Cell Metabolism
Short Article

Targeted Expression of Catalase to Mitochondria Prevents Age-Associated Reductions in Mitochondrial Function and Insulin Resistance

Hui-Young Lee,[1,2] Cheol Soo Choi,[2,5] Andreas L. Birkenfeld,[2] Tiago C. Alves,[2] Francois R. Jornayvaz,[2] Michael J. Jurczak,[1,2] Dongyan Zhang,[1,2] Dong Kyun Woo,[3] Gerald S. Shadel,[3] Warren Ladiges,[6] Peter S. Rabinovitch,[7] Janine H. Santos,[8] Kitt F. Petersen,[2] Varman T. Samuel,[2] and Gerald I. Shulman[1,2,4,*]
[1]Howard Hughes Medical Institute
[2]Department of Internal Medicine
[3]Department of Pathology
[4]Department of Cellular and Molecular Physiology
Yale University School of Medicine, New Haven, CT 06510, USA
[5]Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, 406-840 Incheon, Korea
[6]Department of Comparative Medicine
[7]Department of Pathology
University of Washington, Seattle, WA 98195, USA
[8]Department of Pharmacology & Physiology, UMDNJ - New Jersey Medical School, Newark, NJ 07101, USA
*Correspondence: gerald.shulman@yale.edu
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SUMMARY

Aging-associated muscle insulin resistance has been hypothesized to be due to decreased mitochondrial function, secondary to cumulative free radical damage, leading to increased intramyocellular lipid content. To directly test this hypothesis, we examined both in vivo and in vitro mitochondrial function, intramyocellular lipid content, and insulin action in lean healthy mice with targeted overexpression of the human catalase gene to mitochondria (MCAT mice). Here, we show that MCAT mice are protected from age-induced decrease in muscle mitochondrial function (~30%), energy metabolism (~7%), and lipid-induced muscle insulin resistance. This protection from age-induced reduction in mitochondrial function was associated with reduced mitochondrial oxidative damage, preserved mitochondrial respiration and muscle ATP synthesis, and AMP-activated protein kinase-induced mitochondrial biogenesis. Taken together, these data suggest that the preserved mitochondrial function maintained by reducing mitochondrial oxidative damage may prevent age-associated whole-body energy imbalance and muscle insulin resistance.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) and impaired glucose tolerance affect ~40% of the population over the age of 65 (Harris et al., 1998), and more than half of the 16 million Americans estimated to have T2DM are over age 60 (NDIC, 2002). However, the underlying mechanism for the increased prevalence of T2DM associated with aging is unknown. Using multinuclear magnetic resonance spectroscopy (MRS) to directly assess rates of muscle mitochondrial oxidative phosphorylation activity and intramyocellular lipid content in vivo, Petersen et al. found that healthy lean elderly individuals had an ~35% reduction in basal rates of muscle mitochondrial oxidative phosphorylation activity, which was associated with an ~30% increase in intramyocellular lipid content and severe muscle insulin resistance (Petersen et al., 2003). These results led to the hypothesis that age-associated reductions in muscle insulin sensitivity may be secondary to reduced mitochondrial activity, resulting in increased intramyocellular lipid content, leading to defective insulin signaling (Griffin et al., 1999; Shulman, 2000; Yu et al., 2002). However, it remains to be determined whether the increased intramyocellular lipid content and muscle insulin resistance associated with aging is a cause or consequence of the mitochondrial dysfunction. Furthermore, the nature of the mitochondrial dysfunction associated with aging remains unknown. Although cumulative oxidative stress has been proposed to cause age-associated reductions in mitochondrial function (Cadenas and Davies, 2000; Jang et al., 2009; Pérez et al., 2008; Shigenaga et al., 1994; Stadtman, 2002; Wei et al., 1998), this remains a controversial topic (Anderson et al., 2009; Bashan et al., 2009; Bonnard et al., 2008; Evans et al., 2005; Houstis et al., 2006; Loh et al., 2009; Ristow et al., 2009), and in vivo studies assessing the potential role of oxidative stress in causing muscle mitochondrial function are lacking.

In order to directly examine whether age-associated reductions in mitochondrial function were due to cumulative oxidative damage, and whether age-associated reductions in muscle mitochondrial function would lead to intramyocellular lipid accumulation and muscle insulin resistance, we examined both in vitro and in vivo mitochondrial function as well as intramuscular lipid content and insulin action in young and lean healthy old mice with targeted overexpression of human catalase to the mitochondria (MCAT). Whole-body and tissue-specific effects of insulin were assessed in awake young and old wild-type (WT)

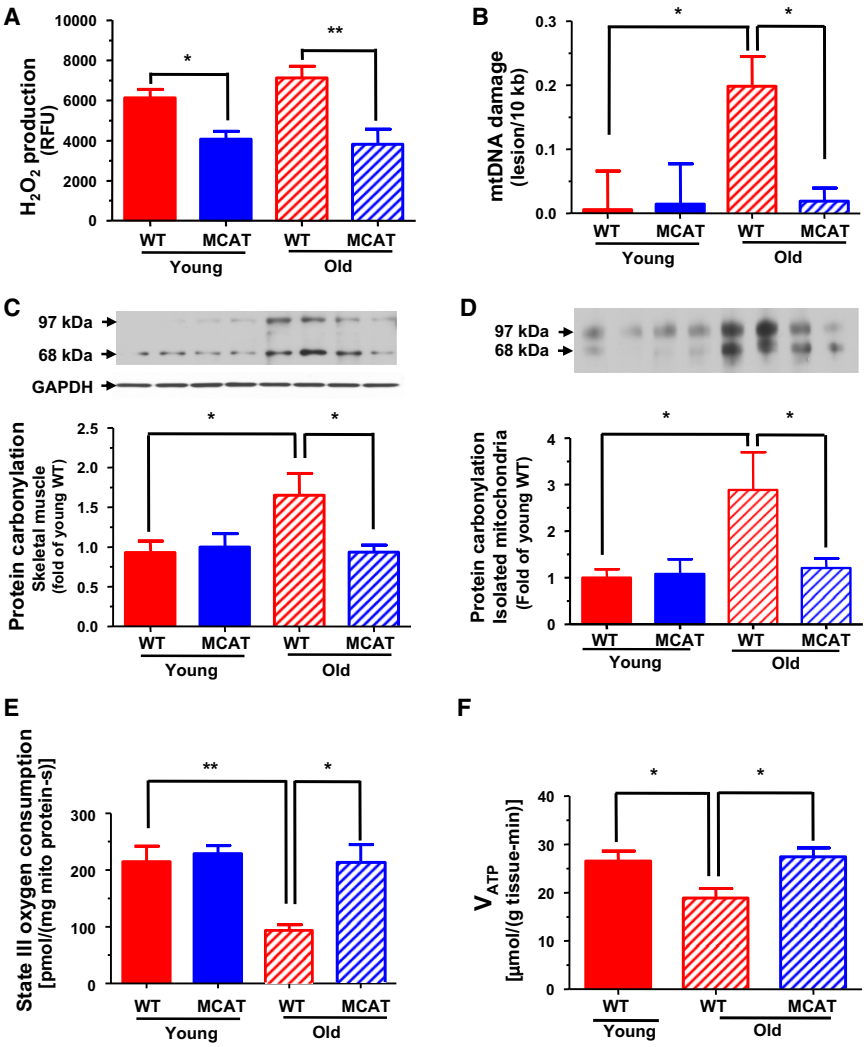

Figure 1. Hydrogen Peroxide Production, Mitochondrial Damage, and Mitochondrial Function, Both In Vitro and In Vivo, in Skeletal Muscle of Young and Old WT and MCAT Mice

(A) Maximal H_2O_2 emission of mitochondria derived from skeletal muscle (n = 10–15 per group).

(B) Mitochondrial DNA damage in skeletal muscle (n = 4–6 per group).

(C and D) Oxidative protein carbonylation in skeletal muscle extracts (C) (n = 6 per group) and in skeletal muscle mitochondria (D) (n = 6 per group).

(E) State III oxygen consumption of mitochondria derived from skeletal muscle (n = 6 per group).

(F) Basal rates of ATP synthesis in skeletal muscle assessed by in vivo ^{31}P-MRS (n = 6 per group). All data are mean ± SEM. *$p < 0.05$ and **$p < 0.01$ by ANOVA with post hoc analysis.

and MCAT mice using a hyperinsulinemic-euglycemic clamp in combination with $^{3}H/^{14}C$-labeled glucose, and in vivo rates of muscle mitochondrial ATP synthesis were assessed in vivo using ^{31}P-MRS.

RESULTS

MCAT Protects Mitochondria from Cumulative Oxidative Damage and Age-Associated Reductions in Mitochondrial Function

As an index of reactive oxygen species (ROS)-induced mitochondrial damage and oxidative phosphorylation activity, we examined muscle mitochondrial hydrogen peroxide (H_2O_2) production, mitochondrial DNA (mtDNA) damage, oxidative protein carbonylation, mitochondrial oxygen consumption, and in vitro and in vivo muscle mitochondrial ATP synthesis in age/weight-matched young and old WT and MCAT mice. The mitochondrial targeted catalase is mainly overexpressed in both slow- and fast-twitch muscle tissues (Figure S1A), and catalase overexpression attenuated muscle mitochondrial H_2O_2 production by ~45% in both young and old MCAT mice compared to WT mice (Figure 1A). There were no differences in superoxide dismutase 2 or glutathione peroxidase 1 protein expression (Figure S1B) or genomic DNA damage in gastrocnemius muscle (Figure S1C). The mtDNA damage (Figure 1B) and mitochondrial protein carbonylation (Figures 1C and 1D) were markedly increased in the old WT mice compared to the young WT and young MCAT mice. In contrast, old MCAT mice were protected from these age-associated increases in mtDNA damage (Figure 1B) and protein carbonylation (Figures 1C and 1D). These age-associated increases in mtDNA damage and protein carbonylation were associated with reductions in both state III and state IV oxygen consumption (Figures 1E and S1D) and decreased rates of muscle mitochondrial ATP synthesis (Figure 1F) assessed by in vivo ^{31}P-MRS. In contrast, old MCAT mice were protected from these age-associated reductions in both in vitro and in vivo mitochondrial function (Figures 1E and 1F).

MCAT Protects Mice from Age-Associated Reductions in Whole-Body Energy Metabolism

To assess the impact of preserved mitochondrial function on age-associated changes in whole-body energy metabolism, body composition-matched young and old MCAT and WT mice were studied by indirect calorimetry. Consistent with the decreased mitochondrial oxygen consumption and ATP synthesis observed in the old WT mice, whole-body oxygen consumption (VO_2) and energy expenditure were decreased by 6.8% and 6.9%, respectively, in old WT mice compared to young WT mice (Figures 2A and S2A). In contrast, old MCAT mice were protected from these age-associated reductions in oxygen consumption, CO_2 production, and energy expenditure (Figures 2B, 2D, and S2B). Body weights and body fat composition were identical between young MCAT and WT mice, with similar

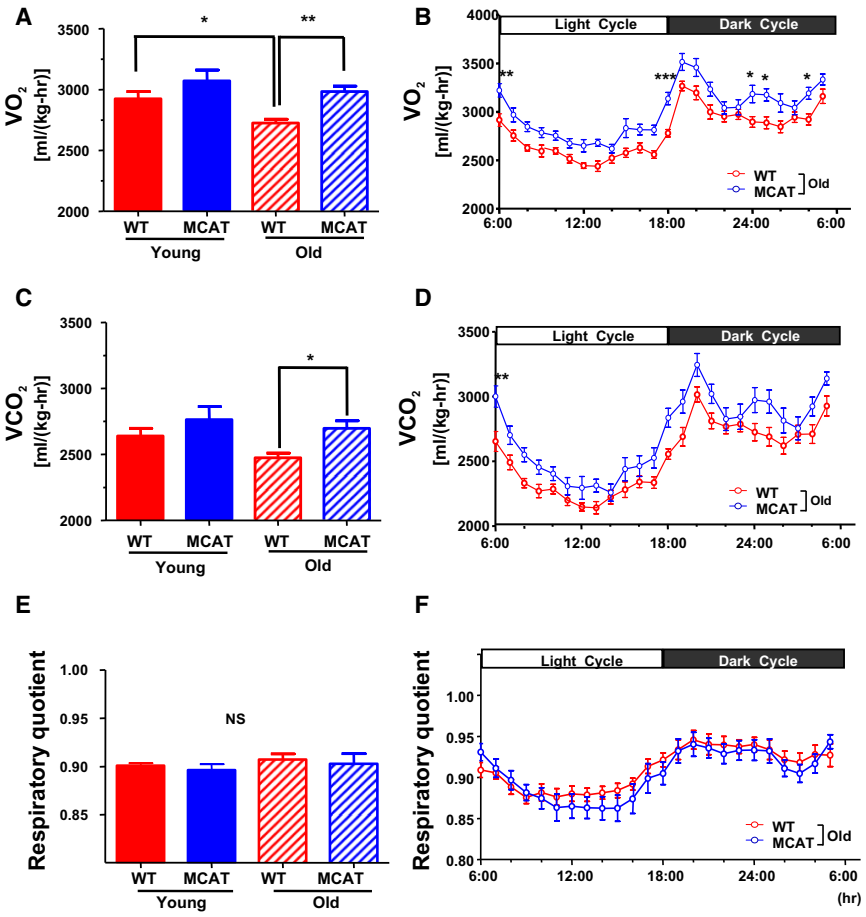

Figure 2. Whole-Body Energy Metabolism

(A–F) Whole-body oxygen consumption (VO_2) (A), CO_2 production (VCO_2) (C), and respiratory quotient (E) in young and old WT and MCAT mice during 72 hr analysis. Also shown are hour-to-hour average VO_2 (B), VCO_2 (D), and respiratory quotient (F) in old WT and MCAT mice during light/dark period. All data are mean ± SEM. n = 8 in young groups; n = 12–15 in old groups. *p < 0.05; **p < 0.01; ***p < 0.001 by ANOVA with post hoc analysis.

increases in body weight occurring with aging in both groups (Table S1). There were no differences in respiratory exchange rate (Figures 2E and 2F) or locomotor activity (Figures S2E and S2F) between the young and old WT and MCAT mice, although there was a tendency for the old MCAT mice to have a slight increase in food intake compared to the old WT mice (Figures S2C and S2D), which may explain the similar body weights and body composition in the old WT and MCAT mice despite an ~7% increase in whole-body energy expenditure in the old MCAT mice. Taken together, these data demonstrate that aging is associated with ROS-induced mitochondrial damage, which is associated with reductions in muscle mitochondrial function and whole-body energy metabolism, and that these changes are prevented by overexpression of catalase in mitochondria.

Age-Associated Reductions in Mitochondrial Function Predispose Aged Mice to Intramyocellular Lipid Accumulation and Insulin Resistance

To determine whether protection from age-associated reductions in muscle mitochondrial function would result in protection from age-associated muscle insulin resistance, we performed hyperinsulinemic-euglycemic clamp studies in the body composition-matched young and old WT and MCAT mice. Old WT mice were markedly insulin resistant, as reflected by a 35% reduction in the glucose infusion rate (GIR) required to maintain euglycemia during the hyperinsulinemic-euglycemic clamp (Figure 3A). In contrast, old MCAT mice were totally protected from age-associated whole-body insulin resistance, as reflected by a GIR, which was indistinguishable from that of the young WT and MCAT mice (Figure 3A). Whole-body insulin resistance in the old WT mice was due to decreased insulin-stimulated peripheral glucose uptake (Figure 3B), which could be attributed to decreased insulin-stimulated muscle glucose uptake, which were both normal in the old MCAT mice (Figures 3B and 3C). Although basal rates of hepatic glucose production were slightly increased in the old versus young WT mice, there were no differences in basal rates of hepatic glucose production (Table S1) or suppression of hepatic glucose production during the hyperinsulinemic-euglycemic clamp between the WT and MCAT mice (Table S2). Fasting plasma glucose and insulin concentrations were similar between WT and MCAT mice (Table S1).

Insulin resistance in skeletal muscle has been attributed to increases in diacylglycerol (DAG) content, which in turn activates protein kinase C-θ (PKCθ) and inhibits insulin signaling at the level of IRS-1 tyrosine phosphorylation (Griffin et al., 1999; Shulman, 2000; Yu et al., 2002). Consistent with this hypothesis, skeletal muscle membrane DAG content was increased by ~70% in the old WT mice (Figure 3D) and was associated with increased PKCθ activation, as reflected by increased PKCθ translocation to the plasma membrane (Figure 3E) and by a ~30% reduction in insulin activation of AKT2 (Figure 3F). In contrast, MCAT mice exhibited no increases in membrane DAG content or PKCθ activation and similar activation of AKT2 compared to young WT and MCAT mice (Figures 3D–3F). Thus, catalase overexpression in the mitochondria prevented muscle mitochondrial dysfunction, which in turn prevented increases in intramuscular DAG content, PKCθ activation, and muscle insulin resistance.

MCAT Reduce the Aging-Associated Declines in Mitochondrial Biogenesis

AMP-activated protein kinase (AMPK) is a critical regulator of mitochondrial biogenesis (Bergeron et al., 2001; Hardie, 2007;

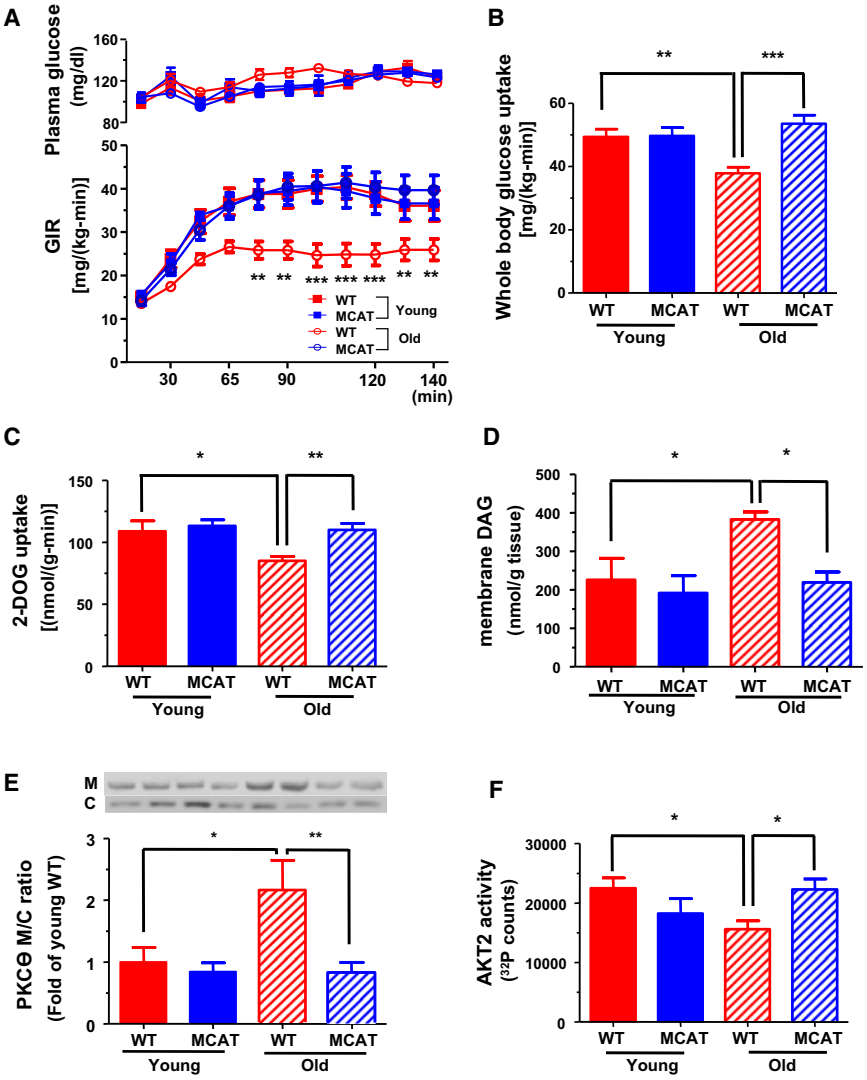

Figure 3. Insulin Sensitivity, Intramyocellular Lipid Accumulation, and PKCθ Activation in Young and Old WT and MCAT Mice during Hyperinsulinemic-Euglycemic Clamp Studies

(A and B) Glucose infusion rate during the hyperinsulinemic euglycemic clamp (n = 8–10 per group) (A) and insulin-stimulated whole-body glucose uptake (n = 8–10 per group) (B).

(C) Insulin-stimulated 2-DOG uptakes in gastrocnemius muscle (n = 8 per group).

(D and E) Membrane DAG concentration (n = 5–8 per group) (D) and membrane translocation of PKCθ (n = 6–10 per group) (E) in quadriceps muscle. M, membrane; C, cytosol.

(F) AKT2 activity in gastrocnemius skeletal muscle (n = 5–6 per group). All data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 by ANOVA with post hoc analysis.

DISCUSSION

The role of ROS in causing age-associated reduction in mitochondrial function is controversial (Bashan et al., 2009; Bonnard et al., 2008; Evans et al., 2005; Houstis et al., 2006; Loh et al., 2009; Ristow et al., 2009). Furthermore, it is unclear whether age-associated reductions in muscle mitochondrial function are responsible for the age-associated increases in intramyocellular lipid content and muscle insulin resistance or whether they are secondary in nature. In order to examine these fundamental questions, we assessed muscle mitochondrial function and liver and muscle insulin responsiveness in young and old WT and MCAT mice. Using this approach, we found that overexpression of catalase in mitochondria prevented age-associated mitochondrial damage and age-associated reductions in muscle mitochondrial function. Furthermore, we found that prevention of age-associated reduction in muscle mitochondrial function prevented age-associated increases in muscle DAG content, PKCθ activation, and muscle insulin resistance. These studies support the hypothesis that age-associated reductions in mitochondrial function predispose to intramyocellular lipid accumulation, PKCθ activation, and muscle insulin resistance (Lowell and Shulman, 2005; Morino et al., 2006; Petersen et al., 2003). We also found that the overexpression of catalase in mitochondria protected mice from age-associated reductions in muscle AMPK activity and mitochondrial biogenesis (Reznick et al., 2007). This data demonstrates that overexpression of catalase in mitochondria prevents age-associated (1) reduction in muscle mitochondrial function in vivo; (2) decreases in whole-body oxygen consumption; (3) increases in muscle DAG content, PKCθ activity, and muscle insulin resistance; and (4) decreases in AMPK activity and reductions in mitochondrial biogenesis.

Zong et al., 2002), and aging is associated with a reduction in AMPK-induced mitochondrial biogenesis (Reznick et al., 2007). In the present study, age-associated decline in AMPK activation was also observed. Specifically, the ratio of phosphorylated AMPK^{T172} (p-AMPK^{T172}) to AMPKα protein levels in skeletal muscle of WT mice was decreased and was accompanied by decreased acetyl-CoA carboxylase (ACC2) phosphorylation and decreased PGC-1α expression (Figure 4A). Furthermore, there were morphological changes in mitochondrial structure during aging, mainly in size and density (Figure 4B). The intramyofibrillar mitochondrial density in soleus muscle was reduced by ~27% in old WT mice compared to young WT mice (Figures 4C and 4D). In contrast, MCAT mice were protected from these age-associated declines in AMPK activity (Figure 4A) and mitochondrial density (Figures 4C and 4D). Consistent with this finding of protection from age-associated reductions in mitochondrial density, we found a similar pattern of reduced VDAC mitochondrial membrane protein expression in the gastrocnemius and quadriceps muscles of the old WT mice, which in contrast was normal in the old MCAT mice (Figure S3).

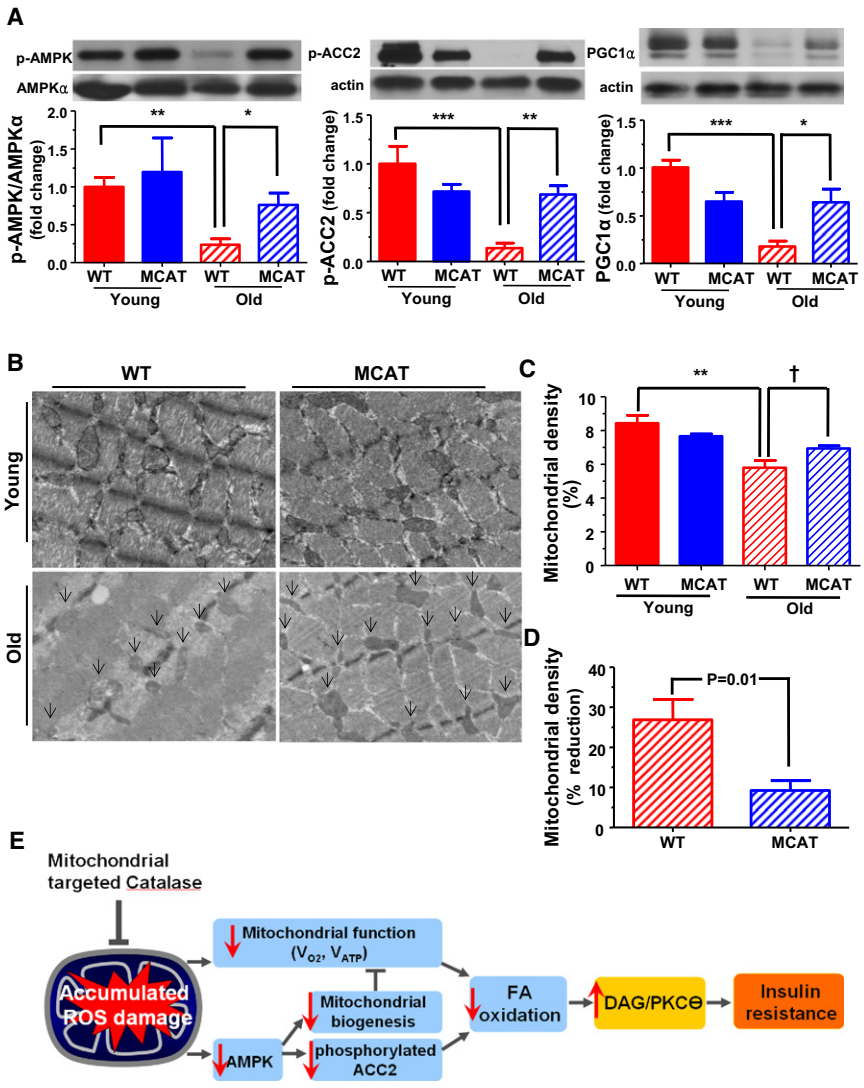

Figure 4. AMPK Activity and Mitochondrial Density in Skeletal Muscle in Young and Old WT and MCAT Mice

(A) Representative immunoblots and densitometric quantification for p-AMPK^{T172}, p-ACC2^{S212}, and PGC-1α in quadriceps muscle (n = 4–6 per group).

(B) Representative figures for the intramyofibrillar mitochondria (arrows) in soleus skeletal muscle.

(C and D) Mitochondrial density in soleus muscle (C) and the percent of declines in mitochondrial density between WT and MCAT during aging (n = 3–5 per group) (D).

(E) Schematic figure of the potential effect of mitochondrial oxidative damage on insulin sensitivity. All data are mean ± SEM; †p < 0.05 by two-tailed unpaired Student's t tests. *p < 0.05; **p < 0.01; ***p < 0.001 by ANOVA with post hoc analysis.

Taken together, these findings support the hypothesis that age-associated reductions in mitochondrial function are due to mitochondrial-generated ROS production, which contributes to the pathogenesis of age-associated muscle insulin resistance and T2DM. Furthermore, these data suggest that therapies targeted to reduce mitochondrial oxidative damage may prevent these age-associated changes.

EXPERIMENTAL PROCEDURES

Animals

Details of the generation of the mice overexpressing mitochondrial catalase (MCAT) used in this study have been described previously (Schriner et al., 2005). The ~3- to 6-month-old (young) and ~15- to 18-month-old (old) male MCAT mice and their age-matched littermate WT control male mice were used. To minimize environmental differences, mice were individually housed for at least 2 weeks before each experiment. Fat and lean body composition were assessed by ^{1}H-nuclear-MRS (Bruker BioSpin; Billerica, MA) on WT and MCAT mice and expressed as percentages of total body weight. A comprehensive animal metabolic monitoring system (CLAMS) (Columbus Instruments; Columbus, OH) was used to evaluate VO_2, VCO_2, respiratory quotient, energy expenditure, food consumption, and activity for body weight- and fat-matched animals, as previously described (Zhang et al., 2010). The study was conducted at the NIH-Yale Mouse Metabolic Phenotyping Center. All procedures were approved by the Yale University Animal Care and Use Committee.

Mitochondrial ROS Production

Fresh skeletal muscle mitochondria were isolated from mixed hindlimb skeletal muscle (quadriceps, gastrocnemius) from overnight-fasted WT and MCAT mice using MITO-ISO kit (Sigma) according to the manufacturer's instructions. Maximal mitochondrial H_2O_2 production was measured using dichlorodihydrofluorescein diacetate in the presence of horseradish peroxidase as described (Andrews et al., 2008). Data are expressed as arbitrary fluorescence units (RFU). Protein concentration was determined by the Bradford method.

Mitochondrial DNA Damage and Oxidative Protein Carbonylation

Total genomic DNA was isolated from snap-frozen gastrocnemius skeletal muscle, and mtDNA copy number and integrity were determined using the QPCR method established by Van Houten and colleagues (Santos et al., 2006). Specific primers were used to amplify a fragment of the β-globin gene (13.5 kb) to determine nuclear DNA integrity, a large fragment of mtDNA (8.9 kb) to determine mtDNA integrity, and a small fragment (221 bp) of the mitochondrial genome to monitor changes in mtDNA copy number and to normalize the data obtained when amplifying the 8.9 kb fragment. Relative amplifications were calculated, comparing each group with the average of young WT, and used to estimate the lesion frequency, expressed as number of lesions per 10 kb, assuming a Poisson distribution of lesions on the template. Sensitivity limit of the technique is 1 lesion/10^5 bases. Oxidative protein carbonylation in both skeletal muscle (quadriceps) lysate and the isolated mitochondrial protein were measured by a western blot method using OxyBlot Protein Oxidation Detection Kit (Millipore).

Mitochondrial Oxygen Consumption

Skeletal muscle mitochondria were isolated from mixed hindlimb skeletal muscle (quadriceps and gastrocnemius) from overnight-fasted animals, and oxygen consumption was assessed as previously described (Andrews et al., 2008) using a Clark-type oxygen electrode (Hansatech Instruments; Norfolk, UK) at 37°C with succinate (10 mM). State III respiration was obtained by adding ADP, and state IV respiration was obtained by adding oligomycin.

Unidirectional Rate of Muscle ATP Synthesis by In Vivo ^{31}P-MRS

After an overnight fast, the mouse was sedated using ~1% isoflurane, and the left hindlimb was positioned under a 15 mm diameter ^{31}P surface coil. The unidirectional rate of muscle ATP synthesis (V_{ATP}) was assessed by ^{31}P saturation-transfer MRS using a 9.4 T superconducting magnet (Magnex Scientific; Oxford, UK) interfaced to a Bruker BioSpec console as described previously (Choi et al., 2008).

Hyperinsulinemic-Euglycemic Clamp Study

Seven days prior to the hyperinsulinemic-euglycemic clamp studies, indwelling catheters were placed into the right internal jugular vein extending to the right atrium. After an overnight fast, [3-^{3}H]glucose (HPLC purified) (PerkinElmer) was infused at a rate of 0.05 μCi/min for 2 hr to assess the basal glucose turnover, and a hyperinsulinemic-euglycemic clamp in awake mice was conducted for 140 min with a primed/continuous infusion of human insulin (152.8 pmol/kg prime, 21.5 pmol/kg/min infusion) (Novo Nordisk; Princeton, NJ) as described previously (Samuel et al., 2006). During the clamp, plasma glucose was maintained at basal concentrations (~120 mg/dl). Rates of basal and insulin-stimulated whole-body glucose fluxes and tissue glucose uptake were determined by bolus (10 μCi) injection of 2-deoxy-D-[1-^{14}C]glucose (2-DOG) (PerkinElmer) as described (Samuel et al., 2006; Zhang et al., 2010).

Tissue Lipid Measurements

DAG extraction and analysis in both cytosolic and membrane samples from quadriceps muscle were performed as previously described (Neschen et al., 2005; Yu et al., 2002). Total DAG content was expressed as the sum of individual DAG species. Tissue triglyceride was extracted using the method of Bligh and Dyer (Bligh and Dyer, 1959) and measured using a DCL Triglyceride Reagent (Diagnostic Chemicals Ltd.; Oxford, CT).

Insulin and AMPK Signaling Assays

AKT2 activity and PKCθ membrane translocation were assessed in protein extracts from gastrocnemius and quadriceps muscle, respectively, harvested after hyperinsulinemic-euglycemic clamp study using the methods previously described (Alessi et al., 1996; Choi et al., 2007; Samuel et al., 2004).

P-AMPK^{T172}, PGC-1α, and phosphorylated ACC2 (p-ACC2) were assessed in protein extracts from quadriceps muscle harvested after overnight fasting. Immunoblots were quantified from multiple exposures using ImageJ (NIH). Relative values from band intensities normalized to actin were calculated comparing each sample with the average of young WT. The primary antibodies used in the current study were as follows: PKCθ (Santa Cruz Biotechnology; Santa Cruz, CA), p-AMPK^{T172} (Cell Signaling; Danvers, MA), AMPKα (Cell Signaling), pan-actin (Cell Signaling), GAPDH (Cell Signaling), PGC-1α (Santa Cruz), phospho-ACC2^{S212} (Santa Cruz), and Catalase (Abcam; Cambridge, MA).

Mitochondrial Density Assessed by Transmission Electron Microscopy

Mitochondrial density was assessed by transmission electron microscopy for soleus muscle as described (Morino et al., 2005). For each sample, five images of three random sections of individual muscle were taken. The average volume density of these 15 images was used to estimate the mitochondrial volume density for each muscle.

The study was conducted and analyzed at the Electron Microscopy Core Facility, Yale School of Medicine. Images were acquired using a Tecnai Biotwin TEM at 80 kV, using a Morada CCD and iTEM software.

Statistics

Values are expressed as mean ± SEM. The significance of the differences in mean values between two groups was evaluated by two-tailed unpaired Student's t tests. More than three groups were evaluated by ANOVA, followed by post hoc analysis using the Bonferroni's Multiple Comparison Test. P values less than 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two tables and can be found with this article online at doi:10.1016/j.cmet.2010.11.004.

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