Biochemical and Molecular Actions of Nutrients

Palmitate Activates the NF-κB Transcription Factor and Induces IL-6 and TNFα Expression in 3T3-L1 Adipocytes[1]

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ABSTRACT Fatty acids and their metabolites regulate gene expression and immunological pathways. Furthermore, obese individuals frequently have increased circulating fatty acid concentrations, and localized inflammation in adipose tissue may facilitate the systemic inflammation associated with the insulin resistance of obesity. Although palmitate induces inflammation (i.e., activates proinflammatory pathways) in myotubes, the effects of fatty acids on inflammatory processes in adipocytes have not been established. Therefore, we examined the potential for palmitate, laurate, and docosahexaenoic acid (DHA) to modulate inflammation in 3T3-L1 adipocytes. Palmitate, but not DHA or laurate, induced nuclear factor κB (NF-κB)-driven luciferase activity and interleukin-6 (IL-6) expression ($P < 0.05$). Inhibition of fatty acyl Co-A synthase (FACS) with triacsin C suppressed palmitate-induced NF-κB activation ($P < 0.05$), but caused an additive increase in palmitate-induced IL-6 expression ($P < 0.05$). Disrupting mitogen-activated protein kinase/Erk kinase (MEK) and protein kinase C (PKC) activity with U0126 and Bisindolylmaleimide (Bis), respectively, suppressed palmitate-induced IL-6 expression ($P < 0.05$), but had no effect on NF-κB reporter gene activity ($P > 0.05$). However, the phosphoinositide-3 kinase (PI3K) inhibitor, wortmannin, alone and additively with palmitate, activated the NF-κB reporter gene and induced IL-6 expression ($P < 0.05$). Palmitate also induced the mRNA expression of tumor necrosis factor α (TNFα) ($P < 0.05$), but the increase in mRNA abundance was not reflected in a greater protein concentration in the media ($P > 0.05$). These data indicate that palmitate induces inflammation in adipocytes, and that this is not a generalized effect of all SFA. Furthermore, PI3K may act constitutively to suppress inflammation. Consequently, inhibition of this enzyme may promote and exacerbate the inflammation in adipose tissue that is associated with obesity and insulin resistance. J. Nutr. 135: 1841–1846, 2005.

KEY WORDS: • *NF-κB* • *fatty acids* • *cytokines* • *adipocyte* • *inflammation*

The potential contribution of fatty acids to the mechanisms underlying impaired glucose metabolism has been described in the so-called Randle effect (1). In addition, fatty acids suppress insulin-stimulated glucose uptake in multiple cell types (2), and it is well established that metabolic disorders, including dyslipidemias, obesity, and diabetes, are often accompanied by elevated serum concentrations of fatty acids (3). Because these diseases are also characterized by marked increases in serum concentrations of inflammatory mediators, including tumor necrosis factor α (TNFα),[3] interleukin-6 (IL-6), sialic acid, and C-reactive protein (4–6), they are commonly referred to as inflammatory states (7,8).

Recent publications showed that palmitate induces insulin resistance in myotube models (9,10), and that the nuclear factor κB (NF-κB) transcription factor is a key mediator of this effect (10,11). However, insulin-induced glycogen synthesis and activation of Akt/protein kinase B (PKB) are not responsive to palmitate in 3T3-L1 adipocytes (9), but are clearly suppressed by this fatty acid in myotubes (10). Therefore it is apparent that the mechanisms underlying fatty acid–induced insulin resistance in myofibers and adipocytes may differ.

Adipose tissue is a major source of proinflammatory cytokines, particularly in obesity (8). Considering this link between obesity and adipose production of inflammatory mediators, it is of great interest that lauric acid (12) and palmitate (11) induce cyclooxygenase (i.e., COX-2) and IL-6 expression in macrophages and muscle, respectively. Some fatty acids also oppose insulin-mediated glucose uptake (13) and activate inhibitor κB kinase (IKK) (14), the kinase that marks inhibitor κB (IκB) for proteolytic degradation to enable NF-κB function. Whether the same regulatory linkages exist in the adipocyte has not been addressed experimentally. We showed previously that NF-κB is a major inflammatory mediator in primary porcine adipocytes (15) and in 3T3-L1 adipocytes (16). Thus, considering that palmitate activates NF-κB in myotube cultures (10,11), we hypothesized that fatty acids would activate NF-κB in the adipocyte and induce TNFα and

[1] Supported by Purdue University Agricultural Research Program # 2005–17559.

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[3] Abbreviations used: AP-1, activator protein-1; Bis, Bisindolylmaleimide; BSA, bovine serum albumin; COX, cyclooxygenase; DHA, docosahexaenoic acid; FACS, fatty acyl Co-A synthase; FBS, fetal bovine serum; IκB, inhibitor κB; IKK, inhibitor κB kinase; IL-6, interleukin-6; MEK, mitogen-activated protein kinase/Erk kinase; NF-κB, nuclear factor κB; PI3K, phosphoinositide-3 kinase; PKB, protein kinase B; PKC, protein kinase C; TNFα, tumor necrosis factor α.

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Manuscript received 15 February 2005. Initial review completed 3 March 2005. Revision accepted 17 May 2005.

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IL-6 production. The data presented herein indicate clearly that palmitate, at physiological concentrations, activates NF-κB and induces proinflammatory cytokine production in adipocytes. Furthermore, the importance of specific kinase-regulated pathways is indicated.

MATERIALS AND METHODS

3T3-L1 adipocyte culture. Cells (3T3-L1) were obtained from the American Type Culture Collection and were grown in 24-well plates according to standard conditions. Briefly, cells were grown in 5% CO_2 in DMEM (GIBCO) containing 10% calf serum (GIBCO) in the presence of a 1% penicillin/streptomycin mixture (Invitrogen). At 2 d postconfluence (d 0), cells were induced to differentiate with a medium containing 10% fetal bovine serum (FBS; GIBCO), 1.7 μmol/L insulin, 1 μmol/L dexamethasone, and 0.5 mmol/L isobutylmethylxanthine for 48 h. Thereafter, fresh medium containing only insulin was added every 2 d for another 4 d. On d 8, cells were fully differentiated and the medium was changed to an insulin-free medium containing 10% FBS.

Cell transfection and reporter assay. On d 5 after induction of differentiation, cells were transfected with Fugene 6 reagent (Roche) for 48 h with pNF-κB-Luc plasmid (0.25 μg/well), with the firefly luciferase gene under the control of 5 repeats of the NF-κB (TGGGGACTTTCCGC) enhancer element (Stratagene). Transfection efficiency was normalized by cotransfecting cells (0.05 μg/well) with pRL-CMV vector (Promega), a plasmid encoding the renilla luciferase gene under the CMV promoter. For all experiments, the firefly luciferase luminescence was normalized with the renilla using the Dual luciferase assay system (Promega). Experiments on the fully differentiated cells were conducted on d 8. Cells were pretreated with serum-poor medium (0.25% FBS) for 12 h before treatments were applied. Media and the cell lysate were recovered for ELISA and the luciferase assay, respectively. A fatty acid–bovine serum albumin (BSA) complex (ratio 3:1) was prepared according to the method of Rakatzi et al. (17). Briefly, 45 μmol of palmitate was converted into its sodium salt in the presence of 0.1 mol/L NaOH. This was added to a prewarmed 10% fatty acid–free BSA solution. The solution was sonicated in quick bursts, allowed to cool to room temperature, and then sterile filtered.

Cytokine ELISA. Media concentrations of IL-6 and TNFα were measured using mouse-specific ELISA kits (Pierce Endogen) according to the manufacturer's protocols.

Real time quantitative PCR. Total RNA was recovered from cells using the Trizol reagent (Invitrogen) and Dnase treated using Turbo Dnase® (Ambion). RNA was reverse transcribed using the Superscript™ first-strand cDNA synthesis kit (Invitrogen). Primer sequences for mouse IL-6 were 5′-AACGATGATGCACTTGCAGA-3′ and 5′-GAGCATTGGAAATTGGGGTA-3′, for the sense and antisense primers, respectively (18). Sense and antisense primers for β-actin and TNFα were, for β-actin, 5′-ATG GGT CAG AAG GAC TCC TAC G-3′ and 5′-AGT GGT ACG ACC AGA GGC ATA C-3′, respectively, and for TNFα, 5′-TCC CCA AAG GGA TGA GAA GTT C-3′ and 5′-TCA TAC CAG GGT TTG AGC TCA G-3′, respectively, as reported earlier (19). Thermal cycling conditions for the PCR reactions were 94°C for 5 min followed by 40 cycles of 94°C for 45 s, 61°C for 30 s, and 72°C for 30 s. Polymerase reaction products amplified by these primers (283, 308, and 411 bp for IL-6, β-actin, and TNFα, respectively) were cloned into pGEMT vector (Promega) and sequenced for verification. Real-time reactions were carried out on an iCycler real-time machine (Biorad) using the IQ™ SYBR® Green Supermix kit (Biorad). The abundance of each gene product was calculated by regressing against the standard curve generated in the same reaction with their respective plasmids. The IL-6 and TNFα values for each sample were normalized to β-actin.

Statistical analyses. Data were examined for normality and analyzed using the mixed-model analysis of a split-plot design. The fixed effect was the treatment, and the random effect was the replicate. The main effects (treatment and replicate) were tested against the treatment × replicate interaction term. When protected by a significant *F*-test, mean separation was accomplished using the least-squares mean separation (pdiff) procedure (20). Differences were considered significant at $P < 0.05$. Values in the text are means ± SEM.

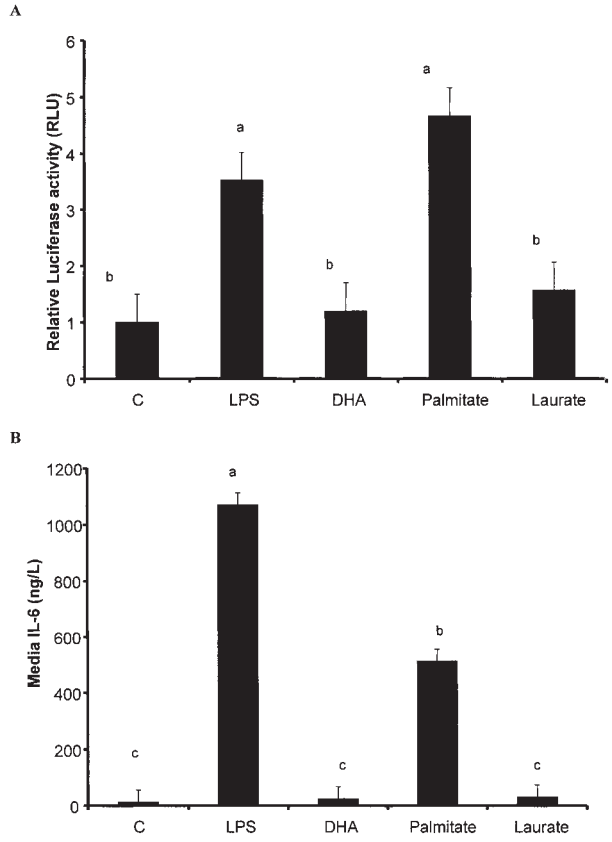

FIGURE 1 Activation of an NF-κB–driven luciferase reporter construct and induction of IL-6 media accumulation by fatty acids. (*A*) Fully differentiated 3T3-L1 adipocytes kept in a serum-poor medium (0.25% FBS) overnight were treated as indicated with LPS (100 μg/L), and 250 μmol/L DHA, palmitate, and laurate for 24 h. Firefly luminescence was normalized to renilla. Data are expressed relative to control. (*B*) Media IL-6 concentration in adipocytes treated as in *A*. Bars represent means + SEM, $n = 4$ replicates. Means without a common letter differ, $P < 0.05$.

RESULTS

The NF-κB transcription factor is critical for the activation for cytokine gene expression; to characterize its regulation by fatty acids, we tested the potential for DHA, laurate, and palmitate to activate an NF-κB–driven luciferase reporter gene construct. We also used *Escherichia coli*–derived LPS as a positive control. LPS and palmitate caused ~2.5- and 3.5-fold increases, respectively, in luciferase activity ($P < 0.05$), whereas DHA and laurate had no effect (**Fig. 1***A*). Thereafter, we examined the induction of cytokine release by these treatments (Fig. 1*B*). Both LPS and palmitate increased the accumulation of IL-6 in the culture media ($P < 0.05$); consistent with the lack of stimulation of NF-κB reporter gene activity, DHA and laurate again had no effect. Because DHA antagonizes the onset and progression of inflammation in some cell types (21), we determined the extent to which DHA modulates inflammatory responses to LPS and palmitate in adipocytes

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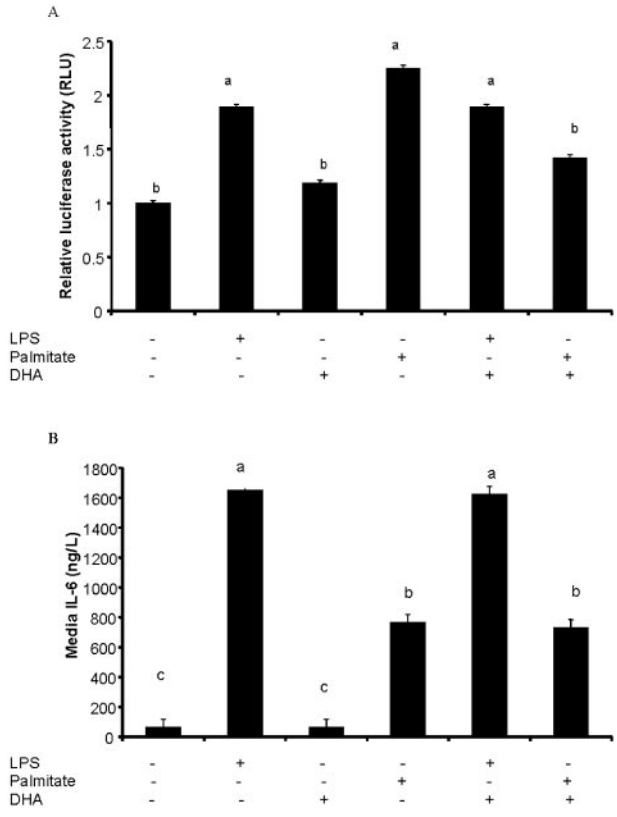

FIGURE 2 Regulation of LPS and palmitate-induced NF-κB luciferase activity and IL-6 media accumulation by DHA. Adipocytes in a serum-poor medium were pretreated for 6 h with 250 μmol/L DHA, and thereafter treated as indicated with LPS (100 μg/L) and 250 μmol/L palmitate for 24 h. (*A*) Normalized firefly luciferase activity. (*B*) Media IL-6 concentration. Bars represent means + SEM, $n = 3$ (*A*) or 4 (*B*) replicates. Means without a common letter differ, $P < 0.05$.

(**Fig. 2***A*). DHA attenuated ($P < 0.05$) the induction of the NF-κB reporter gene by palmitate, but did not antagonize the LPS response. Furthermore, the attenuation of palmitate- or LPS-induced NF-κB activation by DHA occurred without a concomitant reduction in IL-6 mRNA abundance (Fig. 2*B*).

To determine whether the effects of palmitate are dose dependent, we performed a dose titration with regard to IL-6 production. Palmitate increased ($P < 0.05$) both the expression (mRNA abundance, **Fig. 3***A*) and release of IL-6 into the culture medium (Fig. 3*B*). However, the magnitude of the increase was lower at 500 μmol/L than at 125 or 250 μmol/L. As with IL-6, palmitate increased TNFα mRNA abundance ($P < 0.05$), albeit to a lesser extent that that seen with IL-6 (Fig. 3C). Furthermore, there was no concomitant increase in the accumulation of this proinflammatory cytokine at the lower (125 μmol/L) concentration, and an actual reduction ($P < 0.05$) in media TNFα when palmitate was added at 250 and 500 μmol/L (Fig. 3*D*).

Nonesterified fatty acids must be acylated before metabolism. Therefore, we determined whether inhibition of fatty acyl Co-A synthase (FACS) with triacsin C altered the effect of palmitate on NF-κB reporter gene activity or the accumulation of IL-6 in the culture medium. The inhibitor clearly blocked ($P < 0.05$) the ability of palmitate to induce reporter gene activity (**Fig. 4***A*); however, the presence of the inhibitor actually augmented ($P < 0.05$) the palmitate-induced accumulation of IL-6 in the culture medium (Fig. 4*B*).

Finally, we sought to gain initial insights concerning the biochemical mechanisms underlying the enhancement of NF-κB activity and IL-6 production by palmitate by testing these responses in the presence of known inhibitors of mitogen-activated protein kinase/Erk kinase (MEK), protein kinase C (PKC) and phosphoinositide-3 kinase (PI3K). Inhibition of MEK with U0126, or PKC with Bisindolylmaleimide (Bis), did not alter the induction of NF-κB activity by palmitate (**Fig. 5***A*). Inhibition of PI3K with wortmannin increased ($P < 0.05$) reporter gene activity, and there was a marked additive effect ($P < 0.05$) of wortmannin and palmitate. Inhibition of MEK and PKC suppressed ($P < 0.05$) the palmitate-induced increase in IL-6 accumulation in the media (Fig. 5*B*). Wortmannin alone increased media IL-6 accumulation and also enhanced the effect of palmitate ($P < 0.05$).

DISCUSSION

Fatty acids and their metabolites act directly and indirectly to regulate metabolism and immune function through their interactions with specific enzymes (22,23) and receptors (20,24). Recent evidence indicates that palmitate activates the NF-κB transcription factor and induces the expression and secretion of IL-6 in human myotube cultures (11). Because IL-6 may act locally and systemically to induce insulin resistance (25), and because adipocytes are a major source of IL-6 (26,27,15) and a target for insulin-mediated glucose disposal (28), we hypothesized that palmitate would activate NF-κB and induce IL-6 production in these cells. Indeed, the data presented herein are the first to show that palmitate induces NF-κB transcriptional activity and increases IL-6 mRNA abundance and protein accumulation in the culture media of 3T3-L1 adipocytes.

Collectively, the data indicate that the mechanism by which palmitate activates NF-κB in adipocytes is independent of that by which IL-6 release into the culture medium is induced. DHA disrupted NF-κB activation by palmitate, whereas it was inconsequential in stimulating IL-6 release. Similarly, the kinase inhibitor data indicate that activation of NF-κB was largely independent of MEK and PKC activity, but IL-6 release was blocked by inhibition of either kinase. Finally, the activation of NF-κB by palmitate required FACS activity (and presumably, formation of palmitoyl CoA), whereas the stimulation of IL-6 release was enhanced by inhibition of this enzyme. However, when the regulation of NF-κB and IL-6 were discordant, we acknowledge the possibility that NF-κB or cytokine mRNA changes were perhaps transient. Nonetheless, it is quite likely that dietary and pharmacological strategies aimed at alleviating inflammation in adipocytes must of necessity target multiple inflammatory pathways.

Mechanistically, several points are pertinent to the activation of NF-κB and induction of IL-6. Palmitate is readily incorporated into diacylglycerol and activates PKC in some cell types (29–30). Furthermore, the activation of IKK, a pivotal step in the activation of NF-κB, by PKC in 3T3-L1 adipocytes is well established (14). Palmitate is also required for ceramide synthesis (9), and ceramide activates NF-κB in macrophages (31). However, the IL-6 promoter is regulated by multiple transcription factors, including C/CAAT enhancer binding protein, activator protein-1 (AP-1), and cyclic AMP response element binding protein, with NF-κB acting as the terminal signal for the activation of the enhanceosome (32). In the present study,

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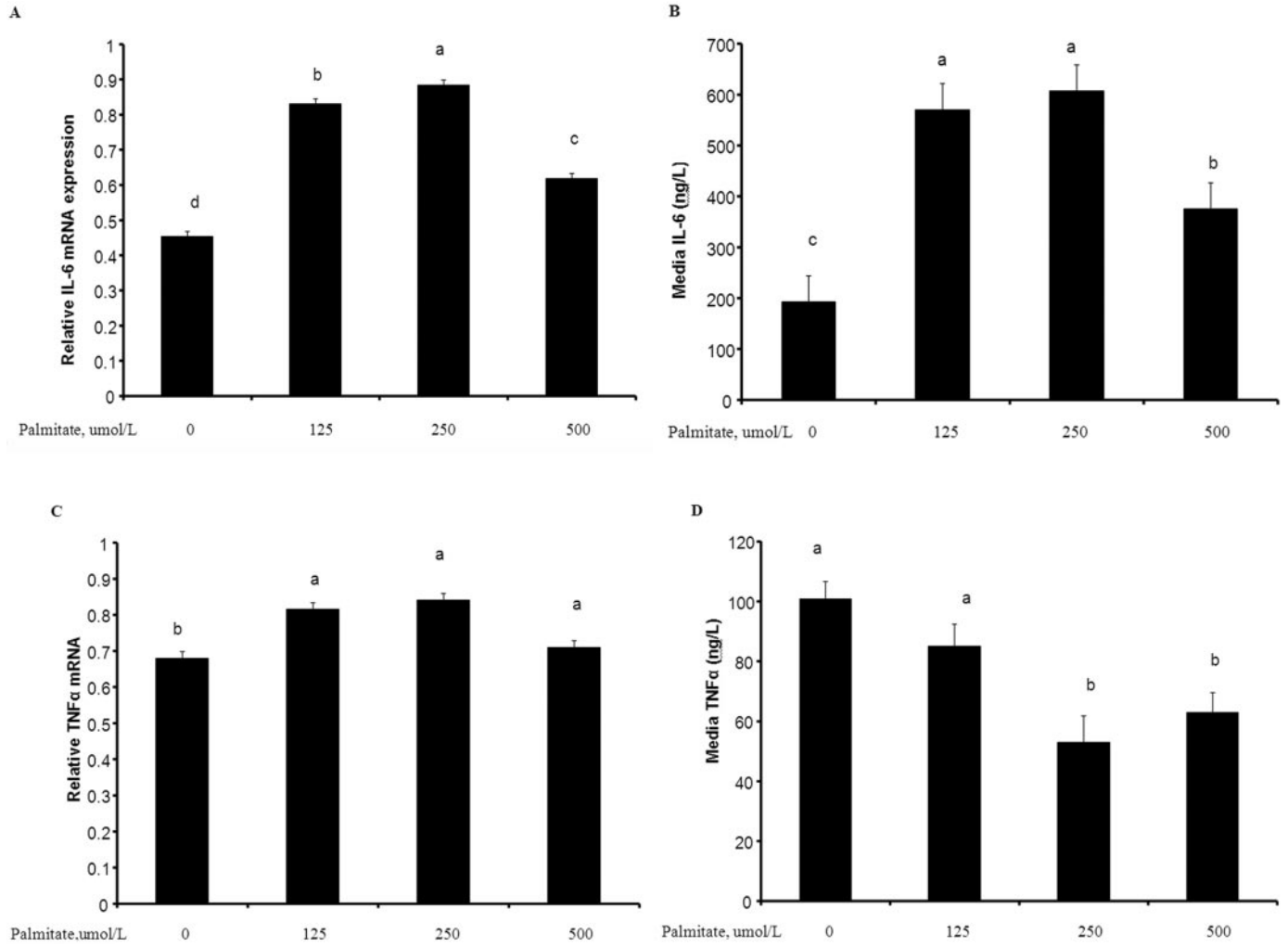

FIGURE 3 Dose dependency of induction of IL-6 and TNFα protein accumulation and mRNA expression by palmitate. Adipocytes were treated in serum-poor medium with palmitate (0.125, 250, and 500 μmol/L) for 24 h. Media and mRNA were collected. (*A*) IL-6 mRNA expression. Expression was normalized to β-actin. (*B*) Media IL-6 concentration. (*C*) TNFα mRNA expression. TNFα mRNA was normalized to β-actin. (*D*) TNFα accumulation in the media. Bars represent means + SEM, $n = 4$ replicates. Means without a common letter differ, $P < 0.05$.

the palmitate-induced accumulation of IL-6 was regulated independently of NF-κB activation, and earlier work in our laboratory, albeit with a primary pig adipocyte model, showed a clear disruption of the LPS-induced IL-6 expression by inhibition of MEK with no apparent reduction in NF-κB activation. This seemingly indicates the importance of AP-1 or other components of the enhanceosome in the regulation of IL-6 production in adipocytes. Thus, strategies to control IL-6 production in these cells must extend beyond the regulation of NF-κB; given that the accumulation of IL-6 in the media was blocked by inhibition of MEK or PKC, these pathways may be particularly important to the overall control of inflammation in adipocytes.

Weigert et al. (11) showed that free (i.e., nonacylated) palmitate per se is responsible for the induction of IL-6 expression in human myotubes, and because activation of NF-κB was required for the IL-6 response, it would seem that free palmitate also led to the activation of NF-κB. However, in our adipocyte model, disrupting acylation of palmitate with the FACS inhibitor actually antagonized the NF-κB–driven reporter gene activity while augmenting the accumulation of IL-6 in the culture media. As noted above, these findings in adipocytes indicate the discoordinate regulation of NF-κB and IL-6 production, and underscore the importance of other pathways. These potential regulatory differences between adipocytes and myocytes also highlight the necessity of detailed mechanistic studies of inflammation in specific cell types.

An additional major finding of this study pertains to the regulation of NF-κB and IL-6 production by palmitate and wortmannin. Lee et al. (21) determined that lauric acid activated NF-κB and induced COX-2 expression via a Tlr4 signaling pathway that is coupled to PI3K-Akt signaling in RAW264.7 macrophages. Our findings in 3T3-L1 adipocytes are inconsistent with these results obtained using the RAW264.7 macrophage and 293T cell models. In adipocytes, lauric acid was an ineffective activator of NF-κB and did not induce IL-6 accumulation in the medium. However, palmitate and wortmannin, a potent inhibitor of PI3K, acted additively to stimulate both NF-κB reporter gene activity and IL-6 accumulation in the medium. Others (33) obtained similar results in that inhibition of the PI3K-Akt pathways enhances the transcriptional activity of NF-κB (p65) in human monocytic cells. Thus, inhibition of PI3K,

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rather than activation, is associated with activation of NF-κB and the induction of IL-6 in adipocytes and human monocytes. It is possible that palmitate partially inhibits PI3K, and that wortmannin further suppresses activity of this enzyme. This is consistent with the induction of insulin resistance by palmitate, and we are currently testing this hypothesis.

Finally, we also determined that palmitate induces the mRNA expression of TNFα in adipocytes. However, the release of this proinflammatory cytokine into the culture medium was actually attenuated by palmitate in a dose-dependent manner, at least within the time line of the experiments conducted. Although we cannot rule out the possibility that degradation of the cytokine in the media affected measurable concentrations at the chosen endpoint, several additional points are pertinent. Xu et al. (34) showed that obesity is associated with suppressed cleavage of transmembrane TNFα from adipocytes. It seems possible that although the higher concentrations of palmitate induced TNFα expression, there was a concomitant disruption of transmembrane cleavage that was reflected in lower concentrations in the media. It is also possible that IL-6 released into the culture medium acted directly or indirectly in autocrine fashion to suppress palmitate-induced release of TNFα into the culture medium. IL-6 was shown previously to exert an anti-inflammatory effect through suppression of TNFα production in skeletal muscle (35). However, these possibilities must be addressed directly in adipocytes.

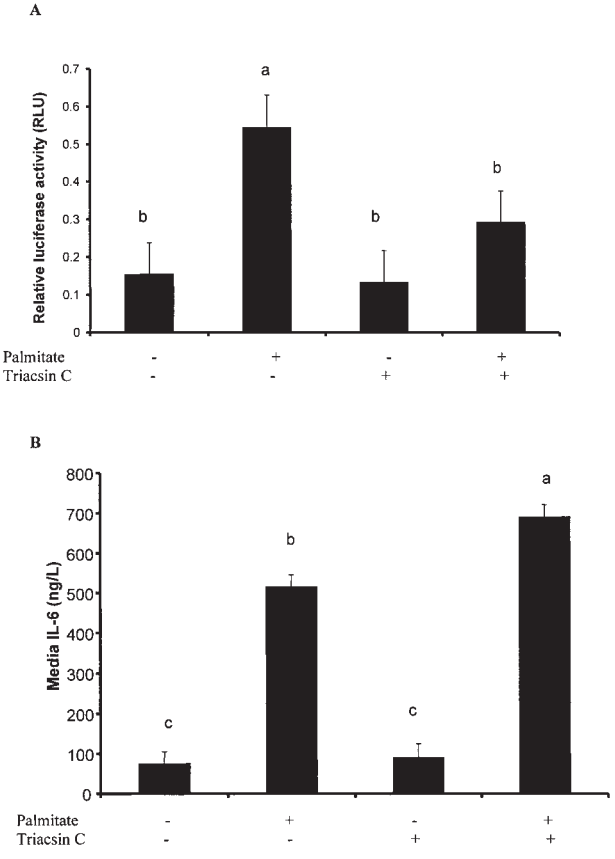

FIGURE 4 Regulation of palmitate-induced NF-κB luciferase activity and IL-6 media accumulation by triacsin C. 3T3-L1 adipocytes in serum-poor medium were pretreated for 2 h with 10 μmol/L triacsin C and thereafter treated as indicated with 250 μmol/L palmitate for 24 h. (*A*) NF-κB luciferase activation. (*B*) Media IL-6 concentration. Bars represent means + SEM, $n = 6$ replicates. Means without a common letter differ, $P < 0.05$.

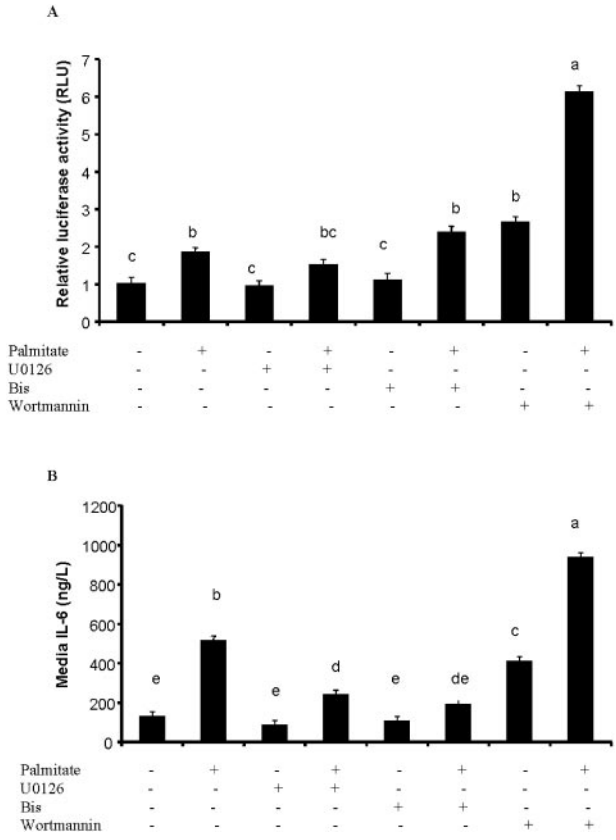

FIGURE 5 Regulation of palmitate-induced NF-κB activation and IL-6 media accumulation by kinase inhibitors. Pharmacological inhibitors to MEK (U0126), PKC (Bis), and PI3K (wortmannin) were used to pretreat cells for 1 h at concentrations of 15, 20, and 1 μmol/L, respectively, before adding palmitate (250 μmol/L) for 24 h. (*A*) Normalized NF-κB luciferase activation. (*B*) Media IL-6 concentration. Bars represent means + SEM, $n = 3$ (*A*) or 6 (*B*) replicates. Means without a common letter differ, $P < 0.05$.

In summary, the data presented herein support 3 major points regarding inflammation in adipocytes. First, the induction of markers of inflammation by palmitate, but not laurate or DHA, indicates that fatty acid–induced inflammation in adipocytes is not a generalized feature of fatty acids, but rather a specific one. Second, that the activation of the NF-κB and the induction of IL-6 release into the media are differentially regulated by inhibition of FACS indicates that 2 independent mechanisms are responding to acylated vs. nonacylated (free) palmitate. Finally, our data indicate clearly that the PI3K pathway is linked directly to the regulation of inflammation in adipocytes. The relevance of these findings in vivo remains to be determined, but our results implicate palmitate in the inflammation in adipose tissue that accompanies obesity.

ACKNOWLEDGMENTS

The authors are grateful to Paul Collodi, Young-Cheul Kim, and Jill Hutchcroft for helpful discussions of the data and their evaluations of the manuscript. We also appreciate the advice of Young-Cheul Kim and Nick Gabler with respect to the 3T3-L1 culture system.

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