

Peroxisome Proliferator–Activated Receptor- α Regulates Fatty Acid Utilization in Primary Human Skeletal Muscle Cells

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In humans, skeletal muscle is a major site of peroxisome proliferator–activated receptor- α (PPAR- α) expression, but its function in this tissue is unclear. We investigated the role of hPPAR- α in regulating muscle lipid utilization by studying the effects of a highly selective PPAR- α agonist, GW7647, on [¹⁴C]oleate metabolism and gene expression in primary human skeletal muscle cells. Robust induction of PPAR- α protein expression occurred during muscle cell differentiation and corresponded with differentiation-dependent increases in oleate oxidation. In mature myotubes, 48-h treatment with 10–1,000 nmol/l GW7647 increased oleate oxidation dose-dependently, up to threefold. Additionally, GW7647 decreased oleate esterification into myotube triacylglycerol (TAG), up to 45%. This effect was not abolished by etomoxir, a potent inhibitor of β -oxidation, indicating that PPAR- α -mediated TAG depletion does not depend on reciprocal changes in fatty acid catabolism. Consistent with its metabolic actions, GW7647 induced mRNA expression of mitochondrial enzymes that promote fatty acid catabolism; carnitine palmityltransferase 1 and malonyl-CoA decarboxylase increased \sim 2-fold, whereas pyruvate dehydrogenase kinase 4 increased 45-fold. Expression of several genes that regulate glycerolipid synthesis was not changed by GW7647 treatment, implicating involvement of other targets to explain the TAG-depleting effect of the compound. These results demonstrate a role for hPPAR- α in regulating muscle lipid homeostasis. *Diabetes* 51: 901–909, 2002

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ACO, acyl-CoA oxidase; ASM, acid-soluble metabolite; CPT1, carnitine palmityltransferase 1; DFM, differentiation media; DGAT, diacylglycerol acyltransferase; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; GM, growth media; GPAT, glycerol-3-phosphate acyltransferase; HS-KMC, human skeletal muscle cell; MCAD, medium-chain acyl-CoA dehydrogenase; MCD, malonyl-CoA decarboxylase; PDH, pyruvate dehydrogenase; PDHK, PDH kinase; PPAR, peroxisome proliferator–activated receptor; RTQ-PCR, real-time quantitative PCR; SREBP1, sterol regulatory element binding protein 1; TAG, triacylglycerol; TCA, tricarboxylic acid.

Peroxisome proliferator–activated receptor (PPAR)- α , δ , and γ belong to a family of nuclear hormone receptors that are bound and activated by fatty acids and/or their derivatives, and they regulate genes that are involved in lipid metabolism. PPAR- γ , which is expressed primarily in adipose tissue, promotes adipocyte differentiation and activates transcription of genes involved in lipogenesis and fatty acid esterification (1). Conversely, PPAR- α , initially identified as the molecular mediator of a class of chemical compounds that induces peroxisomal proliferation in rodent liver, is expressed most abundantly in tissues that are characterized by high rates of β -oxidation (1). Studies in PPAR- α -null mice indicate that the α subtype plays a critical role in maintaining constitutive activity of β -oxidative pathways in liver and heart, as well as mediating adaptive metabolic responses to starvation (2,3). Furthermore, in response to stresses that perturb fatty acid metabolism, PPAR- α null mice accumulate massive levels of neutral lipids in hepatic and cardiac tissues (2,3). These studies demonstrate that in rodents, PPAR- α deficiency results in profound dysregulation of systemic lipid homeostasis.

The role of PPAR- α in humans is less clear. Drugs that are PPAR- α activators are used therapeutically as potent hypolipidemic agents (4), and new evidence suggests that they might also prove effective for treating obesity and insulin resistance (5); however, the precise mechanisms that underlie the efficacy of these compounds still remain obscure. Investigations of PPAR- α -mediated responses have historically focused on the liver, which in rodents is the tissue that expresses PPAR- α most abundantly (1). However, unlike their effect in rodents, PPAR- α -selective drugs do not induce peroxisomal proliferation in human liver (6), indicating some degree of species specificity with regard to target genes and/or tissues. Recent animal studies have shown that treatment with PPAR- α agonists affects gene expression in skeletal muscle (7–10), suggesting that these compounds might target skeletal muscle directly. Importantly, in humans, skeletal muscle is a major site of PPAR- α expression (11), but its function in this tissue has not been well investigated. Skeletal muscle represents a principal tissue responsible for lipid uptake and utilization, and it contributes significantly to whole-body lipid homeostasis. Thus, we hypothesized that hPPAR- α plays a key role in regulating muscle lipid homeostasis, and that the efficacy of PPAR- α -selective

TABLE 1
Primer/probes sets used for real-time quantitative PCR

Gene	Forward	Reverse	Probe
ACO	CCTGAGTGAAGTGCCTGAGCT	TTGCAGTCCAGGAGGTGAAAG	CATGCCCTCACCGCTGGACTGAAGA
CPT-1	CTGCAGTGGGACATTCCAAA	CAACGCCTTGCCACCT	TGCCAGGCGGTCATCGAGAGTTC
DGAT	GTTCTGGCCTCGGCTTT	AGGCGGAACATTGCGAGA	TTCCACGAGTACCTGGTGAGCGTCC
GPAT	ACTCCTTGGGCCTTTGCTG	TTCTGGAACAGGACCACTGAAGT	CCTACAGCTCTGCTGCCATCTTTGTTCA
MCD	CACGTGGCACTGACTGGTG	TTCTGTTTCTGATGGAGGATGTTT	CCAGCAACATCCAGGCAATCGTGAA
PDHK2	CAC ACC CTC ATC TTT GAT G	GGT CGA TGC TGC CGA TGT	CAC CAA CCC AGC CCA TCC C
PDHK4	TGCATTTTTGCGACAAGAATTG	TTGGGTCGGGAGGATATCAA	CTGTGAGACTCGCCAACATTTGAAGGA
PGC-1	GTCTCTCCTTGACGACAAGAA	GATGACCGAAGTGCTTGTTCAG	CCAAGACCAGAAAATCCGAGCCG
SREBP-1	CAAGGCCATCGACTACATT	TTGCTTTTGTGGACAGCAGT	TGCAACACAGCAACCAGAA

PGC1, PPAR co-activator 1.

drugs in humans might be mediated by direct effects on skeletal muscle fuel metabolism. To test these hypotheses, we studied the effects of a highly selective hPPAR- α agonist, GW7647 (12), on lipid metabolism and gene regulation in primary human skeletal muscle cells (HSKMCs). Here, we report that hPPAR- α regulates expression of genes that control muscle lipid utilization, and that PPAR- α activation results in profound effects on fuel metabolism, favoring lipid catabolism over neutral lipid storage. These results contribute new information regarding the function of PPAR- α in human skeletal muscle and may present important therapeutic implications for treating lipid metabolic disorders.

RESEARCH DESIGN AND METHODS

Materials. BSA (essentially fatty acid-free), carnitine, sodium oleate, and oil red O stain were from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Hank's balanced salt solution were from Life Technologies (Gaithersburg, MD). Heat-inactivated horse serum was from Hyclone (Logan, UT). Growth media (GM) and differentiation media (DFM) consisted of Dulbecco's modified Eagles medium (DMEM) from Life Technologies supplemented with human skeletal muscle SingleQuots from BioWhittaker (Walkersville, MD). Biocoat tissue culture plates were from Becton Dickinson (Franklin Lakes, NJ). PCR reagents were from PE Applied Biosystems (Foster City, CA). GW7647 was obtained from Dr. Peter Brown at GlaxoSmithKline.

Primary cultures of human skeletal muscle cells. Protocols were approved by the institutional review board at East Carolina University. Muscle samples weighing 50–200 mg, which were obtained from vastus lateralis by needle biopsy or from rectus abdominis muscle that was excised during surgical procedure, were immediately transferred to ice-cold DMEM and cleaned free of adipose and connective tissues. Satellite cells were isolated by trypsin digestion (13), preplated 1–3 h in 3.0 ml GM on an uncoated T-25 tissue culture flask to remove fibroblasts, and then transferred to a type I collagen-coated T25 flask for attachment. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in GM supplemented with 10% FBS, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, 20 ng/ml human epidermal growth factor, 0.39 μ g/ml dexamethasone, and 50 μ g/ml gentamicin/amphotericin B. After reaching ~70% confluence, myoblasts were subcultured onto 6-, 12-, and 24-well type I collagen-coated plates at densities of 100, 50, and 20 \times 10³ cells per well, respectively. When cells reached 80–90% confluence, differentiation was induced by changing to low-serum DFM consisting of 2% heat-inactivated horse-serum, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, and 50 μ g/ml gentamicin/amphotericin B. Media was changed every 2–3 days, and the PPAR- α -selective compound, GW7647, or DMSO vehicle (0.1% vol/vol) was added to developing myotubes on differentiation days 1–6 or to mature myotubes on days 6–7. Cells were harvested in 1.0 ml Trizol reagent for RNA extraction or 500 μ l NP-40 lysis buffer for Western analyses.

Western blot analyses. Protein (30–50 μ g) prepared from total cell lysates was separated by 10% SDS-PAGE, transferred to PVDF membranes (Biorad, Hercules, CA), and then incubated with antibodies diluted in 5% milk in Tris-buffered saline with 0.1% Tween. Proteins were visualized by horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G from Santa Cruz Biotechnology (Santa Cruz, CA) using a chemiluminescence Western-blotting detection kit from Pierce (Rockford, IL). Myosin and Myo-D polyclonal antibodies were from Santa Cruz, antibody against medium-chain

acyl-CoA dehydrogenase (MCAD) was a gift from Dan Kelly (Washington University, St. Louis, MO), and monoclonal anti-hPPAR- α was synthesized as previously described (11).

Determination of fatty acid metabolism. Myocytes maintained in differentiation medium for 0 days (myoblasts) or 3–9 days (developing myotubes) were incubated at 37°C in sealed 12- or 24-well plates containing 500 or 750 μ l serum-free DFM plus 12.5 mmol/l HEPES, 0.2% BSA, 1.0 mmol/l carnitine, 100 μ mol/l sodium oleate, 50 μ g/ml gentamicin, and 1.0 μ Ci/ml [¹⁴C]oleate (NEN, Boston, MA). After 3 h, the incubation media was transferred to new dishes and assayed for labeled oxidation products (CO₂ and acid-soluble metabolites [ASMs]) (14). The cells were placed on ice, washed twice with PBS, scraped into a 1.5-ml eppendorf tube in two additions of 0.30 ml 0.05% SDS lysis buffer, and then stored at –80°C. Cell lysates were later assayed for protein, and then total cell lipids were extracted (15). Aliquots of the lipid extracts were spotted on 0.25-mm silica gel G plates from Whatman (Maidstone, Kent, U.K.) and chromatographed with hexane:diethyl ether:acetic acid (80:20:1 vol/vol) in parallel with authentic standards. [¹⁴C]oleate-labeled lipid products were quantified using a BioScan Image 200 System (Bioscan, Washington, DC). All assays were performed in triplicate, and data are presented as the means \pm SE of results from 5 to 10 subjects.

Real-time quantitative PCR. Total RNA was prepared using TriZol reagent according to the manufacturer's protocol (Life Technology), treated with DNase I (Ambion, Austin, TX), and quantified using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR). Real-time quantitative PCR (RTQ-PCR) was performed using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA), and primer/probe sets (Table 1) were designed using the manufacturer's software and the sequences available in GenBank. Expression levels of selected genes were compared between myotubes from the same subject, treated with either vehicle or 1.0 μ mol/l GW7647. RNA samples were normalized for comparison by determining 18S rRNA levels by RTQ-PCR. Expression levels were quantified (arbitrary units) by generating a seven-point serial standard curve, as previously described (16). Results are expressed as the fold change, as determined by the ratio of calculated units of RNA in GW7647-treated samples to those in vehicle controls, and are presented as the means \pm SE from four subjects per group. For comparison, relative quantitation was also calculated by using the 2^{– Δ C_T} formula, in which Δ C_T equals the difference between C_T (cycle threshold) values for control and treated cells. Results using this formula (not shown) were essentially identical to those generated using the standard curves.

Statistics. Statistical analyses were performed using JMP Statistical Software (SAS, Cary, NC). Differences between vehicle- and GW7647-treated cells were analyzed by Student's *t* test for paired data, and one- or two-way ANOVAs were performed using a standard least squares model to test both the main and interaction effects of GW7647 and etomoxir (where appropriate) on parameters of lipid metabolism.

RESULTS

Differentiation-dependent induction of PPAR- α in HSKMCs. Preconfluent myoblasts exhibit a mononucleated, spindle-shaped morphology (Fig. 1A). Confluent myoblasts withdraw from the cell cycle, align with one another, and then attach, fuse, and differentiate into multinucleated myotubes (Fig. 1B), a process that is facilitated by switching to a low-serum DFM. Similar to other reports (13), we found that >80% of HSKMCs

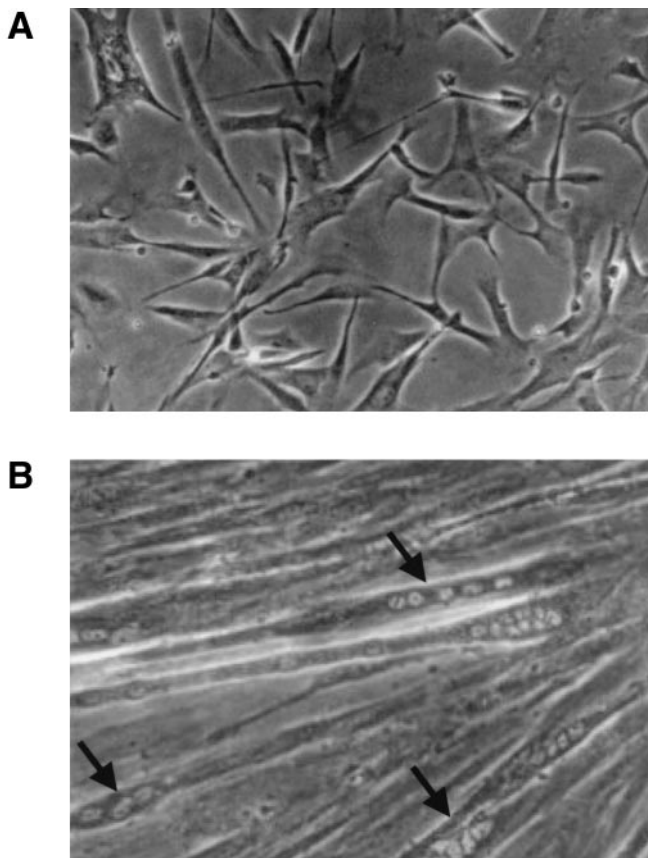


FIG. 1. Phase contrast microscopy of developing myotubes. *A*: Phase contrast photomicrographs (200 \times) showing pre-confluent myoblasts that exhibit mononucleated morphology. When cells reach confluence, myoblasts align and begin to fuse. *B*: Within 6 days after switching to low-serum DFM, >80% of cells exhibit multinucleated (arrows) morphology that is characteristic of mature myotubes.

exhibited elongated, multinucleated myotube morphology within 6–8 days after switching to DFM, indicating low levels of fibroblast contamination in the initial cultures. Although PPAR- α is expressed abundantly in human skeletal muscle (11), it is absent from some stable muscle cell lines (17); thus, we first evaluated PPAR- α protein expression in undifferentiated myoblasts and developing myotubes (Fig. 2*A*). Expression of PPAR- α protein was low in myoblasts, but it increased markedly by day 3 in DFM and was then maintained throughout myotube development at levels that were similar to those in intact human skeletal muscle (Fig. 2*B*). Differentiation-dependent expression of PPAR- α coincided with induction of the muscle-specific proteins myosin and Myo-D and the fatty acid oxidative enzyme MCAD. Myocyte differentiation and concomitant induction of PPAR- α were also associated with increased fatty acid oxidative capacity (Fig. 2*C*).

Effects of GW7647 on fatty acid oxidation. GW7647 is a novel PPAR- α ligand with an EC_{50} value of 6 nmol/l, as determined in PPARx/gal4 chimeric transfection assays (12). GW7647 exhibits 1,000-fold selectivity for PPAR- α over PPAR- γ and - δ and is therefore more potent and selective than many commonly used PPAR- α -targeted compounds. To evaluate the role of PPAR- α in regulating muscle lipid metabolism, we treated day 6 myotubes for 48 h with 0–1,000 nmol/l GW7647, and then day 8 myocytes were incubated for 3 h with 100 μ mol/l [14 C]oleate. Fatty

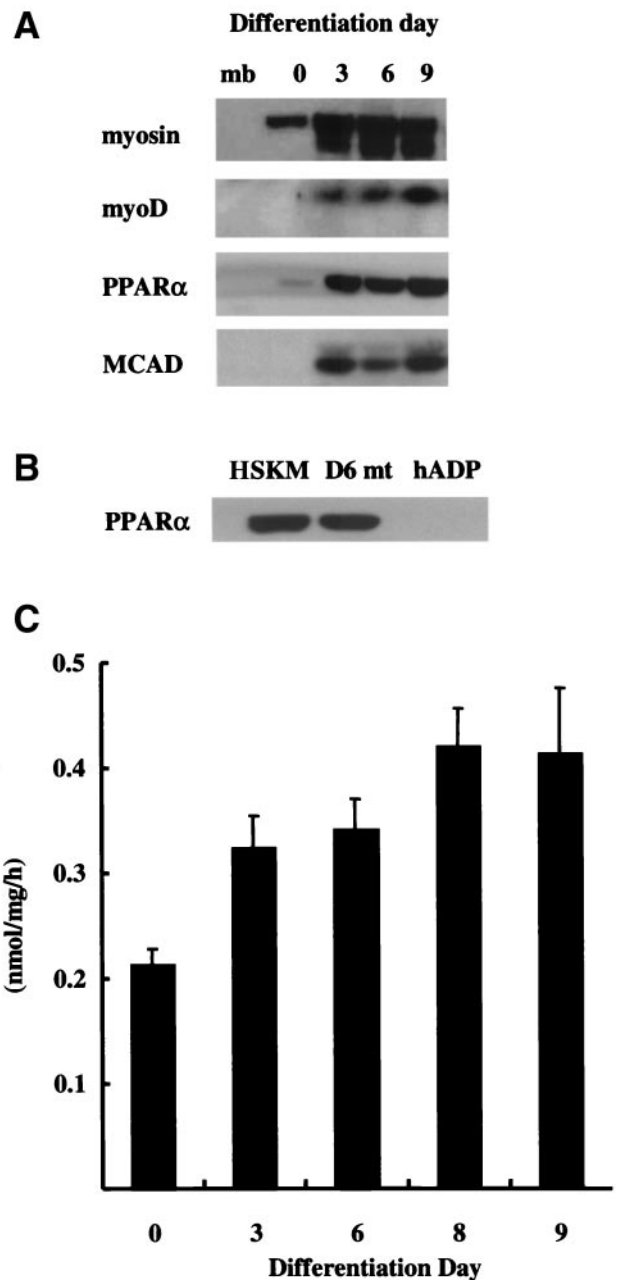


FIG. 2. Expression of PPAR- α in myotubes coincides with induction of muscle-specific proteins and increased fatty acid oxidation. *A*: Western blots showing muscle-specific protein expression in pre-confluent myoblasts (mb), confluent myoblasts (day 0), and myotubes (days 3, 6, and 9). Protein (30–50 μ g) from total cell lysates was separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies against myosin, Myo-D, PPAR- α , or MCAD. *B*: PPAR- α protein expression in HSKM, day 6 human myotubes (D6 mt), and human adipose tissue lysate (hADP). *C*: Myocytes were incubated for 3 h at 37 $^{\circ}$ C with 100 μ mol/l [14 C]oleate and 0.2% BSA. Oleate oxidation was determined by measuring [14 C]CO₂ release into the media. Assays were performed in triplicate. Values are the means \pm SE from three subjects, and the effect of differentiation was analyzed by one-way ANOVA at the $P < 0.05$ level.

acid oxidation, measured as the sum of [14 C]oleate oxidized to CO₂ (complete oxidation) plus ASM (incomplete oxidation), was 1.52 ± 0.14 nmol \cdot mg⁻¹ \cdot h⁻¹ in vehicle-treated myotubes. GW7647 increased myotube oleate oxidation to CO₂ and ASMs in a dose-dependent manner ($P < 0.001$) (Fig. 3*A* and *B*). Myotubes responded maximally at

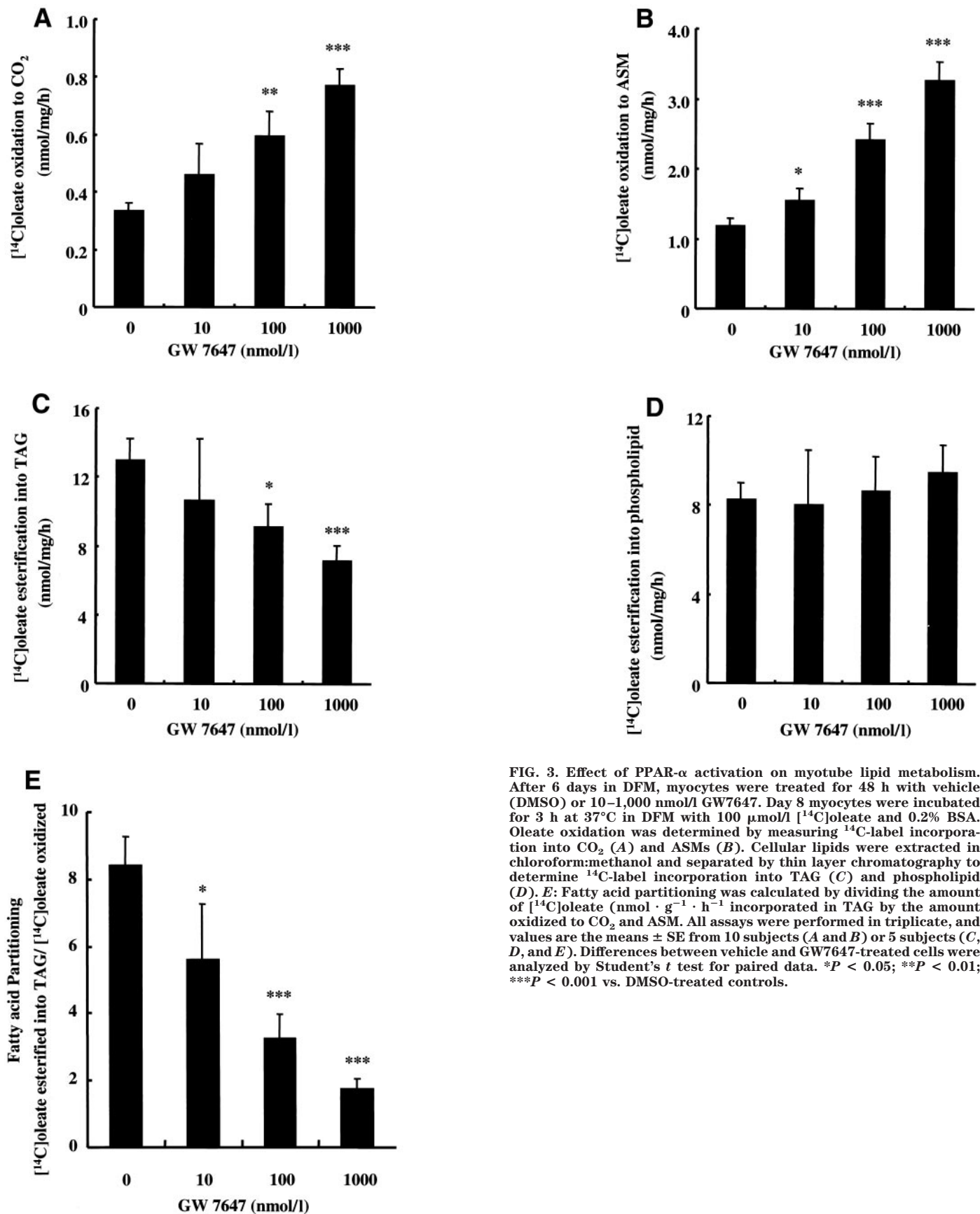


FIG. 3. Effect of PPAR- α activation on myotube lipid metabolism. After 6 days in DFM, myocytes were treated for 48 h with vehicle (DMSO) or 10–1,000 nmol/l GW7647. Day 8 myocytes were incubated for 3 h at 37°C in DFM with 100 μ mol/l [¹⁴C]oleate and 0.2% BSA. Oleate oxidation was determined by measuring ¹⁴C-label incorporation into CO₂ (A) and ASMs (B). Cellular lipids were extracted in chloroform:methanol and separated by thin layer chromatography to determine ¹⁴C-label incorporation into TAG (C) and phospholipid (D). E: Fatty acid partitioning was calculated by dividing the amount of [¹⁴C]oleate (nmol · g⁻¹ · h⁻¹ incorporated in TAG by the amount oxidized to CO₂ and ASM. All assays were performed in triplicate, and values are the means \pm SE from 10 subjects (A and B) or 5 subjects (C, D, and E). Differences between vehicle and GW7647-treated cells were analyzed by Student's *t* test for paired data. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 vs. DMSO-treated controls.

1.0 $\mu\text{mol/l}$ GW7647 by increasing oleate oxidation 2.7-fold to $4.01 \pm 0.32 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$. This response was similar in cells from different muscle sources (vastus lateralis versus rectus abdominus); therefore, results were pooled together.

Effects of GW7647 on triacylglycerol homeostasis. In PPAR- α -null mice, pharmacological inhibition of fatty acid oxidation results in marked accumulation of triacylglycerol (TAG) in liver and heart (18). In contrast, when wild-type mice are treated with the same inhibitor, they exhibit only minor increases in tissue TAG (18). These findings suggest not only that PPAR- α is required for adaptive adjustments in TAG homeostasis, but also that TAG regulation occurs via mechanisms that are independent of lipid catabolism. To determine whether PPAR- α might play a similar role in human muscle, we evaluated the effects of GW7647 on [^{14}C]oleate esterification into glycerolipids. Basal rates of oleate incorporation into TAG and phospholipids were 12.95 ± 1.26 and $8.25 \pm 0.76 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$, respectively, and only small amounts (<10% of the total) were incorporated into diacylglycerol and other minor lipid species (not shown). Opposite to its effect on fatty acid oxidation, GW7647 decreased oleate esterification into TAG, up to 45% ($P < 0.001$), without affecting labeling of phospholipids (Fig. 3C and D).

To quantify the partitioning of fatty acid between opposing metabolic pathways, we divided the rate ($\text{nmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$) of oleate esterified into TAG by the rate of oleate oxidized, thereby providing an index of fatty acid utilization. Because GW7647 regulated oxidation and esterification in opposite directions, the partitioning index decreased 75%, from a TAG-to-oxidation ratio of 8:1 to 2:1 (Fig. 3E). The impact of this marked adjustment in fatty acid partitioning on myotube lipid homeostasis was then examined histologically, by oil red O staining of neutral lipids (Fig. 4). In vehicle-treated myocytes exposed to an overnight fatty acid load, the intensity of the stain increased as a function of increasing oleate concentration (Fig. 4A). In comparison, 48-h pretreatment with GW7647 attenuated fatty acid-induced accumulation of neutral lipids. In Fig. 4B, higher magnification of stained cells confirmed that neutral lipids had accumulated in mature myotubes and indicated that PPAR- α activation decreased the number cells exhibiting large lipid droplets.

Our observation that GW7647 specifically reduced TAG, but not phospholipid, suggested that this was not a mass action effect caused by changes in the myocellular [^{14}C]oleate pool. To determine whether the TAG-depleting effects of GW7647 depended on reciprocal changes in oleate oxidation, we repeated previous experiments but performed the 3-h [^{14}C]oleate incubation in either the presence or absence of etomoxir, a potent inhibitor of fatty acid oxidation. In the absence of etomoxir, 1.0 $\mu\text{mol/l}$ GW37647 increased oleate oxidation approximately threefold, recapitulating our earlier result. As expected, etomoxir inhibited oleate oxidation to negligible levels in both groups (Fig. 5A). During short-term exposures (3 h), blocking oxidation with etomoxir did not result in a compensatory increase in [^{14}C]oleate esterification (Fig. 5B). Moreover, [^{14}C]oleate incorporation into myocyte TAG was lower in GW7647-treated cells, regardless of whether etomoxir was present. These data are consistent

with lipid studies in PPAR- α -null mice (18) and indicate that the TAG-depleting effects of PPAR- α activation do not require opposing changes in fatty acid oxidation.

Effects of GW7647 on regulation of fatty acid oxidative genes. In these experiments, vehicle or GW7647 was administered to developing myotubes (days 1–6) or to mature myotubes for 48 h (days 6–8). Using RTQ-PCR, we evaluated changes in steady-state mRNA expression of several candidate target genes that are known or predicted to regulate muscle energy utilization. Pyruvate dehydrogenase kinase (PDHK), which phosphorylates and inactivates the pyruvate dehydrogenase (PDH) complex, is thought to facilitate preferential oxidation of lipid over glucose substrates. Remarkably, GW7647 increased expression of PDHK4 12- and 45-fold in cells that were treated for 48 h and 6 days, respectively (Table 2). GW7647 also induced highly consistent but less robust increases (1.7- to 2.4-fold) in mRNA expression of malonyl-CoA decarboxylase (MCD) and the muscle isoform of carnitine palmityltransferase 1 (CPT1), enzymes that promote uptake of fatty acid into the mitochondria for β -oxidation. Surprisingly, mRNA abundance of PPAR coactivator 1 was decreased 15% ($P < 0.05$) in mature myotubes that were treated with GW7647 for 48 h; however, this effect was reversed when GW7647 was added to developing myotubes for 6 days. Notably, we found that mRNA expression of the peroxisomal marker enzyme acyl-CoA oxidase (ACO) was unaffected by treatment with GW7647. PPAR- α mRNA abundance was similar in vehicle and GW7647-treated cells; thus, hPPAR- α does not appear to regulate its own expression.

Effects of GW7647 on regulation of TAG-biosynthetic genes. Data from our metabolic studies suggested that GW7647 might repress genes that promote myotube TAG biosynthesis. To address this result, we examined expression of genes involved in TAG synthesis, including sterol regulatory element binding protein 1 (SREBP1), mitochondrial glycerol-3-phosphate acyltransferase (GPAT), and diacylglycerol acyltransferase (DGAT). Contrary to our expectations, mRNA expression of GPAT was similar between control and GW7647-treated cells, and DGAT mRNA abundance was low or undetectable (C_T values >35) in HSKMCs from six of eight subjects, regardless of the treatment (Table 2). SREBP1 actually showed a tendency to increase in response to GW7647, but this effect was statistically significant ($P < 0.05$) only when results from both the 48-h and 6-day treatments were pooled. Collectively, these data suggest the TAG-depleting effects of GW7647 were not caused by direct repression of glycerolipid biosynthetic enzymes.

DISCUSSION

In this study, we used a primary HSKMC system to investigate the role of hPPAR- α in regulating muscle fuel metabolism. In contrast to previous animal studies that examined the effects of PPAR- α activators in vivo, the use of primary HSKMCs allowed us to discern direct, muscle-specific effects of hPPAR- α activation. To our knowledge, this is the first report that characterizes PPAR- α expression and its metabolic target genes in developing human myotubes. Our results indicate that hPPAR- α is robustly

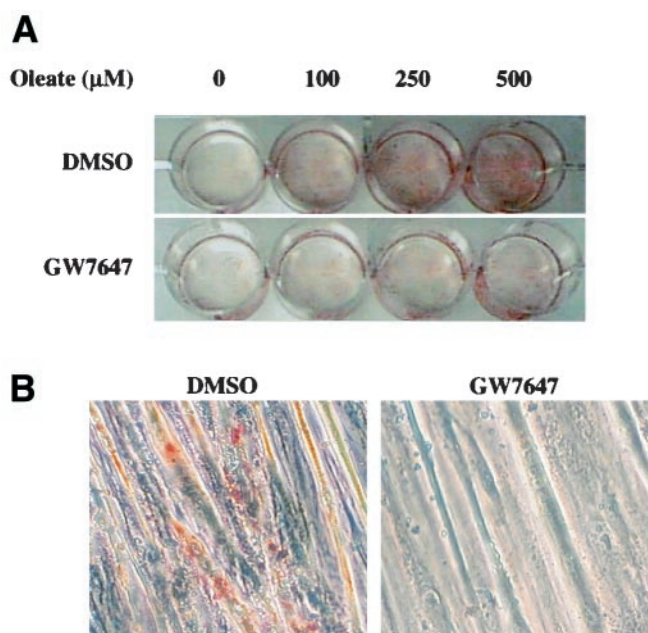


FIG. 4. Effect of PPAR- α activation on neutral lipid accumulation in HSKMCs. **A:** Oil red O staining of neutral lipids in day 8 myocytes that had been pretreated 48 h with vehicle (DMSO) or 1.0 $\mu\text{mol/l}$ GW7647 and then exposed to an overnight fatty acid load (0–500 $\mu\text{mol/l}$ oleate) in serum-free DFM. **B:** Phase contrast photomicrograph (200 \times) showing intramyocellular neutral lipid droplets in vehicle-treated myotubes incubated overnight with 250 $\mu\text{mol/l}$ oleate.

induced upon myocyte differentiation and plays an important role in regulating lipid utilization in human muscle.

Metabolic effects of PPAR- α activation in HSKMCs.

The therapeutic utility of PPAR- α activators in treating dyslipidemia has been well established, but their mechanisms of action, particularly in humans, are still unclear. We found that treating HSKMCs with the highly selective PPAR- α agonist GW7647 increased fatty acid oxidation by approximately threefold, suggesting that the hypolipidemic actions of PPAR- α activators are at least partly mediated by increased muscle clearance and utilization of circulating lipids. In addition to stimulating β -oxidation, GW7647 also decreased fatty acid esterification into myocyte TAG. Similarly, animal studies have shown that PPAR- α agonists ameliorate diet-induced increases in muscle TAG content (5,19), but it was unclear whether this effect was due to primary targeting of muscle gene regulation per se or was secondary to the systemic lipid-lowering actions of the drug. Our results are the first to show that PPAR- α -selective compounds regulate TAG content in human myocytes directly, a finding that has considerable clinical relevance (discussed below).

PPAR- α target genes in HSKMCs. In the present study, we also examined several candidate target genes that are involved in regulating muscle fatty acid oxidation. Most remarkable was our finding that treating developing myotubes with GW7647 induced a 45-fold increase in PDHK4 mRNA expression. This result is consistent with previous studies showing that PDHK4 mRNA levels respond more robustly to changes in muscle fatty acid flux than other PPAR- α target genes (9,16,20,21). Together with these reports, our results indicate that the PDHK4 gene is highly sensitive to both endogenous and pharmacological PPAR- α ligands. Whether this regulatory property of the

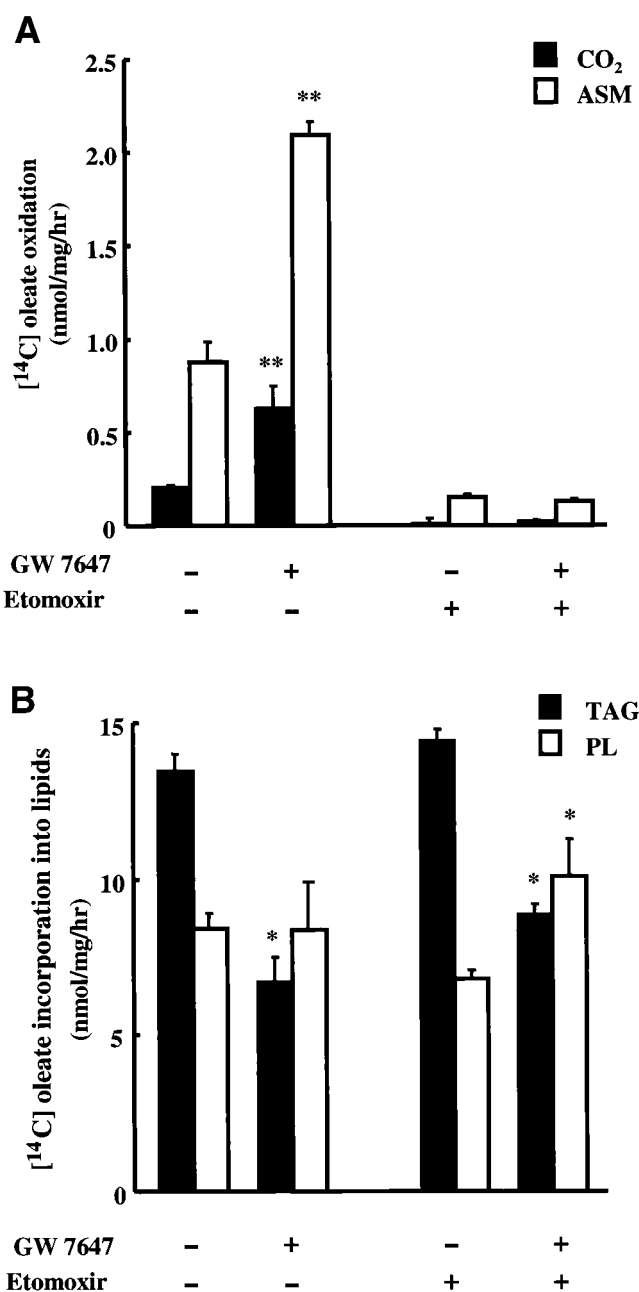


FIG. 5. The TAG-depleting effects of GW7647 do not require reciprocal changes in fatty acid oxidation. After 6 days in DFM, myocytes were treated 48 h with vehicle (DMSO) or 1.0 $\mu\text{mol/l}$ GW7647. Day 8 myocytes were incubated 3 h at 37°C in DFM with 100 $\mu\text{mol/l}$ [^{14}C]oleate and 0.2% BSA in the presence or absence of 100 $\mu\text{mol/l}$ etomoxir. Lipids were extracted in chloroform:methanol and separated by thin layer chromatography to determine [^{14}C]label incorporation into TAG and phospholipid (PL). Assays were performed in triplicate, and values are the means \pm SE from three subjects. Two-way ANOVA was performed to test both the main and interaction effects of GW7647 and etomoxir. Differences between vehicle and drug-treated cells were also analyzed by Student's *t* test for paired data. **P* < 0.05 vs. DMSO-treated controls under the same conditions.

gene might be related to distinguishing features of the PPAR binding element within the PDHK4 promoter remains to be determined. PDHK4 functions by phosphorylating and inactivating PDH, which is a multienzyme complex that catalyzes the oxidation of pyruvate to acetyl-CoA (22). Because this is an irreversible reaction that prevents conversion of acetyl-CoA back to glucose, inac-

TABLE 2
Effects of GW7647 on expression of genes involved in fatty acid homeostasis

	GW7647 treatment			
	48 h in mature myotubes		6 days in developing myotubes	
	Fold vehicle control	<i>P</i> *	Fold vehicle control	<i>P</i> *
FAO genes				
ACO	0.97 ± 0.13	NS	1.05 ± 0.07	NS
CPT1	1.70 ± 0.20	<0.05	2.41 ± 0.43	<0.05
MCAD	1.00 ± 0.001	NS	1.18 ± 0.15	NS
MCD	1.75 ± 0.12	<0.05	1.73 ± 0.02	<0.001
PDHK2	1.04 ± 0.10	NS	1.05 ± 0.14	NS
PDHK4	11.7 ± 3.43	<0.02	45.0 ± 11.0	<0.001
PGC-1	0.85 ± 0.02	<0.05	1.28 ± 0.18	NS
PPAR α	0.91 ± .03	NS	0.91 ± .05	NS
TAG synthetic genes				
DGAT	0.87 \dagger	NS	1.15 \dagger	—
GPAT	1.17 ± 0.11	NS	1.12 ± 0.16	NS
SREBP1	1.26 ± 0.05	NS	1.33 ± 0.17	NS

Total RNA was isolated and quantified by RTQ-PCR as described in RESEARCH DESIGN AND METHODS, and differences in C_T values between treated and control cells were analyzed by Student's *t* test for paired data. Gene expression levels in cells that were treated with 1.0 μ M GW7647 for 48 h (mature myotubes) or 6 days (developing myotubes), relative to vehicle (DMSO) controls, are presented as the means \pm SE of cells from four different subjects. FAO, fatty acid oxidative genes; PGC1, PPAR co-activator 1. *Compared with DMSO controls; \dagger mRNA levels in cells from three of four subjects were too low to measure.

tivation of the PDH complex facilitates glucose sparing during states of energy depletion. There are at least four PDHK isoenzymes that exhibit distinct tissue distributions and kinetic properties (23). Skeletal muscle expresses PDHK2, a lower specific activity enzyme that is ubiquitously expressed in the fed state, and PDHK4, a higher specific activity enzyme that is less sensitive to inhibition by pyruvate and is robustly induced by increased fatty acid flux (21,24). Our finding that GW7647 selectively upregulated PDHK4 without affecting expression of PDHK2 is consistent with the physiological regulation of these genes in vivo. Increased expression and activity of PDHK4, which occurs in response to exercise (20), starvation (21), and a low-carbohydrate diet (25), is thought to promote fatty acid oxidation and spare pyruvate for nonoxidative, anaplerotic entry into the tricarboxylic acid (TCA) cycle. Conversely, refeeding (21) and insulin treatments (9) decrease PDHK4 expression, which favors preferential oxidation of carbohydrates (26). These studies indicate that the PDH reaction is positioned at a key metabolic branch point. Furthermore, our results, together with these earlier studies, support the hypothesis that PDHK isoform switching imparts a mechanism by which the muscle can rapidly adjust the source of substrate that supplies acetyl-CoA to the TCA cycle (21).

RTQ-PCR analyses also showed that GW7647 increased expression of muscle CPT1 and MCD approximately twofold. The magnitude of these responses is highly consistent with the in vivo effects of pharmacological (10) and physiological (20) activation of PPAR- α . CPT1 catalyzes the initial and rate-limiting step in the transport of fatty acid into mitochondria, whereas MCD disposes of the potent CPT1 inhibitor malonyl-CoA. Thus, activation of these genes increases fatty acid catabolism by promoting their entry into the mitochondria. In contrast to the effects of PPAR- α activation in mouse liver (3) and heart (18), but consistent with reports in humans (6), treating HSKMCs with GW7647 did not induce mRNA expression of the peroxisomal marker enzyme ACO. Although this result

suggests that GW7647 might preferentially upregulate mitochondrial over peroxisomal β -oxidation, it does not rule out the possibility that other extramitochondrial pathways might participate in PPAR- α -mediated regulation of cellular lipid balance.

Our finding that GW7647 reduced HSKMC esterification into TAG, even when fatty acid oxidation was inhibited by etomoxir, suggests that the TAG-depleting effects of the compound do not depend on reciprocal increases in lipid catabolism. However, we were unable to explain this observation at the molecular level. GPAT and DGAT catalyze the first and final committed steps, respectively, in the pathway of TAG biosynthesis, and SREBP1 is a transcription factor that coordinately stimulates expression of several lipogenic enzymes (27). Expression levels of these candidate genes were unchanged by the PPAR- α agonist. Thus, we found no evidence that PPAR- α represses expression of TAG biosynthetic enzymes. Alternatively, PPAR- α activation might decrease TAG content by reducing the supply of lipogenic precursors, stimulating hydrolysis, or perhaps by stimulating lipid export via lipoprotein particles, a pathway known to operate primarily in liver but that has also been described in heart (28). These possibilities warrant further investigation.

Clinical implications. Recently, there has been heightened interest in the lipid oversupply hypothesis that links increased muscle lipid content with the development of insulin resistance (29). Several rodent studies have shown that insulin sensitivity indexes correlate inversely with changes in muscle lipid content (5,30). Similarly, in humans, [^1H - ^{13}C] nuclear magnetic resonance studies have demonstrated that intramyocellular TAG content correlates inversely with insulin resistance, and that multiple regression analyses select muscle TAG as the strongest predictor of insulin resistance, independent of BMI, adiposity, and waist-to-hip ratio (31,32). These data suggest that muscle lipid dysregulation, which is marked by increased muscle TAG content, is causally related to insulin resistance. The underlying factors contributing to muscle

lipid accumulation are still obscure but may be related to reduced oxidative capacity (rev. in 33). We and others have reported that fatty acid oxidation rates (14) and fatty acid oxidative enzyme activities (14,33) are up to 50% lower in muscle from obese compared with lean subjects, and that markers of fatty acid oxidative capacity, including CPT1, correlate inversely with insulin resistance (34). These reports imply that diminished lipid oxidation precedes muscle lipid accumulation and insulin resistance; thus, pharmacological interventions designed to enhance muscle lipid oxidation might facilitate weight loss, lower muscle lipid content, and promote insulin sensitivity. Although this prediction contradicts the classic model by Randle et al. (35), which hypothesizes that increased fatty acid oxidation contributes to insulin resistance through product inhibition of hexokinase, recent studies have challenged this convention by showing that glucose-6-phosphate does not accumulate during increased fatty acid substrate utilization (29). Moreover, in rodent models of obesity and insulin resistance, administration of PPAR- α agonists has been shown to increase whole-body lipid catabolism (8,36) while improving glucose tolerance (5,19,36). Clinical trials in humans have shown either improvement (37,38) or no change (39) in insulin sensitivity indexes. Clearly, future studies using the HSKMC model to evaluate the effects of PPAR- α activation on myocyte glucose homeostasis should provide additional insight into the therapeutic potential of PPAR- α activators in treating patients with type 2 diabetes.

In summary, PPAR- α is a nuclear hormone receptor that is expressed abundantly in human skeletal muscle, but until now, its function in this tissue was essentially unknown. In the present study, we demonstrated that PPAR- α was induced during myocyte differentiation and that its activation stimulated lipid oxidation and decreased TAG accumulation in primary human myotubes. Consistent with these biochemical observations, we identified three hPPAR- α target genes, CPT1, MCD, and PDHK4, each of which is involved in promoting muscle lipid catabolism. These results not only suggest a mechanism for the hypolipidemic effects of PPAR- α -selective drugs in humans but also implicate hPPAR- α as a transcription factor that mediates fatty acid-induced upregulation of these genes in vivo. Additionally, we found that hPDHK4 mRNA expression was more responsive to PPAR- α activation than other known target genes, suggesting a critical role for this gene in regulating muscle's adaptive response to changes in fuel availability.

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