Cyclooxygenase 2 Inhibition Exacerbates Palmitate-Induced Inflammation and Insulin Resistance in Skeletal Muscle Cells

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Palmitate-induced inflammation is involved in the development of insulin resistance in skeletal muscle cells. Here we evaluated the effect of the saturated fatty acid palmitate and the monounsaturated fatty acid oleate on Toll-like receptors (TLR)-2 and -4 and cyclooxygenase 2 (COX-2) expression and examined whether the inhibition of this enzyme modulates fatty acid-induced inflammation. Skeletal muscle cells exposed to palmitate showed enhanced TLR-2 and COX-2 mRNA levels, whereas oleate did not modify their expression. Palmitate-induced expression of these genes was dependent on nuclear factor (NF)-κB activation, because expression was reduced in the presence of the NF-κB inhibitor parthenolide. Co-incubation of palmitate-exposed cells with oleate also prevented the increase in the expression of TLR-2 and COX-2, through a mechanism that may involve activation of peroxisome proliferator-activated receptor-α (PPARα) by this monounsaturated fatty acid. COX-2 inhibition by NS-398 enhanced IL-6 and TNF-α expression and IL-6 protein secretion induced by palmitate. NF-κB binding activity and TNF-α mRNA levels were enhanced in palmitate-exposed cells in the absence or in the presence of NS-398, whereas coincubation of palmitate-exposed cells with NS-398 and prostaglandin E₂ (PGE₂) prevented these changes. In contrast, 12-lipoxygenase and cytochrome P450 hydroxylase pathways were not involved in these changes. Similarly, COX-2 inhibition impaired insulin-stimulated Akt phosphorylation and 2-deoxy-D-[14C]glucose uptake in palmitate-exposed skeletal muscle cells, and this effect was abolished in the presence of PGE₂. These findings indicate that COX-2 activity, through the production of PGE₂, attenuates the fatty acid-induced inflammatory process and insulin resistance. (Endocrinology 151: 537–548, 2010)

Plasma free fatty acids (FFAs) are elevated in obese individuals and animals and play a critical role in the development of insulin resistance (1). Experimental evidence indicates that FFAs are a primary trigger for insulin resistance in both animal models and humans (1, 2). FFAs may cause insulin resistance in skeletal muscle through several mechanisms, including activation of proinflammatory pathways, linking the development of this pathology with a low-grade chronic systemic inflammatory response (3). Thus, evidence suggests that high FFA levels increase their flux in skeletal muscle, exceeding its oxidation (4). This in turn leads to increased FFA metabolites that stimulate inflammatory pathways, including the proinflammatory transcription factor nuclear factor (NF)-κB, leading to impaired insulin signaling (5). In addition, recent reports indicate that FFAs also stimulate inflammatory
pathways in skeletal muscle cells through direct activation of plasma membrane receptors (6). Consistent with this, it has been reported that FFAs may serve as ligands for several members of the Toll-like receptor (TLR) family (7, 8). TLRs 2 and 4 play an important role in the innate immune system by activating inflammatory pathways in response to a wide spectrum of exogenous and endogenous ligands, including the lipopolysaccharide (LPS) from Gram-negative bacterial cell walls (9). The critical component of LPS involved in the activation of TLRs is the lipid A moiety, which consists almost entirely of fatty acids (10). In macrophages, adipocytes, and skeletal muscle cells, saturated fatty acids are potent activators of TLR-2 and TLR-4 (6, 11). In the presence of ligands, the TLR-4 complex (including its coreceptors cluster of differentiation 14 and myeloid differentiation protein 2) recruits the adaptor protein myeloid differentiation factor-88 (MyD88), which in turn leads to the auto-phosphorylation of IL-1 receptor-associated kinase (IRAK). Finally, when IRAK is activated, it interacts with TNF-associated factor 6 (TRAF6), which leads to NF-κB activation.

Thus, whatever the mechanism involved, whether intracellular FFA metabolite accumulation or TLR activation, elevated FFAs finally result in NF-κB activation. Once activated, NF-κB stimulates the transcription of several inflammatory genes including IL-6, cyclooxygenase 2 (COX-2), and TNF-α (12), which may modulate insulin signaling. NF-κB activation by the saturated fatty acid palmitate has also been implicated in the increase in TLR-4 and TLR-2 expression observed in skeletal muscle of type 2 diabetic subjects (13). In these patients, the increased availability of these receptors may potentiate the effect of fatty acids on inflammation and insulin resistance. Thus, those strategies aimed at preventing NF-κB activation may reduce inflammation in skeletal muscle cells. Of these strategies, we recently reported that the monounsaturated fatty acid oleate prevents inflammation caused by palmitate in skeletal muscle cells (14). However, further research is necessary to ascertain whether oleate prevents the increase in TLR and COX-2 expression caused by saturated fatty acids.

COX-2, also known as prostaglandin-endoperoxide synthase-2, synthesizes pro- and anti-inflammatory prostanoids that may affect the development of inflammation and, therefore, insulin resistance. Chronic COX-2-mediated inflammation seems to be involved in the development of insulin resistance because type 2 diabetes mellitus in Pima Indians has been associated with a promoter variant in the inducible COX-2 gene (15), and COX-mediated inflammation and oxidative stress has been related to type 2 diabetes mellitus in elderly men (16). Moreover, a recent report suggests that chronic COX-2-mediated inflammation in fat is crucial for obesity-linked insulin resistance (17). However, an increasing number of recent reports have revealed that prostaglandins can exert a protective role during inflammation (18). In particular, some prostaglandins increase during the resolution phase of inflammation and alleviate this process in animal models (19–21). Moreover, reduced production of prostaglandins due to genetic deficiency or through administration of COX-2 inhibitors may even worsen inflammation (22–24). Furthermore, inhibition of COX-2 may increase the availability of arachidonic acid to be transformed by lipoxigenase and cytochrome P450 and by no enzymatic oxidative modification (25), leading to enhanced synthesis of active compounds that may modulate the inflammatory process. Therefore, COX-2 may differently modulate inflammation.

The purpose of this research was to study the effects of saturated and monounsaturated fatty acids on COX-2 and TLR expression in skeletal muscle cells and to investigate whether COX-2 inhibition modulates fatty acid-induced inflammation. Our results indicate that whereas palmitate increases the expression of COX-2 and TLR-2 through a NF-κB-dependent mechanism, oleate does not. Oleate prevented palmitate-induced up-regulation of COX-2 and TLR-2 through a mechanism that involves peroxisome proliferator-activated receptor-α (PPARα) activation. Finally, COX-2 inhibition enhanced palmitate-induced IL-6 and TNF-α expression and IL-6 secretion in skeletal muscle cells, indicating that COX-2 activity attenuates fatty acid-induced inflammation.

Materials and Methods

Materials

Fatty acids, Wy-14,643, MK886, prostaglandin E2 (PGE2), baicalein, and 17-octadecynoic acid (17-ODYA) were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture

Mouse C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum, 50 U/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 an additional period of 4 d, the differentiated C2C12 cells had fused into myotubes. Lipid-containing media were prepared by conjugation of FFA with FFA-free BSA, using a modified version of the method described by Chavez and Summers (26). Briefly, FFAs were dissolved in ethanol and diluted 1:100 in DMEM containing 2% (wt/vol) fatty acid-free BSA. Myotubes were incubated for 16 h in serum-free DMEM containing 2% BSA in either the presence (FFA-treated cells) or absence (control cells) of FFAs. After incubation, RNA was extracted from myotubes as
described below. Culture supernatants were collected, and the secretion of IL-6 was assessed by ELISA (Invitrogen, Carlsbad, CA) and the levels of PGE$_2$ by EIA (Cayman Chemical, Ann Arbor, MI).

**Measurements of mRNA**

Levels of mRNA were assessed by RT-PCR, as described elsewhere (14). Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston, TX). 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 as follows: IL-6, 5'-TCCAGCCAGTGCTCCCGGA-3' and 5'-TTGTTCCCCCTTCTTCT-3'; TNF-$
\alpha$, 5'-GGGCTCACACACTCCACCA-3' and 5'-GGTGGTGAGTTGGAACTGG-3'; COX-2, 5'-ATGGGTGAGTGGGTTGAC-3' and 5'-TCGCCATAGTAGGACTGCCCCCC-3'; and Aprt (adenosyl phosphoribosyl transferase), 5'-GCCCTGGGCAATCTGAGGACA-3' and 5'-CCAGGCYCTACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (TLR-2, 151 bp; COX-2, 163 bp; TNF-$
\alpha$, 284 bp; IL-6, 229 bp; and Aprt, 329 bp). Preliminary experiments were carried out with varying amounts of cDNA to determine nonsaturating conditions of PCR amplification (TLR-2, 22 cycles; COX-2, 21 cycles; TNF-$
\alpha$, 25 cycles; IL-6, 32 cycles) for all the genes studied. Thus, under these conditions, relative quantification of mRNA was assessed using the RT-PCR method described in this study (27). Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging, Marne-la-Vallée, France). The results for the expression of specific mRNAs are always given in relation to the expression of the control gene (Aprt).

**Isolation of nuclear extracts and EMSA**

Nuclear extracts isolation and EMSA were performed as described elsewhere (14).

**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 $\mu$g/ml aprotinin). The homogenate was centrifuged at 10,000 x g for 30 min at 4°C. Protein concentration was measured by the Bradford method. Total and nuclear proteins (30 $\mu$g) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against total (Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-Akt (Ser473). Detection was achieved using the EZ-CL Immulocimesence detection kit (Biological Industries, Beit Hemeek, Israel). The equal loading of proteins was assessed by red phenol staining. The size of detected proteins was estimated using protein molecular mass standards (Invitrogen, Barcelona, Spain).

2-Deoxy-d-$[^{14}$C]glucose (2-DG) uptake experiments

Determination of 2-DG uptake was performed as reported elsewhere (28).

**Statistical analyses**

Results are expressed as the mean ± sd of six separate experiments. Significant differences were established by one-way ANOVA using the computer program GraphPad Instat version 2.03 (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**

Palmitate increases TLR-2 mRNA levels through a mechanism that does not require COX-2 activity

First we studied the time-response effects of 0.5 mM palmitate on the mRNA levels of several inflammatory markers in skeletal muscle cells (Fig. 1A). Whereas palmitate treatment for 1 h caused a nonsignificant reduction in COX-2 mRNA levels, a significant increase was observed after 8 h (3.3-fold, P < 0.01) and 16 h (22-fold, P < 0.001) in palmitate-exposed cells compared with control cells. In contrast, the mRNA levels of TNF-$\alpha$ were barely detectable until cells were exposed to palmitate for 16 h, when a strong induction was observed. IL-6 mRNA levels followed a similar profile to that reported for COX-2, with huge inductions in cells exposed to palmitate for 8 and 16 h.

When we analyzed TLR-4 and TLR-2 mRNA levels, no changes were observed in the former, whereas TLR-2 mRNA levels showed a transient increase (2-fold induction, P < 0.001) after 8 h and then returned to basal levels in cells exposed to palmitate for 16 h (Fig. 1B).

Because it has been reported that several prostaglandins may modulate TLR-2 expression (29), we then evaluated whether the effect of palmitate on TLR-2 expression was dependent on COX-2 activity. To this end, cells were incubated with palmitate for 8 h either in the presence or in the absence of the COX-2-specific inhibitor NS-398. Inhibition of COX-2 activity by NS-398 did not affect either TLR-2 or COX-2 mRNA levels (Fig. 2), indicating that in this cellular model, the increase in the expression of these genes was independent of prostaglandin synthesis. As a control, we measured PGE$_2$ secretion to the culture media as a surrogate marker of COX-2 activity. In agreement with the increase in COX-2 mRNA levels, palmitate exposure caused a 2.3-fold increase (P < 0.001 vs. control cells) in PGE$_2$ levels, whereas in cells exposed to palmitate plus NS-398, this increase was abolished.

**Induction of COX-2 and TLR-2 mRNA levels by palmitate are mediated through NF-$\kappa$B activation**

It has been reported that activation of NF-$\kappa$B is both sufficient and necessary to induce maximal expression of COX-2 (30). Therefore, we investigated whether palmitate-induced COX-2 expression was mediated through the activation of NF-$\kappa$B in skeletal muscle cells by using the NF-$\kappa$B inhibitor parthenolide, which specifically inhibits
the activity of this proinflammatory transcription factor by preventing the degradation of inhibitor of NF-κB (31). In the presence of parthenolide, the palmitate-mediated induction of COX-2 was almost completely abolished (Fig. 3A). Likewise, the NF-κB inhibitor prevented the increase in TLR-2 mRNA caused by palmitate (Fig. 3B). These findings are consistent with those of previous studies (13, 30) and demonstrate that the saturated fatty acid palmitate increases COX-2 and TLR-2 mRNA levels through a mechanism involving NF-κB activation.

**Oleate prevents palmitate-mediated increases in COX-2 and TLR-2 mRNA levels**

In contrast to the effects of the saturated fatty acid palmitate, we previously reported elsewhere that the monounsaturated fatty acid oleate does not stimulate NF-κB in skeletal muscle cells (14). In addition, coincubation of palmitate-exposed cells with oleate prevented NF-κB activation (14). Therefore, in this research we investigated whether oleate affected COX-2 and TLR-2 mRNA levels and whether this fatty acid prevented palmitate-induced expression of these genes. Exposure of skeletal muscle cells to 0.5 mM oleate affected neither COX-2 nor TLR-2 mRNA levels compared with control cells (Fig. 3, C and D). Moreover, when cells were coincubated with 0.5 mM palmitate and 0.3 mM oleate, the increase in COX-2 and TLR-2 mRNA levels caused by palmitate was completely abolished. These data indicate that low concentrations of oleate prevent palmitate-mediated inflammation in skeletal muscle cells.

**PPARα activation inhibits the palmitate-mediated increase in COX-2 mRNA**

Because we have previously reported elsewhere that oleate activates PPARα in skeletal muscle cells (14) and
PPARα agonists prevent phorbol myristate acetate-induced expression of COX-2 in human colorectal carcinoma cells (32), in this study, we evaluated whether the PPARα agonist Wy-14,643 prevented the induction in COX-2 and TLR-2 mRNA levels caused by palmitate. When palmitate-exposed cells were coincubated with Wy-14,643, a significant reduction was observed in COX-2 mRNA expression (9.8-fold induction compared with 18-fold induction in cells exposed to palmitate only (P < 0.05) (Fig. 4A). Similarly, coincubation with Wy-14,643 significantly reduced the induction in TLR-2 mRNA levels caused by palmitate (2-fold vs. 2.6-fold induction, P < 0.05) (Fig. 4B). To demonstrate that oleate reduces palmitate-mediated induction of COX-2 mRNA levels by activating PPARα, we used MK886, an antagonist of this transcription factor. In the presence of MK886, the effect of oleate on COX-2 mRNA levels in cells exposed to palmitate was partially reversed, suggesting that COX-2 expression was down-regulated by oleate, at least in part, through a PPARα-dependent mechanism (Fig. 5C).

**COX-2 inhibition enhances palmitate-induced IL-6 and TNF-α expression**

Because it has been reported that prostaglandins may limit stimulus-induced cytokine secretion in macrophages (33) we evaluated the contribution of COX-2 to palmitate-induced inflammation. Skeletal muscle cells co-incubated with palmitate and the COX-2 inhibitor NS-398 showed higher IL-6 and TNF-α mRNA levels than those exposed to palmitate alone (Fig. 5, A and B). In addition, when we evaluated the secretion of IL-6 protein levels, we observed that coincubation of the cells with palmitate and NS-398 caused a 22-fold increase in the secretion of this cytokine compared with control cells (813 ± 49 vs. 37 ± 2 pg/ml, P < 0.001), which was 3-fold higher than the induction attained in cells exposed to palmitate alone (259 ± 18 vs. 37 ± 2 pg/ml, P < 0.001) (Fig. 5C).

**PGE2 prevents the proinflammatory effect and ameliorates insulin resistance caused by COX-2 inhibition in palmitate-exposed skeletal muscle cells**

The proinflammatory effect caused by COX-2 inhibition in palmitate-exposed cells can be related to the reduction in PGE2 synthesis or to the increased availability of arachidonic acid to be transformed by 12-lipoxygenase and cytochrome P450 hydroxylase, leading to increased levels of 12-hydroxyeicosatetraenic acid (12-HETE) and 20-HETE, respectively, which can elicit proinflammatory actions (34, 35). We examined the contribution of these mediators to palmitate-mediated inflammation by measuring NF-κB DNA-binding activity by EMSA. NF-κB formed three complexes with nuclear proteins (Fig. 6A), and specificity of the DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide. Palmitate-exposed cells in the presence or in the absence of NS-398 showed an increase in NF-κB DNA-binding activity, mainly of complex I, compared with control cells, whereas in cells coincubated with palmitate plus NS-398 and PGE2, the increase in NF-κB DNA-binding activity was abolished. No changes were observed in the NF-κB DNA-binding activity of control cells.
DNA-binding activity when cells were incubated with palmitate and NS-398 and baicalein or 17-ODYA, inhibitors of the 12-lipoxygenase and cytochrome P450 hydroxylase, respectively. Similarly, cells incubated with 12-HETE and 20-HETE did not show increased NF-κB DNA-binding activity. Addition of antibody against the p65 subunit of NF-κB supershifted the complexes, indicating that these bands mainly consisted of this subunit. In agreement with these changes, PGE2 addition to skeletal muscle cells incubated with palmitate plus NS-398 prevented the increase in TNF-α mRNA levels (Fig. 6B). Finally, we assessed whether COX-2 inhibition impaired insulin sensitivity in palmitate-exposed skeletal muscle cells by measuring insulin-stimulated Akt phosphorylation. Palmitate exposure reduced insulin-stimulated Akt phosphorylation, and this reduction was more intense in palmitate-exposed cells coincubated with the COX-2 inhibitor NS-398 (Fig. 6C). Interestingly, insulin sensitivity was restored in cells coincubated with palmitate plus NS-398 and PGE2, whereas inhibitors of 12-lipoxygenase and cytochrome P450 hydroxylase had no effect. A similar trend was observed when we assessed 2-DG uptake (Fig. 6D). Palmitate reduced insulin-stimulated 2-DG uptake, and the reduction was more intense in cells exposed to palmitate plus NS-398, whereas in the presence of PGE2, the effect of NS-398 was blunted.

Discussion

In this research, we evaluated the effect of the most abundant saturated and monounsaturated fatty acids in plasma (36), palmitate, and oleate, respectively, on the mRNA levels of TLR-2 and COX-2 in skeletal muscle cells. TLR-2 and TLR-4 are considered the receptors that mediate FFA-induced activation of inflammatory pathways and metabolic signaling in insulin resistance (6, 11), mainly through NF-κB activation. Once activated, either by TLR receptors or intracellular accumulation of FFA metabolites, NF-κB increases the transcription of proinflammatory genes, such as IL-6 and TNF-α, which modulate insulin signaling. COX-2 gene expression is also under the control of NF-κB, and it may modulate the inflammatory process in skeletal muscle cells through the synthesis of prostaglandins. Long regarded as proinflammatory molecules, some prostaglandins also have antiinflammatory effects (37). However, the contribution of COX-2 to fatty acid-induced inflammation in skeletal muscle cells remains unknown.

Exposure of skeletal muscle cells to palmitate increased TLR-2 and COX-2 mRNA levels. However, whereas a transient increase in TLR-2 expression was observed after 8 h palmitate exposure, the increase in COX-2 mRNA levels began at 8 h and peaked at 16 h. The induction of
other inflammatory genes, such as IL-6, also began at 8 h, indicating that the increase in the transcription of NF-κB target genes mediated by palmitate requires this time period. However, 16 h palmitate treatment was necessary for the induction of TNF-α. Although it has been reported that activation of apoptotic pathways by palmitate may contribute to explain part of its effects (38), we found that α-spectrin protein levels were not different between control and palmitate-exposed cells for 16 h (data not shown), suggesting that neither caspase-3 nor calpain pathways were increased by palmitate, making unlikely the involvement of apoptosis in the changes observed. In contrast, we have demonstrated that the increase in TLR-2 mRNA levels by palmitate is dependent on NF-κB activation, whereas prostaglandins are unlikely to be involved. Contrary to TLR-2, TLR-4 mRNA levels were not affected by palmitate treatment. In fact, it has been reported that TLR-2 is essential for the development of palmitate-induced inflammation and insulin resistance in C2C12 skeletal muscle cells (11). Similarly, it has been reported that in 3T3-L1 adipocytes palmitate also increased TLR-2 mRNA levels, whereas TLR-4 expression was not altered (39). In addition, knockdown of TLR-2 prevented the increase in TNF-α caused by FFAs (40), and in vivo inhibition of TLR-2 improved skeletal muscle insulin sensitivity (41). All these data suggest that TLR-2 contributes to fatty acid-induced inflammation and insulin resistance in several tissues, which is consistent with the significantly higher frequency of polymorphisms in the TLR-2 gene that seems to correlate to populations at higher risk of insulin resistance (42). Furthermore, the mRNA levels of both TLR-2 and TLR-4 in human myotubes were significantly higher in obese and type 2 diabetic patients than in lean patients, and the increase in TLR-4 expression after palmitate treatment was dependent on NF-κB activation (13). In this study, we also observed that the palmitate-mediated increase in COX-2 mRNA levels was dependent on NF-κB activation, which is consistent with the fact that human and murine COX-2 genes contain two putative NF-κB binding sites in their 5′-flanking regions (43). Therefore, in our cell culture model, palmitate-mediated activation of the inhibitor of NF-κB kinase β (IKKβ)-NF-κB pathway contributes to the increase in TLR-2 and COX-2 mRNA levels. In addition, activation of this pathway in vitro contributes to insulin resistance, because its inhibition prevents the detrimental effects of palmitate on the metabolic actions of insulin (44). However, in vivo, activation of the IKKβ-NF-κB pathway in skeletal muscle does not seem to be an important local mediator of insulin resistance. Thus, Polkinghorne et al. (45) found no effect of IKKβ activation on insulin-stimulated glucose disposal into muscle. Similarly, transgenic overexpression of IKKβ showed no effect on whole-body glucose tolerance (46), and muscle-specific deletion of IKKβ did not prevent obesity-induced insulin resistance in mice (47). These studies do not rule out the possibility of a NF-κB-mediated induction of TLR-2 and COX-2 expression in vivo, because in

FIG. 4. Oleate prevents the palmitate-mediated increase in COX-2 mRNA levels by activating PPARα. A and B, Analysis of the mRNA levels of COX-2 (A) and TLR-2 (B) in C2C12 myotubes incubated with 0.5 mM palmitate for 8 h in the absence or in the presence of the PPARα activator Wy-14,643 (10 μM); C, analysis of the mRNA levels of COX-2 in C2C12 myotubes incubated with 0.5 mM palmitate for 8 h in the absence or in the presence of different fatty acids (0.5 mM palmitate, 0.5 mM oleate, or 0.5 mM palmitate supplemented with 0.3 mM oleate) and the PPARα antagonist MK886 (10 μM). Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as means ± sd of six independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. control cells; #, P < 0.05; ###, P < 0.001 vs. palmitate-treated cells; @, P < 0.05 vs. cells exposed to palmitate plus oleate.
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human skeletal muscle from obese and type 2 diabetic patients, Reyna et al. (13) found reduced inhibitor of \( \kappa \)B levels, suggesting activation of the \( \text{IKKB-NF-\( \kappa \)B} \) pathway. However, additional studies are necessary to evaluate how changes in TLR and COX-2 expression and activity in vivo affect skeletal muscle insulin resistance.

Fatty acids differ in their contribution to inflammation and insulin resistance. In fact, whereas saturated fatty acids promote insulin resistance (48, 49), oleic acid improves insulin sensitivity (36, 50, 51). However, the mechanisms by which oleate may ameliorate insulin sensitivity are not well understood. Here we report that oleate, in contrast to palmitate, does not induce the diacylglycerol/protein kinase \( \text{C0H}\) NF-\( \kappa \)B pathway, and as a result, the expression of inflammatory markers (IL-6 and TNF-\( \alpha \)) was not induced (14). In addition, the lack of effect of oleate on inflammation could be also attributed to its effects on TLR-2 signaling, because it has been reported that unsaturated FFAs do not activate this pathway (8). Therefore, oleate, in contrast to palmitate, is not proinflammatory. Interestingly, oleate, even at a lower concentration (0.3 mm), abolished the induction in COX-2 and TLR-2 mRNA levels caused by 0.5 mm palmitate, which is in agreement with previous studies showing that oleate prevents the deleterious effects of palmitate on insulin signaling in muscle (52, 53). Although several mechanisms may be involved, here we show that PPAR\( \alpha \) activation by Wy-14,643 reduces palmitate-mediated expression of COX-2 and TLR-2. In fact, in the presence of the PPAR\( \alpha \) antagonist MK886, the effect of oleate on COX-2 mRNA levels was partially reversed. These findings indicate that oleate may prevent palmitate-mediated NF-\( \kappa \)B activation and COX-2 mRNA up-regulation by activating PPAR\( \alpha \), as we reported previously (14). In fact, it is well known that PPAR\( \alpha \) inhibits NF-\( \kappa \)B activation through several mechanisms (54).

Additional mechanisms not explored in this work may also contribute to prevent palmitate-induced inflammation by oleate. In macrophages, it has been reported that unsaturated fatty acids, including oleic acid, inhibit lauric acid-induced activation of NF-\( \kappa \)B and COX-2 expression (7, 43, 55). In the case of unsaturated fatty acids, such as docosahexaenoic acid, the target of this inhibition was TLR-2 itself or its associated molecules, but not downstream signaling components (7). Further research is needed to determine whether oleate acts in a similar way in skeletal muscle cells to prevent palmitate-induced inflammation. A recent report indicates that LPS and lipopeptide contamination of some fatty acid-free-BSA preparations is responsible for the activation of TLR receptors by saturated fatty acids, casting doubts on whether saturated fatty acids induce TLR signaling (56). In our conditions, we found huge differences in COX-2 expression between palmitate and oleate, both fatty acids complexed to BSA, indicating that it was the kind of fatty acid, and not the BSA, that was responsible for the changes observed.

Finally, we explored the contribution of COX-2 to the development of palmitate-induced inflammation in skeletal muscle cells by using a selective inhibitor of this enzyme. Our findings demonstrate that COX-2 inhibition amplifies the inflammatory process caused by palmitate. This is consistent with previous studies showing that PGE\(_2\) and 15-deoxy-\( \Delta^{12,14}\)-prostaglandin J2 inhibit TNF-\( \alpha \) expression in macrophages (33, 57). Therefore, these data suggest that prostaglandins can limit the acute inflammatory process and that COX-2 inhibition may amplify it. Furthermore, inhibition of COX-2 may increase the availability of arachidonic acid to be transformed by lipooxygenase and cytochrome P450 and by no enzy-
matic oxidative modification (25). The increase in these transformations may lead to enhanced synthesis of active compounds that may modulate the inflammatory process. Thus, 12-lipoxygenase and its major product 12-HETE have been reported to increase the expression of IL-6 and TNF-α/H9251 in macrophages (34). Likewise, the product of the cytochrome P450 hydroxylase, 20-HETE, stimulates NF-κB and the production of inflammatory cytokines in human endothelial cells (35). Our findings suggest that the increase in inflammation and insulin resistance attained by COX-2 inhibition in palmitate-exposed cells is caused by the reduction in PGE2, because coincubation with this prostaglandin prevents these changes. PGE2, the best known and most well-studied prostaglandin, plays a critical role in inflammation. However, its role is complex, and both pro- and antiinflammatory properties have been reported for this prostaglandin. For instance, most of the effects of PGE2 in macrophages are antiinflammatory (58). Interestingly, peritoneal macrophages from diabetes-prone Bio-Breeding (BB) rats secrete more TNF-α/H9251 than macrophages from diabetes-resistant BB or normal Wistar rats (59), and this increased secretion has been related to a defect in PGE2 production, making feedback inhibition by this prostaglandin insufficient and leading to prolonged secretion of TNF-α/H9251. Interestingly, it has been reported that inhibition of COX-2-derived PGE2 may enhance LPS-induced atherosclerosis by increasing macrophage production of TNF-α/H9251 (60). In addition, in dendritic cells, it has been reported that COX-2-generated PGE2 also inhibits IL-6 and TNF-α production (61).
increase in inflammation and insulin resistance after COX-2 inhibition in palmitate-exposed skeletal muscle cells.

Based on the data shown here, we hypothesize that during acute inflammation, COX-2 up-regulation in skeletal muscle cells leads to increased synthesis of PGE2, which limits the extension of inflammation by reducing the expression of IL-6 and TNF-α. Because COX-2 expression is under the control of NF-κB, the more potent the inflammatory process, the higher the synthesis of these prostaglandins. Likewise, those strategies that reduce inflammation (oleate and PPAR activators) also down-regulate the expression of COX-2 and the synthesis of these prostaglandins. It remains to be studied whether in populations prone to suffer insulin resistance (e.g., obese patients) the use of COX-2 inhibitors might worsen inflammation and insulin sensitivity. However, although the action of prostaglandins may limit the extension of acute inflammation in some cells (macrophages and skeletal muscle cells), it has been reported that when inflammation becomes chronic, COX-2 up-regulation in white adipose tissue contributes to an increase in inflammation (17), suggesting that the effects resulting from COX-2 inhibition can be cell type specific.

To summarize, the findings of this study imply the possibility that cellular expression of COX-2 and TLR-2 in skeletal muscle cells can be differentially regulated by palmitate and oleate. Furthermore, COX-2 inhibition enhances palmitate-induced inflammation in skeletal muscle cells, suggesting that prostaglandin synthesis may attenuate this process, at least during the acute phase of the inflammatory process.

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