Alterations in Skeletal Muscle Fatty Acid Handling Predisposes Middle-aged Mice to Diet-induced Insulin Resistance

Running Title: Skeletal Muscle Metabolism and Insulin Resistance

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Objective—Although advanced age is a risk factor for type 2 diabetes (T2D), 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 WT and CD36 knockout (KO) mice fed either a standard or a high fat (HF) diet for 12 weeks. Molecular signaling pathways, intramuscular triglycerides (IMTG) accumulation as well as targeted metabolomics of in vivo mitochondrial substrate flux were also analyzed in the skeletal muscle of all aged mice.

Results—Middle-aged mice fed a standard diet demonstrated an increase in IMTGs without a concomitant increase in insulin resistance. However, middle-aged mice fed a HF 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 HF diet.
Over the past few decades type 2 diabetes (T2D) has increased in prevalence largely due to 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 T2D (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 (FA) 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 FA oxidation but might actually result from excessive skeletal muscle mitochondrial FA 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 FAs into the skeletal muscle plays a central role in diet-induced insulin resistance.

As advanced age is a significant risk factor in the etiology of T2D (15; 16), the accompanying loss of mitochondrial function observed with normal aging has been proposed to contribute to the increased risk of T2D 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 T2D in the western world is expected to increase dramatically over the next 5-10 years (16; 18). Based on this, the study herein was designed to investigate how middle-age impacts whole-body glucose utilization, FA 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 AMPK, pAMPK, ACC, pACC, Akt, pAkt 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 Inc.

Mice— This study was performed with the approval of The University of Alberta Animal Policy and Welfare Committee. Experiments were carried out on male WT (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 (5001; LabDiet). At 32-34 weeks of age, a subset of mice was randomly divided into a LF diet group (D12450B, Research Diets, Inc.) and a HF diet group (D12492, Research Diets, Inc) 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 hours to complete a 12-h dark (active)/12-h light (inactive) cycle. The respiratory exchange
ratio (RER; RER=VCO₂/VO₂) 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, scratching).

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

**Metabolic Profiling**— Overnight fasted mice were anaesthetized 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 (MS/MS) as previously described (14) and organic acids were quantified according to (22).

**Statistical Analysis**— Data are expressed as mean ± SEM. Comparisons between groups were performed using the unpaired Student's two-tailed t-test or analysis of variance (ANOVA) with a Bonferroni post-hoc test of pairwise comparisons where appropriate. A probability value of <0.05 is considered significant.

**Supplemental Information**— For further descriptions of the materials and methods, refer to the supplementary material available in the online appendix which can be found in an online appendix at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org).

**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. 1A), substrate use was altered slightly with reductions in RER indicating that middle-aged mice used more FA throughout the day compared to young mice (Fig. 1B). In addition, significant reductions in oxygen consumption (VO₂; Fig. 1C) and carbon dioxide production (VCO₂; Fig. 1D) were observed during both the dark (active) and light (inactive) phase in middle-aged mice compared to young mice. Consistent with this, heat production (Fig. 1E) was decreased in middle-aged mice compared to young mice, whereas activity measurements were not significantly different between age groups (Fig. 1F). Taken together our data indicate that middle-aged mice have a lower metabolic rate compared to young mice and that this might increase the susceptibility towards 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**— As 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. 2A) was elevated in the middle-aged.
compared to young mice and citrate synthase (CS; Fig 2B) followed a similar upward trend, suggesting that β-oxidation and TCA cycle activity were not directly compromised in the middle-aged mice. As mitochondrial number (Fig. 2C) and/or function did not appear to be altered at middle-age, we next assessed whether peroxisome proliferator-activated receptor (PPAR)-α responsive genes and/or molecular signaling cascades known to regulate skeletal muscle FA flux and FA entry into the mitochondria were associated with this overall reduction in whole-body basal metabolic rate observed in middle-age. While a more comprehensive assessment of the multiple mediators of FA 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 P-AMPK were significantly reduced in skeletal muscle of middle-aged mice compared to young mice (Fig. 2D), whereas total AMPK levels remained unchanged (Fig. 2E). Moreover, the phosphorylation status of acetyl CoA carboxylase (ACC), the downstream target of AMPK that indirectly regulates FA entry into the mitochondria and ultimately β-oxidation, was significantly decreased in skeletal muscle from middle-aged mice (Fig. 2F). 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 P-AMPK 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 TG were significantly elevated in middle-aged mice compared to young mice (Fig. 2G). As 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 IMTG (Fig. 2G) and impaired basal and insulin-stimulated Akt phosphorylation (Fig. 2H and 2I, respectively) in skeletal muscle of middle-aged mice, whole-body glucose tolerance (Fig. 2J) and fasted insulin levels (Fig. 2K) were not different in middle-aged compared to young mice, suggesting that age-induced alterations in skeletal muscle FA handling and increased TG storage precede development of insulin resistance and metabolic disease.

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

As HF diet-induced insulin resistance in young rodents is associated with an increased efficiency of FA uptake into skeletal muscle (26), protein expression of CD36, a protein that facilitates FA transport, was determined in skeletal muscle of mice fed a HF 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 HF diet, as well as an increase in IMTG levels (data not shown). Consistent with our hypothesis, CD36 expression was significantly elevated in skeletal muscle of middle-aged mice fed a HF diet compared to a LF diet (Fig. 3L). In accordance with increased CD36 protein expression, skeletal muscle TG levels were significantly elevated in HF fed middle-aged mice compared to LF fed mice (Fig. 3M), as were long-chain acyl CoA esters (LCCoA; Fig. 3N) and ceramides (Fig. 3O). Together, these data suggest that increased CD36-mediated FA transport may contribute to lipid accumulation and impaired insulin sensitivity in skeletal muscle of middle-aged mice fed a HF diet.

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

**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 WT and CD36 KO mice fed a LF or HF diet, we used mass spectrometry to measure a broad range of intermediary metabolites, including acylcarnitines of various chain lengths, organic acids and amino acids. Acylcarnitine are byproducts of
fuel catabolism that respond to changes in substrate availability and/or flux limitations at specific mitochondrial enzymes (14; 33; 34). Middle-aged CD36 KO mice fed a LF diet had elevated levels of acetyl-carnitine (C2), and β-hydroxybutyryl-carnitine (C4OH) compared to their WT counterparts (Supplemental Fig. 1A; Supplemental Table 1). Whereas several short-chain acylcarnitine species, including C2 and C4OH, as well as propionyl-carnitine (C3) and succinyl-carnitine (C4DC) tended to increase in response to HF diet, these same metabolites trended downward in CD36 KO mice fed a HF diet (Supplemental Fig. 1A; Supplemental Table 1).

In addition, several long chain (LC) acylcarnitine species were reduced in muscle from WT mice fed a HF diet, while at the same time levels of hydroxylated acylcarnitine species (LCOH) were increased, resulting in a robust increase in the LC to LCOH acylcarnitine ratio (Fig. 5A-5C). As LC acylcarnitines accumulate when their production by mitochondrial CPT1 exceeds flux through β-oxidation enzymes, such as long chain acyl-CoA dehydrogenase (LCAD) and βHAD (35), this pattern is consistent with a diet-induced shift in flux limitation from LCAD to βHAD. Notably, levels of many LC and LCOH acylcarnitines were lower in the CD36 KO mice fed a HF diet compared to WT mice fed a HF diet (Fig. 5A; 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. 1B and 1C; Supplemental Table 2). Muscle levels of amino acids were higher in WT mice fed a LF diet, but were dramatically decreased following HF feeding. By comparison, amino acid levels remained unchanged in CD36 KO mice in response to a HF diet (Supplemental Fig. 1D; 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 prevents 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 HF diet were not protected from decreased P-AMPK or P-ACC levels (Fig. 5D and 5E) compared to young WT mice (Fig. 2D and 2F), 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 FA levels were elevated in HF fed CD36 KO mice (Fig. 5F), CD36 ablation resulted in a significant reduction in skeletal muscle TG (Fig. 5G) and LCCoA (Fig. 5H) accumulation. By contrast, ceramide levels remained similar between HF fed groups (Fig. 5I), 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 mice was associated with both improved whole-body glucose tolerance (Fig. 5J) and significantly lower fasting blood glucose levels (Fig. 5K) compared to HF fed middle-aged WT mice. Moreover, fasted insulin levels were significantly reduced (Fig. 5L) and insulin-induced glucose clearance was dramatically improved (Fig. 5M) in HF fed middle-aged CD36 KO mice compared to HF fed middle-aged WT 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, P-Akt was similar in skeletal muscle from middle-aged WT & CD36 KO mice on HF diet (Fig. 5N). Furthermore, we found no difference in glycogen content in livers from CD36 KO mice fed a LF or HF 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. 1B-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 towards FAs. 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. 2A 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. 2D and 2F). Still unclear is whether reduced P-AMPK 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. 2H and 2I) whole-body glucose tolerance and plasma insulin levels remained normal (Fig. 2J 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 HF diet compared to their younger counterparts, we subjected middle-aged mice to 12 weeks of a LF or HF diet. As expected, middle-aged mice gained significantly more weight than young mice when fed a HF diet (middle-aged, 22.8 ± 1.9 g vs. young, 12.0 ± 1.1 g; p<0.01). Although impairment of glucose tolerance was similar between age groups (Fig. 3C, 3H and 3I), HF diet-induced hyperinsulinemia was only observed in middle-aged mice (Fig. 3E and 3F), indicating that increased β-cell insulin secretion was sufficient to offset overt hyperglycemia in this age group (Fig. 3G). 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 T2D (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 2-fold increase in CD36 protein expression in skeletal muscle of HF fed middle-aged mice compared to LF fed middle-aged mice (Fig. 3L), suggesting FA 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 FA transport and metabolism could rescue the HF 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 HF diet as compared to age-matched WT mice (Fig. 4E and 4F). Since food intake was similar between groups (Fig. 4C), we propose that increased energy expenditure in HF fed middle-aged CD36 KO mice contributes to their protection against diet-induced obesity (Fig. 4B).

The blunted decline in metabolic rate in the middle-aged CD36 KO mice fed a HF diet (Fig. 4E and 4F) was accompanied by alterations in muscle concentrations of several metabolic intermediates (Fig. 5A–5C; Supplemental Fig. 1), but no improvement in AMPK and ACC phosphorylation (Fig. 5D 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 FA oxidation. Under these conditions, HF feeding lowered several LC acylcarnitines but increased LCOH acylcarnitines. The drop in LC acylcarnitines could reflect decreased FA availability, lower CPT1 activity or increased LCCoA flux through LCAD. Several lines of evidence support the latter possibility. First, the HF 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. 5A), suggesting upregulation of the isomerase enzyme that catalyzes conversion of the double bond (42). Third, the generalized rise in LCOH species (Fig. 5B) along with the high LCOH/LC ratio (Fig. 5C) 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. 5J-5M) in mice fed a HF diet. Although the mechanisms responsible for this are not known, excessive intramuscular lipid accumulation induced by HF feeding was prevented in skeletal muscle of middle-aged CD36 KO mice (Fig. 5G and 5H). Whereas elevated intramuscular TG levels were not associated with insulin resistance in young mice (Fig. 2J-2K), preventing the more dramatic age- and diet-induced accumulation of intramuscular TG and LCCoA 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 FA uptake (31; 32), it is also possible that the effects that we report using the CD36 KO mice are secondary to changes in FA metabolism. Notwithstanding this later possibility, our data demonstrate that limiting CD36-mediated skeletal muscle FA transport guards against whole body and muscle insulin resistance in middle-aged mice fed a HF diet. This finding infers a potential therapeutic strategy for combating metabolic disease in the face of age-related abnormalities.

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REFERENCES:

30. Smith AC, Mullen KL, Junkin KA, Nickerson J, Chabowski A, Bonen A, Dyck DJ: Metformin and exercise reduce muscle FAT/CD36 and lipid accumulation and blunt the
42. de Fourmestraux V, Neubauer H, Poussin C, Farmer P, Falquet L, Burcelin R, Delorenzi M, Thorens B: Transcript profiling suggests that differential metabolic adaptation of mice to a high fat diet is associated with changes in liver to muscle lipid fluxes. *J Biol Chem* 279:50743-50753, 2004
FIGURE LEGENDS:

**Figure 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), oxygen consumption (VO2; c), carbon dioxide production (VCO2; d), heat production adjusted for bodyweight (e), and total activity (f) was measured in both the dark (active) and light (inactive) phase. Values are the mean ± S.E.M. 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 phase (Mann Whitney U Test). Two-way ANOVA was performed for RER (b) and indicated main effect for age (p<0.01).

**Figure 2.** Alterations in fatty acid handling and reduced insulin signaling in skeletal muscle of middle-aged mice does not result in impaired whole-body glucose tolerance. Activity of two mitochondrial enzymes β-hydroxyacyl CoA dehydrogenase (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 complexes 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 acetyl-CoA carboxylase (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 (10U/kg), and immunoblots were performed to detect phosphorylation status of Akt in gastrocnemius muscle lysates (i). Glucose tolerance test (j) was performed in young (open circles) and middle-aged mice (closed circles) following a 6 hr fast. Serum insulin was detected in young and middle-aged mice after an overnight fast (k). Values are the mean ± S.E.M. of n = 5-7 mice in each group. * P<0.05 indicates comparisons performed between young and middle-aged mice (Mann Whitney U Test).

**Figure 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) was measured in gastrocnemius muscle from young (12-14 weeks of age) mice following 12 weeks of high fat (HF) feeding. Serum insulin levels in fasted (e) and fed (f) state obtained from young (12-14 weeks of age) and middle-aged (48-52 weeks of age) mice fed a low fat (LF) and HF 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 HF feeding. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) as a surrogate marker of insulin resistance in young and middle-aged mice fed a HF diet (k). Immunoblot analysis using anti-CD36 antibody was performed in gastrocnemius muscle lysates from middle-aged mice fed a LF and HF 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 LF or HF diet. Values are the mean ± S.E.M. of n = 8 mice in each group. * P<0.05 indicates comparisons performed between young and middle-aged mice or between LF and HF fed mice in fed state or fasted state (Mann Whitney U Test). Two-way ANOVA was performed to detect main effects of age, diet as well as age x diet interactions on insulin levels (e, f) and HOMA-IR (k). Significant effect of age, diet and age x diet interaction (p<0.05) was observed for e, f and k.

Figure 4. Protection of diet-induced obesity in middle-aged CD36 KO mice fed a high fat diet for 12 weeks. Representative image of middle-aged (48-52 weeks of age) WT and CD36 KO (KO) mice fed a high fat (HF) diet for 12 weeks (a). Weight gain (b) and food intake adjusted for body weight (BW) (c) in WT and KO mice fed a HF diet. Respiratory Exchange Ratio (RER; d), oxygen consumption (VO2; e) and carbon dioxide production (VCO2; f) in both the dark (active) and light (inactive) phase following 12 weeks of HF feeding in WT and KO mice. Total activity for a complete dark/light cycle (g), heat production (h) and heat production adjusted for body weight (i) in WT and KO mice following 12 weeks of HF feeding. Values are the mean ± S.E.M. of n = 6-10 mice in each group. * P<0.05 indicates comparisons performed between LF fed mice and between HF fed mice (Mann Whitney U test (b, g) or ANOVA with Bonferroni posthoc test for pairwise comparison).

Figure 5. Altered skeletal muscle lipid handling prevents development of insulin resistance in middle-aged CD36 KO mice fed a high fat diet for 12 weeks. Acylcarnitine levels were measured in gastrocnemius muscle from overnight fasted middle-aged (48-52 weeks of age) WT and CD36 KO (KO) mice fed a LF or HF diet for 12 weeks. Levels of individual long chain (LC) and hydroxylated-long acylcarnitine (LCOH) species (a), the sum total of all LC or LCOH species (b), and the ratio of total LCOH/LC species (c). Phosphorylation status of AMPK (Thr172; d) and acetyl-CoA carboxylase (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 FFA from HF fed WT and CD36 KO mice were determined after 12 weeks of diet (f). Intramuscular levels of triglyceride (TG; g), long-chain acyl CoA (LCCoA; h) and C18-ceramide (i) were determined in gastrocnemius muscle obtained from WT and KO mice fed a HF diet for 12 weeks. Glucose tolerance test (j) was performed in HF-fed WT (open circles) and KO (closed circles) mice fasted for 6h. Fasted blood glucose levels (k) and serum insulin levels (l) obtained from middle-aged WT and KO mice fed a HF diet for 12 weeks. Insulin tolerance test with blood glucose levels expressed as % change of blood glucose at time zero in middle-aged WT and KO mice fed a HF diet for 12 weeks (m). Immunoblots were performed on gastrocnemius muscle isolated from middle-aged WT and KO mice following HF diet and phosphorylation status of Akt measured and normalized to total Akt levels (n). Values are the mean ± S.E.M. of n = 6-10 mice in each group. * P<0.05 indicates comparisons performed between LF fed WT and LF fed KO mice and between HF fed WT and HF fed KO mice (Mann Whitney U Test or ANOVA with Bonferroni posthoc test (j, m). Main effects of genotype and diet as well as genotype x 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.
Figure 1

A. Body Weight (g)

B. RER (VCO2/VO2)

C. VO2 (ml/kg/hr)

D. VCO2 (ml/kg/hr)

E. Heat (kcal/kg/hr)

F. Activity (Beam Brakes)
Figure 2

A. β-HAD Activity (µmol/mg ww/min) for Young and Aged groups.

B. CS Activity (µmol/mg ww/min) for Young and Aged groups.

C. Western blot images of Complexes V, COX I, Complex II, Complex I, and Tubulin.

D. p-AMPK/AMPK (relative to young) for Young and Aged groups.

E. AMPK/Tubulin (relative to young) for Young and Aged groups.

F. p-AKT/AKT (relative to young) for Young and Aged groups.

G. Muscle TG (µg/mg protein) for Young and Aged groups.

H. p-AKT/AKT (relative to young) for Young and Aged groups.

I. p-AKT/Tubulin (relative to young) for Young and Aged groups.

J. Blood Glucose (mmol/L) over time (min) for Young and Aged groups.

K. Serum Insulin (ng/ml) for Young and Aged groups.
Figure 3

A. Body Weight (g) - Young vs. Aged, LF vs. HF

B. Blood Glucose (mmol/L) - Fed vs. Fasted, Young vs. Aged

C. Blood Glucose (mmol/L) over Time (min), Young

D. p-AKT/AKT (relative to LF) - Young

E. Serum Insulin-Fasted (ng/ml) - Young vs. Aged, LF vs. HF

F. Serum Insulin-Fed (ng/ml) - Young vs. Aged, LF vs. HF

G. Blood Glucose (mmol/L) over Time (min), Aged

H. Blood Glucose (mmol/L) over Time (min), Aged

I. AUC - Young vs. Aged, LF vs. HF

J. p-AKT/AKT (relative to LF) - Aged

K. HOMA-IR - Young vs. Aged, LF vs. HF

L. CD36 Expression (relative to LF) - Aged

M. Muscle TG (µg/mg protein) - Aged

N. LCCoA (pmol/mg ww) - LF vs. HF

O. Ceramide (pmol/mg ww) - LF vs. HF
Figure 4

A. WT vs CD36 KO

B. Heat Production (kcal/hr)

C. Food Intake 24 hr (kcal/kg BW)

D. RER (VCO2/VO2)

E. VO2 (ml/kg/hr)

F. VCO2 (ml/kg/hr)

G. Total Activity (counts)

H. Heat Production (kcal/hr)

I. Heat Production (kcal/hr)
Figure 5

A

Acylcarnitines-MC & LC (pmol/mg protein)

B

Acylcarnitines (pmol/mg protein)

C

Sum LC-OH/Sum LC

D

p-AMPK/AMPK (relative to WT LF)

E

p-ACC/ACC (relative to WT LF)

F

Serum FFA (mmol/L)

G

Muscle TG (µg/mg protein)

H

LC-CoA (pmol/mg ww)

I

Ceramide (pmol/mg ww)

J

Blood Glucose (mmol/L)

K

Blood Glucose (mmol/L)

L

Serum Insulin-Fasted (ng/dl)

M

Blood Glucose (% initial at t=0)

N

p-AKT/AKT (relative to WT LF)