THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 3, pp. 1607–1614, January 19, 2007


# Calcineurin Regulates Skeletal Muscle Metabolism via Coordinated Changes in Gene Expression*[S]

Received for publication, September 28, 2006, and in revised form, November 14, 2006 Published, JBC Papers in Press, November 15, 2006, DOI 10.1074/jbc.M609208200

**Yun Chau Long**[1], **Stephan Glund, Pablo M. Garcia-Roves, and Juleen R. Zierath**[2]

*From the Department of Molecular Medicine and Surgery, Section of Integrative Physiology, Karolinska Institutet, von Eulers väg 4, 4th Floor, Stockholm S-171 77, Sweden*

**The metabolic property of skeletal muscle adapts in response to an increased physiological demand by altering substrate utilization and gene expression. The calcium-regulated serine/threonine protein phosphatase calcineurin has been implicated in the transduction of motor neuron signals to alter gene expression programs in skeletal muscle. We utilized transgenic mice that overexpress an activated form of calcineurin in skeletal muscle (MCK-CnA*) to investigate the impact of calcineurin activation on metabolic properties of skeletal muscle. Activation of calcineurin increased glucose incorporation into glycogen and lipid oxidation in skeletal muscle. Activated calcineurin suppressed skeletal muscle glucose oxidation and increased lactate release. The enhancement in lipid oxidation was supported by increased expression of genes for lipid metabolism and mitochondrial oxidative phosphorylation. In a reciprocal fashion, several genes of glycolysis were down-regulated, whereas pyruvate dehydrogenase kinase 4 was markedly induced. This expression pattern was associated with decreased glucose utilization and enhanced glycogen storage. The peroxisome proliferator-activated receptors (PPARs) and PPARγ coactivator 1α (PGC1α) are transcription regulators for the expression of metabolic and mitochondrial genes. Consistent with changes in the gene-regulatory program, calcineurin promoted the expression of PPARα, PPARδ, and PPARγ coactivator 1α in skeletal muscle. These results provide evidence that calcineurin-mediated skeletal muscle reprogramming induces the expression of several transcription regulators that coordinate changes in the expression of genes for lipid and glucose metabolism, which in turn alters energy substrate utilization in skeletal muscle.**

The metabolic property of skeletal muscle is highly flexible and adapts to various physiological demands by altering energy substrate utilization (1, 2). Skeletal muscle can be classified into two categories based on the distinct metabolic and contractile activity: fast glycolytic fibers that derive energy primarily from glycolysis for sudden rapid movement and slow oxidative fibers that rely mainly on oxidation of energy substrates for sustained recurring activity. Although such diversity has been long recognized, the cellular mechanisms that regulate skeletal muscle metabolic properties remain largely elusive.

Signals from the motor neurons are critical factors in regulating the metabolic properties of skeletal muscle (3, 4). Chronic low frequency electrical stimulation of skeletal muscle (resembling the firing pattern of slow motor neurons) activates the expression of *Lpl* (lipoprotein lipase) (5), *Cd36* (fatty acid transporter) (6), *Had* (hydroxyacyl-CoA dehydrogenase) (7), and *Cs* (citrate synthase) (7, 8). Furthermore, the expression of Glut4 (glucose transporter 4) and activity of Hk2 (hexokinase 2), two proteins that are essential for glucose uptake and storage, are increased in skeletal muscle after electrical stimulation (9). Although electrical stimulation represents an artificial model of contractile activity-induced motor neuron activity (3), this model provides evidence that signals evoked by motor neurons can induce substantial changes in metabolic gene expression profiles in skeletal muscle.

Calcineurin is a calcium-regulated serine/threonine protein phosphatase implicated in the transduction of calcium signals elicited by the motor neurons to the myofibers (10–12). Calcineurin dephosphorylates nuclear factor of activated T cell transcription factors and promotes their translocation into the nucleus for transcription of target genes of the slow fiber program (10). Transgenic mice expressing activated calcineurin in fast glycolytic skeletal muscle have increased expression of slow contractile machinery, including troponin I and slow myosin ATPase (13). Conversely, pharmacological inhibition of calcineurin activity induces a slow to fast myosin ATPase transformation in rat soleus muscle (10). In addition, several transcription factors and coactivators have been implicated in the metabolic adaptation of skeletal muscle. Overexpression of peroxisome proliferator-activated receptor α (PPARα)[3] in skeletal muscle increases the expression of regulatory genes involved in lipid metabolism and mitochondrial oxidative phosphorylation (14). Skeletal muscle-specific overexpression of PPARγ coactivator 1α (PGC1α) (15) or activation of PPARδ (16) drives the formation of slow oxidative fibers, concomitant with increased mitochondrial biogenesis.

We previously reported that activation of calcineurin enhances glucose transport and insulin action in skeletal mus-

* This work was supported by the Swedish Research Council, the Swedish Diabetes Association, Swedish Center for Sports Research, the Foundation for Scientific Studies of Diabetology, the Strategic Research Foundation (INGVAR II), and the Commission of the European Communities (Contract LSHM-CT-2004-005272 EXGENESIS, Contract LSHM-CT-2004-512013 EUGENE2, and Contract LSHM-CT-2004-512013 EUGENEHEART). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

[1] Supported by a scholarship from the Karolinska Institutet.

[2] To whom correspondence should be addressed. Tel.: 46-8-524-875-81; Fax: 46-8-33-54-36; E-mail: Juleen.Zierath@ki.se.

[3] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PGC1α, PPARγ coactivator 1α; EDL, extensor digitorum longus.

cle (17). In this study, we investigated the impact of calcineurin-mediated skeletal muscle reprogramming on the energy substrate utilization and determined whether calcineurin induces metabolic adaptations via coordinated changes in gene expression. We tested the hypothesis that calcineurin activates the slow fiber gene regulatory program by activating PPARα, PPARδ, and PGC1α.

## EXPERIMENTAL PROCEDURES

*Transgenic Mice*—A line of transgenic mice expressing a constitutively active form of calcineurin (18) driven by skeletal muscle creatine kinase promoter/enhancer was established at the Karolinska Institutet, using MCK-CnA* mice (a kind gift from Dr. Eric N. Olson) that were originally developed at the Department of Molecular Biology, University of Texas Southwestern Medical Center at Dallas (13). Female MCK-CnA* mice and wild-type littermates were maintained on a 12-h light-dark cycle and allowed free access to water and standard rodent chow. Mice were anesthetized via intraperitoneal injection of 2.5% avertin (0.02 ml/g of body weight), and extensor digitorum longus (EDL) and soleus muscles were removed for *in vitro* incubation or expression analysis. We studied EDL and soleus muscles because they display different contractile and metabolic properties. EDL muscles from wild-type mice contain predominantly fast twitch fibers and ~2% type I (slow oxidative) fibers, whereas soleus muscles are composed of ~50% type I fibers, with the remaining percentage attributed mainly to type IIa (fast oxidative) fibers (19). The mice were humanely killed by cervical dislocation immediately after muscle dissection. The Ethics Committee on Animal Research in Northern Stockholm approved all experimental procedures.

*RNA Purification and cDNA Synthesis*—EDL was homogenized in 800 μl of Trizol reagent (Sigma), and RNA was purified according to recommendations of the manufacturer. Purified RNA was then treated with DNase I, using a DNA-free kit (Ambion, Huntingdon, Cambridgeshire, UK) according to the manufacturer's protocol. DNase-treated RNA was used as a template for cDNA synthesis using the SuperScript first strand synthesis system (Invitrogen) with oligo(dT) primers. A reaction without reverse transcriptase was included for each sample to serve as reverse transcriptase-minus control.

*Quantitative Real Time PCR*—The quantity of cDNA for each transcript was measured using real time PCR with the ABI PRISM 7000 sequence detector system and fluorescence-based SYBR-green technology (Applied Biosystems, Warrington, UK). PCR was performed in a final volume of 25 μl, consisting of diluted cDNA sample, 1× SYBR-green PCR Master Mix (Applied Biosystems), primers optimized for each target gene, and nuclease-free water. All samples were analyzed in duplicate. Relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against a housekeeping gene (acidic ribosomal phosphoprotein PO) using the standard curve method. Primers were designed using Primer Express computer software (Applied Biosystems). Transcript sequences obtained from the ENSEMBL data base were *Cpt1* (carnitine palmitoyl-CoA transferase 1; ENSMUST00000023287), *Cd36* (ENSMUST00000003024), *Cs* (citrate synthase; ENSMUST00000005826), *Hadsc* (short chain HAD; ENSMUST00000029610), *Lpl* (ENSMUST00000015712), *Pfkm* (muscle 6-phosphofructokinase; ENSMUST00000043950), *Pdk4* (pyruvate dehydrogenase kinase 4; ENSMUST00000019721), and sequences from the NCBI GenBank™ data base were acidic ribosomal phosphoprotein PO (BC003833), *Aldoa* (aldolase A; NM007438), *Cpt2* (NM009949), *Decr1* (2,4-dienoyl-CoA reductase 1; NM026172), *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase; BC095932), *Ldh* (lactate dehydrogenase; NM008492), *Slc25a20* (mitochondrial carnitine/acylcarnitine translocase; NM020520), and *Acadvl* (very long chain acyl-CoA dehydrogenase; NM017366).

*Muscle Incubations*—Incubation media were prepared from stocks of pregassed (95% $O_2$, 5% $CO_2$) Krebs-Henseleit buffer supplemented with 5 mM HEPES and 0.1% bovine serum albumin (radioimmunoassay grade). EDL and soleus muscle were excised and incubated at 30 °C in a shaking water bath under a constant gas phase (95% $O_2$/5% $CO_2$) unless stated otherwise.

*Glucose Oxidation, Glucose Incorporation into Glycogen, and Lactate Release*—Muscles were incubated (30 °C for 60 min) in 1 ml of Krebs-Henseleit buffer containing 8 mM [U-$^{14}$C]glucose (0.3 μCi/ml) with or without insulin (12 nM). Vials were sealed with a rubber stopper, which was fitted with a center well. Muscles were oxygenated for the first 60 min of the incubation period via a needle inserted through the stopper. After 60 min, muscles were removed from the vial, trimmed of excess tendon, and weighed. Thereafter, 0.2 ml of Protosol (PerkinElmer Life Sciences) was injected through the rubber stopper into the center well, and the medium was acidified by injection of 0.5 ml of 15% perchloric acid. Liberated $CO_2$ was collected for 60 min, and center wells were transferred to vials for liquid scintillation counting. Results were expressed as mmol of oxidized glucose/g of wet weight/h. The rate of glucose incorporation into glycogen was determined by accumulation of $^{14}$C into glycogen. The muscles were homogenized in 500 μl of 1 M NaOH and subsequently neutralized with 500 μl of 20% trichloroacetic acid. The homogenates were centrifuged at 3500 × *g* for 15 min, and the supernatant was collected. Glycogen of the supernatant was precipitated by the addition of 200 μl of 110 mM glycogen and 2 ml of 95% ethanol. The glycogen precipitate was collected after centrifugation (2000 × *g* for 15 min) and dissolved in water for liquid scintillation counting. For the measurement of lactate release, nonradiolabeled glucose was used. After 60 min of incubation with or without insulin, medium was collected, and the lactate concentration was measured by using a colorimetric lactate assay kit (Biomedical Research Service Center, University at Buffalo) according to the manufacturer's instructions.

*Oleate Oxidation*—Muscles were trimmed of excess tendon and weighed before preincubation for 40 min in Krebs-Henseleit buffer supplemented with 5 mM HEPES, 3.5% fatty acid-free bovine serum albumin, 5 mM glucose, and 12 nM insulin. Muscles were then transferred to vials containing 1 ml of identical medium with the addition of 0.3 mM [1-$^{14}$C]oleate (0.2 μCi/ml) and incubated for 60 min. Vials were sealed with a rubber stopper, which was fixed with a center well. Muscles were oxygenated for the first 15 min of the incubation period via a needle inserted through the stopper. Thereafter, the oxygen needle was removed to close the system. After 60 min, 0.2 ml of Solvable (PerkinElmer Life Sciences) was injected through the rub-

ber stopper into the center well, and 0.5 ml of 15% perchloric acid was injected into the medium. Released $CO_2$ was collected for 60 min, and center wells were transferred to vials for liquid scintillation counting after the addition of 47 $\mu$l of 5 M HCl. Results are expressed as nmol of oxidized oleate/g of wet weight/h.

*Western Blot Analysis*—Muscles were pulverized in microcentrifuge tubes over liquid nitrogen and homogenized by a motor-driven pestle in 0.3 ml of ice-cold lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 10 mM NaF, 1 mM MgCl, 1 mM $Na_3VO_4$, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% Triton X 100, 1 $\mu$g/ml aprotinin, and 1 $\mu$g/ml leupeptin. Homogenates were solubilized by end-over-end mixing at 4 °C for 60 min and subjected to centrifugation for 10 min at 12,000 × *g* and 4 °C. Total protein was determined using a commercially available kit (Pierce), and proteins (50 $\mu$g) solubilized in Laemmli sample buffer were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed using the following antibodies against actin (Cell Signaling), aldolase (Biodesign, Saco, ME), HK2 (a kind gift from O. Pedersen, Steno Memorial Hospital, Gentofte, Denmark), PDK4 (Abgent, San Diego, CA), PGC1$\alpha$ (Chemicon, Temecula, CA), GAPDH, PPAR$\alpha$, and PPAR$\delta$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies against NADH-ubiquinol oxidoreductase, succinate-ubiquinol oxidoreductase, ubiquinol-cytochrome *c* oxidoreductase subunit II, cytochrome *c* oxidase subunit I, and ATP synthase $\alpha$ subunit were from Molecular Probes, Inc. (Eugene, OR). Proteins were visualized by chemiluminescence and quantified by densitometry.

*Statistical Analysis*—Data are expressed as means ± S.E. Differences among groups were determined by two-way analysis of variance followed by Fisher's least significant differences *post hoc* analysis. Differences between two groups were determined by unpaired Student's *t* test. Significance was accepted at $p < 0.05$.

## RESULTS

*Rate of Glucose Incorporation into Glycogen and Glucose Oxidation*—The rate of glucose incorporation into glycogen was determined in skeletal muscle from wild-type mice and MCK-CnA* mice. In wild-type mice, insulin increased the rate of glucose incorporation into glycogen 10.1- and 4.3-fold in EDL and soleus muscles, respectively (Fig. 1*A*). Under insulin-stimulated conditions, the rate of glucose incorporation into glycogen was 98 and 34% greater in EDL and soleus muscles from MCK-CnA* mice respectively, compared with wild-type mice (Fig. 1*A*). Rates of glucose incorporation into glycogen of soleus muscles were not significantly different between wild-type and MCK-CnA* mice under the basal condition. Insulin increased the rate of glucose oxidation 2.1-fold in EDL muscles from wild-type mice (Fig. 1*B*). However, the effect of insulin on glucose oxidation was blunted in EDL muscles from MCK-CnA* mice (Fig. 1*B*). The basal and insulin-stimulated rate of glucose oxidation was not significantly different between soleus muscles from wild-type and MCK-CnA* mice.

*Regulatory Genes of Glucose Metabolism*—In EDL muscles from MCK-CnA* mice, mRNA abundance of *Pfkm* (42%), *Aldoa* (33%), and *Gapdh* (24%) was reduced compared with

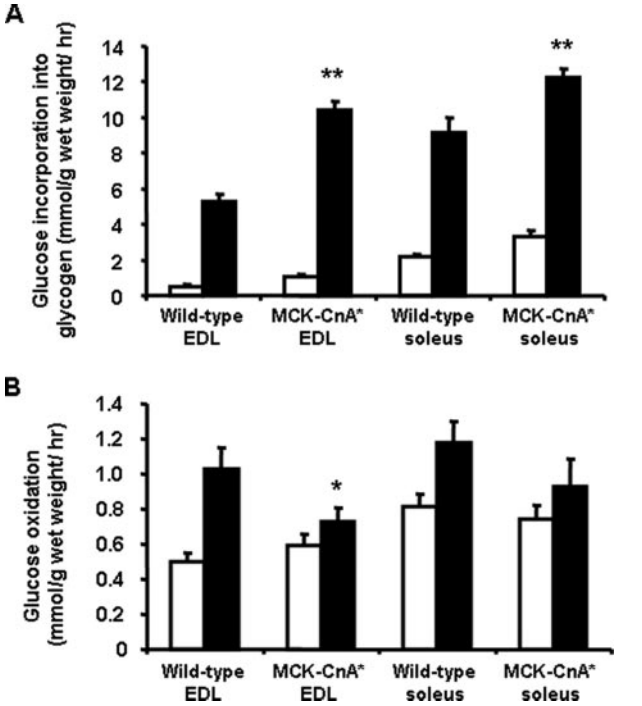


FIGURE 1. **Glucose metabolism in EDL muscle from MCK-CnA* mice.** The rates of glucose incorporation into glycogen (*A*) and glucose oxidation (*B*) were assessed in EDL and soleus muscle from wild-type and MCK-CnA* mice under basal (*open bars*) and insulin-stimulated (*closed bars*) conditions. Data are means ± S.E. for $n = 5–7$ muscles. *, $p < 0.05$; **, $p < 0.005$ compared with wild type.

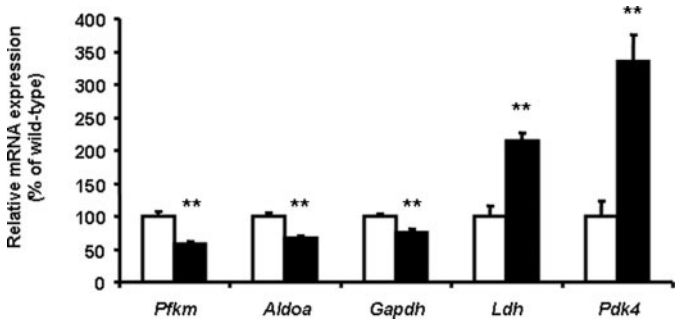


FIGURE 2. **Coordinated changes in expression of genes essential for glucose metabolism in EDL muscle from MCK-CnA* mice.** The level of mRNA expression of genes essential for glucose metabolism in EDL muscle from wild-type (*open bars*) and MCK-CnA* (*closed bars*) mice was determined by real time PCR. Data are expressed as percentage of EDL muscle from wild-type mice. Data are means ± S.E. for $n = 8–9$ muscles. **, $p < 0.005$ compared with wild type.

wild-type mice (Fig. 2). In contrast, mRNA abundance of *Ldh* and *Pdk4* was increased 210 and 340%, respectively, compared with wild-type mice. Protein content of HK2 was increased 37% in EDL muscles from MCK-CnA* mice, relative to wild-type mice (Fig. 3*A*). In contrast, protein content of aldolase and GAPDH was down-regulated in EDL muscles from MCK-CnA* mice 59 and 45% compared with wild-type mice (Fig. 3, *B* and *C*). Expression of activated calcineurin in EDL muscle increased PDK4 protein content 260% compared with wild-type mice (Fig. 3*D*). No significant change was observed for protein content of these enzymes in soleus muscle between wild-type and MCK-CnA* mice.

*Rate of Oleate Oxidation and Lactate Release*—Rate of lipid oxidation was determined in skeletal muscle from wild-type

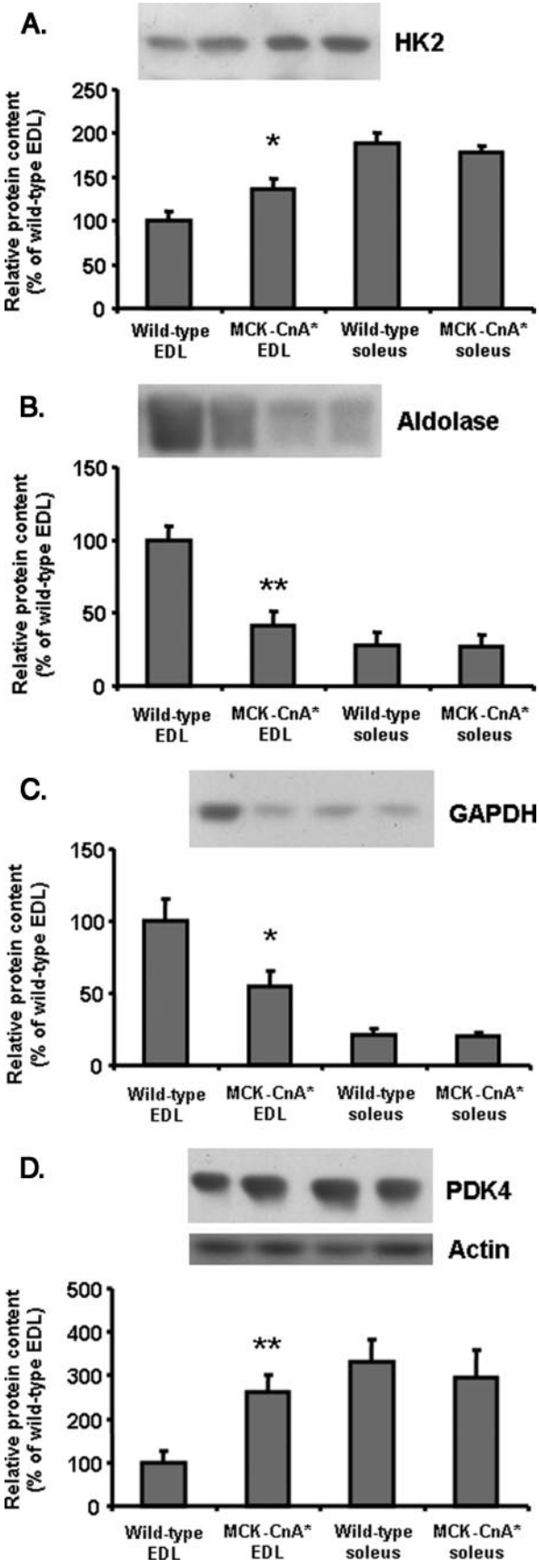


FIGURE 3. **Changes in protein content of enzymes of glucose metabolism in EDL muscle from MCK-CnA* mice.** Protein content of HK2 (*A*), aldolase (*B*), GAPDH (*C*), and PDK4 (*D*) in EDL and soleus muscle of wild-type and MCK-CnA* mice was determined by Western blot analysis (equal sample loading was confirmed by blotting for actin). Data are expressed as a percentage of EDL muscle from wild-type mice. Data are means ± S.E. for $n = 5–6$ muscles. *, $p < 0.05$; **, $p < 0.005$ compared with wild type.

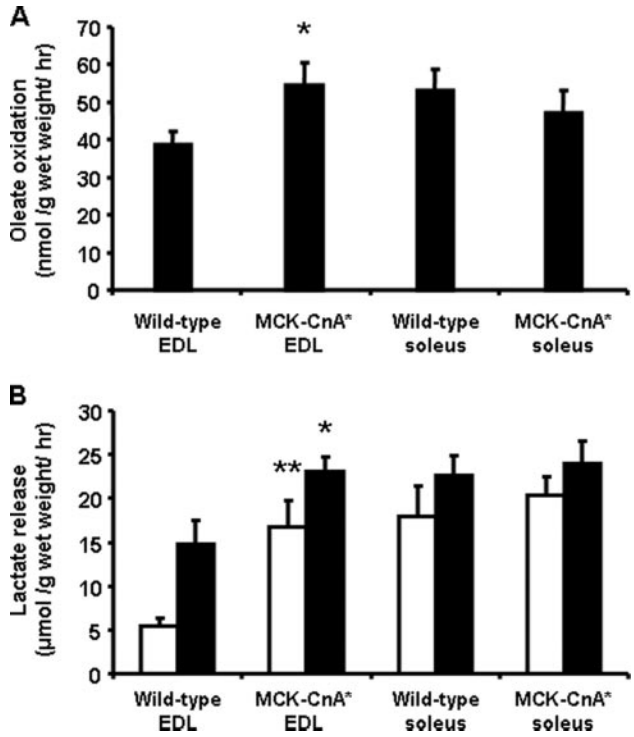


FIGURE 4. **Lipid oxidation and lactate release in EDL muscle from MCK-CnA* mice.** EDL and soleus muscle from wild-type and MCK-CnA* mice were incubated in the absence (*open bars*) or presence (*closed bars*) of insulin, and oleate oxidation (*A*) or lactate release (*B*) was determined. Data are means ± S.E. for $n = 6–7$ muscles. *, $p < 0.05$; **, $p < 0.005$ compared with wild type.

and MCK-CnA* mice. Oleate oxidation was 41% greater in EDL muscles of MCK-CnA* mice compared with wild-type mice under the insulin-stimulated condition (Fig. 4*A*), consistent with our previous finding of elevated basal oleate oxidation in EDL muscles from MCK-CnA* mice (17). Insulin increased the rate of lactate release in EDL muscles from wild-type mice 280% (Fig. 4*B*). The basal and insulin-stimulated rate of lactate release was increased in EDL muscles from MCK-CnA* mice 310 and 156% respectively, compared with wild-type mice (Fig. 4*B*). No significant further increase in lactate release was observed in soleus muscle from MCK-CnA* mice, relative to wild-type mice.

*Regulatory Genes of Lipid and Mitochondrial Oxidative Phosphorylation*—Expression of activated calcineurin in EDL skeletal muscle induced a consistent increase in the mRNA level of regulatory genes for lipid metabolism. The mRNA level of *Lpl* and *Cd36* (genes involved in transport of lipid into the myofiber) was elevated 185 and 116%, respectively, in MCK-CnA* mice compared with wild-type mice (Fig. 5). Genes essential for transport of acyl-CoA into the mitochondria, including *Cpt1* (65%), *Cpt2* (127%), and *Slc25a20* (89%), were up-regulated in EDL muscles from MCK-CnA* mice, compared with wild-type mice. The mRNA abundance of mitochondrial

enzymes involved in oxidation of fatty acid, including *Decr1* (113%), *Acadvl* (77%), *Hadhsc* (62%), and *Cs* (24%), were also augmented in EDL muscle from MCK-CnA* mice compared with wild-type mice.

Expression of activated calcineurin induced a marked increase in protein content of mitochondrial proteins for oxidative phosphorylation in EDL muscle from MCK-CnA* mice relative to wild-type mice, including NADH-ubiquinol oxidoreductase (193%), succinate-ubiquinol oxidoreductase (27%), ubiquinol-cytochrome *c* oxidoreductase subunit II (135%), cytochrome *c* oxidase subunit I (216%), and ATP synthase $\alpha$ subunit (71%) (Table 1, Fig. 6). There was no further increase in the protein expression of these genes in soleus muscle of MCK-CnA* mice.

*Induction of Transcription Factor and Co-activator by Calcineurin*—Protein content of PPAR$\alpha$ (160%) and PPAR$\delta$ (90%) was increased in EDL muscle from MCK-CnA* mice, relative to wild-type mice (Fig. 7). Consistent with our previous finding (17), protein content of PGC1$\alpha$ was also increased 191% in EDL muscle from MCK-CnA* mice, compared with the wild-type mice. Comparable levels of protein content were observed in soleus muscle of MCK-CnA* and wild-type mice.

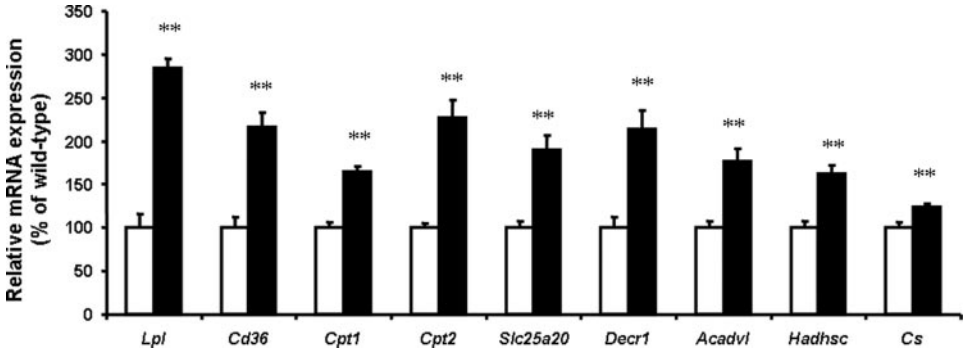


FIGURE 5. **Calcineurin-induced expression of genes essential for lipid metabolism.** The level of mRNA expression of genes critical for lipid metabolism in EDL muscle from wild-type (*open bars*) and MCK-CnA* (*closed bars*) mice was determined by real time PCR. *Cs*, citrate synthase. Data are expressed as percentage of EDL muscle from wild-type mice. Data are means ± S.E. for $n = 8–9$ muscles. **, $p < 0.005$ compared with wild type.

## DISCUSSION

Our results provide evidence that expression of activated calcineurin in fast glycolytic skeletal muscle induces a marked shift in glucose and lipid metabolism. Moreover, insulin-induced glucose oxidation was suppressed, concomitant with a coordinated decrease in the expression of glycolytic enzymes (PFKM, aldolase, and GAPDH) and an increase in the expression of LDH and PDK4, a potent inhibitory enzyme for glucose oxidation (20). The decrease in glucose oxidation was accompanied by a striking increase in insulin-stimulated glucose incorporation into glycogen. This is consistent with our previous report of increased expression of Glut4 and glycogen synthase (17) and our current finding of increased HK2 expression. Therefore, expression of activated calcineurin in skeletal muscle reduces the partitioning of glucose for oxidation but increases glucose sparing for glycogen synthesis (supplemental Fig. S1). These calcineurin-regulated metabolic changes are partly mediated by coordinated changes in gene expression.

Lipid oxidation in EDL muscles from MCK-CnA* mice under the insulin-stimulated condition was elevated compared with wild-type mice, consistent with our previous report of increased basal lipid oxidation (17). The enhanced capacity for lipid oxidation and increased expression of PDK4 markedly increased lactate release from EDL muscle of MCK-CnA* mice under basal and insulin-stimulated conditions, further supporting decreased entry of glucose into the Krebs cycle. Therefore, expression of activated calcineurin induced a shift in substrate metabolism from glucose utilization to lipid oxidation in skeletal muscle.

The enhancement of lipid oxidation in EDL muscle of MCK-CnA* mice is supported by a consistent increase in the expression of genes involved in lipid metabolism. The expression of *Lpl* and *Cd36*, two

**TABLE 1**

**Protein content of enzymes involved in mitochondrial oxidative phosphorylation**

Shown is protein content of enzymes involved in mitochondrial oxidative phosphorylation in EDL and soleus muscle from wild-type and MCK-CnA* mice, as determined by Western blot analysis. Data are expressed as the percentage of EDL muscle from wild-type mice. Values ($n = 5–6$) are reported as means ± S.E. NS, not significant.

| | Wild type | MCK-CnA* | *p* value |
|---|---|---|---|
| | % | % | |
| **NADH-ubiquinol oxidoreductase** | | | |
| EDL | 100 ± 17 | 293 ± 24 | $p < 0.005$ |
| Soleus | 351 ± 20 | 341 ± 14 | NS |
| **Succinate-ubiquinol oxidoreductase** | | | |
| EDL | 100 ± 6 | 127 ± 11 | $p < 0.05$ |
| Soleus | 131 ± 8 | 123 ± 7 | NS |
| **Ubiquinol -cytochrome *c* oxidoreductase subunit II** | | | |
| EDL | 100 ± 4 | 235 ± 12 | $p < 0.005$ |
| Soleus | 254 ± 7 | 250 ± 18 | NS |
| **Cytochrome *c* oxidase subunit I** | | | |
| EDL | 100 ± 10 | 316 ± 34 | $p < 0.005$ |
| Soleus | 424 ± 56 | 339 ± 24 | NS |
| **ATP synthase $\alpha$ subunit** | | | |
| EDL | 100 ± 22 | 171 ± 21 | $p < 0.05$ |
| Soleus | 176 ± 22 | 179 ± 14 | NS |

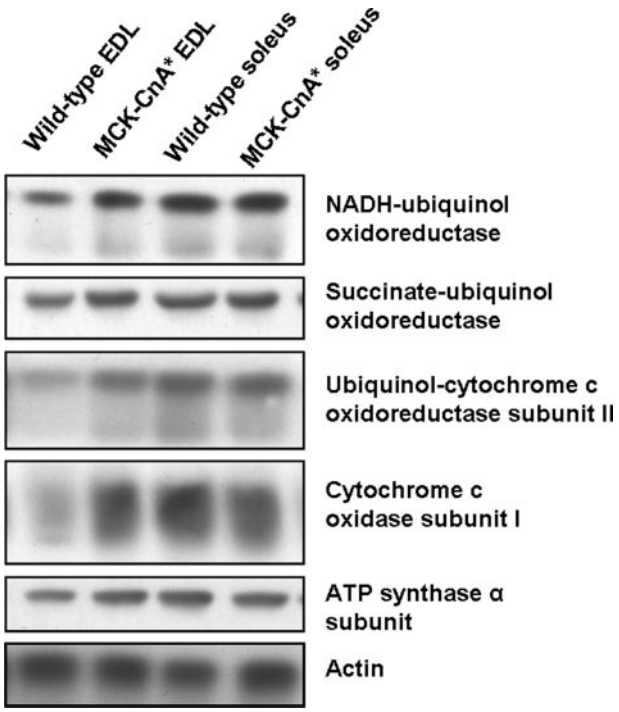


FIGURE 6. **Calcineurin-induced increase in protein content of enzymes involved in mitochondrial oxidative phosphorylation.** Representative immunoblot for protein content of NADH-ubiquinol oxidoreductase, succinate-ubiquinol oxidoreductase, ubiquinol-cytochrome *c* oxidoreductase subunit II, cytochrome *c* oxidase subunit I, and ATP synthase $\alpha$ subunit was determined by Western blot analysis (equal sample loading was confirmed by blotting for actin). Quantification of results is summarized in Table 1.

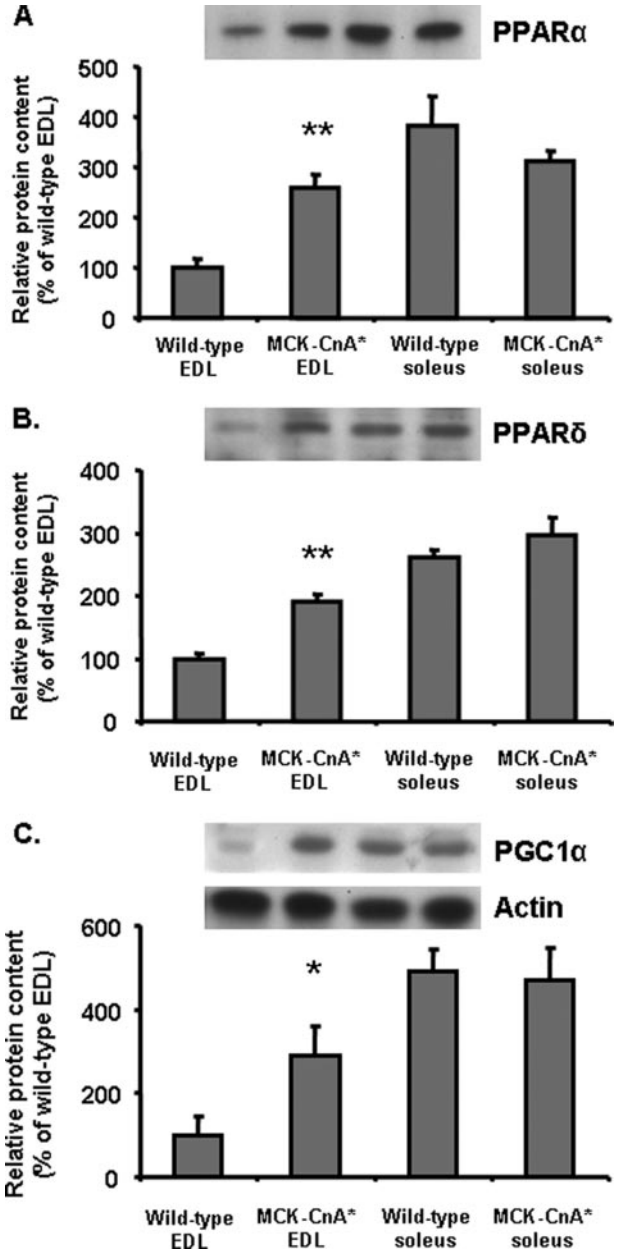


FIGURE 7. **Protein content of PPAR$\alpha$, PPAR$\delta$, and PGC1$\alpha$ in skeletal muscle of MCK-CnA* mice.** Protein content of PPAR$\alpha$ (*A*), PPAR$\delta$ (*B*), and PGC1$\alpha$ (*C*) was determined in EDL and soleus muscle from wild-type and MCK-CnA* mice (equal sample loading was confirmed by blotting for actin). Data are expressed as percentage of EDL muscle from wild-type mice. Data are means $\pm$ S.E. for $n = 5–6$ muscles. *, $p < 0.05$; **, $p < 0.005$ compared with wild-type.

genes required for the uptake of lipid from extracellular space into the myofiber was up-regulated in EDL muscle of the transgenic mice. The entry of lipid-derived acyl-CoA into the mitochondrial is facilitated by multiple genes, including *Cpt1*, *Cpt2*, and *Slc25a20*, and expression of activated calcineurin increased the mRNA level of these genes in support of lipid utilization. Furthermore, several genes essential for $\beta$-oxidation, namely *Decr1*, *Acadvl*, and *Hadhsc* were also up-regulated in skeletal muscle harboring activated calcineurin. In cultured primary cardiac myocytes, activation of calcineurin induces the aforementioned lipid metabolic genes (21), further underlining the role of calcineurin in the regulation of lipid metabolic gene expression. Therefore, activated calcineurin is sufficient to increase the transcription of multiple enzymes important for lipid metabolism to achieve a profound up-regulation of skeletal muscle lipid oxidation.

The expression of genes regulating lipid metabolism, including *Lpl*, *Cd36*, *Cpt1*, *Cpt2*, *Acadvl*, and *Hadhsc*, as well as *Pdk4* are regulated by PPAR$\alpha$ (22). Consistent with the increase in mRNA expression of these genes, expression of activated calcineurin in skeletal muscle markedly increased the *in vivo* expression of PPAR$\alpha$. Skeletal muscle-specific overexpression of PPAR$\alpha$ in mice also promotes the expression of *Cd36*, *Cpt2*, *Slc25a20*, *Decr1*, *Acadvl*, *Hadhsc*, and *Pdk4* (14). In cultured C2C12 myotubes and cardiac myocytes, expression of *Ppar*$\alpha$ is directly induced by calcineurin via activation of the *Ppar*$\alpha$ promoter (21). Here, we provide evidence that activated calcineurin induced the expression of PPAR$\alpha$, in support of lipid oxidation via consistent increase in lipid metabolic gene expression.

In addition to PPAR$\alpha$, PPAR$\delta$ has also been implicated as a transcription regulator of skeletal muscle lipid metabolism. Both PPAR subtypes have a redundant and compensatory function in the regulation of lipid oxidation and gene regulatory events (23). We therefore cannot exclude the role of PPAR$\delta$ (apart from PPAR$\alpha$) in mediating the effects of calcineurin on lipid oxidation. The induction of lipid oxidation and metabolic genes in human and rat cultured myotubes observed in response to a selective PPAR$\delta$ agonist is similar to the effects

observed in response to a PPARα-selective agonist (23). Moreover, there was no additive effect on lipid oxidation and gene expression when maximum doses of both agonists were used concurrently, suggesting that both PPARs might bind to the same response element within a particular promoter (23). Moreover, starvation and exercise elicit an appropriate response on skeletal muscle *Pdk4* and *Ucp3* expression in PPARα knock-out mice. Given that the expression of skeletal muscle *Pparδ* is severalfold higher than *Pparα*, as well as the finding that there is functional similarity between these two transcription regulators, PPARδ has been proposed to compensate for the loss of PPARα in these mice (23). Consistently, *in vivo* pharmacological activation of PPARδ increases lipid oxidation in skeletal muscle via induction of lipid metabolic genes, including *Cpt1*, *Pdk4*, and *Ucp3* (24), a response that is also achieved by activation of PPARα. Our results provide evidence for the role of both PPARα and -δ in calcineurin-induced skeletal muscle lipid oxidation and metabolic gene expression.

NADH and $FADH_2$ generated from β-oxidation are reoxidized via oxidative phosphorylation to produce ATP. Our results show that expression of activated calcineurin is associated with increased lipid oxidation in skeletal muscle and a concomitant increase in protein content of the mitochondrial oxidative phosphorylation machinery. Skeletal muscle mitochondrial biogenesis is promoted by overexpression of either PGC1α or activated PPARδ. Overexpression of either PGC1α (15) or activated PPARδ (16) in mouse skeletal muscle also induces the formation of slow oxidative fibers. In transfected cells, PGC1α coactivates the calcineurin-mediated activation of myoglobin and troponin I gene promoter, suggesting a synergistic effect of calcineurin and PGC1α on the activation of the slow oxidative gene expression program (15). Furthermore, in skeletal muscle of PGC1α knock-out mice, mitochondrial gene expression (25, 26) and respiration (25) are impaired. Transgenic or pharmacological approaches to activated PPARδ also increase mitochondrial gene expression, including *Cpt1* and various *Cox* genes in skeletal muscle (16). However, the signaling pathways that regulate the expression of these transcription factors and coactivator are largely unresolved. Our results support the hypothesis that calcineurin induces mitochondrial biogenesis via induction of PGC1α and PPARδ expression.

Calcineurin has been proposed to play a role in the regulation of exercise-induced gene expression in human skeletal muscle (27–29). Exercise activates skeletal muscle calcineurin and PGC1α expression (27–29), and this is correlated with mitochondrial biogenesis (29). However, inhibition of calcineurin by cyclosporin treatment in rats did not abolish exercise-induced mRNA expression of PGC1α and multiple mitochondrial genes (30). The regulation of mitochondrial biogenesis in skeletal muscle is complex and likely to involve additional signaling pathways. For example, AMP-activated protein kinase and myogenin (a muscle-specific transcription factor) have also been proposed to mediate exercise-induced metabolic adaptations. Activation of AMP-activated protein kinase in mice by genetic manipulation and pharmacological approaches induces a similar pattern of exercise-induced gene expression (31, 32), including lipid metabolic genes (33, 34) and mitochondrial proteins (35, 36) in skeletal muscle. In myogenin transgenic mice, there is a shift from glycolytic to oxidative metabolism, concomitant with increased mitochondrial proteins in skeletal muscle (37). Clearly, multiple signaling pathways and nuclear effectors are involved in the regulation of the metabolic gene expression program in skeletal muscle.

Our results support the hypothesis that calcineurin regulates pathways controlling lipid and glucose metabolism in skeletal muscle via coordinated changes in the expression of metabolic genes, as well as transcription regulators, including PPARα, PPARδ, and PGC1α. Furthermore, activation of calcineurin markedly induced skeletal muscle mitochondrial biogenesis. Therefore, we provide evidence that activated calcineurin alters metabolic and mitochondrial gene expression as well as various transcription regulators, leading to profound alterations in skeletal muscle lipid and glucose metabolism.

## REFERENCES


1. Andres, R., Cader, G., and Zierler, K. L. (1956) *J. Clin. Invest.* **35,** 671–682
2. Kelley, D. E., Reilly, J. P., Veneman, T., and Mandarino, L. J. (1990) *Am. J. Physiol.* **258,** E923–E929
3. Pette, D., and Vrbova, G. (1999) *Muscle Nerve* **22,** 666–677
4. Hughes, S. M. (1998) *Curr. Biol.* **8,** R892–R894
5. Hamilton, M. T., Etienne, J., McClure, W. C., Pavey, B. S., and Holloway, A. K. (1998) *Am. J. Physiol.* **275,** E1016–E1022
6. Bonen, A., Dyck, D. J., Ibrahimi, A., and Abumrad, N. A. (1999) *Am. J. Physiol.* **276,** E642–E649
7. Theriault, R., Theriault, G., and Simoneau, J. A. (1994) *J. Appl. Physiol.* **77,** 1885–1889
8. Nuhr, M., Crevenna, R., Gohlsch, B., Bittner, C., Pleiner, J., Wiesinger, G., Fialka-Moser, V., Quittan, M., and Pette, D. (2003) *Eur. J. Appl. Physiol.* **89,** 202–208
9. Kong, X., Manchester, J., Salmons, S., and Lawrence, J., Jr. (1994) *J. Biol. Chem.* **269,** 12963–12967
10. Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, H., Zhu, W., Bassel-Duby, R., and Williams, R. S. (1998) *Genes Dev.* **12,** 2499–2509
11. Dunn, S. E., Simard, A. R., Bassel-Duby, R., Williams, R. S., and Michel, R. N. (2001) *J. Biol. Chem.* **276,** 45243–45254
12. Serrano, A. L., Murgia, M., Pallafacchina, G., Calabria, E., Coniglio, P., Lomo, T., and Schiaffino, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13108–13113
13. Naya, F. J., Mercer, B., Shelton, J., Richardson, J. A., Williams, R. S., and Olson, E. N. (2000) *J. Biol. Chem.* **275,** 4545–4548
14. Finck, B. N., Bernal-Mizrachi, C., Han, D. H., Coleman, T., Sambandam, N., LaRiviere, L. L., Holloszy, J. O., Semenkovich, C. F., and Kelly, D. P. (2005) *Cell Metabolism* **1,** 133–144
15. Lin, J., Wu, H., Tarr, P. T., Zhang, C.-Y., Wu, Z., Boss, O., Michael, L. F., Puigserver, P., Isotani, E., Olson, E. N., Lowell, B. B., Bassel-Duby, R., and Spiegelman, B. M. (2002) *Nature* **418,** 797–801
16. Wang, Y. X., Zhang, C. L., Yu, R. T., Cho, H. K., Nelson, M. C., Bayuga-Ocampo, C. R., Ham, J., Kang, H., and Evans, R. M. (2004) *PLoS Biol.* **2,** e294
17. Ryder, J. W., Bassel-Duby, R., Olson, E. N., and Zierath, J. R. (2003) *J. Biol. Chem.* **278,** 44298–44304
18. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., and O'Neill, E. A. (1992) *Nature* **357,** 692–694
19. Tsao, T. S, Li, J., Chang, K. S., Stenbit, A. E., Galuska, D., Andersson, J. E., Zierath, J. R., McCarter, R. J., and Charron, M. J. (2001) *FASEB J.* **15,** 958–969
20. Sugden, M. C., and Holness, M. J. (2003) *Am. J. Physiol.* **284,** E855–E862
21. Schaeffer, P. J., Wende, A. R., Magee, C. J., Neilson, J. R., Leone, T. C., Chen, F., and Kelly, D. P. (2004) *J. Biol. Chem.* **279,** 39593–39603
22. Mandard, S., Muller, M., and Kersten, S. (2004) *Cell Mol. Life Sci.* **61,** 393–416
23. Muoio, D. M., MacLean, P. S., Lang, D. B., Li, S., Houmard, J. A., Way, J. M.,

Winegar, D. A., Corton, J. C., Dohm, G. L., and Kraus, W. E. (2002) *J. Biol. Chem.* **277,** 26089–26097
24. Tanaka, T., Yamamoto, J., Iwasaki, S., Asaba, H., Hamura, H., Ikeda, Y., Watanabe, M., Magoori, K., Ioka, R. X., Tachibana, K., Watanabe, Y., Uchiyama, Y., Sumi, K., Iguchi, H., Ito, S., Doi, T., Hamakubo, T., Naito, M., Auwerx, J., Yanagisawa, M., Kodama, T., and Sakai, J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15924–15929
25. Leone, T. C., Lehman, J. J., Finck, B. N., Schaeffer, P. J., Wende, A. R., Boudina, S., Courtois, M., Wozniak, D. F., Sambandam, N., Bernal-Mizrachi, C., Chen, Z., Holloszy, J. O., Medeiros, D. M., Schmidt, R. E., Saffitz, J. E., Abel, E. D., Semenkovich, C. F., and Kelly, D. P. (2005) *PLoS Biol.* **3,** e101
26. Arany, Z., He, H., Lin, J., Hoyer, K., Handschin, C., Toka, O., Ahmad, F., Matsui, T., Chin, S., and Wu, P.-H. (2005) *Cell Metabolism* **1,** 259–271
27. Pilegaard, H., Saltin, B., and Neufer, P. D. (2003) *J. Physiol.* (*Lond.*) **546,** 851–858
28. Norrbom, J., Sundberg, C. J., Ameln, H., Kraus, W. E., Jansson, E., and Gustafsson, T. (2004) *J. Appl. Physiol.* **96,** 189–194
29. Garnier, A., Fortin, D., Zoll, J., N'Guessan, B., Mettauer, B., Lampert, E., Veksler, V., and Ventura-Clapier, R. (2005) *FASEB J.* **19,** 43–52
30. Garcia-Roves, P. M., Huss, J., and Holloszy, J. O. (2006) *Am. J. Physiol.* **290,** E1172–E1179
31. Long, Y. C., and Zierath, J. R. (2006) *J. Clin. Invest.* **116,** 1776–1783
32. Winder, W. W. (2001) *J. Appl. Physiol.* **91,** 1017–1028
33. Long, Y. C., Barnes, B. R., Mahlapuu, M., Steiler, T. L., Martinsson, S., Leng, Y., Wallberg-Henriksson, H., Andersson, L., and Zierath, J. R. (2005) *Diabetologia* **48,** 2354–2364
34. Barnes, B. R., Long, Y. C., Steiler, T. L., Leng, Y., Galuska, D., Wojtaszewski, J. F., Andersson, L., and Zierath, J. R. (2005) *Diabetes* **54,** 3484–3489
35. Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., Young, L. H., Semenkovich, C. F., and Shulman, G. I. (2001) *Am. J. Physiol.* **281,** E1340–E1346
36. Zong, H., Ren, J. M., Young, L. H., Pypaert, M., Mu, J., Birnbaum, M. J., and Shulman, G. I. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 15983–15987
37. Hughes, S. M., Chi, M. M.-Y., Lowry, O. H., and Gundersen, K. (1999) *J. Cell Biol.* **145,** 633–642