

# Palmitate-Mediated Downregulation of Peroxisome Proliferator-Activated Receptor- $\gamma$ Coactivator 1 $\alpha$ in Skeletal Muscle Cells Involves MEK1/2 and Nuclear Factor- $\kappa$ B Activation

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The mechanisms by which elevated levels of free fatty acids cause insulin resistance are not well understood. Previous studies have reported that insulin-resistant states are characterized by a reduction in the expression of peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC)-1, a transcriptional activator that promotes oxidative capacity in skeletal muscle cells. However, little is known about the factors responsible for reduced PGC-1 expression. The expression of PGC-1 mRNA levels was assessed in C2C12 skeletal muscle cells exposed to palmitate either in the presence or in the absence of several inhibitors to study the biochemical pathways involved. We report that exposure of C2C12 skeletal muscle cells to 0.75 mmol/l palmitate, but not oleate, reduced PGC-1 $\alpha$  mRNA levels (66%;  $P < 0.001$ ), whereas PGC-1 $\beta$  expression was not affected. Palmitate led to mitogen-activated protein kinase (MAPK)-extracellular signal-related kinase (ERK) 1/2 (MEK1/2) activation. In addition, pharmacological inhibition of this pathway by coinubation of the palmitate-exposed cells with the MEK1/2 inhibitors PD98059 and U0126 prevented the downregulation of PGC-1 $\alpha$ . Furthermore, nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation was also involved in palmitate-mediated PGC-1 $\alpha$  downregulation, since the NF- $\kappa$ B inhibitor parthenolide prevented a decrease in PGC-1 $\alpha$  expression. These findings indicate that palmitate reduces PGC-1 $\alpha$  expression in skeletal muscle cells through a mechanism involving MAPK-ERK and NF- $\kappa$ B activation. *Diabetes* 55:2779–2787, 2006

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Received for publication 14 November 2005 and accepted in revised form 11 July 2006.

DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-related kinase; FFA, free fatty acid; I $\kappa$ B, inhibitor of  $\kappa$ B; IL, interleukin; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK-ERK 1/2; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PMA, phorbol myristic acid; PGC, PPAR- $\gamma$  coactivator; PPAR, peroxisome proliferator-activated receptor; PKC, protein kinase C.

DOI: 10.2337/db05-1494

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Different lines of evidence support the contention that elevated plasma free fatty acids (FFAs) are responsible for much of the insulin resistance present in type 2 diabetic patients. In fact, several studies (1–3) have consistently demonstrated that elevated plasma FFA levels generate insulin resistance in healthy subjects. Although insulin-resistant subjects exhibit chronic increases in plasma FFAs, due in part to excess lipolysis (4), their rates of fatty acid oxidation in skeletal muscle are reduced (5). As a result of these changes, fatty acids accumulate in skeletal muscle of insulin-resistant subjects and lead to enhanced levels of triglycerides (6), fatty acyl CoA (7), diacylglycerol (7), and ceramides (8). It is worth noting that a positive correlation has been reported between increased intramyocellular triglyceride content and insulin resistance (9), suggesting that prevention of lipid accumulation in skeletal muscle may improve insulin resistance. Despite such data, it is not known how elevated levels of FFAs result in lipid accumulation in skeletal muscle cells.

Exposure of skeletal muscle cells to elevated FFAs may affect the expression of several genes, contributing to the development of insulin resistance. We (10) and other researchers (11–13) have reported that insulin-resistant states are characterized by a reduction in skeletal muscle of peroxisome proliferator-activated receptor (PPAR) $\gamma$  coactivator-1 (PGC-1), the transcriptional coactivator driving the expression of many genes coding for proteins in mitochondria. PGC-1 was initially identified as a protein that interacted with PPAR $\gamma$  (14). Several isoforms of PGC-1 have been cloned, including PGC-1 $\alpha$ , PGC-1 $\beta$ , and the PGC-related coactivator (15). PGC-1 $\alpha$  is highly expressed in tissues with high metabolic rates, such as the heart, muscle, and brown adipose tissue (15). PGC-1 $\alpha$  acts as a transcriptional coactivator for PPARs and activates the expression of genes involved in fatty acid metabolism. It has recently been reported that lipid infusion decreases the expression of nuclear encoded mitochondrial genes and PGC-1 in human skeletal muscle (16). This discovery suggests that enhanced lipid levels in plasma may be responsible for the downregulation of PGC-1 in insulin-resistant states. Consistent with this finding, it has been reported that PGC-1 expression has been shown to be inversely correlated with plasma FFA concentration (13). However, nothing is yet known either about the kind of

fatty acid that may reduce PGC-1 expression or the mechanisms involved. To gain a better understanding of the mechanism by which skeletal muscle cell exposure to fatty acids may result in PGC-1 downregulation, we used the myoblast C2C12 cell line, which develops biochemical and morphological properties characteristic of skeletal muscle and which has been proven useful in studies of skeletal muscle metabolism (17). Exposure of skeletal muscle C2C12 cells to the saturated fatty acid palmitate, but not to the monounsaturated fatty acid oleate, reduced PGC-1 $\alpha$  mRNA expression, whereas PGC-1 $\beta$  was not significantly affected. The mechanism by which palmitate reduced the expression of PGC-1 $\alpha$  seems to involve mitogen-activated protein kinase (MAPK)–extracellular signal-related kinase (ERK) 1/2 (MEK1/2), since inhibition of this pathway prevented PGC-1 $\alpha$  downregulation. These results identify high plasma levels of the saturated fatty acid palmitate as one of the factors potentially contributing to the downregulation of PGC-1 $\alpha$  in skeletal muscle.

## RESEARCH DESIGN AND METHODS

Anti-interleukin (IL)-6 antibody, C<sub>2</sub>-ceramide, ISP1, and pyrrolidine dithiocarbamate were obtained from Sigma (St. Louis, MO) and PD98059 and calphostin C from Biomol Research Labs (Plymouth Meeting, PA). Other chemicals were purchased from Sigma.

**Cell culture.** Mouse C2C12 myoblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin. When cells reached confluence, the medium was switched to the differentiation medium containing DMEM and 2% horse serum, which was changed every other day. After 4 additional days, the differentiated C2C12 cells had fused into myotubes. Lipid-containing media were prepared by conjugation of FFAs with FFA-free BSA, using a method modified from that described by Chavez et al. (18). Briefly, FFAs were dissolved in ethanol and diluted 1:100 in DMEM containing 2% (wt/vol) fatty acid-free BSA. Myotubes were incubated for 16 h in serum-free DMEM containing 2% BSA in either the presence (FFA-treated cells) or absence (control cells) of FFAs. Cells were then incubated with 100 nmol/l insulin for 10 min. Following incubation, RNA was extracted from myotubes as described below.

**Measurements of mRNA.** Levels of PGC-1 $\alpha$  and - $\beta$  mRNA were assessed by RT-PCR, as previously described (19). Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston). The total RNA isolated by this method was undegraded and free of protein and DNA contamination. The sequences of the sense and antisense primers used for amplification were PGC-1 $\alpha$ , 5'-CCCGTGGATGAAGACGGATTG-3' and 5'-GTGGGTGTGGTTTGTGCATG-3'; PGC-1 $\beta$ , 5'-CCTTTATCTGTGCCCCCCAGC-3' and 5'-CAAGGCCGTGACTTCTGGAA-3'; and *Aprt* (adenosyl phosphoribosyl transferase), 5'-GCCTCTTGGCCAGTCACTGA-3' and 5'-CCAGGCTCACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (PGC-1 $\alpha$ : 228 bp, PGC-1 $\beta$ : 185 bp, and *Aprt*: 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method described in this study (20). Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*Aprt*).

**Determination of ceramide levels.** The content of ceramides in skeletal muscle and C2C12 myotubes was determined by the diacylglycerol kinase method. Briefly, lipids were extracted from ~50 mg skeletal muscle with 600  $\mu$ l chloroform, methanol, and 1 N HCl (100:100:1). After agitation and centrifugation, the lower phase containing the chloroform-extracted lipids was transferred to a new microfuge tube. Chloroform was evaporated under an N<sub>2</sub> stream. Dried lipids were resuspended in 300  $\mu$ l of 0.1 N KOH in methanol and incubated for 1 h at 37°C to eliminate diacylglycerol. Then, 300  $\mu$ l PBS were added and lipid extraction was repeated as indicated above. Lipids were resuspended in 100  $\mu$ l of reaction buffer (150  $\mu$ g/100  $\mu$ l cardiolipin, 280  $\mu$ mol/l diethylenetriaminepentaacetic acid, 51 mmol/l octyl  $\beta$ -D-glucopyranoside, 50 mmol/l NaCl, 51 mmol/l imidazol, 1 mmol/l EDTA, 12.5 mmol/l MgCl<sub>2</sub>, 2 mmol/l dithiothreitol, 0.7% glycerol, 70  $\mu$ mol/l  $\beta$ -mercaptoethanol, 500  $\mu$ mol/l ATP, and 5  $\mu$ Ci/100  $\mu$ l [ $\gamma$ -<sup>32</sup>P]ATP), and 35 ng of diacylglycerol kinase was added to each sample. Reactions were incubated at 30°C for 30 min and stopped by the addition of 170  $\mu$ l of stop buffer (135

mmol/l NaCl, 1.5 mmol/l CaCl<sub>2</sub>, 0.5 mmol/l glucose, and 10 mmol/l HEPES, pH 7.2) and 30  $\mu$ l of 100 mmol/l EDTA. Lipids were extracted again with 1 ml chloroform, methanol, and 1 N HCl (100:100:1), resuspended in 40  $\mu$ l chloroform, spotted onto silica gel TLC plates (Whatman), and resolved using chloroform:methanol:acetic acid (65:15:5) as a solvent. Plates were measured in a PhosphorImager (BioRad). Quantification of ceramide mass was obtained by comparison with a standard curve ranging from 0 to 1,000 pmol ceramide-1-phosphate (Sigma), which was processed in parallel to the samples.

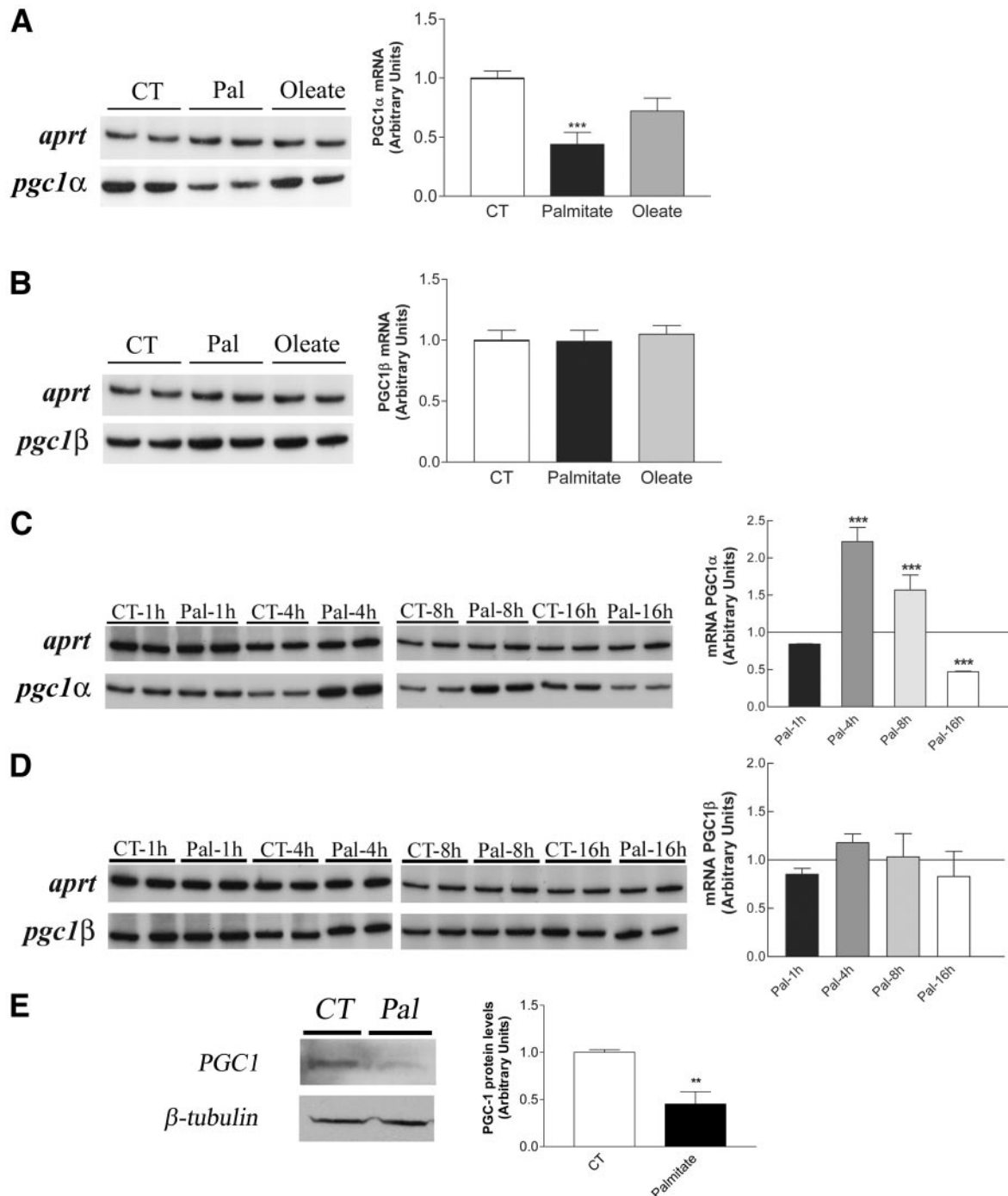
**Isolation of nuclear extracts and electrophoretic mobility shift assay.** Nuclear extracts were isolated according to Andrews et al. (21). Electrophoretic mobility shift assay (EMSA) was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) nucleotide (5'AGTTGAGGGGACTTTC CCAGGC-3'). Oligonucleotides were labeled in the following reaction: 2  $\mu$ l of oligonucleotide (1.75 pmol/ $\mu$ l), 2  $\mu$ l of 5 $\times$  kinase buffer, 1  $\mu$ l of T4 polynucleotide kinase (10 units/ $\mu$ l), and 2.5  $\mu$ l [ $\gamma$ -<sup>32</sup>P] ATP (3,000 Ci/mmol at 10 mCi/ml) incubated at 37°C for 1 h. The reaction was stopped by adding 90  $\mu$ l of TE buffer (10 mmol/l Tris-HCl, pH 7.4, and 1 mmol/l EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia, Sant Cugat, Spain) according to the manufacturer's instructions. Five micrograms of crude nuclear protein were incubated for 10 min on ice in binding buffer [10 mmol/l Tris-HCl, pH 8.0, 25 mmol/l KCl, 0.5 mmol/l dithiothreitol, 0.1 mmol/l EDTA, pH 8.0, 5% glycerol, 5 mg/ml BSA, 100  $\mu$ g/ml tRNA, and 50  $\mu$ g/ml poly(dI-dC)] in a final volume of 15  $\mu$ l. The labeled probe (~60,000 cpm) was added and the reaction incubated for 15 min at room temperature. Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. p65 antibody was added 15 min before incubation with the labeled probe at 4°C. Protein-DNA complexes were resolved by electrophoresis at 4°C on a 5% acrylamide gel and subjected to autoradiography.

**Immunoblotting.** To obtain total proteins, C2C12 myotubes were homogenized in cold lysis buffer (5 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l sodium orthovanadate, and 5.4  $\mu$ g/ml aprotinin). The homogenate was centrifuged at 10,000g for 30 min at 4°C. Protein concentration was measured by the Bradford method. Total and nuclear proteins (30  $\mu$ g) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against total MEK1/2, phospho-MEK1/2 (Ser<sup>217/221</sup>), total ERK1/2, and phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), pan-protein kinase C (PKC) (Cell Signaling Technology, Beverly, MA), PGC-1 (Chemicon, Temecula, CA),  $\alpha$ -actin, and  $\beta$ -tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek, Israel). The equal loading of proteins was assessed by red phenol staining. The size of detected proteins was estimated using protein molecular mass standards (Invitrogen, Barcelona, Spain).

**Statistical analyses.** Results are expressed as the mean  $\pm$  SD of five separate experiments. Significant differences were established by either the Student's *t* test or one-way ANOVA, according to the number of groups compared, using the computer program GraphPad Instat (GraphPad Software V2.03; GraphPad Software, San Diego, CA). In the latter case, when significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Differences were considered significant at *P* < 0.05.

## RESULTS

**Palmitate, but not oleate, reduces PGC-1 $\alpha$  mRNA levels in skeletal muscle cells.** To study the effects of FFA on PGC-1 expression in C2C12 skeletal muscle cells, we chose the saturated FFA palmitate (16:0) and the monounsaturated FFA oleate (18:1 n-9), which are among the most common fatty acids (22). Because we recently observed that exposure of C2C12 skeletal muscle cells to palmitate for 16 h leads to reduced insulin-stimulated glucose uptake (19), we used this experimental condition to evaluate the effects of palmitate and oleate on PGC-1 $\alpha$  expression. Skeletal muscle cells exposed to 0.75 mmol/l palmitate showed a 66% (*P* < 0.001) reduction in PGC-1 $\alpha$  expression compared with control cells, whereas the reduction attained by the same concentration of oleate did not reach significance (Fig. 1A). The effects of palmitate on PGC-1 $\alpha$  expression in skeletal muscle cells were specific, since neither palmitate nor oleate affected the expression of PGC-1 $\beta$  (Fig. 1B). Further, we studied the time response



**FIG. 1.** Palmitate, but not oleate, downregulates PGC-1 $\alpha$  mRNA levels in skeletal muscle cells. Analysis of the mRNA levels of PGC-1 $\alpha$  (A) and PGC-1 $\beta$  (B) in C2C12 myotubes incubated in the presence or in the absence of the indicated FFAs (16 h, 0.75 mmol/l). A total of 0.5  $\mu$ g of total RNA was analyzed by RT-PCR. A representative autoradiogram and quantification, normalized to the APRT mRNA levels, are shown. Data are expressed as the mean  $\pm$  SD of five different experiments. \*\*\* $P$  < 0.001 vs. control cells. Time course of palmitate effects on PGC-1 $\alpha$  (C) and PGC-1 $\beta$  (D) mRNA levels in C2C12 myotubes incubated with 0.75 mmol/l palmitate for the indicated time points. A representative autoradiogram and quantification, normalized to the APRT mRNA levels, are shown. Data (mean  $\pm$  SD of five different experiments) are expressed as the relative increase to control cells at the same time point. \*\*\* $P$  < 0.001 vs. control cells. E: Nuclear extracts from C2C12 myotubes incubated in the presence or in the absence of 0.75 mmol/l palmitate for 16 h were assayed for Western blot analysis with PGC-1 and  $\beta$ -tubulin antibodies. Data are expressed as means  $\pm$  SD of three different experiments normalized to  $\beta$ -tubulin levels. \*\* $P$  < 0.01 vs. control cells.

effects of palmitate on PGC-1 mRNA levels. Whereas palmitate treatment for 1 h did not significantly affect PGC-1 $\alpha$  mRNA expression, a slight increase was observed after four (twofold,  $P$  < 0.001) and 8 h (1.5-fold,  $P$  < 0.001), reaching a deep reduction after 16 h of treatment (Fig. 1C). In contrast to PGC-1 $\alpha$ , a time course study with palmitate did not show changes in PGC-1 $\beta$  expression

(Fig. 1D). The reduction in mRNA expression was correlated with protein levels, since Western immunoblot analysis revealed that skeletal muscle cells exposed to palmitate for 16 h showed a significant reduction (55%,  $P$  < 0.01) in PGC-1 protein expression (Fig. 1E).

**Ceramides and PKC activation are not involved in palmitate-mediated PGC-1 $\alpha$  downregulation in skel-**

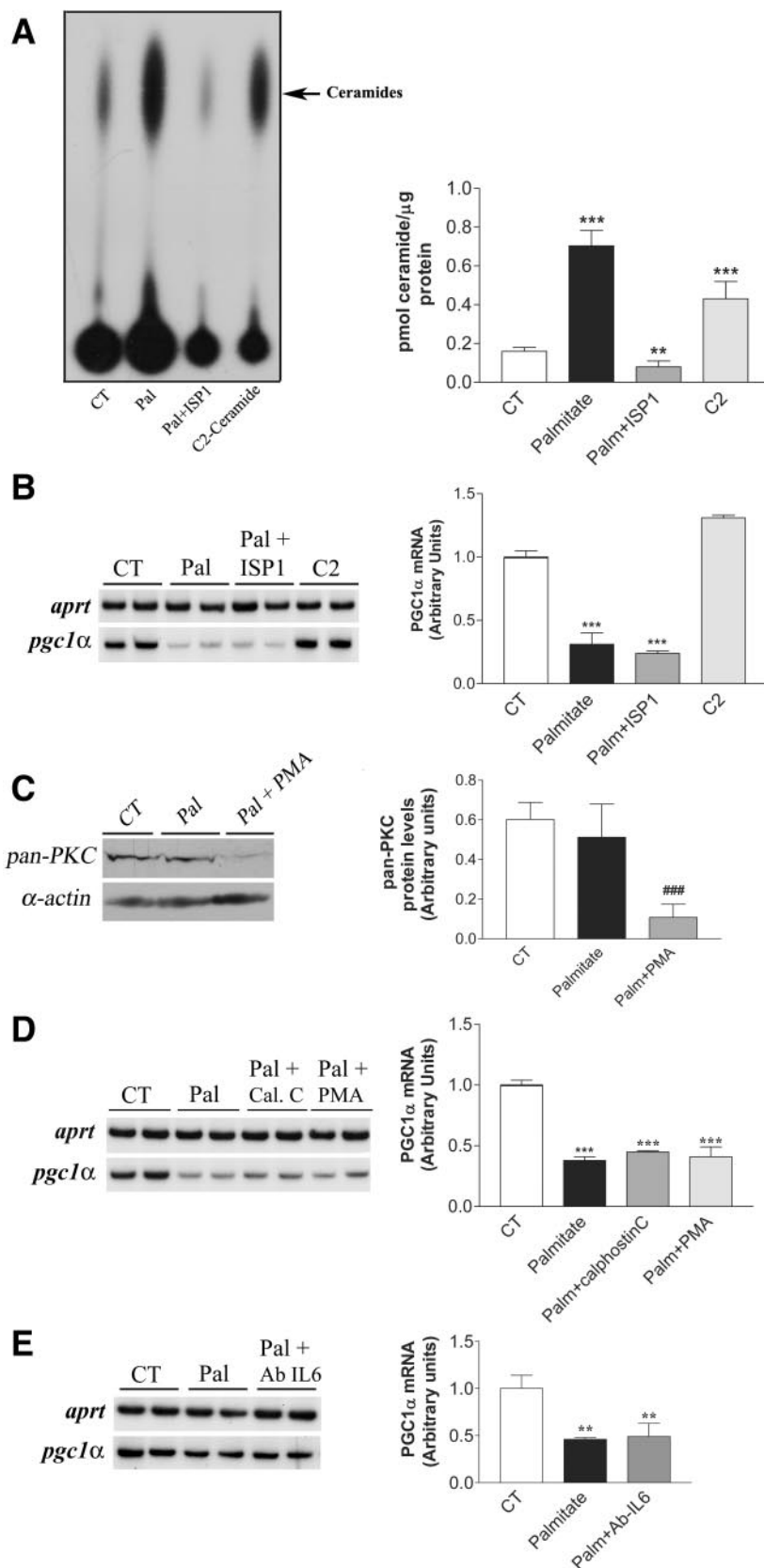
**etal muscle cells.** Since palmitoyl-CoA is a precursor of sphingolipid synthesis, palmitate treatment may result in enhanced ceramide synthesis and apoptosis (23). Thus, to gain further insight into the mechanism by which palmitate downregulates PGC-1 $\alpha$  mRNA levels, we tested the effects of one inhibitor of de novo ceramide synthesis. The initial step in ceramide synthesis is the formation of 3-ketodihydrosphingosine from palmitoyl-CoA and L-serine. This step is inhibited by the sphingosine analog ISP1 at picomole concentrations (24). To further clarify the potential involvement of ceramides, we treated C2C12 skeletal muscle cells with C<sub>2</sub>-ceramide, a cell-permeable ceramide analog. First, we evaluated the effects of palmitate, ISP1, and C<sub>2</sub>-ceramide on the intracellular concentrations of ceramides. Incubation of C2C12 myotubes with palmitate and the C<sub>2</sub>-ceramide analog enhanced ceramide levels (4.5- and 2.7-fold induction, respectively), whereas coincubation of the cells with palmitate and ISP1 prevented the increase in the levels of these lipid mediators (Fig. 2A). Regarding the expression of PGC-1 $\alpha$ , coincubation of the cells with palmitate in the presence of ISP1 did not affect the fall (69%,  $P < 0.001$ ) in the mRNA levels of this coactivator attained by palmitate (Fig. 2B). Similarly, the addition of 50  $\mu\text{mol/l}$  C<sub>2</sub>-ceramide did not modify PGC-1 $\alpha$  mRNA levels (Fig. 2B). Overall, these data suggest that de novo ceramide synthesis is not involved in the effects of palmitate on PGC-1 $\alpha$  expression.

As elevations in plasma FFAs may lead to diacylglycerol-mediated activation of PKC (25), we subsequently investigated whether palmitate-mediated PGC-1 $\alpha$  downregulation involved PKC activation. To test this hypothesis, we used two approaches. We first verified the effects of calphostin C, a strong and specific inhibitor of PKC (26), on PGC-1 $\alpha$  downregulation caused by palmitate in C2C12 myotubes. Cells were preincubated with calphostin C (100  $\mu\text{mol/l}$ ) for 30 min and subsequently stimulated with 0.75  $\mu\text{mol/l}$  palmitate for 16 h. We then pretreated cells with 0.5  $\mu\text{mol/l}$  phorbol myristic acid (PMA) for 24 h before stimulation with palmitate. This long-term pretreatment with PMA causes PKC downregulation (27,28) (Fig. 2C). As shown in Fig. 2D, no changes were observed in PGC-1 $\alpha$  mRNA levels in those cells exposed to palmitate coincubated with either calphostin C or PMA. These data seem to rule out the involvement of PKC activation in palmitate-mediated PGC-1 $\alpha$  downregulation in skeletal muscle cells. Finally, since we have recently reported that skeletal muscle cells exposed to palmitate secrete IL-6 into culture media (19), and that this IL-6 may affect cell metabolism, we determined whether IL-6 was involved in palmitate-mediated PGC-1 $\alpha$  downregulation using an anti-IL-6 antibody. While treatment with palmitate led to a reduction in PGC-1 $\alpha$  mRNA levels, coincubation with anti-IL-6 antibody did not prevent this change (Fig. 2E).

**Activation of the ERK-MAPK pathway and NF- $\kappa$ B are involved in palmitate-mediated PGC-1 $\alpha$  downregulation.** Since activation of the ERK-MAPK cascade is reportedly activated by palmitate (29), we next evaluated its involvement in the downregulation of PGC-1 $\alpha$  following palmitate exposure. We first confirmed that under our conditions, exposure of the cells to palmitate led to MEK1/2 activation. Subsequent immunoblotting detection of total and phosphorylated MEK1/2 (Fig. 3A) revealed that palmitate treatment activated this pathway (1.75-fold induction,  $P < 0.05$ ), whereas no significant changes were observed in cells exposed to oleate. To clearly demonstrate the involvement of the ERK-MAPK cascade in PGC-1 $\alpha$  downregulation in those cells incubated with

palmitate, we utilized pharmacological inhibitors to block the upstream regulators of ERK1/2. We assayed the effects of two MEK1/2 inhibitors: PD98059, a more general inhibitor of this pathway, and U0126, a more potent and specific ERK1/2 inhibitor that binds to MEK, thereby inhibiting its catalytic activity as well as phosphorylation of ERK1/2. The reduction in PGC-1 $\alpha$  mRNA levels achieved by palmitate (58%,  $P < 0.001$  vs. control cells) was abrogated when the cells were coincubated with either PD98059 (19%,  $P < 0.01$  vs. palmitate-treated cells) or U0126 (9%,  $P < 0.001$  vs. palmitate-treated cells) (Fig. 3B), indicating that activation of the MAPK-ERK cascade was involved in the decrease of this coactivator following palmitate treatment. As control, we verified that U0126 treatment condition inhibited ERK1/2 activation (Fig. 3C).

Since we have previously reported that C2C12 exposure to palmitate activates NF- $\kappa$ B (19), and activation of the ERK-MAPK cascade may influence NF- $\kappa$ B activation in skeletal muscle (30), we next determined whether activation of this transcription factor was involved in palmitate-mediated PGC-1 $\alpha$  downregulation. We first confirmed whether palmitate-mediated NF- $\kappa$ B activation involved activation of the ERK-MAPK cascade by performing EMSA studies using nuclear extracts from palmitate-treated cells incubated either in the presence or in the absence of U0126. The NF- $\kappa$ B probe formed two complexes with nuclear proteins (complexes I and II) (Fig. 4A). Specificity of the two DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF- $\kappa$ B oligonucleotide. NF- $\kappa$ B binding activity, primarily involving specific complex II, increased in nuclear extracts from palmitate-treated cells. However, U0126 prevented this increase in NF- $\kappa$ B binding activity, indicating that in the presence of this MEK1/2 inhibitor, palmitate failed to induce this transcription factor. The addition of antibody against the p65 subunit of NF- $\kappa$ B supershifted complex II, indicating that this band was mainly comprised of this subunit. No changes were observed in the DNA binding of nuclear proteins to an Oct-1 probe, indicating that the increase observed for the NF- $\kappa$ B probe was specific (data not shown). As control, we verified that the NF- $\kappa$ B inhibitor parthenolide prevented palmitate-induced NF- $\kappa$ B activation (Fig. 4A). Since palmitate-induced NF- $\kappa$ B activation is mediated by degradation of inhibitor of  $\kappa$ B (I $\kappa$ B)  $\alpha$  (19,31), we examined the effects of palmitate and U0126 on I $\kappa$ B $\alpha$  protein levels. Whereas cells exposed to palmitate showed reduced I $\kappa$ B $\alpha$  protein levels, this reduction was prevented in cells coincubated with palmitate and U0126 (Fig. 4B). The effect of palmitate on I $\kappa$ B $\alpha$  protein levels was specific for this saturated fatty acid, given that oleate did not significantly affect the levels of this protein (Fig. 4C). To clearly demonstrate whether NF- $\kappa$ B activation was involved in palmitate-mediated PGC-1 $\alpha$  downregulation, we used the NF- $\kappa$ B inhibitor parthenolide, which specifically inhibits NF- $\kappa$ B activation by preventing I $\kappa$ B degradation (32). The reduction in PGC-1 $\alpha$  mRNA expression levels attained by palmitate (51%,  $P < 0.001$  vs. control cells) was prevented when C2C12 cells were coincubated with parthenolide (27%,  $P < 0.05$  vs. palmitate-exposed cells) (Fig. 4D). In agreement with the results obtained for mRNA expression, coincubation of the cells with palmitate, in either the presence of U0126 or parthenolide, prevented the reduction in PGC-1 protein levels (Fig. 4E). Overall, these results suggest that PGC-1 $\alpha$  downregulation in skeletal muscle cells following palmitate exposure is regulated by the ERK-MAPK-NF- $\kappa$ B pathway.

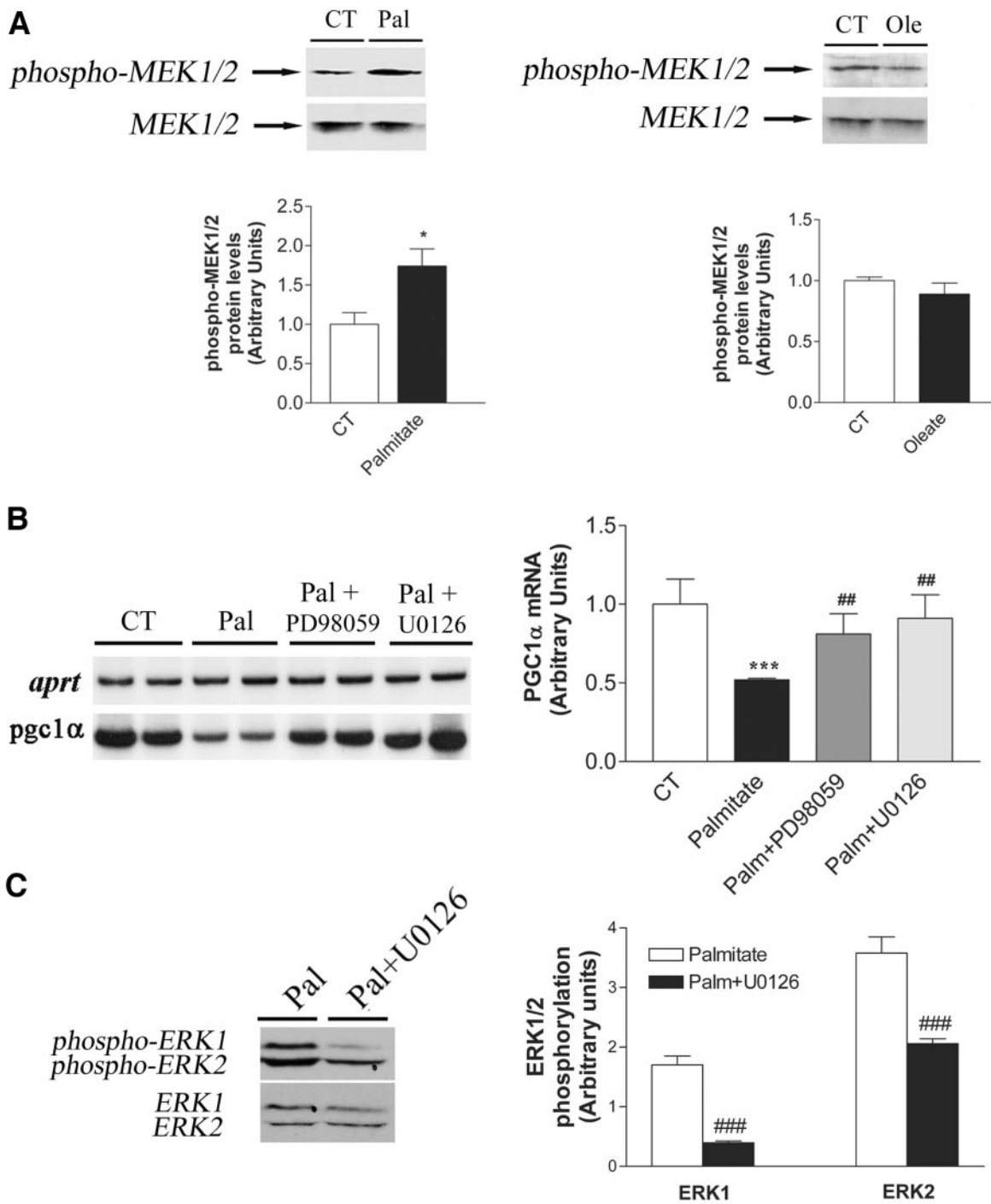


**FIG. 2.** Ceramides and PKC activation are not involved in palmitate-mediated downregulation of PGC-1 $\alpha$  in skeletal muscle cells. Measurement of ceramide (A) and PGC-1 $\alpha$  mRNA (B) levels in C2C12 myotubes incubated (16 h) with 0.75 mmol/l palmitate in the presence or in the absence of 100 nmol/l ISP1, an inhibitor of de novo ceramide synthesis. Cells were also treated with 50  $\mu$ mol/l C<sub>2</sub>-ceramide, a cell-permeable ceramide analog. Lipid extracts were prepared and assayed for ceramides as detailed under RESEARCH DESIGN AND METHODS. Data are expressed as the mean  $\pm$  SD of three different experiments. A total of 0.5  $\mu$ g of total RNA was analyzed by RT-PCR. A representative autoradiogram and quantification, normalized to the APRT mRNA levels, are shown. C: Total protein extracts from C2C12 myotubes incubated with 0.75 mmol/l palmitate for 16 h in the presence or in the absence of 500 nmol/l PMA were assayed for Western blot analysis with pan-PKC and  $\alpha$ -actin antibodies. Data are expressed as the mean  $\pm$  SD of three different experiments. C2C12 myotubes were incubated (16 h) with 0.75 mmol/l palmitate in the presence or in the absence of either 100  $\mu$ mol/l calphostin C, 500 nmol/l PMA (D), or 5  $\mu$ g/ml of anti-IL-6 antibody (E), and the mRNA levels of PGC-1 $\alpha$  were analyzed. Data are expressed as the mean  $\pm$  SD of five different experiments. \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 vs. control cells; ### $P$  < 0.001 vs. palmitate-treated cells.

## DISCUSSION

Skeletal muscle insulin resistance is the major characteristic of type 2 diabetes (33). Insulin resistance correlates more strongly with intramyocellular lipid levels than with any other factor, including BMI or percent body fat

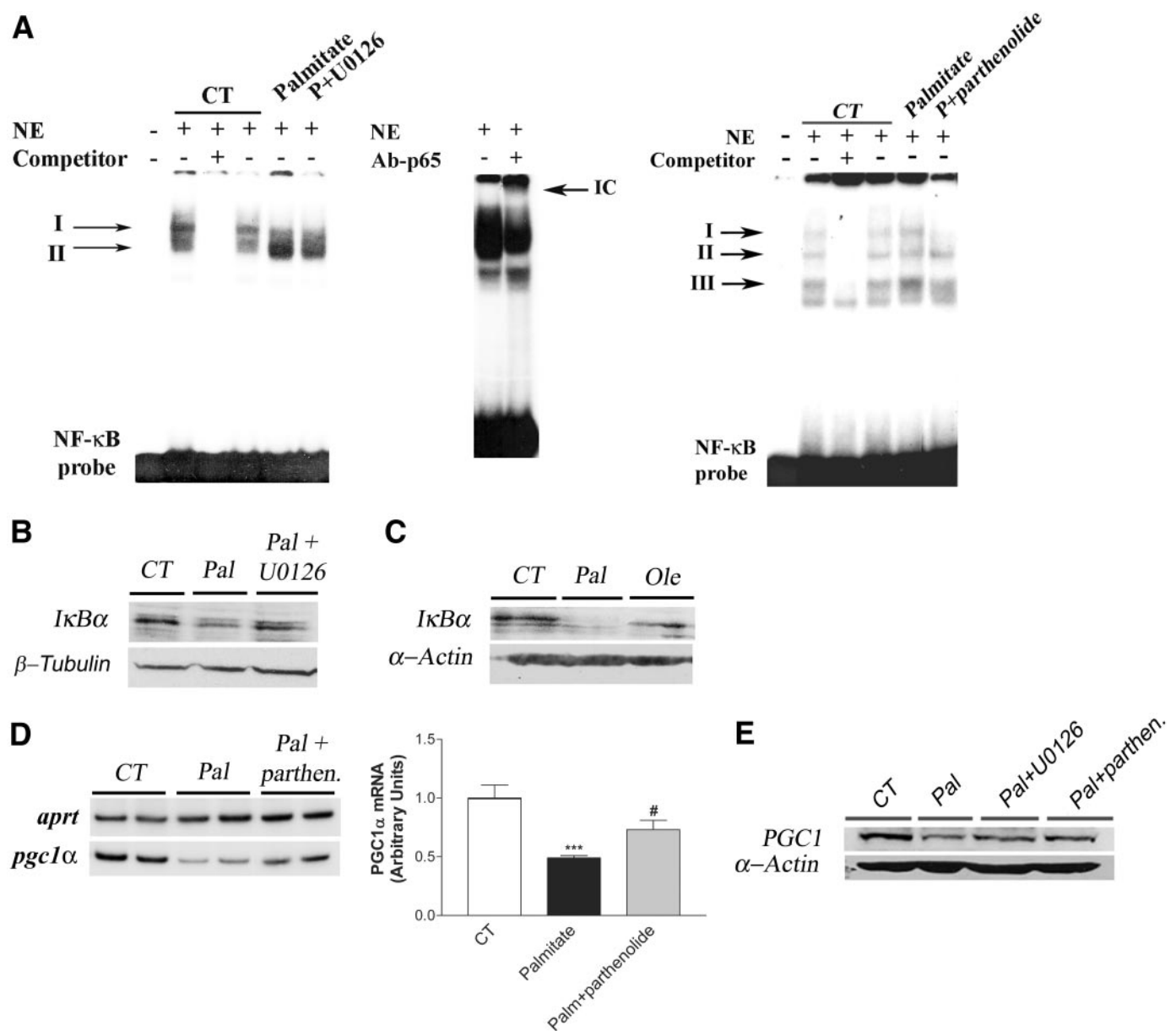
(7,34,35). Despite these data, the mechanisms by which elevated FFA levels cause intramyocellular lipid accumulation and insulin resistance are not well understood. Elevated FFAs may affect the expression of several genes, contributing to intramyocellular lipid accumulation and,



**FIG. 3.** The ERK-MAPK pathway is involved in palmitate-mediated PGC-1 $\alpha$  downregulation in skeletal muscle cells. C2C12 myotubes were incubated (16 h) with 0.75 mmol/l fatty acids in the presence or in the absence of 100  $\mu$ mol/l PD98059 or 10  $\mu$ mol/l U0126, inhibitors of the ERK-MAPK pathway. **A:** Analysis of MEK 1/2 activity determined by immunoblotting. The level of phosphorylated MEK1/2 was normalized against total MEK1/2 in each sample. **B:** Analysis of the mRNA levels of PGC-1 $\alpha$ . A total of 0.5  $\mu$ g total RNA was analyzed by RT-PCR. A representative autoradiogram and quantification, normalized to the APRT mRNA levels, are shown. Data are expressed as the mean  $\pm$  SD of five different experiments. \* $P$  < 0.05 and \*\*\* $P$  < 0.001 vs. control cells; ## $P$  < 0.01 vs. palmitate-treated cells. **C:** Analysis of ERK and phospho-ERK by immunoblotting of total protein extracts from C2C12 myotubes incubated with 0.75 mmol/l palmitate for 16 h in the presence or in the absence of 10  $\mu$ mol/l U0126. The level of phosphorylated ERK1/2 was normalized against total ERK1/2 in each sample. Data are expressed as the mean  $\pm$  SD of three different experiments. ### $P$  < 0.001 vs. palmitate-treated cells.

as a result, to the development of insulin resistance. Interestingly, insulin-resistant states have been associated in skeletal muscle with decreased expression of PGC-1 $\alpha$ , a transcriptional coactivator promoting oxidative capacity in this tissue (10,13,16). Thus, overexpression of PGC-1 $\alpha$  in cultured myoblasts increases mitochondrial biogenesis

and oxidative respiration (36). Moreover, muscle-specific overexpression of PGC-1 $\alpha$  in transgenic mice results in enhanced mitochondrial biogenesis and more slow-twitch (type I) fiber formation (37). Although PGC-1 expression has been inversely correlated with plasma FFA levels (13) and lipid infusion decreased PGC-1 in skeletal muscle (16),



**FIG. 4.** NF- $\kappa$ B activation is involved in palmitate-mediated PGC-1 $\alpha$  downregulation in skeletal muscle cells. **A:** The MEK1/2 inhibitor U0126 blocks the action of NF- $\kappa$ B by palmitate. C2C12 myotubes were incubated for 16 h with 0.75 mmol/l palmitate in the presence or in the absence of either 10  $\mu$ mol/l U0126 or 10  $\mu$ mol/l parthenolide. An autoradiograph of EMSA performed with a  $^{32}$ P-labeled NF- $\kappa$ B nucleotide and crude nuclear protein extract (NE) is shown. Specific complexes, based on competition with a molar excess of unlabeled probe, are shown. A supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF- $\kappa$ B is also shown. **B** and **C:** total protein extracts from C2C12 myotubes incubated for 16 h with either 0.75 mmol/l palmitate or oleate in the presence or in the absence of 10  $\mu$ mol/l U0126 were assayed for Western blot analysis with I $\kappa$ B $\alpha$  antibody. **D:** C2C12 myotubes were incubated (16 h) with 0.75 mmol/l palmitate in the presence or in the absence of the NF- $\kappa$ B inhibitor (10  $\mu$ mol/l) parthenolide. Analysis of PGC-1 $\alpha$  mRNA levels. A total of 0.5  $\mu$ g of total RNA was analyzed by RT-PCR. A representative autoradiogram and quantification, normalized to the APRT mRNA levels, are shown. Data are expressed as the mean  $\pm$  SD of five different experiments. \*\*\* $P$  < 0.001 vs. control cells; # $P$  < 0.05 vs. palmitate-treated cells. **E:** ERK-MAPK and NF- $\kappa$ B inhibitors prevent the decrease in PGC-1 protein levels in skeletal muscle cells exposed to palmitate. Nuclear extracts from C2C12 myotubes incubated with 0.75 mmol/l palmitate for 16 h in the presence or in the absence of U0126 (10  $\mu$ mol/l) or parthenolide (10  $\mu$ mol/l) were assayed for Western blot analysis with PGC-1 and  $\alpha$ -actin antibodies.

little was known about the type of FFAs that may reduce PGC-1 $\alpha$  or the biochemical pathways involved. Here, we report that the FFA palmitate reduces PGC-1 $\alpha$  expression in skeletal muscle cells through a mechanism involving activation of the ERK-MAPK-NF- $\kappa$ B pathway.

The results presented here confirm the role that elevated FFA plays in the downregulation of PGC-1 $\alpha$  in skeletal muscle cells. It is worth noting that the type of fatty acid may be central to the development of insulin resistance. In this study, we used the saturated fatty acid

palmitate. Intramuscular triglycerides and their palmitate fraction of these triglycerides, though not the oleate fraction, negatively correlated with insulin-stimulated glucose uptake (38). Interestingly, simultaneous to the effects of PGC-1 $\alpha$  on mitochondrial respiration in skeletal muscle cells, this coactivator also induces gene expression for the insulin-sensitive glucose transporter (GLUT4) and increases glucose uptake (39). These data suggest that the palmitate fraction of fatty acids present in intramuscular triglycerides, which is elevated in the muscle of obese

patients compared with lean subjects (38), may also have deleterious effects on muscle glucose and fatty acid metabolism through the decrease in the expression of PGC-1 $\alpha$ .

The mechanism by which palmitate reduces PGC-1 $\alpha$  mRNA levels in skeletal muscle cells seems to involve activation of the ERK-MAPK cascade, which in turn activates NF- $\kappa$ B, whereas enhanced ceramide levels and PKC activation seem not to be implicated. Our results demonstrate for the first time that exposure of skeletal muscle cells to palmitate leads to PGC-1 $\alpha$  downregulation through a mechanism involving MEK1/2 activation, whereas inhibition of this cascade prevents the fall in PGC-1 $\alpha$  expression caused by palmitate. The results reported here suggest that MEK activation depends on the type of fatty acids, since oleate treatment did not result in MEK activation. However, the mechanisms through which palmitate treatment results in MEK activation remain to be established. Interestingly, it has been reported that the conjugated linoleic acid activates MEK in human adipocytes through unidentified upstream signal that may involve activation of membrane proteins or generation of metabolites, which, in turn, triggers a signal that activates this pathway (40). To our knowledge, there are no reports in the literature addressing the direct effect of either MEK activation or inhibition on PGC-1 $\alpha$  mRNA levels. However, Watt et al. (41) reported that enhanced PGC-1 $\alpha$  mRNA levels in human skeletal muscle following exercise was accompanied by a reduction in phospho-ERK levels, supporting the contention that these kinases play a role in controlling PGC-1 $\alpha$  mRNA levels.

Furthermore, our results suggest that NF- $\kappa$ B activation by palmitate is also involved in the regulation of PGC-1 $\alpha$  expression in skeletal muscle cells, thereby bolstering the considerable evidence supporting a link between inflammation and type 2 diabetes. This relationship between palmitate-activated NF- $\kappa$ B and PGC-1 $\alpha$  downregulation in skeletal muscle cells is interesting since NF- $\kappa$ B has been linked to the fatty acid-induced impairment of insulin action in rodent skeletal muscle (42,43), while palmitate-induced insulin resistance is reportedly prevented by inhibition of this transcription factor (44). Palmitate may activate NF- $\kappa$ B through several mechanisms. First, it has been suggested that palmitate activates NF- $\kappa$ B by proteasome-mediated degradation of I $\kappa$ B $\alpha$  (31). This is consistent with the reported reduction in I $\kappa$ B $\alpha$  mass after palmitate treatment shown in this study (Fig. 4B). Second, MEK1/2 is important for NF- $\kappa$ B activation (45), and in this study we report that palmitate-mediated activation of MEK1/2 contributes to NF- $\kappa$ B activation, a process that is prevented when MEK1/2 is inhibited. Here, we do not provide an explanation about how palmitate-mediated NF- $\kappa$ B activation results in PGC-1 $\alpha$  downregulation, but several mechanisms could be involved. Interestingly, while this study was under evaluation, Hondares et al. (46) reported the presence of a PPAR $\gamma$  response element in the promoter of the PGC-1 $\alpha$  gene. Since MEK activation decreases PPAR $\gamma$  activity by phosphorylating this nuclear receptor and/or by interfering its transactivation activity (47), this provides a potential mechanism for the MEK-mediated downregulation of PGC-1 $\alpha$  after palmitate exposure. Similarly, NF- $\kappa$ B activation results in reduced PPAR $\gamma$  activity (48), suggesting that this mechanism may also contribute to the reduction in PGC-1 $\alpha$  expression after palmitate treatment. In agreement with this possibility, we have reported that skeletal muscle cells exposed to palmi-

tate show reduced PPAR binding activity (49). Further, since the NF- $\kappa$ B inhibitor parthenolide, compared with MEK inhibitors, only partially reversed the effect of palmitate on PGC-1 $\alpha$  expression, this may indicate that a blockade of upstream targets, i.e., MEK, is required to completely reverse the effects attained by palmitate or that additional proteins, such as Akt (50), are involved in the changes observed.

In summary, we report here that exposure of skeletal muscle cells to palmitate results in a reduction in PGC-1 $\alpha$  expression via a mechanism involving the activation of the ERK-MAPK-NF- $\kappa$ B axis. These results not only confirm the hypothesis that enhanced plasma FFAs reduce PGC-1 $\alpha$  expression but also identify the biochemical pathway involved, providing an attractive target for therapeutics in diabetes and related diseases.

#### ACKNOWLEDGMENTS

This study was partly supported by grants from the Fundació Privada Catalana de Nutrició i Lípids, Fundació Ramón Areces, Ministerio de Educación y Ciencia, Spain (SAF2003-01232 and SAF2006-01475) and European Union FEDER funds. We also thank the Generalitat de Catalunya for grant 2001SGR00141. T.C., M.J., and X.P. (programa Juan de la Cierva) were supported by grants from the Ministerio de Educación y Ciencia, Spain. R.R.-C. was supported by a grant from the Fundació Ramón Areces.

We thank the University of Barcelona's Language Advisory Service for their helpful assistance.

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