The transcription factor FoxO1 contributes to the metabolic adaptation to fasting by suppressing muscle oxidation of glucose, sparing it for glucose-dependent tissues. Previously, we reported that FoxO1 activation in C2C12 muscle cells recruits the fatty acid translocase CD36 to the plasma membrane and increases fatty acid uptake and oxidation. This, together with FoxO1 induction of lipoprotein lipase, would promote the reliance on fatty acid utilization characteristic of the fasted muscle. Here, we show that CD36-mediated fatty acid uptake, in turn, up-regulates protein levels and activity of FoxO1 as well as its target PDK4, the negative regulator of glucose oxidation. Increased fatty acid flux or enforced CD36 expression in C2C12 cells is sufficient to induce FoxO1 and PDK4, whereas CD36 knockdown has opposite effects. In vivo, CD36 loss blunts fasting induction of FoxO1 and PDK4 and the associated suppression of glucose oxidation. Importantly, CD36-dependent regulation of FoxO1 is mediated by the nuclear receptor PPARα/β. Loss of PPARα/β phenocopies CD36 deficiency in blunting fasting induction of muscle FoxO1 and PDK4 in vivo. Expression of PPARα/β in C2C12 cells, like that of CD36, robustly induces FoxO1 and suppresses glucose oxidation, whereas co-expression of a dominant negative PPARα/β compromises FoxO1 induction. Finally, several PPRe sites were identified in the FoxO1 promoter, which was responsive to PPARα/β. Agonists of PPARα/β were sufficient to confer responsiveness and transactivate the heterologous FoxO1 promoter but not in the presence of dominant negative PPARα/β. Taken together, our findings suggest that CD36-dependent FA activation of PPARα/β results in the transcriptional regulation of FoxO1 as well as PDK4, recently shown to be a direct PPARα/β target. FoxO1 in turn can regulate CD36, lipoprotein lipase, and PDK4, reinforcing the action of PPARα/β to increase muscle reliance on FA. The findings could have implications in the chronic abnormalities of fatty acid metabolism associated with obesity and diabetes.

Fatty acids (FAs)2 supply a major fraction of the energy required for muscle function and contribute to the intricate regulation of muscle glucose utilization. However, excess FA or diminished FA oxidation can lead to chronic accumulation of metabolites that impair insulin responsiveness of glucose utilization (1, 2). Several reports have implicated high sarcolemmal levels of the FA translocase CD36 and persistently increased FA uptake in muscle insulin resistance (3–6). CD36 facilitates a large fraction of FA uptake by muscle (7), impacting glucose metabolism and insulin sensitivity (3, 8, 9). Muscle CD36 protein levels are modulated by insulin, leptin, resistin, contraction, obesity, and diabetes (3, 10). In addition, CD36 is induced by the PPAR transcription factors (11, 12), which function as nutrient sensors and metabolic regulators (13, 14). CD36-facilitated FA flux may in turn be a part of a feedback loop activating the PPARs; CD36 deficiency reverses myocardial lipotoxicity and functional impairments of the heart caused by PPARα overexpression (15). CD36 expression is also important for PPARγ activation by dietary fat in adipose tissue (16).

Optimal functioning and insulin responsiveness of muscle are linked to its ability to adjust fuel preference, suppressing glucose utilization, with more reliance on FA during nutrient shortage, and rapidly reversing these changes with feeding. The fasting/feeding adaptation is impaired in obesity and diabetes, described as diseases of metabolic inflexibility (17–19). Several pathways contribute to the fasting/feeding response, notably those involving the PPARs and the AMP-activated protein kinase (AMPK). The major PPAR isoform in muscle, PPARα/β, regulates FA catabolism and plays a central role in the adaptation to fasting (20). AMPK, on the other hand, is activated by an increase in the AMP/ATP ratio, a sensitive indicator of cellular energy, and functions to restore ATP levels by enhancing oxidation of glucose and FA (21). AMPK activation is especially important for the exercising muscle.

Recent evidence supports involvement of the transcription factor FoxO1 (Forkhead box O1A) in regulating the adaptive metabolism of muscle. FoxO1 is activated by nutrient shortage and inhibited by insulin/growth factor signaling (22, 23). Inhi-
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Activity alteration of FoxO1 activity mediates many effects of insulin on gene expression (24–26). Fasting activates muscle FoxO1, contributing to induction of PDK4 (pyruvate dehydrogenase kinase 4) (27), which then phosphorylates and inactivates pyruvate dehydrogenase (28). This inhibits pyruvate transition to acetyl-CoA and glucose oxidation. Although pyruvate dehydrogenase is acutely inhibited by FA oxidation products (high NADH/NAD+ and acetyl-CoA/CoA), chronically it is inactivated by PDK4. In addition to fasting, muscle PDK4 is increased by high fat diets (29), diabetes, and obesity, suggesting that it is a “lipid status” pyruvate dehydrogenase kinase isoform that facilitates FA oxidation (30).

As FoxO1 induces PDK4 to suppress glucose oxidation, it also acts to increase sarcoplasmic content of CD36, enhancing FA uptake and oxidation (31). FoxO1 also suppresses expression of acyl-CoA carboxylase (ACC), which reduces levels of the FA oxidation inhibitor malonyl-CoA. Thus, FoxO1 contributes to regulating muscle glucose and FA preference during fasting-feeding. Many pathways integrate feedback loops that optimize long term regulation (32–34), so we asked whether regulation of muscle FoxO1 and PDK4 was in turn responsive to CD36 function. This would be consistent with the reportedly high levels of CD36 (5, 35, 36) and PDK4 in diabetic muscle (29). We examined how CD36 overexpression or knockdown impact FoxO1 level, ability to induce PDK4, and muscle adaptation to fasting. Using in vitro and in vivo systems where expression of CD36, FoxO1, and PPARβ/δ was manipulated, we document that CD36-facilitated FA uptake via regulating PPARβ/δ and FoxO1 positively reinforces muscle FA utilization. This provides a potential mechanism by which FA uptake can chronically alter muscle bioenergetics.

EXPERIMENTAL PROCEDURES

Mouse Models—CD36-null (37) mice and mice with muscle-targeted CD36 overexpression (MCK-CD36) (9) (overexpressing CD36 in skeletal and heart muscles) were crossed five times to the C57Bl/6 background. PPARβ/δ-null (38) mice were on average 75% C57BL/6N. MKR mice (overexpressing a dominant-negative IGF-I receptor in muscle) and MKR-CD36 mice (bigenic, expressing muscle-specific CD36 on the MKR background) were on a FVB/N background.

Sample Preparation and Protein Analysis—Hearts were excised immediately after mice sacrifice, minced, and homogenized on ice (Fisher PowerGen 125) in SDS sample buffer (60 mM Tris-HCl at pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol) followed by boiling for 5 min. Protein concentration was quantified (Bio-Rad), and 30 μg of lysate were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore, Billerica, MA) membranes, which were blotted with the appropriate antibodies: FoxO1 (1:1000; 9462; Cell Signaling Technology, Danvers, MA), Phospho-FoxO1 (1:1000; 9461, Cell Signaling Technology), PDK4 (dilution 1:1000; AP704ib; Abgent, San Diego, CA), AKT (1:1000; 9272; Cell Signaling Technology), phospho-AKT Ser473 (1:1000; 9271), phospho-SPAK/JNK (1:500, Thr183/Tyr185, 9255; Cell Signaling Technology), p53 (1:1000, CM5p, Novoceastra), Ran (1:2000; sc-1156, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), α-tubulin (1:2000; B-5–1-2; Sigma). Anti-mouse horseradish peroxidase (1858413; Amersham Biosciences), and anti-rabbit horseradish peroxidase (1858415; Amersham Biosciences) were used as secondary antibodies. Signal was visualized using ECL detection (RPN 2132; Amersham Biosciences).

Membrane CD36 Content—Hearts were dissected from fed (n = 3) and fasted (n = 3) mice and homogenized in 1.5 ml of buffer (100 mM KCl, 50 mM Tris, 5 mM Na2HPO4, 100 μM phenylmethylsulfonyl fluoride, pH 7.4) on ice three times for 15 s. An aliquot of homogenate (0.2 ml) was frozen in liquid nitrogen for protein analysis, and the remainder was centrifuged at 4 °C for 10 min (800 × g) and then at 9000 × g to pellet out nuclei and mitochondria. A crude membrane fraction was then obtained by centrifugation at 4 °C for 1 h (190,000 × g) and was resuspended in 0.1 ml of buffer. Protein concentration in heart homogenate and plasma membranes using a modified Bradford assay (DC Protein Assay; Bio-Rad). For Western blotting, 40 μg of plasma membrane were processed by 10% SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Whatman, Florham, NJ). Membranes were incubated with monoclonal antibody CD36 (1:1500; Cascade Biosciences, Winchester, MA) or monoclonal antibody glycerolaldehyde-3-phosphate dehydrogenase (1:2000; Abcam, Cambridge, MA) overnight at 4 °C in 5% milk in TBST and then with secondary antibodies for 2 h at room temperature. Immunodetection was with chemiluminescence and Ultra Blue Autorad Film (ISC BioExpress, Kaysville, UT). glycerolaldehyde-3-phosphate dehydrogenase was the loading control.

Real Time PCR Analysis—Total RNA was isolated from tissues and cells using TriZol (Invitrogen) as recommended by the manufacturer. RNA pellets were washed in 75% ethanol, dried at room temperature, and resuspended in UltraPure distilled water (Invitrogen), and content was quantified by spectrophotometry. Samples were amplified using the Superscript III Platinum SYBR Green one-step quantitative reverse transcription-PCR kit (Invitrogen) on the SmartCycler system (Cepheid, Sunnyvale, CA). Results were analyzed by comparing the threshold crossing (Ct) of each sample after normalization to control genes (ΔCt). Changes in the threshold crossing (ΔCt) were used to calculate relative levels of each mRNA using the formula 2^-ΔCt. The intron-spanning primer pairs used for amplification were as follows: 18S, AGTCCCTTCCCTTTTGTACACA and GATCCAGGGGTCTCATTAAAOX; FoxO1, CTTGCTTCAAGTAGAT and GGGGTGAGGCATCGTTC; PDK4, TTTCTGCTTCTAGCGCAG and GATACACAGTCTAGTTCG; UCP3 (uncoupling protein 3), CAGAGCCATCTGGGATCCGTTGTTAC and GAGTGAAGCTCCAGACTACTTGT; PPR8/β, AGATGGTGGCGAGTCTAGTCC and TCTCTTGATGTCCTGTTCC.

Cell Culture—C2C12 myoblasts were maintained in low glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 200 units/ml penicillin, and 50 μg/ml streptomycin. Confluent myoblasts (90% confluence) were differentiated into myotubes by switching cells to Dulbecco’s modified Eagle’s medium supplemented with 1% horse serum, 200 units/ml penicillin, and 50 μg/ml streptomycin. Polynucle-
ated myotubes were obtained within 4 days. For FA treatment and FA time course, C2C12 myoblasts were incubated with oleic acid (400 μM acid, FA/BSA = 1:1) for 16 h. In some experiments, wortmannin (9951; Cell Signaling) was added (500 nM) to inhibit PI 3-kinase, N-Ac-Leu-Leu-norleucinal (40 mM) to inhibit proteosomal activity and cycloheximide (CHX) (10 μg/ml) to inhibit protein synthesis.

Retroviral Infection and RNA Interference (Short Hairpin RNA)—RNA sequences were designed using the Cold Spring Harbor Laboratory RNAi OligoRetriever data base and pSHAG vectors. Retroviral constructs with drug-selectable markers were transfected into Phoenix packaging cells using calcium phosphate. High titer viruses were used to infect cells (39, 40). Retroviruses encoding shCD36 were generated as described (41).

**Glucose Oxidation**—For glucose oxidation, U-14C-labeled glucose was used, and the amount oxidized was evaluated by measuring 14CO2 production as described (31). Cells were washed three times with Krebs Ringer Hepes containing 40 μM FA-free BSA and incubated for 1–2 h in the same buffer containing [U-14C]glucose (1 μCi/80 μl). 14CO2 trapping, using flasks with wells containing benzenthionium hydroxide-soaked filters, was overnight at 30 °C.

**Fatty Acid Uptake**—Cells were washed with Krebs Ringer Hepes buffer with 0.5% FA-free BSA, and uptake was started by the addition of transport buffer (Krebs Ringer HEPES with 80 μM [3H]palmitate; 0.5 μCi/ml; FA/BSA ratio 0.5–2). Uptake was performed at room temperature and stopped by the addition of cold buffer. Cells were lysed in 0.1 N NaOH, and aliquots were used for determining total counts, for protein assay (Life Science Research, Hercules, CA), and for FA incorporation into cell lipids.

**Bioinformatic Analysis of FoxO1 Promoter**—mRNA sequences of mouse FoxO1 (NM_019739) were retrieved from NCBI and the proximal promoter sequence ((−700, +300) about the transcription start site) was obtained from the CSHLmpd mammalian promoter data base (42). The MATCH program associated with the TRANSFAC data base (43), which minimizes the false negative rate, was used to identify the potential PPRE.

**Luciferase Assay**—Genomic fragments corresponding to the mouse FoxO1 promoter were amplified by PCR from mouse genomic DNA using sequence-specific primers (available upon request) to introduce KpnI/XhoI restriction sites. After purification and digestion cycles, amplicons were cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI) at KpnI/XhoI sites. C2C12 cells were transfected using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions with 1 μg of the reporter construct, 200 ng of the PPARδ/β expression plasmid or a vector control, and 1.2 μg of the pRL-β-globin control plasmid (Promega). Cells were harvested 36 h after transfection (or as indicated). PPARδ/β agonist GW0742 was used at 40–120 nM. Luciferase activity, assayed using a luminometer (Promega 20/20) was normalized to the control pRL-β-globin.

**Statistical Analysis**—Values shown are means ± S.E. Differences were analyzed for statistical significance (p < 0.05) by Student’s t test, one-way analysis of variance, or, in some cases, repeated measures analysis of variance. All experiments were repeated at least three times and included a minimum of three biological samples.

**RESULTS**

**Long Chain FAs Up-regulate FoxO1 Expression in Muscle Cells**—Excessive FA influx inhibits glucose utilization in muscle and contributes to the development of insulin resistance. We determined the effects of oleic acid on muscle glucose oxidation in C2C12 myoblasts. Fig. 1A shows that a 16-h incubation with 400 μM oleic acid markedly reduces glucose oxidation. We asked whether this effect correlates with induction or activation of known negative regulators of muscle glucose oxidation, such as FoxO1. Western blot analysis of C2C12 myoblasts exposed to oleic acid and the proteosome inhibitor N-Ac-Leu-Leu-norleucinal (LLnL; 40 μM) or DMSO as a control, p53, regulated by proteosomal degradation, is a positive control. Data are representative of three experiments carried in triplicates.

![FIGURE 1. Long chain fatty acids compromise glucose oxidation and induce FoxO1. A, glucose oxidation in C2C12 myoblasts treated for 16 h with oleic acid (400 μM; FA/BSA ratio = 1) or a BSA control. Cells were incubated with [U-14C]glucose for 2 h, and glucose oxidation was determined from 14CO2 production (31). Data are plotted as arbitrary units (AU), and values are expressed as means ± S.E. (n = 3). Statistical significance was by Student’s t test with p values <0.05 as significant. B, Western blot analysis of FoxO1 protein expression in C2C12 cell lysates using FoxO1 and phosphospecific FoxO1 (Ser256) antibodies. Phosphorylation at serine 256 is inhibitory and reflects inactive FoxO1. C2C12 myoblasts were treated with oleic acid as in A in the presence or absence of the PI 3-kinase inhibitor wortmannin (0.5 μM). Time course of total FoxO1 in C2C12 myoblasts exposed to oleic acid and the proteosome inhibitor N-Ac-Leu-Leu-norleucinal (LLnL; 40 μM) or DMSO as a control. p53, regulated by proteosomal degradation, is a positive control. Data are representative of three experiments carried in triplicates.](http://www.jbc.org/content/283/21/14319.f1)
in myotubes (Fig. 1B), and FA exposure for 16 h did not alter expression of genes associated with myotube differentiation (data not shown) or myotube morphology. A corresponding hypophosphorylation (at Ser308) of FoxO1 protein was detected in both myotubes and myoblasts, suggesting FoxO1 activation (Fig. 1B). Since FoxO1 inhibitory phosphorylation at Ser256 is typically mediated by activated AKT (22), we determined AKT status by assaying total and phospho-Ser256 AKT protein levels. A marked reduction in phosphorylation at Ser256 was observed (Fig. 1C), consistent with the decrease in phospho-FoxO1 with no detectable difference in total AKT (Fig. 1C) or phospho- 

AKT(Thr308) levels (data not shown). Next, we investigated the some-mediated proteolysis. FoxO1 protein expression concomitant with a decrease in cel-

Fig. 1 analysis in the presence of OA (data not shown). Collectively, these results indicated that oleic acid triggers the induction of total FoxO1 levels and examined whether they are altered by inhibition of the PI 3-kinase pathway. It has been reported that in the presence of PI 3-kinase inhibitors like wortmannin, growth factors or insulin fail to affect total FoxO1 protein expression (23). Myoblasts were treated with FA, in the presence or absence of wortmannin. As shown in Fig. 1D, wortmannin reduces phospho-FoxO1 levels as expected but has no effects on total FoxO1 expression. The same results were obtained with another PI 3-kinase inhibitor LY294002 (data not shown). Apparently, FA-induced FoxO1 protein accumulation is not inhibited by compromising PI 3-kinase signaling. We asked if the FA effect involved the proteosomal system with possible alteration in FoxO1 protein stability. Myoblasts were incubated in the presence or absence of N-Ac-Leu-Leu-norleucinal, a potent proteosome inhibitor. As shown in Fig. 1E, N-Ac-Leu-Leu-norleucinal treatment and inhibition of proteosomal degradation mechanisms were sufficient to induce accumulation of p53, a proteosomal target. However, no alteration in FoxO1 protein expression was observed. The same results were obtained in a similar time course analysis in the presence of OA (data not shown). Collectively, our data indicate that oleic acid triggers the induction of total FoxO1 protein expression concomitant with a decrease in cellular glucose oxidation. The FA up-regulation of total FoxO1 does not seem to involve the PI 3-kinase pathway or proteosome-mediated proteolysis.

Effect of CD36 on FoxO1 Level and Activation—Fatty acid transfer into muscle in vivo is facilitated in large part by the membrane protein CD36 (7, 45). Previously, we reported that enforced expression of CD36 in C2C12 muscle cells increases FA uptake and oxidation (46). We examined the effects of CD36 overexpression in these cells on FoxO1 levels. Fig. 2A shows robust induction of FoxO1 protein with CD36 overexpressing. In vivo relevance was shown by the observation that transgenic mice (MCK-CD36) with muscle-specific CD36 overexpression had higher FoxO1 mRNA (Fig. 2B). To determine the specific contribution of CD36, we generated a C2C12 cell line stably expressing a CD36 RNAi construct (shCD36) and examined if CD36 loss of function can alter FoxO1 expression. Fig. 2C shows that shCD36 expression resulted in marked knockdown of CD36 protein and decreased FA uptake (Fig. 2D). Importantly, these effects were associated with robust down-modulation of FoxO1 protein. An increase in glucose oxidation was also measured in shCD36-expressing cells (Fig. 2E), consistent with the reduction in FoxO1 level and activity. Thus, enforced expression of CD36 or its knockdown significantly induce or reduce FoxO1 expression, respectively. By influencing FoxO1 content, muscle CD36 expression would contribute to long-term regulation of muscle glucose oxidation.

**Contribution of CD36 to Fasting Induction of FoxO1 and PDK4**—Short term fasting in muscle is characterized by reduced glucose utilization and by increased reliance on FA (17). FoxO1 expression in muscle is enhanced by fasting (27), and FoxO1 activation in muscle cells increases membrane CD36 content and promotes FA uptake and oxidation (31). We examined the adaptation to fasting and the contribution of CD36 in several relevant mouse models. As shown in Fig. 3A, a 16-h overnight fast of WT mice is associated with a 3-fold increase in muscle membrane CD36 content. Furthermore, as shown in Fig. 3B, fasting is associated with down-regulation of glucose utilization, measured by 2-[18F]fluorodeoxyglucose uptake. This effect of fasting on glucose utilization was abolished by loss of CD36, as shown in the CD36−/− mice (Fig. 3B). Of note, basal glucose utilization was similar in the fed states in WT and CD36-null hearts and diaphragms, underscoring perhaps the more significant role for CD36 under states of elevated FA flux, such as fasting. FoxO1 levels were examined, and, as shown in Fig. 3C, FoxO1 protein expression is induced in the WT muscle (diaphragm) with fasting, whereas this induction is blunted in age- and gender-matched CD36-deficient muscle. Similar effects, albeit more modest, are detected in heart tissues (Fig. 3D). Analysis of FoxO1 mRNA also reveals diminished induction with fasting in CD36-null muscle as compared with WT (Fig. 3E). Importantly and in parallel with the changes in FoxO1 levels, induction of mRNA for PDK4, also induced with fasting (27), was markedly blunted with CD36 loss (Fig. 3F).

The role for CD36 in FoxO1 regulation in vivo was confirmed in another mouse model of altered CD36 expression in muscle,
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the double transgenic MKR-CD36. The MKR mouse has impaired IGF and insulin signaling in muscle as a result of muscle-targeted expression of a dominant negative IGF-1 receptor that hybridizes with both the IGF and insulin receptors (47). The mouse exhibits reduced IGF-1 and insulin-stimulated glucose uptake in muscle and global insulin resistance. This mouse was also shown to have reduced muscle FA oxidation with enhanced accumulation of intramuscular triglycerides. Muscle-targeted CD36 overexpression in the MKR enhanced FA oxidation, reduced muscle lipid accumulation, and was associated with a reversal of the diabetes (47). To determine whether some of the defects in muscle FA metabolism in the MKR mouse may reflect an abnormal CD36-FoxO1 interaction, we examined muscle FoxO1 levels in MKR and MKR/CD36 mice. As shown in Fig. 3G, FoxO1 and PDK4 protein levels were very low in the MKR muscle (lanes 1 and 2) and were induced by CD36 expression (compare lanes 1 and 2 with lanes 5 and 6) to levels comparable with those observed with fasting. Thus, expression of CD36 was sufficient to induce FoxO1 and PDK4 in the presence of compromised PI 3-kinase signaling. This is consistent with our findings (Fig. 1) that FA induction of total FoxO1 protein is not altered by inhibiting PI 3-kinase. The findings that CD36 loss attenuates fasting induction of FoxO1 and PDK4 suggest that CD36 function is required for full induction of these proteins independent of the PI 3-kinase pathway.

Kinetics for FA Induction of FoxO1 and PDK4—We and others reported that FoxO1 is sufficient to induce PDK4 (27, 31). Binding of FoxO1 to the PDK4 promoter was suggested in C2C12 myoblasts (27). To gain further insight into the FoxO1 regulation of PDK4, C2C12 cells expressing exogenous FoxO1 fused to a modified estrogen receptor (FoxO1:ER) were used. In this cell system, the FoxO1 protein is relocated to the nucleus by hydroxytamoxifen treatment (31). We tested whether induction of PDK4 mRNA is altered in presence of CHX, an inhibitor of protein biosynthesis that blocks translational elongation. If PDK4 induction is a direct result of FoxO1 transcripational activity, it will not be affected by CHX in this system. C2C12 myoblasts stably expressing FoxO1:ER were treated with hydroxytamoxifen for 8 h in the presence or absence of CHX. As shown in Fig. 4A, PDK4 mRNA was induced by FoxO1 activation, as previously described (31), but this induction was prevented by CHX. Apparently, activation of the FoxO1:ER is not sufficient to induce PDK4 in the absence of de novo protein synthesis, supporting requirement of additional factors and indirect transcriptional activation.
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To further understand how FAs regulate the interaction of FoxO1 and PDK4 in muscle cells, we determined the changes in mRNA and protein expression for FoxO1 and PDK4 as a function of time following FA treatment. We first confirmed that PDK4, like FoxO1, is induced in response to FA (Fig. 4B). Next, we analyzed the time course for this induction. Fig. 4C shows that PDK4 mRNA is rapidly induced preceding that of FoxO1. Similar patterns were obtained for protein induction (Fig. 4, D and E). Collectively, these data support the interpretation that although FoxO1 can induce PDK4, under conditions of FA flux, FoxO1 and PDK4 are induced separately. Of note, JNK kinase, which is potently activated by FA (48) and is implicated in acutely activating FoxO1 (49) was quickly phosphorylated (activated) in these cells with no change in total JNK levels (data not shown). JNK phosphorylation is consistent with the effects of FA on phospho-AKT (Ser473) (Fig. 1), since JNK modulates AKT activity and reduces phospho-AKT (Ser473) levels (49).

PPARδ/β Contributes to FA Regulation of FoxO1 in Vivo—Free FAs and their derivatives are activating ligands for the PPAR transcription factors (50, 51). Recent data in heart (15) and adipose tissue (16) suggest that CD36-facilitated FA uptake is linked to PPAR activation. PPARδ/β expression in skeletal muscle is high, favoring oxidative fibers (52, 53), and this isoform has been implicated in regulating FA oxidation and in muscle adaptation to fasting (54). Importantly, a new report showed that the PPARδ/β isoform regulates PDK4 expression via a direct transcriptional mechanism (55). In addition, the PPARδ/β targets ADRP and UCP2 were elevated early after FA addition to C2C12 cells, as shown in Fig. 4F. Similar early induction was also observed for the PPARδ/β target UCP3 (data not shown). As such, we asked whether PPARδ/β could be involved in induction of FoxO1 expression by CD36-mediated FA flux. We determined the expression of adipophilin (ADRP) and UCP3 in the feeding/fasting cycle. As shown in Fig. 5A, expression of these targets was responsive to fasting, and this response was significantly diminished in CD36-deficient as compared with WT muscle. Expression of ADRP and UCP3 was similarly blunted in PPARδ/β-deficient muscle, as expected. In contrast, expression of these PPARδ/β targets was increased in skeletal muscle from transgenic MCK-CD36 mice, where muscle CD36 expression is enhanced (data not show) and where FoxO1 is induced by fasting (data not shown). Apparently, conditions of elevated FA flux or CD36 overexpression correlate with enhanced PPARδ/β activity, as reflected by target genes, whereas CD36 deficiency is associated with opposite changes. Therefore, we determined the effect of PPARδ/β loss on FoxO1 in vivo. A reduction of FoxO1 induction would be expected if PPARδ/β is required for FoxO1 expression. As shown in Fig. 5B, homozygous loss of PPARδ/β compromises myocardial expression of FoxO1 and its induction with fasting. Fasting induction of FoxO1 was also blunted in diaphragms of PPARδ/β-deficient mice compared with WT (Fig. 5C), and similar effects were also observed with CD36 deficiency (Fig. 5C). Induction of PDK4 expression was markedly suppressed in tissues deficient in either CD36 or PPARδ/β (Fig. 5D). In summary, loss of PPARδ/β, like that of CD36, suppresses induction of FoxO1 and PDK4 in response to fasting. The data suggest that CD36 facilitated FA activation of PPARδ/β is required for fasting induction of FoxO1 and PDK4.

PPARδ/β Is Sufficient to Induce FoxO1 and Inhibits Glucose Oxidation—We confirmed that the PPARδ/β targets are induced in C2C12 cells, where FoxO1 and PDK4 are elevated in response to FA treatment. As shown in Fig. 6, oleic acid induced expression of the PPARδ/β targets UCP2, UCP3, and ADRP in C2C12 myoblasts and myotubes (Fig. 6, A and B, respectively). We then stably introduced PPARδ/β into C2C12 myoblasts and asked whether this would be associated with FoxO1 induction. PPARδ/β overexpression was sufficient to robustly induce FoxO1 (Fig. 6C, compare lanes 1 and 3 or lanes 2 and 4). FA treatment did not increase FoxO1 levels further in PPARδ/β-overexpressing cells, supporting the interpretation that PPARδ/β mediates to a large extent such effects. Glucose oxidation levels were reduced in cells treated with FA or expressing PPARδ/β (Fig. 6D) in line with the elevated FoxO1 levels. Finally, we asked whether reducing PPARδ/β can attenuate FoxO1 expression. We stably introduced a dominant negative construct into PPARδ/β-overexpressing cells. Indeed, the DN PPARδ/β reduced FoxO1 expression in a manner that correlates with PPARδ/β expression levels (Fig. 6E, compare lane 1

![Figure 5. PPARδ/β can mediate the regulation of FoxO1 and PDK4 in vivo.](image-url)
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with lane 3 or lane 2 with lane 4). Taken together, muscle FoxO1 expression is regulated by PPARβ/δ, and this could mediate, at least in part, the effect of CD36-facilitated FA uptake.

PPARβ/δ Regulates FoxO1 through a Transcriptional Mechanism—The mechanism of PPARβ/δ regulation of FoxO1 was examined further. To determine whether the FoxO1 promoter contains PPRE binding sites, the MATCH program associated with the TRANSFAC data base of cis-regulatory motifs (56) was used. This unbiased approach identifies transcription factor binding sites on the basis of position weight matrices constructed from previously characterized binding sites and assigns scores for similarity between a candidate site and the position weight matrices. As shown in Fig. 7, A and B, PPAR response elements, including at least one predicted PPRE site, were identified in the FoxO1 promoter. The genomic fragment containing the predicted PPARβ/δ sites (sequence +732 to −237 relative to the transcriptional start site) were cloned into a luciferase reporter plasmid (FoxO1-luc), which was co-transfected into C2C12 myoblasts with either an empty vector control or a PPARβ/δ expression plasmid and a normalization control. As shown in Fig. 7C, PPARβ/δ induces significant transactivation of the FoxO1-luc reporter. Co-transfection in the presence or absence of the PPARβ agonist, GW0742, is shown in Fig. 7D. As expected, the FoxO1 promoter was responsive to increasing doses of GW0742 (0, 40, and 120 nM). Similar results were obtained when we measured FoxO1 transactivation as a function of time (0, 6, and 16 h; Fig. 7E) in the presence of PPARβ agonist. Finally, and as expected, cells expressing a DN PPARβ/δ failed to respond to agonist stimulation (Fig. 7E).

Together, the data support existence of a transcriptional mechanism for PPARβ/δ regulation of FoxO1, probably acting in concert with that of PPARβ/δ on PDK4 (55).

DISCUSSION

Muscle tissue accounts for one-third of the resting metabolic rate and for a major fraction of insulin-stimulated glucose uptake. Thus, its flexibility with respect to fuel preference is crucial for glucose homeostasis. In insulin resistance, muscle adaptation to fasting and feeding appears dysfunctional, with features of the fasted state, such as enhanced FA utilization, persisting into the fed state (19). A better understanding of the molecular mechanisms mediating normal muscle adaptation may provide...
CD36 Regulation of Muscle FoxO1 and PDK4

insight into the etiology of metabolic diseases. This study shows that CD36-facilitated FA uptake modulates PPARδ/β and FoxO1 function to reinforce muscle reliance on FA and the adaptation to fasting. First, CD36 overexpression or knockdown in vitro correlates with FoxO1 and PDK4. Second, CD36 deficiency or overexpression, in vivo, has opposite effects on fasting induction of FoxO1 and PDK4, which are paralleled with altered substrate utilization. Third, regulatory effects of CD36-FA uptake on FoxO1 and PDK4 involve PPARδ/β as shown by its overexpression or knockdown in vitro and by its deletion in vivo. Fourth, FA activated PPARδ/β directly induces FoxO1 expression. The data suggest that CD36-FA uptake in fasting up-regulates PPARδ/β activity, levels, and activity of FoxO1 and PDK4. As a result it contributes to determining muscle fuel preference and ability to adapt to metabolic stress.

The FoxO1 transcription factor has been implicated in regulating various aspects of cellular metabolism (57–59). In the liver, FoxO1 suppresses glycogenesis and lipogenesis while increasing gluconeogenesis (60, 61). In muscle, fasting up-regulates FoxO1, which contributes to induction of PDK4, inhibiting glucose oxidation (27). FoxO1 activation also recruits CD36 to the sarcolemma (31) and induces lipoprotein lipase (62) to coordinate increase FA uptake. Regulation of FoxO1 appears to integrate several signaling inputs. Post-translational modifications that acutely alter the FoxO1 proteins are the most understood events (63). Among these, the role of JNK (activation) and AKT (inhibition) in FoxO1 regulation are well documented (23). However, the mechanisms that may promote increased steady state FoxO1 levels, as documented with fasting or diabetes, remain poorly defined. One of the findings of this study is that FoxO1 level is sensitive to increased CD36-facilitated FA flux. Since FoxO1 promotes enrichment of membrane CD36, the ensuing enhancement in FA uptake is probably maintained via positive feedback regulation of both FoxO1 activity (via AKT inactivation and JNK activation) and expression (via PPARδ/β). Thus, it is conceivable that conditions that chronically change muscle CD36 content would alter the adaptive response of FoxO1 and PDK4 to metabolic challenges, which may relate to how FAs induce muscle insulin resistance. In this context, the CD36-null mouse where FA uptake into muscle is impaired has blunted fasting induction of muscle FoxO1 and PDK4 (Figs. 3 and 5), and this is associated with enhanced insulin sensitivity and glucose uptake in this tissue (64). On the other hand, the mouse with muscle-targeted CD36 overexpression (MCK-CD36) with higher muscle FoxO1 (Fig. 2B) has features of insulin resistance that include high glucose and insulin levels (9). Thus, the increased FoxO1 and PDK4 expression in obesity and diabetes (28, 29) may reflect in part the high sarcolemmal CD36 in these conditions (3) and possibly could be reversed by CD36 down-regulation.

Our findings indicate that PPARδ/β, an FA-activated nuclear receptor, induces transcription of FoxO1, and loss-of-function experiments in vitro and in vivo (PPARδ/β-null mouse) demonstrate its pivotal role in FoxO1 regulation. Recent evidence shows that PDK4 is also a direct target of PPARδ/β (55), further supporting the primary role of this isoform in regulating muscle oxidative metabolism. Activation of PPARδ/β in muscle induces a fasting-like phenotype characterized by increased FA oxidation and suppressed glucose oxidation (65). This phenotype is similar to that induced by FoxO1, which together with our data suggests that some PPARδ/β effects in muscle may be mediated via FoxO1. This would be consistent with the report that PPARδ/β agonists initiate a muscle atrophy program (66) that is regulated by the PI 3-kinase/AKT/FoxO1 pathway (67). It is important to emphasize that findings of this study, especially results described in Fig. 4, favor the interpretation that PDK4 expression under conditions of FA flux is not FoxO1-dependent. Given the transcriptional regulation of both PDK4 and FoxO1 by PPARδ/β, these genes are probably regulated independently by this PPAR as further depicted in the model in Fig. 8.

Conceivably, some metabolic effects of CD36 facilitated FA flux in the fasted muscle may involve the AMPK pathway, which contributes to adaptive regulation of muscle glucose and FA oxidation. We did not detect changes in levels of phosphorylated AMPK or ACC in response to CD36 deletion (data not shown). However, this does not rule out a role for AMPK, since FA flux may alter the interaction between PPARδ/β and AMPK (68), which was not examined. FA effects on the interaction of PPARδ/β and PGC-1α, which plays a key role in regulating mitochondrial biogenesis and oxidative capacity (13, 69, 70), were also not considered and will need to be examined. Our data, which focused on PPARδ/β, do not rule out contribution of PPARα to the observed effects of FA, and there is evidence for significant redundancy in the regulatory effects of the two isoforms in muscle (71). Of note, although fasting induction of PDK4 is unaltered in skeletal muscle or slightly reduced in heart from PPARα-null mice (71), it is markedly blunted in both tissues of the PPARδ/β-null mouse (Fig. 5). This suggests that PPARδ/β is more directly involved in regulating muscle fuel preference in response to fasting.

In conclusion, the functional interplay between CD36, a major FA uptake protein in muscle, PPARδ/β, FoxO1, and
PDK4, key modulators of glucose and FA metabolism provides a framework for long term regulation of muscle fuel preference. Dysfunction in either of these factors by virtue of their interdependence would lead to an abnormal metabolic profile and alter adaptability of the tissue to energy challenges. Suppressing CD36 expression and hence reducing FA flux could improve metabolism of insulin-resistant muscle by restoring regulation of PDK4 and ability to oxidize glucose. Although this has not been directly tested, it would be consistent with the phenotypes of the CD36-null (64) or the transgenic MCK-CD36 mice (9).

However, in certain contexts, enforced CD36 expression may have beneficial effects in activating PPARβ/δ to promote FA uptake and oxidation, as in the MKR muscle (47). Activation of PPARβ/δ can improve muscle FA oxidation and the plasma lipid profile and has insulin-sensitizing effects (72). The fact that CD36 overexpression is beneficial in the context of absent insulin and IGF-1 signaling (MKR) suggests that glucose metabolism and insulin action may contribute to the long term negative effects of excess FA flux by inhibiting FA oxidation (73–75). The ensuing imbalance between FA uptake and oxidation would predispose to insulin resistance and is consistent with the findings that insulin-resistant muscle exhibits impaired FA oxidation (76–78). Thus, therapies targeting CD36 or PPARβ/δ would have to carefully consider the context involved.

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