Emerging evidence documents a key function for the forkhead transcription factor FoxO1 in cellular metabolism. Here, we investigate the role of FoxO1 in the regulation of fatty acid (FA) metabolism in muscle cells. C2C12 cells expressing an inducible construct with either wild type FoxO1 or a mutant form (FoxO1/TSS) refractory to the protein kinase B inhibitory effects were generated. FoxO1 activation after myotube formation altered the expression of several genes of FA metabolism. Acyl-CoA oxidase and peroxisome proliferator-activated receptor δ mRNA levels increased 2.2-fold and 1.4-fold, respectively, whereas mRNA for acetyl-CoA carboxylase decreased by 50%. Membrane uptake of oleate increased 3-fold, and oleate oxidation increased 2-fold. Cellular triglyceride content was also increased. The enhanced FA utilization induced by FoxO1 was mediated by a severalfold increase in plasma membrane level of the fatty acid translocase FAT/CD36 and eliminated by cell treatment with the CD36 inhibitor sulfo-N-succinimidyl-oleate. We conclude that FoxO1 activation induces coordinate increases in FA uptake and oxidation and that these effects are mediated, at least in part, by membrane enrichment in CD36. The data suggest that FoxO1 contributes to preparing the muscle cell for the increased reliance on FA metabolism that is characteristic of fasting. Dysregulation of FoxO1 in muscle could contribute to intramuscular lipid accumulation and insulin resistance by maintaining activation of FA uptake.

Maintaining the appropriate balance between glucose and fatty acid (FA) metabolism is essential for muscle cells as they undergo nutritional transitions from feeding to fasting. As circulating levels of glucose and insulin fall, the muscle cell increases its reliance on FA oxidation. The decline in muscle cell glucose utilization that is associated with fasting allows glucose sparing because glycogen body stores are limited and can be depleted in a short period of time. In contrast to glycogen, the amount of stored fat is substantial, and FA released by adipocytes is a more sustainable substrate. As a result, the ability of muscle cells to accomplish the substrate shift from glucose to FA is a physiologically important adaptation for surviving caloric restriction.

FoxO1 is a member of the evolutionarily conserved FoxO subfamily of forkhead transcription factors (1, 2), which are thought to be important in mediating effects of insulin and growth factors on glucose homeostasis and processes such as apoptosis and the cell cycle (2–4). FoxO1 is expressed in tissues involved in energy metabolism such as liver, muscle, and adipose tissue, where its function is inhibited by insulin and insulin-like growth factor I (5). Phosphorylation of three highly conserved protein kinase B sites, corresponding to Thr24, Ser256, and Ser319 in human FoxO1, suppresses transactivation and promotes nuclear exclusion of FoxO proteins by multiple mechanisms (6). In muscle, FoxO1 expression is increased with fasting (7), which induces the key regulatory enzyme pyruvate dehydrogenase kinase (PDK) 4 (8), a FoxO1 target gene. PDK4 phosphorylates and inactivates pyruvate dehydrogenase, which inhibits oxidation of glucose in muscle (9), sparing it for glucose-dependent tissues including brain. Recently, FoxO1 has also been shown to promote protein turnover through proteasomal mechanisms in response to glucocorticoids and during fasting (10), an important step in providing substrate for glucose production by the liver.

Muscle FA utilization, although sensitive to FA supply, is not simply a function of blood FA levels and has been shown to be regulated at the tissue level (11–13). In particular, the membrane protein CD36 facilitates an important fraction of muscle FA uptake (11, 12). Recent studies with mice overexpressing a dominant negative insulin-like growth factor I receptor in muscle documented low FA oxidation levels in this tissue that were reversed by muscle-targeted overexpression of CD36 (14). This suggested that the insulin-like growth factor I pathway, possibly via its regulation of FoxO protein activation, modulates FA metabolism. Here, we examined the possibility that the increased expression and function of FoxO1 that occur with fasting may contribute to the adaptive response of FA metabolism in muscle. We measured the effect of FoxO1 activation on FA uptake and oxidation by C2C12 myotubes. To avoid confounding effects of FoxO on cell differentiation (15), we used TAM-inducible wild type and mutated forms of FoxO1, which were...
activated after myotube formation was complete. Our results indicate that FoxO1 increases FA uptake and oxidation in muscle cells by increasing membrane CD36 content and by regulating expression of key genes of FA utilization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium, fetal bovine serum, antibiotics, and other cell culture products were from Invitrogen and Roche Applied Science. [9,10(H)-H]oleate, [1-14C]oleate, and t-(U-14C)glucosamine were from ICN Biochemicals (Costa Mesa, CA). FoxO1 (FKHR) antibody was from Cell Signaling Technology (Beverly, MA). CD36 antibody was from Cascade Biosciences (Winchester, MA), and the estrogen receptor (ER) antibody was from Santa Cruz Biotechnology. 4-Hydroxytamoxifen was obtained from Sigma. Inmmobilon-P membranes used for Western blots were from Millipore. Primers for quantitative PCR were from Invitrogen. Fatty acid-free bovine serum albumin (BSA) was obtained from Sigma and used for all experiments.

**C2C12 Cells with Stable Expression of FoxO1**—The constitutively active FoxO1/TSS was generated by site-directed mutagenesis in pBluescript; the substitution of Thr24, Ser256, and Ser319 by alanines was verified by deoxy sequencing, as previously reported (16). To create a DNA binding-defective protein, His[Ser256] was replaced by an inactive variant containing Ile[Ser256] (17). The C-terminal end for cloning in-frame with the modified ligand-binding domain of the estrogen receptor in the pBABE retroviral vector containing the puromycin selection marker (provided by Dr. Nissim Hay, University of Illinois at Chicago). The ligand-binding domain of the estrogen receptor in this vector has been mutated such that it specifically responds to TAM but not to endogenous estrogens (17). Retroviruses were produced by transfecting eotopic Phoenix packaging cells with pBabe plasmids expressing FoxO1-ER, FoxO1-TSS-ER, FoxO1/H215, or the empty pBabe vector. After 8 h, cells were washed with phosphate-buffered saline (PBS) and incubated for 48 h in fresh medium. Medium containing the recombinant virus was recovered and filtered (0.45-μm Nalgene filters) prior to infection of C2C12 myoblasts at 50% confluence in the presence of 10 μg/ml Polybrene. After 48 h, myotubes were incubated with 4-hydroxytamoxifen to the culture medium. Myotubes that specifically responded to TAM but not to endogenous estrogens were isolated and the supernatant was centrifuged at 16,000 × g. The pellet containing plasma membrane was resuspended in buffer containing 50 mm Tris-HCl, pH 7.4, 100 mm NaCl, 50 mm LiCl, 5 mm EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, and 0.02% sodium azide.

For Western blotting, proteins (40 μg) were separated on a 4–20% polyacrylamide-reduced gradient gel and electroblotted onto Immobilon-P polyvinylidene difluoride membranes. Blots were blocked with 5% milk in Tris-buffered saline for 60 min at room temperature and then incubated overnight at 4 °C with antibodies against ER, FoxO1 (1:10,000), or CD36 (1:2000) in Tris-buffered saline and 0.05% Tween 20 (TBST) containing 5% milk. Blots were washed in TBST and incubated with goat anti-rabbit (for FoxO1) or goat anti-mouse (for CD36) horse-radish peroxidase-conjugated secondary antibodies (1:30,000) for 30 min at room temperature. Membranes were washed in TBST and antiserum-antibody complexes were visualized by chemiluminescence using an ECL kit (Amersham Biosciences).

**Glucose Uptake**—Myotubes were washed three times with 2 ml of Krebs-Ringer solution buffered with HEPES (KRH). Uptake was assayed at an incubation temperature, as previously described (19). Briefly, it was started by addition of 2 ml of transport buffer (0.5 μM 2-deoxy-o-(1,2,3-[3H]glucose in KRH with 5 mm glucose) with 4-hydroxytamoxifen as indicated. Uptake was stopped by medium aspiration and washing three times with ice-cold KRH. Cells were lysed (30 min) in 1 ml of 0.1 N NaOH before aliquots were taken for scintillation counting (Beckman LS3801) and protein measurement using a Bio-Rad kit.

**Glycogen Accumulation**—Glycogen formation was assayed as described previously (19). Washed cells were scraped into 300 μl of 1× KOH. Extracts were heated (100 °C, 10 min), and an aliquot was taken for protein assays. Saturated Na2SO4 (40 μl) was added, and glycogen was precipitated at −70 °C (30 min) with 700 μl of ice-cold acetone. Pellets (20,000 × g, 30 min) were air dried, and redissolved in 100 μl of water before adding scintillant for counting.

**Fatty Acid Uptake**—Myotubes were washed three times with 2 ml of KRH, pH 7.4, 0.1% BSA and once with 2 ml of KRH. Uptake was performed at room temperature for the indicated times (19). It was started by addition of 2 ml of transport buffer, KRH containing 5 mm glucose and 50 μM [3H]H-glucose bound to BSA (0.5 μM/ml; FA/BSA 2). It was stopped by medium aspiration and washing three times with ice-cold KRH. Myotubes were lysed in 1 ml of 0.1 N NaOH for 30 min, and aliquots were taken for scintillation counting and protein determination.

**Fatty Acid Oxidation**—FA oxidation assays were performed as described previously (19, 20). Briefly, cells were washed three times with KRH containing 40 μM BSA and then incubated for 2 h in 1 ml of the same buffer containing [1,2-3H]oleate (1 μCi/ml) with/without 1 μM 4-hydroxytamoxifen. CO2 trapping was performed overnight at 30 °C with gentle shaking. The acid-soluble metabolic (ASM) fraction was evaluated after a perchloric acid precipitation of the medium. Oxidation rates (CO2 + ASM) were expressed as nmol/mg protein/h or as indicated.

**FA Partitioning and Triglyceride Content**—Myotubes pre-incubated for 12 h in the presence or absence of 1 μM TAM were washed and differentiated as described. The medium with TAM was removed, and the medium without TAM containing [1,2-3H]oleate (1 μCi/ml; 80 μl) was added. Cells were then washed at 3 °C (three washes with PBS/0.5% BSA, followed by two washes with buffer lacking BSA) and scraped in 500 μl of PBS. Aliquots were taken for Tolc extraction and protein assays. Tolc extracts were analyzed by TLC on Silica Gel 60A plates (Whatman, Clifton, NJ) using a two solvent system. The first solvent (diethyl ether:benzene:ethanol:acetic acid, 40:50:2:0.2) was run up to three-fourths of the plate. Plates were air dried, heated briefly to remove traces of acetic acid, and run in the second solvent (diethyl ether:hexane, 6:94) from 1 cm to the top. Plates were again dried at 60 °C for 30 min. Spots corresponding to TG, identified by standards run simultaneously and visualized by iodine vapors, were scraped and counted.

For triglyceride content, Tolc extracts were evaporated and redissolved in isopropanol. TG content was measured using a triglyceride assay kit (GPO-Trinder) from Sigma.

**Quantitative PCR**—Total RNA was prepared using the High-Pure RNA Isolation kit (Roche Diagnostics). Real-time quantitative PCR was performed as described previously (21). Briefly, complementary DNA was synthesized from 10 μg of total RNA with Superscript II Reverse Transcriptase (Invitrogen). Quantitative PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) in the presence of SYBR Green I (Sigma-Aldrich) with Taq DNA polymerase (Roche Diagnostics).

The annealing temperature of each oligonucleotide pair (Table I) was determined using a T-Gradient thermal cycler (Biotema, Göttingen, Germany) to ensure synthesis of only one DNA product. Amplion mass at the cycle threshold was determined by comparing fluorescence to a
or presence of 1 nM tamoxifen and then incubated for 12 h in the absence or presence of 1 μM TAM. FoxO1-ER distribution between cytoplasmic (C) and nuclear (N) compartments was evaluated by Western blotting using an antibody against the ER ligand binding domain (top row) or FoxO1 (bottom row). C, luciferase activation by TAM in FoxO1-ER- and FoxO1/TSS-ER-expressing cells. Data in A–C are representative of two experiments. D, expression of the differentiation markers MyoD1 and myogenin in TAM versus basal cells. Quantitative PCR analysis was performed under the same conditions described for A. Data are means ± S.D. (n = 9).

**RESULTS**

**C2C12 Cells Expressing FoxO1**—To assess the role of FoxO1 in regulating FA metabolism in muscle cells, C2C12 myocytes stably expressing the inducible FoxO1 fusion proteins FoxO1-ER and the mutant FoxO1/TSS-ER were generated (Fig. 1A). Unlike its wild type counterpart, the FoxO1/TSS-ER protein is refractory to negative regulation by protein kinase B because the three protein kinase B phosphorylation sites (Thr24, Ser256, and Ser319) that promote nuclear exclusion have been mutated. Fig. 1A shows the expression levels achieved for both fusion proteins in C2C12 cells. Using an antibody against FoxO, we determined in separate experiments that levels of exogenously expressed FoxO1 were comparable to those of endogenous FoxO for FoxO1/TSS or about 2-fold higher for FoxO1 (data not shown). To confirm the responsiveness of the fusion proteins to tamoxifen, their distribution was examined in the presence or absence of tamoxifen. When immunoblotted with an ER-specific antibody, 100-kDa bands corresponding to FoxO1-ER and FoxO1/TSS-ER were detected in the cytoplasmic fractions (Fig. 1B). Upon addition of TAM, both proteins were detected in the nuclear fraction, reflecting the ability of TAM to induce nuclear localization. Of note, a small residual level of FoxO1-ER remains cytoplasmic after treatment with TAM, perhaps reflecting the antagonistic effect of basal protein kinase B activity and phosphorylation of FoxO1-ER on localization. In contrast, the effect of TAM on FoxO1/TSS-ER localization is complete. In separate studies using transient transfections, we documented that the effects of TAM were specific for FoxO-ER fusion proteins and did not alter the function of wild type FoxO1 (data not shown). Fig. 1C shows that TAM treatment is associated with transcriptional activation by both FoxO1-ER and FoxO1/TSS-ER and consistent with changes in the cellular distribution of these proteins. In addition, as shown, TAM treatment did not have any effect on the activity of endogenous FoxO proteins (control bars) in C2C12 cells. The tight regulation and activation of both fusion proteins (Fig. 1, B and C) by TAM make it possible to study how acute modulation of FoxO1 function impacts FA metabolism.

Acute activation of FoxO1 subsequent to myotube formation was not associated with obvious changes in the terminal differentiation status of C2C12 cells examined microscopically. In line with this, as shown in Fig. 1D, activation of FoxO1-ER or FoxO1/TSS-ER did not alter expression of the differentiation markers MyoD and myogenin in myotubes. This indicated that activation of FoxO1 after myotube formation was completed could be used to examine effects on metabolism without confounding effects on cell differentiation.

**Glucose Metabolism Is Only Modestly Altered in C2C12 Cells Expressing FoxO1**—FoxO1 has been implicated in the regulation of glucose metabolism, especially in the liver (3, 4, 22). In muscle, although increases in FoxO1 expression are observed with fasting, the role of these increases in regulating glucose metabolism has not been studied. Here, we examined the effects of targeting FoxO1 to the nucleus of C2C12 myotubes on glucose uptake, oxidation, and incorporation into glycogen and on expression of several key regulatory genes. As shown in Fig. 2, A and B, glucose uptake and oxidation were not significantly altered by TAM. Glucose incorporation into glycogen was significantly decreased, although the effect was modest (20%) (Fig. 2C). No changes were observed in expression of the major muscle glucose transporters, Glut1 and Glut4. The enzyme PDK4, which regulates activity of pyruvate dehydrogenase, has been shown to be a FoxO1 target (8). In line with this, a small but significant increase in PDK4 expression was observed with FoxO1 activation. Together, these data indicate that FoxO1 alone may not play a major role in regulating glucose metabolism in C2C12 muscle cells.

**FoxO1 Increases FA Oxidation and the Expression of FA Oxidation-Related Genes**—We speculated that if FoxO1 is an important regulator of muscle cell adaptation to fasting, it would be expected to promote FA utilization. To address this question, we first examined the effects of activating FoxO1 in C2C12 cells on FA oxidation. As shown in Fig. 3A, oleate oxidation was significantly increased by FoxO1 activation. This effect was apparent 4 h after addition of TAM and increased in magnitude as the length of TAM treatment was extended to 12 h (Fig. 3B). In
contrast, TAM addition alone did not affect FA oxidation in C2C12 cells stably transfected with the empty retroviral vector (data not shown), indicating that the effect on FA oxidation is mediated through FoxO1.

Fig. 3C shows that TG content was increased after FoxO1 activation from 12 to 18 H9262 g/mg protein. However, FA incorporation into TG and glycerol release were similar in cells before and after TAM treatment, indicating that turnover of TG was unaltered by FoxO1 activation (data not shown).

The effect of FoxO1 activation on the expression of several key genes involved in the regulation of FA oxidation in C2C12 cells was examined. As shown in Fig. 3D, mRNA abundance for the first enzyme of FA metabolism, acyl-CoA synthase (ACS), was not altered. The enzyme of peroxisomal FA oxidation, acyl-CoA oxidase, was increased more than 2-fold by TAM treatment. Expression of the mitochondrial enzyme of oxidation carnitine palmitoyl transferase-1, examined for both the H9251 and H9252 isoforms, which are known to be present in muscle, was unchanged. However, mRNA for the enzyme acetyl-CoA carboxylase (ACC), which catalyzes formation of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyl transferase-1, was reduced by 50%. Expression of the malonyl-CoA decarboxylase enzyme, which acts to process malonyl-CoA, was not affected.

This suggests that FoxO1 activation may promote FA oxidation in part by limiting the formation of malonyl-CoA and thereby enhancing the function of carnitine palmitoyl transferase-1. Expression of PPARγ, which has been shown to play an important role in regulating FA oxidation in muscle (23, 24) was moderately increased after TAM addition, whereas no effect was observed on either PPARα or PPARγ (data not shown). Thus, activation of FoxO1 may stimulate FA oxidation at least in part by reducing the expression of ACC and increasing that of acyl-CoA oxidase, promoting FA oxidation in mitochondria and peroxisomes, respectively.

**FoxO1 Increases FA Uptake**—Intramyocellular TGs reflect a balance between uptake and oxidation of FA. The finding that TG content increases in myotubes after FoxO1 activation indicated that the enhancement in FA oxidation was not sufficient to limit TG accumulation and suggested that FoxO1 may be increasing FA uptake directly. To examine this, we determined initial rates of FA uptake into FoxO1 myotubes after a 12-h incubation with or without 1 H9262 M TAM. As shown in Fig. 4A, activation of FoxO1 increased oleate uptake by about 3-fold as compared with the basal state (no TAM). The effect could be detected as early as 45 min after addition of TAM, and its magnitude increased with time (Fig. 4B). To determine whether DNA binding by FoxO1

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**Fig. 2. Effects of FoxO1 activation on glucose utilization in C2C12 myotubes.** Myotubes were incubated in the presence or absence of 1 μM TAM for 12 h, washed, and used for the different assays. A, glucose uptake. Uptake was at room temperature in KRH with 5 mM glucose and 2-deoxy-D-[1,2-3H]glucose. B, glucose oxidation. Cells were incubated with n-[U-14C]glucose for 2 h. Glucose oxidation was evaluated from production of 14CO2. C, glucose into glycogen. Cells incubated with glucose as described in B were washed with ice-cold PBS and then lysed with 1 M KOH, and glucose incorporation into glycogen was performed as described under “Experimental Procedures.” D, expression of genes of glucose metabolism. Quantitative PCR analysis of Glut-1, Glut-4, and PDK gene expression in myotubes incubated with or without TAM for 12 h. All data are means ± S.D. (n = 9). *, p < 0.05.
was required for this effect, studies were performed with cells expressing FoxO/H215-ER, a fusion protein that is defective in DNA binding activity due to mutation of His215 within helix 3 of the DNA binding domain. No enhancement of uptake was observed after TAM treatment in FoxO/H215-expressing cells (Fig. 4C), indicating that DNA binding is likely important for the effect of FoxO1 on FA uptake.

FoxO1 Increases Membrane CD36 Content—The membrane protein CD36 (or FAT) facilitates the major fraction of FA uptake in muscle (11, 25), and its membrane recruitment is acutely regulated, allowing rapid adaptations in muscle FA uptake. For example, muscle contraction is associated with CD36 recruitment to the plasma membrane, favoring an increase in FA uptake and utilization that is important for the working muscle (26). We examined whether FoxO1 activation was associated with regulation of CD36 expression or localization. Fig. 4D shows that the CD36 mRNA level was not changed after a 12-h activation of FoxO1 by TAM, and the possibility that early and transient induction of CD36 expression could have occurred was ruled out in separate experiments where levels were measured at shorter time points (data not shown).

On the other hand, FoxO1 activation increased CD36 in the plasma membrane (Fig. 4E). CD36 protein content in membranes increased 5- and 10-fold after 3 and 6 h of treatment with TAM, respectively. There was no effect on the total cellular content of CD36 (data not shown), which was in line with the lack of change in CD36 mRNA. The effect of FoxO1 to enhance membrane CD36 is consistent with the role of the protein in facilitating FA uptake by muscle cells.

CD36 Mediates the Effects of FoxO1 on FA Oxidation—Previous work showed that the FA uptake step rate-limits oxidation of exogenous FA in muscle (27). To determine whether CD36-facilitated uptake is required for the ability of FoxO1 to enhance FA oxidation, we tested the effect of a CD36 inhibitor on FoxO1-induced activation of oxidation.

Sulfosuccinimidyl oleate (SSO) reacts covalently and specifically with CD36 (28) and is an irreversible inhibitor of FA uptake into myocytes and other cells (29). The effect of SSO treatment on FoxO1 induction of FA oxidation in C2C12 cells expressing either FoxO1 or FoxO1/TSS is shown in Fig. 5. Under all conditions, SSO inhibited the increase in FA oxidation after TAM, and inhibition was similar for FoxO1- or FoxO1/TSS-expressing cells. These data indicate that FA availability via the CD36-facilitated pathway is important for the ability of FoxO1 to up-regulate oxidation of exogenous FA.

**DISCUSSION**

Our findings indicate that activation of FoxO1 contributes to the adaptive response of muscle during the transition from feeding to fasting. Insulin induces phosphorylation of FoxO1 at several sites, which results in exclusion of FoxO1 from the nucleus and inhibition of its transcriptional activity (6, 16). In fasting, insulin signaling declines, and FoxO1 is transported to the nucleus, where it can affect the transcription of target genes. A previous report has shown that FoxO1 can stimulate
expression of lipoprotein lipase in muscle cells, which would serve to promote increased FA availability (30, 31). Here, we found that FoxO1 enhances FA metabolism through additional mechanisms, including CD36 recruitment to the plasma membrane and the enhancement of FA oxidation. The finding that enhanced oxidation of exogenous FA is prevented by the CD36 inhibitor SSO indicates that regulation of CD36-facilitated FA uptake is critical for this effect of FoxO1. This is in line with the rate-limiting role of uptake, as documented in human (30, 31) and rodent (11, 12) muscle. Our data suggest that FoxO1 regulation of membrane CD36 is important in promoting increased muscle FA uptake and utilization with fasting. Membrane CD36 content was shown to be increased by contraction and other conditions or agents (26, 32–34). The effect of phosphatidylinositol 3-kinase inhibitors (35) is the most relevant with respect to the present study because we speculate it is likely mediated by reduced phosphorylation and enhanced nuclear translocation and activity of FoxO1 (36–38).

In addition to increasing FA uptake by membrane recruitment of CD36, FoxO1 promoted an intracellular environment that favored FA oxidation by suppressing expression of ACC, the enzyme responsible for malonyl-CoA synthesis. Malonyl-CoA is an inhibitor of carnitine palmitoyl transferase-1 that mediates the transfer of long chain fatty acyl-CoAs into mitochondria, where β-oxidation can occur. This reduction in ACC may help explain the reported decrease in muscle malonyl-CoA with fasting (39–41). Regulation of malonyl-CoA levels is likely important for sustaining increased FA oxidation under conditions in which FA uptake is enhanced, and muscle ACC (ACC2 or ACCβ) has been shown by immunofluorescence microscopic analysis to be associated with the mitochondria (42). Acutely, ACC is activated allosterically by citrate and inhibited by AMP-activated protein kinase-dependent phosphorylation (43). The 50% reduction in ACC expression with FoxO1 activation provides a novel mechanism for the chronic regulation of this enzyme. Interestingly, previous data by Saha et al. (44) suggested the existence of such regulation in vivo by showing that increasing plasma insulin in rodents using a chronic (1–4 days) glucose infusion was associated with a significant and progressive enhancement of soleus ACC activity. In line with this, a 50% decrease in ACC2 gene expression was measured in muscle of formerly obese subjects who underwent weight loss after bariatric surgery. Because the decrease correlated to the drop in fasting plasma insulin, the authors speculated that reduction in insulin may inhibit ACC2 expression (45).

Other effects of FoxO1 appear to contribute to enhanced FA oxidation. Increased expression of acyl-CoA oxidase suggests that microsomal FA oxidation may be promoted. Similarly, increased PPARα expression is consistent with the reported importance of this PPAR isoform in up-regulating genes linked
to muscle oxidative metabolism (46, 47).

The mechanism by which FoxO1 activation recruits CD36 to the membrane may reflect FoxO modulation of proteasomal activity in muscle cells (10). Such a mechanism is suggested by the findings of Liang et al. (35), who showed that CD36 undergoes degradation in both lysosomal and proteasomal pathways and that decreased entry of CD36 into these pathways may be associated with increased recycling to the membrane.

Whereas FoxO1 activation had significant effects on FA uptake and utilization, its impact on glucose metabolism was limited to a modest effect on glucose incorporation into glycogen. Although FoxO1 stimulated expression of PDK4, an enzyme that acts to inhibit glucose oxidation by phosphorylating and inhibiting pyruvate dehydrogenase (48), no significant reduction in glucose oxidation could be measured. Activation of PDK4 by FoxO1 has been reported before in C2C12 cells (7), and the resulting effect on glucose oxidation was not determined. The lack of inhibition that we measured in C2C12 cells treated with TAM may reflect the modest regulation of PDK4 that we observed. It is also possible that more robust effects of FoxO on PDK4 and glucose metabolism may require the presence of glucocorticoids to stimulate the PDK4 promoter (49). Thus, it is important to note that our data highlight the specific effects of FoxO1 on glucose and FA metabolism. It remains to be determined how these effects are integrated into the in vivo adaptation of fasting muscle, which likely involves multiple mechanisms.

In conclusion, fasting, as insulin levels fall, increased activity of muscle FoxO1 promotes enhanced CD36 recruitment to the plasma membrane, thereby increasing FA uptake into the cell. In parallel, down-regulation of ACC reduces malonyl-CoA, promoting mitochondrial FA oxidation, whereas up-regulation of acyl-CoA oxidase would contribute to enhanced peroxisomal FA oxidation. These effects of FoxO1 are appropriate in the fasting state at a time when it is essential to conserve glucose but would be deleterious under conditions in which glucose is plentiful and insulin signaling is impaired. Impaired ability of insulin to suppress FoxO1 activity may contribute to the increased content of sarcolemmal CD36 observed in insulin-resistant subjects (50). Dysfunctional FoxO1 may also contribute to accumulation of intracellular triglycerides in muscle. In C2C12 cells, FoxO1 effects on FA uptake exceeded those on oxidation, resulting in net triglyceride accumulation. Insulin resistance is associated with increased intramyocellular lipid in human subjects (51), and intracellular lipid is also increased in simpler organisms (Caenorhabditis elegans) when insulin signaling is impaired (52).

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