

# Defining the role of DAG, mitochondrial function, and lipid deposition in palmitate-induced proinflammatory signaling and its counter-modulation by palmitoleate<sup>S</sup>

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**Abstract** Chronic exposure of skeletal muscle to saturated fatty acids, such as palmitate (C16:0), enhances proinflammatory IKK-NFκB signaling by a mechanism involving the MAP kinase (Raf-MEK-ERK) pathway. Raf activation can be induced by its dissociation from the Raf-kinase inhibitor protein (RKIP) by diacylglycerol (DAG)-sensitive protein kinase C (PKC). However, whether these molecules mediate the proinflammatory action of palmitate, an important precursor for DAG synthesis, is currently unknown. Here, involvement of DAG-sensitive PKCs, RKIP, and the structurally related monounsaturated fatty acid palmitoleate (C16:1) on proinflammatory signaling are investigated. Palmitate, but not palmitoleate, induced phosphorylation/activation of the MEK-ERK-IKK axis and proinflammatory cytokine (IL-6, CINC-1) expression. Palmitate increased intramyocellular DAG and invoked PKC-dependent RKIP<sup>Ser153</sup> phosphorylation, resulting in RKIP-Raf1 dissociation and MEK-ERK signaling. These responses were mimicked by PMA, a DAG mimetic and PKC activator. However, while pharmacological inhibition of PKC suppressed PMA-induced activation of MEK-ERK-IKK signaling, activation by palmitate was upheld, suggesting that DAG-sensitive PKC and RKIP were dispensable for palmitate's proinflammatory action. Strikingly, the proinflammatory effect of palmitate was potently repressed by palmitoleate. This repression was not due to reduced palmitate uptake but linked to increased neutral lipid storage and enhanced cellular oxidative capacity brought about by palmitoleate's ability to restrain palmitate-induced mitochondrial dysfunction.—Macrae, K., C. Stretton, C. Lipina, A. Blachnio-Zabielska, M. Baranowski, J. Gorski, A. Marley, and H. S. Hundal. **Defining the role of DAG, mitochondrial function, and lipid deposition in palmitate-induced proinflammatory signaling and its counter-modulation by palmitoleate.** *J. Lipid Res.* 2013. 54: 2366–2378.

**Supplementary key words** saturated fatty acid • monounsaturated fatty acid • diacylglycerol • nuclear factor kappa-B • interleukin-6 • cytokine

Chronic, low-grade inflammation is a characteristic feature of obesity (1) that involves enhanced activation of proinflammatory signaling in tissues, such as skeletal muscle, and is associated with increased expression and secretion of cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) (2, 3). An important factor contributing to the enhanced inflammatory drive in skeletal muscle is an increase in the circulating concentration of fatty acids, in particular saturated fatty acids (SFA), such as palmitate, which are notably elevated during obesity (4). Increases in SFAs are thought to be of significance given that numerous studies have demonstrated that these possess a greater pathogenic potential than mono- or polyunsaturated fatty acids (MUFA and PUFA) (5–8), which, in contrast, not only confer beneficial metabolic effects (9) but also possess the capacity to counter some of the deleterious effects associated with SFA oversupply (6, 10). Indeed, palmitoleate, a MUFA, released from white adipose tissue has been coined a “lipokine” based on its ability to promote systemic metabolic homeostasis (11). How fatty acids exert their differential effects upon signaling pathways

Abbreviations: CINC-1, cytokine-induced neutrophil chemoattractant-1; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; IκB, inhibitor of NFκB; IKK, inhibitor of NFκB kinase; IL-6, interleukin 6; MAPK, mitogen-activated protein kinase; MEK, mitogen/ERK-activated protein kinase; NFκB, nuclear factor kappa-B; OCR, oxygen consumption rate; PA, palmitate; PKB, protein kinase B; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PO, palmitoleate; RKIP, raf-kinase inhibitor protein; TLR, Toll-like receptor; TNFα, tumor necrosis factor α.

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<sup>S</sup>The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of four figures.

This work was supported by a Biotechnology and Biological Sciences Research Council (BBSRC)/Industrial (AstraZeneca) CASE Quota Award, by BBSRC, and by Diabetes UK.

Manuscript received 19 February 2013 and in revised form 3 July 2013.

Published, JLR Papers in Press, July 6, 2013

DOI 10.1194/jlr.M036996

that impact upon cellular metabolism and inflammation remains unclear, but such effects may be attributable, in part, to the observation that SFAs are less well oxidized than MUFAs and PUFAs (12, 13). Consequently, sustained exposure of muscle cells to elevated SFAs will result in greater intramyocellular conversion to fatty-acid-derived metabolites, such as diacylglycerol (DAG) and ceramide, whose accumulation is known to dysregulate proximal signaling events downstream of receptor tyrosine kinases (14–17) and induce expression of proinflammatory genes that are regulated in a nuclear factor kappa-B (NF $\kappa$ B)-dependent manner (2, 18, 19).

There has been considerable interest in delineating how palmitate or its lipid derivatives trigger inflammatory signaling in skeletal muscle. Toll-like receptors (TLR) of the innate immune system, in particular TLR2 and TLR4 (for which SFAs may be ligands), have been implicated in the activation of NF $\kappa$ B (20, 21), but there is also considerable evidence showing that members of the protein kinase C (PKC) (22, 23) and MAP kinase families (7, 18) can promote SFA-induced NF $\kappa$ B signaling independently of TLR involvement in skeletal muscle cells. We recently reported that palmitate induces activation of JNK, p38 MAPK, and MEK-ERK signaling but that only pharmacological inhibition of the MEK-ERK pathway attenuated SFA-induced NF $\kappa$ B signaling and IL-6 expression in L6 skeletal muscle cells (18). The activation of the classical MAP kinase (ERK) pathway by palmitate is unlikely to be initiated by TLRs in these muscle cells as it does not manifest for at least 6–8 h (18), whereas TLR-induced MAP kinase signaling is typically initiated within minutes of receptor activation in other tissues (24). The lag period of 6–8 h that we observe with respect to SFA-induced activation of the MEK-ERK-NF $\kappa$ B signaling axis in muscle cells may reflect the time required to accumulate palmitate-derived lipid intermediates to a threshold that promotes activation of molecules that subsequently stimulate the classical MAP kinase pathway. One family of molecules that may respond in this manner are the novel and conventional PKC isoforms, which can be activated by increases in intracellular DAG occurring in response to palmitate oversupply (25). PKCs have been linked to activation of MEK-ERK signaling via their ability to modulate the activity of the serine/threonine kinase Raf, which lies immediately upstream of MEK (26, 27). Modulation of Raf relies, in part, upon regulating its association with raf kinase inhibitory protein (RKIP), which when bound to Raf, suppresses Raf-mediated phosphorylation/activation of MEK (28, 29). RKIP is a physiological target for PKCs, and phosphorylation of RKIP on Ser<sup>153</sup> induces its dissociation from Raf thereby enabling increased phosphorylation/activation of MEK and consequently that of ERK (30). However, it remains currently unknown whether DAG generated in response to palmitate overprovision helps drive MEK-ERK-NF $\kappa$ B signaling in response to palmitate in muscle cells, and if so, whether DAG-sensitive PKCs and RKIP are crucial regulators within this process and whether they are subject to counter-modulation by MUFAs, such as palmitoleate. Addressing these questions was the over-arching objective of the studies presented here.

## Materials

$\alpha$ -Minimal Essential Medium ( $\alpha$ -MEM), fetal bovine serum (FBS), Trypsin/EDTA solution, and penicillin/streptomycin solution were purchased from Life Technologies (Paisley, UK). Other reagent-grade chemicals were purchased from Sigma-Aldrich (Poole, UK), including palmitate, palmitoleate, lauric acid, oleate, stearic acid, dimethyl sulphoxide (DMSO), etomoxir, and antibodies to  $\beta$ -actin and GAPDH. Fraction V fatty acid-free bovine serum albumin (BSA) and complete protein phosphatase inhibitor tablets were purchased from Boehringer-Roche Diagnostics (Basel, Switzerland). [<sup>3</sup>H]palmitate (0.5 mCi/ml) was from Perkin Elmer UK. G66976 and G66983 were from Tocris Bioscience (Bristol, UK), and phorbol 12-myristate 13-acetate (PMA) was from Ascent Scientific (Bristol, UK). Triglyceride assay kit was purchased from Sigma-Aldrich. Antibodies to IKK $\alpha$ / $\beta$  phospho-Ser<sup>180/181</sup>, ERK1/2, ERK 1/2 phospho-Thr<sup>202</sup>/Tyr<sup>204</sup>, Raf-1 phospho-Ser<sup>338</sup>, MEK 1/2 phospho-Ser<sup>217/221</sup>, and PKC $\theta$  were purchased from Cell Signaling Technology (MA). Antibodies to I $\kappa$ B $\alpha$  and Raf-1 were from Santa Cruz Biotechnology (CA). Antibody to RKIP was from Upstate Biotechnology (VA) and to RKIP phospho-Ser<sup>153</sup> was from Epitomics (CA). Anti- $\alpha$ 1 Na/K-ATPase ( $\alpha$ 6F) antibody was obtained from the University of Iowa Hybridoma Bank. The anti-CD36 mAb clone 63 (anti-CD36-cl63; also known as clone CRF D2717) was from BD Biosciences (Oxford, UK).

## Cell culture and fatty acid treatment

L6 muscle cells were maintained as myoblasts and differentiated into myotubes in  $\alpha$ -MEM containing 2% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution (100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 250 ng/ml amphotericin B) at 37°C with 5% CO<sub>2</sub> as described previously (31). Myotubes were exposed to fatty acids (or vehicle) that were pre-conjugated to BSA for the times and at the concentrations indicated in the figure legends.

## Cell lysis and isolation of total membranes

Following the incubations stated in the figure legends, cells were washed three times in ice-cold phosphate-buffered saline (PBS) and lysed using lysis buffer (50 mM TRIS at pH 7.4, 0.27 M sucrose, 1 mM Na-Orthovanadate at pH 10, 1 mM EDTA, 1 mM EGTA, 10 mM Na- $\beta$ -glycerophosphate, 50 mM NaF, 5 mM N-pprophosphate, 1% (v/v) Triton X-100, 0.1% 2-mercaptoethanol, and protease inhibitors). Whole-cell lysates were centrifuged at 4,000 *g* at 4°C, snap-frozen in liquid nitrogen, and stored at –20°C. In some experiments, total-cell membranes were isolated as described previously (31).

## Generation of recombinant adenovirus and transduction of L6 cells

The RKIP sequence was amplified from L6 cDNA and cloned into the vector pCR2.1-TOPO (Invitrogen). Site-directed mutagenesis was carried out using the QuickChange II kit (Stratagene) and was used to mutate serine 153 to valine. RKIP S153V was then subcloned into the shuttle vector pShuttleCMV. The resultant plasmid was linearized by restriction endonuclease PmeI and cotransformed with the adenoviral vector pAdEasy1 into the *Escherichia coli* strain BJ5183. Recombinants were selected for by kanamycin resistance and screened by endonuclease digestion with BamHI, PacI, and SpeI. The final recombinant adenoviral construct was linearized with the restriction endonuclease PacI and transfected into the packaging cell line Ad293 using Lipofectamine (Life Technologies). After seven days, cells were lysed by four

freeze-thaw cycles, centrifuged to pellet cell debris, and then the supernatant containing virus was used to further propagate the recombinant adenovirus in Ad293 cells. Adenovirus was collected when approximately 70% of the cell monolayer showed cytopathic effect. Cell culture supernatant containing virus was collected and stored at  $-80^{\circ}\text{C}$ . Viral titers were determined by plaque assay in Ad293 cells. L6 cells at day 4 of differentiation were transduced for 2 h with  $86.8 \times 10^6$  pfu/ml of virus (either containing or lacking the RKIP S153V construct) and then incubated until fully differentiated as described previously (31).

### Immunoblotting

Cell lysates or total membranes (30  $\mu\text{g}$  protein) were diluted in Laemmli buffer and subjected to SDS-PAGE on 10% resolving gels and transferred onto Immobilon-P membranes as described previously (31). Membranes were probed with primary antibodies against proteins of interest prior to incubation with the appropriate peroxidase-conjugated IgG antibodies. Proteins were detected using chemiluminescence involving exposure to autoradiographic film (31).

### Immunoprecipitation

Coimmunoprecipitations of Raf-1 and RKIP were carried out in detergent-free, mild conditions as described previously (32). Protein-G sepharose beads were incubated with anti-RKIP antibody for 2 h at  $4^{\circ}\text{C}$  on an orbital platform shaker. L6 myotubes were lysed by sonication in PBS containing protease inhibitors, and 500  $\mu\text{g}$  of the resulting lysate was incubated with the beads for a further 2 h at  $4^{\circ}\text{C}$ . The immunoprecipitates were washed four times in ice-cold PBS and then boiled for 5 min in Laemmli buffer.

### RNA extraction and PCR

Total RNA extraction and quantitative real-time PCR were carried out as previously described (18). The sequences for the primers used were as follows: IL-6 forward, 5'-GACTGATGTGTTGACAGCCA-3'; IL-6 reverse, 5'-ATGCTTAGGCATAACGC-ACTAGGTT-3'; CINC-1 forward, 5'-ACCCGCTCGCTTCTCTGTGC-3'; CINC-1 reverse, 5'-CAGCGCAGCTCATGGCGAC-3'; UCP2 forward, 5'-CCTACAAGACCATTGCACGAGAG-3'; UCP2 reverse, 5'-GGCAAAGGGAGGTCGTCTGTC-3'; GAPDH forward, 5-TGGAAAGCTGTGGCGTGAT-3; and GAPDH reverse 5-GCT-TCACCACCTTCTTGAT-3.

### Analysis of DAG and ceramide content

DAGs and ceramides were extracted by the use of an extraction mixture composed of isopropanol:water:ethyl acetate (35:5:60; v/v/v). Quantitative measurement was made using an Agilent 6460 triple quadrupole mass spectrometer. Diacylglycerols and ceramides were analyzed using positive ion electrospray ionization (ESI) source with multiple reaction monitoring (MRM). The chromatographic separation was performed using an Agilent 1290 Infinity Ultra Performance Liquid Chromatography (UPLC) as recently described (33). The analytical column was a reverse-phase Zorbax SB-C8 column  $2.1 \times 150$  mm,  $1.8 \mu\text{m}$  (Agilent). Chromatographic separation was conducted in binary gradient using 2 mM ammonium formate, 0.15% formic acid in methanol as Solvent A, and 1.5 mM ammonium formate, 0.1% formic acid in water as Solvent B at the flow rate of 0.4 ml/min. 1,3-dipentadecanoyl-rac-glycerol was used as an internal standard.

### Measurement of intramyocellular triglyceride, fatty acid uptake, and whole-cell oxygen consumption

Intramyocellular triglyceride accumulation was assayed using an enzyme (lipase)-coupled colorimetric assay kit (MAK040, Sigma-Aldrich) as per manufacturer's instructions. Palmitate

uptake in L6 myotubes was investigated as described previously (8). Oxygen consumption rate (OCR) was assayed by plating out L6 myoblasts onto 24-well plates at a density of 6,000 cells/well and allowing them to differentiate into myotubes over a seven-day period with media changes every two days. For chronic fatty acid treatments, myotubes were treated for 14 h with palmitate and/or palmitoleate in 2% BSA  $\alpha$ -MEM and then transferred into Krebs-Henseleit buffer (111 mM NaCl, 4.7 mM KCl, 2 mM  $\text{Mg}_2\text{SO}_4$ , 1.2 mM  $\text{Na}_2\text{HPO}_4$ , 2.5 mM glucose, 0.5 mM carnitine) containing 2% (w/v) BSA and the relevant fatty acids. After 1 h, the OCR was measured continuously over a period of 2 h using a Seahorse Extracellular Flux (XF) Analyzer.

### Statistical analyses

For multiple comparisons, statistical analysis was performed using one-way ANOVA (ANOVA) followed by a Bonferroni post-test. For individual comparisons, statistical analysis was performed using a *t*-test. Data analysis was performed using GraphPad Prism software and considered statistically significant at  $P < 0.05$ .

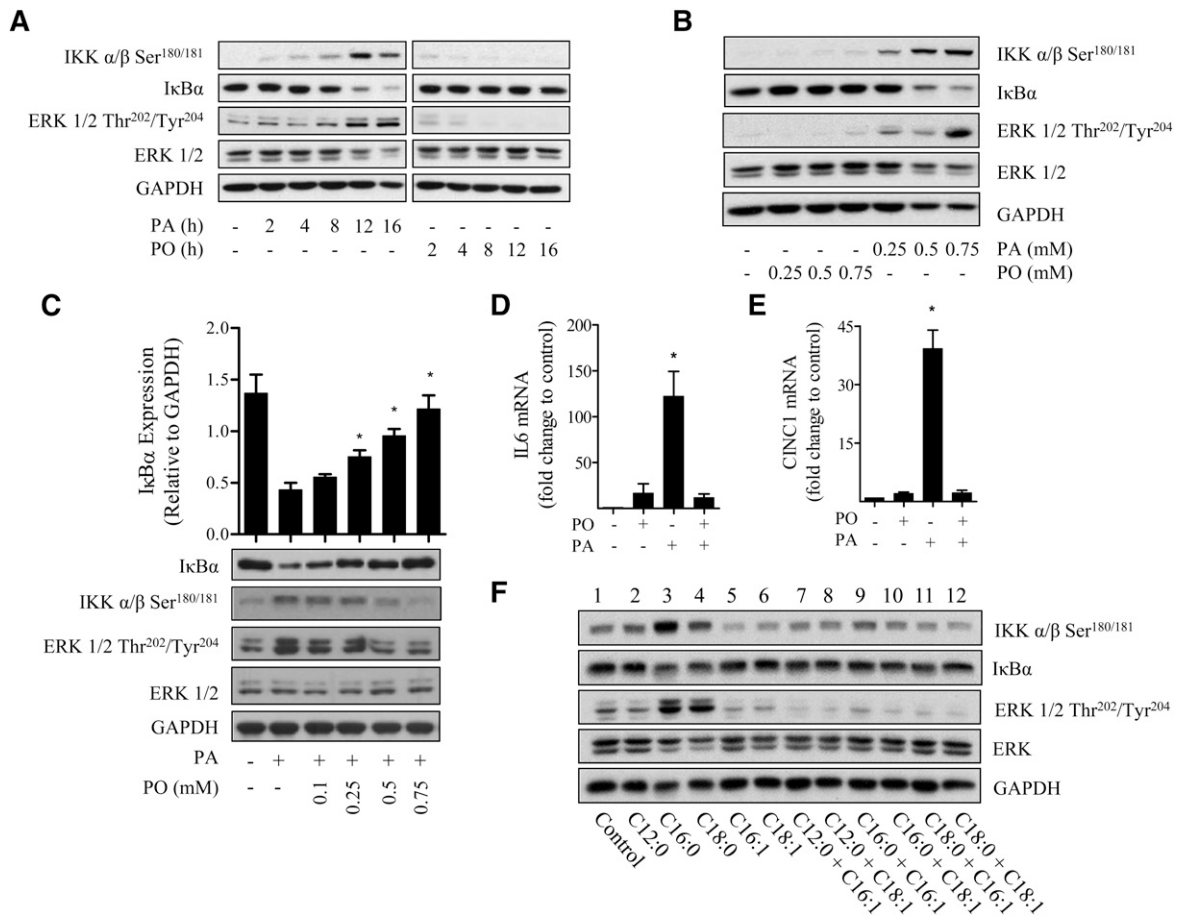
## RESULTS

### Effects of palmitate and MUFAs on ERK, IKK $\alpha$ / $\beta$ phosphorylation, and I $\kappa$ B $\alpha$ abundance

To explore the mechanisms underpinning the effect of increased SFA availability upon proinflammatory signaling, we initially monitored the phosphorylation/activation status of IKK $\alpha$ / $\beta$  and the cellular abundance of its downstream target I $\kappa$ B $\alpha$ , which serves as a readout of NF $\kappa$ B activation. In line with our recent work (18), palmitate (C16:0) induced a dose- and time-dependent increase in IKK phosphorylation/activation that was associated with a corresponding loss in I $\kappa$ B $\alpha$ . These responses were maximal after 16 h incubation with 0.75 mM palmitate (Fig. 1A, B). In contrast, palmitoleate (a MUFA, C16:1) did not exert any detectable effect upon IKK $\alpha$ / $\beta$  phosphorylation or upon I $\kappa$ B $\alpha$  abundance over the same dose and time range. We have previously demonstrated that the increase in NF $\kappa$ B signaling in response to palmitate is dependent upon the upstream activation of ERK (18). Consistent with these observations, palmitate but not palmitoleate enhanced ERK phosphorylation (Fig. 1).

Because palmitoleate did not elicit any increase in NF $\kappa$ B signaling, we subsequently assessed whether it could affect the proinflammatory drive induced by palmitate. Fig. 1C shows that palmitoleate antagonized the palmitate-induced activation/phosphorylation of ERK and IKK $\alpha$ / $\beta$  and restrained I $\kappa$ B $\alpha$  loss in a dose-dependent manner. Subsequent real-time quantitative PCR analysis of two NF $\kappa$ B-regulated muscle genes, IL-6 and CINC-1 (the rat homolog of IL-8) (34, 35), revealed that while palmitate induced expression of IL-6 and CINC-1 by  $\sim$ 120-fold and 40-fold, respectively (Fig. 1D, E), this was fully repressed in cells simultaneously incubated with equivalent concentrations (0.75 mM) of both palmitate and palmitoleate.

Although palmitoleate is a rather minor circulating MUFA, the data presented in Fig. 1A–E highlight the contrasting effects of introducing a single double-bond into an otherwise very prevalent C16 SFA. We therefore compared



**Fig. 1.** Effects of palmitate, palmitoleate, and select MUFAs on proinflammatory signaling in L6 myotubes. (A, B) L6 myotubes (A) were treated with fatty acid-free BSA (vehicle control) or BSA conjugated to PA (0.75 mM) or PO (0.75 mM) for times indicated. Muscle cells (B) were incubated for 16 h with the fatty acids at the indicated concentrations. Cells were lysed and lysates subjected to immunoblot analysis using antibodies against IKK $\alpha/\beta$  Ser<sup>180/181</sup>, I $\kappa$ B $\alpha$ , and ERK 1/2 Thr<sup>202</sup>/Tyr<sup>204</sup>. (C) L6 myotubes were incubated in the absence or presence of 0.75 mM palmitate and/or palmitoleate at concentrations indicated for 16 h prior to lysis and immunoblotting for ERK 1/2 Thr<sup>202</sup>/Tyr<sup>204</sup>, IKK $\alpha/\beta$  Ser<sup>180/181</sup>, I $\kappa$ B $\alpha$ , and GAPDH. The cellular abundance of I $\kappa$ B $\alpha$  was quantified and expressed relative to GAPDH, which was used as a gel loading control. (D, E) Alternatively, following treatment in the absence or presence of 0.75 mM palmitate and/or 0.75 mM palmitoleate, RNA was extracted from L6 myotubes and (D) IL-6 and (E) CINC-1 mRNA were determined by real time qPCR. Bars represent mean  $\pm$  SEM from three separate experiments, and asterisks signify a significant difference from the untreated control values ( $P < 0.05$ ). (F) Effect of different fatty acids upon proinflammatory signaling was assessed. L6 myotubes were treated for 16 h with 0.75 mM lauric acid (C12:0), 0.75 mM palmitate (C16:0), or 0.1 mM stearic acid (C18:0) alone or in combination with 0.75 mM palmitoleate (C16:1) or 0.75 mM oleate (C18:1). Lysates were immunoblotted to assess the phosphorylation status of IKK $\alpha/\beta$  and ERK 1/2 and total protein abundance of I $\kappa$ B $\alpha$ . Equal gel loading was ascertained by immunoblotting with an antibody against total ERK 1/2 and GAPDH. The blots are representative of three separate experiments.

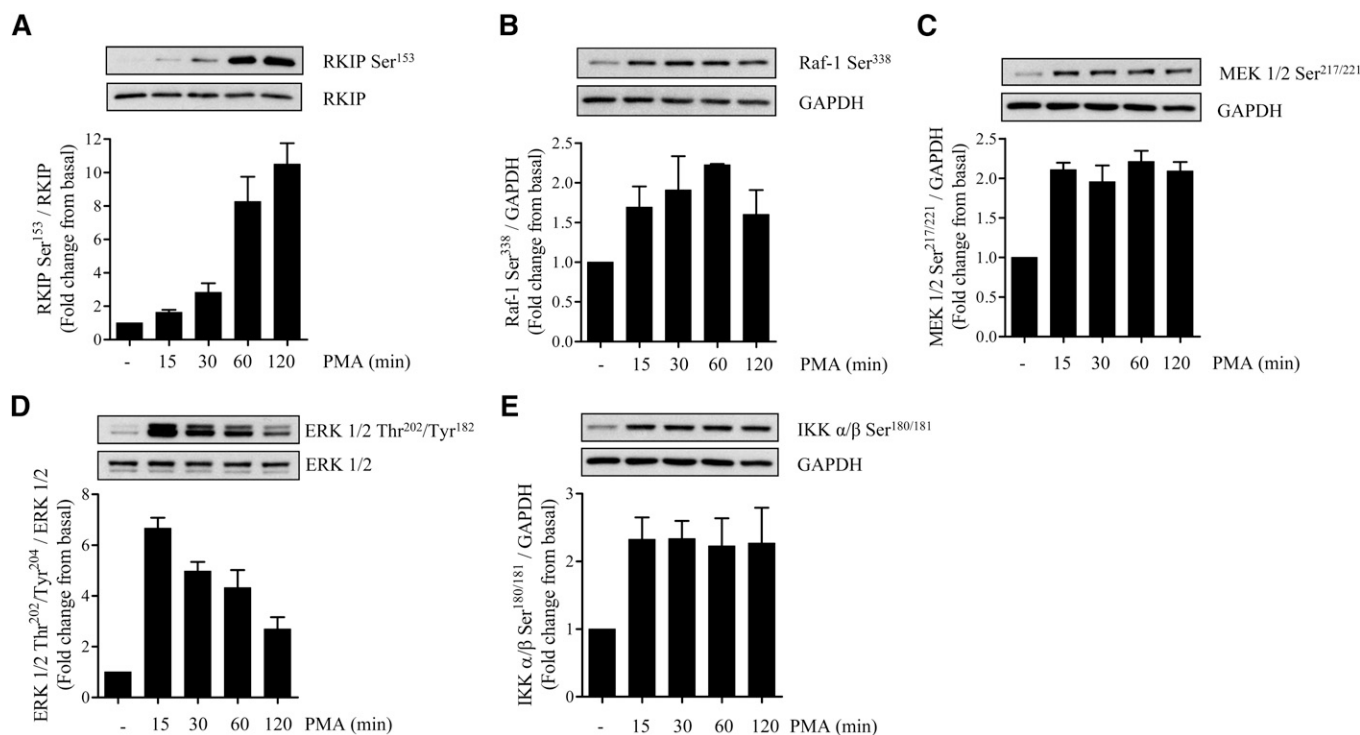
the extent to which fatty acyl chain length and saturation were important with respect to inducing their pro- or anti-inflammatory effects. L6 muscle cells were chronically incubated with laurate (C12:0, SFA), palmitate (C16:0), stearate (C18:0, SFA), palmitoleate (C16:1), or oleate (C18:1, MUFA) either alone or with SFA and MUFA in the combinations shown in Fig. 1F. Our data indicate that, unlike long chain (C16:0 and C18:0) SFAs, laurate does not induce phosphorylation/activation of ERK or IKK $\alpha/\beta$ , and it does not promote loss of I $\kappa$ B $\alpha$  (Fig. 1F). In line with much of the palmitoleate data presented in Fig. 1, muscle cells did not exhibit an increase in proinflammatory signaling when chronically exposed to oleate, a very prevalent circulating MUFA. Furthermore, like palmitoleate, oleate was able to attenuate the proinflammatory effects of both palmitate and stearate based on the reduced phosphorylation of

ERK and IKK $\alpha/\beta$  and retention of I $\kappa$ B $\alpha$  (Fig. 1F, compare lanes 10 and 12 with lanes 3 and 4).

### Palmitate induces RKIP phosphorylation and its dissociation from Raf1

In an attempt to understand the mechanisms contributing to the differential effect that SFAs and MUFAs have upon the ERK-IKK $\alpha/\beta$ -NF $\kappa$ B signaling axis, we focused on regulation of molecules upstream of ERK. We have shown that intramyocellular accumulation of DAG is a significant feature associated with incubation of muscle cells with palmitate under conditions that result in proinflammatory NF $\kappa$ B signaling (14, 18). RKIP is an inhibitor of Raf1 activity and phosphorylation of RKIP on Ser<sup>153</sup> by DAG-sensitive PKC promotes its dissociation from Raf1 and consequently alleviates RKIP-mediated inhibition of Raf1 (28, 29). **Fig. 2A, B**





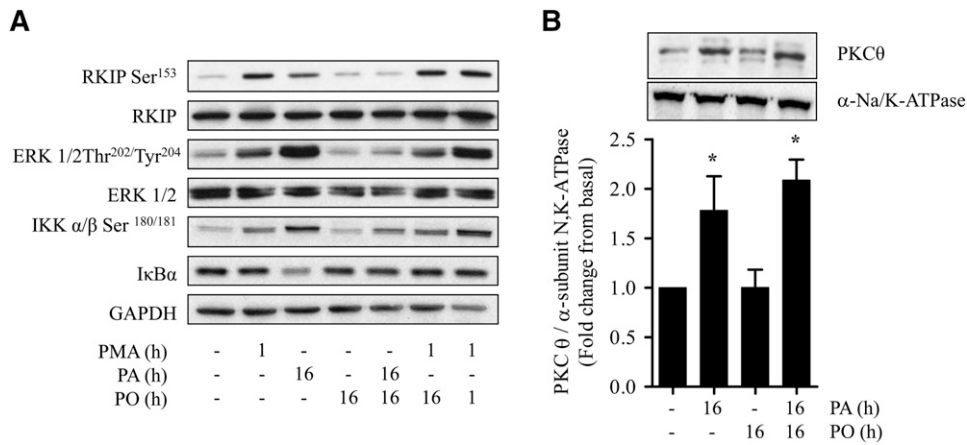
**Fig. 3.** PMA, a DAG mimetic and novel/conventional PKC activator, induces phosphorylation of RKIP and of the ERK-IKK signaling axis. L6 myotubes were treated with 100 nM PMA for the times indicated. Cells were lysed and subsequently immunoblotted to assess the phosphorylation status of (A) RKIP, (B) Raf-1, (C) MEK 1/2, (D) ERK 1/2, and (E) IKK $\alpha/\beta$ . Phospho signals were either normalized to the unphosphorylated native protein or to GAPDH. Values shown are the mean  $\pm$  SEM from three separate experiments.

suggesting that both stimuli are likely to act via a common mechanism (i.e., PKC activation) to promote RKIP phosphorylation (supplementary Fig. 1A). To assess the contribution of PKC to these signaling events, we subsequently tested the effect of two PKC inhibitors; Gö6983 (which inhibits both novel and conventional PKCs) and Gö6976 (which selectively targets conventional PKCs) (38). **Fig. 4A** shows that phosphorylation of RKIP in response to palmitate was suppressed by Gö6983 (compare lanes 4 and 5) but not by Gö6976 (compare lanes 4 and 6). However, despite the observed sensitivity of RKIP phosphorylation toward Gö6983, the inhibitor did not antagonize the fatty acid-induced phosphorylation of ERK or IKK $\alpha/\beta$  or curtail the loss of I $\kappa$ B $\alpha$  (Fig. 4B–D, compare lanes 4 and 5 in each panel). In contrast, both Gö6983 and Gö6976 were effective in negating the effects of PMA upon RKIP, ERK, and IKK $\alpha/\beta$  (Fig. 4A–D, compare lanes 8 and 9 with lane 7 in each panel). It should be stressed that PMA did not induce any appreciable proteosomal loss of I $\kappa$ B $\alpha$  in these experiments given that, unlike palmitate (which was present for 16 h), muscle cells were only exposed to PMA for 30 min in these studies, which is likely to be an insufficient period of time to observe depletion of this protein. The notion that RKIP may serve as an important intermediate in PKC-directed activation of the MEK-ERK pathway in response to PMA but that it is dispensable in response to palmitate is further supported by our finding that expression of dominant interfering RKIP<sup>Ser153Val</sup> mutant (28) suppressed phosphorylation of ERK in response to PMA but not to palmitate (Fig. 4E).

### Effects of palmitoleate upon proinflammatory signaling and PKC activation

The findings presented in Fig. 4 imply that although palmitate induces activation of novel PKCs in L6 myotubes and these can mediate phosphorylation of RKIP, this signaling event appears not to be a crucial requirement for activation of the ERK-IKK proinflammatory pathway. To validate this proposition further, we compared the effect of palmitoleate on proinflammatory signaling induced by palmitate and PMA. **Fig. 5A** shows that while palmitoleate antagonizes palmitate-induced phosphorylation of RKIP, ERK, and IKK $\alpha/\beta$ , phosphorylation of these proteins in response to PMA was not suppressed by the MUFA. This latter observation suggests that the ability of palmitoleate to antagonize the proinflammatory effect of palmitate was unlikely to be the result of a targeted reduction in the activation of DAG-sensitive PKCs. This view is supported by the finding that PKC $\theta$ , a DAG-sensitive novel PKC that participates as an effector of fatty acid-induced signaling (39), was activated in response to palmitate as judged on the basis of its increased membrane association. However, unlike activation of the ERK-IKK $\alpha/\beta$  signaling axis, the SFA-induced activation of PKC $\theta$  could not be repressed by palmitoleate (Fig. 5B). In line with these findings, quantitative analysis of the fatty acid composition of different intramyocellular DAG species revealed that cell incubation with palmitate induced significant increases in DAG lipids with both saturated and unsaturated fatty acyl chains (Fig. 6A–K). Our analysis revealed that, with the





**Fig. 5.** Palmitoleate does not prevent PMA-induced activation of the RKIP/ERK signaling axis and does not block PKC $\theta$  activation in palmitate-treated cells. L6 myotubes were incubated with fatty acid-free BSA (vehicle) alone or with BSA conjugated for the times indicated with PA (0.75 mM), PO (0.75 mM), or PMA (100 nM) alone or in combination. Cells were harvested, and (A) total cell lysates were immunoblotted using antibodies against the proteins indicated or (B) total membranes were immunoblotted with antibodies against PKC $\theta$  and the  $\alpha$ -subunit of the N,K-ATPase, a plasma membrane marker protein. The blots are representative of three separate experiments, and bars represent mean  $\pm$  SEM from 3 separate experiments. Asterisks signify a significant difference from the control values ( $P < 0.05$ ).

low as 0.1 mM can antagonize the effects of palmitate on these molecules (Fig. 7B). These observations imply that modest MUFA provision confers beneficial effects not only upon cellular respiration but also on proteins implicated in proinflammatory signaling and that enhanced fuel oxidation may help offset some of the lipotoxic effects associated with SFA oversupply. Indeed, the finding that inhibiting mitochondrial fatty acid uptake using etomoxir blunted palmitoleate's ability to fully antagonize loss of I $\kappa$ B $\alpha$  in response to palmitate underscores the importance of sustaining mitochondrial function (Fig. 7D).

In addition to promoting cellular respiration and maintaining mitochondrial function, palmitoleate provision may induce metabolic benefits by facilitating increased storage of fatty acids as neutral lipid. To assess this, we quantified intramyocellular triglyceride content and found that while treatment with palmitate or palmitoleate alone induced a significant increase in neutral lipid, it was enhanced in a greater than additive manner when myotubes were coincubated with both fatty acids together (Fig. 7E).

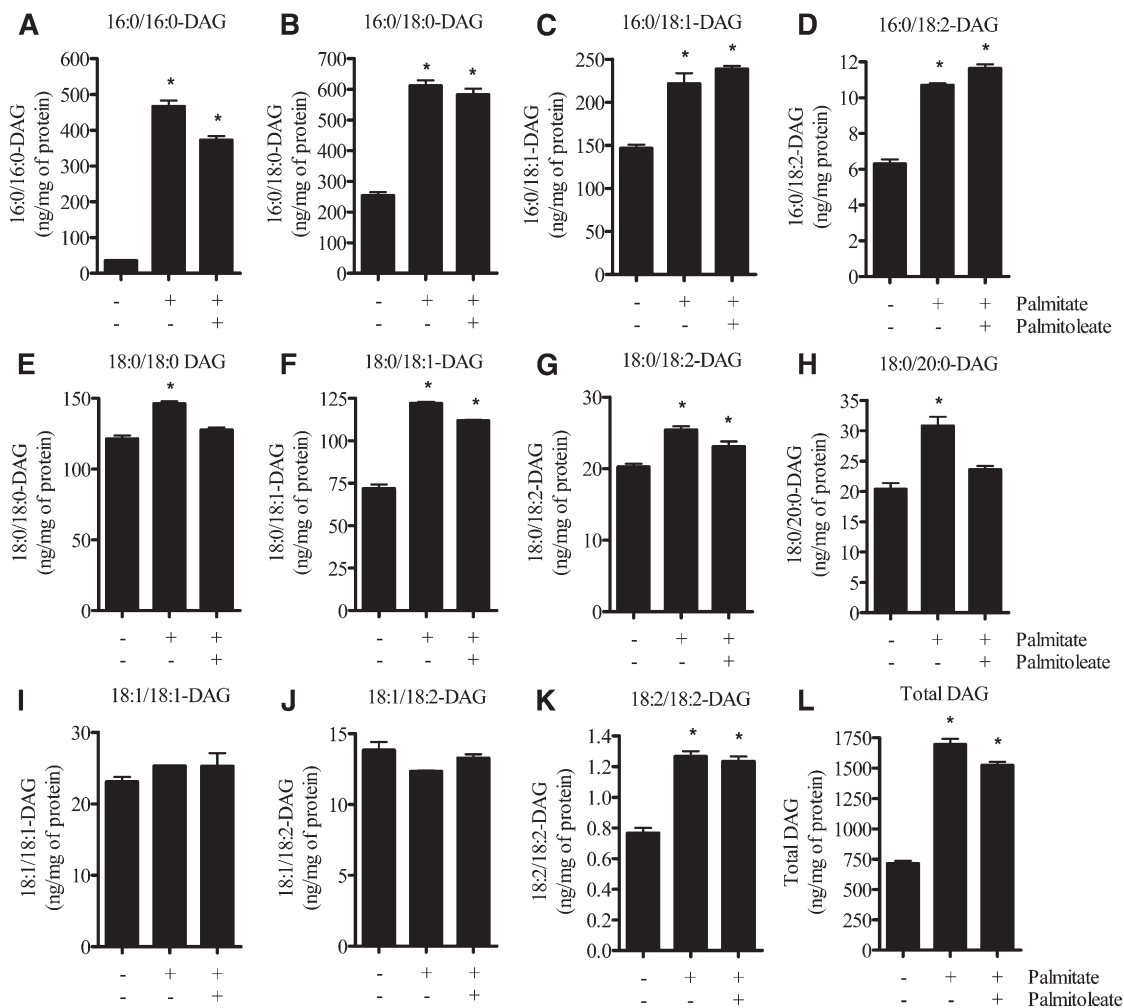
## DISCUSSION

Studies in rodent models of obesity indicate that chronic, low-grade inflammation is a characteristic feature of the obese state that may, in part, be a consequence of a sustained increase in the circulating concentration of SFAs (e.g., palmitate) (40, 41), which can induce a potent inflammatory response in numerous tissues, including skeletal muscle. By contrast, MUFAs do not elicit this response and, intriguingly, are able to counter the proinflammatory effect of SFAs by as yet poorly understood mechanisms. Delineating how MUFAs modulate SFA-induced inflammatory

tone in skeletal muscle in vivo is a major challenge given that, in addition to direct effects that SFAs may have upon muscle, tissue responses may be confounded by other systemic consequences of obesity. These include, for example, changes in adipokine levels (TNF $\alpha$ , IL-6, and IL-1 $\beta$ ) (42), interorgan nutrient flow, and activation of both resident and infiltrating macrophages (43) that could affect diverse signaling pathways within muscle. Thus, in an attempt to gain a clearer understanding of how MUFAs may antagonize palmitate-induced proinflammatory signaling in muscle, this study utilized an established cultured rat skeletal muscle cell line (L6) that exhibits a robust SFA-induced proinflammatory response (18).

We recently reported that chronic exposure of L6 skeletal muscle cells to palmitate induces proinflammatory NF $\kappa$ B signaling in a manner dependent upon the upstream activation of the MEK-ERK signaling axis (18). Given that a rise in intramyocellular DAG is a major outcome associated with sustained oversupply of palmitate, we postulated that DAG-sensitive PKCs may feature prominently in the activation of the Raf-MEK-ERK pathway, a supposition based on evidence in the literature showing that PKCs can promote activation of this pathway in a number of different cell types (26, 28, 44). One mechanism linking activated PKCs to the MAP kinase cascade involves modulation of RKIP, a molecule whose association with Raf1 helps maintain the kinase in a repressed state (28). PKC-mediated phosphorylation of RKIP on Ser153 results in its dissociation from Raf1, thus facilitating activation of MEK-ERK signaling. Consistent with such a mechanism, we show that cell treatment with palmitate or the DAG mimetic PMA results in phosphorylation of RKIP<sup>Ser153</sup> and subsequent activation of Raf1, MEK, ERK, and IKK. Intriguingly, phosphorylation of RKIP induced in response to palmitate and PMA displays differential sensitivity to Gö6983 and Gö6976, the



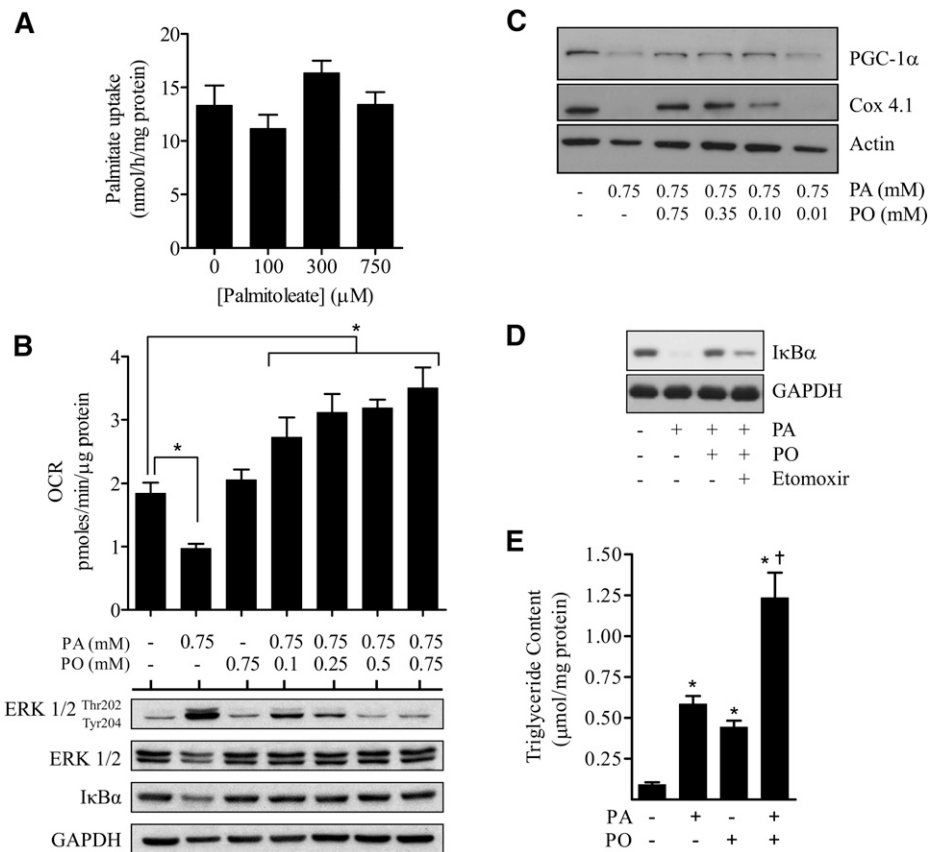


**Fig. 6.** Effects of palmitate and palmitoleate upon intramyocellular abundance of different DAG species. L6 myotubes were incubated with palmitate (0.75 mM) and/or palmitoleate (0.75 mM) for 16 h. Following incubation, muscle cells were harvested in ice-cold PBS, and lipids were extracted to assess DAG content as described in Methods. Bars represent mean  $\pm$  SEM from three separate experiments, and asterisks signify a significant difference from the untreated control values ( $P < 0.05$ ).

two PKC inhibitors used in our study. Both inhibitors suppress the PMA-induced phosphorylation of RKIP, but only Gö6983 inhibited phosphorylation of RKIP in response to palmitate. Since Gö6983 inhibits both novel and conventional PKC isoforms and Gö6976 is considered more selective against conventional PKC isoforms (45, 46), our findings imply that palmitate-induced phosphorylation of RKIP is mediated by novel PKCs, whereas PMA invokes a broad-spectrum response involving both novel and conventional PKC isoforms. Surprisingly, our findings indicate that the PKC-mediated phosphorylation of RKIP triggered by palmitate is dispensable with respect to the downstream activation of ERK and IKK, unlike the response to PMA. This proposition is based on two separate lines of evidence. First, while PKC inhibitors suppress RKIP<sup>Ser153</sup> phosphorylation in response to both PMA and palmitate, the downstream activation of ERK was only halted in response to PMA. Second, stable overexpression of a phosphorylation-resistant dominant negative RKIP mutant (RKIP<sup>Ser153Val</sup>) only suppresses PMA and not palmitate-induced ERK activation. Collectively, these observations imply that while

the Raf-MEK-ERK signaling axis can be stimulated in a PKC- and RKIP-dependent manner by PMA and palmitate, the stimulation of this pathway in response to palmitate is not exclusively mediated via the PKC-RKIP-Raf link. Our observations thus implicate a separate, as yet undefined, palmitate-derived signal that feeds into the MEK-ERK cascade at a point beyond RKIP/Raf that helps sustain palmitate's proinflammatory signaling potential.

Previous studies have demonstrated that oleate (a C18:1 MUFA) reduces intramyocellular DAG accumulated in response to palmitate oversupply (6). In such studies, DAG content was determined by assaying incorporation of radiolabeled fatty acids into lipid extracts that were resolved by thin layer chromatography, which does not allow quantification of different DAG species. In contrast, the present study utilized an ESI mass spectrometry-based approach that allows profiling and quantification of multiple DAG species. Our analysis focused on the most abundant DAG species and revealed, somewhat surprisingly, that while chronic exposure of L6 myotubes to palmitate induced a greater than 2-fold increase in total DAG content,



**Fig. 7.** Fatty acid uptake and effects of palmitate and palmitoleate on oxygen consumption, mitochondrial protein expression, and accumulation of neutral lipids. (A) L6 myotubes were incubated in the absence and presence of palmitoleate (PO) at the indicated concentrations and uptake of [ $^3\text{H}$ ]palmitate determined as described in Methods. (B) L6 skeletal muscle cells were treated with the stated concentrations of fatty acid for 16 h, at which point cells were either used for analysis of oxygen consumption rate (OCR) which was assayed using Seahorse Bioscience technology as described in Methods, or L6 skeletal muscle cells lysed and lysates immunoblotted using antibodies against phospho or native proteins indicated. (C) L6 myotubes were treated with fatty acids at concentrations indicated as in (B) and lysates immunoblotted using antibodies against PGC1 $\alpha$  and Cox 4.1. (D) Myotubes were incubated with fatty acids (0.75 mM) as indicated in the absence or presence of etomoxir (100  $\mu\text{M}$ ) prior to lysis and immunoblotting using antibodies against I $\kappa$ B $\alpha$  and GAPDH. (E) L6 muscle cells were treated with 0.75 mM PA and/or PO for 12 h prior to assaying TAG content as indicated in Methods. Bars represent mean  $\pm$  SEM from three separate experiments. Asterisks signify a significant difference from the untreated control bar ( $P < 0.05$ ), and the cross signifies a significant difference from the single fatty acid treatments alone ( $P < 0.05$ ).

cosupplementation of palmitoleate did not significantly reduce total DAG abundance. Intriguingly, however, the MUFA did promote a selective reduction in the palmitate-induced increase of C18:0/C18:0 DAG and C18:0/C20:0 DAG. Since DAG-sensitive PKCs mediate phosphorylation of RKIPSer<sup>153</sup> and palmitoleate blocks SFA-induced phosphorylation of this site, it is tempting to speculate that increases in these two DAG species may specifically participate in palmitate-induced regulation of RKIP. If so, given that abundance of numerous other DAG species in response to palmitate was not altered by palmitoleate, our results may indicate that activation of novel PKCs (induced by increases in C18:0/C18:0 DAG and C18:0/C20:0 DAG) and phosphorylation of RKIP may occur in specific cell compartments. We cannot exclude the possibility that other MUFAs, such as oleate, may exert a broader suppressive effect on SFA-induced accumulation of different DAG lipids


that may potentially help explain the discrepancy between our findings and those reported in the literature (6). In any event, our data indicate that the ability of palmitoleate to inhibit the proinflammatory potential of palmitate in muscle cells is independent of any suppressive effect the MUFA has upon DAG-mediated RKIP modulation.

Increased provision of SFAs is also known to promote intramyocellular accumulation of ceramide and induce endoplasmic reticulum (ER) stress, both of which have been implicated in the cellular proinflammatory response (47, 48). However, while we find that sustained exposure of L6 myotubes to palmitate elevates total cell ceramides (attributable to specific increases in numerous long-chain ceramide species), this increase, unlike the increase in NF $\kappa$ B-mediated proinflammatory signaling, is not antagonized by palmitoleate (supplementary Fig. II). This finding is consistent with our previous work showing that sustained

(16 h), low-grade exposure to 10  $\mu$ M C2 ceramide or short (2 h) exposure to 100  $\mu$ M C2 ceramide fails to increase proinflammatory signaling in muscle cells (18). In line with work of other groups (48), we also find that palmitate induces expression of ER stress markers ATF3 and CHOP. Expression of these stress genes was also markedly enhanced in response to thapsigargin, a known ER stress-inducing agent. Intriguingly, however, while palmitoleate repressed palmitate-induced expression of ATF3 and CHOP, the MUFA failed to avert expression of these ER stress markers in response to thapsigargin (supplementary Fig. III). Moreover, cotreatment of muscle cells with palmitate and two separate ER stress inhibitors (4-phenylbutyric acid and tauroursodeoxycholic acid) at concentrations previously shown to prevent ER stress in L6 myotubes (49) failed to suppress palmitate-induced I $\kappa$ B $\alpha$  loss or IL-6 gene expression (supplementary Fig. IV). These observations collectively suggest that, while ER stress is a feature associated with palmitate overprovision, it is unlikely to be a major driver of palmitate's proinflammatory potential in muscle cells.

While numerous studies have demonstrated that increased fatty acid availability results in elevated intramyocellular lipid deposition and that this correlates with loss of skeletal muscle insulin sensitivity (50–53), there is also evidence that suggests that intramuscular accumulation of neutral lipid (triacylglycerol, TAG) need not be lipotoxic or insulin desensitizing (54–56). Such observations have invariably been reported in skeletal muscle of chronically exercised individuals who remain markedly insulin-sensitive despite having high intramyocellular triglycerides (i.e., the so called “athletes paradox”) and most likely reflects an adaptive state in which skeletal muscle of such individuals has become more efficient in “turning over” stored lipid (57). Partitioning of fatty acids into storage or oxidative pathways is, however, a highly variable process. Compared with MUFAs, SFAs have been shown to be less well oxidized (58) and more poorly incorporated into TAG (56), but intriguingly, when copresented with MUFAs, their esterification into TAG (6, 59) as well as their mitochondrial oxidation has been shown to be enhanced (6). We found that muscle cells incubated with palmitate exhibited a lower OCR than those exposed to palmitoleate alone but that cellular respiration and TAG accumulation was augmented in the presence of both fatty acids together. The reduced oxidative capacity associated with oversupply of SFA is likely to be a consequence of mitochondrial dysfunction given that *i*) we observed a reduction in Cox4.1 and PGC1 $\alpha$  (two proteins with important but distinct roles in mitochondrial biology) and *ii*) recent studies show that palmitate causes mitochondrial damage/fragmentation in muscle cells by inducing a greater inflammatory drive, insulin resistance, and apoptosis (60, 61). In contrast, promoting oxidation and/or storage of SFAs by coprovision of MUFAs reduces the proapoptotic drive of palmitate (58), and as we and others (6) found, it also suppresses NF $\kappa$ B-mediated proinflammatory signaling.

In summary, the current study excludes any direct involvement of DAG-sensitive PKCs and RKIP in the

palmitate-induced activation of the MEK-ERK-IKK proinflammatory signaling axis in L6 myotubes. Our results suggest that it is highly likely that the heightened proinflammatory response triggered by palmitate oversupply is primarily a manifestation of not being able to effectively oxidize and/or channel the fatty acid for storage as neutral triglyceride. These findings add to the growing body of evidence showing that the presence of MUFA stimulates metabolic handling of palmitate and that this helps subvert some of the lipotoxic (i.e., proinflammatory) effects associated with palmitate overload. Precisely how MUFAs achieve this protective effect remains unclear, but recent work in SRD-13A cells (a CHO-derived cell line) has identified the presence of Ubx $\delta$ , a protein capable of sensing availability of unsaturated fatty acids and regulating triglyceride synthesis (62). Ubx $\delta$  is an ubiquitin-like (UBX) domain containing protein involved in ER-associated degradation of Insig-1, a protein involved in ER membrane retention of sterol regulatory element binding protein-1 (SREBP-1). SREBP-1 functions as a transcription factor regulating expression of numerous genes involved in lipid homeostasis. Under conditions of low fatty acid availability when synthesis of TAG would be low, Ubx $\delta$  promotes degradation of Insig-1, thus enabling SREBP1 to be transported to the golgi where it is cleaved and then routed to the nucleus to activate genes promoting fatty acid synthesis. However, the presence of unsaturated fatty acids leads to oligomerization and inactivation of Ubx $\delta$ , thereby preserving Insig1 and promoting ER retention of SREBP1. Under such circumstances, expression of genes supporting fatty acid synthesis would be suppressed in favor of a greater shift toward TAG and lipid droplet formation (62). Intriguingly, SFAs do not bind to or promote oligomerization of Ubx $\delta$  and are very inefficient in repressing Ubx $\delta$ , but their incorporation into TAG is likely to be enhanced when presented to cells alongside unsaturated fatty acids. It is currently unknown whether Ubx $\delta$  or closely related homologs are expressed in skeletal muscle and whether they function in a similar manner to that reported in SRD-13A cells, but it is conceivable that pharmacological modulation of such a sensor may induce dynamic changes in fatty acid metabolism that could help combat lipid-induced metabolic disorders. 

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