Inhibition of Carnitine Palmitoyltransferase-1 Activity Alleviates Insulin Resistance in Diet-Induced Obese Mice

Wendy Keung, John R. Ussher, Jagdip S. Jaswal, Monique Raubenheimer, Victoria H.M. Lam, Cory S. Wagg, and Gary D. Lopaschuk

Impaired skeletal muscle fatty acid oxidation has been suggested to contribute to insulin resistance and glucose intolerance. However, increasing muscle fatty acid oxidation may cause a reciprocal decrease in glucose oxidation, which might impair insulin sensitivity and glucose tolerance. We therefore investigated what effect inhibition of mitochondrial fatty acid uptake has on whole-body glucose tolerance and insulin sensitivity in obese insulin-resistant mice. C57BL/6 mice were fed a high-fat diet (60% calories from fat) for 12 weeks to develop insulin resistance. Subsequent treatment of mice for 4 weeks with the carnitine palmitoyltransferase-1 inhibitor, oxfenicine (150 mg/kg i.p. daily), resulted in improved whole-body glucose tolerance and insulin sensitivity. Exercise capacity was increased in oxfenicine-treated mice, which was accompanied by an increased respiratory exchange ratio. In the gastrocnemius muscle, oxfenicine increased pyruvate dehydrogenase activity, membrane GLUT4 content, and insulin-stimulated Akt phosphorylation. Intramyocellular levels of lipid intermediates, including ceramide, long-chain acyl CoA, and diacylglycerol, were also decreased. Our results demonstrate that inhibition of mitochondrial fatty acid uptake improves insulin sensitivity in diet-induced obese mice. This is associated with increased carbohydrate utilization and improved insulin signaling in the skeletal muscle, suggestive of an operating Randle Cycle in muscle. ***Diabetes*** **62:711–720, 2013**

Obesity is a major problem in Western society, with 10% of the population being overweight or obese (1). It imposes health risks on individuals, including insulin resistance and type 2 diabetes, leading to an increased risk for hypertension, dyslipidemia, and cardiovascular diseases such as heart failure (2).

Insulin resistance occurs when there is an inability of the body to take up and use glucose as a source of energy upon insulin stimulation. Insulin resistance affects a number of tissues, including liver, skeletal muscle, pancreas, adipose tissue, and the heart. Skeletal muscle accounts for more than 70% of whole-body glucose utilization (3) and is therefore the most important organ system controlling blood glucose levels and overall insulin sensitivity. Thus, any therapeutic approach that can improve the responsiveness to insulin in skeletal muscle may be beneficial to whole-body insulin sensitivity and glucose tolerance.

Insulin resistance in skeletal muscle is accompanied by an imbalance between fatty acid uptake and fatty acid β-oxidation (4,5). Excess intracellular accumulation of fatty acids and their metabolites has been implicated as a key mediator of insulin resistance. These metabolites include diacylglycerol (DAG) (6), ceramide (7,8), and long-chain acyl CoA (9), all of which have been shown to be elevated in obesity and/or diabetes. Indeed, one therapeutic approach for treatment of insulin resistance is to increase fatty acid oxidation, thereby decreasing the levels of these metabolites. Furthermore, genetic and pharmacological manipulation of certain fatty acid oxidation–related genes to promote fatty acid oxidation has been shown to improve insulin sensitivity (10–12).

Although increasing fatty acid oxidation may alleviate insulin resistance via decreasing lipid metabolites, other evidence suggests that increasing fatty acid oxidation may not be beneficial for the treatment of insulin resistance in obese and diabetic individuals. First, fatty acid oxidation rates have been shown to be increased in obesity and diabetes (13,14). Second, increased fatty acid oxidation is also associated with an increase in incomplete fatty acid oxidation (15,16), which has been shown to promote insulin resistance. Furthermore, increasing fatty acid oxidation may also potentially decrease the oxidation of glucose in muscle due to the reciprocal relationship between fatty acid and glucose oxidation, termed the Randle Cycle (17). The Randle Cycle was first demonstrated in the isolated heart and in diaphragm strips. However, its operation in muscle still remains controversial (18).

Carnitine palmitoyltransferase-1 (CPT-1) is an important enzyme involved in the regulation of mitochondrial fatty acid oxidation. CPT-1 catalyzes the conversion of cytoplasmic long-chain acyl CoA to acylcarnitine, which then enters into the mitochondria for fatty acid β-oxidation. This enzyme is located on the outer mitochondrial membrane and is the rate-limiting enzyme for mitochondrial fatty acid uptake (19–21). Although genetic knockouts of the liver (22) and the muscle (23) isoforms of CPT-1 have been shown to be embryonically lethal, pharmacological inhibition of CPT-1 has been shown to effectively reduce fatty acid oxidation (16,24).

Oxfenicine (4-hydroxy-L-glycine) is an inhibitor of fatty acid oxidation that acts by inhibiting CPT-1. Transamination of oxfenicine to its metabolite, 4-hydroxyphenylglyoxylate, is required for its pharmacological actions (24). Heart mitochondrial CPT-1 is more sensitive to oxfenicine and 4-hydroxyphenylglyoxylate inhibition than the liver isoform of CPT-1 (25). Because the muscle isoform of CPT-1 is the predominant isoform in the heart and skeletal muscle (26), administration of oxfenicine in vivo would preferentially inhibit fatty acid oxidation in skeletal muscles.

From the Cardiovascular Research Centre, Mazankowski Alberta Heart Institute, Department of Pediatrics, University of Alberta, Edmonton, Alberta, Canada.

Corresponding author: Gary D. Lopaschuk, gary.lopaschuk@ualberta.ca.

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In this study, we sought to determine whether decreasing rather than increasing fatty acid oxidation in skeletal muscle may alleviate whole-body insulin resistance. We hypothesized that the pharmacological inhibition of CPT-1 would decrease fatty acid oxidation while increasing glucose oxidation via a Randle Cycle mechanism in skeletal muscle. We also investigated whether this decrease in fatty acid oxidation is accompanied by an improvement in insulin sensitivity.

RESEARCH DESIGN AND METHODS

Animal handling. All studies were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and conformed to the guidelines of the Canadian Council on Animal Care. Male C57/BL6 mice (12 weeks old; 20–25 g) were randomly assigned to a low-fat diet (LFD) of 13% calories from fat (4% fat by weight) or a high-fat diet (HFD) of 60% calories from fat (Research Diets Inc.) and fed the respective diet for 12 weeks. Mice were housed one per cage in a temperature-controlled room and maintained on a 12/12-h light–dark cycle. Mice had ad libitum access to food and water. Body weights and food intake were measured at the same time each day.

At the end of 12 weeks, animals were injected daily with the CPT-1 inhibitor, oxfenicine (150 mg/kg i.p.) suspended in 1× PBS, or vehicle control for 4 weeks. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed after 2 weeks of oxfenicine treatment. Mice were fasted for 16 h and given an intraperitoneal glucose injection at a dose of 2 g/kg body weight. Blood glucose was measured with Accu Check Advantage system (Roche) at 15-min intervals for 90 min. Forty eight hours after the GTT, mice were fasted for 16 h and given an intraperitoneal insulin injection at a dose of 0.3 units/kg body weight. Blood glucose was measured with Accu Check Advantage system at 15-min intervals for 90 min.

Whole-body in vivo metabolic assessment. In vivo metabolic assessment via indirect calorimetry was performed using the Oxymax CLAMS (Columbus Instruments). Animals were initially acclimatized in the system for 24 h, and the subsequent 24-h period was used for data collection.

Exercise capacity testing. Exercise capacity was performed by running animals on a calibrated, motor-driven treadmill (Columbus Instruments) at a speed of 3 m/min for 1 min, followed by increasing speeds of 4 m/min for 1 min, 5 m/min for 1 min, 6 m/min for 3 min, 8 m/min for 14 min, 9 m/min for 10 min, 10 m/min for 7 min, 12 m/min for 7 min, and 14 m/min until exhaustion. The first 6 min were used as an acclimatization period for the animals. Exhaustion was determined as the animal spending >10 consecutive seconds on the shock grid.

Tissue handling. At the end of the 4-week treatment protocol, animals were killed via an intraperitoneal injection of sodium pentobarbital (12 mg) in the fed state in the middle of the dark cycle. Tissues were excised and immediately frozen in liquid nitrogen.

Determination of intramyocellular lipid intermediates. Short-chain CoA esters were determined in 6% perchloric acid extracts from frozen tissues, as described previously (27). Long-chain acyl CoA esters were analyzed with high-performance liquid chromatography, as described previously (28). Triacylglycerol (TAG) was extracted from tissue according to the method of Folch (29). Tissue diacylglycerol (DAG) and ceramides were determined using an assay previously described (7,30).

Determination of mitochondrial enzyme activities. Citrate synthase activity was measured in tissue homogenates by monitoring the rate of reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) at 412 nm. Activity of β-hydroxyacyl CoA dehydrogenate (β-HAD) was measured in tissue homogenates by monitoring the rate of disappearance of NADH. Pyruvate dehydrogenase (PDH) complex activity was determined using a modification of the radioisotope-coupled enzyme assay described previously (31,32).

Membrane fractionation and immunoblot analysis. Frozen powdered tissue was homogenized using a Polytron homogenizer for 30 s at 4°C in homogenization buffer containing 50 mmol/L Tris HCl (pH 8), 1 mmol/L EDTA, 10% (w/v) glycerol, 0.02% Brij-35, phosphatase inhibitors I and II (1:100), protease inhibitor (1:1000), and 1 mmol/L dithiothreitol. After centrifugation at 10,000*g* for 20 min at 4°C, protein content was measured using the Bradford protein assay.

In experiments where detection of membrane contents of proteins was required, powdered tissues were homogenized and subfractionated by differential centrifugation using a commercially available protein extraction kit (Calbiochem). To determine the expression of enzyme proteins, homogenates underwent SDS-PAGE. After gel electrophoresis, the fractionated proteins were transferred to a nitrocellulose membrane using a wet transfer method. The transfer buffer contained 25 mmol/L Tris, 192 mmol/L glycine, and 20% (v/v) methanol. Membranes were blocked with 10% (w/v) skim milk powder in Tris-buffered saline with 0.05% Tween 20 for 1 h at room temperature.

For immunoblotting, membranes were incubated with an appropriate amount of monoclonal or polyclonal antibodies against the protein of interest at 4°C overnight. Membranes were washed three times with Tris-buffered saline with 0.05% Tween 20, then probed with a horseradish peroxidase-conjugated secondary antibody. Membranes were then washed with Tris-buffered saline with 0.05% Tween 20. Target proteins were visualized using an ECL Western blotting kit (PerkinElmer Inc, Waltham, MA).

Statistical analysis. All data are presented as the mean ± SEM. Student *t* test was used to evaluate the statistical significance of differences among the HFD mice administered vehicle (HF+vehicle) and those that received oxfenicine (HF+oxfenicine). One-way ANOVA with a Bonferroni post hoc test was used to evaluate the statistical significance of differences among multiple groups. Values of $P < 0.05$ were considered significant.

RESULTS

Inhibition of CPT-1 improves glucose tolerance and insulin sensitivity. As expected, feeding C57/BL6 mice an HFD (60% kCal from fat) for 12 weeks resulted in a significant increase in body weight (Supplementary Fig. 1*A*) and an impairment in glucose tolerance (Supplementary Fig. 1*B*) compared with mice fed an LFD. Mice from the HF+vehicle and the HF+oxfenicine groups lost a small but significant amount of weight during the experimental protocol due to handling (as analyzed by a paired *t* test). However, treatment of the HFD mice with oxfenicine had no significant effect on body weight ($P = 0.13$ between the HF+vehicle and HF+oxfenicine group by unpaired *t* test; Fig. 1*A*). The GTT administered after treatment of HFD-fed mice with oxfenicine (150 mg/kg/day) for 2 weeks showed an improved glucose clearance in the treated mice to a level that was similar to the LFD control group (Fig. 1*B* and *C*). Moreover, the ITT results showed a small but significant increase in insulin sensitivity in the oxfenicine-treated mice (data not shown). This was also corroborated with the finding that plasma insulin was decreased in oxfenicine-treated mice compared with vehicle control-treated HFD-fed mice (Fig. 1*D*).

Inhibition of CPT-1 increases whole-body carbohydrate utilization while decreasing fatty acid utilization. Indirect calorimetry assessment revealed an increase in the respiratory exchange ratio (RER) in the oxfenicine-treated mice during the dark cycle, indicating an increased utilization of carbohydrates for energy metabolism (Fig. 2*A*). Food intake, ambulatory activity, and heat production were not altered in oxfenicine-treated mice (data not shown). However, there was a small but significant decrease in oxygen consumption (Fig. 2*C*), which is consistent with the oxygen-sparing effect of increased carbohydrate use. Plasma glucose (Fig. 2*D*) was also lower in oxfenicine-treated mice, although it only reached significance under fasting conditions. Plasma levels of free fatty acids and TAG were higher (Fig. 2*E* and *F*) in mice treated with oxfenicine, which was consistent with a decrease in whole-body fatty acid oxidation. Consistent with the increase in plasma free fatty acid and TAG levels, CPT-1 activity in the gastrocnemius muscles was also decreased in oxfenicine-treated mice (Fig. 2*G*). Liver TAG levels were not significantly different in the oxfenicine-treated groups, indicating that oxfenicine does not exert a significant effect on the liver isoform of CPT-1.

Exercise capacity is decreased in obese and insulin-resistant mice. Interestingly, together with the improvement in glucose tolerance, oxfenicine treatment of mice also resulted in an increased exercise capacity (Fig. 3*A* and *B*).

Inhibition of CPT-1 improves muscle glucose metabolism and insulin sensitivity. At the end of the 4-week oxfenicine treatment period, mice were killed and skeletal muscle

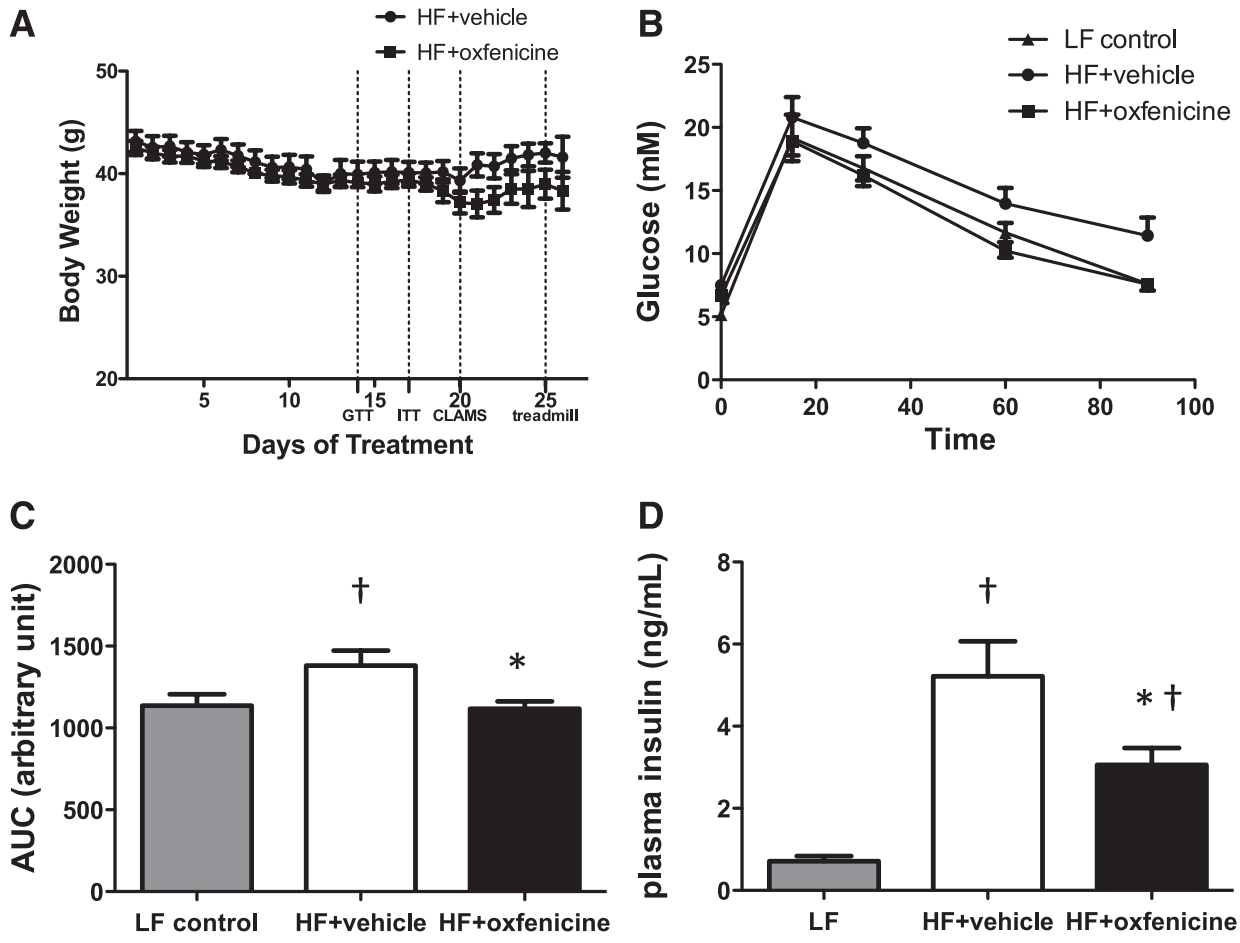

FIG. 1. Inhibition of CPT-1 by oxfenicine in HFD-fed (HF) mice improves glucose tolerance and reverses insulin resistance. *A*: Body weight changes during drug treatment in HF mice treated with vehicle or oxfenicine (150 mg/kg/day). *B*: GTT in HF mice treated with vehicle or oxfenicine (150 mg/kg/day). *C*: Area under the curve (AUC) of GTT in LFD-fed (LF) mice and HF mice treated with vehicle or oxfenicine. *D*: Plasma insulin levels in LF mice and HF mice treated with vehicle or oxfenicine. Values represent mean ± SEM, n = 6–9. GTT and ITT curves were significantly different between vehicle and oxfenicine groups using two-way ANOVA. Differences were determined using ANOVA, followed by the post hoc Bonferroni test. *$P < 0.05$ significantly different from HF vehicle control. †$P < 0.05$ significantly different from LF control.

metabolism was analyzed. Treatment with oxfenicine for 4 weeks resulted in a decrease in CPT-1 activity in the gastrocnemius muscle of HFD mice (Fig. 2*G*), despite a marked decrease in malonyl CoA levels (Supplementary Fig. 2). Interestingly, together with the decrease in CPT-1 activity in the gastrocnemius muscle, there was a concomitant increase in active PDH activity (Fig. 4*A*), with no change in total activity (Fig. 4*B*). This resulted in an increase in the percentage active PDH complex (Fig. 4*C*). Phosphorylation of Akt was also increased when normalized to total Akt content and also when normalized to plasma insulin levels (Fig. 4*D*), confirming the improvement in insulin sensitivity. Downstream of Akt activation, insulin-stimulated plasma membrane GLUT4 content was increased with oxfenicine treatment (Fig. 4*E*), suggesting an increase in muscle insulin sensitivity.

Inhibition of CPT-1 did not decrease fatty acid β-oxidation capacity but decreased fatty acid transport in skeletal muscle. Although there was a clear decrease in CPT-1 activity in skeletal muscle (Supplementary Fig. 2*A*) as well as an increase in RER (Fig. 2*B*), indicative of decreased fatty acid utilization, we did not observe a decrease in the expression of the fatty acid β-oxidation enzymes, 3-ketoacyl CoA thiolase (3-KAT; Fig. 5*A*) or β-HAD (Fig. 5*B*). Interestingly, there was an increase in the tricarboxylic acid cycle (TCA) enzyme, citrate synthase (CS) (Fig. 5*C*), resulting in a decrease in the ratio of β-HAD to CS activity (Fig. 5*D*). There was also an increase in peroxisome proliferator–activated receptor-γ coactivator-1α (PGC-1α) expression in the oxfenicine-treated HFD mice (Supplementary Fig. 3). With regards to muscle fatty acid transport proteins, oxfenicine treatment resulted in a decreased expression of CD36, which is the major fatty acid transport protein in muscle (Fig. 5*E*). Moreover, the level of CD36 in the plasma membrane, which is indicative of fatty acid transport rate (33), was also lower in the oxfenicine-treated mice (Fig. 5*F*).

Inhibition of CPT-1 decreases fatty acid intermediates in skeletal muscle. We also measured the levels of different fatty acid intermediates in gastrocnemius muscle. Long-chain acyl CoA (Fig. 6*A*), ceramide (Fig. 6*B*), and DAG (Fig. 6*C*) levels were all decreased in the gastrocnemius muscle of oxfenicine-treated mice. However, intramyocellular TAG levels were comparable to nontreated mice (Fig. 6*D*), indicating there was no accumulation of fatty acids despite a decrease in other fatty acid intermediates.

DISCUSSION

It has been postulated that insulin resistance can be alleviated by increasing fatty acid oxidation in skeletal muscle

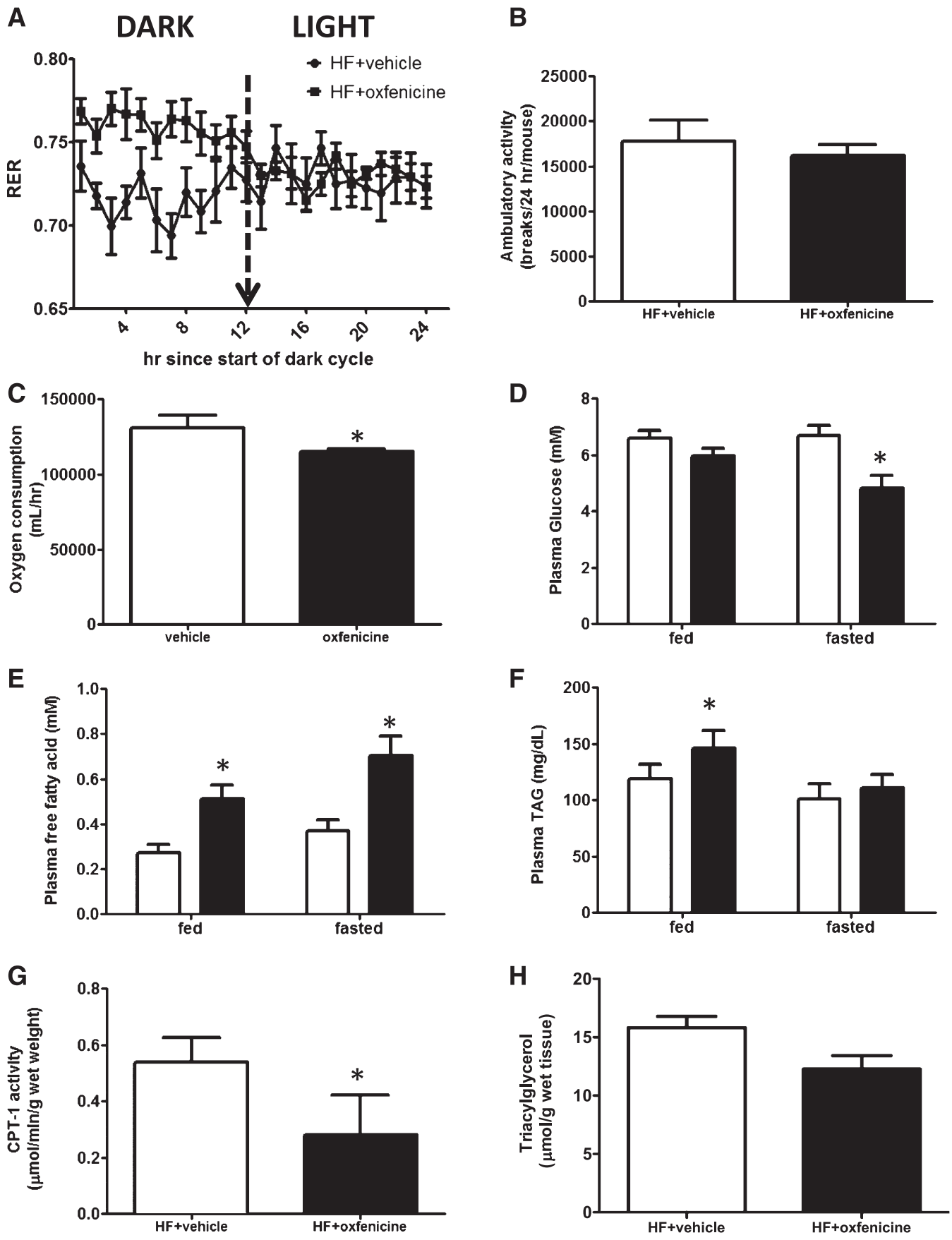

FIG. 2. Energy substrate utilization in HFD-fed (HF) mice is shifted toward carbohydrate oxidation after oxfenicine-treatment. hr, hours. *A*: Dark cycle RER curves during a 24-hour period were significantly different between HF mice treated with vehicle and oxfenicine (two-way ANOVA). Twenty-four-hour average ambulatory activity (*B*) and average oxygen consumption (*C*) are shown in HF mice treated with vehicle or oxfenicine.

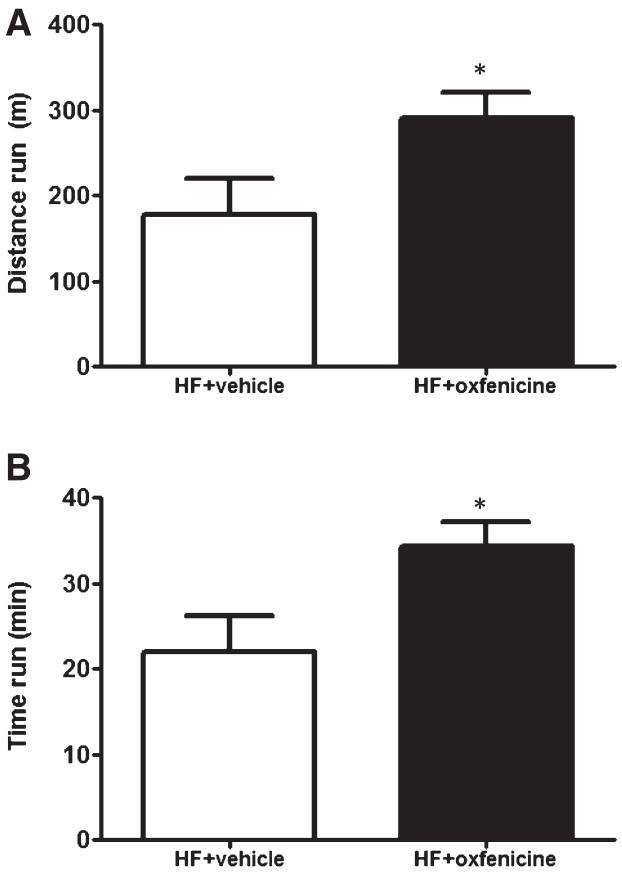

FIG. 3. Oxfenicine treatment reverses the impairment of exercise capacity in HFD-fed (HF) mice. Distance (*A*) and time (*B*) run during exercise challenge on treadmill. Values represent mean ± SEM, $n = 6–9$. Differences were determined using two-tailed Student t test. *$P < 0.05$ significantly different from control.

(10,11,34). In contrast, we demonstrate that lowering fatty acid oxidation via pharmacological inhibition of CPT-1 enhances not only glucose metabolism but also insulin sensitivity in mice with diet-induced obesity (DIO) and insulin resistance. Furthermore, inhibition of fatty acid oxidation did not cause an accumulation of fatty acid intermediates.

In the 1960s, Randle et al. (17) proposed a reciprocal relationship between glucose and fatty acid oxidation. It was demonstrated primarily in the heart that inhibition of fatty acid oxidation could increase glucose metabolism upon insulin stimulation (17). However, recent studies have suggested that this reciprocal relationship does not hold true in skeletal muscle because changes in glucose uptake can be dissociated from increases in fatty acid oxidation (18,35). Here, we report that inhibition of mitochondrial fatty acid uptake can improve glucose oxidation at the level of PDH, as well as GLUT4 translocation, supporting the notion of an operating Randle Cycle in skeletal muscle. Not only is glucose metabolism increased, as evident from the increase in the RER in oxfenicine-treated mice, insulin signaling is also improved, as seen from the decreased plasma insulin levels, increased whole-body insulin sensitivity, and the increased insulin-stimulated Akt phosphorylation in skeletal muscle. Furthermore, muscle glycogen levels (Supplementary Fig. 5*A*) and phosphorylation-induced inhibition of glycogen synthase kinase (GSK), which inhibits glycogen synthase, are also increased in oxfenicine-treated mice (Supplementary Fig. 5*B*). Because skeletal muscle accounts for at least 70% of total insulin-stimulated glucose disposal (3), an improvement of insulin signaling in skeletal muscle itself would translate into an improvement in whole-body glucose tolerance and insulin sensitivity. Importantly, we observed no difference in body weight, food intake, heat production, or ambulatory activity between oxfenicine-treated obese mice and their control counterparts, suggesting that there is no change in energy intake or expenditure and that the improvement in glucose tolerance is due to the switch in energy substrate preference.

Exercise capacity has been shown to be decreased in DIO mice (7). We show that there is a significant improvement in treadmill exercise capacity in oxfenicine-treated mice. This is associated with an increase in the RER value as well as a decrease in oxygen consumption. Previous studies have shown that inhibition of CPT-1 with oxfenicine may induce cardiac toxicity in the form of hypertrophy (36,37). This has been suggested to be due to its inhibition of fatty acid oxidation, which limits cardiac energy reserve and ultimately translates into decreased exercise capacity (37). In our study, however, we did not observe signs of increased heart weight (Supplementary Table 1) nor was there any change in cardiac function when the hearts were perfused in vitro in the working mode (data not shown). Thus, it is unlikely that oxfenicine-treatment caused cardiac hypertrophy in our DIO mouse model. This is consistent with the findings of Okere et al. (38), where rats fed an HFD and treated with oxfenicine exhibited no signs of cardiac hypertrophy. Furthermore, mice deficient for malonyl CoA decarboxylase have an elevation in malonyl CoA content and subsequent inhibition of CPT-1 and fatty acid oxidation rates but do not exhibit any signs of cardiac hypertrophy after DIO (39). We hypothesize that because carbohydrates are more oxygen-sparing substrates for metabolism with a higher phosphate-to-oxygen ratio relative to fatty acids (40), an increase in the RER and a decrease in oxygen consumption may translate to an increase in metabolic efficiency.

Increasing fatty acid oxidation has been demonstrated to alleviate insulin resistance in a number of studies. In particular, increasing CPT-1 activity by its overexpression in the extensor digitorum longus muscles of rats has been demonstrated to improve insulin stimulated glucose uptake (11). At first glance, the findings from these two studies seem to be contradictory. However, the increase in CPT-1 activity in the above-mentioned study is limited to white muscles, including the extensor digitorum longus and the tibialis anterior, which are predominantly glycolytic and have relatively low rates of fatty acid oxidation. It is also unknown whether increasing CPT-1 activity further in red muscle, where there is already a higher rate of fatty acid oxidation and higher abundance of fatty acid transporters, may improve or worsen insulin sensitivity. Furthermore,

Plasma glucose (*D*), plasma free fatty acid (*E*), and plasma TAG (*F*) are shown under fed and fasting conditions in HF mice treated with vehicle or oxfenicine. *G*: CPT-1 activity in gastrocnemius muscles of HF mice treated with vehicle or oxfenicine. *H*: TAG in livers of HF mice treated with vehicle or oxfenicine. Values represent mean ± SEM, $n = 6–9$. Differences were determined using two-tailed Student t test. *$P < 0.05$ significantly different from control.

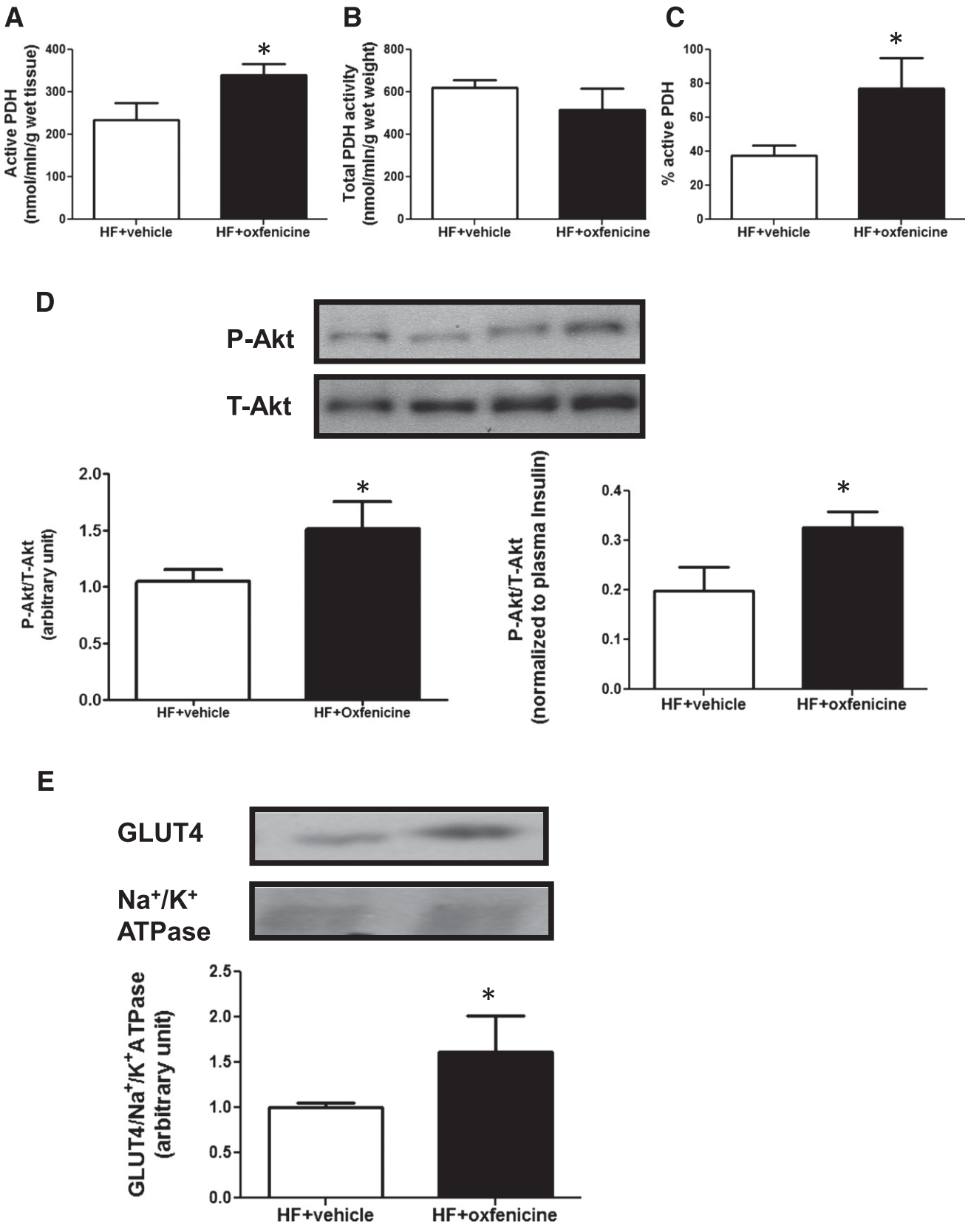

FIG. 4. Oxfenicine treatment improves glucose metabolism and insulin signaling in gastrocnemius muscles of HFD-fed (HF) mice. Active (*A*), total (*B*), and percentage (%) active activity (*C*) of PDH complex in gastrocnemius muscles of HF mice treated with vehicle or oxfenicine. Phosphorylation status (Ser 435) of Akt (*D*) and levels of phosphorylated Akt normalized to plasma insulin in gastrocnemius muscles of HF mice treated with vehicle or oxfenicine. *E*: Membrane content of GLUT4 in gastrocnemius muscles of HFD mice treated with vehicle or oxfenicine. Values represent mean ± SEM, n = 6–9. Differences were determined using a two-tailed Student t test. *$P < 0.05$ significantly different from control.

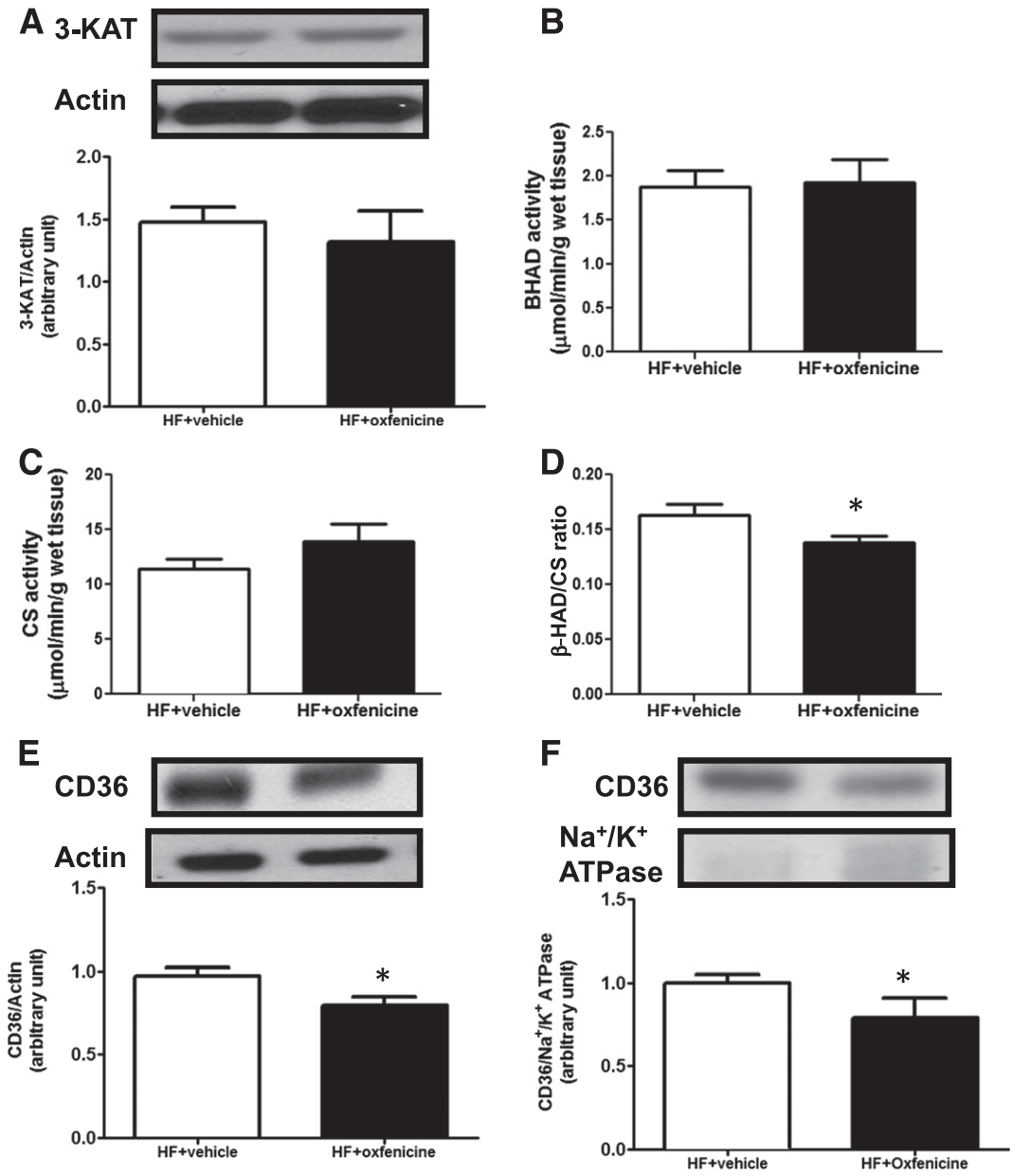

FIG. 5. Oxfenicine treatment did not decrease fatty acid oxidation capacity in gastrocnemius muscles of HFD-fed (HF) mice. *A*: Representative blot and quantification of 3-KAT expression in gastrocnemius muscle of HF mice treated with vehicle or oxfenicine. β-HAD (BHAD) activity (*B*), CS activity (*C*), and ratio of β-HAD to CS activity (*D*) in HF mice treated with vehicle or oxfenicine. Total CD36 expression (*E*) and membrane expression of CD36 (*F*) in gastrocnemius muscles of HF mice treated with vehicle or oxfenicine. Values represent mean ± SEM, n = 6–9. Differences were determined using a two-tailed Student t test. *$P < 0.05$ significantly different from control.

increased insulin-stimulated glucose uptake was only demonstrated in the muscles of interest in that particular study. Indeed, a recent study by Hoehn et al. (41) showed that a chronic increase in fatty acid oxidation by the deletion of acetyl CoA carboxylase was ineffective in improving insulin resistance, nor could it increase energy expenditure or promote leanness. These authors also concluded that increasing the metabolism of one fuel would only lead to a compensatory downregulation in the other with no net change in energy balance, as initially suggested by Randle et al. (17). Increasing fatty acid oxidation in skeletal muscle by intracerebroventricular injection also did not improve whole-body insulin sensitivity (42). In the current study, inhibition of CPT-1 activity in muscle is associated with increased whole-body glucose disposal and whole-body insulin sensitivity. This is also concomitant with an increase in insulin stimulated GLUT4 translocation.

Inhibition of CPT-1 by specific CPT-1 inhibitors has been shown to improve glucose tolerance in studies in diabetic patients (43). This effect has been suggested to be the result of inhibition of hepatic glucose production in response to decreased fatty acid oxidation in the liver (44,45). Indeed, etomoxir and other CPT-1 inhibitors have been shown to be more specific for the liver isoform of CPT-1, decreasing liver fatty acid oxidation, leading to an increase in liver TAG (46), a decrease in liver energy production, and increasing food intake (47). In this study, we used oxfenicine to inhibit CPT-1, which has been shown to be more specific to the muscle type CPT-1 rather than the liver isoform of CPT-1 (24). We did not observe an

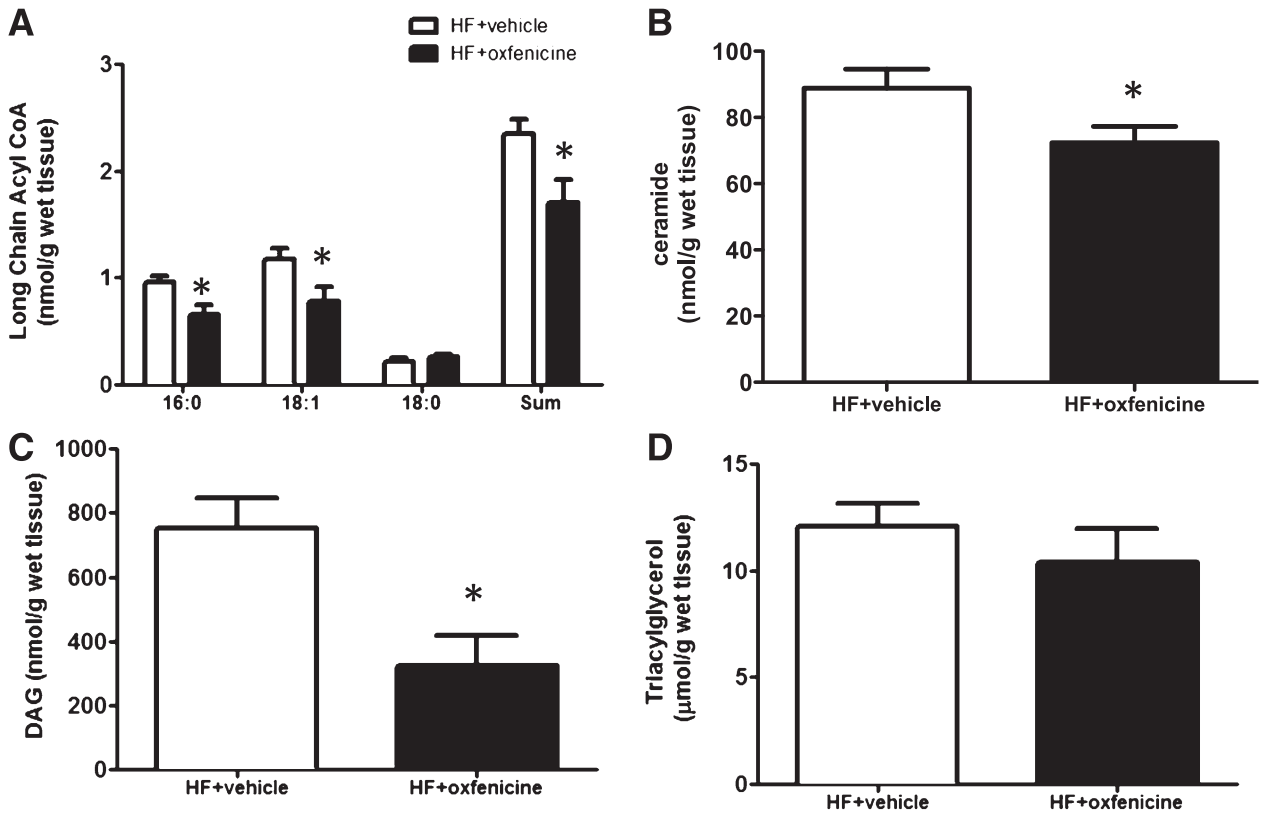

FIG. 6. Oxfenicine treatment reduces intramyocellular levels of lipid metabolites in gastrocnemius muscles of HFD-fed (HF) mice. Levels of long-chain acyl CoA (*A*), ceramide (*B*), DAG (*C*), and TAG (*D*) in gastrocnemius muscles of HF mice treated with vehicle or oxfenicine. Values represent mean ± SEM, n = 6–9. Differences were determined using a two-tailed Student t test. *$P < 0.05$ significantly different from control.

increase in food intake as reported by others using etomoxir. This is also consistent with the finding that oxfenicine treatment improves glucose tolerance with no change in food intake in muscle-specific peroxisome proliferator–activated receptor-α–expressing mice (48). This, together with the lack of change in liver weight (Supplementary Table 1) and hepatic TAG content (Fig. 2*H*) in oxfenicine-treated mice, suggests that oxfenicine exerts its effect on glucose tolerance and insulin sensitivity predominantly via action on the skeletal muscle rather than the liver.

Insulin resistance has been shown to be correlated with mitochondrial dysfunction and a decrease in the rates of fatty acid oxidation, leading to a decrease in the ability of the muscle to convert reducing equivalents of flavin adenine dinucleotide and NADH toward ATP production. As with a previous study using the same DIO protocol, we did not observe mitochondrial dysfunction or a decrease in fatty acid oxidation capacity (7). Treatment with oxfenicine does not alter the expression and activity of fatty acid oxidation proteins including 3-KAT and β-HAD, which are already increased upon high-fat feeding. However, there is an increase in the capacity of CS activity in the oxfenicine-treated mice, which catalyzes the first committed step of the TCA cycle. The reduced ratio of β-HAD to CS activity in the oxfenicine-treated group is suggestive of improved coupling between fatty acid oxidation and the TCA cycle. Koves and colleagues (16) demonstrated that the coupling of TCA cycle with fatty acid oxidation depends on PGC-1α–dependent muscle gene programming. We did observe a significant increase in PGC-1α expression in the oxfenicine-treated mice (Supplementary Fig. 3*A*). However, whether transcriptional activity of PGC-1α is changed is unknown, because it has also been suggested that the activity of PGC-1α is dependent not only on its abundance but also on its posttranslational modification, including phosphorylation (49) and acetylation (50), which we have yet to assess.

Our finding that fatty acid intermediates in skeletal muscle are decreased in the oxfenicine-treated mice is somewhat surprising. Indeed, the rationale for therapeutic approaches to alleviate insulin resistance by increasing fatty acid oxidation in skeletal muscle advocated by a number of research groups is based on the observed increase in fatty acid intermediates such as long-chain acyl CoA (51), ceramide (7,8) and DAG (6) in the skeletal muscle of insulin-resistant animal models, which have all been implicated as key mediators of insulin resistance. A number of studies aiming at increasing fatty acid oxidation have demonstrated success at improving glucose tolerance (10–12). In contrast, treatment of high-fat fed rats with etomoxir, another CPT-1 inhibitor, results in an increase in intramyocellular lipid intermediates as opposed to the decrease we see in the current study (52). However, the increase in intramyocellular lipid metabolites is also accompanied by a worsening of insulin resistance in HFD-fed rats. The decrease in fatty acid intermediates in the current study may be attributed to the compensatory decrease in long-chain fatty acid uptake at the plasma membrane due to negative feedback (53,54), which has previously been shown to be absent in the hearts of etomoxir-treated rats (53). However, it should be noted that the decrease in fatty acid transport into the cell is consistent with the observation that CPT-1 is the major flux control enzyme for long-chain acyl CoA in tissues with high rates of fatty acid

oxidation, including the liver and skeletal muscle (53,55). Indeed, we have observed a decrease in the expression and membrane translocation of CD36, the major plasma membrane fatty acid transporters in the muscle cells. The decrease in fatty acid uptake into the muscle cells is also evident from the increased plasma fatty acid levels in oxfenicine-treated mice.

It has been demonstrated that fatty acid intermediates such as DAG may activate proinflammatory proteins, including Jun NH_2-terminal kinase (JNK) and IκB kinase (IKK), to phosphorylate and inactivate insulin receptor substrate-1 proteins (6,56), leading to insulin resistance. However, we observed no change in the phosphorylation of JNK and IKK in oxfenicine-treated DIO mice (Supplementary Fig. 3*B*), suggesting that the increase in insulin sensitivity in these mice is not due to the decrease in the levels of these metabolites or the activation of proinflammatory proteins.

In conclusion, we herein report that reducing fatty acid oxidation by pharmacological inhibition of CPT-1 with oxfenicine improves whole-body glucose tolerance and insulin sensitivity in DIO mice. Insulin signaling in skeletal muscle is enhanced, as evident from the increased levels of active PDH and membrane GLUT4 expression. The improved glucose tolerance is also accompanied by a decrease in lipid metabolites in the DIO mice. These findings suggest that therapeutic strategies aiming at reducing excessive fatty acid oxidation in muscle may improve insulin resistance.

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W.K. designed the research, researched data, and wrote the manuscript. J.R.U. researched data, contributed to discussion, and reviewed and edited the manuscript. J.S.J. researched data and contributed to discussion. M.R., V.H.M.L., and C.S.W. researched data. G.D.L. designed the research and wrote the manuscript. G.D.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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