Increased Reactive Oxygen Species Production Down-regulates Peroxisome Proliferator-activated α Pathway in C2C12 Skeletal Muscle Cells*

Received for publication, October 26, 2001, and in revised form, December 18, 2001 Published, JBC Papers in Press, January 15, 2002, DOI 10.1074/jbc.M110321200

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Generation of reactive oxygen species may contribute to the pathogenesis of diseases involving intracellular lipid accumulation. To explore the mechanisms leading to these pathologies we tested the effects of etomoxir, an inhibitor of carnitine palmitoyltransferase I which contains a fatty acid-derived structure, in C2C12 skeletal muscle cells. Etomoxir treatment for 24 h resulted in a down-regulation of peroxisome proliferator-activated receptor α (PPARα) mRNA expression, achieving an 87% reduction at 80 μM etomoxir. The mRNA levels of most of the PPARα target genes studied were reduced at 100 μM etomoxir. By using several inhibitors of de novo ceramide synthesis and Cα-ceramide we showed that they were not involved in the effects of etomoxir. Interestingly, the addition of triacsin C, a potent inhibitor of acyl-CoA synthetase, to etomoxir-treated C2C12 skeletal muscle cells did not prevent the down-regulation in PPARα mRNA levels, suggesting that the active form of the drug, etomoxir-CoA, was not involved. Given that saturated fatty acids may generate reactive oxygen species (ROS), we determined whether the addition of etomoxir resulted in ROS generation. Etomoxir increased ROS production and the activity of the well known redox transcription factor NF-κB. In the presence of the pyrrolidine dithiocarbamate, a potent antioxidant and inhibitor of NF-κB activity, etomoxir did not down-regulate PPARα mRNA in C2C12 skeletal muscle cells. These results indicate that ROS generation and NF-κB activation are responsible for the down-regulation of PPARα and may provide a new mechanism by which intracellular lipid accumulation occurs in skeletal muscle cells.

Skeletal muscle insulin resistance is the major characteristic of non-insulin-dependent diabetes mellitus (1). The mechanisms responsible for the reduced sensitivity of muscle to insulin still remains unclear, but there is a strong correlation between insulin resistance and the presence of increased lipid levels in skeletal muscle (2). Data from different studies are consistent with this idea. In humans it has been reported that insulin resistance correlates more tightly with intramyocellular lipid levels than with any other factor, including body mass index or percent body fat (3–5). Moreover, insulin resistance appears after exposure of rat skeletal muscle to elevated free fatty acids, which is associated with the accumulation of fatty acyl-CoA (6) and triglycerides (7, 8). Similar results have been obtained with cells exposed to increased lipid levels (9).

Fatty acid catabolism is mainly regulated by the peroxisome proliferator-activated receptor α (PPARα) (10). This PPAR subtype is expressed primarily in tissues with a high level of fatty acid catabolism such as liver, kidney, heart, and skeletal muscle (11, 12). To be transcriptional active, PPARα needs to heterodimerize with the retinoid X receptor. PPARα-retinoid X receptor heterodimers bind to DNA specific sequences called peroxisome proliferator-response elements consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (DR-1). Recently it has been reported that PPARα activation in skeletal muscle increases expression of enzymes involved in fatty acid β oxidation. These changes lead to prevention of diet-induced obesity and insulin resistance (13). On the other hand, hypertrophic growth in cardiac myocytes showed reduced PPARα expression, and its activity is altered at the transcriptional level via the extracellular signal-regulated kinase mitogen-activated protein kinase pathway. These hypertrophied myocytes, with reduced PPARα expression, showed a reduced capacity for cellular lipid homeostasis, resulting in intracellular lipid accumulation in response to fatty acids (14).

To gain a better understanding of the mechanism by which exposure of skeletal muscle cells to fatty acids results in lipid accumulation, we have used the myoblast C2C12 cell line, which develops biochemical and morphological properties characteristic of skeletal muscle and has been proven useful for studies of skeletal muscle metabolism (15, 16). To promote fatty acyl-CoA accumulation in these cells, we have taken advantage of the use of etomoxir. Etomoxir is an irreversible inhibitor of CPT-I and, therefore, of fatty acid β-oxidation (17). Treatment of C2C12 skeletal muscle cells for 24 h with etomoxir strongly decreased PPARα mRNA levels. Overall,

*This study supported in part by a grant from the Fundació Privada Catalana de Nutrició; Lipids, Fondo de Investigaciones Sanitarias Grant 00/1124, and Ministerio de Ciencia y Tecnología of Spain Grant SAF00-0201. This work was also supported by Generalitat de Catalunya, Grants SGR96–84 and 1998SGR-33. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Supported by a grant from the Ministerio de Educación of Spain.

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1The abbreviations used are: PPARα, peroxisome proliferator-activated receptor α; CPT-I, carnitine palmitoyltransferase I; ROS, reactive oxygen species; PDTC, pyrrolidine dithiocarbamate; ACO, acyl-CoA oxidase; M-CPT-I, muscle-type carnitine palmitoyltransferase I; UCP-3, uncoupling protein 3; MCAD, medium chain acyl-CoA dehydrogenase; APRT, adenosylphosphoribosyltransferase; bp, base pair; NF-κB, nuclear factor κB; ACS, acyl-CoA synthetase; DCFH, 2′,7′-dichloro-fluorescein diacetate; RT, reverse transcription.
ROS Down-regulate PPARα in Skeletal Muscle Cells

PPARα target genes showed a biphasic response to etomoxir, with small inductions in their expression at low etomoxir concentrations and reductions at higher concentrations. Surprisingly, the effects of etomoxir on PPARα expression were not mediated through CPT-I inhibition, since inhibition of the formation of the active form of the drug, etomoxir-CoA, did not prevent its effects. In contrast, we show that treatment with etomoxir, which contains a saturated fatty acid-derived structure with an oxirane group, resulted in the generation of reactive oxygen species (ROS) and nuclear factor-κB (NF-κB) activation. Interestingly, in the presence of the pyrrolidine dithiocarbamate (PDTC), a potent antioxidant and inhibitor of NF-κB activity, etomoxir did not down-regulate PPARα expression in C2C12 skeletal muscle cells. These results indicate that intracellular ROS generation and increased NF-κB activity leads to a down-regulation of the fatty acid oxidation metabolism mediated by PPARα, which in turn may result in intracellular lipid accumulation in skeletal muscle cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Etomoxir (2-[6-(4-chlorophenoxo)hexyl]oxirane-2-carboxylic acid ethyl ester; Sigma-Aldrich) was determined to be purified by Dr. Horst Wolf (Germany). Fumonisin B1, triacsin C were from Biomol Research Labs Inc. (Plymouth Meeting, PA), and fumonisin B1, ISP1, and PDTC were from Sigma. PD98059 and triacyls were from Biomedes Laboratories (Burlington, VT). C2-ceramide, (1,2-dioleoyl-sn-glycero-3-phosphorylcholine) was obtained from Dr. Horst Wolf (Germany). C2-ceramide, PPARγ1, PPARγ2, and M-CPT-I were from Biosource (Camarillo, CA), and ACO, APRT, and M-CPT-I were purchased from ScyTek Laboratories (Logan, UT). PPARγ1, PPARγ2, and M-CPT-I were synthesized from RNA samples by mixing 1 μg of total RNA, 125 ng of each dNTP, 5 μg/ml yeast tRNA, 50 units/ml penicillin, and 50 μg/ml streptomycin. When cells reached confluence, the medium was switched to the differentiation medium containing Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. When cells reached confluence, the medium was switched to the differentiation medium containing Dulbecco’s modified Eagle’s medium and 2% horse serum, which was changed every other day. After 4 additional days, the differentiated C2C12 cells had fused into myotubes, which were then treated in serum-free Dulbecco’s modified Eagle’s medium with either vehicle (0.1% methanol) or etomoxir. After the incubation, RNA was extracted from myotubes as described below.

**RNA Preparation and Analysis**—Total RNA was isolated by using the Ultraspec reagent (Biotex). Relative levels of specific mRNAs were assessed by reverse transcription (RT)-PCR. Complementary DNA was synthesized from RNA samples by mixing 1 μg of total RNA, 125 ng of random hexamers as primers in the presence of 50 mM Tris-HCl, 50 mM KCl, and 10 mM MgCl2, 100 units of Taq polymerase (Ec-Taq, Vienna, Austria), 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 20 units of RNasin (Invitrogen), and 0.5 μM of each dNTP (Sigma) in a total volume of 20 μl. Samples were incubated at 37 °C for 60 min. A 5 μl aliquot of the reverse transcription reaction was then used for subsequent PCR amplification with specific primers.

Each 25-μl PCR reaction contained 5 μl of the reverse transcription reaction, 1.25 units of Taq polymerase (6 μg/ml), 0.2 μM of each primer, 200 μM of each dNTP, 10 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 200 units of Taq polymerase (Ec-Taq), 10 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2 μg/ml leupeptin) by flicking the tube. Cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Then samples were centrifuged for 10 s, and the supernatant fraction was discarded. Pellets were resuspended in 50 μl of cold buffer C (20 mM HEPES-KOH, pH 7.9, at 4 °C, 15 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2 μg/ml leupeptin) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 °C, and the supernatant fraction (containing DNA binding proteins) was stored at −80 °C. Nuclear extract containing DNA binding proteins was eluted by the Br Dynamic Binding (Germany) by dialysis. DNA binding proteins were eluted by the Br Dynamic Binding (Germany) by dialysis. DNA binding proteins were eluted by the Br Dynamic Binding (Germany) by dialysis. DNA binding proteins were eluted by the Br Dynamic Binding (Germany) by dialysis. DNA binding proteins were eluted by the Br Dynamic Binding (Germany) by dialysis.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay was performed using double-stranded oligonucleotides (Promega) for the consensus binding site of the NF-κB nucleus (5′-AGTT-5′GCCAACCCCTCCAGG-3′). Oligonucleotides were labeled with 1 μl of oligonucleotide (3.5 pmol/μl), 2 μl of 5× kinase buffer, 5 units of T4 polynucleotide kinase, and 3 μl of [γ-32P]ATP (3000 Ci/mmol at 10 μCi/ml) incubated at 37 °C for 1 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Amersham Biosciences, Inc.) according to the manufacturer’s instructions. Five micrograms of crude nuclear proteins were incubated for 10 min on ice in binding buffer (10 mM Tris-HCl, pH 8.0, 25 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, pH 8.0, 5% glycerol, 5 mg/ml bovine serum albumin, 100 μg/ml RNase, and 50 μg/ml poly(dI-dC)) in a final volume of 15 μl. Labeled probe (∼40,000 cpm) was added, and the reaction was incubated for 15 min at room temperature. Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. p65 antibody was added 15 min before incubation with the labeled probe at 4 °C. Protein-DNA complexes were resolved by electrophoresis at 4 °C on a 5% acrylamide gel and subjected to autoradiography.

**Detection of Programmed Cell Death**—Nuclear fragmentation was analyzed on a Epics XL (Coultier Corp.) flow cytometer. Briefly, cells were collected with 0.25% trypsin in phosphate-buffered saline and fixed with 70% ethanol for 2 h. Fixed cells were rinsed in phosphate-buffered saline and then resuspended in a solution containing 0.02 mg/ml propidium iodide (0.2 μg/ml DNAase-free DNase II) and 40 μg/ml tRNA, 10 mM Triton X-100 in phosphate-buffered saline. Thereafter, cells were incubated at room temperature for 30 min in the dark and analyzed by flow cytometry.

**Analysis of Reactive Oxygen Species**—After 24 h of treatment with etomoxir (1 μM), 30 μM PDTC, or 10 μM etomoxir with or without 5 mA 2-Me, cells were scraped into phosphate-buffered saline, and the pellet was resuspended in 1 ml with phosphate-buffered saline and stained for 1 h at 37 °C in the dark with 10 μM DCFH-DA. Cells were then pelleted and resuspended in phosphate-buffered saline, and cell viability was assessed by using propidium iodide (1 μM). After incubation with fluorochrome, cells were kept in the dark at 4 °C, and fluorescence intensity was measured in a Epics EPICS ELITE cytometer (Coultier Corp., Hialeah, FL). A minimum of 10,000 cells/sample were acquired and analyzed.

**Statistical Analyses**—Results are usually expressed as means ± S.D. of three experiments. Significant differences were established by Student’s t test or analysis of variance according to the number of groups compared. When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. All the statistical analyses were performed using the computer program GraphPad Instat.

**RESULTS**

Effects of Etomoxir on the mRNA Levels of PPARα and Its Target Genes in C2C12 Skeletal Muscle Cells—C2C12 myoblasts differentiated morphologically to fuse into myotubes
when cultured in the presence of 2% horse serum 4 days after reaching confluence. The effects of different concentrations of etomoxir on PPARα mRNA expression were assessed in C2C12 myotubes for 24 h, incubated in serum-free conditions. Under these culture conditions, concentrations of etomoxir ranging from 10 to 100 μM strongly decreased PPARα mRNA levels (Fig. 1A). At low etomoxir concentrations, ranging from 10 to 40 μM, the reduction in PPARα mRNA levels was about 50%, with respect to the control values. At 80 μM, etomoxir reduced PPARα mRNA levels by 87% (p < 0.001). To test whether PPARα reduction resulted in down-regulation of genes regulated by this transcription factor, we analyzed the transcript levels of several well known PPARα target genes, aco, m-cpt-I, mcad, and ucp-3 (12). The effects of the different concentrations of etomoxir on ACO expression, which catalyzes the rate-limiting step of peroxisomal β-oxidation, showed a similar profile to that previously reported for PPARα (Fig. 1B). At low concentrations of etomoxir (10–40 μM), no significant changes in ACO mRNA levels were observed with respect to the control values. However, at 80 μM, a 69% reduction was observed in ACO transcripts (p < 0.04). In contrast to the effects on ACO mRNA levels, etomoxir treatment increased M-CPT-I mRNA levels, achieving a 2.6-fold induction at 80 μM, which is consistent with the reported increase in M-CPT-I in heart after etomoxir treatment (21). However, no change in M-CPT-I mRNA levels was observed at 100 μM etomoxir, with respect to the control cells. The mRNA levels of MCAD, an enzyme catalyzing a rate-limiting step in the mitochondrial β-oxidation, were not modified by etomoxir at concentrations ranging from 10 to 40 μM. In contrast, at higher concentrations, etomoxir caused a fall in MCAD transcript levels, achieving an 80% (p < 0.001) and a 98% reduction at 80 and 100 μM, respectively. UCP-3, a mitochondrial proton transporter that may be involved in fatty acid oxidation, was up-regulated by low concentrations of etomoxir, reaching a maximal induction of 3-fold at 40 μM. At 80 μM no change was observed with respect to the control values, whereas 100 μM etomoxir caused a 70% reduction. Etomoxir treatment did not affect the mRNA expression of
neither PPARβ/δ nor PPARγ, indicating that the effects of this drug etomoxir were specific for the PPARα isotype (data not shown).

Ceramides Are Not Involved in the Effects Caused by Etomoxir on PPARα mRNA Levels in C2C12 Skeletal Muscle Cells—CPT-I inhibition by etomoxir prevents the entrance of palmitoyl-CoA into mitochondria, leading to its accumulation in the cytoplasm. Because palmitoyl-CoA is a precursor of sphingolipid synthesis, etomoxir treatment may result in enhanced ceramide synthesis and apoptosis (22). Thus, to gain further insight into the mechanism by which etomoxir down-regulates PPARα mRNA levels, we tested the effects of several inhibitors of de novo ceramide synthesis. The initial step in ceramide synthesis is the formation of 3-ketodihydrosphingosine from palmitoyl-CoA and L-serine. This step is inhibited by the sphingosine analog ISP1 at picomole concentrations (23). Similarly, fumonisin B1 suppresses ceramide synthetase activity (24), the final step in de novo synthesis of ceramide. Neither ISP1 nor fumonisin B1 treatment prevented the down-regulation in PPARα mRNA levels produced by etomoxir (Fig. 2A). To further clarify the potential involvement of ceramides in the down-regulation of PPARα caused by etomoxir, we treated C2C12 skeletal muscle cells with C2-ceramide, a cell-permeable ceramide analog, for 24 h. The addition of 5 μM C2-ceramide did not modify PPARα mRNA levels (Fig. 2B). These data suggest that de novo ceramide synthesis is not involved in the effects of etomoxir. In addition, etomoxir treatment did not increase apoptosis, as observed by the incidence of nuclear fragmentation events (Fig. 2C). Likewise, no changes were observed in DNA ladder formation after etomoxir treatment (data not shown).

Effects of Triacsin C on the Effects Mediated by Etomoxir on PPARα mRNA Levels in C2C12 Skeletal Muscle Cells—Long chain fatty acids should be previously activated by acyl-CoA synthetase (ACS) to be available as CPT-I substrates. Moreover, in cells etomoxir is metabolized to etomoxir-CoA, which is the active form of the drug, and it is also formed by this enzyme (25). To study whether the effects of etomoxir were mediated through etomoxir-CoA, we tested the effects of a potent inhibitor of ACS activity, triacsin C. The addition of triacsin C to the etomoxir-treated C2C12 skeletal muscle cells did not prevent the down-regulation in PPARα mRNA levels (Fig. 3A), suggesting that etomoxir and not etomoxir-CoA was responsible for the effects caused by this drug. Interestingly, triacsin C alone significantly reduced PPARα mRNA levels by 33% (p < 0.001). Similarly, the effects of 80 μM etomoxir on M-CPT-I and UCP-3 mRNA levels were not significantly altered by triacsin C (Figs. 3B and C). Treatment with triacsin C alone caused a 2.3- and 1.7-fold induction in M-CPT-I and UCP-3 mRNA levels, respectively, which is consistent with the activation of PPARα by free fatty acids (26).

Effects of Inhibitors of Mitogen-activated Protein Kinase on PPARα Down-regulation by Etomoxir—It has been shown that during cardiac hypertrophic growth, PPARα activity is reduced at the levels of gene expression as well as by rapid post-translational effects involving phosphorylation by the extracel-
lular signal-regulated kinase mitogen-activated protein kinase pathway (14). Therefore, to study whether phosphorylation was involved in the deactivation of PPARα regulatory pathways caused by etomoxir, we studied the effect of PD98059, a known inhibitor of the extracellular signal-regulated kinase mitogen-activated protein kinase pathway, on the mRNA expression of PPARα and several of its target genes after etomoxir treatment (Fig. 4). In the presence of PD98059, the 81% reduction in PPARα mRNA levels caused by etomoxir alone did not reverse. Similarly, PD98059 did not affect the reduction in ACO mRNA levels, whereas the 87% reduction in MCAD mRNA partially reversed to a 67% reduction (p < 0.05 respect to etomoxir-treated cells). These results suggest that phosphorylation was not involved in the effects caused by etomoxir in the PPARα pathway, although it can regulate MCAD expression.

**PPARα Down-regulation by Etomoxir in C2C12 Skeletal Muscle Cells Requires ROS Generation and Results in NF-κB Activation**—Finally, we attempted to identify the mechanism whereby etomoxir treatment results in a reduction in PPARα mRNA levels. Because etomoxir contains a saturated fatty acid-derived structure with an oxirane group and saturated fatty acids such as palmitate generate ROS (27), probably through protein kinase C-dependent activation of NAD(P)H oxidase (28), we determined whether etomoxir addition resulted in ROS generation. To determine ROS, we used DCFH, which shows increased fluorescence upon reaction with intracellular oxygen radicals, which can be detected by flow cytometry. Supplementation of C2C12 skeletal muscle cells with 80 μM etomoxir resulted in an increase in DCFH fluorescence, indicating that reactive oxygen intermediates were generated by etomoxir treatment (Fig. 5A). Increased DCFH fluorescence was observed in 28% of total cell population compared with the 2% in untreated cells. The addition of 5 mM PDTC, a potent antioxidant, to etomoxir-treated cells resulted in a decrease in DCFH fluorescence to values similar to those observed in control cells. These findings demonstrate that etomoxir treatment leads to ROS generation. Because etomoxir treatment of C2C12 skeletal muscle cells results in the generation of ROS and PDTC inhibits NF-κB activity (29), we next studied whether this well known redox-regulated transcription factor was involved in the changes caused by etomoxir. Electrophoretic mobility shift assay demonstrated that NF-κB formed four complexes with nuclear proteins (complexes I to IV) (Fig. 5B). Specificity of the three DNA binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide. NF-κB binding activity mainly of specific complex I increased in nuclear extracts from etomoxir-treated cells. The addition of anti-p65 antibody completely supershifted complex I, indicating that this band corresponds to the NF-κB p65 subunit.

Finally, to determine whether the generation of ROS is essential for the etomoxir-induced changes in the PPARα pathway, we measured the ability of the antioxidant PDTC to inhibit PPARα mRNA down-regulation in C2C12 skeletal muscle cells. In the presence of 5 mM PDTC, etomoxir was unable to significantly decrease the mRNA expression of PPARα (Fig. 5C). Thus, the addition of PDTC, which avoids ROS generation and NF-κB activation by etomoxir, prevented the reduction in PPARα mRNA expression.

**DISCUSSION**

The results presented here demonstrate that PPARα pathway down-regulation after etomoxir treatment in C2C12 skeletal muscle cells is mediated by ROS generation and increased NF-κB activity. The reduction in PPARα expression was reversed by PDTC, a potent antioxidant and inhibitor of NF-κB. This finding indicates that ROS generation and NF-κB are involved in the fall in PPARα expression. Previous studies suggest that age-associated reductions in PPARα mRNA levels are mediated through enhanced cellular redox stress and NF-κB activation (30). In fact, the NF-κB-driven cytokines tumor necrosis factor-α, interleukin-1β, and interleukin-6 have been demonstrated to cause a reduction in the expression of...
PPARα (31, 32). These cytokines are present at high levels in cells from aged animals (33), which present reduced mRNA levels of ppara and aco genes (30). When antioxidants were administered to aged rats, an increase in the mRNA levels of PPARα and ACO in splenocytes was observed, reaching similar values to those present in young animals (30). In the present
work we show that an increase in the cellular redox state in skeletal muscle cells results in a fall in PPARα mRNA levels. In addition to the effects of NF-κB on PPARα transcript levels, a reciprocal transcriptional interference has been reported between PPARα and the p65 subunit of NF-κB (34). p65 repressed PPARα transactivation of a peroxisome proliferator response element-driven promoter in COS cells, and it was suggested that cross-talk between PPARα and p65 occurs mainly via the ligand binding domain of PPARα (34). Therefore, according to these mechanisms is likely that the increase in NF-κB activity by ROS generation after etomoxir treatment may repress both PPARα mRNA expression and PPARα transactivation. We propose that ROS generation and subsequent NF-κB activation may contribute to the accumulation of intracellular lipid accumulation in skeletal muscle cells.

Etomoxir belongs to the family of CPT-I inhibitors, which activate PPARα, and their transcriptional activity correlate with their ability to bind this nuclear receptor (35). The mechanism by which these drugs activate PPARα, direct binding to this receptor and, indirectly, acting as a metabolic inhibitor, may lead to the accumulation of endogenous fatty acid lipids. Because in the present study C2C12 skeletal muscle cells were incubated without exogenous fatty acids, accumulation of fatty acyl-CoA derivatives under these conditions should be negligible. Therefore, PPARα activation by etomoxir in C2C12 skeletal muscle cells should be assigned only to direct binding to this receptor. Interestingly, our data show a different dual function of etomoxir depending on the concentration of etomoxir used. At low concentrations ranging from 10 to 40 μM the PPARα reduction in mRNA levels was about 50%. However, this reduction was not sufficient to avoid the transcriptional induction of several PPARα target genes such as UCP-3 or M-CPT-I, whereas other PPARα target genes such as ACO or MCAD were not modified. When the concentrations of etomoxir used were higher than 40 μM, the down-regulation in PPARα mRNA levels was of such intensity that a fall in the mRNA expression of all the PPARα target genes studied except M-CPT-I was observed. Therefore, the data presented here indicate that at low concentrations, when the reduction in PPARα expression is small, etomoxir activates PPARα target genes. However, at higher concentrations, another mechanism appears, leading to a fall in PPARα expression, and as a result, the expression of its target genes is reduced.

It has been reported that CPT-I inhibition by etomoxir results in enhanced palmitate-induced cell death and led to a further increase in ceramide synthesis in LyD9 and WEHI-231 cells (22). Therefore, we determined whether the effects of etomoxir on PPARα mRNA levels were the result of programmed cell death through increase ceramide synthesis. By using inhibitors of the de novo ceramide synthesis and a ceramide analog we have demonstrated that ceramides were not involved in the effects of etomoxir on PPARα down-regulation. In addition, etomoxir treatment did not result in increased apoptosis. In fact, in the work of Paumen et al. (22), the addition of etomoxir alone at a concentration of 400 μM did not cause nonspecific cell damage, and 200 μM etomoxir did not compromise cell viability nor increased nuclear fragmentation events in LyD9 cells. In contrast, the combined addition of etomoxir and palmitate resulted in a dramatic increase in DNA ladder formation and nuclear fragmentation. In our study, C2C12 skeletal muscle cells were treated with etomoxir in the absence of exogenous fatty acids. Therefore, as it is shown, ceramides and apoptosis does not contribute to the effects elicited by etomoxir on PPARα expression. On the contrary, we have previously reported (36) that 40 μM etomoxir up-regulates UCP-3 and M-CPT-I mRNA levels in C2C12 skeletal muscle cells. Given that C2-ceramide treatment caused a similar induction in the expression of these genes, we suggested that de novo ceramide synthesis could be the mechanism underlying the induction in UCP-3 and M-CPT-I caused by etomoxir treatment. Therefore, although ceramide de novo synthesis is not involved in the down-regulation of PPARα after etomoxir treatment, it may be implicated in the up-regulation of UCP-3 and M-CPT-I observed after treatment with etomoxir.

Long chain fatty acids are not available as CPT-I substrates until they are activated by acyl-CoA synthetase. The ability of etomoxir to block CPT-I activity depends also on this enzyme, which forms the active form of the drug, etomoxir-CoA. Surprisingly, the effects of etomoxir were not prevented in the presence of the acyl-CoA synthetase inhibitor, triacsin C. These results show that the effects of etomoxir do not depend on acyl-CoA synthetase to gain access to the mitochondrial CPT system, suggesting that CPT-I inhibition is not involved in the effects of this drug on PPARα expression.

It remains to study whether etomoxir treatment may result in ROS generation in vitro at pharmacological doses. However, because the IC_{50} value of etomoxir-CoA for inhibiting CPT-I activity in rat heart is 14 μM (37), it is unlikely that etomoxir administration at pharmacological doses led to ROS generation.

It is important to remark that generation of ROS, independent of ceramide synthesis, is important for the lipotoxic response and may contribute to the pathogenesis of diseases involving intracellular lipid accumulation (27). Here we propose a regulatory mechanism through which the inhibition of the PPARα pathway by ROS and NF-κB activation may contribute to intracellular lipid accumulation in skeletal muscle cells. Because cellular enrichment with both saturated and polyunsaturated fatty acids initiates an increase in ROS (27, 38) and activates NF-κB binding (38), it remains to study whether skeletal muscle exposition to elevated free fatty acids results in PPARα down-regulation.

Acknowledgment—We thank Robin Rycroft (Language Advisory Service of the University of Barcelona) for helpful assistance.

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18. We thank Robin Rycroft (Language Advisory Service of the University of Barcelona) for helpful assistance.
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