Loss of Pgc-1α expression in aging mouse muscle potentiates glucose intolerance and systemic inflammation

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ABSTRACT:
Diabetes risk increases significantly with age and correlates with lower oxidative capacity in muscle. Decreased expression of peroxisome proliferator-activated receptor-gamma coactivator-1alpha (Pgc-1α) and target gene pathways involved in mitochondrial oxidative phosphorylation are associated with muscle insulin resistance, but a causative role has not been established. We sought to determine whether a decline in Pgc-1α and oxidative gene expression occurs during aging and potentiates the development of age-associated insulin resistance. Muscle-specific Pgc-1α knock-out (MKO) mice and wild-type littermate controls were aged for two years. Genetic signatures of skeletal muscle (microarray and mRNA expression) and metabolic profiles (glucose homeostasis, mitochondrial metabolism, body composition, lipids, and indirect calorimetry) of mice were compared at 3, 12, and 24 months of age. Microarray and gene set enrichment analysis highlighted decreased function of the electron transport chain as characteristic of both aging muscle and loss of Pgc-1α expression. Despite significant reductions in oxidative gene expression and succinate dehydrogenase activity, young mice lacking Pgc-1α in muscle had lower fasting glucose and insulin. Consistent with loss of oxidative capacity during aging, Pgc-1α and Pgc-1β expression were reduced in aged wild-type mouse muscle. Interestingly, the combination of age and loss of muscle Pgc-1α expression impaired glucose tolerance and led to increased fat mass, insulin resistance, and inflammatory markers in white adipose and liver tissues. Therefore, loss of Pgc-1α expression and decreased mitochondrial oxidative capacity contributes to worsening glucose tolerance and chronic systemic inflammation associated with aging.

KEYWORDS: aging, mitochondria, PGC-1, muscle, diabetes, inflammation
ABBREVIATIONS: peroxisome proliferator-activated receptor-gamma coactivator-1 (Pgc-1), Muscle-specific Pgc-1α knock-out (MKO), electron transport chain (ETC), gene set enrichment analysis (GSEA).

INTRODUCTION:
Age is a significant risk factor for sarcopenia, insulin resistance, and the development of type 2 diabetes (37, 40). Mitochondria are central to maintaining muscle health, yet controversy still surrounds whether mitochondrial dysfunction contributes to declining metabolic function and insulin sensitivity associated with advanced age (reviewed in (23, 41, 42)). Thus, it remains unclear whether deficiencies in mitochondrial health play a significant causative role, or whether they are simply consequence of disease progression.

Altered mitochondrial function can lower ATP synthesis, decrease lipid oxidation, and increase reactive oxygen species, causing energy imbalances and oxidative damage. Many genes encoding mitochondrial electron transport chain (ETC) components are decreased in aged muscle (56), consistent with observed decreases in oxidative capacity (47). Aged muscle has lower mitochondrial density (7) and mitochondria are often depolarized or non-functional (41). Similarly, patients with diabetes and/or insulin resistance have decreased expression of gene pathways involved in oxidative phosphorylation (34, 38). Thus, diabetic and aging muscle share similar deficiencies in mitochondrial function, linking ETC dysfunction to glucose intolerance.

Pgc-1α expression is decreased in aging human muscle (13, 44) and dysregulation of this transcriptional coactivator may underlie lower mitochondrial function and muscle pathologies associated with age (8). PGC-1α is considered a master regulator of mitochondrial biogenesis and function, acting as an upstream regulatory switch for gene pathways controlling oxidative
phosphorylation, fatty acid oxidation, reactive oxygen species detoxification, and mitochondrial density. Over-expression of PGC-1α in muscle protects aging mice from age-related muscle wasting and glucose intolerance (50, 52); yet tight regulation of PGC-1α expression is required to maintain efficient glucose and lipid handling in obese mice (6, 19, 51). Expression of Pgc-1α in muscle is low in type 2 diabetics and related family members (2, 21, 33, 38), but can be increased with exercise to possibly prevent or reverse metabolic abnormalities (3, 25, 44, 49). Thus, a decline in Pgc-1α expression in aging muscle (8, 13), brought on by factors such as diet, obesity, or decreased physical activity, may play a significant role in the age-associated development of glucose intolerance.

In the current study, we address whether decreasing muscle Pgc-1α and oxidative gene expression can serve as a foundation for age-related glucose intolerance. We hypothesized that long-term reductions in muscle Pgc-1α expression, causing persistent, yet moderate loss of mitochondrial content and function, would exacerbate glucose intolerance later in life.

METHODS:

Generation of new Pgc-1α floxed mouse line

Previously generated male Pgc-1α floxed mice are sterile (29), prohibiting generation of homozygous floxed mice (19). As reduced Pgc-1α in multiple tissues can have potentially confounding effects on whole body glucose metabolism (27, 29), a new line of floxed Pgc-1α mice was generated lacking the Neomycin selection cassette. Briefly, floxed Pgc-1α mice (29) were bred to mice expressing low-efficiency cre-recombinase (EIIa-cre, Jackson Labs #003724). Selected progeny with an intact Pgc-1α floxed locus, but lacking the neomycin cassette, were bred to C57B/6N mice to remove the EIIa-cre transgene. Excision of exons 3-5 in these floxed
mice by cre-recombinase prevents protein expression of all currently identified PGC-1α isoforms (Jackson Labs, B6.129-Ppargc1atm2Brsp/J, #009666).

**Muscle-specific Pgc-1α knock-out mice**

The regenerated line of Pgc-1α \( ^{fl/fl} \) mice was bred to a Myogenin-cre line (28) to generate skeletal muscle-specific Pgc-1α knock-out mice (Pgc-1α \( ^{fl/fl} \) + myo-cre, MKO). Test groups consisted of male mice homozygous for the floxed Pgc-1α alleles, expressing myogenin-cre (MKOs, 50%) or no transgene (50%, age-matched, littermate controls). Aging groups started with \( n=13-17 \). Mice with the myogenin-cre transgene alone responded similarly to wild-type (WT) (not shown). Mice were on a mixed C57B/6J/6N/129 background and maintained on a rodent chow (5008I, PharmaServ) for two years. All experiments were performed in accordance with the BIDMC animal facility Institutional Animal Care and Use Committee regulations.

**Expression array**

Genome-wide gene expression profiles were generated using Affymetrix Mouse Genome 430 2.0 Arrays. Gene expression profiles of gastrocnemius muscle mRNA from young (10 week old) and old (24 month old) WT or MKO mice were compared (\( n = 5 \) mice, three arrays analyzed per genotype. Arrays 1 and 2 contained RNA pooled from two mice, Array 3 was a single mouse). Raw and normalized gene expression data are available on GEO (accession number provided by GEO once series is accepted). A multivariate linear model was fit for each gene. Raw CEL files from Genome 430 2.0 Array chips were normalized using RMA [PMID: 12925520] and annotated using biomaRt [PMID: 16082012]. When multiple probe sets matched the same Entrez Gene ID, the probe set exhibiting the highest variance was used for further analysis; reducing set to 16,617 unique genes.

\[
Y_i = \beta_0 + \beta_A + \beta_G + \beta_{AG} \times G
\]
Where $Y_i$ is the variable representing expression of gene $i$, $A$ and $G$ are variables representing age and genotype respectively, $A^*G$ is the interaction term for age and genotype, and $\beta$s are the regression coefficients. Significance for the contribution of age and genotype are estimated by t-test on corresponding $\beta$. To correct for multiple testing, we computed false discovery rate (FDR) for age and genotype from the nominal $p$-values, using a FDR of 25% as cut-off for significance. Full results are provided in Supplementary File S1.

**Gene set enrichment analysis (GSEA)**

To identify pathways significantly associated to age and genotype, genes were ranked based on corresponding t-statistics (see statistical comparison of phenotypes) and pre-ranked gene set enrichment analyses were performed (GSEA version 2.0.10 [PMID: 16199517]). Pathways were defined by gene ontology terms [PMID: 10802651] curated in the mouse genome database (MGD [PMID: 22075990]). Only pathways whose corresponding gene sets contained at least 15 genes and less than or equal to 500 genes, were considered for further analysis (Supplementary File S2). Only pathways with a FDR equal to or less than 25% were considered. The full results of the gene set enrichment analyses are provided in Supplementary File S3.

**Indirect Calorimetry**

Oxygen consumption, carbon dioxide production, movement, respiratory exchange ratios (RER), and cumulative food intake were measured in live mice (*ad libitum* chow diet with a 12-hour light/dark cycle) over 3 days using the Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments). Air sampling was performed in 32-minute intervals. Number of laser breaks (movement along the X- and Y-axis) were totaled and plotted for each interval. Food intake is shown as cumulative grams of chow missing from a pre-weighed chamber at each interval.
Tissue dissection and isolation

Mice were sacrificed by CO$_2$ and the following tissues were isolated by dissection: skeletal muscle (gastrocnemius, quadriceps femoris, tibialis anterior, and soleus) from both legs, epididymal white fat pad, small intestine (jejunum), colon, left brain hemisphere, anterior right lobe of liver, heart, interscapular brown fat, and pancreas. In general, whole muscle from left leg was used for mRNA and right leg for histology. For gastrocnemius, the lateral head was used.

RNA isolation and quantitative RT-PCR

Snap frozen tissue was homogenized in TRIzol® (Invitrogen). 1 μg of DNAse-treated RNA was reverse transcribed and cDNA was amplified and quantified using Sybr-green PCR master mix (Applied Biosystems). Gene expression levels were normalized to Hprt and relative expression calculated by $\Delta\Delta$ct threshold cycle method. PCR efficiency was identical for endogenous control and target genes and Hprt was unaffected experimental conditions. Primer sequences listed in Table S1.

SDH staining

Succinate dehydrogenase activity was visualized in cross-sections of gastrocnemius muscle frozen in OCT following incubation in a phosphate buffer containing sodium succinate and tetrazoliumand (Sigma). Color development is proportional to SDH activity.

Serum glucose, lipids, body composition and glucose / insulin tolerance tests

Blood glucose was measured in tail blood using a standard glucometer at the times indicated. Serum insulin, IL-6, and TNFα were determined by ELISA, and triglycerides and NEFA by colorimetric assay (Specialized Assay Core, Joslin Diabetes Center). Mice were fasted for 16 or 6 hours prior to i.p. injection of 1 g/kg D-glucose or 0.8 U/kg insulin (Humulin®), respectively. Fat and lean mass was determined by nuclear magnetic resonance in live mice (EchoMRI).
Islet histology and insulitis score

Formalin fixed pancreata were mounted and sectioned to capture maximal surface area. Following H&E staining, lymphocyte infiltration for all islets was scored and an insulitis score calculated as previously described (17). Briefly, scoring was assigned as following: 0 – no lymphocytes present; 1 – peri-islet infiltration (lymphocytes on the periphery, not fully surrounding the islet); 2 – immune infiltration surrounding the entire islet, but islet appeared mostly intact; 3 – half the islet was infiltrated (engulfed) by lymphocytes; 4 – the entire islet was engulfed (few beta-cells evident). Insulitus per islet was calculated as

\[
\text{Insulitis per islet} = \frac{\sum \text{inflammation score \times Number of islets}}{\text{total number of islets}}
\]

representing the average inflammation state of each islet per mouse. Two independent reviewers scored blinded sections from each mouse.

Statistical analysis

Statistical significance (p<0.05) for tolerance tests and mRNA expression was assessed by two-way ANOVA (with repeated measures for GTTs), followed by Fisher’s LSD post-hoc test. Area under the curve (AUC) was calculated with baseline set at fasting glucose. Comparisons between two independent groups were performed using either a two-tailed unpaired Student’s t-test or a Mann & Whitney U-test if the distribution was not Gaussian (by Shapiro-Wilk normality test). Two-way ANOVA was used when two factors (age and genotype) effect on gene expression was assessed followed by a Holm-Sidak’s multiple comparison’s test for significance of each gene. To determine effect of one factor (genotype or age) on multiple genes, we performed an unpaired Student’s t test for each gene and corrected our analysis for multiple comparisons (FDR=10%). Analysis was completed using GraphPad Prism. All data points represent means ± SEM.
RESULTS:

Aging abolishes compensatory increases of skeletal muscle \textit{Pgc-1}\(\beta\) expression

As expected, young male MKO mice exhibited a >95% knockdown of \textit{Pgc-1}\(\alpha\) mRNA in skeletal muscle compared to WT littermates (Fig 1A). Interestingly, there was a compensatory increase in \textit{Pgc-1}\(\beta\) mRNA in all skeletal muscles that were tested (Fig 1B). PGC-1\(\beta\) is a closely related family member that may share significant overlap in function to PGC-1\(\alpha\) (45, 57).

Expression of \textit{Pgc1}\(\alpha\) and \textit{Pgc1}\(\beta\) were unchanged in other tissues examined, including cardiac muscle, confirming specificity of the knock-down to skeletal muscle only. We next compared muscle RNA from young (6 month old) and old (24 month old) WT and MKO mice to determine whether \textit{Pgc-1} gene expression was impacted by age. Interestingly, expression of both \textit{Pgc-1}\(\alpha\) and \textit{Pgc-1}\(\beta\) mRNA decreased in aged WT skeletal muscle (Fig. 1C) and the compensatory increase in \textit{Pgc-1}\(\beta\) seen in young MKO muscle was lost in all skeletal muscle groups with aging (Fig 1C - quadriceps, other muscles not shown).

Decreased mitochondrial oxidative capacity is a major effector pathway shared by aged muscle and muscle lacking PGC-1\(\alpha\).

To determine if biological processes impacted by loss of \textit{Pgc-1}\(\alpha\) intersect with those altered during aging, global changes in muscle mRNA expression in gastrocnemius muscle from young (10 week old) and old (24 month old) wild-type (WT) and muscle-specific \textit{Pgc-1}\(\alpha\) knock-out mice (MKO) mice were compared using genome-wide Affymetrix GeneChip arrays.

Although >2500 genes were differentially expressed in young versus old muscle samples (FDR<25%, Fig. 2A and Supplementary File S1), only 11 molecular pathways were enriched following GSEA (FDR<25%, Fig. 2B and Supplementary File S3). In comparison, 475
individual genes were different between WT and MKO mice regardless of age (FDR<25%, Figure 2A), with GSEA identifying 95 gene sets (FDR<25%, Fig. 2B). Strikingly, 43% of the genes affected by loss of Pgc-1α were also changed in aging muscle (8% of total age-affected genes). Within this pool of 206 genes shared by both Pgc-1α loss and aging (Fig. 2A), 80% changed in the same direction (Supplementary File S4), suggesting that a significant proportion of genes regulated by Pgc-1α (~35%) may be involved in the molecular program of muscle aging.

While PGC-1α has been implicated in multiple aspects of muscle biology, the top 8 gene sets affected by Pgc-1α loss were pathways of mitochondrial metabolism (Fig. 2C). Of note, only 2 gene sets were shared by genotype and age, “electron transport chain” function and “regulation of TGF-β signaling” (Fig. 2C), illustrating that altered oxidative phosphorylation is indeed a hallmark of both reduced Pgc-1α and aging muscle in mice.

**Aging exaggerates decreased oxidative capacity in muscle**

As predicted by expression array data, mitochondrial genes were sensitive to both aging and loss of Pgc-1α (Fig. 3A). It is already established that PGC-1α is an important regulator of nuclear-encoded mitochondrial gene and protein expression (12, 55). To illustrate whether aging exaggerated decreases in MKO mice, we directly compared young versus old mice by normalizing data to age-matched controls (Fig. 3B). This demonstrated that aging further lowered expression of many nuclear-encoded mitochondrial genes. Although Tfam expression was not significantly affected by Pgc-1α loss, mtDNA-encoded Ndl and Atp6 were decreased in young MKO muscle, suggesting an early decrease in mitochondrial mass (Fig. 3A). Aging alone
decreased mtDNA transcripts (Fig. 3A); however, unlike nuclear-encoded mitochondrial genes, aging did not further exacerbate mtDNA decreases caused by *Pgc-1α* ablation (Fig. 3B). SDH activity (an indicator of mitochondrial oxidative capacity) in muscle was decreased by age alone and loss of *Pgc-1α* in both young and old gastrocnemius muscle (Fig. 3C).

The combination of reduced muscle *Pgc-1* and aging worsened glucose intolerance

As advanced age is a significant risk factor for diabetes, and low muscle ETC activity is linked to insulin resistance, we tested whether long-term loss of muscle *Pgc-1α* aggravated glucose intolerance. Chow-fed, wild type and MKO were followed over a period of two years. At 3 months of age, MKO mice had significantly decreased fasting glucose and circulating insulin (Table 1), and blood glucose was significantly lower 20 minutes following an i.p. glucose challenge (Fig. 4A). At 12 months of age, although glycemia remained lower in MKO mice at fasting and 20 minutes post-glucose challenge, overall glucose tolerance tended to worsen (Fig. 4B). For both 3- and 12- month old mice, despite similar AUCs (Fig. 4A, B), the shapes of the MKO glucose disposal curves were different compared to WT controls (significant interaction between time and genotype, two-way ANOVA, p<0.05), suggesting that loss of skeletal muscle *Pgc-1α* impacted glucose excursion kinetics. After 24 months, MKO mice were significantly less glucose tolerant than controls (Fig. 4C) and total AUC was increased. Thus, despite reduced mitochondrial gene expression and oxidative capacity at all ages, significant glucose intolerance manifested only after prolonged aging.
MKO mice developed insulin resistance with advanced age

We next assessed peripheral insulin sensitivity by insulin tolerance test (ITT). Similar to published reports (20, 57), younger MKOs (6 months old) showed no differences in response to exogenous insulin (Fig. 5A). In contrast to young mice, 24-month old MKOs could not lower glucose as effectively in response to exogenous insulin (Fig. 5B). Moreover, while body composition was not different in young mice, old MKO mice had increased fat and decreased lean masses (Fig. 5C) with no significant difference in circulating triglycerides or free fatty acids in serum (Fig. 5D,E). Despite decreased fasting insulin (Table 1) in MKOs, fed levels of serum insulin were similar (Fig. 5F), suggesting no defects in post-prandial insulin secretion. Despite decreased oxidative capacity in MKO muscle, intramuscular triglyceride levels trended lower in old MKOs (Fig. 5G), arguing against insulin-resistance due to high intramuscular lipids. The GLUT4 insulin-sensitive glucose transporter is a target of PGC-1α (32); however, Glut4 mRNA levels were unchanged in young mice and significantly lower only in aged MKO soleus, not gastrocnemius or other metabolically active tissues (Fig. 5H,I). No significant differences were noted in total body weight between WT and MKO mice (Table 1), although the MKOs tended to be heavier.

There were also no significant differences between genotypes in VO₂, VCO₂, heat production, activity, or food intake by indirect calorimetry, regardless if data was corrected for total body weight (Fig. 6A-E) or lean body mass (data not shown). However, aged MKO mice exhibited significantly lower respiratory exchange ratios (RER) during dark phase only (Fig. 6F), indicating a shift toward lipid metabolism. As mice generally eat during dark phase, increased catabolism of lipids when circulating insulin levels are highest can support insulin resistance (4,
While defects in post-prandial insulin secretion could also contribute to the shift in fuel utilization, fed insulin levels in 6-month old MKOs were similar to WT (Fig. 5F).

**Loss of muscle Pgc-1α causes age-dependent inflammation in white adipose and liver tissue**

Low-grade, chronic inflammation is associated with aging and insulin resistance. As loss of muscle Pgc-1α may increase circulating IL-6 (19), we hypothesized that increased inflammatory signaling in muscle contribute to glucose intolerance in old MKO mice. However, there was no significant increase in mRNA levels of *Il-6, Tnfα, Socs3*, or *Mcp-1 (Ccl2)* in sedentary young or aged MKO muscle (Fig. 7A). *Cd68* expression (a marker of macrophage infiltration) and circulating TNFα were also similar between genotypes (Fig. 7A,B).

Since it was hypothesized that loss of muscle Pgc-1α leads to inflammatory signaling in islets (19), we assessed lymphocytic infiltration. The number of inflamed islets, the severity of lymphocyte infiltration, and the insulitus score of young MKO islets trended higher in younger mice, but was not significant (Fig. 7C). Unexpectedly, quantitative histological analysis demonstrated that old MKO islets were actually significantly less inflamed than old WT littermates. No overt differences in islet size were noted (not shown). Thus, to investigate other possible mechanisms contributing to glucose intolerance and insulin resistance in aged MKO mice, we investigated inflammatory gene expression in other tissues that play a role in glucose clearance. In contrast to muscle, there was a significant increase in *Il-6* and *Mcp-1* expression in the white adipose tissue of young MKOs that became more pronounced with age (Fig. 7D). Similarly, inflammatory markers increased with age in livers of mice lacking *Pgc-1α* in skeletal muscle (Fig. 7E).
DISCUSSION:

Reduced expression of *Pgc-1* co-activators in muscle leads to significant defects in mitochondrial oxidative capacity (45, 55, 57). We show this is not sufficient to worsen insulin sensitivity, but when combined with advanced age, promotes glucose intolerance, insulin resistance, and inflammation in white adipose and liver. Thus, both genotype and chronological age played a role, highlighting an interaction between the two variables. Defects in mitochondrial function in muscle as a cause of insulin resistance is widely debated (14, 22). In fact, we show that young MKO mice had modestly improved metabolic parameters (fasting glucose, insulin, and glycemia immediately following glucose challenge) despite lower mitochondrial gene expression and function. This is consistent with other mouse models of disrupted mitochondrial function, including muscle-specific knock-out of the *Tfam* and *Aif* genes (43, 53). The mechanism for improved glucose homeostasis in young MKOs and these other mouse models remains unclear, but may involve increased glucose uptake or 5' adenosine monophosphate-activated protein kinase (AMPK) activity.

It is important to note that most studies are performed in young mice (2 to 4 months), while we investigated the long-term effects of modestly reduced muscle mitochondrial function. Our model provides mechanistic evidence that decreased *Pgc-1α* and deficiencies in electron transport chain function can indeed precede and lead to insulin resistance and impaired glucose clearance over time, which is particularly relevant as age is a significant risk factor for diabetes. Consistent with our observations, overexpression of *Pgc-1α* in muscle improves glucose handling in sedentary, aged mice (52), but causes insulin resistance in young, high-fat fed mice (6) that can be reversed by exercise (50). While these studies support targeting muscle PGC-1s and mitochondria to improve insulin sensitivity, they highlight the importance of considering
confounding variables such as physical activity, diet, and age. In fact, detrimental effects of age on metabolism can be improved by regular exercise (25, 31). In our current study, we limited the ability of the mice to exercise to better mimic the long-term, sedentary lifestyle associated with metabolic disease. However, since muscle PGC-1α is intimately linked to exercise physiology, it would be interesting to evaluate how increased physical activity impacts the metabolic homeostasis of aging MKOs.

Our results in young MKOs contrast those of Handschin et al. 2007 (19), who report decreased body weight and fat mass, increased energy expenditure, and resistance to diet-induced obesity despite glucose intolerance. One potential explanation for the discrepancy is their use of control mice lacking one allele of Pgc-1α in all tissues (due to breeding problems of their floxed line). Reduced Pgc-1α in metabolically active tissues such as liver, adipose, and brain has multiple confounding effects on baseline whole body glucose metabolism (11, 24, 26, 29, 30). In fact, aged whole body Pgc-1 knock-out mice have increased circulating TNFα and IL-6 with no appreciable increase in muscle inflammatory signaling (36). We do note a trend toward increased islet lymphocyte infiltration in our young MKOs, consistent with evidence of pancreatic inflammation. However, we did not observe altered islet size or morphology, and interestingly, old MKO islets had less age-induced lymphocyte infiltration. While this data suggests that MKO islets are protected from increased systemic inflammatory signaling associated with aging, we did not assess β-cell function in aged MKOs and it is possible that secretory capacity was also affected.

mRNA expression analysis in muscle uncovered a potentially cooperative role for PGC-1β in muscle glucose homeostasis. Pgc-1β mRNA levels were higher in all skeletal muscle groups of young MKO mice. While the mechanism for this seemingly compensatory increase is
not known, increased Pgc-1β expression in young mice may limit or delay consequences of losing Pgc-1α. Since complete loss of both Pgc-1α and -1β decreases the oxidative capacity of muscle, but does not in itself worsen glucose tolerance in young mice (45, 57), our data suggest that a combination of advanced age and reduction of both Pgc-1 family members is required for impairment of glucose handling. This is consistent with a growing body of evidence suggesting that reduced mitochondrial function or content in muscle does not directly impair glucose tolerance, but may intersect with other genetic or environmental factors to worsen disease (25). It is also possible that loss of muscle Pgc-1β impacts unique gene targets not shared with Pgc-1α and not involved in mitochondrial oxidation. Thus, it will be important to evaluate the individual and/or synergistic contributions of each family member to the observed phenotype.

Our model suggests inflammation in white adipose and liver tissues as an underlying mechanism of insulin resistance related to decreased mitochondrial oxidative capacity in aging muscle. Increased inflammation in these tissues is believed to contribute to the pathogenesis of type 2 diabetes and the metabolic syndrome (9, 16, 39, 48) and provides a plausible explanation for the age-dependent development of glucose intolerance, insulin resistance, and decreased circulating insulin in aged MKO mice. Important unanswered questions are how loss of Pgc-1α in muscle impacts inflammatory signaling in other tissues and whether these effects are due to defects in mitochondrial metabolism or non-mitochondrial actions of PGC-1. Over-expressed Pgc-1α or -1β inhibits NF-κB signaling in muscle cells (1, 10) and forced exercise potentiates inflammation in muscle lacking muscle Pgc-1α (18, 54). However, lack of increased inflammatory markers in muscle of sedentary MKO mice suggests that muscle may not be the primary site of inflammation and implicates alternative mechanisms of inter-organ cross-talk. PGC-1α was recently shown to regulate the expression of myokines such as FNDC5, Myostatin,
and IGF-1 (5, 35, 46) and our GSEA analysis identifies altered muscle TGF-β signaling in both aged and MKO muscle, indicating that alterations in hormone expression, secretion, or action may mediate the non-cell autonomous effects of losing muscle Pgc-1α on whole body glucose homeostasis.

Our gene expression analysis also illustrated that a significant proportion of muscle Pgc-1α activity (~35%) may be involved in molecular mechanisms underlying aging. However, only 6% of total age-related gene changes were mimicked by loss of Pgc-1α. Thus, while decreased Pgc-1α may not be sufficient to drive muscle aging, it appears to play a significant role in age-associated loss of muscle oxidative capacity and glucose intolerance. Of note, GSEA identified only altered ETC function within aging muscle, while other pathways of mitochondrial biology (lipid oxidation, ATP metabolism, or tricarboxylic acid cycling) were not significantly enriched. While this may be a function of the large aging data set and limits of our analysis, it is consistent with studies specifically identifying oxidative phosphorylation as a major player in deregulated muscle metabolism during aging (40, 56).

Diabetes is a progressive and chronic disorder in humans, with diagnosis commonly made in mid-late life. Evidence suggests that decreased physical activity, increased adiposity, and gradually declining mitochondrial function, act synergistically to regulate insulin sensitivity during aging. Importantly, reduced mitochondrial function in MKO mice preceded increased adiposity, glucose intolerance, and insulin resistance. Our study highlights that pre-existing, modest disruption of mitochondrial gene expression and function early in life, when combined with a sedentary lifestyle, can disrupt glucose homeostasis in later years. Identification of mitochondrial deficiencies in young adults may help predict diabetes risk and augmentation of mitochondrial function could be a valid approach to delay or prevent disease progression.
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DUALITY OF INTEREST:
The authors declare no conflict of interest.

CONTRIBUTION STATEMENT:
JLE and JLR designed experiments. BHK was responsible for bioinformatic analysis and interpretation. JLE, SS, AA, SK, DLB, and CDW performed experiments and analyzed data. ABP and JLE performed statistical analysis. JLE and BHK wrote the manuscript. SS, ABP, JLR, SK, CDW, and JLE reviewed/revised the manuscript.

LEGENDS:
Table 1: Metabolic parameters in WT versus MKO mice at different ages. Data are expressed as mean ± SEM (n=9-12 mice for 3-12 months old groups, n=5 for 24 month old).
*p<0.05 comparing WT and MKO of similar age.
Figure 1: Aging promotes loss of both Pgc-1α and Pgc-1β in skeletal muscle. Relative mRNA expression of A) Pgc-1α or B) Pgc-1β in tissues from young WT or MKO mice (6 months, n=8). Expression is normalized to levels in tissues of age-matched WT mice. C) Expression of Pgc-1 coactivators in young (6 month, n=8) versus aged quadriceps muscle (24 months, n=5) WT and MKO mice expressed relative to mRNA levels in young WT mice. Data are means ± SEM (*p<0.05 WT vs MKO of similar age, #p<0.05 young vs. old mice of similar genotype).

Figure 2: mRNA expression and gene set enrichment analysis in MKO versus aging muscle. A) Unique and over-lapping transcript number and B) enriched gene sets identified by multivariant analysis of expression arrays (n=3, p<0.05, FDR 25%) of gastrocnemius muscle RNA from WT vs. MKO (genotype), or 10-week old vs. 24-month old (age) mice. C) The top 10 significantly enriched gene sets (ranked by FDR) for genotype or age. Solid red (genotype) or blue (age) bars represent relative strengths of gene set enrichment (FDR, x-axis). Red hatched line represents cut-off of significance (FDR 25%) and green boxes denote pathways shared across genotype and age data.

Figure 3: Reduced Pgc-1α expression exacerbated decreasing mitochondrial gene expression, function, and oxidative fibers in aging muscle. A) Relative mRNA expression of nuclear- and mitochondrial(mt)-encoded modulators of mitochondrial metabolism in gastronemius muscle of young (10 week old, n=8) versus aged (24 months, n=5). Data are means ± SEM normalized to young WT mice. B) Percentage decreases in mitochondrial gene expression in young versus old MKO mice (Data are means ± SEM normalized to relative age-
matched WT controls. \(*p<0.05\) WT vs. MKO of similar age, \(^*p<0.05\) young vs. old mice of similar genotype. C) Succinate dehydrogenase activity in muscle sections (gastrocnemius) visualized by nitro blue tetrazolium staining.

**Figure 4: MKOs develop glucose intolerance with advanced age.** A-C) Glucose tolerance tests performed in WT versus MKO mice over 24 months (n=8-9). Area under the curve calculated from baseline glucose represented under each curve. Data are means ± SEM, \(*p<0.05\) WT vs. MKO.

**Figure 5: Aging promoted insulin resistance, increased adipose tissue mass, and lower RER in MKO mice.** Insulin tolerance tests in (A) 6 months and (B) 24-month old WT and MKO mice (n=5). C) Body composition of mice by NMR (n=6-8). D) Triglycerides, E) free fatty acids, F) insulin, and G) intramuscular triglyceride (TG) content measured by colorimetric assay using serum from fed WT and MKO mice at 24 months old (n=5-9). *Glut4* mRNA expression in tissues from H) young (3 months, n=8-9) and I) aged (24 months, n=5) WT vs. MKO mice. Data points represent mean ± SEM, \(*p<0.05\) WT vs. MKO. Gene expression is normalized to mean expression of corresponding tissue in age-matched WT mice. White adipose tissue (WAT), brown adipose tissue (BAT).

**Figure 6: MKO mice had decreased respiratory exchange ratios during dark phase only.** Indirect calorimetry by CLAMS measuring A) oxygen consumption (VO\(_2\)), B) carbon dioxide production (VCO\(_2\)), C) heat, D) activity (laser breaks), E) cumulative food intake and F) respiratory exchange ratio (RER) in WT and MKO mice (24 months old) over the course of three
days. Solid bars represent dark phase (6pm to 6am), dashed lines represent light phase (6am-6pm). Data points represent means ± SEM (n=8). For RER, *p<0.05 by Repeated Measure ANOVA analysis of values in dark phase only.

Figure 7: Aged, sedentary MKO mice have increased inflammation in liver and white adipose tissue. mRNA expression of inflammatory markers in A) gastrocnemius muscle, D) white adipose tissue (WAT), and E) liver from young (10 week old, n=8) or old (24 month, n=5) WT or MKO mice. Data are normalized to age-matched WT mice and are means ± SEM, *p<0.05. B) Circulating TNFα in 6-month-old mice. C) Percentage of islets scored for severity of lymphocyte inflammation and insulitis score per age and genotype.

REFERENCES:


Table 1: Metabolic parameters in WT versus MKO mice at different ages.

<table>
<thead>
<tr>
<th></th>
<th>3 months</th>
<th>12 months</th>
<th>24 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (g)</td>
<td>KO (g)</td>
<td>WT (g)</td>
</tr>
<tr>
<td>Weight (grams)</td>
<td>23.4 ± 0.9</td>
<td>25.7 ± 0.7</td>
<td>36.6 ± 1.2</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dL)</td>
<td>96.7 ± 5.7</td>
<td>79.3 ± 6.8*</td>
<td>97.6 ± 4.3</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.49 ± 0.05</td>
<td>0.37 ± 0.02*</td>
<td>---</td>
</tr>
</tbody>
</table>
FIGURE 1:

A. Relative mRNA levels of *Pgc-1α* in WT and MKO mice across different tissues.

B. Relative mRNA levels of *Pgc-1β* in WT and MKO mice across different tissues.

C. Relative mRNA levels of PGC-1α and PGC-1β in young and old mice.
FIGURE 2:

A  INDIVIDUAL GENES

B  GENE SET ENRICHMENT

C  TOP ENRICHED PATHWAYS

GENOTYPE

- Electron transport chain
- Tricarboxylic acid cycle
- ATP biosynthetic process
- Fatty acid beta oxidation
- ATP metabolic process
- Fatty acid metabolic process
- Mitochondrial respiratory chain complex I assembly
- Mitochondrion organisation
- Proton transport
- Response to DNA damage stimulus

AGE

- Regulation of mitotic cell cycle
- Mammary gland development
- Water transport
- Embryonic skeletal system development
- Wnt receptor signalling pathway
- Neural tube development
- Regulation of TGF beta receptor signalling pathway
- Blood vessel remodelling
- Positive regulation of angiogenesis

GENOTYPE AGE
FIGURE 3:

(A) Bar chart showing relative mRNA levels of various genes in different groups: Young WT, Old WT, Young MKO, and Old MKO.

(B) Graph depicting the percentage change compared to age-matched WT for various genes.

(C) Images comparing WT and MKO groups in young and old conditions.
FIGURE 4:

GTT: 3 months

- WT
- MKO

AUC

GTT: 12 months

- WT
- MKO

AUC

GTT: 24 months

- WT
- MKO

AUC

Two-way ANOVA p<0.05, interaction

Two-way ANOVA p<0.01, interaction

Two-way ANOVA p<0.05, WT vs MKO
FIGURE 5:

A. 6 months

**ITT**

- WT
- MKO

Blood glucose (mg/dL)

Time (minutes)

Two-way ANOVA p<0.05 WT vs MKO

B. 24 months

**ITT**

Blood glucose (mg/dL)

Time (minutes)

Two-way ANOVA p<0.05 WT vs MKO

C. 3 months

**% body weight**

- WT
- MKO

Fat
Lean

D. Triglycerides

- WT
- MKO

E. FFAs

- WT
- MKO

F. Insulin (ad libitum)

- WT
- MKO

G. Muscle TG

- WT
- MKO

H. **Glut4**

- WT young
- MKO young

- Relative mRNA levels

- Soleus
- Gastrocnemius

I. **Glut4**

- WT old
- MKO old

- Relative mRNA levels

- Soleus
- Gastroc
- WAT
- Heart
- Liver
- BAT

p=0.08
FIGURE 6:

A and B: VO₂ and VCO₂ over time. The graphs show the metabolic rates of WT and MKO mice during the dark phase.

C: Heat Movement. The graph illustrates the heat generated by WT and MKO mice over time.

D: Movement. The graph depicts the cumulative movement counts of WT and MKO mice.

E: Cumulative Food Intake. The graph shows the cumulative food intake of WT and MKO mice over time.

F: RER. The graph displays the respiratory exchange ratio (RER) for WT and MKO mice during the dark phase, with a statistical difference indicated by RM ANOVA p<0.05 WT vs MKO.
FIGURE 7:

A. GASTROCNEMIUS

B. TNFα

C.

D. YOUNG

E. LIVER

Two-way ANOVA p<0.001

Two-way ANOVA p<0.01