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Role of AMP Kinase and PPARδ in the Regulation of Lipid and Glucose Metabolism in Human Skeletal Muscle*

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The peroxisome proliferator-activated receptor (PPAR)δ has been implicated in the regulation of lipid metabolism in skeletal muscle. Furthermore, activation of PPARδ has been proposed to improve insulin sensitivity and reduce glucose levels in animal models of type 2 diabetes. We recently demonstrated that the PPARδ agonist GW501516 activates AMP-activated protein kinase (AMPK) and stimulates glucose uptake in skeletal muscle. However, the underlying mechanism remains to be clearly identified. In this study, we first confirmed that incubation of primary cultured human muscle cells with GW501516 induced AMPK phosphorylation and increased fatty acid transport and oxidation and glucose uptake. Using small interfering RNA, we have demonstrated that PPARδ expression is required for the effect of GW501516 on the intracellular accumulation of fatty acids. Furthermore, we have shown that the subsequent increase in fatty acid oxidation induced by GW501516 is dependent on both PPARδ and AMPK. Concomitant with these metabolic changes, we provide evidence that GW501516 increases the expression of key genes involved in lipid metabolism (FABP3, CPT1, and PDK4) by a PPARδ-dependent mechanism. Finally, we have also demonstrated that the GW501516-mediated increase in glucose uptake requires AMPK but not PPARδ. In conclusion, the PPARδ agonist GW501516 promotes changes in lipid/glucose metabolism and gene expression in human skeletal muscle cells by PPARδ- and AMPK-dependent and -independent mechanisms.

The peroxisome proliferator-activated receptors (PPARs)[4] have been the focus of attention within the field of metabolic research because of their lipid-sensor properties and functional role in transcriptional regulation. PPARs regulate lipid utilization and storage and influence metabolic substrate utilization in relation to energy supply during fasting (1) or energy demand during physical exercise (2). PPAR isoforms display tissue-specific expression and gene regulatory profiles. PPARγ is a key regulator of adipose development and adipose insulin sensitivity (3), whereas PPARα regulates genes involved in hepatic lipid oxidation (4). PPARδ is the predominant isoform in skeletal muscle, and studies in transgenic mice show that targeted expression of activated PPARδ increases the predominance of oxidative type 1 muscle fibers, enhances whole-body insulin sensitivity, and increases exercise endurance capacity (5). Because pharmacological activation of PPARδ with the specific agonist GW501516 improves insulin sensitivity in aged rhesus monkeys (6) and rodents (7, 8), PPARδ agonism may offer an efficacious strategy for the management of metabolic disorders.

The PPARδ-mediated enhancement in whole-body insulin sensitivity primarily reflects improvements in the lipid profile. PPARδ activation can improve glucose homeostasis by increasing lipid oxidation to lower plasma-free fatty acid levels, which thereby relieves negative feedback on the canonical insulin signaling cascade to enhanced glucose uptake and reduce plasma glucose levels (9). Skeletal muscle is a key target tissue in orchestrating this scenario, because it is a key organ for lipid oxidation and glucose uptake. Indeed, improvements in skeletal muscle insulin sensitivity are beneficial to control glucose homeostasis (10).

Genetic data in humans provide evidence for a role for PPARδ in the regulation of skeletal muscle glucose metabolism. Single nucleotide polymorphisms of the human *PPARD* gene are associated with enhanced whole-body insulin sensitivity. The use of positron emission tomography to determine tissue-specific glucose disposal rates *in vivo* that suggest single nucleotide polymorphisms in the *PPARD* gene mainly affect glucose uptake in skeletal muscle but not adipose tissue (11). Furthermore, single nucleotide polymorphisms in the *PPARD* gene modify the conversion from impaired glucose tolerance to type 2 diabetes, particularly in combination with single nucleotide polymorphisms identified in PPAR gamma co-activaor (PGC)1α and PPARγ2 (12). Interestingly, improvements in insulin sensitivity are correlated with increased PPARδ expression in type 2 diabetic patients performing moderate walking

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[4] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; CPT1, carnitine palmitoyltransferase 1; PDK4, pyruvate dehydrogenase kinase 4; FABP3, fatty acid-binding protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; PGC1, PPARγ co-activator 1; FBS, fetal bovine serum; siRNA, small interfering RNA.

exercise (13). In addition, PPARδ expression is associated with an increased proportion of insulin-sensitive oxidative skeletal muscle fiber types in human (14) and rodent (5) skeletal muscle.

The effects of PPARs on metabolic responses may involve the AMP-activated protein kinase (AMPK). Indirect evidence to support this hypothesis arises from the observation that the metabolic profile achieved in response to AMPK activation overlaps with the metabolic phenotype observed with PPARδ activation. AMPK is a heterotrimeric protein kinase that participates in cellular energy homeostasis (15). Once activated under conditions of low energy status, AMPK enhances cellular nutrient uptake, activates ATP-producing catabolic pathways, and down-regulates energy-consuming processes (15). AMPK-mediated effects on lipid and glucose metabolism partly involve activation of PPARs through diverse signaling pathways (16, 17) and/or direct phosphorylation (18). The PPARδ agonist GW501516 increases basal and insulin-stimulated glucose uptake in cultured primary human myotubes, C2C12 cells, and 3T3-L1 adipocytes, with effects correlated with AMPK phosphorylation in human skeletal myotubes (19). The contribution of AMPK to the described effects of PPAR agonists on the regulation of lipid and glucose metabolism is unknown.

The aim of this study was to determine whether PPARδ has a direct role in the regulation of glucose and lipid metabolism in skeletal muscle. In addition, we endeavored to determine whether AMPK contributes to the enhanced metabolic phenotype observed in skeletal muscle conferred by PPARδ agonists.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, fetal bovine serum (FBS), penicillin, streptomycin, and fungizone were obtained from Invitrogen. Radiochemical 2-[G-^{3}H]deoxy-D-glucose (6.0 Ci/mmol/l) and D-[U-^{14}C]glucose (310 mCi/mmol/l) were from Amersham Biosciences. All other chemicals were analytical grade and from Sigma.

Human Primary Skeletal Muscle Cell Cultures—Skeletal muscle biopsies were obtained from healthy individuals who underwent general surgery. None of the subjects had known metabolic disease. Satellite cells were isolated, and primary muscle cultures were established (20). The ethical committee at the Karolinska Institutet approved the protocols. Cells were grown in DMEM (1000 mg/liter glucose) with 10% FBS and 1% penicillin/streptomycin in non-coated dishes. To differentiate human myoblasts into myotubes, dishes with a cell density of 80–90% were grown in DMEM with 4% FBS for 2 days to induce myotube formation, and then grown in DMEM with 2% FBS for 2 days. Before utilization, the cells were controlled optically for the formation of elongated myotubes and serum-starved overnight.

Metabolic Analysis—Glucose uptake and glucose incorporation into glycogen in primary human muscle cells was determined as previously described previously (21). Determination of free fatty acid uptake and oxidation were performed as described previously (22).

RNA Purification and Quantitative Reverse Transcription-coupled Real-time PCR—Myoblasts were cultured in 100-mm dishes, and the differentiation was initiated at >90% confluence. Five days after differentiation, myotubes were FBS-starved for 24 h (except in the FBS withdrawal experiment, where FBS starvation times were 0, 8, 16, and 24 h) and then incubated with 100 nM GW501516 for 60 min or FBS-starved for 24 h with 100 nM GW501516 in the medium for the last 18 h of the starvation period. Control cells were treated with the vehicle (Me_2SO) in an identical manner. At the end of the incubation, cells were washed three times with RNase-free phosphate-buffered saline and then harvested directly for RNA extraction (RNAeasy minikit, Qiagen, Crawley, UK). All RNA was DNase-treated before reverse transcription (RQ1 RNase-free DNase, Promega, Southampton, UK). Total RNA concentration was measured, and cDNA was prepared using the TaqMan reverse transcription reagent. Real-time PCR (ABI-PRISMA 7000 Sequence Detector, Perkin-Elmer Life Sciences) was performed for quantification of specific mRNA content, and data were collected and analyzed by ABI Prism 7000 SDS software version 1.1. mRNA content was normalized for β-actin mRNA (unchanged expression after GW501516 treatment) and expressed relative to that in control cells treated with vehicle. Oligonucleotide primers (sequences available upon request) and TaqMan probes were purchased from Applied Biosystems.

Western Blot Analysis—Expression of AMPK, acetyl-CoA carboxylase (ACC), PPARγ co-activator (PGC)1, respiratory chain complex I (NADH-ubiquinol oxidoreductase and complex IV (cytochrome *c* oxidase I)) were assessed by immunoblot analysis using the AMPK pan-α-subunit (Cell Signaling Technology), ACC (Upstate Biotechnology), PGC1 (Chemicon), NADH-ubiquinol oxidoreductase respiratory chain complex I (Invitrogen), and cytochrome *c* oxidase respiratory chain complex IV (Invitrogen) antibodies, respectively. PPARδ antibody was from Santa Cruz Biotechnology. The phosphorylation state of AMPK and ACC was measured with anti-phospho-Thr^{172} (Cell Signaling Technology)- and anti-phospho-Ser^{79} (Upstate Biotechnology)-specific antibodies, respectively. Briefly, cell lysates were rotated for 60 min at 4 °C in homogenization buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM Na3VO4, 10 mM NaF, 30 mM Na4P2O7, 10% (v/v) glycerol, 1 mM benzamidine, 1 mM dithiothreitol, 10 μg/ml 1 leupeptin, 200 mM phenylmethylsulfonyl fluoride, and 1 μM microcystin) and then subjected to centrifugation (20,000 × *g* for 10 min at 4 °C). Samples of cell lysate supernatant were resuspended in Laemmli buffer, and proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), blocked with 7.5% nonfat milk, washed with TBST (10 mM Tris HCl, 100 mM NaCl, 0.02% Tween 20), and finally incubated with the appropriate primary antibodies overnight at 4 °C. Membranes were washed with TBST and incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G, from Bio-Rad Laboratories. Western blots were visualized by chemiluminescence (ECL, Amersham Biosciences) and quantified by densitometry.

Nuclear Extraction—Myotubes were grown in 10-cm Petri dishes, stimulated as described above, and then washed immediately with ice-cold phosphate-buffered saline. The nuclear extraction procedure was essentially as previously described (23).

Small Interfering RNA (siRNA) Transfection in Myotubes—Myotubes were transfected using Lipofectamine 2000 (Invitrogen). Differentiation media were changed to antibiotic-free

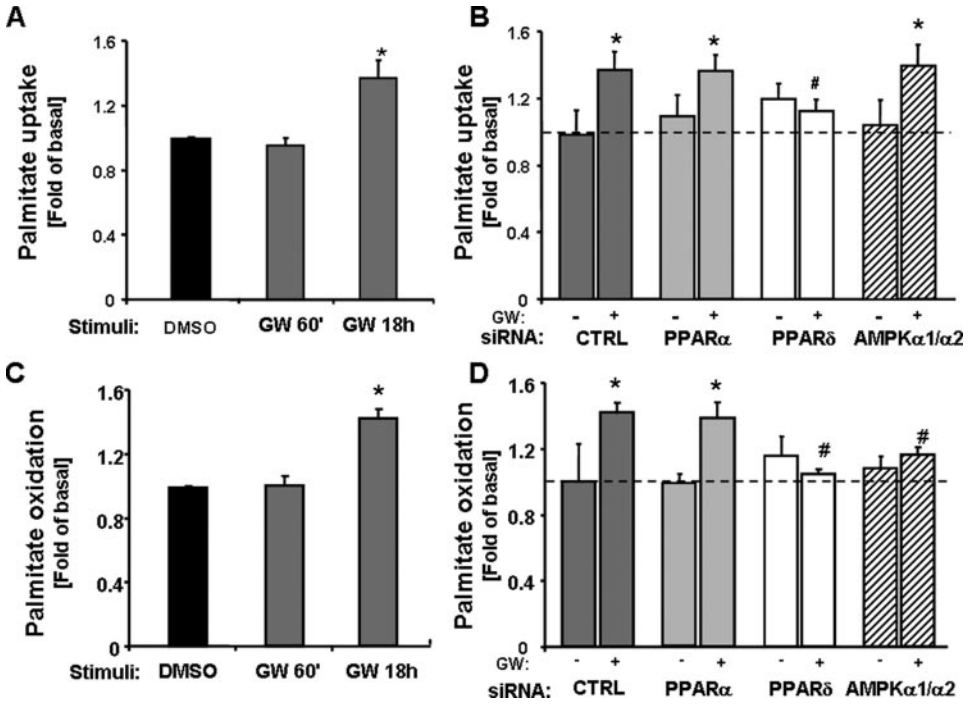

FIGURE 1. **Palmitate uptake in primary cultures of human skeletal muscle.** *A*, intracellular accumulation of [^{14}C]palmitate was determined as described under "Experimental Procedures." The results are means ± S.E. for data obtained from cultures from six individuals following exposure to 100 nM GW501516 for 1 and 18 h or in cells where siRNA-has been used to knock-down PPARδ, PPARα, or AMPKα1/α2 expression. *B*, basal palmitate uptake in these cells was 0.61 ± 0.25 counts/min/mg protein. *C*, palmitate oxidation in human skeletal muscle cells was determined by release of [^{14}C]CO_2 as described under "Experimental Procedures." The results are means ± S.E. for cultures from six individuals following exposure to 100 nM GW501516 for either 1 or 18 h, or (*D*) in cells where siRNA has been used to knock down PPARδ, PPARα, or AMPKα1/α2 expression. Basal palmitate oxidation in these cells was 1.0 ± 0.5 counts/min/mg protein; *, $p < 0.05$ as compared with vehicle-treated controls; #, $p < 0.05$ as compared with 100 nM GW501516-treated cells for 18 h with random control siRNA.

growth media on day 2 of myotube differentiation. On day 3, individual siRNAs (1 μg/ml) were transfected using Lipofectamine in serum-free DMEM (incubating time >16 h). Myotubes were washed with phosphate-buffered saline, and 2 ml of DMEM containing 2% FBS was added to each well. On day 5, the cells were used for experiments. The siRNA reagents for the control and various PPAR isoforms (details of sequences available upon request) were from Dharmacon (Perbio Science, Erembodegem-Aalst, Belgium). The siRNA reagents for AMPK α1 and α2 were from Ambion (Austin, TX) and were designed as previously described (24).

High Performance Liquid Chromatography Measurement of ATP, ADP, and AMP—Adenine nucleotides were separated by high performance liquid chromatography using a Spherisorb column with a 5-μm outer diameter (0.46 × 18 cm). Elution was done with 25 mM sodium pyrophosphate/pyrophosphoric acid, pH 5.75, with a flow rate of 1 ml min^{-1}. Absorbance was measured at 254 nm (25).

Statistics—Groups were compared using analysis of variance, and differences were identified with Fischer's post hoc analysis. Statistical significance was accepted when $p < 0.05$.

RESULTS

GW501516-induced Stimulation of Fatty Acid Uptake in Primary Cultured Human Myotubes Requires PPARδ—Previous results obtained with animal models indicated that activation of PPARδ leads to increased lipid metabolism in skeletal muscle. To determine the effects of PPARδ activation in human muscle, differentiated primary myotubes were exposed to 100 nM GW501516 for 1 or 18 h. Palmitate uptake, as assessed by intracellular accumulation of ^{14}C-labeled palmitate, was significantly increased 37% in response to long term GW501516 treatment (Fig. 1*A*). Conversely, GW501516 did not increase palmitate uptake after the short term 60-min exposure, suggesting that transcriptional effects are required to mediate metabolic responses.

To assess the requirement for PPARδ for the GW501516 effect on fatty acid uptake, the expression of either PPARα or PPARδ was specifically reduced using siRNA technology. Efficiency and specificity of the siRNA transfection on the expression of the targeted gene was determined using quantitative real-time PCR. mRNA expression was reduced 71% for PPARα ($p < 0.001$) and 78% ($p < 0.001$) for PPARδ, as compared with random siRNA control constructs (Fig. 2*A*). Protein expression of PPARδ was determined in nuclear extracts, and confirmed siRNA mediated reductions by 65% (Fig. 2*B*). The siRNA-mediated reduction in PPARδ expression, abolished the stimulation of palmitate uptake induced by GW501516, whereas the reduction in PPARα expression was without effect. Furthermore, the siRNA-mediated reduction of AMPK (65%, $p < 0.01$) (Fig. 2) was without effect on GW501516-mediated palmitate uptake (Fig. 1*B*).

GW501516-induced Increase in Palmitate Oxidation Requires Both PPARδ and AMPK—In accordance with results obtained for palmitate uptake, incubation of differentiated human myotubes for 18 h with GW501516 resulted in a 50% increase in palmitate oxidation, whereas no effect was observed after the shorter 1-h exposure to the agonist (Fig. 1*C*). The siRNA-mediated reduction of either PPARδ or AMPKα1/α2 totally prevented the GW501516-induced stimulation of palmitate oxidation, although the siRNA-mediated reduction of PPARα did not blunt this effect (Fig. 1*D*).

GW501516 Increases mRNA Expression of Candidate Genes Involved in Lipid Metabolism by a PPARδ-dependent Mechanism—Incubation of differentiated human myotubes for 18 h with GW501516 resulted in a significant increase in CPT1 (5.5-fold), PDK4 (4-fold), and FABP3 (1.7-fold) expression compared with vehicle-treated cells (Fig. 3*A*). In contrast, expression of other genes, including DGKδ, GAPDH, nuclear respiratory factor 1, and cytochrome *c* were unaltered by

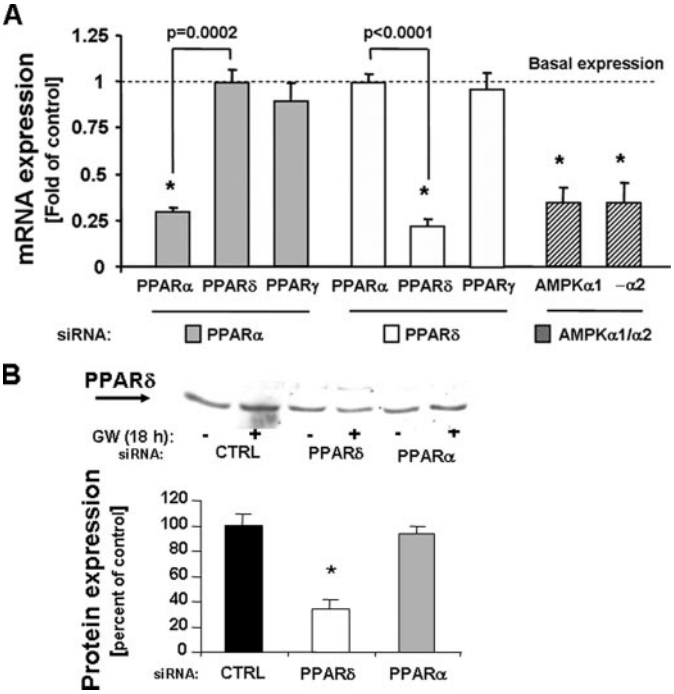

FIGURE 2. *A*, mRNA content was determined using quantitative PCR as described under "Experimental Procedures." Cells were pretreated with siRNA to silence the expression of PPARδ, PPARα, or AMPKα1/α2. Results are means ± S.E. for data from cultures from seven individuals. *, $p < 0.05$ as compared with cells treated with random control siRNA. *B*, protein expression of PPARδ in skeletal muscle cells following siRNA to silence the expression of PPARδ, or PPARα. Representative immunostaining shows protein expression of PPARδ following siRNA to silence the expression of PPARδ or PPARα in cells exposed to vehicle or 100 nM GW501516 for 18 h. The graph shows the summarized quantification.

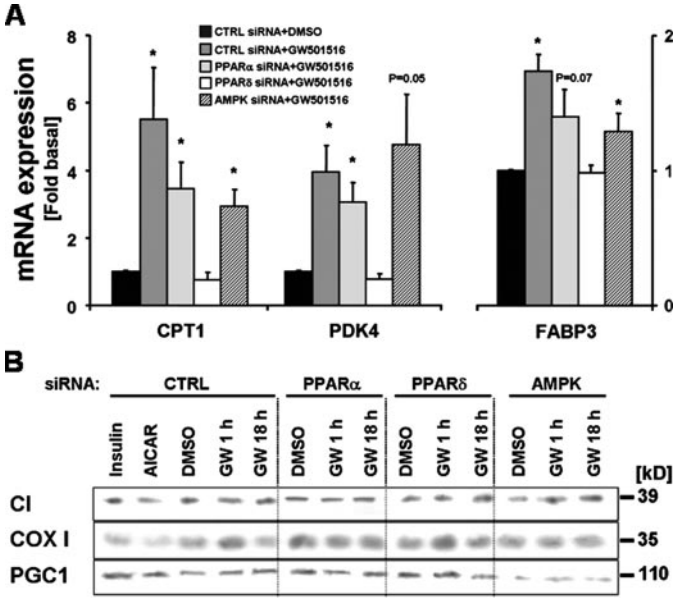

FIGURE 3. **Expression of key metabolic genes was determined by quantitative PCR as described under "Experimental Procedures."** The results are means ± S.E. for data from cultures from four individuals. Cells were pretreated with siRNA to silence PPARδ, PPARα, or AMPKα1/α2 expression. Cells were exposed to vehicle or 100 nM GW501516 for 18 h. *, $p < 0.05$ as compared with vehicle (Me_2SO) treated cells with random control siRNA. *A*, summary of mRNA changes in key metabolic proteins. *B*, expression of mitochondrial markers NADH-ubiquinol oxidoreductase respiratory chain complex I, cytochrome *c* oxidase respiratory chain complex IV, and PGC1 was determined in cells where siRNA was used to silence PPARδ, PPARα, or AMPKα1/α2 expression following treatment with vehicle or 100 nM GW501516 for 1 or 18 h. Representative blots (of seven individuals) are shown.

GW501516 treatment (data not shown). The GW501516-mediated transcriptional effect on CPT1, PDK4, and FABP3 was abolished when PPARδ expression was reduced using siRNA. The siRNA-mediated reduction in PPARα or AMPKα1/α2 did not alter the GW501516-mediated induction of these genes. Taken together, our results indicate that activation of PPARδ in cultured primary human skeletal muscle increases lipid metabolism concomitant with an increase in mRNA content of key regulators of the fatty acid transport (FABP3) and oxidation (CPT1 and PDK4).

GW501516 Does Not Alter Protein Expression of Mitochondrial Markers—Expression of activated PPARδ in mouse skeletal muscle increases the expression of several mitochondrial markers and induces a transformation in skeletal muscle fiber type to an oxidative type I phenotype (5). Protein expression of PGC1, a target that is involved in mitochondrial biogenesis (26) and the formation of slow twitch muscle fibers (27), was unaffected by GW501516 regardless of the condition studied (Fig. 3*B*). There was a non-significant trend for reduced protein expression of PGC1 in myotube siRNA-mediated reduction of AMPK (Fig. 3*B*). Furthermore, protein expression of NADH-ubiquinol oxidoreductase respiratory chain complex I and cytochrome *c* oxidase respiratory chain complex IV, two subunits of the main mitochondrial respiratory chain complexes, was also unaltered by exposure to the PPARδ agonist for either 1 or 18 h.

AMPK Phosphorylation Is Increased after GW501516 Treatment by a PPARδ-independent Mechanism and Involves Changes in the Cellular Energy Status—As previously reported (19), AMPK phosphorylation was increased by the specific PPARδ agonist GW501516 in primary cultured human skeletal muscle. Treatment of primary human muscle cultures with 100 nM GW501516 for either 1 or 18 h resulted in a significant 2- and 2.3-fold AMPK phosphorylation, respectively (Fig. 4*A*). In cells whereby siRNA against PPARα and δ was employed to inhibit PPAR expression, the GW501516-induced AMPK phosphorylation was unaltered, demonstrating that this effect was independent of either PPARα or PPARδ. The siRNA mediated-reduction in AMPK expression blunted the GW501516 effect on AMPK phosphorylation. Similarly, the phosphorylation state of ACC, a downstream target of AMPK, was significantly increased 1.4- and 1.7-fold after 1 or 18 h of GW501516 treatment, respectively (Fig. 4*B*). Silencing of either PPARα or PPARδ expression by siRNA did not alter this effect, whereas inhibition of AMPKα1/α2 expression led to a significant reduction in the effect of GW501516 on ACC phosphorylation.

To further investigate the underlying mechanism of the PPARδ-independent effect of GW501516 on AMPK phosphorylation, we measured adenine nucleotide concentrations by high performance liquid chromatography in human skeletal myotubes to determine the cellular ATP:ADP and AMP:ATP ratios. Following a short term exposure (60 min) to GW501516, ATP levels were significantly reduced and ADP levels increased in a dose-dependent manner compared with control cells exposed to the vehicle (data not shown). This was associated

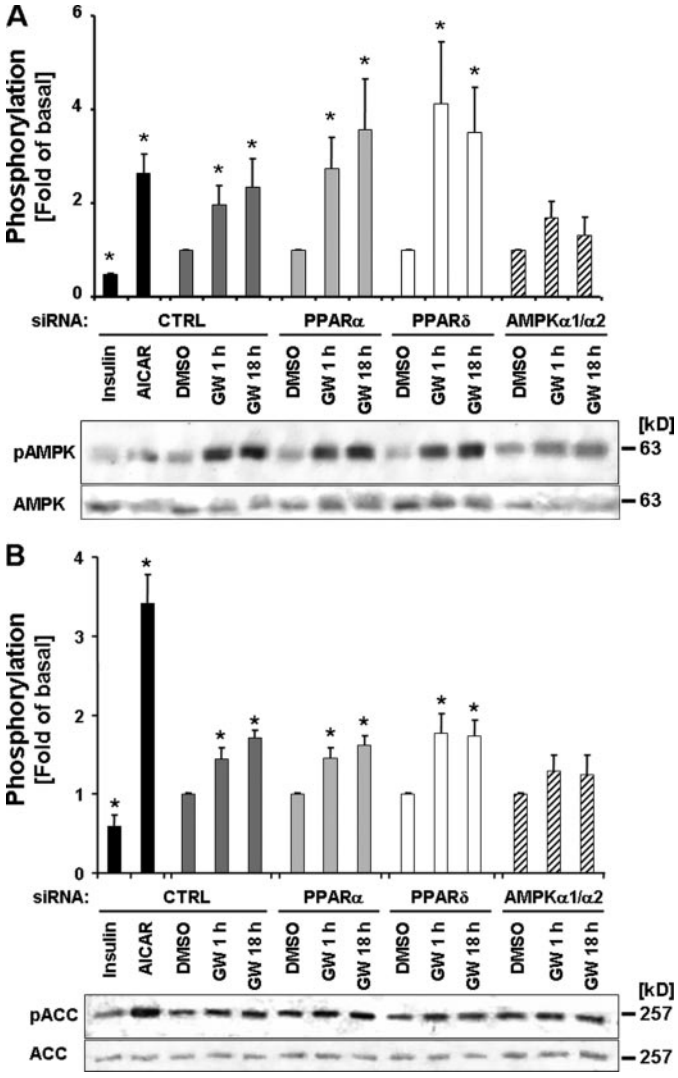

FIGURE 4. **Phosphorylation of AMPK and ACC in cells following siRNA-mediated silencing of PPARδ, PPARα, or AMPKα1/α2 expression in cells exposed to vehicle or 100 nM GW501516 for 1 or 18 h.** AMPK and ACC phosphorylation was measured and quantified as described under "Experimental Procedures." The results are means ± S.E. for data from cultures from seven individuals. Representative blots are shown for phosphorylation of AMPK phosphorylation (*A*) and ACC phosphorylation (*B*). *, $p < 0.05$ as compared with vehicle (Me_2SO)-treated cells with random control siRNA.

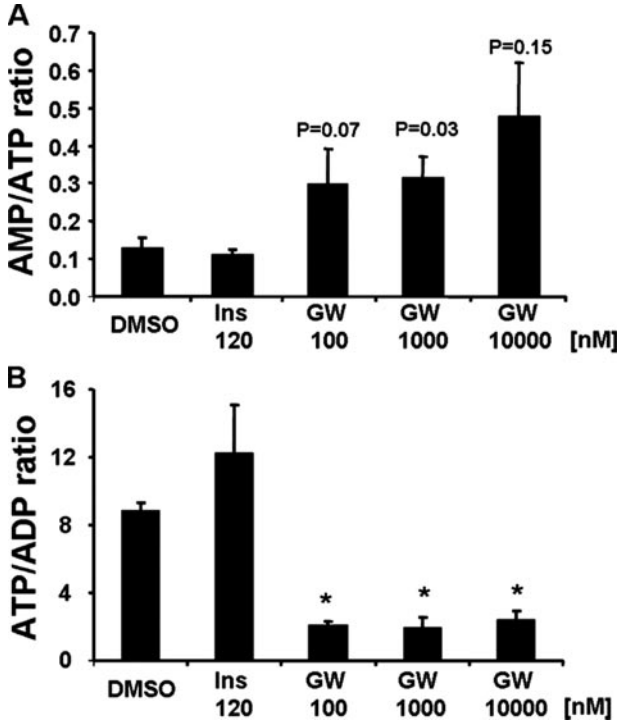

FIGURE 5. **High performance liquid chromatography measurements of AMP, ADP, and ATP.** *A*, the AMP:ATP ratio after 60 min treatment with vehicle or GW501516. Results are means ± S.E. for data from cultures from four individuals. *B*, ATP:ADP ratio after 1 h of GW501516 treatment as compared with vehicle-treated cells. Results are means ± S.E. for data from cultures from four individuals. *, $p < 0.05$ or as presented in the figure, as compared with vehicle-treated controls.

with a significant decrease in ATP:ADP ratio for all of the concentrations of the PPARδ agonist used (76% at 100 nM, 79% at 1 μM, and 73% at 10 μM; $p < 0.05$ (Fig. 5*B*). In addition, although intracellular AMP concentrations were low and close to the limit of detection, a trend toward an increase in AMP levels in the presence of GW501516 was noted (data not shown), leading to a concomitant increase in AMP:ATP ratio (Fig. 5*A*). Taken together, these results provide evidence that the increase in AMPK phosphorylation (and presumably activity) observed after GW501516 treatment was because of a decrease in cellular energy status in the cultured human myotubes.

The GW501516-induced Stimulation of Glucose Uptake Requires AMPK but Not PPARδ—As previously reported (19), GW501516 stimulates glucose uptake in primary cultured human myocytes after 1 and 18 h of incubation (Fig. 6, *A* and *B*). To determine signaling specificity, cells were transfected with siRNA against AMPKα1/α2, PPARα, or PPARδ to reduce the respective protein expression. As expected, control and PPARα siRNA was without effect on the GW501516-induced stimulation of glucose uptake at either 1 h (34%; $p < 0.01$) (Fig. 6*A*) or 18 h (22%; $p < 0.05$) (Fig. 6*B*). The siRNA-mediated reduction of PPARδ expression was also without effect on the stimulation of glucose uptake by GW501516. Conversely, inhibition of AMPK expression abolished the GW501516 effect on glucose uptake after 1 and 18 h as compared with the random siRNA control. Thus, the effect of GW501516 on glucose uptake appears to be PPARδ-independent and requires AMPK activation.

GW501516 Has No Effect on Glucose Incorporation into Glycogen—Glucose incorporation into glycogen was measured by determining the [^{14}C]glycogen content in differentiated human myotubes following incubation in the presence or absence of insulin (1 or 120 nM) with or without 100 nM GW501516. Exposure to 120 nM insulin resulted in a significant increase in glycogen synthesis (75%; $p < 0.01$), as compared with non-insulin-stimulated cells, and 1 nM insulin treatment resulted in a trend toward increased glycogen synthesis (Fig. 6*C*). Although treatment with GW501516 for 18 h did not increase glycogen synthesis, when combined with 120 nM insulin, glycogen synthesis was increased but to a lesser extent compared with 100 nM insulin (12%; $p = 0.055$).

DISCUSSION

Activation of PPARδ has beneficial effects on whole-body metabolism and improves several parameters of the metabolic

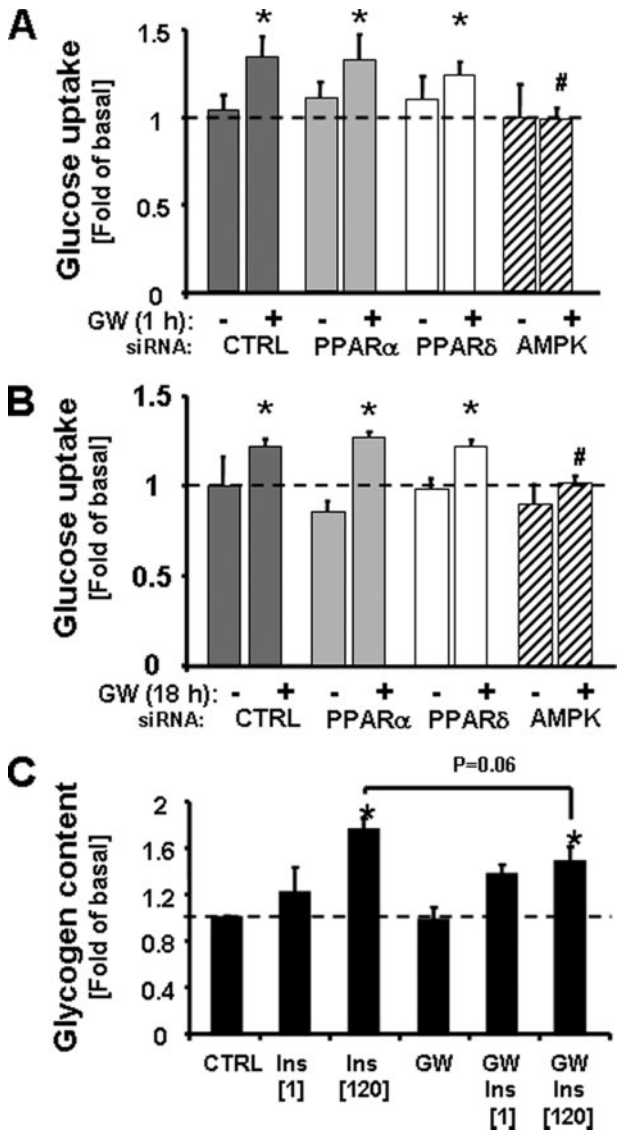

FIGURE 6. Cells were pretreated with siRNA to silence PPARδ, PPARα, or AMPKα1/α2 expression and subsequently exposed to vehicle or 100 nM GW501516 for 1 h (*A*) or 18 h (*B*) and effects on glucose uptake measured. The results are means ± S.E. for data from cultures from six different individuals. Basal glucose uptake in these cells was 60 ± 15 counts/min/mg of protein/min. *, $p < 0.05$ as compared with vehicle-treated controls. #, $p < 0.05$ as compared with 100 nM GW501516-treated cells for 1 or 18 h with random control siRNA. *C*, glycogen content was measured in response to 1 or 120 nM insulin with or without pretreatment of 100 nM GW501516 for 18 h. Results are means ± S.E. for data from cultures from five individuals. *, $p < 0.05$ as compared with vehicle-treated controls.

syndrome (28–31). We investigated the direct effect of the PPARδ agonist GW501516 on metabolic and gene regulatory response in human skeletal muscle. Exposure of differentiated primary human myotubes to GW501516 increased fatty acid uptake and oxidation. This effect required PPARδ, changes in gene expression, and functional AMPK. Furthermore, GW501516 leads to a PPARδ-independent activation of AMPK, which mediates the stimulatory effects on glucose uptake.

Effects on Lipid Metabolism—Transgenic mice expressing an activated form of PPARδ have enhanced fatty acid utilization and are protected against high fat diet-induced obesity (32). Similarly, expression of an activated form of PPARδ in C2C12 myocytes enhances β-oxidation (32). Here we provide evidence that activation of PPARδ using a synthetic activator increases lipid uptake and utilization in primary human skeletal muscle cells. This effect requires PPARδ and is likely to be dependent on changes in gene expression, because acute stimulation (1 h) was without effect. The siRNA-mediated reduction in PPARδ expression in human muscle myotubes prevented the GW501516-induced changes in mRNA expression of numerous genes that are likely to be important for the regulation of lipid metabolism following PPARδ activation. Indeed, GW501616 treatment increased mRNA expression of the fatty acid transporter FABP3, a cytosolic protein involved in uptake and transport of fatty acids (33), carnitine palmitoyltransferase 1 (CPT1), a rate-limiting step in mitochondrial fatty acid oxidation and PDK4, a key enzyme that mediates the shift from glycolytic to fatty acid oxidative metabolism. Thus activation of PPARδ in human skeletal muscle has direct effects on lipid metabolism.

The siRNA-mediated reduction in AMPK also led to a significant reduction in the GW501516-mediated effect on fatty acid oxidation but not lipid uptake. This response likely reflects the key role of ACC in regulating fatty acid oxidation, because ACC phosphorylation was reduced as a consequence of AMPK silencing. Conversely, the reduction in AMPK expression did not alter the GW501516 effect on mRNA expression of select target genes, indicating that AMPK does not appear to mediate these transcriptional events. Our results suggest that some aspects of PPARδ-regulated lipid metabolism require functional AMPK and/or ACC. In human myotubes, siRNA-mediated reduction of AMPK reduced the protein expression of PGC1α, which may affect signals from several nuclear receptors for which PGC1α is a co-factor. Although not observed in this study, recent evidence from transgenic mice indicates that PPARδ may also influence the expression of PGC1α (34). Thus, the relationship between AMPK, PGC1, and PPARδ is complex and requires further elucidation.

Effects on Glucose Metabolism—Several lines of evidence suggest that activation of PPARδ leads to enhanced insulin sensitivity and/or increased glucose uptake (5–7, 9, 13, 19). The precise mechanism(s) for these effects remain to be explained. According to the glucose-fatty acid cycle as proposed by Randle (35), enhanced utilization of lipids is predicted to lead to a reduction in carbohydrate usage. Thus, the combined effects of enhanced lipid and glucose utilization in response to activation of PPARδ are intriguing. Recent evidence in primary hepatocytes isolated from GW501516-treated *db/db* mice demonstrate an increase in the conversion rate of ^{14}C-labeled glucose into organically extractable lipids, suggesting that PPARδ activation increases the utilization of glucose in hepatic *de novo* lipogenesis (9). Furthermore, genetic association studies suggest that PPARδ polymorphisms may play an important role in glucose metabolism in skeletal muscle and may be important in the conversion from impaired glucose tolerance to T2DM (12).

We have previously demonstrated that GW501516 has direct effects on glucose transport in human skeletal muscle cells (19). Here we provide evidence that the effect of GW501516 on glucose uptake is independent of PPARδ activation. Indeed,

knockdown of either PPARδ or PPARα expression did not alter the GW501516-mediated increase in glucose uptake. However, a reduction in the expression of AMPK inhibited the GW501516 effect on glucose transport. The effect of the PPARδ agonist on the stimulation of glucose transport is mediated via AMPK by phosphorylation/activation. Because we observed an increase in the AMP:ATP ratio in cells incubated with GW501516, the GW501516-induced phosphorylation of AMPK could be due to a modification of cellular energy status. Thus, GW501516, in a manner analogous to a number of other chemical compounds including thiazolidinediones (36, 37) or metformin (38), exerts direct and indirect effect(s) on mitochondrial machinery. Indeed, the GW501516-induced decrease in ATP levels could be due to a specific inhibition of one or more complexes of the respiratory chain and/or to an effect on the ATP synthase system (complex V itself, adenine nucleotide translocator and/or inorganic phosphate transporter). Furthermore, an uncoupling effect of GW501516 on the mitochondrial oxidative phosphorylation could account for the observed effects on metabolism, thereby altering the yield of ATP synthesis and leading to AMPK activation (39). A short term effect on mitochondrial metabolism is supported by the fact that AMPK phosphorylation was markedly evident, together with a drop in ATP levels even following an acute incubation (60 min) with GW501516.

The PPARδ agonist GW501516 has been reported to increase glucose uptake in intact muscle incubated *ex vivo*. In isolated rat soleus and epitrochlearis muscle incubated with 10 nM GW501516, no effect on glucose uptake was noted (40). However, rat soleus muscle strips exposed to 1 μmol/liter GW501516 for 24 h responded differently, depending on the presence or absence of fatty acids in the incubation medium. Insulin-mediated glucose transport rates have been reported to be increased in the absence of and decreased in the presence of fatty acids (39). Interestingly, we note that withdrawal of serum from the incubation media enhances the response to GW501516 on glucose uptake in cultured human muscle cells (data not shown). Taken together, the presence of fatty acids in serum may influence the action of GW501516 on glucose uptake in human skeletal muscle cells. The mechanism by which fatty acids affect the putative GW501516-mediated mitochondrial effect(s) and/or AMPK phosphorylation remains to be determined. Using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling in cultured L6 myotubes, no toxic effects of GW501516 were noted at concentrations up to 10 μM (41).

Attention has recently been drawn to the effect of different synthetic PPAR compounds on mitochondrial dysfunction (42). Direct "non-receptor" effects have been described for thiazolidinedione activators of PPARγ (43). Mitochondrial uncoupling, resulting in increased AMP:ATP concentrations in the cell leads to activation of AMPK (43, 44). Targeted activation of AMPK has been an attractive strategy for treatment of the metabolic dysfunction associated with type 2 diabetes (15, 37). AMPK activation in skeletal muscle may constitute a crucial property for the clinical effects of thiazolidinediones in skeletal muscle (37). In contrast to thiazolidinediones, GW501516 is a more potent specific activator of PPARδ than thiazolidinediones are for PPARγ (45), hence the concentrations required for clinical activation of PPARδ may be below the threshold required to trigger mitochondrial uncoupling. In line with this, PPARδ knock-out mice placed on a high fat diet are insensitive to the PPARδ agonist GW501516 and fail to correct metabolic abnormalities compared with wild type control mice, suggesting that PPARδ-directed effects are required for metabolic improvements (32). The effects of GW501516 on glucose uptake in cultured cells may reflect a faster accumulation of the compound in the mitochondria cell monolayer as compared with responses noted in individual tissues. The PPARδ-independent effects of GW501516 on AMPK will require further evaluation.

In summary, we provide evidence that the specific PPARδ activator GW501516 enhances lipid uptake and utilization in primary human skeletal muscle cells. These effects are mediated, in part, via PPARδ-specific transcriptional effects. GW501516 also exhibits PPARδ-independent effects. We have demonstrated that changes in the cellular energy status, as reflected by an increase in the AMP:ATP ratio, occurs following an acute GW501516 treatment. Moreover, we have shown that activation of AMPK constitutes a mechanistic cornerstone in the mediation of the effects of the PPARδ agonist GW501516 on glucose metabolism in skeletal muscle.

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