PGC-1α

Control
Caffeine
Caffeine + SB202190

Significant difference (*)
Figure 6

The figure shows Western blot analysis comparing control and caffeine conditions for phosphorylated PKB (p-PKB), phosphorylated AMPK (p-AMPK), and AMPK. The blot images depict the protein bands, with bars indicating the quantitative comparison in arbitrary units.
Figure 7

[Bar chart showing the phosphorylation levels of ATF-2 under different conditions: basal, caffeine, caffeine + SB202190, caffeine + KN93. The chart includes error bars for each condition.]

* Significant difference compared to basal.
Figure 8

The graph shows the change in PGC-1α Protein (Arbitrary Units) over different durations of exposure to caffeine. The x-axis represents the duration of exposure to caffeine (hours), and the y-axis represents the protein levels. The control group is shown on the left, and the 6 hours and 2 hours recovery group is marked with an asterisk. The graph indicates a significant increase in PGC-1α protein levels with increased duration of exposure to caffeine, with a notable difference between the control group and the 6 hours + 2 hr recovery group.
Calcium induces increases in peroxisome proliferator-activated receptor γ coactivator-1 α and mitochondrial biogenesis by a pathway leading to p38 mitogen activated protein kinase activation

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