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Oleate Reverses Palmitate-induced Insulin Resistance and Inflammation in Skeletal Muscle Cells*

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Here we report that in skeletal muscle cells the contribution to insulin resistance and inflammation of two common dietary long-chain fatty acids depends on the channeling of these lipids to distinct cellular metabolic fates. Exposure of cells to the saturated fatty acid palmitate led to enhanced diacylglycerol levels and the consequent activation of the protein kinase Cθ/nuclear factor κB pathway, finally resulting in enhanced interleukin 6 secretion and down-regulation of the expression of genes involved in the control of the oxidative capacity of skeletal muscle (peroxisome proliferator-activated receptor (PPAR)γ-coactivator 1α) and triglyceride synthesis (acyl-coenzyme A: diacylglycerol acyltransferase 2). In contrast, exposure to the monounsaturated fatty acid oleate did not lead to these changes. Interestingly, co-incubation of cells with palmitate and oleate reversed both inflammation and impairment of insulin signaling by channeling palmitate into triglycerides and by up-regulating the expression of genes involved in mitochondrial β-oxidation, thus reducing its incorporation into diacylglycerol. Our findings support a model of cellular lipid metabolism in which oleate protects against palmitate-induced inflammation and insulin resistance in skeletal muscle cells by promoting triglyceride accumulation and mitochondrial β-oxidation through PPARα- and protein kinase A-dependent mechanisms.

Insulin resistance is a major characteristic of type 2 diabetes mellitus and is also associated with obesity, hypertension, and cardiovascular disease (1). Skeletal muscle accounts for most insulin-stimulated glucose utilization and is, therefore, the main site of insulin resistance. Impairment of glucose utilization and insulin sensitivity during this process has been related to the presence of high free fatty acids (FFA)[4] in plasma. Along these lines, several studies have consistently demonstrated that a rise in plasma FFA produces insulin resistance in both diabetic patients and non-diabetic subjects (2–5). High FFA levels presumably increase FFA uptake, exceeding its oxidation, which in turn leads to increased intramuscular triglycerides and diacylglycerol (DAG), the latter being a potent allosteric activator of both conventional and novel PKC isoforms. Interestingly, it has been reported that the incubation of skeletal muscle cells with the saturated fatty acid palmitate results in the activation of PKCθ, which is the most abundant PKC isoform in skeletal muscle (6–8). This PKC isoform phosphorylates insulin receptor substrate 1 (IRS-1) (9), the main mediator of insulin response in muscle (10), leading to impaired insulin signaling. In addition, PKCθ has the unique ability among the PKC isoforms to activate pro-inflammatory NFκB (6), which has been linked to fatty acid-induced impairment of insulin action in skeletal muscle in rodents (12, 13). The activation of this pathway during insulin resistance supports a link between inflammation and type 2 diabetes (for review, see Ref. 14). In fact, markers of inflammation, including pro-inflammatory cytokines (such as tumor necrosis factor α, interleukin (IL) 1, interferon-γ, and IL-6) have been reported to be high in type 2 diabetes (15, 16). Of these cytokines, IL-6 correlates most strongly with insulin resistance and type 2 diabetes (15–17), and its plasma levels are increased 2–3-fold in patients with obesity and type 2 diabetes compared with lean control subjects (16). Further, recent evidence suggests that skeletal muscle cells generate IL-6 production when exposed to the saturated fatty acid palmitate (18, 19) through activation of the PKCθ-NFκB pathway.

Interestingly, saturated and monounsaturated fatty acids differ significantly in their contribution to insulin resistance (20, 21). Thus, it is generally accepted that saturated fatty acids induce insulin resistance (21–23), whereas monounsaturated fatty acids increase insulin sensitivity in diabetic patients (24, 25) and healthy subjects (21). However, the mechanisms by which enrichment with oleate favors insulin sensitivity are still unknown. The present study was designed to characterize the cellular mechanisms by which the two most common fatty acids, palmitate and oleate (26), exert their differential effects on fatty acid-induced impairment of insulin signaling and

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[4] The abbreviations used are: FFA, free fatty acids; PGC-1, peroxisome proliferator-activated receptor γ coactivator 1; NFκB, nuclear factor κB; PKA, cAMP-dependent protein kinase; PKCθ, protein kinase C θ; PPAR, peroxisome proliferator-activated receptor; DAG, diacylglycerol; TG, triglyceride; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; DGAT, acyl-coenzyme A:diacylglycerol acyltransferase.

inflammation in skeletal muscle cells and to find whether oleate prevents the deleterious effects of palmitate. We report that the different effects of these fatty acids are related to their ability to promote DAG accumulation. Thus, exposure to palmitate increased DAG levels and activated the PKCθ-NFκB pathway, resulting in enhanced secretion of IL-6 and down-regulation of PPARγ coactivator 1α (PGC-1α) and acyl-coenzyme A:diacylglycerol acyltransferase 2 (DGAT2), enzyme that controls the rate of triglyceride (TG) synthesis from DAG. In contrast, coincubation of palmitate-exposed cells with oleate reversed these changes by promoting TG accumulation and mitochondrial β-oxidation, thus preventing DAG synthesis and activation of the PKCθ-NFκB pathway. The different effects of these two fatty acids seem to be related to the ability of oleate to activate PPARα and protein kinase A (PKA).

EXPERIMENTAL PROCEDURES

Reagents—Wy-14,643, H89, and MK886 were obtained from Sigma and GW501516 from Biomol Research Labs Inc. (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 μg/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 FFA with FFA-free bovine serum albumin, using a method modified from that described by Chavez and Summers (27). Briefly, FFAs were dissolved in ethanol and diluted 1:100 in DMEM containing 2% (w/v) fatty acid-free bovine serum albumin. Myotubes were incubated for 16 h in serum-free DMEM containing 2% bovine serum albumin in either the presence (FFA-treated cells) or absence (control cells) of FFAs. Cells were then incubated with 100 nM insulin for 10 min. Following incubation, RNA was extracted from myotubes as described below. Culture supernatants were collected, and the secretion of IL-6 was assessed by ELISA (Amersham Biosciences).

Measurements of mRNA—Levels of mRNA were assessed by reverse transcription-polymerase chain reaction (RT-PCR), as previously described (28). 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: Il-6, 5′-TCCAGCCAGTTGCCTTCTTGG-3′ and 5′-TCTGACAGTGCATCATCGCTG-3′; Pgc-1α, 5′-CCCGTGGATGAAGACGGATTG-3′ and 5′-GTGGGTGTGGTTTGCTGCATG-3′; Cpt-I, 5′-TATGTGAGGATGCTGCTTCC-3′ and 5′-CTCGGAGAGCTAAGCTTGTC-3′; Dgat1, 5′-GCGACGGCTACTGGGATCTGA-3′ and 5′-CAGGCCAGCTGTAGGGGTCCT-3′ and 5′-GAGGCCAGCTGTAGGGGTCCT-3′; Dgat2, 5′-ATGCCTGGCAAGAACGCAGTC-3′ and 5′-CAGAGGAGAAGAGGCCTCGGC-3′ and Aprt (adenosyl phosphoribosyl transferase), 5′-GCCTCTTGGCCAGTCACCTGA-3′ and 5′-CCAGGCTCACACACTCCACCA-3′. Amplification of each gene yielded a single band of the expected size (Il-6; 229 bp; Pgc-1α: 228 bp, Cpt-I: 629 bp; Dgat1: 219 bp; Dgat2: 226 bp and Aprt: 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine non-saturating 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 (29). Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always given in relation to the expression of the control gene (Aprt).

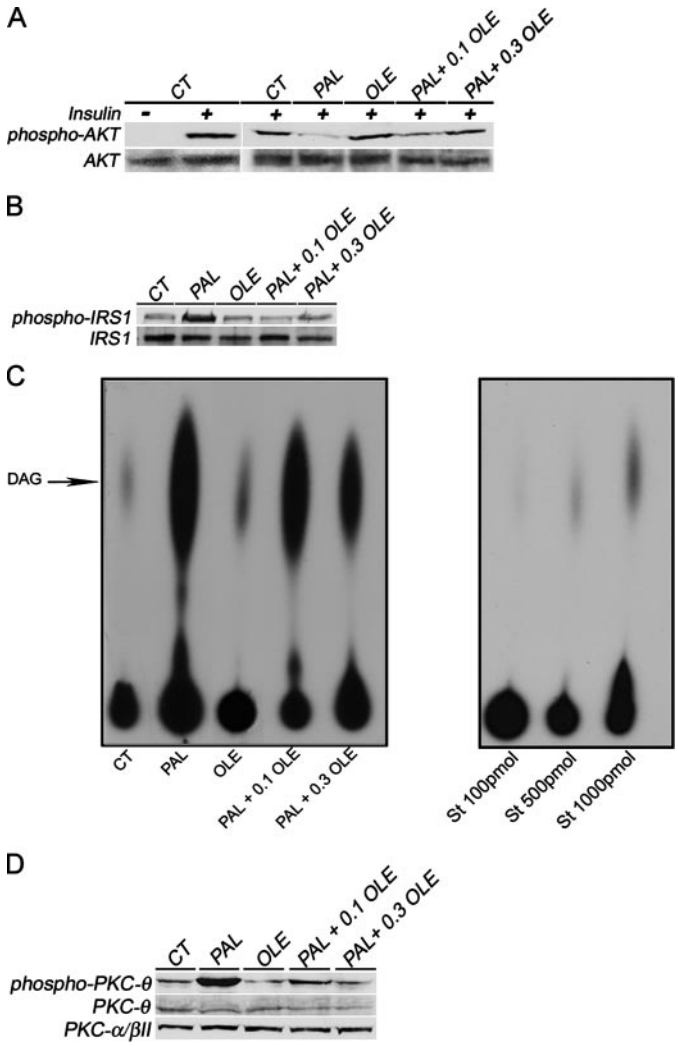


FIGURE 1. **Oleate prevents palmitate-induced impairment of insulin signaling pathway and DAG accumulation in skeletal muscle cells.** C2C12 myotubes were incubated for 16 h in the presence or absence of different fatty acids (0.5 mM palmitate, 0.5 mM oleate, or 0.5 mM palmitate supplemented with either 0.1 or 0.3 mM oleate). Cell lysates were assayed for Western blot analysis with antibodies against (*A*) total and phospho-Akt (Ser^{473}) and (*B*) total and phospho-IRS1 (Ser^{307}). The blot data are the result of three separate experiments. *C*, measurement of DAG levels. Lipid extracts were prepared and assayed for DAG as detailed under "Experimental Procedures." *D*, analysis of PKCθ levels. Total membrane protein extracts from C2C12 myotubes incubated in the presence or absence of fatty acids for 16 h were assayed for Western blot analysis with specific antibodies against total and phospho-PKCθ (Thr^{538}) and phospho-PKC-α/βII (Thr$^{638/641}$). The blot data are the result of three separate experiments. *CT*, control; *PAL*, palmitate; *OLE*, oleate; *St*, standard.

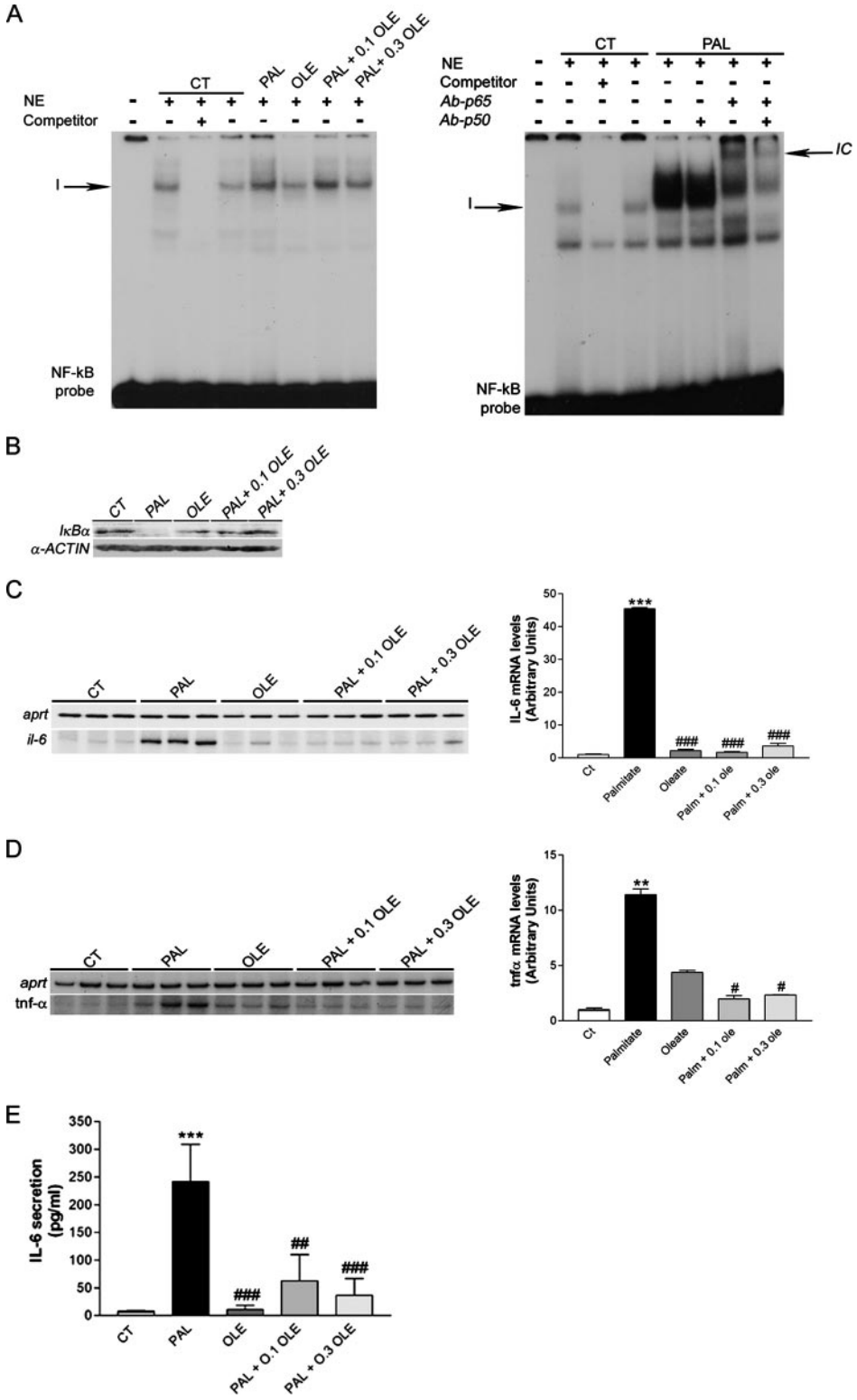


FIGURE 2. **Oleate prevents palmitate-induced NFκB activation and IL-6 expression and secretion in skeletal muscle cells.** C2C12 myotubes were incubated for 16 h in the presence or absence of different fatty acids (0.5 mM palmitate, 0.5 mM oleate, or 0.5 mM palmitate supplemented with either 0.1 or 0.3 mM oleate). *A*, autoradiograph of EMSA performed with a ^{32}P-labeled NFκB 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 NFκB is also shown. *B*, protein levels of IκBα. Protein extracts from C2C12 myotubes were assayed for Western blot analysis with IκBα and α-actin antibodies. The blot data are the result of three separate experiments. Analysis of the mRNA levels of Il-6 (*C*) and Tnf-α (*D*). 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram normalized to the APRT mRNA levels is shown. *E*, determination by ELISA of IL-6 secretion to the culture medium. Data are expressed as mean ± S.D. of six experiments. ***, $p < 0.001$ *versus* control; ##, $p < 0.01$; ###, $p < 0.001$ *versus* palmitate-treated cells.

Determination of Diacylglycerol Levels—Diacylglycerol and ceramide levels were determined by the diacylglycerol kinase method, as described elsewhere (28).

Incorporation of [^{14}C]FA into Lipid Fractions—Cells were incubated with [1-^{14}C]palmitic acid and/or [1-^{14}C]oleic acid for 16 h. The cell monolayers were then washed three times with phosphate-buffered saline, and the lipids were extracted twice with $CHCl_3$/MeOH (2:1) and 0.1 M KOH. After drying under nitrogen stream, the lipid extract was re-dissolved in chloroform-methanol (2:1) and separated on thin-layer chromatography (TLC), using hexane-diethyl ether-acetic acid (70:30:1). Plates were measured in a PhosphorImager (Bio-Rad). DAG and TG were identified by comparison with standards (Sigma) processed in parallel to the samples.

Isolation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extract isolation and EMSA were performed as described elsewhere (28).

Immunoblotting—To obtain total proteins, C2C12 myotubes were homogenized in cold lysis buffer (5 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5.4 μg/ml aprotinin). The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C. Protein concentration was measured by the Bradford method. Total and nuclear proteins (30 μg) 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 and phospho-PKCθ (Thr^{538}), PKC-α/βII (Thr$^{638/641}$) (Cell Signaling Technology Inc.), total (Santa Cruz Biotechnology) and phospho-Akt (Ser^{473}), total and phospho-IRS1 (Ser^{307}) (Cell Signaling), IκBα (Santa Cruz Biotechnology), PPARα, PPARβ/δ (provided by W. Wahli), and α-actin (Sigma). Detection was achieved using the EZ-ECL chemi-

luminescence detection kit (Biological Industries, Beit Haemek Ltd.). The equal loading of proteins was assessed by red phenol staining. The size of detected proteins was estimated using protein molecular mass standards (Invitrogen).

Statistical Analyses—Results are expressed as the mean ± S.D. of six separate experiments. Significant differences were established by one-way analysis of variance using the computer program GraphPad Instat (GraphPad Software V2.03) (GraphPad Software Inc., San Diego, CA). When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Differences were considered significant at $p < 0.05$.

RESULTS

Oleate Prevents Palmitate-induced Insulin Resistance and IL-6 Secretion by Inhibiting the DAG-PKCθ-NFκB Pathway—As exposure of skeletal muscle cells to the saturated fatty acid palmitate leads to both phosphorylation of IRS-1 on serine residues and inhibition of insulin-stimulated Akt phosphorylation, thereby attenuating insulin signaling (14), we first evaluated whether oleate prevented these effects. As expected, insulin stimulated Akt phosphorylation, whereas this process was inhibited by 0.5 mM palmitate (Fig. 1*A*). However, no changes were observed when cells were exposed to the same concentration of oleate. Interestingly, co-incubation of palmitate-exposed cells with increasing concentrations of oleate (0.1 and 0.3 mM) prevented this effect in a concentration-dependent manner. Likewise, palmitate exposure enhanced Ser^{307} phosphorylation of IRS-1 (Fig. 1*B*), whereas exposure to oleate did not, and co-incubation with oleate reversed the effect of the saturated fatty acid.

Because phosphorylation of IRS1 on Ser^{307} after exposure of skeletal muscle cells to palmitate is mediated by DAG-mediated activation of PKCθ, we next assessed whether oleate prevented these changes. As previously reported (27), palmitate treatment led to enhanced DAG levels (Fig. 1*C*), whereas cells exposed to oleate showed DAG amounts similar to those observed in control cells. When palmitate-exposed cells were co-incubated with oleate, a concentration-dependent reduction was observed in the content of this complex lipid. Consistent with the changes in the levels of DAG, palmitate induced phosphorylation of PKCθ, unlike cells exposed to bovine serum albumin (Fig. 1*D*). However, oleate did not affect phospho-PKCθ levels, and co-supplementation of palmitate-treated cells with oleate prevented the phosphorylation of this PKC isoform.

Palmitate-induced inflammation in skeletal muscle cells occurs through a mechanism involving NFκB activation by PKCθ (30). To determine whether oleate prevented palmitate-induced inflammation, we measured NFκB binding activity by EMSA. NFκB formed one complex with nuclear proteins (Fig. 2*A*). Specificity of the DNA binding complexes was assessed in competition experiments by adding an excess of unlabeled NFκB oligonucleotide. NFκB binding activity increased in nuclear extracts from palmitate-treated cells, whereas in those from oleate-exposed cells, the binding activity was similar to that observed in control cells. Notably, co-incubation of palmitate-treated cells with oleate reduced NFκB binding activity, especially at 0.3 mM. Addition of antibody against the p65 subunit of NFκB supershifted the complex, indicating that this band mainly consisted of this subunit. Further, the p50 subunit of NFκB was also present in this complex, although in minor quantities. No changes were observed in the DNA binding of nuclear proteins from control and palmitate-treated cells to an

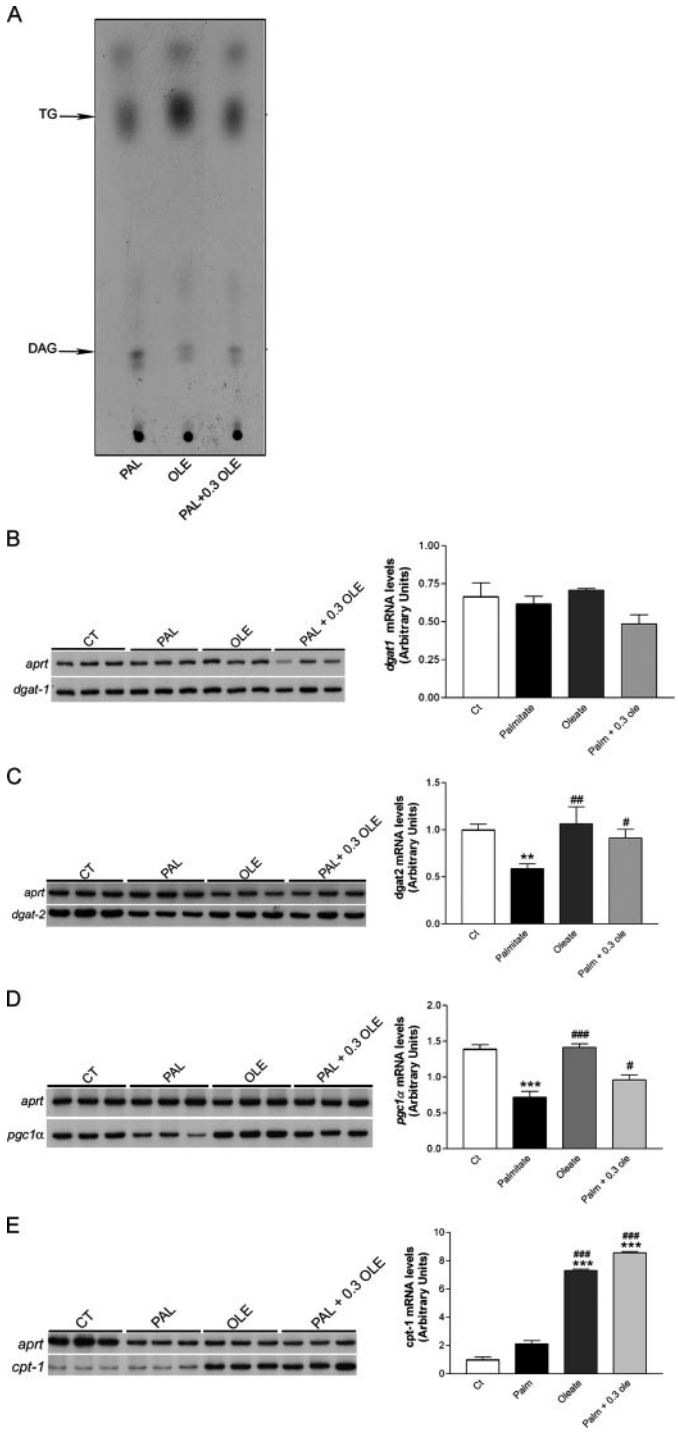


FIGURE 3. **Palmitate and oleate differ in their incorporation into complex lipids and in their effects on the expression of genes involved in fatty acid metabolism in skeletal muscle cells.** C2C12 myotubes were incubated for 16 h in the presence or absence of different fatty acids. *A*, incorporation of [^{14}C]palmitate, [^{14}C]oleate, and a mixture of both fatty acids into DAG and TG. Lipid extracts were prepared and assayed as detailed under "Experimental Procedures." Analysis of the mRNA levels of *Dgat1* (*B*), *Dgat2* (*C*), *Pgc-1α* (*D*), and *Cpt-I* (*E*). 0.5 μg 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 mean ± S.D. of six experiments. **, $p < 0.01$; ***, $p < 0.001$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ *versus* palmitate-treated cells.

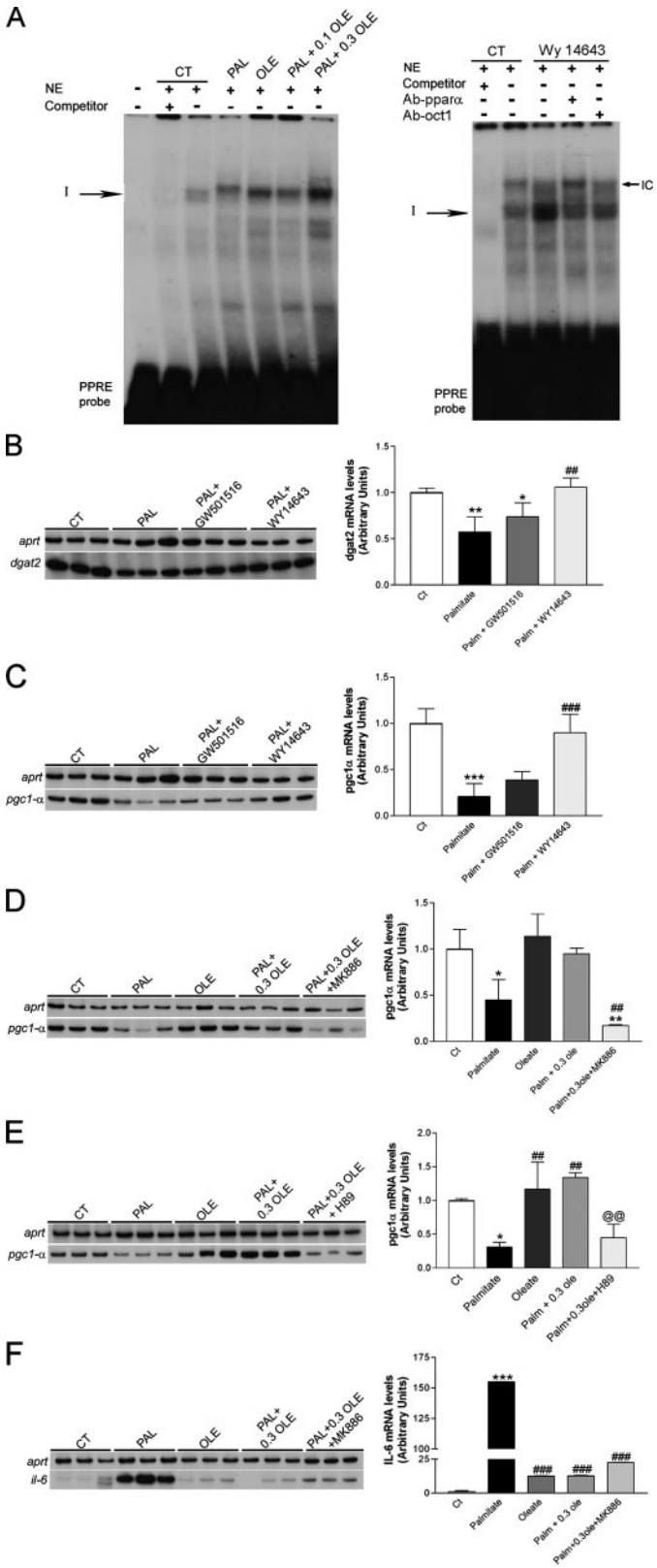


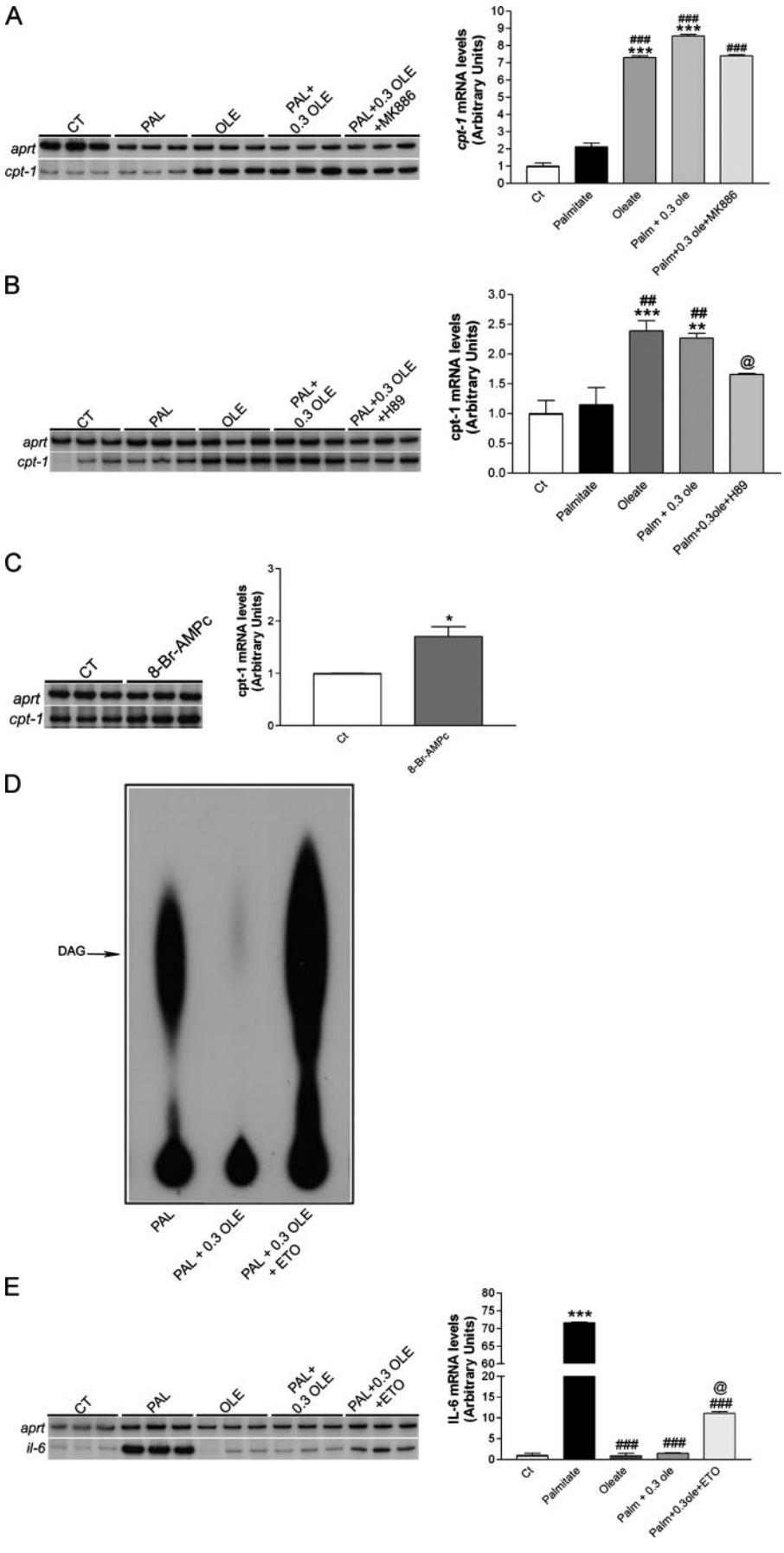
FIGURE 4. **Oleate, but not palmitate, increases PPAR DNA binding activity in skeletal muscle cells.** *A*, autoradiograph of EMSA performed with a ^{32}P-labeled PPRE nucleotide and nuclear extracts (*NE*) from C2C12 myotubes incubated for 16 h with different fatty acids. Two specific complexes (I to II), based on competition with a molar excess of unlabeled probe, are shown. The supershift immune complex (*IC*) obtained by incubating NE with an antibody directed against PPARα and β/δ is also shown. The autoradiograph data are the result of three separate experiments. mRNA levels of Dgat2 (*B*), Pgc-1α (*C*, *D*, *E*), and Il-6 (*F*) were analyzed in skeletal muscle cells incubated for 16 h with different fatty acids in the presence or absence of the PPARα antagonist MK886 (10 μM), the PPARα agonist Wy-14,643 (10 μM), the PPARβ/δ agonist (1 μM), or the PKA inhibitor H89 (10 μM). 0.5 μg 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 mean ± S.D. of six experiments. **, $p < 0.01$; ***, $p < 0.001$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ *versus* palmitate-treated cells; @@, $p < 0.01$ *versus* palmitate plus oleate exposed cells.

Oct-1 probe, indicating that the changes observed for the NFκB probe were specific (data not shown). NFκB is located in the cytosol bound to inhibitor κB (IκB), and palmitate supplementation causes phosphorylation and degradation of IκBα, thus liberating and activating NFκB (18, 19). When we examined the effects of the fatty acids on the protein levels of IκBα (Fig. 2*B*), we observed that palmitate caused a fall in its levels, whereas supplementation with oleate did not. Finally, in cells co-incubated with palmitate and oleate, a recovery was observed in the levels of this NFκB inhibitor, which is consistent with the reduction in NFκB binding activity.

Palmitate-induced activation of NFκB in skeletal muscle cells results in enhanced expression and secretion of pro-inflammatory cytokines, such IL-6, that contribute to the development of insulin resistance (18, 19). Because co-incubation of palmitate-treated cells with oleate prevented activation of the DAG-PKCθ-NFκB pathway, we next explored whether oleate prevented palmitate-induced Il-6 expression and secretion. As Fig. 2*C* shows, palmitate strongly induced Il-6 mRNA levels, whereas supplementation with oleate showed expression levels similar to those observed in control cells. In addition, oleate supplementation prevented the increase in Il-6 expression caused by palmitate. The same pattern of expression was observed for TNF-α (Fig. 2*D*). Consistent with the changes in mRNA levels of *Il-6*, incubation with palmitate led to a 31-fold induction in the levels of IL-6 protein secreted into the culture media (control 7.7 ± 1.0 *versus* palmitate 241 ± 68 pg/ml, $p < 0.001$, Fig. 2*E*). Oleate did not significantly modify secretion of this interleukin (control 7.7 ± 1.0 *versus* oleate 10.9 ± 3.3 pg/ml), whereas oleate co-supplementation prevented the increase in IL-6 secretion caused by palmitate (74% reduction at 0.1 mM and 85% at 0.3 mM, $p < 0.01$ and $p < 0.001$ *versus* palmitate-treated cells, respectively). Overall, these findings indicate that oleate reverses palmitate-induced inflammation in skeletal muscle cells by preventing activation of the DAG-PKCθ-NFκB pathway.

Oleate Reduces Palmitate-mediated DAG Accumulation by Promoting Triglyceride Synthesis and Fatty Acid Oxidation—Because DAG accumulation in skeletal muscle cells exposed to palmitate is the first step leading to palmitate-induced insulin resistance and inflammation, we explored the potential mechanisms by which oleate prevents the accumulation of this complex lipid. Incubation of cells exposed to palmitate and oleate with a diacylglycerol kinase inhibitor did not result in changes in Il-6 mRNA levels, suggesting that oleate did not affect DAG degradation (data not shown). On the other hand, it has been reported that palmitate and oleate are differentially utilized by myotubes (18). Thus, whereas saturated fatty acid seems to be incorporated into TG and DAG, monounsaturated fatty acid is

channeled toward TG (31). When we analyzed the incorporation of palmitate, oleate, and the mixture of these two fatty acids into DAG and TG (Fig. 3*A*), we found that the saturated fatty acid was mainly incorporated into DAG and TG (the TG:DAG ratio expressed as percentage of total radioactivity was 16:1), the monounsaturated fatty acid was incorporated mainly into TG (TG/DAG ratio was 273:1, 17 times greater induction than palmitate), whereas cells exposed to both palmitate and oleate were incorporated more into TG (TG/DAG ratio was 88:1, 5.5 times greater than cells exposed only to palmitate). These differences in the channeling of fatty acids into TG and DAG may be caused by changes either in the expression of genes involved in TG synthesis or in fatty acid oxidation. Given that DGAT is the enzyme that catalyzes the final reaction in the synthesis of TG from DAG, we assessed the effects of palmitate and oleate on the expression of this gene. Fatty acids did not affect the expression of *Dgat1* (Fig. 3*B*). However, Dgat2 mRNA levels were lower in cells exposed to palmitate (36% reduction, $p < 0.01$) (Fig. 3*C*), whereas oleate did not affect the expression of this gene and supplementation of palmitate-exposed cells with oleate prevented the effect of the saturated fatty acid. This finding suggests that the channeling of palmitate into DAG may be the result of a reduction in the expression of *Dgat2*, whereas supplementation with oleate restores both the expression of this gene and TG synthesis. We also evaluated whether additional mechanisms may account for the effects of oleate on DAG levels. Because up-regulation in the expression of genes involved in the oxidation of fatty acids may reduce their availability for DAG synthesis (32), we focused on the expression of genes, such as *Pgc-1α* and *Cpt-I*. The former is a co-activator of peroxisome proliferator-activated receptors (PPARs) involved in the control of fatty acid oxidation (33), whereas the second allows the



transport of fatty acids into mitochondria for β-oxidation (34). Consistent with previous studies (28), we observed a reduction in the mRNA levels of *Pgc-1α* in cells exposed to palmitate, whereas oleate did not affect the expression of this gene (Fig. 3*D*). When cells exposed to palmitate were co-supplemented with oleate, no changes were observed in the levels of *Pgc-1α*. Nor did palmitate treatment significantly affect Cpt-I mRNA levels. However, cells exposed to oleate and those treated with palmitate co-supplemented with oleate showed a 7-times greater induction ($p < 0.001$) in the transcript levels of *Cpt-I* (Fig. 3*E*).

Oleate Increases the DNA Binding Activity of PPAR—The expression of both *Pgc-1α* (35, 36) and *Cpt-I* (34) is regulated by PPARs, suggesting that oleate may affect the activity of these transcription factors. EMSA were performed to examine the interaction of PPARs with its *cis*-regulatory element using a ^{32}P-labeled PPRE (peroxisome proliferator response element) probe and nuclear extracts from C2C12 myotubes exposed to different fatty acids. The PPRE probe formed a single main complex with nuclear proteins (Fig. 4*A*). Competition studies performed with a molar excess of unlabeled probe revealed that this complex represented a specific PPRE-protein interaction. Nuclear extracts from skeletal muscle cells incubated with 20 μM Wy-14,643, a selective PPARα activator at this concentration (37), were used as a positive control to demonstrate that enhanced binding activity was due to increased PPARα activity. Incubation of nuclear extracts with an antibody against PPARα supershifted the complex, indicating that this band contained this nuclear receptor. In contrast, an unrelated antibody, directed to Oct-1 protein, did not supershift the complex. In nuclear extracts from cells exposed to oleate and palmitate plus 0.3 mM oleate, a significant increase was observed in the binding activity of complex I than in nuclear extracts from cells exposed to palmitate.

Oleate Affects the Expression of Genes Involved in TG Synthesis and Fatty Acid Oxidation through Mechanisms Involving PPARα and PKA—We next explored whether enhanced PPAR activation by oleate was responsible for the effects of this fatty acid on DAG amounts using PPAR activators and antagonists. When skeletal muscle cells were co-incubated with palmitate and the PPARα activator Wy-14,643, reduction in the mRNA levels of *Dgat2* was prevented (Fig. 4*B*), which is in agreement with the reported regulation of *Dgat2* by PPARα (38, 39). This finding suggests that the increase in PPARα activation caused by oleate may prevent the fall in the expression of *Dgat2* and, as a result, in the synthesis of TG. Like *Dgat2*, in the presence of the PPARα activator Wy-14,643, the reduction in the expression of *Pgc-1α* caused by palmitate was prevented, whereas in the presence of the PPARβ/δ activator there was a slight increase that did not reach statistical significance (Fig. 4*C*). Interestingly, in the presence of the PPARα antagonist MK886, the effect of oleate on *Pgc-1α* mRNA levels in cells exposed to palmitate was abolished, clearly demonstrating that *Pgc-1α* expression was up-regulated by oleate through a PPARα-dependent mechanism (Fig. 4*D*). Because it is has been reported that PKA activation increases PPARα and PPARβ/δ DNA binding activity (40–42) and that oleate may activate this kinase (43), we explored whether this mechanism was involved in the effects of oleate. In the presence of the PKA inhibitor H89, the effect of oleate on *Pgc-1α* in palmitate-exposed cells was also abolished (Fig. 4*E*), suggesting that PKA activation was involved in the effects of the monounsaturated fatty acid. Finally, the involvement of PPARα activation in the effects of oleate was demonstrated in those cells co-incubated with palmitate, oleate, and MK886 (Fig. 4*F*). In the presence of this PPARα antagonist, the expression of *Il-6* was partially recovered indicating that PPARα activation by oleate contributes to prevent palmitate-induced expression of this cytokine. Unlike the effects of oleate on Pgc-1α, its effects on Cpt-I mRNA levels were not abolished by the PPARα antagonist MK886 (Fig. 5*A*), whereas co-incubation of cells with the mixture of palmitate, oleate and H89 reversed the effect of the monounsaturated fatty acid (Fig. 5*B*), which corroborates the reported regulation of Cpt-I by PKA (44). Similar to H89, the PKA inhibitor KT5720 (1 μM) significantly abolished the effect of palmitate plus oleate on Cpt-I up-regulation (data not shown), and the PKA activator 8-Br-AMPc significantly enhanced Cpt-I mRNA levels (Fig. 5*C*). Finally, to demonstrate the contribution of the increase in fatty acid oxidation with the effects of oleate, palmitate-exposed cells co-supplemented with oleate were treated with the CPT-I inhibitor etomoxir. In the presence of this inhibitor of fatty acid oxidation, the effects of oleate on DAG amounts and on *Il-6* expression were partially abolished (Fig. 5, *C* and *D*), indicating that the increase in fatty acid oxidation caused by oleate contributes, at least in part, to prevent the activation of the DAG-PKCθ-NFκB pathway.

DISCUSSION

Skeletal muscle insulin resistance correlates more strongly with intramuscular lipid levels than with any other factor, including BMI or percentage body fat (45, 46). Furthermore, intramuscular lipid accumulation may lead to inflammation in skeletal muscle, a process that has been linked to the development of type 2 diabetes (14). Despite these data, the mechanisms by which intramuscular lipid accumulation results in inflammation and insulin resistance in skeletal muscle are not well understood. Interestingly, although high fat diets are known to affect glucose metabolism, their contribution to insulin resistance depends on dietary fatty acids. Thus, whereas saturated fatty acids promote insulin resistance (21–23), the monounsaturated oleic acid improves insulin sensitivity (24, 25, 47). These data have led to suggestions that the dietary

FIGURE 5. **Up-regulation of *Cpt-I* expression by oleate is mediated by PKA and is necessary to prevent palmitate-mediated induction of *Il-6*.** mRNA levels of Cpt-I (*A* and *B*) were analyzed in skeletal muscle cells incubated for 16 h with different fatty acids in the presence or absence of the PPARα antagonist MK886 (10 μM) and the PKA inhibitor H89 (10 μM). Cpt-I mRNA levels were analyzed in skeletal muscle cells incubated for 16 h in the presence or absence of 1 mM 8-Br-AMPc (*C*). DAG levels (*D*) and Il-6 mRNA levels (*E*) were analyzed in skeletal muscle cells incubated for 16 h with different fatty acids in the presence or absence of the CPT-I inhibitor etomoxir (40 μM). 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of six experiments. ***, $p < 0.001$ *versus* control; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ *versus* palmitate-treated cells; @, $p < 0.05$ *versus* palmitate plus oleate exposed cells.

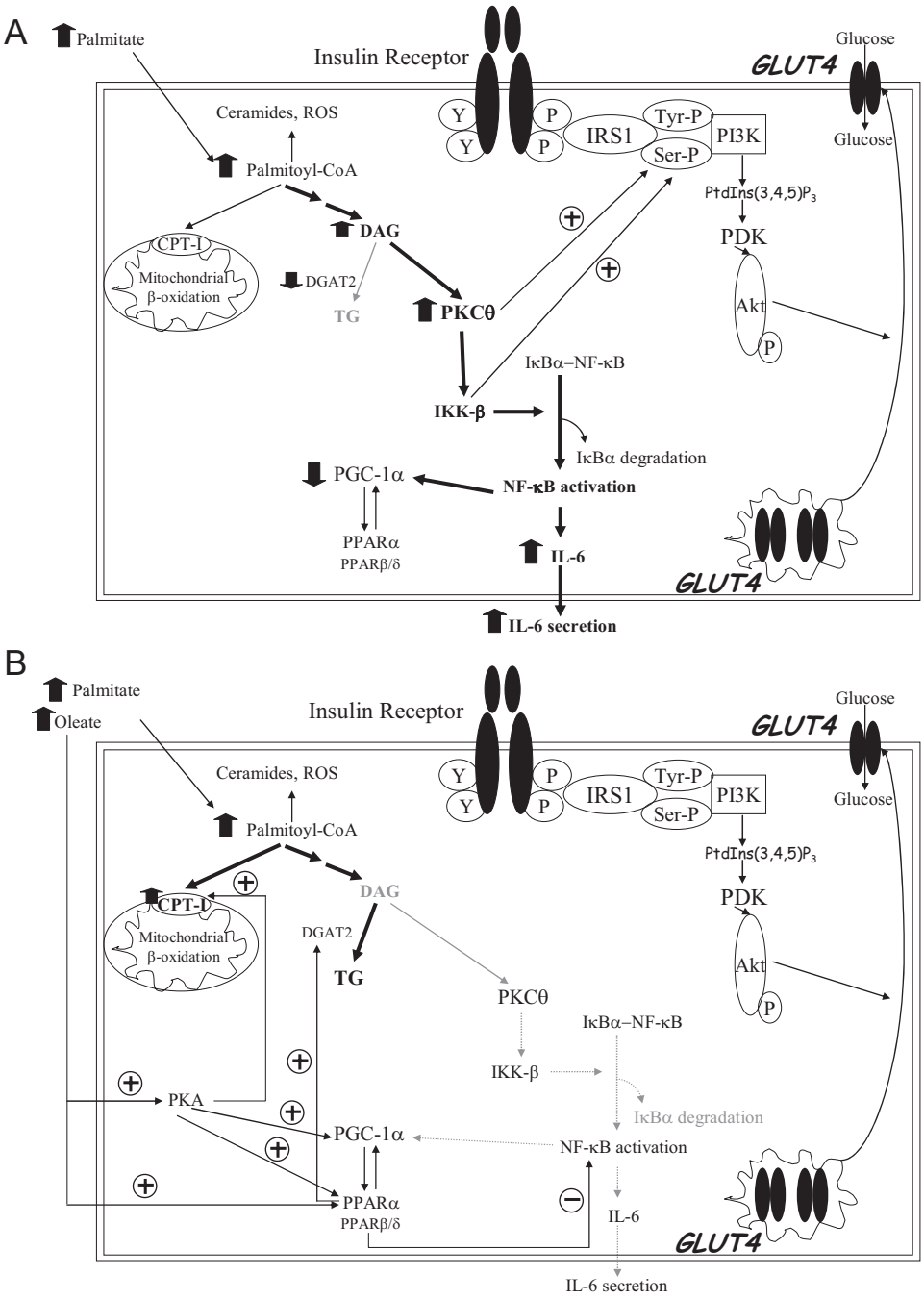


FIGURE 6. **Potential mechanisms by which oleate prevents palmitate-induced impairment of insulin signaling and inflammation in skeletal muscle cells.** *A*, saturated fatty acid palmitate induces insulin resistance and inflammation in skeletal muscle cells by promoting DAG accumulation, which in turn activates PKCθ and NFκB, leading to phosphorylation of IRS-1 on Ser^{307} and inhibition of insulin-stimulated Akt phosphorylation and IL-6 secretion. Under these conditions, palmitate is poorly incorporated into the cellular triglyceride pool by a mechanism that may involve a reduction in the expression of *Dgat2*, the enzyme that controls the rate of TG synthesis from DAG. The monounsaturated fatty acid oleate does not affect the expression of *Dgat2* and is mainly incorporated into TG. As a result, this fatty acid does not activate the DAG-PKCθ-NFκB pathway. *B*, presence of oleate prevents palmitate-induced insulin resistance and inflammation by restoring *Dgat2* expression and TG synthesis and by up-regulating *Cpt-1* and *Pgc-1*α expression. Both mechanisms reduce the availability of fatty acids for incorporation into DAG. These effects of oleate are the result of enhanced PPARα and PKA activation.

intake of oleic acid should be increased in the management of type 2 diabetes mellitus (48). However, the mechanisms by which oleate may improve insulin resistance are unknown. Here we report that oleate prevents palmitate-induced insulin resistance and inflammation in skeletal muscle cells. Exposure of skeletal muscle cells to palmitate increased DAG levels, which in turn activates the PKCθ-NFκB pathway, leading to both phosphorylation of IRS-1 in serine residues and inhibition of insulin-stimulated Akt phosphorylation, thereby attenuating insulin signaling (Fig. 6). Further, as a result of NFκB activation, a large increase was observed in *Il-6* expression and secretion. In contrast, oleate did not activate this pathway, and when cells exposed to palmitate were supplemented with oleate, a concentration-dependent reduction was observed in the activation of this pathway. The key point in the activation of this pathway seems to be accumulation of DAG, because this accumulation allows activation of PKCθ that could lead to insulin resistance by phosphorylating IRS-1 or by activating the pro-inflammatory transcription factor NFκB. In fact, it has been reported that PKCθ mice are protected from fat-induced insulin resistance (49). Thus, DAG is accumulated in skeletal cells when exposed to palmitate, but not when exposed to oleic acid, which corroborates with previous studies (27, 31, 50). The reasons for the different channeling toward DAG of palmitate and oleate were unknown. In this study we provide a potential explanation for the different behavior of these fatty acids. Thus, palmitate exposure led to a fall in the expression of *Dgat2*, whereas oleate did not. *Dgat2* is essential for TG synthesis, because mice with a disruption of this gene have severely reduced TG content in their tissues (51). Further, it has been reported that the close association of stearoyl-CoA desaturase 1 and Dgat2 increases the efficiency of palmitate conversion to oleate, which is then preferentially used for TG synthesis (52). Notably, when palmitate-exposed cells were incubated with oleate, *Dgat2* expression was not affected, which is consistent with the shift in the incorporation of palmitate from DAG to TG. In line with previous studies showing that PPARα up-regulates *Dgat2* expression in skeletal muscle and heart (38, 39), the decrease in the expression levels of *Dgat2* caused by palmitate exposure was prevented in the

presence of PPARα activators, suggesting that oleate may act through a similar mechanism. The findings of our study support this possibility, because oleate exposure led to enhanced PPAR DNA-binding activity. Overall, the results of this study indicate that palmitate is mainly incorporated into DAG because its incorporation into TG is reduced by the fall in the expression of *Dgat2*. However, oleate is mainly incorporated into TG and when palmitate-exposed cells are co-supplemented with oleate the expression of *Dgat2* is not reduced and palmitate is then diverted toward TG instead of DAG. It is worth saying that increased TG accumulation in obese and type 2 diabetic muscle fibers *in vivo* has been considered an adaptive event (50), which initially is not itself toxic. Rather, TG accumulation reduces the formation of additional lipid species with more deleterious effects and thus may serve to prevent lipotoxicity. In line with this hypothesis, it has been reported that TG accumulation protects against fatty acid-induced lipotoxicity (53). However, when lipid availability is prolonged, lipotoxicity may occur when cellular capacity for TG accumulation is exceeded or when TG is hydrolyzed. In addition, two recent studies have elegantly demonstrated that either acute exercise or overexpression of *Dgat1* leads to increased triglyceride synthesis in skeletal muscle, preventing fatty acid-induced insulin resistance and inflammation (54, 55). In concordance with the results of our study, the protection against insulin resistance and inflammation reported in these studies was associated with attenuated activation of DAG-responsive PKCs and enhanced IκBα abundance. Therefore, oleate supplementation and physical activity prevent fatty acid-induced inflammation and insulin resistance through similar mechanisms.

Additional mechanisms may also contribute to the effects of oleate on DAG levels. Our data demonstrate that the effect of oleate on the DAG-PKCθ-NFκB pathway depends on enhanced fatty acid oxidation, because in the presence of etomoxir, an inhibitor of CPT-I, DAG and *Il-6* levels were enhanced. Oleate, unlike palmitate, led to enhanced expression of *Cpt-I*, which catalyzes the entry of long-chain fatty acids into the mitochondrial matrix, through a mechanism that was not dependent on PPARα, because in the presence of the antagonist MK886 it was not reversed. However, the up-regulation of *Cpt-I* caused by oleate was prevented in the presence of the PKA inhibitor H89. As previously reported (44, 56), PKA activation may lead directly to enhanced *Cpt-I* expression and activity. Further, because PKA activators increase ligand-activated and basal activity of PPARα and PPARβ/δ (40, 41, 57) through a mechanism that seems to stabilize binding of the liganded PPAR to DNA, the oleate-mediated activation of *Cpt-I* may be secondary to the increase in the PPAR DNA binding activity caused by this monounsaturated fatty acid. *Pgc-1α*, a transcriptional co-activator promoting oxidative capacity in skeletal muscle (33), is also under the control of PPARs and PKA (35, 36). This is consistent with the abolition of the effects of oleate on *Pgc-1α* expression in palmitate-exposed cells in the presence of MK886 and H89. Further, we have previously reported that palmitate down-regulates *Pgc-1α* in skeletal muscle cells through a NFκB-dependent mechanism (28). Given that PPARα activators prevent NFκB activation (58, 59), it is likely that the reduction in NFκB activity caused by oleate could also be involved in the effects of this fatty acid on *Pgc-1α* expression.

Overall, these findings indicate that oleate may prevent the deleterious effects of palmitate on skeletal muscle cells by increasing *Cpt-I* expression through a PKA-dependent mechanism. The involvement of CPT-I in the changes caused by oleate were confirmed by the use of the CPT-I inhibitor, etomoxir. It should be noted that these findings are consistent with previous studies reporting that overexpression of *Cpt-I* contributes to protecting skeletal muscle cells from fatty acid-induced insulin resistance (32, 60).

In addition to the reduction of DAG accumulation, additional mechanisms may also contribute to the prevention of palmitate-induced inflammation and insulin resistance by oleate. For instance, exposure of skeletal muscle cells to palmitate leads to the accumulation of ceramides, which are key players in the development of insulin resistance. In fact, a recent study clearly demonstrated the involvement of increased ceramide synthesis in response to excessive saturated fatty acids in the development of insulin resistance by inhibiting Akt phosphorylation and activation (61). Interestingly, Pickersgill *et al.* (11) recently suggested that oleate may also prevent palmitate-induced ceramide synthesis. However, increased ceramide synthesis seems not to be involved in palmitate-induced inflammation because inhibition of ceramide synthesis fails to prevent lipid induction of the inflammatory cytokine *Il-6*, suggesting that ceramides do not affect the PKCθ-NFκB pathway. This is in agreement with a previous study of our group showing that inhibition of palmitate-induced ceramide synthesis did not prevent the increase in *Il-6* expression (19). Therefore, although the involvement of increased ceramide levels in the development of insulin resistance has been clearly demonstrated, the contribution of this lipid mediator to fatty acid-induced inflammation in skeletal muscle cells is less clear. Moreover, it remains to be studied whether oleate affects the activity of JNK, which is involved in fatty acid-induced insulin resistance and inflammation (55).

In summary, the results reported here demonstrate that oleate protects against palmitate-induced inflammation and insulin resistance in skeletal muscle cells by promoting TG accumulation and mitochondrial β-oxidation, thus preventing DAG synthesis and activation of the PKCθ-NFκB.

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