Different impacts of saturated and unsaturated free fatty acids on COX-2 expression in C\textsubscript{2}C\textsubscript{12} myotubes

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Kadotani A, Tsuchiya Y, Hatakeyama H, Katagiri H, Kanzaki M. Different impacts of saturated and unsaturated free fatty acids on COX-2 expression in C\textsubscript{2}C\textsubscript{12} myotubes. Am J Physiol Endocrinol Metab 297: E1291–E1303, 2009. First published September 15, 2009; doi:10.1152/ajpendo.00293.2009.—In skeletal muscle, saturated free fatty acids (FFAs) act as proinflammatory stimuli, and cyclooxygenase-2 (COX-2) is a pro/anti-inflammatory enzyme induced at sites of inflammation, which contributes to prostaglandin production. However, little is known about the regulation of COX-2 expression and its responses to FFAs in skeletal muscle. Herein, we examined the effects of saturated and unsaturated FFAs, including a recently identified lipid (lipid hormone derived from adipocytes), palmitoleate, on COX-2 expression in C\textsubscript{2}C\textsubscript{12} myotubes as a skeletal muscle model. Exposure of myotubes to saturated FFAs [palmitate (16:0) and stearate (18:0)], but not to unsaturated FFAs [palmitoleate (16:1), oleate (18:1), and linoleate (18:2)], led to a slow-onset induction of COX-2 expression and subsequent prostaglandin E\textsubscript{2} production via mechanisms involving the p38 MAPK and NF-κB but not the PKC\textsubscript{δ} signaling cascades. Pharmacological modulation of mitochondrial oxidative function failed to interfere with COX-2 expression, suggesting the mitochondrial overload/excessive β-oxidation contribution to this event to be minimal. On the contrary, unsaturated FFAs appeared to effectively antagonize palmitate-induced COX-2 expression with markedly different potencies (linoleate > oleate > palmitoleate), being highly associated with the suppressive profile of each unsaturated FFA toward palmitate-evoked intracellular signals, including p38, JNK, ERK1/2 MAPKs, and PKC\textsubscript{δ}, as well as IkB degradation. In addition, our data suggest little involvement of PPAR in the protective actions of unsaturated FFAs against palmitate-induced COX-2 expression. No direct contribution of the increased COX-2 activity in generating palmitate-induced insulin resistance was detected, at least in terms of insulin-responsive Akt phosphorylation and GLUT4 translocation. Taken together, our data provide a novel insight into the molecular mechanisms responsible for the FFA-induced COX-2 expression in skeletal muscle and raise the possibility that, in skeletal myocytes, COX-2 and its product prostaglandins may play an important role in the complex inflammation responses caused by elevated FFAs, for example, in the diabetic state.

cyclooxygenase-2; inflammation; signal transduction; glucose transporter 4; insulin resistance

ACCUMULATING EVIDENCE DEMONSTRATES A POTENTIAL LINK BETWEEN METABOLIC DISORDERS (such as type 2 diabetes, obesity, and atherosclerosis) and chronic low-grade inflammation, characterized by abnormal cytokine production and activation of a network of inflammatory signaling pathways in insulin target tissues such as fat, liver, and skeletal muscle (26, 54). Although these inflammatory responses have been recognized as being triggered by lipid-laden adipose tissue under obese conditions (20), recent studies demonstrate that skeletal muscle also secretes a number of proinflammatory cytokines, [e.g., interleukin (IL)-6, tumor necrosis factor (TNF)\textalpha] in response to various stimuli including exercise (39) and elevated levels of free fatty acids (FFAs) (29, 30). Indeed, it has been reported that exposure of skeletal muscle cells to saturated FFAs, especially palmitate (C16:0), induces insulin resistance and increased secretion of these proinflammatory cytokines; however, oleate (C18:1), an unsaturated FFA, does not have such deleterious effects, instead antagonizing these palmitate-induced responses (9, 10, 29, 30). Intriguingly, palmitoleate (C16:1), another unsaturated FFA derived from adipose tissue and thereby termed a lipokine (lipid hormone), has been suggested to directly regulate skeletal muscle metabolism and insulin responsiveness (5). However, details of the mechanisms underlying the actions of saturated and unsaturated FFAs and the potential role of palmitoleate in pro/anti-inflammatory responses of skeletal muscle remain unclear.

Several lines of evidence have also indicated the involvement of inducible cyclooxygenase (COX-2) and its proinflammatory prostaglandin (PG) products in metabolic disorders, including type 2 diabetes (28, 32). On the other hand, some COX-2 metabolites, such as cyclopentenone PGs, have been shown to exert anti-inflammatory actions (18, 45). In contrast, constitutively expressed COX-1, COX-2 is promptly induced by various proinflammatory stimuli (17). COX-2 can convert arachidonate (20:4) released by cytosolic phospholipase A2 from membrane phospholipids into PGG\textsubscript{2}, which is further metabolized into biologically active end products such as PGE\textsubscript{2} by multiple specific enzymes in a cell type-restricted fashion (17). In the context of metabolic disorders, elevated PGE\textsubscript{2} synthesized via macrophage COX-2 contributes to plaque rupture in atherosclerosis (4). Also, COX-2 upregulations in the pancreas, mesangial tissue, and peripheral tissue are associated with chronic diabetic pancreatitis (35), diabetic nephropathy (33), and diabetic peripheral neuropathy (46), respectively. In addition, COX-2 (+/−) heterozygote mice are reportedly obese, although the underlying mechanism remains unclear (15). In skeletal muscle, physical exercise has been shown to induce COX-2 expression (31, 52), which appeared to play a role in the regulation of muscle functions, including muscle differentiation, healing, and regeneration (2, 25). However, no study has investigated the effects of saturated and unsaturated FFAs on COX-2 expression in skeletal muscle cells. In addition, detailed signaling mechanisms directly involved in the induction of COX-2 expression in skeletal muscle are poorly understood.
The detrimental effects of saturated FFAs such as palmitoleate (C16:0) in skeletal muscle cells have been attributed to abnormal accumulation of palmitoyl-CoA, diacylglycerol, and/or ceramide, which in turn leads to deleterious activation of various serine/threonine kinases such as PKCθ (19, 30, 49). PKCθ, a novel PKC isoform expressed abundantly in skeletal muscle cells, has a unique ability to activate transcriptional factor NF-κB, and the PKCθ-NF-κB signaling cascade has been shown to play an important role in the production of IL-6 and TNFα in skeletal muscle cells (9, 29, 30, 53). Intriguingly, the antagonizing action of oleate (18:1) against palmitate-induced responses has been at least partially explained by a mechanism involving suppression of the PKCθ-NF-κB signaling cascades by oleate (9, 29, 30). On the other hand, saturated FFAs also reportedly activate members of the MAPK family, including ERK1/2, p38, and JNK (10, 11, 51), although their possible involvement in proinflammatory responses in skeletal muscle cells is poorly understood. Furthermore, a detailed analysis of the different impacts of saturated and unsaturated FFAs on these intracellular signaling cascades in skeletal muscle cells has yet to be reported.

In this study, we evaluated the effects of saturated and unsaturated FFAs on proinflammatory COX-2 expression and the involved intracellular signals, such as p38, JNK, ERK1/2 MAPKs, PKCθ, and NF-κB, in C2C12 skeletal muscle cells. We also examined the protective action of unsaturated FFAs against the palmitate-evoked events. Our findings are the first to shed light on the regulation of FFA-induced COX-2 expression and intracellular signals in skeletal muscle.

MATERIALS AND METHODS

Materials. The Western blot detection kit (West super femto detection reagents) and Immobilon-P were purchased from Pierce Biotechnology (Rockford, IL) and Millipore (Bedford, MA), respectively. Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and trypsin-EDTA were purchased from Sigma Chemical (St. Louis, MO). Cell culture equipment was obtained from BD Biosciences (San Jose, CA). Calf Serum (CS) and fetal bovine serum (FBS) were purchased from BioWest (Nuaille, France). FFA free-bovine serum albumin (BSA) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-COX-2, anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-phospho-PKCθ (Thr68/Tyr67), anti-JNK, anti-phospho-JNK (Thr183/Tyr185), anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-IκBa, and anti-phospho-activating transcription factor-2 (ATF-2) (Thr277) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin antibody was purchased from Sigma. The ELISA kit for PGE2 was purchased from R & D Systems (Minneapolis, MN). Fatty acids, pyridine dithiocarbamate (PDTC), SB-203580, rottlerin, etomoxir, thenoyltrifluoroacetone (TFFA), and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma, Wye-14643, GW-501516, and 2O4 were purchased from Cayman Chemicals (Ann Arbor, MI), Alexis Biochemicals (Lausen, Switzerland), and MP Biomedicals (Solon, OH), respectively. Other chemicals were purchased from Sigma or Wako.

Cell culture. Mouse skeletal muscle cell lines, C2C12 myoblasts, were maintained in DMEM supplemented with 10% FBS, 30 μg/ml penicillin, and 100 μg/ml streptomycin (growth medium) at 37°C under a 5% CO2 atmosphere. For biochemical study, the cells were grown on six-well plates (BD Biosciences) at a density of 3 × 104 cells/well in 3 ml of growth medium. Three days after plating, cells had reached ~80–90% confluence (day 0). Differentiation was then induced by replacing the growth medium with DMEM (4.5 g/l glucose) supplemented with 2% CS, 1 mM insulin, 30 μg/ml penicillin, and 100 μg/ml streptomycin (differentiation medium) (40). The differentiation medium was changed every 24 h, and the differentiated cells (on days 4 and 5) were used for subsequent experiments. For immunofluorescence analysis, cells were grown on 22-mm glass coverslips (CO22221; Matsunami, Osaka, Japan) in six-well plates. FFA-containing media were prepared by preincubation of FFA with DMEM supplemented with 2% FFA-free BSA, as described previously (30). Inhibitors were prepared in dimethylsulfoxide and then added to media at 0.1% (vol/vol).

Quantitative real-time PCR. Total RNA was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. Quantitative real-time PCR analysis was performed using the Light Cycler instrument and SYBR Green detection kit according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). PCR primers for COX-2 were 5′-AGA TCA TAA GCG AGG ACC TG-3′ and 5′-TAC ACC TCT CCA CTA ATG AC-3′.

Western blot analysis. Cells lysates were prepared using lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% EDTA, 1% NP-40, 1 μg/ml peptatin, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 6,500 IU/ml aprotinin, phosphatase inhibitor cocktail-1 (Sigma)), and the protein concentrations of cell lysates were then measured using the BCA protein assay kit (Pierce Biotechnology). Proteins (20 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane (Immobilon-P), and the membranes were then blocked for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20. The membranes were next immunoblotted with primary antibodies at dilutions of 1:500 to 1:1,000. Specific total or phospho-proteins were visualized after subsequent incubation with a 1:10,000 dilution of anti-mouse or rabbit IgG conjugated to horseradish peroxidase and the SuperSignal Chemiluminescence detection procedure (Pierce Biotechnology). Three independent experiments were performed for each condition.

ELISA for PGE2. PGE2 levels in media were evaluated using the PGE2 Parameter Assay Kit (R & D Systems).

Immunofluorescence analysis. C2C12 myotubes were cultured on coverslips placed on six-well plates. After differentiation, the cells were treated with or without 1 mM palmitate for 16 h. Then the cells were fixed with 2% paraformaldehyde in PBS (~), and permeabilized using 0.4% Triton X-100 in PBS (~), followed by immunocytochemistry using anti-COX-2 antibody (Cell Signaling Technology), and anti-rabbit IgG antibody conjugated with Alexa 594 (Invitrogen). F-actin was visualized by Oregon Green 488 phalloidin (Invitrogen). Images were monitored and analyzed using Olympus Fluoview FV1000 confocal microscopy and the associated application program ASW Ver1.3 (Olympus, Tokyo, Japan).

Anti-c-myc antibody uptake assay. Glucose transporter 4 (GLUT4) translocation was analyzed as described previously (41). Briefly, C2C12 myotubes expressing myc-GLUT4-enhanced cyan fluorescent protein (ECFP) were serum-starved, washed three times with Krebs-Ringer-Pi-HEPES buffer, and then placed in a CO2 incubator with 2 ml of Krebs-Ringer-Pi-HEPES buffer. After 10 min of incubation, 4 μg/ml anti-c-myc antibody was added to the buffer, and the cells were stimulated with or without 100 nM insulin for 30 min. After incubation for 30 min with the anti-myc antibody, the cells were plated on ice to stop the reaction and washed five times with PBS. The cells were harvested using 1× Laemmli’s buffer and subjected to Western blot analysis using anti-mouse IgG antibody and anti-c-myc antibody.

Statistical analysis. Data are expressed as means ± SE. Statistical significance was determined by ANOVA followed by the Bonferroni-Dunn post hoc test using StatView software (SAS Institute). Differences were considered significant when P values were <0.05.

RESULTS

Saturated, but not unsaturated, FFAs induce COX-2 expression in C2C12 myotubes. Utilizing mouse skeletal muscle line C2C12 cells, we examined the effects of FFAs on the mRNA expression of COX-2 (Fig. 1A). Treatment with palmitate (C16:0) or stearate (C18:0) for 16 h dramatically induced COX-2 mRNA expression in a dose-dependent manner in
C2C12 myotubes, and the effective dose was >0.75 mM. Expression of COX-1 mRNA was not increased by palmitate treatment (data not shown). In contrast to saturated FFAs, unsaturated FFAs [palmitoleate (C16:1), oleate (C18:1), and linoleate (C18:2)] had no effects on the COX-2 mRNA level. Consistent with these mRNA data, Western blot analysis demonstrated that only saturated FFAs, especially palmitate (C16:0), strongly induce COX-2 protein (Fig. 1B), and this was
further confirmed by immunofluorescent staining using anti-COX-2 antibody (Fig. 1C).

Time course experiments revealed COX-2 protein to be detectable after 16 h of palmitate treatment (Fig. 1D), apparently accompanied by enhanced phosphorylation of p38 (Thr\(^{180}/\text{Y}r^{182}\)) and slight degradation of I\(\kappa\)B\(x\) (Fig. 1D). On the contrary, slight PKC\(\theta\) phosphorylation (Thr\(^{38}\)) was detected after as little as 1 h of treatment and was further augmented thereafter, reaching a plateau after 6 h of stimulation. Differentiated C\(_{2}\)C\(_{12}\) myotubes were highly responsive to palmitate, in terms of this COX-2 expression, whereas myoblasts were far less responsive.

To further characterize the palmitate-induced COX-2 expression and the intracellular signal(s) possibly involved, we conducted detailed dose-response analyses of the effects of palmitate on the amount of COX-2 protein, the phosphorylation status of p38 and PKC\(\theta\), and the amount of I\(\kappa\)B protein in C\(_{2}\)C\(_{12}\) myotubes (Fig. 2, A–E). Consistent with the mRNA data, 16-h treatment with pathophysiologically relevant concentrations of palmitate (>0.75 mM) increased cellular COX-2 protein contents (Fig. 2, A and B), resulting in increased accumulation of PGE\(_{2}\), a bioactive product of COX-2, in conditioned media (Fig. 2F). The palmitate-induced phosphorylations of p38 (Fig. 2, A and C) and PKC\(\theta\) (Fig. 2, A and D) were both sensitively detected even when relatively low concentrations of palmitate were applied and peaked at 0.75 mM of palmitate. The degradation of I\(\kappa\)B as assessed by the total amount of I\(\kappa\)B was also observed at palmitate concentrations >0.75 mM (Fig. 2E). Since physiological levels of circulating FFAs are reportedly between 0.3 and 0.4 mM, raising to 0.6–0.8 mM in obese or type 2 diabetes patients (1, 30), these data indicate that the saturated FFA-induced COX-2 expression observed herein may be a phenomenon occurring only at hyperphysiological FFA levels, approximating pathophysiological conditions.

Taken together, these results indicate that saturated FFAs, but not unsaturated FFAs, induce COX-2 expression in C\(_{2}\)C\(_{12}\) myotubes. Our data also raise the possibility that the p38- and/or PKC\(\theta\)-NF-\(\kappa\)B pathway may be involved in palmitate-induced COX-2 expression and PGE\(_{2}\) production in C\(_{2}\)C\(_{12}\) myotubes.

p38 and NF-\(\kappa\)B are involved in palmitate-induced COX-2 expression in C\(_{2}\)C\(_{12}\) myotubes. To define the involvement of p38 and PKC\(\theta\) as well as that of NF-\(\kappa\)B in palmitate-induced COX-2 expression, C\(_{2}\)C\(_{12}\) myotubes were incubated with 1 mM palmitate for 16 h in the presence or absence of selective pharmacological inhibitors for NF-\(\kappa\)B (PDTC), p38 (SB-203580), or PKC\(\theta\) (rottlerin) (Figs. 3 and 4). PDTC, an NF-\(\kappa\)B inhibitor, significantly reduced the COX-2 protein expression induced by palmitate in a dose-dependent manner, and this expression was almost entirely suppressed by a high dose of PDTC (Fig. 3, A and B). Similarly, SB-203580, a p38 inhibitor, dose-dependently blocked palmitate-induced COX-2 expression (Fig. 3, C and D), whereas phosphorylations of PKC\(\theta\) and p38 as well as I\(\kappa\)B degradation were unchanged (Fig. 3C). As expected, phosphorylation of ATF-2, a substrate of p38, was also significantly suppressed in the presence of SB-203580 (Fig. 3E), indicating that SB-203580 did indeed inhibit p38 enzymatic activity under these experimental conditions. On the contrary, rottlerin (an inhibitor of novel PKCs) failed to affect the COX-2 protein expression induced by palmitate (Fig. 4, A and B), although PKC\(\theta\) phosphorylation was dose-dependently decreased by rottlerin (Fig. 4, A and C). The phosphorylation and total amount of p38 were, as expected, unaffected by rottlerin (Fig. 4A). Although phosphorylation of JNK, another stress-activated MAPK, was increased by palmitate treatment (Fig. 6A), inhibition of JNK by SP600125 failed to interfere with COX-2 expression (data not shown). Overall, these results indicate that p38 and NF-\(\kappa\)B are involved in regulation of the COX-2 expression induced by palmitate, but our experimental evidence revealed no contribution of PKC\(\theta\) to this COX-2 expression despite PKC\(\theta\) phosphorylation being strongly augmented by palmitate treatment.

Mitochondrial fatty acid oxidation may not be involved in COX-2 expression. Mitochondrial overload and incomplete fatty acid oxidation may contribute to insulin resistance (36) as well as to inflammatory responses in skeletal muscle. Thus, we next investigated the possibility that fatty acid oxidation is involved in COX-2 expression by using pharmacological inhibitors for enzymes related to fatty acid oxidation (Fig. 5). However, etomoxir ( carnitine palmitoyltransferase I inhibitor), TTFA (electron transport complex II inhibitor), and CCCP (an uncoupler of oxidative phosphorylation) all failed to exert any inhibitory effect on palmitate-induced COX-2 expression (Fig. 5A).

We also examined the effect of 2-bromopalmitate (2-BP) on COX-2 expression, since 2-BP is a nonmetabolizable palmitate analog (8). One mM of 2-BP had no ability to induce COX-2 expression (Fig. 5B). Taken together, these results indicate that palmitate metabolism is apparently required for COX-2 expression, whereas fatty acid oxidation in mitochondria is not directly involved in this event in C\(_{2}\)C\(_{12}\) myotubes.

Unsaturated fatty acids reverse palmitate-induced COX-2 expression. Physiologically, saturated and unsaturated FFAs are both present in blood and cells, and previous studies demonstrated that oleate (C18:1) reverses palmitate-induced insulin resistance and IL-6 production (9). Recently, palmitoate (C16:1) was proposed to serve as a lipokine that communicates with both the liver and skeletal muscles and regulates systemic metabolic homeostasis (5). Therefore, we evaluated the impacts of unsaturated fatty acids on palmitate-induced COX-2 protein expression as well as on the related intracellular signals activated by palmitate (Fig. 6). Unsaturated FFAs displayed remarkable dose-dependent inhibitory effects on all of the palmitate-induced events analyzed (Fig. 6). Importantly, potencies against palmitate-induced responses differed significantly among these unsaturated fatty acids, although their patterns were similar (linoleate > oleate > palmitoate), and sufficient concentrations of each unsaturated fatty acid completely abolished palmitate-induced COX-2 protein expression (Fig. 6, A and B) as well as the related PGE\(_{2}\) production (Fig. 6J).

Unexpectedly, despite clear dose-responsive suppression of COX-2 protein expression by these unsaturated FFAs, the phosphorylation status of p38 did not correspond well to this change. This phenomenon was exemplified by 30 \(\mu\)M linoleate producing a slight but significant enhancement of the palmitate-induced phosphorylation of p38 instead of inhibiting it. Moreover, these unsaturated FFAs failed to dampen p38 phosphorylation status to the basal level (Fig. 6C), whereas they clearly abolished the palmitate-induced phosphorylation of ATF-2 (Fig. 6D) as well as that of JNK (Fig. 6F), another upstream kinase responsible for ATF-2 phosphorylation (21). Although the molecular mechanism(s) underlying the bell-shaped dose-response profile of p38 phosphorylation at Thr\(^{180}/\text{Y}r^{182}\) under mixed FFA conditions remains to be clarified, the observation that unsaturated FFAs...
Fig. 2. Effects of palmitate on signaling pathways via p38 or PKC and prostaglandin E2 (PGE2) production. 

A: C2C12 myotubes were treated with 0.25–1 mM palmitate for 16 h, and the cell lysates were then subjected to Western blot analysis (A). Specific bands of COX-2, phospho-p38 (Thr180/Tyr182), phospho-PKCδ (Thr538), and IκB-α (E) were quantified using a densitometer. Data are expressed as means ± SE of 3 independent experiments (*P < 0.005, **P < 0.0005 vs. control).

B: C2C12 myotubes were treated with 0.25–1 mM palmitate for 16 h, and the cell lysates were then subjected to Western blot analysis (B). COX-2 protein level was quantified using a densitometer. Data are expressed as means ± SE of 3 independent experiments (**P < 0.005 vs. control).

C: Phospho-p38 protein level was quantified using a densitometer. Data are expressed as means ± SE of 3 independent experiments (*P < 0.005, **P < 0.0005 vs. control).

D: Phospho-PKCδ protein level was quantified using a densitometer. Data are expressed as means ± SE of 3 independent experiments (*P < 0.005, **P < 0.0005 vs. control).

E: IκB-α protein level was quantified using a densitometer. Data are expressed as means ± SE of 3 independent experiments (**P < 0.005 vs. control).

F: C2C12 myotubes were treated with 0.25–1 mM palmitate for 16 h, and the media were then collected. PGE2 levels in the media were measured by ELISA. Data are expressed as means ± SE of 6 independent experiments (**P < 0.005 vs. control).
abolish palmitate-induced ATF-2 phosphorylation (Fig. 6D) strongly suggests that signaling cascades reaching ATF-2 are effectively hampered by administering unsaturated FFAs, although they have markedly different potencies.

Neither PPARα nor PPARβ/δ is involved in the protective actions of unsaturated FFAs against palmitate-induced COX-2 expression and p38 MAPK activation. To explore possible mechanisms underlying the protective effects of unsaturated
FFAs on the palmitate-induced events in C2C12 myotubes, we examined the effects of several agonists of peroxisome proliferator-activated receptor (PPAR) on palmitate-induced events, since unsaturated FFAs have been shown to serve as potent ligands for these PPARs (16). The palmitate-induced events were reversed completely by arachidonate (20:4; also known as an endogenous PPAR pan-agonist) (16) but not by Wy-14643 (PPARγ agonist) or GW-501516 (PPARγ/β agonist) (34, 42), indicating that PPARs may not be involved in the protective actions of unsaturated FFAs against palmitate-induced events.

Effect of pharmacological inhibition of PKCθ on palmitate-induced insulin resistance in C2C12 myotubes. Finally, we examined the possible involvement of COX-2 and its metabolites in the generation of insulin resistance in C2C12 myotubes.

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myotubes. We employed a myc-GLUT4 translocation assay using anti-myc antibody, as reported previously (41). Insulin-induced Akt phosphorylation was also examined to monitor insulin receptor signals. As reported previously (6, 50), palmitate (1 mM) treatment obviously suppressed insulin-induced Akt (Ser473) phosphorylation, which was not restored by NS-398, a COX-2-selective inhibitor (Fig. 8A). As expected, insulin-responsive GLUT4 translocation was also impaired by this palmitate treatment, with impaired insulin responsiveness being partially attributable to the increased GLUT4 translocation even in the basal state (Fig. 8B). Consistent with the Akt phosphorylation data, NS-398 failed to restore the impaired insulin-induced GLUT4 translocation in palmitate-treated C2C12 myotubes (Fig. 8, B and C).

### DISCUSSION

Palmitate-induced COX-2 expression requires the activities of p38 MAPK and NF-κB but not that of PKCθ. In the present study, we observed phosphorylation of PKCθ (Thr538) by palmitate treatment within 1 h (Fig. 1D). However, a relatively longer period (~16 h) was required for COX-2 expression, and pharmacological inhibition of this enzyme by rottlerin failed to inhibit palmitate-induced COX-2 expression (Fig. 4), although the NF-κB activity was apparently required for this event (Fig. 3, A and B). These results strongly suggest that activation of PKCθ alone was insufficient for rapid induction of COX-2 expression via activation of NF-κB in C2C12 myotubes instead indicating that delayed activation of p38 is additionally required for COX-2 expres-

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**Fig. 6.** Effects of unsaturated fatty acids (USFA) on palmitate-induced COX-2 expression, its signaling pathways, and PGE2 production. A–I: C2C12 myotubes were treated with 1 mM palmitate (C16:0) alone or in combination with palmitoleate (C16:1), oleate (C18:1), or linoleate (C18:2) for 16 h, and the cell lysates were then subjected to Western blot analysis (A). Specific bands in the Western blots were quantified using a densitometer. Data are expressed as means ± SE of 3 independent experiments (*P < 0.05, **P < 0.005, ***P < 0.0005 vs. control; B–I). J: PGE2 levels in the media were measured by ELISA. Data are expressed as means ± SE of 4 independent experiments (**P < 0.005, ***P < 0.0005 vs. control).
sion (Fig. 3, C–E). In this regard, a recent study demonstrated that both DNA binding and transcriptional activation of NF-κB began to rise in cultured myotubes after an 8-h incubation with palmitate (24). Moreover, p38 has been shown to directly regulate NF-κB phosphorylation and its nuclear localization in cardiac myocytes (55), and direct involvement of the p38-ATF-2 pathway in the regulation of COX-2 expression has also been reported in other cell types (23, 43). Supporting this notion is our observation that SB-203580 failed to reverse the IkB degradation elicited by palmitate treatment (Fig. 3C), suggesting p38 activity to be crucial for COX-2 expression to function independently of
IkB degradation. Taking these observations together, although PKC0-mediated NF-κB activation via palmitate-induced intracellular lipid accumulation has been implicated in the increased secretion of IL-6 and TNFα (9, 29, 30, 53), our present data provide compelling evidence that p38 MAPK, but not PKC0, serves as the crucial signaling intermediate for the COX-2 expression in C2C12 skeletal muscle cells. A similar mechanism was also observed for the palmitate-induced suppression of PGC-1α expression in skeletal muscle cells (10, 11).

Mitochondrial overload, ceramide, and Toll-like receptors do not play a crucial role in palmitate-induced COX-2 expression. An interesting observation reported herein is that inhibition of fatty acid oxidation by various reagents that modulate mitochondrial respiration and reactive oxygen species (ROS) generation totally failed to influence palmitate-induced COX-2 expression (Fig. 5), which would appear to contradict a recent report showing that mitochondrial overload with excessive β-oxidation could be a major cause of insulin resistance in skeletal muscle (36). Because 1 mM of a halogenated analog of palmitate, 2-BP (22), has no COX-2-inducible effect, a specific fatty acyl-CoA moiety and/or palmitate metabolism is apparently required for COX-2 expression (Fig. 5). In addition, it has been reported that mitochondria-derived ROS is a key initiator for the phosphorylation of p38 (37). Since we have not examined the effects of supplemental carnitine, which reportedly tends to be deficient in cell cultures and thereby partially obliterates actual mitochondrial performance (36), we cannot as yet exclude the involvement of mitochondrial ROS generation in palmitate-induced COX-2 expression. Elucidation of this possibility requires further experiments.

Although it has been reported that palmitate can activate the proinflammatory pathway via a Toll-like receptor-dependent mechanism(s) (12, 51), we observed no inhibitory effects of blocking antibodies against Toll-like receptor-2/4 on palmitate-induced COX-2 expression (data not shown). We also examined the possible involvement of ceramide in COX-2 expression by utilizing a cell-permeable C2-ceramide (50). However, 100 μM C2-ceramide did not induce COX-2 expression in C2C12 myotubes (data not shown), indicating ceramide itself to be insufficient for reproducing palmitate-induced COX-2 expression, although ceramide accumulation might contribute to this event in conjunction with other palmitate-induced component(s).

Effects of unsaturated FFAs on COX-2 expression and intracellular signals evoked by palmitate treatment. Although a recent report proposed the importance of palmitoleate (16:1) serving as an adipose-derived lipid hormone (lipo-kinase) (5), our findings indicate that palmitoleate, in contrast
to oleate (18:1) and linoleate (18:2), has relatively fewer beneficial effects in terms of suppressing COX-2 expression as well as having little impact on the intracellular signals evoked by palmitate treatment (Fig. 6). These observations are quite novel and raise an important question concerning the significance of palmitoleate serving as a lipokine, at least in terms of exerting protective effects against the COX-2 expression triggered by palmitate treatment in skeletal muscle cells. Given that the serum palmitoleate concentration is usually quite low, and oleate and linoleate are the most prevalent unsaturated FFAs in the circulation (7), our results suggest oleate and/or linoleate, but not palmitoleate, to potentially be major contributors to the protective effects against palmitate-induced COX-2 expression in vivo via dampening of a wide array of the palmitate-inducible intracellular signaling cascades (Fig. 6).

It is well established that unsaturated FFAs function as ligands for PPARs, including PPARβ/δ, a predominant skeletal muscle PPAR isoform, with different binding affinities (16). For example, oleate (18:1) has recently been shown to provide protection from palmitate-induced detrimental responses by promoting mitochondrial β-oxidation as well as by shunting excess palmitate to triacylglycerol instead of diacylglycerol accumulation through a mechanism involving a PPARβ/δ-dependent upregulation of the related metabolic genes, which in turn results in the prevention of inappropriate activation of the PKCθ-NF-κB cascades in skeletal muscle cells (9). We also observed the palmitate-induced phosphorylation of PKCθ to be inhibited by each unsaturated FFA examined (Fig. 6). However, in contrast to this notion, we could not reproduce the protective action against palmitate-induced events using a selective PPARβ/δ activator, GW-50156, or a PPARα activator, Wy-140143 (Fig. 7), suggesting that PPARs may not be a major contributor to the protective actions. Consistent with this, neither Wy-14643 nor GW-50156 had any suppressive effect on palmitate-induced phosphorylation of PKCθ (Fig. 7). Since arachidonate (20:4), a polyunsaturated fatty acid serving as a major substrate for COXs, has also been shown to potently activate both PPARα and PPARβ/δ (16), we utilized arachidonate as an endogenous pan-PPAR agonist. Unexpectedly, arachidonate strongly suppressed palmitate-induced events (Fig. 7), raising the possibility that arachidonate and its metabolite(s) play an important role in exerting the protective actions. In line with this, linoleate, which is preferentially metabolized to arachidonate, exhibited a stronger suppressive effect on palmitate-induced events than oleate or palmitoleate (Fig. 6). Together, these observations suggest a potential role for the arachidonate cascade, including synthesis of bioactive lipids in the protective actions of unsaturated FFAs.

**p38 Phosphorylation induced by palmitate in combination with unsaturated FFAs shows a bell-shaped dose-response relationship.** Our data demonstrated that p38 MAPK is required for COX-2 expression and that unsaturated FFAs potently abolished this palmitate-induced COX-2 expression. Intriguingly, however, we found that all three unsaturated FFAs similarly induced slight enhancement of palmitate-induced p38 phosphorylation (Thr180/Tyr182), showing a bell-shaped dose-response relationship (Fig. 6C), whereas they all diminished the phosphorylations of JNKs (Fig. 6, F and G) and ATF-2 (Fig. 6E), a common substrate for p38 and JNK MAPKs (21). This discrepancy between the phosphorylation status of p38 MAPK (Thr180/Tyr182) and its substrate ATF-2 (Thr69/71) (Fig. 6D) apparently reflects the complex regulatory mechanism of p38 by multiple phosphorylation events and by

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**Fig. 8. Effects of NS-398 on palmitate-induced insulin resistance.**

A: C2C12 myotubes were treated with 1 mM palmitate (C16:0) alone or in combination with 30 μM NS-398 for 16 h. The cells were serum-starved and then treated with the indicated concentration of insulin for 5 min. The cell lysates were then subjected to densitometric analysis for quantification. Three independent experiments were performed, and representative results were obtained.

B: differentiation C2C12 myotubes expressing myc-glucose transporter 4 (GLUT4)-enhanced cyan fluorescent protein (ECFP) were treated with 100 nM insulin for 30 min in the presence of 4 μg/ml anti-myc antibody. The cells were then washed 5 times with PBS and analyzed by Western blotting with the use of anti-mouse IgG horseradish peroxidase-conjugated antibody. C: the results from B. uptake of anti-myc antibody in response to insulin, were subjected to densitometric analysis for quantification. Three independent experiments were performed, and representative results were obtained.
binding of unsaturated FFAs or their metabolites (e.g., arachidonic acid) to the lipid-binding site of this enzyme (14, 44, 48). Thus, an interesting aspect of unsaturated FFA actions on p38 regulation might be further involvement in the beneficial effects of suppressing inflammatory responses.

Potential role of COX-2 and subsequent PG production in skeletal muscle. Although palmitate treatment clearly induced PGE₂ production in C₂C₁₂ myotubes (Figs. 2F and 6J), NS-398, a COX-2-selective inhibitor, failed to reverse the palmitate-induced impairments in both insulin-induced Akt phosphorylation and insulin-responsive GLUT4 translocation (Fig. 8). These data indicate that palmitate-induced COX-2 and its metabolites may make little, if any, direct contribution to the development of insulin resistance in C₂C₁₂ myotubes. However, it is highly likely that COX-2 metabolites are involved in inflammatory responses in a paracrine fashion by inducing migration of immune cells (13, 27, 38, 47), which may contribute indirectly to the generation of insulin resistance as well as to complex inflammatory responses in skeletal muscles in vivo. In this regard, a recent study demonstrated that administration of a COX-2 inhibitor improved whole body and muscle insulin resistance in sucrose-fed rats (28). In addition, recent studies utilizing COX-2-deficient mice have also demonstrated that COX-2 plays a crucial role in the regeneration of injured muscle (2) and growth of atrophied muscle (3), indicating that inflammation, induced at least in part by COX-2-mediated PG production, is an important process of skeletal muscle healing. Analogous to this, our results indicate that inducible expression of COX-2 and subsequent PGE₂ production may contribute to muscle healing/regeneration after cell damage caused by treatment with pathophysiologically high FFA concentrations.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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