Genetic and pharmacological inhibition of malonyl CoA decarboxylase does not exacerbate age-related insulin resistance in mice

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ABSTRACT

Aging is associated with the development of chronic diseases such as insulin resistance and type 2 diabetes. A reduction in mitochondrial fat oxidation is postulated to be a key factor contributing to the progression of these diseases. Our aim was to investigate the contribution of impaired mitochondrial fat oxidation towards age-related disease. Mice deficient for malonyl CoA decarboxylase (MCD−/−), a mouse model of reduced fat oxidation, were allowed to age while lifespan and a number of physiological parameters (glucose tolerance, insulin tolerance, indirect calorimetry) were assessed. Decreased fat oxidation in MCD−/− mice resulted in the accumulation of lipid intermediates in peripheral tissues, but this was not associated with a worsening of age-associated insulin resistance and conversely, improved longevity. This improvement was associated with reduced oxidative stress and reduced acetylation of the antioxidant enzyme, superoxide dismutase 2 (SOD2) in muscle but not the liver of MCD−/− mice. These findings were recapitulated in aged mice treated with an MCD inhibitor (CBM-3001106), and these mice also demonstrated improvements in glucose and insulin tolerance. Therefore, our results demonstrate that in addition to decreasing fat oxidation, MCD inhibition also has novel effects on protein acetylation. These combined effects protect against age-related metabolic dysfunction, demonstrating that MCD inhibitors may have utility in the battle against chronic disease in the elderly.
INTRODUCTION

It is well established that aging is associated with the development of a number of chronic diseases, some of which include insulin resistance, type 2 diabetes, and cardiovascular disease (1; 2). The development of chronic diseases with aging is due in part to a reduction in mitochondrial function, which manifests as a decline in fatty acid oxidative capacity, and is believed to result in the accumulation of intracellular lipid intermediates that interfere with normal organ function (3; 4).

Malonyl CoA decarboxylase (MCD), is an important enzyme involved in the regulation of fatty acid oxidation, since it controls cellular levels of malonyl CoA, a potent endogenous inhibitor of carnitine palmitoyl transferase 1, the rate-limiting enzyme of mitochondrial fatty acid uptake (5). We have shown that acute inhibition of MCD decreases fatty acid oxidation rates in the heart and improves functional recovery during reperfusion following ischemia (6). We observed similar results in a mouse model of MCD deficiency (MCD-/-) (7), and have shown that these mice are also protected against skeletal muscle and cardiac insulin resistance induced by high fat feeding (8; 9).

Although MCD inhibition protects against acute disease, due to the suggestion that impaired mitochondrial fatty acid oxidative capacity can contribute to disease progression in the elderly (3; 4), it became important to determine the potential consequences of lifelong MCD deficiency on whole body physiology, which was examined in aged MCD-/- mice and their wild type (WT) littermates.
RESEARCH DESIGN AND METHODS

Animal Studies

All animals received care according to the Canadian Council on Animal Care and the University of Alberta Health Sciences Animal Welfare Committee. MCD-/- mice and their WT littermates were placed on a standard chow/low fat diet (4% kcal from lard) and separated into two cohorts. In the first cohort, WT and MCD-/- mice were monitored until death to determine average lifespan. In the second cohort, male WT and MCD-/- mice at 70-75 wks of age were subjected to a number of experimental procedures described below. In a separate study, 16-month-old C57BL/6J mice were treated with either vehicle control or an MCD inhibitor (CBM-3001106 dissolved in a 1:1:1:7 mixture of ethanol:dimethylsulfoxide:cremophore:water, 10 mg/kg via daily oral gavage for 4 weeks) and subjected to a number of experimental procedures described below. Once all *in vivo* physiological parameters were assessed, animals were euthanized via an intraperitoneal injection of sodium pentobarbital (12 mg) in the random fed state 4 hrs into their dark cycle. Tissues were excised and immediately frozen in liquid N\textsubscript{2} for biochemical analyses.

**Palmitate Oxidation Rates**

A separate cohort of WT and MCD-/- mice were treated with either vehicle control or CBM-3001106 (10 mg/kg) via oral gavage once daily for 2 days (injections at 5:00 pm). The following morning mice were euthanized and hearts extracted for isolated working heart perfusion with 5 mM glucose and 0.4 mM \([9,10-^3\text{H}]\)palmitate bound to 3% bovine serum albumin, and palmitate oxidation rates were assessed as previously described (10).
Glucose/Pyruvate/Insulin Tolerance Tests

Glucose, pyruvate, and insulin tolerance tests were performed 6 hr after food withdrawal in mice using glucose, pyruvate and insulin doses of 2 g/kg, 2 g/kg, and 0.7 U/kg, respectively. Plasma insulin concentrations were determined via use of a commercially available kit (Alpco Diagnostics) as previously described (11).

Whole body in vivo Metabolic Assessment and Exercise Tolerance

In vivo metabolic assessment via indirect calorimetry was performed using the Oxymax CLAMS (Columbus Instruments). Animals were initially acclimatized in the system for a 24 hr period, the subsequent 24 hr period was utilized for data collection. Exercise capacity was performed by running animals on a calibrated, motor-driven treadmill (Columbus Instruments) as previously described (12).

Enzyme Activity Assays

Citrate synthase activity was determined in frozen gastrocnemius (10 mg) as previously described (12). PDH activity was determined in frozen gastrocnemius (20 mg) using a radiometric assay described by Constantin-Teodosiu et al. (13).

Determination of Lipid Intermediates

Tissue TAGs were extracted with a 2:1 chloroform-methanol solution and quantified with a commercially available enzymatic assay kit (Roche), whereas DAGs were extracted in 0.8 ml of 1 mM NaCl and quantified via TLC utilizing the DAG kinase assay (8; 12). Tissue was extracted for ceramide with 1 mL of a 1:1:1 chloroform-methanol-1 N HCl in the presence of 0.3 mL
saline solution, while long chain acyl CoA was extracted in 200 µL of cold 100 mM KH$_2$PO$_4$ for HPLC analysis as previously described (8; 12). To determine malonyl CoA content, tissue was extracted in 300 µL of 6% (v/v) perchloric acid and 2 mM DTT for HPLC analysis as previously described (8; 12).

**Tissue Homogenate Preparation and Immunoblot Analysis**
Frozen gastrocnemius or liver tissue (25-30 mg) was homogenized in buffer containing 50 mM Tris HCl (pH 8 at 4°C), 1 mM EDTA, 10% glycerol (w/v), 0.02% Brij-35 (w/v), 1 mM DTT, protease and phosphatase inhibitors (Sigma), and subjecting to Western blotting protocols as previously described (11). Primary antibodies were prepared in a 1/1000 dilution in 5% BSA. Immunoprecipitation with anti-acetyl-lysine antibodies followed by Western blotting methods was also used to detect overall protein acetylation and superoxide dismutase 2 (SOD2) acetylation as previously described (14).

**Protein Carbonylation and Lipid Peroxidation**
Protein carbonylation was assessed in gastrocnemius muscle and liver extracts following instructions from a kit from Millipore (S7150). Overall carbonylation was normalized against β-actin protein expression. Lipid peroxidation was assessed in gastrocnemius muscle extracts following the instructions from a lipid hydroperoxide assay kit from Millipore (437639).

**Statistical Analysis**
All values are presented as mean ± standard error (SE). The significance of differences was determined by the use of a Kaplan Meier survival analysis, an unpaired two-tailed Student’s t-
test, or a two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis where appropriate.

RESULTS

Increased Malonyl CoA Content in Aged MCD-/- Mice is Associated with a Substrate Switch in Metabolism.

As predicted based on the increase in malonyl CoA content in muscle and liver (Figure 1A/B), use of a comprehensive lab animal monitoring system (CLAMS) revealed that whole body fatty acid oxidation rates are decreased in aged MCD-/- mice, evidenced by the reduction in whole body oxygen consumption rates and elevation in respiratory exchange ratio (Figure 1C/D). Further support for a reduction in fatty acid oxidation rates in aged MCD-/- mice was also seen during aerobic isolated working heart perfusion experiments, where a significant decrease in palmitate oxidation rates was observed (Figure 1E). Consistent with a fuel substrate switch favoring carbohydrate oxidation, gastrocnemius muscle pyruvate dehydrogenase (PDH) activity, the rate-limiting enzyme of glucose oxidation, was elevated in aged MCD-/- mice (Figure 1F), and was in a more active-dephosphorylated state (Supplementary Figure 1A). The decreased oxygen consumption rates in aged MCD-/- mice could not be explained by changes in gastrocnemius mitochondrial citrate synthase activity (Supplementary Figure 1B) or protein expression of PGC1α (Supplementary Figure 1C), a key transcriptional regulator of mitochondrial biogenesis. Furthermore, the decrease in oxygen consumption and fatty acid oxidation in aged MCD-/- mice did not impair their exercise tolerance (Supplementary Figure 1D/E), affect overall body weight (Figure 1G), or increase their adiposity, as epididymal and
perirenal fat mass were comparable to that observed in their aged WT littermates (Figure 1H/I). Although malonyl CoA levels in the brain are important regulators of appetite and peripheral activity (15), food intake and ambulatory activity were not different between aged MCD−/− mice and their aged WT littermates (Supplementary Figure 2A/B).

**Muscle and Hepatic Lipid Intermediate Accumulation in Aged MCD−/− Mice does not Impair Glycemic Control.**

Aged MCD−/− mice demonstrated no accumulation of TAG or ceramide levels in gastrocnemius muscle, but did accumulate certain species of long chain acyl CoA and showed a trend towards an accumulation of DAG (Figure 1J-M). Although liver weights and plasma levels of free fatty acids and TAGs were not altered in aged MCD−/− mice (Supplementary Figure 3), hepatic TAG levels were elevated with no change in long chain acyl CoA esters or ceramide content (Figure 2A-C). In contrast, hepatic DAG content was significantly decreased in aged MCD−/− mice (Figure 2D). Despite these alterations in muscle and hepatic lipid content, random fed and fasted glucose and insulin levels remained similar (Figure 2E/F), while both glucose and pyruvate tolerance were not impaired in aged MCD−/− mice (Figure 2G/H). Conversely, the age-related impairment in insulin tolerance was not as severe in MCD−/− mice (Figure 2I). Furthermore, Akt and GSK3β phosphorylation, key elements in the insulin signaling cascade, were similar in the liver and muscle of aged MCD−/− mice (Figure 2J/K). Although we did not directly assess glycolysis rates, protein expression of a number of glycolytic enzymes was similar in gastrocnemius muscles and livers from aged MCD−/− mice and their WT littermates (Supplementary Figure 4A/B). Additional support indicating that MCD deficiency does not affect glycolysis was seen in young MCD−/− mice and their WT littermates, where we
observed similar glycolysis rates during aerobic isolated working heart perfusions (Supplementary Figure 4C), reproducing our previous findings (7).

**Improved Lifespan in MCD-/- mice is Associated with Decreased Oxidative Stress.**

Reduced fatty acid oxidation rates and lipid accumulation in MCD-/- mice were not associated with reduced longevity, on the contrary, we observed an increased lifespan in MCD-/- mice (28% increase) versus their WT littermates (Figure 3A). In addition, we observed an increased expression of MCD protein and subsequent reduction in malonyl CoA content in the gastrocnemius muscle of aged WT mice versus their young counterparts (Figure 3B/C). Expression of sirtuin 3 (SIRT3), a critical factor influencing mitochondrial function, was not altered in gastrocnemius muscle and liver of aged MCD-/- mice (Figure 3D). Interestingly, we observed a significant decrease in oxidative stress in gastrocnemius muscle from aged MCD-/- mice (Figure 3E/F), which was associated with a significant reduction in the acetylation of the antioxidant enzyme SOD2 (Figure 3G). These results appeared to be specific to skeletal muscle, as SOD2 acetylation and markers of oxidative stress were not different in livers from aged MCD-/- mice versus their WT littermates (Supplementary Figure 5A/B & 6).

**MCD Inhibition Decreases Muscle Acetylation and Improves Age-Related Impairments in Glycemic Control.**

Recent studies have suggested that in addition to controlling fatty acid oxidation rates via malonyl CoA, MCD may also function as an acetylase (16). In support of this, similar to SOD2 acetylation, overall protein acetylation was also reduced in gastrocnemius muscle from aged MCD-/- mice (Supplementary Figure 7). Moreover, we treated C2C12 myotubes with an MCD
inhibitor (CBM-3001106, 10 µM) that we previously showed to decrease fatty acid oxidation rates (17), and observed a decrease in overall protein acetylation (Figure 4A). Next, we treated aged C57BL/6J mice with CBM-3001106 for 4 weeks (10 mg/kg via daily oral gavage, a dose that did not affect food intake or animal activity (Supplementary Figure 2C/D)), which recapitulated the acetylation effects observed in gastrocnemius muscles from aged MCD-/- mice, though SOD2 acetylation only showed a trend towards a reduction (Figure 4B/C). Also similar to aged MCD-/- mice, no differences were observed for total liver protein acetylation or liver SOD2 acetylation in CBM-3001106 treated aged mice (Supplementary Figure 5C/D). In contrast to findings observed in aged MCD-/- mice, MCD inhibition via CBM-3001106 treatment for 3 weeks did not reduce oxygen consumption in aged C57BL/6J mice (Figure 4E), though we did observe a mild increase in RER in the latter half of the dark cycle (Figure 4F). In addition, gastrocnemius muscle long chain acyl CoA and ceramide content were unaltered in aged mice treated with CBM-3001106 (Figure 4G/H). Interestingly, pharmacological MCD inhibition with CBM-3001106 for 4 weeks yielded a significant improvement in both glucose and insulin tolerance in aged mice (Figure 4I/J). These findings were independent of changes in body weight (Figure 4K) and adiposity, as nuclear magnetic resonance imaging demonstrated similar body fat content in aged mice treated with CBM-3001106 (Figure 4L). Illustrating that our results treating aged mice with CBM-3001106 were likely due to an inhibition of MCD, isolated working heart perfusion studies from MCD-/- mice and their WT littermates treated for 2 days with CBM-3001106 demonstrated that CBM-3001106 only reduced palmitate oxidation rates in the WT mouse heart, but not hearts from MCD-/- mice (Figure 4M). Furthermore, treatment of MCD-/- mice with CBM-30011006 had no effect on protein acetylation in gastrocnemius muscle (Supplementary Figure 8).
DISCUSSION

Recent studies postulate that decreased mitochondrial fatty acid oxidation is responsible for the accumulation of intracellular lipid intermediates that contribute to the development of numerous chronic diseases in the elderly (3; 18). Therefore, MCD-/- mice, which have decreased fatty acid oxidation rates should be at an increased risk for developing insulin resistance, heart failure, and hepatic steatosis. All of these risks in combination would be expected to shorten the overall lifespan of these animals.

In contrast, MCD-/- mice displayed a significant increase in lifespan of ~30%, which was unexpected based on their lower overall fatty acid oxidative capacity. However, it is important to note that our n number for these experiments is not optimal for a true lifespan study, despite our findings being statistically significant (19). Nevertheless, the critical observation that must be highlighted from our study is that we did not observe any premature metabolic disease development or early death in MCD-/- mice, despite the accumulation of lipid intermediates in key metabolic tissues. Indeed, we observed a strong trend towards an increase in muscle DAG content, which numerous studies have implicated as a key lipid intermediate in mediating skeletal muscle insulin resistance (20). Despite this trend, glucose tolerance was not worse in aged MCD-/- mice, and their age-related impairment in insulin tolerance was not as severe in comparison to their WT littermates. This may be due to the fact that muscle ceramide content was not altered in aged MCD-/- mice, and we (12) and others (21) have shown that increased ceramide content is a key factor precipitating skeletal muscle insulin resistance. With regards to insulin signaling, the aging-associated reduction in skeletal muscle and hepatic Akt and GSK3β
phosphorylation were comparable in aged MCD-/- mice versus their aged WT littermates, but these findings would be strengthened if measured in the fasted and insulin-stimulated state (our assessment was carried out in tissues from animals sacrificed 4 hrs into their dark cycle).

Our findings in aged MCD-/- mice are not meant to insinuate that the accumulation of tissue lipid intermediates does not cause insulin resistance, as there is a mounting body of evidence supporting that ceramide and DAG are critical to the pathogenesis of insulin resistance (22-24). However, it is likely that some other factor or mechanism outweighs the harmful effects of increased lipid intermediates, and somehow protects aged MCD-/- mice from developing metabolic disease. Indeed, we have previously shown that young MCD-/- mice are actually protected from obesity-induced skeletal muscle insulin resistance, which may arise from a reduction in the incomplete oxidation of fatty acids observed during obesity (9). Thus, it is possible that incomplete fatty acid oxidation contributes to the insulin resistance in elderly patients, and may explain why the age-related impairment in insulin tolerance is not as severe in aged MCD-/- mice. On the other hand, elegant experiments from Bouzakri et al. demonstrate a critical role for MCD in regulating substrate selection in human muscle, whereby inhibition of MCD decreases palmitate oxidation, which is associated with a corresponding increase in insulin-stimulated GLUT4 translocation, glucose uptake, and glucose oxidation (25). Accordingly, our RER data and gastrocnemius PDH activity in aged MCD-/- mice are consistent the observations of Bouzakri et al., as aged MCD-/- mice demonstrate a switch in substrate preference for carbohydrates.

Another significant concern with reductions in fatty acid oxidation in the elderly population is that it may increase their risk of developing hepatic steatosis and non-alcoholic fatty liver disease (20). Although hepatic TAG content was greater in aged MCD-/- mice, they had a
subsequent reduction in hepatic DAG content. This finding is of particular interest, as DAG accumulation has been proposed to be a key player in the development of hepatic insulin resistance observed in elderly patients and those with type 2 diabetes (20). These differences we observed with regards to DAG content in the muscle and liver illustrate and support the notion that the most powerful effect of lowering DAG content to improve insulin sensitivity appears to take place in the liver (26). To our surprise, despite this marked lowering in hepatic DAG content, the reduction in oxidative stress in aged MCD-/- mice was only observed in skeletal muscle and not the liver. However, these findings do not rule out the decreased hepatic DAG content contributing to the overall metabolic health benefits seen in aged MCD-/- mice.

Since our findings in MCD-/- mice could potentially be model-specific and due to compensatory adaptations to global MCD deletion throughout development, we examined the effect of CBM-3001106, an MCD inhibitor, in aged mice. Our findings in this study are the first ever published in vivo findings with this MCD inhibitor, and similar to genetic MCD deficiency, we observed protection against age-related insulin resistance with pharmacological MCD inhibition. On the contrary, we also observed protection against glucose intolerance following pharmacological MCD inhibition. Reasons for this discrepancy between our genetic and pharmacological data are unclear at the moment, but could be due to the fact that pharmacological MCD inhibition produces robust effects on both inhibition of fatty acid oxidation and stimulation of glucose oxidation (6), whereas genetic MCD deficiency produces a metabolic phenotype that only becomes apparent under metabolic stress (7; 8). Nevertheless, in spite of these minor discrepancies, a key critical take-home message from our findings is that an inhibition of fatty acid oxidation, either via genetic or pharmacological MCD inhibition, does not worsen age-related metabolic disease as would be predicted based on current dogma (3; 20; 24).
Of interest, our results support the recent suggestion that MCD may be a bifunctional enzyme that also acts as an acetylase regulating protein acetylation (16). However, our findings do not confirm whether MCD acts as an acetylase itself, or simply controls protein acetylation via controlling acetyl CoA content (Supplementary Figure 9). It will be important for future studies to investigate this further, and determine whether our MCD inhibitor, CBM-3001106, which inhibits MCD’s decarboxylase activity, also reduces protein acetylation via inhibiting an independent acetylase activity of MCD. We believe that the reduced protein acetylation following treatment with CBM-3001106 is likely a result of a reduction in acetyl CoA supply for mitochondrial acetylases (Supplementary Figure 9). Indeed, MCD localizes to both the mitochondria and peroxisomes (5), and one target we observed to have lower acetylation following genetic or pharmacological MCD inhibition, SOD2, primarily localizes to the mitochondria in mammals (27). Our results are limited though by encompassing measurements of acetylation in whole cellular extracts versus mitochondrial extracts, which would be the important compartment to measure regarding SOD2 acetylation. In spite of this limitation, because ~95% of acetyl CoA, which is the substrate for protein acetylation, is localized to the mitochondria (28), we do believe our total protein and SOD2 acetylation results likely reflect changes within the mitochondrial compartment. Furthermore, the deacetylases that regulate SOD2 acetylation, such as SIRT3, are also regulated via nicotinamide adenine dinucleotide (NAD+) levels (29). Although muscle SIRT3 protein expression was similar in aged MCD-/- mice, our future studies will need to measure mitochondrial NAD+ content specifically, which may potentially mediate the observed changes in SOD2 acetylation by increasing SIRT3 activity.

To our surprise, the observed changes in gastrocnemius protein acetylation were not observed in livers from aged MCD-/- mice or aged mice treated with CBM-3001106. We are not
certain why such changes would only be observed in the muscle and not the liver following MCD deficiency and/or inhibition, but could be related to our finding that MCD protein expression is only increased in muscle during aging, but not in the liver (Supplementary Figure 10). In addition, it should be noted though that our acetylation results were completed via immunoprecipitation with anti-acetyl-lysine antibodies followed by immunoblotting techniques with an SOD2 antibody to detect SOD2 acetylation. Thus, more sophisticated proteome techniques that examine protein-specific changes to the acetylome are necessary before we can truly say that MCD inhibition also affects protein acetylation, in addition to reducing fatty acid oxidation.

In conclusion, our data challenges the hypothesis that decreased fat oxidation accelerates the progression of age-related diseases. Instead, we demonstrate that life-long whole body deficiency of MCD is associated with normal insulin sensitivity, decreased whole body fatty acid oxidation, increased whole body carbohydrate utilization, and normal liver function. Furthermore, pharmacological MCD inhibition produced marked improvements in glucose homeostasis in aged mice, though it will be important to in future studies to determine whether MCD inhibition improves glucose homeostasis in aged obese animals, as the world’s elderly population is frequently also overweight and/or obese. Preliminary studies in our laboratory suggest that pharmacological MCD inhibition also protects against obesity-induced insulin resistance in young mice (data not shown). Whether such findings will translate to aged obese mice remains to be determined, and with the combined elements of both aging and obesity leading to increases in organ lipid accumulation, MCD inhibition may not be favorable in this scenario. Nevertheless, our previous studies have shown that MCD inhibition and/or deficiency in young mice is associated with protection against ischemia/reperfusion injury (6; 7; 10) and high fat diet-
induced insulin resistance (9), while even reducing appetite and body weight gain (15). Taken together with our observations that MCD deficiency and/or inhibition appears to protect against age-related insulin resistance, we illustrate an important paradigm shift, whereby chronic inhibition of fatty acid oxidation does not exacerbate age-related disease, but may instead have overall metabolic health benefits in the elderly.

AUTHOR CONTRIBUTIONS

JRU designed the study, researched data, and wrote the manuscript. NF researched data, contributed to the discussion, and reviewed/edited the manuscript. WK, LZ, JM, VKS, AF, KG, DGL, and CSW researched data and reviewed/edited the manuscript. JSJ and JRBD contributed to the discussion and reviewed/edited the manuscript. GDL designed the research and wrote the manuscript, and is the guarantor of the manuscript and takes responsibility for all aspects of the work.

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DISCLOSURES

GDL and JRBD are shareholders and officers of Metabolic Modulators Research Ltd., a company with commercial interests in the development of MCD inhibitors.
REFERENCES

FIGURE LEGENDS

Figure 1. Increased malonyl CoA content in aged MCD-/- mice is associated with decreased whole body fatty acid oxidation rates and an increase in muscle lipid intermediates.
A: Gastrocnemius malonyl CoA (n = 4, 6) and B: Liver malonyl CoA content (n = 4, 5) in aged male WT and MCD-/- mice. C: Whole body oxygen consumption rates, and D: 24 hr respiratory exchange ratio (RER), in aged male WT and MCD-/- mice (n = 6). E: Palmitate oxidation rates in isolated aerobically perfused working hearts from aged male WT and MCD-/- mice (n = 7, 8). F: Gastrocnemius PDH activity (n = 6), G: Body weights, and H: Epididymal (Epi) fat pad weights and I: Perirenal fat pad weights in aged male WT and MCD-/- mice (n = 7-9). J: Triacylglycerol, K: Long chain acyl CoA, L: Diacylglycerol, and M: Ceramide content in gastrocnemius muscle of aged male WT and MCD-/- mice (n = 6). Values represent mean ± SE. Differences were determined by the use of an unpaired, two-tailed Student’s t-test, or a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P<0.05, significantly different from aged WT littermate.

Figure 2. Hepatic TAG content is increased and DAG content decreased in aged MCD-/- mice, which is associated with a mild attenuation of the age-related impairment of insulin tolerance.
A: Triacylglycerol, B: Long chain acyl CoA, C: Diacylglycerol, and D: Ceramide content in livers of aged male WT and MCD-/- mice (n = 5, 6). E: Random fed and fasted plasma glucose and F: Insulin levels in aged male WT and MCD-/- mice (n = 5-6). G: Glucose tolerance in aged male WT and MCD-/- mice following a 6 hr fast (n = 7, 8). H: Pyruvate tolerance in aged male WT and MCD-/- mice following a 6 hr fast (n = 4, 5). I: Insulin tolerance in aged male WT and
MCD-/− mice following a 6 hr fast (n = 7, 8). J: Akt serine 473 and GSK3β serine 9 phosphorylation in livers of aged male WT and MCD-/− mice (n = 5, 6). K: Akt serine 473 and GSK3β serine 9 phosphorylation in gastrocnemius muscles of aged male WT and MCD-/− mice (n = 5, 6). Values represent mean ± SE. Differences were determined by the use of an unpaired, two-tailed Student’s t-test, or a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P<0.05, significantly different from aged WT littermate. †P<0.05, significantly different from young counterpart.

**Figure 3.** MCD-/− mice exhibit enhanced longevity that is associated with a reduction in muscle oxidative stress.
A: Kaplan-Meier survival curve analysis of MCD-/− mice (n = 16) and their WT littermates (n = 8). B: Gastrocnemius muscle MCD protein expression, and C: Malonyl CoA content in young and aged WT littermates (n = 3). D: SIRT3 protein expression in the liver and gastrocnemius muscle of aged male WT and MCD-/− mice (n = 3). E: Gastrocnemius protein carbonylation and F: lipid peroxidation in aged male WT and MCD-/− mice (n = 3). G: Superoxide dismutase 2 (SOD2) acetylation in gastrocnemius muscle from aged male WT and MCD-/− mice (n = 5). Differences were determined by the use of an unpaired, two-tailed Student’s t-test, or a Kaplan-Meier survival curve analysis. *P<0.05, significantly different from aged WT littermate.

**Figure 4.** Pharmacological MCD inhibition is associated with a reduction in protein acetylation and improves glucose homeostasis in aged mice.
A: Overall protein acetylation in C2C12 myotubes treated with saline or CBM-3001106 (n = 6). B: Overall protein acetylation in gastrocnemius muscle from aged C57BL/6J mice treated with...
saline or CBM-3001106 (n = 5). C: Superoxide dismutase 2 (SOD2) acetylation in gastrocnemius muscle from aged C57BL/6J mice treated with saline or CBM-3001106 (n = 5). D: Whole body oxygen consumption rates, and E: 24 hr respiratory exchange ratio (RER), in aged C57BL/6J mice treated with saline or CBM-3001106 (n = 6). F: Long chain acyl CoA, G: Ceramide, and H: Triacylglycerol (TAG) content in gastrocnemius muscle of aged C57BL/6J mice treated with saline or CBM-3001106 (n = 6). I: Glucose tolerance, and J: Insulin tolerance in aged C57BL/6J mice treated with saline or CBM-3001106 following a 6 hr fast (n = 6, 8). K: Body weights and L: Body composition (fat and lean body mass) in aged C57BL/6J mice treated with saline or CBM-3001106 (n = 6, 7). M: Palmitate oxidation rates in isolated aerobically perfused working hearts from male WT and MCD-/- mice treated with either saline or CBM-3001106 (n = 4, 5). Values represent mean ± SE. Differences were determined by the use of an unpaired, two-tailed Student’s t-test, or a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P<0.05, significantly different from aged saline treated mice. †P<0.05, significantly different from aged pre-MCDi (MCD inhibitor) mice.
Figure 2

A. Triacylglycerol

B. Long Chain Acyl CoA

C. Diacylglycerol

D. Ceramide

E. Blood Glucose (mM)

F. Plasma Insulin (ng/mL)

G. Glucose Tolerance

H. Pyruvate Tolerance

I. Insulin Tolerance

J. Western Blot: P-Akt/Akt and P-GSK3β/GSK3β

K. Western Blot: P-Akt, Akt, P-GSK3β, GSK3β
Figure 3

A

Percent Survival

B

Young WT  Aged WT

MCD  Actin

C

Gastrocnemius Malonyl CoA Content

D

Liver

SIRT3  Actin

Gastrocnemius

SIRT3  Actin

WT  MCD/-

E

Gastrocnemius Protein Carbonylation

F

Gastrocnemius Lipid Peroxidation

G

Ac-SOD2  SOD2

WT  MCD/-
Figure 4

**A** Overall Acetylation in C2C12 Myotubes

**B** Overall Acetylation in Aged C57BL/6J Mice

**C** Ac-SOD2/SOD2 Acetylation in Aged MCDi Mice

**D** Oxygen Consumption in Aged C57BL/6J Mice

**E** RER (Respiratory Exchange Ratio) in Aged C57BL/6J Mice

**F** Long Chain Acyl CoA in Aged C57BL/6J Mice

**G** Ceramide in Aged C57BL/6J Mice

**H** TAG (Triacylglycerol) in Aged C57BL/6J Mice

**I** Glucose Tolerance in Aged C57BL/6J Mice

**J** Insulin Tolerance in Aged C57BL/6J Mice

**K** Body Composition in Aged C57BL/6J Mice

**L** Palmitate Oxidation in Aged C57BL/6J Mice

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*Control vs. MCDi*
Supplementary Figure 2

A. Ambulatory Activity

B. Food Intake

C. Ambulatory Activity

D. Food Intake

- Aged WT Male
- Aged MCD-/- Male
- Aged Vehicle
- Aged MCDi
Supplementary Figure 3

A. Liver Wt. (% of BW)

B. Plasma FFAs (mM)

C. Plasma TAGs (mg/dL)

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<td>Aged MCD-/-</td>
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Ad Libitum Fasted Fasted Ad Libitum

Aged WT Male Aged MCD-/- Male

Supplementary Figure 3
Aged WT Male ■ Aged MCD-/- Male

**A**

<table>
<thead>
<tr>
<th>Protein</th>
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<th>MCD-/-</th>
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<tr>
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<td>Hsp90</td>
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**Gastrocnemius**

**B**

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<td>Hsp90</td>
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**Liver**

**C**

***Myocardial Glycolysis Rates***

- **w/o Insulin**
  - Young WT Male: 1500 μmol/g dry wt/min
  - Young MCD-/- Male: 4500 μmol/g dry wt/min

- **w/ Insulin**
  - Young WT Male: 10000 μmol/g dry wt/min
  - Young MCD-/- Male: 3000 μmol/g dry wt/min
Supplementary Figure 5

(A) Overall Acetylation

(B) Ac-SOD2/SOD2

(C) Overall Acetylation

(D) Ac-SOD2/SOD2

Liver
Supplementary Figure 6

Liver Protein Carbonylation

Aged WT Male  Aged MCD-/- Male

Arbitrary Units (Normalized to Actin)

Diabetes
Overall Acetylation

Tubulin

MCD-/- + Control

MCD-/- + MCDi

Acetylation/Tubulin

4.5

3.0

1.5

0
CHANGES IN MITOCHONDRIAL METABOLISM & FATTY ACID OXIDATION RATES
Liver

Young WT  Aged WT

MCD
Actin

Young WT  Aged WT

MCD/Actin
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Aged MCD-/- mice exhibit normal mitochondrial function and exercise capacity, but an increase in PDH activity.
A: PDH phosphorylation in gastrocnemius muscle of aged male WT and MCD-/- mice B: Gastrocnemius citrate synthase activity (n = 6), and C: PGC1α protein expression in aged male WT and MCD-/- mice. D: Treadmill time (min) and E: distance (meters (m)) in aged male WT and MCD-/- mice ran on a motor-driven exercise treadmill. Values represent mean ± SE. Differences were determined by the use of an unpaired, two-tailed Student’s t-test. *P<0.05, significantly different from aged WT littermate.

Supplementary Figure 2. Activity and food intake during housing in metabolic cages.
A: Ambulatory activity and B: Food intake in aged male WT and MCD-/- mice housed in metabolic cages (n = 6). C: Ambulatory activity and B: Food intake in aged male C57BL/6J mice housed in metabolic cages and treated with either vehicle control or CBM-3001106 (n = 6). Values represent mean ± SE. Differences were determined by the use of an unpaired, two-tailed Student’s t-test.

Supplementary Figure 3. Plasma lipid profiles in aged WT and MCD-/- mice.
A: Liver weights (normalized to animal body weight) in aged male WT and MCD-/- mice (n = 7, 9). B: Ad libitum and C: fasted plasma free fatty acids (n = 5), and D: ad libitum and E: fasted triacylglycerols (n = 4, 5) in aged male WT and MCD-/- mice. Values represent mean ± SE.
**Supplementary Figure 4.** Glycolytic enzyme expression in aged MCD-/- mice.

A: Protein expression of a number of glycolysis enzymes (hexokinase, pyruvate kinase (PKM1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase (PGAM1), aldolase) in gastrocnemius muscle from aged male WT and MCD-/- mice (n = 5-6). B: Protein expression of a number of glycolysis enzymes (PKM1, enolase, GAPDH, PGAM1, aldolase) in livers from aged male WT and MCD-/- mice (n = 5). C: Glycolysis rates in aerobically perfused isolated working hearts from young WT and MCD-/- mice (n = 3-4). Values represent mean ± SE.

**Supplementary Figure 5.** Overall protein and SOD2 acetylation in liver.

A: Overall protein acetylation and B: SOD2 acetylation in livers from aged male WT and MCD-/- mice (n = 4, 5). C: Overall protein acetylation and D: SOD2 acetylation in livers from aged male C57BL/6J mice treated with either vehicle control or CBM-3001106 (n = 4, 5). Values represent mean ± SE.

**Supplementary Figure 6.** Liver protein carbonylation is not altered in aged MCD-/- mice

Liver protein carbonylation in aged male WT and MCD-/- mice (n = 5, 6). Values represent mean ± SE.

**Supplementary Figure 7.** Overall protein acetylation in gastrocnemius muscle is reduced in aged MCD-/- mice.

Overall protein acetylation in gastrocnemius muscle from aged male WT and MCD-/- mice (n = 5). Values represent mean ± SE. Differences were determined by the use of an unpaired, two-
tailed Student’s \( t \)-test, or a Kaplan-Meier survival curve analysis. \( *P<0.05 \), significantly different from aged WT littermate.

**Supplementary Figure 8.** Overall protein acetylation in gastrocnemius muscle from MCD/- mice treated with either vehicle control or CBM-3001106.

Overall protein acetylation is not reduced in gastrocnemius muscles from male MCD/- mice treated with CBM-3001106 (\( n = 4, 5 \)). Values represent mean \( \pm \) SE.

**Supplementary Figure 9.** Proposed dual role of MCD inhibition in modulating mitochondrial metabolism and fatty acid oxidation during aging.

MCD may regulate both fatty acid oxidation and overall mitochondrial metabolism via a dual mechanism, whereby peroxisomal MCD produces malonyl CoA to inhibit CPT-1 on the outer mitochondrial mechanism to inhibit fatty acid oxidation via the classic malonyl CoA – CPT-1 axis, whereas mitochondrial localized MCD produces acetyl CoA for mitochondrial acetylases that regulate mitochondrial metabolism via changing acetylation of mitochondrial proteins. Thus, MCD inhibition inhibits fatty acid oxidation rates by increasing malonyl CoA content, while also altering other aspects of mitochondrial metabolism via decreasing acetyl CoA supply for mitochondrial acetylases, thereby decreasing acetylation of mitochondrial proteins and altering their activity.

**Supplementary Figure 10.** Aging-related changes in liver MCD expression.

MCD protein expression in livers from young and aged male C57BL/6J mice (\( n = 5 \)). Values represent mean \( \pm \) SE.