Alterations in Skeletal Muscle Fatty Acid Handling Predisposes Middle-Aged Mice to Diet-Induced Insulin Resistance

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OBJECTIVE—Although advanced age is a risk factor for type 2 diabetes, a clear understanding of the changes that occur during middle age that contribute to the development of skeletal muscle insulin resistance is currently lacking. Therefore, we sought to investigate how middle age impacts skeletal muscle fatty acid handling and to determine how this contributes to the development of diet-induced insulin resistance.

RESEARCH DESIGN AND METHODS—Whole-body and skeletal muscle insulin resistance were studied in young and middle-aged wild-type and CD36 knockout (KO) mice fed either a standard or a high-fat diet for 12 weeks. Molecular signaling pathways, intramuscular triglycerides accumulation, and targeted metabolomics of in vivo mitochondrial substrate flux were also analyzed in the skeletal muscle of mice of all ages.

RESULTS—Middle-aged mice fed a standard diet demonstrated an increase in intramuscular triglycerides without a concomitant increase in insulin resistance. However, middle-aged mice fed a high-fat diet were more susceptible to the development of insulin resistance—a condition that could be prevented by limiting skeletal muscle fatty acid transport and excessive lipid accumulation in middle-aged CD36 KO mice.

CONCLUSION—Our data provide insight into the mechanisms by which aging becomes a risk factor for the development of insulin resistance. Our data also demonstrate that limiting skeletal muscle fatty acid transport is an effective approach for delaying the development of age-associated insulin resistance and metabolic disease during exposure to a high-fat diet. ***Diabetes*** **59:1366–1375, 2010**

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Over the past few decades, type 2 diabetes has increased in prevalence largely as a result of the obesity epidemic (1). Although it is widely accepted that skeletal muscle insulin resistance is a major determinant in both the onset and progression of type 2 diabetes (2), the exact cause of decreased insulin action in skeletal muscle is not known (3). That said, it is generally believed that skeletal muscle insulin resistance develops secondary to impaired mitochondrial fatty acid oxidation (4,5). However, several other studies have shown that lipid accumulation is not associated with skeletal muscle insulin resistance (6–8) or overall mitochondrial dysfunction (9–13). Consistent with this, a growing body of evidence has suggested that the cause of skeletal muscle insulin resistance may not result from impaired fatty acid oxidation but might actually result from excessive skeletal muscle mitochondrial fatty acid oxidation and ensuing mitochondrial stress (12,14). While it is not known which of these two processes are most relevant in the pathogenesis of skeletal muscle insulin resistance, it is clear that excessive entry of fatty acids into the skeletal muscle plays a central role in diet-induced insulin resistance.

Because advanced age is a significant risk factor in the etiology of type 2 diabetes (15,16), the accompanying loss of mitochondrial function observed with normal aging has been proposed to contribute to the increased risk of type 2 diabetes in the elderly population (17). However, a clear understanding of the physiological changes that occur during the onset of middle age and the influence that this may have on the development of insulin resistance is currently lacking. This is particularly important given that the baby boomer generation, the largest population group in the Western world, is currently classified as middle-aged (18) as well as the fact that the prevalence of type 2 diabetes in the Western world is expected to increase dramatically over the next 5–10 years (16,18). The study herein was designed to investigate how middle age impacts whole-body glucose utilization, fatty acid handling, and triglyceride accumulation within skeletal muscle as well as the susceptibility of middle-aged mice to the development of diet-induced insulin resistance.

RESEARCH DESIGN AND METHODS

Reagents. Antibodies against AMP kinase (AMPK), phosporylated AMPK (pAMPK), acetyl CoA carboxylase (ACC), phosporylated ACC, Akt, phosporylated Akt, and tubulin were from Cell Signaling; anti-Oxphos was from MitoSciences; anti–CD36-[HRP] was purchased from NOVUS Biologicals, and human recombinant insulin (Novolin) from Novo Nordisk Canada.

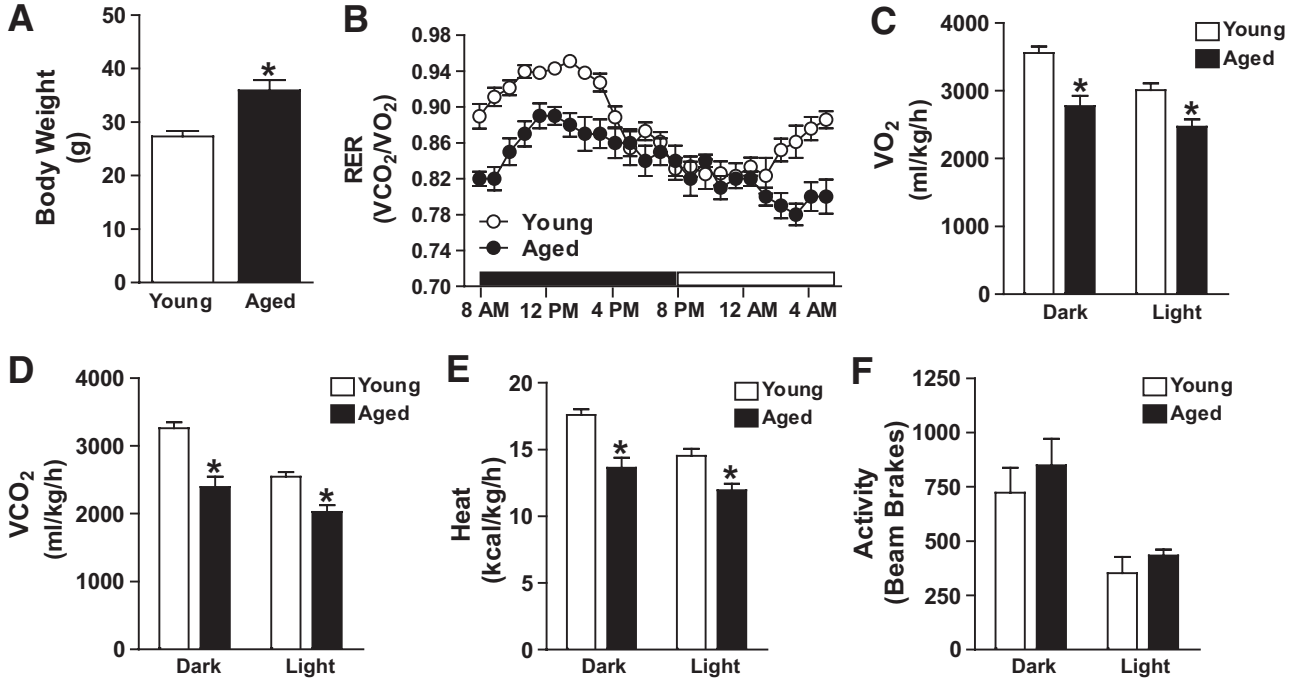

FIG. 1. Depressed metabolic rate in middle-aged mice fed a standard laboratory diet. Body weight of young (12–14 weeks of age) and middle-aged (52–58 weeks of age) mice fed a standard laboratory diet (*A*). Indirect calorimetry was performed to measure respiratory exchange ratio (RER) (*B*), Vo_2 (*C*), VCo_2 (*D*), and heat production adjusted for bodyweight (*E*); total activity (*F*) was measured in both the dark (active) and light (inactive) phases. Values are means ± SEM of n = 5–7 mice in each group. *$P < 0.05$ indicates comparisons performed between young and middle-aged mice in either dark or light phases (Mann-Whitney *U* Test). Two-way ANOVA was performed for RER (*B*) and indicated the main effect for age ($P < 0.01$).

Mice. This study was performed with the approval of the University of Alberta Animal Policy and Welfare Committee. Experiments were carried out on male wild-type (C57BL6) and CD36 knockout (KO) mice (19) maintained in a temperature-controlled room with a reversed 12-h light/12-h dark cycle. Mice were left relatively undisturbed for either 12–14 or 52–58 weeks of age with free access to water and standard rodent diet (category no. 5001; LabDiet). At 32–34 weeks of age, a subset of mice was randomly divided into a low-fat diet group (category no. D12450B; Research Diets) and a high-fat diet group (category no. D12492; Research Diets) for a period of 12 weeks.

Metabolic analysis in vivo. Indirect calorimetry was performed using the Comprehensive Lab Animal Monitoring System (Oxymax/CLAMS; Columbus Instruments, Colombus, OH). Following an initial 24-h acclimatization period, mice were monitored every 13 min for 24 h to complete a 12-h dark (active)/12-h light (inactive) cycle. The respiratory exchange ratio (RER = VCo_2/Vo_2) was used to estimate the percent contribution of fat and carbohydrate to whole-body energy metabolism in mice in vivo. Total activity was calculated by adding *Z* counts (rearing or jumping) to total counts associated with ambulatory movement and stereotypical behavior (grooming and scratching).

Determination of skeletal muscle triglycerides, long-chain acyl-CoA, and ceramides. Upon phospholipid digestion with phospholipase C (2 h at 30°C) and lipid extraction, levels of triglycerides were determined in gastrocnemius muscle lysates by gas-liquid chromatography as previously described (20). Identification and quantification of the major long-chain acyl-CoA molecular species (C16:0, C18:0, and C18:1) and C18-ceramides were performed by high-performance liquid chromatography as previously described (21).

Metabolic profiling. Overnight-fasted mice were anesthetized with sodium pentobarbital, and gastrocnemius muscle was rapidly removed and freeze-clamped in liquid nitrogen and stored at −80°C. Sample preparation was performed as previously described (14). Acylcarnitine measurements were made using flow-injection tandem mass spectrometry as previously described (14), and organic acids were quantified as previously described (22).

Statistical analysis. Data are expressed as means ± SEM. Comparisons between groups were performed using unpaired Student's two-tailed t test or ANOVA with a Bonferroni post hoc test of pairwise comparisons where appropriate. A probability value of <0.05 is considered significant. For further descriptions of the materials and methods, refer to the supplementary material available in an online appendix, available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1142/DC1.

RESULTS

Age-induced slowing of metabolic rate increases susceptibility to metabolic disease. To determine whether an age-related decline in resting metabolic rate and energy expenditure might contribute to the development of insulin resistance, C57Bl6 mice of 12–14 (young) or 52–58 (middle-aged) weeks of age were analyzed using indirect calorimetry. As mice aged and body weight was increased (Fig. 1*A*), substrate use was altered slightly, with reductions in RER indicating that middle-aged mice used more fatty acid throughout the day compared with young mice (Fig. 1*B*). In addition, significant reductions in oxygen consumption (Vo_2) (Fig. 1*C*) and carbon dioxide production (VCo_2) (Fig. 1*D*) were observed during both the dark (active) and light (inactive) phases in middle-aged mice compared with young mice. Consistent with this, heat production (Fig. 1*E*) was decreased in middle-aged mice compared with young mice, whereas activity measurements were not significantly different between age-groups (Fig. 1*F*). Taken together, our data indicate that middle-aged mice have a lower metabolic rate than young mice and that this might increase susceptibility to weight gain, obesity, and metabolic disease.

Age-induced alterations in fatty acid handling and lipid accumulation in skeletal muscle precede the development of whole-body glucose intolerance. Given that skeletal muscle metabolism contributes to whole-body basal metabolic rate, we addressed whether skeletal muscle metabolism was depressed in middle-aged mice by assessing the activities of two enzymes involved in regulating mitochondrial metabolism. Interestingly, the activity of β-hydroxyacyl–CoA dehydrogenase (β-HAD) (Fig. 2*A*) was elevated in the middle-aged compared with the young mice, and citrate synthase (Fig. 2*B*) followed a

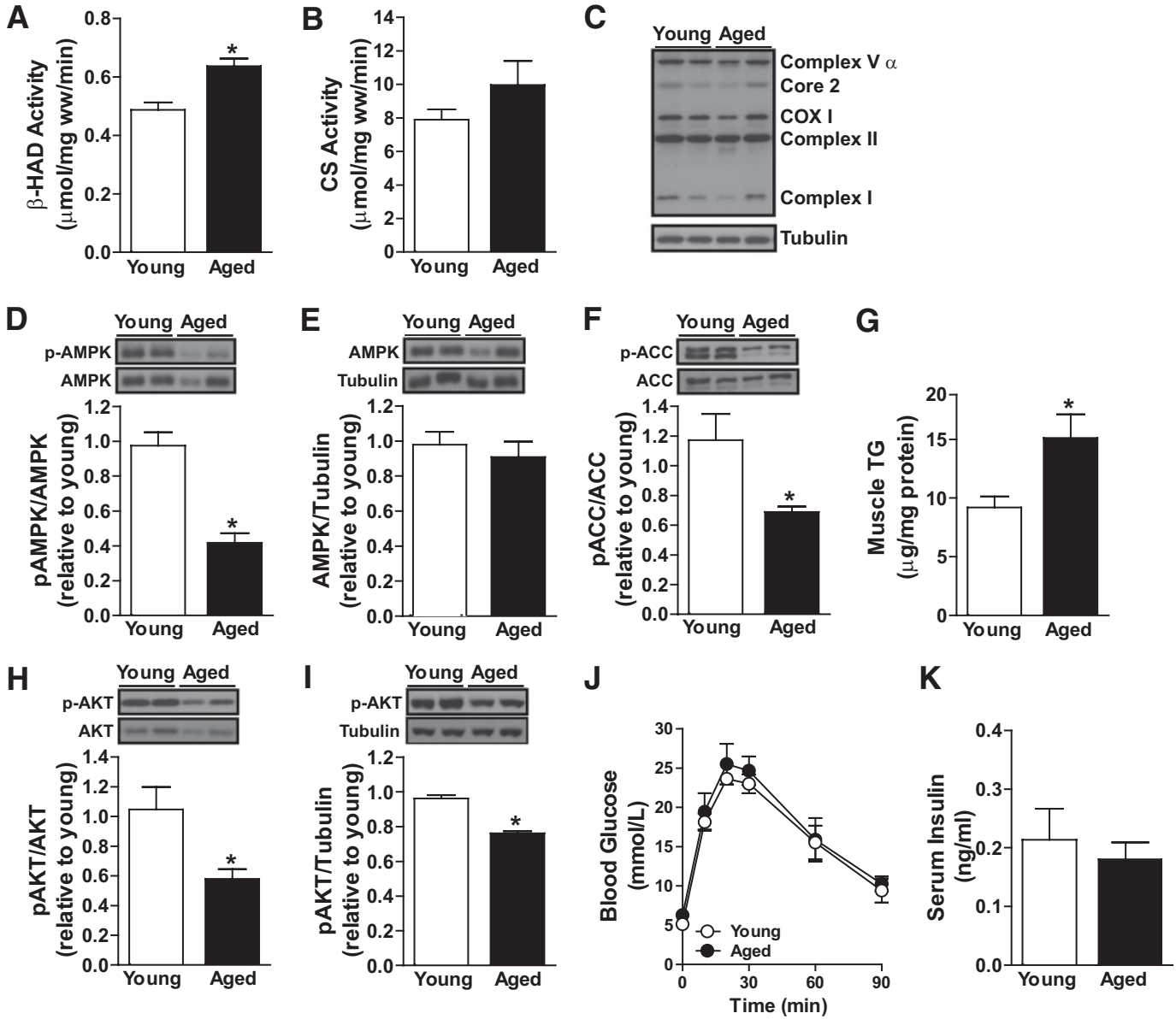

FIG. 2. Alterations in fatty acid handling and reduced insulin signaling in skeletal muscle of middle-aged mice do not result in impaired whole-body glucose tolerance. Activity of two mitochondrial enzymes β-HAD (*A*) and citrate synthase (CS) (*B*) was determined in gastrocnemius muscle from overnight fasted young (12–14 weeks of age) and middle-aged (52–58 weeks of age) mice. Immunoblot analysis using a total OXPHOS complex antibody cocktail was performed in gastrocnemius muscle lysates, and immunoblots were normalized against tubulin as a control for protein loading (*C*). Phosphorylation status of AMPK at threonine 172 (*D*) and ACC at serine 79 (*F*) was detected using immunoblot analysis with phospho-specific antibodies. Immunoblots were quantified by densitometry and normalized against total protein levels of AMPK (*E*) and ACC (*F*). Triglyceride (TG) levels (*G*) and phosphorylation status of Akt (*H*) were determined in gastrocnemius muscle from overnight-fasted young and middle-aged mice. Gastrocnemius muscle was collected from a separate group of overnight-fasted young and middle-aged mice following intraperitoneal injection with either saline or human recombinant insulin (10 units/kg), and immunoblots were performed to detect phosphorylation status of Akt at serine 473 in gastrocnemius muscle lysates (*I*). Glucose tolerance test (*J*) was performed in young (□) and middle-aged (■) mice following a 6-h fast. Serum insulin was detected in young and middle-aged mice after an overnight fast (*K*). Values are means ± SEM of $n = 5–7$ mice in each group. *$P < 0.05$ indicates comparisons performed between young and middle-aged mice (Mann-Whitney *U* Test).

similar upward trend, suggesting that β-oxidation and TCA cycle activity were not directly compromised in the middle-aged mice. Since mitochondrial number (Fig. 2*C*) or function did not appear to be altered at middle age, we next assessed whether peroxisome proliferator–activated receptor (PPAR)α responsive genes or molecular signaling cascades known to regulate skeletal muscle fatty acid flux and fatty acid entry into the mitochondria were associated with this overall reduction in whole-body basal metabolic rate observed in middle age. Although a more comprehensive assessment of the multiple mediators of fatty acid utilization may reveal additional mechanisms, we did not observe any changes in protein levels of known PPARα responsive genes involved in lipid metabolism, including malonyl-CoA decarboxylase and acyl-CoA synthetase 1 (data not shown). As a result, we also examined the energy-sensing kinase, AMPK, which is known for its ability to govern energy metabolism (23). In agreement with previous reports using older rodents (24,25), levels of phosphorylated AMPK were significantly reduced in skeletal muscle of middle-aged mice compared with young mice (Fig. 2*D*), whereas total AMPK levels remained

unchanged (Fig. 2*E*). Moreover, the phosphorylation status of acetyl CoA carboxylase (ACC), the downstream target of AMPK that indirectly regulates fatty acid entry into the mitochondria and ultimately β-oxidation, was significantly decreased in skeletal muscle from middle-aged mice (Fig. 2*F*). Although reduced AMPK phosphorylation could be the result of impaired activity of upstream AMPK kinases or increased AMPK phosphatase activity, it is currently unknown which of these contributes to reduced AMPK phosphorylation in our model. However, consistent with decreased energy expenditure, increased adiposity, and reduced AMPK activity in skeletal muscle from middle-aged mice, the levels of skeletal muscle triglycerides were significantly elevated in middle-aged mice compared with young mice (Fig. 2*G*). Given that lipid accumulation in skeletal muscle has been proposed in rodents (26) and humans (17,27,28) to be one of the primary causes of skeletal muscle insulin resistance, we next investigated whether glucose tolerance was impaired in middle-aged mice. Despite elevated intramuscular triglycerides (Fig. 2*G*) and impaired basal and insulin-stimulated Akt phosphorylation (Fig. 2*H* and *I*, respectively) in skeletal muscle of middle-aged mice, whole-body glucose tolerance (Fig. 2*J*) and fasted insulin levels (Fig. 2*K*) were not different in middle-aged compared with young mice, suggesting that age-induced alterations in skeletal muscle fatty acid handling and increased triglyceride storage precede the development of insulin resistance and metabolic disease.

Aging increases the sensitivity to diet-induced obesity and metabolic disease. Since we speculated that middle-aged mice are more susceptible than young mice to the development of insulin resistance, young and middle-aged mice were subjected to high-fat feeding for 12 weeks. Although young mice fed a high-fat diet displayed weight gain (Fig. 3*A*) and signs of glucose intolerance compared with young mice fed a low-fat diet (Fig. 3*B*, *C*, and *I*), Akt phosphorylation (Fig. 3*D*) and insulin levels (Fig. 3*E* and *F*) remained relatively normal in these mice. In contrast, middle-aged mice fed a high-fat diet showed significant weight gain (body weight: low fat, 30.18 ± 0.70 g, high fat, 55.14 ± 1.56 g; $P < 0.05$) and displayed dramatically elevated insulin levels (Fig. 3*E* and *F*), whereas blood glucose levels remained stable (Fig. 3*G*). In addition, whole-body glucose tolerance in the middle-aged mice fed a high-fat diet was significantly impaired compared with that in middle-aged mice fed a low-fat diet (Fig. 3*H* and *I*). Although activation of insulin signaling, as determined by the phosphorylation status of Akt is not impaired in skeletal muscle of young (Fig. 3*D*) or middle-aged (Fig. 3*J*) mice fed a high-fat diet compared with that in mice fed a low-fat diet, this is likely due to elevated levels of circulating insulin (Fig. 3*E* and *F*) observed in the respective high-fat groups. In support of the glucose tolerance data, homeostasis model assessment of insulin resistance values were significantly higher in the high-fat–fed middle-aged mice (Fig. 3*K*), suggesting that high-fat feeding induces more dramatic insulin resistance in middle-aged mice than in young mice. Interestingly, young mice on a high-fat diet were of the same weight as middle-aged mice on a low-fat diet; yet, only the young mice on a high-fat diet had impaired glucose disposal (data not shown). Although we cannot discriminate between the effects of aging and increased adiposity because these variables coassociate, our findings do suggest that the increased weight gain associated with aging is not sufficient to alter whole-body glucose disposal and that other factors such as high-fat diet are likely involved.

Because high-fat diet–induced insulin resistance in young rodents is associated with an increased efficiency of fatty acid uptake into skeletal muscle (26), protein expression of CD36, a protein that facilitates fatty acid transport, was determined in skeletal muscle of mice fed a high-fat diet. Consistent with previous publications in young mice (26,29,30), we observed a modest increase in CD36 protein expression in the muscle of young mice fed a high-fat diet, as well as an increase in intramuscular triglyceride levels (data not shown). Consistent with our hypothesis, CD36 expression was significantly elevated in skeletal muscle of middle-aged mice fed a high-fat diet compared with that in mice fed a low-fat diet (Fig. 3*L*). In accordance with increased CD36 protein expression, skeletal muscle triglyceride levels were significantly elevated in high-fat–fed middle-aged mice compared with those in low-fat–fed mice (Fig. 3*M*), as were long-chain acyl-CoA esters (Fig. 3*N*) and ceramides (Fig. 3*O*). Together, these data suggest that increased CD36-mediated fatty acid transport may contribute to lipid accumulation and impaired insulin sensitivity in skeletal muscle of middle-aged mice fed a high-fat diet.

Ablation of CD36 protects against diet-induced obesity in middle-aged mice. To investigate whether inhibition of fatty acid transport into the skeletal muscle could alter the observed responses of a middle-aged mouse to a high-fat diet, we utilized the CD36 KO mouse, which has skeletal muscle fatty acid uptake rates ~40–70% of those in wild-type mice (31,32). Interestingly, there was a striking difference in weight gain between middle-aged wild-type and CD36 KO mice following 12 weeks of high-fat feeding (Fig. 4*A*), with middle-aged CD36 KO mice accumulating 51% less weight than the wild-type mice over the same period of time (Fig. 4*B*). Though differences in caloric intake (Fig. 4*C*) or substrate preference (Fig. 4*D*) between groups could not account for this dramatic change in weight gain, indirect calorimetry indicated that energy expenditure (Fig. 4*E* and *F*) and overall activity (Fig. 4*G*) were significantly increased in middle-aged CD36 KO mice fed a high-fat diet compared with those in high-fat–fed middle-aged wild-type mice. Although this increased activity in the middle-aged CD36 KO mouse fed a high-fat diet could be attributed to the absence of obesity, heat production was also increased in middle-aged CD36 KO mice fed a low-fat diet compared with that in low-fat–fed middle-aged wild-type mice (Fig. 4*H*) and in high-fat–fed middle-aged KO mice when normalized for body weight (Fig. 4*I*).

Alterations in muscle metabolites and lipid balance correspond with protection against diet-induced insulin resistance. To gain a more comprehensive metabolic assessment of muscle metabolism in middle-aged wild-type and CD36 KO mice fed a low-fat or high-fat diet, we used mass spectrometry to measure a broad range of intermediary metabolites, including acylcarnitines of various chain lengths, organic acids, and amino acids. Acylcarnitines are by-products of fuel catabolism that respond to changes in substrate availability or flux limitations at specific mitochondrial enzymes (14,33,34). Middle-aged CD36 KO mice fed a low-fat diet had elevated levels of acetyl-carnitine (C2), and β-hydroxybutyryl-carnitine (C4OH) compared with their wild-type counterparts (supplemental Fig. 1*A* and supplemental Table 1). Whereas several short-chain acylcarnitine species, including C2 and C4OH, as

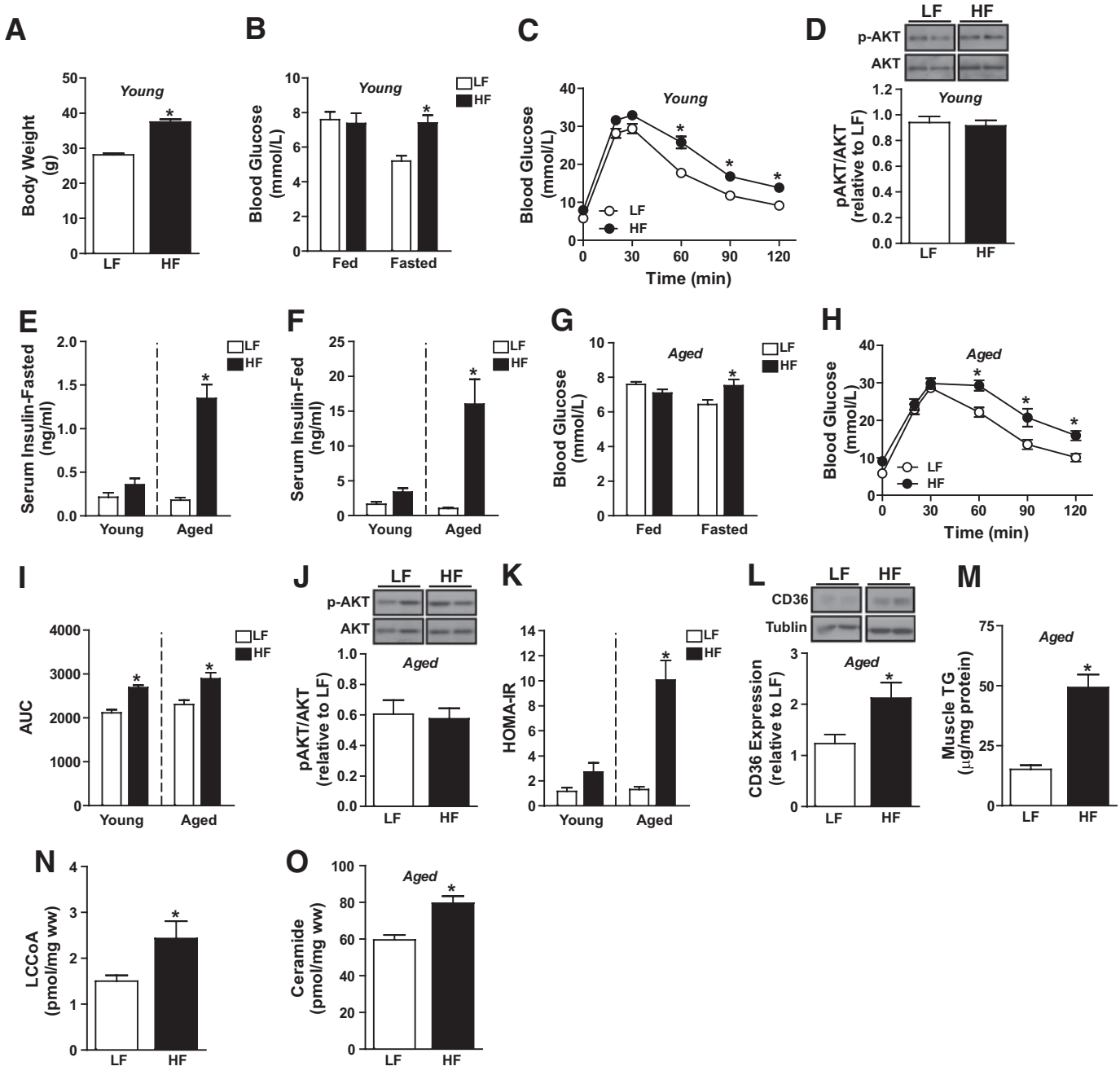

FIG. 3. Aging increases the susceptibility to the development of glucose intolerance and insulin resistance in mice fed a high-fat diet. Body weights (*A*), fed and fasted blood glucose (*B*), glucose tolerance test (*C*), and phosphorylation status of Akt (*D*) were measured in gastrocnemius muscle from young (12–14 weeks of age) mice following 12 weeks of high fat (HF) feeding. Serum insulin levels in the fasted (*E*) and fed (*F*) states obtained from young (12–14 weeks of age) and middle-aged (48–52 weeks of age) mice fed a low-fat (LF) and high-fat diet for 12 weeks. Fed and fasted blood glucose levels (*G*), glucose tolerance test (*H*), area under the curve (AUC) for the glucose tolerance test (*I*), and phosphorylation status of Akt in gastrocnemius muscle (*J*) in middle-aged mice following 12 weeks of high-fat feeding. Homeostasis model assessment of insulin resistance (HOMA-IR) as a surrogate marker of insulin resistance in young and middle-aged mice fed a high-fat diet (*K*). Immunoblot analysis using anti-CD36 antibody was performed in gastrocnemius muscle lysates from middle-aged mice fed a low- and high-fat diet, and immunoblots were quantified by densitometry and normalized against tubulin as a control for protein loading (*L*). Triglyceride (TG) (*M*), long-chain acyl-CoA (LCCoA) (*N*), and C18 ceramide (*O*) levels in gastrocnemius muscle from overnight-fasted middle-aged mice fed a low- or high-fat diet. Values are means ± SEM of $n = 8$ mice in each group. Two-way ANOVA was performed to detect main effects of age, diet, and age × diet interactions on insulin levels (*E* and *F*) and HOMA-IR (*K*). Significant effect of age, diet, and age × diet interaction ($P < 0.05$) was observed for *E*, *F*, and *K*. *$P < 0.05$ indicates comparisons performed between young and middle-aged mice or between low- and high-fat fed mice in fed or fasted state (Mann-Whitney *U* Test).

well as propionyl-carnitine (C3) and succinyl-carnitine (C4DC), tended to increase in response to a high-fat diet, these same metabolites trended downward in CD36 KO mice fed a high-fat diet (supplemental Fig. 1*A* and supplemental Table 1).

In addition, several long-chain acylcarnitine species were reduced in muscle from wild-type mice fed a high-fat diet while at the same time levels of hydroxylated long-chain acylcarnitine (LCOH) species were increased, resulting in a robust increase in the long-chain–to–LCOH acylcarnitine ratio (Fig. 5*A*–*C*). Given that long-chain acylcarnitines accumulate when their production by mito-

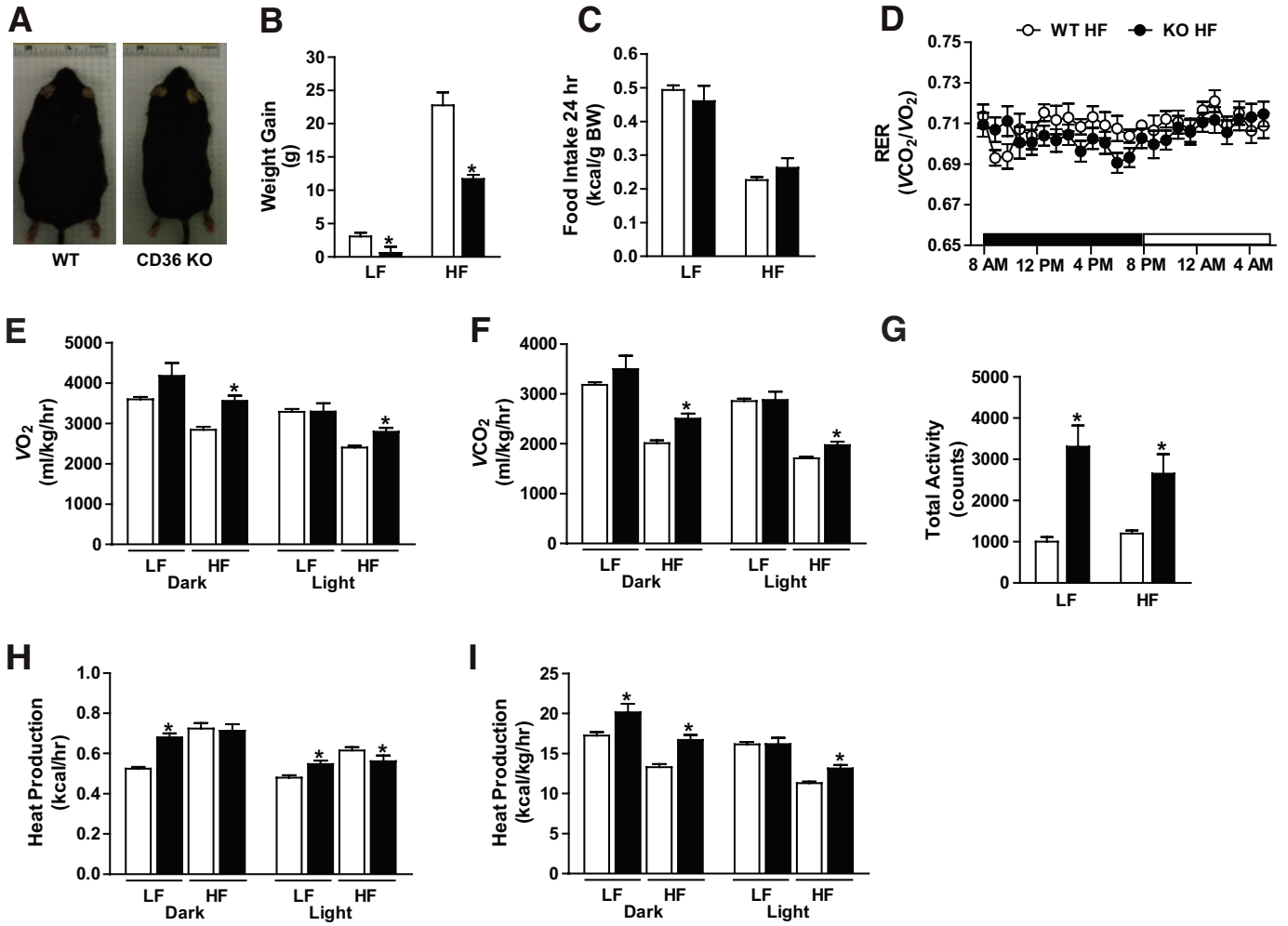

FIG. 4. Protection of diet-induced obesity in middle-aged CD36 KO mice fed a high-fat (HF) diet for 12 weeks. Representative image of middle-aged (48–52 weeks of age) wild-type (□) and CD36 KO (■) mice fed a high-fat diet for 12 weeks (*A*). Weight gain (*B*) and food intake adjusted for body weight (BW) (*C*) in wild-type and KO mice fed a high-fat diet. Respiratory exchange ratio (RER) (*D*), oxygen consumption (VO_2) (*E*), and carbon dioxide production (VCO_2) (*F*) in both the dark (active) and light (inactive) phase following 12 weeks of high-fat feeding in wild-type and KO mice. Total activity for a complete dark/light cycle (*G*), heat production (*H*), and heat production adjusted for body weight (*I*) in wild-type and KO mice following 12 weeks of high-fat feeding. Values are the means ± SEM of n = 6–10 mice in each group. *$P < 0.05$ indicates comparisons performed between the low-fat (LF)-fed mice and between high-fat–fed mice (Mann-Whitney *U* test [*B* and *G*] or ANOVA with Bonferroni post hoc test for pairwise comparison). (A high-quality digital representation of this figure is available in the online issue.)

chondrial carnitine palmitoyl transferase (CPT)1 exceeds flux through β-oxidation enzymes, such as long-chain acyl-CoA dehydrogenase (LCAD) and β-HAD (35), this pattern is consistent with a diet-induced shift in flux limitation from LCAD to β-HAD. Notably, levels of many long-chain and LCOH acylcarnitines were lower in the CD36 KO mice fed a high-fat diet than in the wild-type mice fed a high-fat diet (Fig. 5*A*; supplemental Table 1). The organic acids were less responsive to both diet and genotype, although subtle changes were detected in succinate, fumarate, and citrate levels (supplemental Fig. 1*B* and *C*; supplemental Table 2). Muscle levels of amino acids were higher in wild-type mice fed a low-fat diet but were dramatically decreased following high-fat feeding. By comparison, amino acid levels remained unchanged in CD36 KO mice in response to a high-fat diet (supplemental Fig. 1*D*; supplemental Table 2). Although these metabolite measurements do not fully characterize mitochondrial substrate flux, together the data suggest that ablation of CD36 not only alters baseline mitochondrial and intermediary metabolism but also significantly impacts the muscle response to lipid exposure.

Ablation of CD36 and reduction in intramuscular lipid accumulation prevent the development of diet-induced insulin resistance. Interestingly, despite the changes in muscle acylcarnitine levels, the activity of β-HAD was not altered (data not shown) and middle-aged CD36 KO mice fed a high-fat diet were not protected from decreased levels of AMPK or ACC phosphorylation (Fig. 5*D* and *E*) compared with young wild-type mice (Fig. 2*D* and *F*), suggesting that alternate mechanisms are responsible for the metabolic phenotype observed in CD36 KO mice. Similarly, absolute expression of AMPK and ACC in skeletal muscle from aged CD36 mice is not different between groups (data not shown). Although serum free fatty acid levels were elevated in high-fat–fed CD36 KO mice (Fig. 5*F*), CD36 ablation resulted in a significant reduction in skeletal muscle triglycerides (Fig. 5*G*) and long-chain acyl-CoA's (Fig. 5*H*) accumulation. By contrast, ceramide levels remained similar between high-fat fed groups (Fig. 5*I*), suggesting that accumulation of lipid-derived intermediates other than ceramides, may contribute to impaired insulin sensitivity. Indeed, reduced intramuscular lipid accumulation in middle-aged CD36 KO

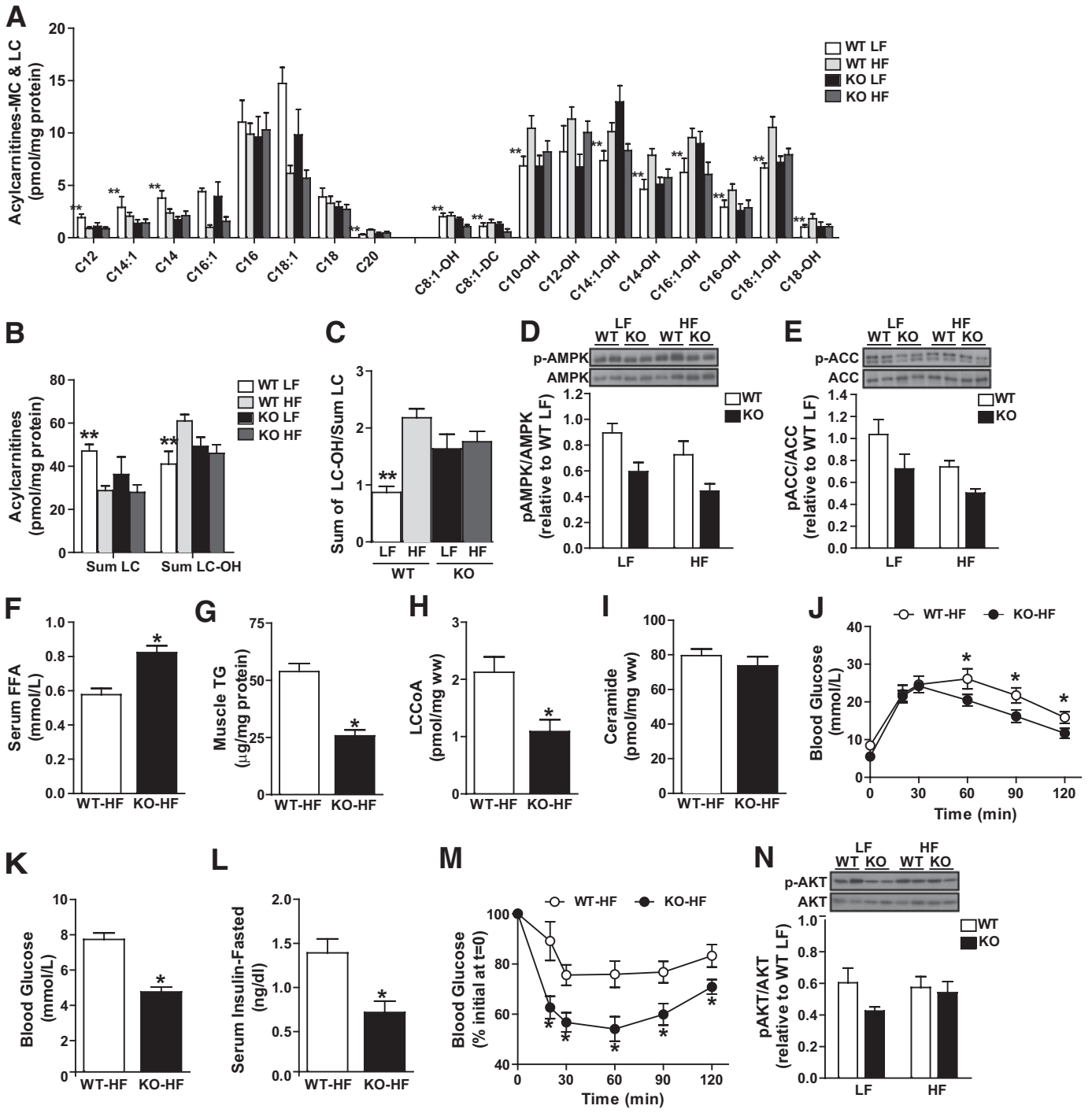

FIG. 5. Altered skeletal muscle lipid handling prevents development of insulin resistance in middle-aged CD36 KO mice fed a high-fat (HF) diet for 12 weeks. Acylcarnitine levels were measured in gastrocnemius muscle from overnight-fasted middle-aged (48–52 weeks of age) wild-type (WT) and CD36 KO mice fed a low- (LF) or high-fat diet for 12 weeks. Levels of individual long chain (LC) and LCOH species (*A*), the sum total of all long-chain or LCOH species (*B*), and the ratio of total LCOH–to–long-chain species (*C*). Phosphorylation status of AMPK (Thr172) (*D*) and ACC (Ser 79) (*E*) was detected in gastrocnemius muscle using immunoblot analysis. Immunoblots were quantified by densitometry and normalized against total AMPK (*D*) and ACC (*E*). Serum levels of free fatty acids (FFAs) from high-fat–fed wild-type and CD36 KO mice were determined after 12 weeks of diet (*F*). Intramuscular levels of triglyceride (TG) (*G*), LCCoA (*H*), and C18 ceramide (*I*) were determined in gastrocnemius muscle obtained from wild-type and KO mice fed a high-fat diet for 12 weeks. Glucose tolerance testing (*J*) was performed in high-fat–fed wild-type and KO mice fasted for 6 h. Fasted blood glucose (*K*) and serum insulin (*L*) levels obtained from middle-aged wild-type and KO mice fed a high-fat diet for 12 weeks. Insulin tolerance test with blood glucose levels expressed as percent change of blood glucose at time zero in middle-aged wild-type and KO mice fed a high-fat diet for 12 weeks (*M*). Immunoblots were performed on gastrocnemius muscle isolated from middle-aged wild-type and KO mice following high-fat diet and phosphorylation status of Akt measured and normalized to total Akt levels (*N*). Values are means ± SEM of $n = 6$–10 mice in each group. Main effects of genotype, diet, and genotype × diet interactions on acylcarnitine levels (*A*–*C*) were detected by two-way ANOVA. For simplicity, symbols indicate metabolites that were affected by genotype, diet, or a genotype-diet interaction. Detailed results of the statistical analysis for all acylcarnitine species are presented in supplemental Table 1. *$P < 0.05$ indicates comparisons performed between low-fat–fed wild-type and low-fat–fed KO mice and between high-fat–fed wild-type and high-fat–fed KO mice (Mann-Whitney *U* Test or ANOVA with Bonferroni post hoc test [*J* and *M*]).

mice was associated with both improved whole-body glucose tolerance (Fig. 5*J*) and significantly lower fasting blood glucose levels (Fig. 5*K*) compared to high-fat fed middle-aged wild-type mice. Moreover, fasted insulin levels were significantly reduced (Fig. 5*L*) and insulin-induced glucose clearance was dramatically improved (Fig. 5*M*) in high-fat fed middle-aged CD36 KO mice compared to high-fat fed middle-aged wild-type mice, suggesting that insulin sensitivity is restored by preventing lipid accumulation in skeletal muscle. Interestingly, despite improved glucose utilization and reduced plasma insulin levels in these mice, the phosphorylation status of Akt was similar in skeletal muscle from middle-aged wild-type and CD36 KO mice on high-fat diet (Fig. 5*N*). Furthermore, we found no difference in glycogen content in livers from CD36 KO mice fed a low-fat or high-fat diet (data not shown), suggesting that the improved glucose tolerance observed in these mice is the result of increased glucose uptake and/or glucose oxidation in skeletal muscle from CD36 KO mice and not alterations in hepatic glucose metabolism.

DISCUSSION

Consistent with previous reports (17), our data show a significant decline in metabolic rate in middle-aged mice when compared to their young counterparts (Fig. 1*B*–1F). Interestingly, although mitochondrial function was not directly assessed in this study, the decline in overall metabolic rate did not appear to stem from compromised mitochondrial function in skeletal muscle of middle-aged mice compared to young mice. Indeed, whole body RER was modestly decreased with aging and muscle activity of β-HAD increased, suggesting there is a shift in substrate selection from carbohydrates toward fatty acids. However, the age-associated reduction in overall metabolic rate did not appear to correlate with changes in maximal activities of mitochondrial enzymes in muscle from young and middle-aged mice (Fig. 2*A* and 2B), suggesting other factors may influence substrate oxidation in muscle, as well as overall metabolic rate. Consistent with this, the AMPK/ACC signaling axis was significantly reduced in skeletal muscle from middle-aged mice (Fig. 2*D* and 2F). Still unclear is whether reduced AMPK phosphorylation in the older mice reflects a cause or consequence of reduced metabolic rate. Although results of a recent study suggest that ACC-mediated shifts in fat oxidation per se do not impact whole body energy expenditure or susceptibility to diet-induced obesity (36), AMPK acts on a broad range of enzymatic and transcriptional targets that could affect energy balance via mechanisms other than substrate selection (25,37). Nonetheless, CD36 deficiency raised metabolic rate without activating AMPK, indicating that other mechanisms are operative in this model (see below).

Although Akt phosphorylation is reduced in skeletal muscle from middle-aged mice compared to young mice (Fig. 2*H* and 2I) whole-body glucose tolerance and plasma insulin levels remained normal (Fig. 2*J* and 2K), suggesting that impaired activation of insulin-signaling parameters in skeletal muscle precede overt changes in whole body glucose disposal and may increase the susceptibility of aged mice to diet-induced insulin resistance. To determine whether middle-aged mice indeed are more susceptible to developing insulin resistance in response to a high-fat diet compared to their younger counterparts, we subjected middle-aged mice to 12 weeks of a low-fat or high-fat diet. As expected, middle-aged mice gained significantly more weight than young mice when fed a high-fat diet (weight gain: middle-aged, 22.8 ± 1.9 g *versus* young, 12.0 ± 1.1 g; $P < 0.01$). Although impairment of glucose tolerance was similar between age-groups (Fig. 3*C*, 3H and 3I), high-fat diet-induced hyperinsulinemia was only observed in middle-aged mice (Fig. 3*E* and 3F), indicating that increased β-cell insulin secretion was sufficient to offset overt hyperglycemia in this age-group (Fig. 3*G*). Given that prolonged hypersecretion of insulin by the β-cells to compensate for peripheral insulin resistance can contribute to β-cell failure (38) and potentially type 2 diabetes (39), our data suggest that middle-aged mice have a heightened susceptibility to the development of insulin resistance. However, this susceptibility might not be due to aging per se, since adiposity was also increased in the older mice. As our study design does not permit to discriminate between the effects of aging and adiposity on skeletal muscle metabolism and the development of insulin resistance, future experiments should be directed at conducting weight loss (food restriction) studies or exercise studies to determine if these 'aging' effects are prevented or mitigated by controlling adiposity. Nevertheless, as aging and increased adiposity co-associate our findings likely reflect the majority of middle-aged humans in the western world who are at risk of developing insulin resistance.

Although intramuscular lipid accumulation associated with aging did not appear to result from mitochondrial dysfunction, we do show a significant twofold increase in CD36 protein expression in skeletal muscle of high-fat fed middle-aged mice compared to low-fat fed middle-aged mice (Fig. 3*L*), suggesting fatty acid transport into muscle exceeded the capacity for their oxidation. Although it is unknown what caused increased CD36 expression in our study, it may have resulted from high levels of plasma glucose levels that have been shown to regulate CD36 expression through both transcriptional and/or translational mechanisms in rodents and humans (40,41). To determine whether reduced fatty acid transport and metabolism could rescue the high-fat diet-induced phenotype, we utilized the CD36 KO mouse (19). Consistent with our prediction, CD36 deficiency prevented the decline in metabolic rate and energy expenditure in middle-aged mice fed a high-fat diet as compared to age-matched wild-type mice (Fig. 4*E* and 4F). Since food intake was similar between groups (Fig. 4*C*), we propose that increased energy expenditure in high-fat fed middle-aged CD36 KO mice contributes to their protection against diet-induced obesity (Fig. 4*B*).

The blunted decline in metabolic rate in the middle-aged CD36 KO mice fed a high-fat diet (Fig. 4*E* and 4F) was accompanied by alterations in muscle concentrations of several metabolic intermediates (Fig. 5*A*–5C; Supplemental Fig. 1), but no improvement in AMPK and ACC phosphorylation (Fig. 5*D* and 5E, respectively). The impact of the diet on muscle metabolites in this study differed to some extent as compared to a previous report (14) in which tissue specimens were harvested from younger animals in the fed state. Herein, tissues were collected after an overnight fast because we sought to evaluate a state of heightened fatty acid oxidation. Under these conditions, high-fat feeding lowered several long-chain acylcarnitines but increased LCOH acylcarnitines. The drop in long-chain acylcarnitines could reflect decreased fatty acid availability, lower CPT1 activity or increased long-chain acyl CoA flux through LCAD. Several lines of evidence support the latter possibility. First, the high-fat

diet increases rather than decreases lipid delivery to muscle. Second, of the two major products of CPT1 (palmitoylcarnitine (C16) and oleylcarnitine (C18:1), only the unsaturated species was reduced by the diet (Fig. 5*A*), suggesting upregulation of the isomerase enzyme that catalyzes conversion of the double bond (42). Third, the generalized rise in LCOH species (Fig. 5*B*) along with the high LCOH/long-chain ratio (Fig. 5*C*) in response to chronic lipid exposure suggest a shift in flux limitation from the earlier to later steps in β-oxidation. Lastly, the diet resulted in a robust drop in whole body RER, indicative of a systemic increase in fat oxidation. Notably, CD36 deficiency altered baseline levels of several muscle metabolites, and in general, tended to mitigate diet-induced changes in several acylcarnitine and amino acid species. This apparent resistance to diet-induced metabolic perturbations in the CD36 KO mice might be directly related to a reduction in fat delivery and/or secondary to enhanced energy expenditure and insulin sensitivity. Although further work is necessary to fully understand the implications of these results, it is clear that loss of CD36 has a global impact on muscle fuel metabolism. In addition, it is possible that other organs such as adipose tissue, liver and brain (43,44) are also involved in maintaining a high level of energy expenditure in the middle-aged CD36 KO mice.

Overall, ablation of CD36 was associated with an improvement in whole-body glucose utilization and insulin sensitivity (Fig. 5*J*–5M) in mice fed a high-fat diet. Although the mechanisms responsible for this are not known, excessive intramuscular lipid accumulation induced by high-fat feeding was prevented in skeletal muscle of middle-aged CD36 KO mice (Fig. 5*G* and 5H). Whereas elevated intramuscular triglyceride levels were not associated with insulin resistance in young mice (Fig. 2*J*–2K), preventing the more dramatic age- and diet-induced accumulation of intramuscular triglyceride and long-chain acyl CoA levels in middle-aged CD36 KO mice correlated with improved whole-body insulin sensitivity. Although there is ample evidence indicating that CD36 ablation significantly reduces skeletal muscle fatty acid uptake (31,32), it is also possible that the effects that we report using the CD36 KO mice are secondary to changes in fatty acid metabolism. Notwithstanding this later possibility, our data demonstrate that limiting CD36-mediated skeletal muscle fatty acid transport guards against whole-body and muscle insulin resistance in middle-aged mice fed a high-fat diet. This finding suggests a potential therapeutic strategy for combating metabolic disease in the face of age-related abnormalities.

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No potential conflicts of interest relevant to this article were reported.

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