Muscle-specific overexpression of PGC-1α does not augment metabolic improvements in response to exercise and caloric restriction

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Running title: Oxidative capacity and energy homeostasis

Word count: 4262

Figures: 7

Tables: 1
Abstract

This study used mice with muscle-specific overexpression of PGC-1α, a transcriptional co-activator that promotes mitochondrial biogenesis, to determine whether increased oxidative potential facilitates metabolic improvements in response to lifestyle modification. MCK-PGC1α mice and non-transgenic (NT) littermates were fed a high fat diet (HFD) for 10 weeks, followed by stepwise exposures to voluntary wheel running (HFD+Ex) and then 25% caloric restriction with Ex (Ex/CR), each for an additional 10 weeks with continued HFD. Running and CR improved weight and glucose control similarly in MCK-PGC1α and NT mice. Sedentary MCK-PGC1α mice were more susceptible to diet-induced glucose intolerance, and insulin action measured in isolated skeletal muscles remained lower in the transgenic compared to the NT group, even after Ex/CR. Comprehensive profiling of over 200 metabolites and lipid intermediates revealed dramatic group-specific responses to the intervention but did not produce a lead candidate that tracked with changes in glucose tolerance irrespective of genotype. Instead, principal components analysis identified a chemically diverse metabolite cluster that correlated with multiple measures of insulin responsiveness. These findings challenge the notion that increased oxidative capacity defends whole body energy homeostasis and suggest that the interplay between mitochondrial performance, lipotoxicity and insulin action is more complex than previously proposed.
The escalating obesity pandemic presents a serious threat to global health and economic stability. Obesity increases risk of numerous comorbidities, including cardiovascular disease, impaired blood glucose control and type 2 diabetes (1). Importantly, even moderate levels of weight loss can improve risk factors for these diseases (2). The most effective, non-invasive strategies for achieving weight control are based on lifestyle modifications that promote balanced nutrition and physical activity. Moreover, habitual exercise imparts metabolic benefits independent of weight loss, including improvements in insulin sensitivity, glucose tolerance and blood lipid profiles. Whereas lifestyle factors clearly influence disease risk, enthusiasm for behavioral modification approaches has been dampened by psychological, economical and/or physical barriers to long term patient compliance.

While the need for novel therapeutics continues to grow, efforts to develop drugs that facilitate weight loss and/or improve weight loss maintenance have thus far met limited success. The compelling association between routine physical activity and favorable health outcomes has fueled strong interest in the development of exercise mimetic compounds. Although the concept of “exercise in a pill” is attractive, the molecular strategy to meet this end remains uncertain because the precise mechanisms responsible for the health benefits of physical activity are poorly understood (3). Prominent among the many molecular and cellular adaptations that occur in response to aerobic exercise training are increased mitochondrial biogenesis and enhanced potential for oxidative metabolism (4). One prevailing view in this field posits that remodeling of skeletal myofibers toward a more oxidative phenotype confers protection against disease by promoting fat catabolism and preventing tissue accumulation of toxic
lipid intermediates such as diacylglycerol (DAG), ceramides and long chain acyl-CoAs. This model stems from numerous human and animal studies reporting that severe obesity and glucose intolerance associate with increased muscle content of lipid intermediates and reduced respiratory function (5-7). Additionally, investigators have suggested that individuals with an inherently high content of oxidative myofibers fare better during a weight loss intervention than those with more glycolytic fibers (8-10).

Although a large body of correlational data supports a link between oxidative potential and health outcomes, direct experimental evidence of cause and effect remains sparse (11). The principal goal of the current study was to test the causal relation between respiratory capacity, energy balance and glucose control using a transgenic mouse model with muscle-specific overexpression of PGC-1α (MCK-PGC1α), a transcriptional co-activator that functions as a master regulator of oxidative metabolism (12). The MCK-PGC1α model was selected as a genetically engineered mimic of exercise training because: 1) PCG-1α expression and activity increase with exercise training (13,14), 2) muscle-specific overexpression of this co-activator augments mitochondrial biogenesis, fat oxidation and exercise endurance (15-17), and 3) activation of PGC-1α is often credited as a unifying mechanism that mediates favorable metabolic responses to various behavioral, pharmacological and genetic maneuvers (18,19).

Herein, MCK-PGC1α mice and non-transgenic control littersmates (NT) (20) were fed a high fat diet, administered alone or in combination with weight loss interventions that encompassed exercise and caloric restriction. The animals were monitored longitudinally and primary outcomes included body weight, glucose tolerance and
comprehensive metabolic profiling of muscle specimens. Our findings challenge the notion that enhanced oxidative potential protects against metabolic disease, and raise new and important questions regarding the roles of mitochondrial function and lipotoxicity in the pathogenesis of obesity and insulin resistance.

**Materials and Methods**

Animal studies were approved by the Duke University Institutional Animal Care and Use Committee. Male mice were housed in a temperature-controlled environment with a 12:12 hr light/dark cycle and weaned on a standard chow (SC-5% fat, 72% carbohydrate) (5001; Harlan Teklad). At 13-16 weeks the mice were placed on a high fat/high sucrose diet (45% fat, 25% sucrose, D03021303; Research Diets) and water. For voluntary exercise studies, mice were individually housed in cages equipped with running wheels (Columbus Instruments). Mice were fasted 3 hours prior to sacrifice.

**Exercise Performance Studies.** Mice were acclimated to the treadmill (Eco3/6 treadmill, Columbus Instruments) three days prior to the experiments by running for 5 min/day at 5 m/min and 10 m/min followed by 15 m/min for one minute. The grade increased by 5% each day, reaching a 10% grade on day three. During the endurance protocol mice ran for 30 minutes at 10 m/min. Speed was increased 2 m/min every 15 min until reaching 28 m/min, and then every ten minutes until exhaustion. During the VO\textsubscript{2} peak test, mice ran at 6 m/min and speed was increased 3 m/min every 3 min until exhaustion, defined as remaining on the shock grid for ten consecutive seconds. RER and VO\textsubscript{2} were monitored using the Oxymax Modular Treadmill System (Columbus Instruments).
**Mitochondrial Oxidation.** Mitochondria were isolated from the gastrocnemius muscle of MCK-PGC1α and NT mice fed a HFD for 6 weeks (18). Oxidation studies were performed using 0.2 mM [1-14C]palmitate ± 1.0 mM sodium pyruvate. 14CO2 trapped in NaOH and 14C-labeled acid-soluble metabolites were assessed by liquid scintillation counting in Uniscint BD (National Diagnostics) (21).

**Citrate Synthase Activity.** Ground tibialis anterior muscles were resuspended in CellLytic MT (Sigma-Aldrich). Assays were performed on unclarified homogenates at 25°C in a reaction buffer containing acetyl CoA, DTNB and tris-HCl as described in (22). Reactions were initiated with oxaloacetate. Data were normalized to ground tissue weight.

**Glucose Tolerance Tests.** Mice were fasted 6 hours before an intraperitoneal injection of glucose (1.5 g/kg lean body weight). Plasma glucose levels were measured at the indicated times.

**Glucose oxidation in isolated muscle strips.** Whole soleus and EDL muscles were dissected from mice and placed in KHB buffer for re-oxygenation and then incubated with 5 mM 14C-glucose ± 100 nM porcine insulin for two hours at 37°C. Reactions were terminated with 70% perchloric acid. 14CO2 was trapped in 1N NaOH and quantified by liquid scintillation counting in Uniscint BD (National Diagnostics). Flash frozen muscles were used for measurement of glycogen synthesis (23).

**Targeted proteomics.** Proteomic analysis was performed as previously described (24). Briefly, mitochondria isolated from the quadriceps were run on an SDS-page gel and underwent in-gel tryptic digestion. Entire lanes were cut and subjected to selected reaction monitoring on a triple quadrupole mass spectrometer. Five assays were run to
measure β-oxidation, TCA cycle, glycolytic and antioxidant proteins. Data were analyzed using the Pinpoint program. Protein levels are expressed as pmol/100ug of protein utilizing a BSA standard.

**Metabolite Measurements.** The fatty acid composition of diacylglycerol (DAG) and triacylglycerol (TAG) in skeletal muscle was analyzed by gas chromatography (25). Acylcarnitines, acyl-CoAs, organic acids and amino acids were analyzed in skeletal muscle and serum by MS/MS (26-28). Ceramides were extracted and analyzed based on published methods using flow-injection mass spectrometry (29).

**Statistical Analysis.** Data are expressed as means ± SEM. Results were analyzed by *Student’s t* test unless otherwise indicated in the figure legends. A *p* value less than or equal to 0.05 was considered statistically significant. Prior to utilizing principal component analysis (PCA) to reduce a large number of correlated metabolites to a smaller number of uncorrelated factors, the metabolite concentrations were log transformed to approximate normal distribution. PCA was performed using orthogonal varimax rotation, and factors with eigenvalues ≥ 1.0 were retained (SAS version 9.3; Cary, NC). Metabolites with a loading score of ≥|0.4| are reported for each factor. Tests of main effects of genotype, diet, and genotype by diet interactions were performed using two-way ANOVA (SAS PROC GLM). Associations between factor scores and physiological measures are reported as Spearman’s rank correlation coefficients.
Results

In preliminary experiments we sought to assess the impact of PGC-1α overexpression on muscle mitochondrial quality and performance in the context of a high fat diet (HFD). Mitochondrial yield from muscle of MCK-PGC1α mice was approximately 3-fold greater than that in the NT controls, consistent with increased mitochondrial biogenesis (13). A targeted proteomics survey of isolated mitochondria from quadriceps muscles of MCK-PGC1α compared to NT control mice fed a HFD for 6 weeks revealed marked upregulation (2-3 fold) of proteins involved in β-oxidation, the TCA cycle, electron transport chain, energy metabolism and antioxidant defense (Table 1). Consistent with these results, in isolated mitochondria from MCK-PGC1α mice compared to the NT controls, rates of complete oxidation of [14C]palmitate to CO₂ (Figure 1A) and incomplete oxidation to ASM (Figure 1B) were likewise increased 3- and 1.5-fold, respectively. Addition of pyruvate as a competing fuel inhibited fat oxidation to a similar degree in both genotypes, although the absolute rates of β-oxidation remained higher in mitochondria from the transgenic mice (Figure 1A). Together, these findings show that PGC-1α overexpression caused mitochondrial remodeling as well as expansion, resulting in a profound increase in the capacity of the muscle to utilize lipid substrate.

To determine whether the effects of mitochondrial reprogramming would manifest at a systemic level, we next evaluated exercise tolerance and whole body substrate selection after six weeks of HF feeding. At rest, energy expenditure and respiratory exchange ratio (22) were similar between genotypes. As expected, MCK-PGC1α mice reached a higher peak oxygen consumption (VO₂) (Figure 1C) and ran
longer (Figure 1D) during a graded maximal exercise test on an enclosed metabolic treadmill. As compared to the NT group, MCK-PGC1α maintained a lower RER (Figure 1E and 1F), indicative of increased muscle fat oxidation during exercise.

Insulin resistance and impaired glucose control are hallmarks of pre-diabetes. In a previous study MCK-PGC1α transgenic mice were found to be more susceptible to insulin resistance when animals were fed a short-term (3 week) HF diet (16). Investigators speculated that the health benefits of increased mitochondrial mass might take effect only when mice were permitted to exercise. We therefore questioned whether the foregoing genotype differences in fat oxidation during exercise would confer an advantage during a weight loss intervention. Accordingly, mice were fed a HFD for 10 weeks in standard cages, followed by stepwise exposure to running wheels (HFD+Ex) and then Ex plus 25% caloric restriction (Ex/CR), each for an additional 10 weeks (Figure 2A). Weight gain and glucose tolerance were followed longitudinally and compared against a control group that remained sedentary. In general, PGC-1α overexpression did not affect changes in body weight; although weight gain was slightly higher in transgenic mice during weeks 11-20 of the HFD, whereas weight gain was slightly lower in this group during the Ex/CR phase of the intervention (Figure 2B-D). Daily caloric intake and food efficiency were also similar between NT and MCK-PGC1α mice (Figure 2E-F). Citrate synthase activity measured in muscle homogenates was 2.3-fold greater in MCK-PGC1α versus NT (156 and 362 µM/min/mg tissue, respectively), but was unaffected by the Ex/CR intervention in both genotypes. Mean running distance was similar between genotypes but highly variable (Figure 2G).
Running distance was unrelated to weight gain when mice were fed ad libitum (Figure 2H), but correlated strongly with weight loss during the Ex/CR phase of the intervention ($r^2=0.7805$, $p< 0.001$) (Figure 2I). In a separate experiment, mice were housed with running wheels at the onset of a HFD for ten weeks. Again, running distance and metabolic outcomes were similar between the NT and MCK-PGC1α mice (Supplemental Data Figure 1A-F), and weight gain correlated negatively with running distance but was unaffected by genotype (Supplemental Figure 1F).

Figure 3A shows results of a glucose tolerance test performed in the age-matched sedentary control groups after 26 weeks of HFD. At this time point, which corresponds with the six week Ex/CR group in Figure 3B, diet-induced glucose intolerance was more severe in the transgenic compared to NT mice. Similar results were observed after only two weeks of HFD (not shown). Figure 3B shows a series of glucose tolerance tests performed longitudinally after 10 weeks of HFD and then at the six week time point of each intervention arm. Glucose tolerance tests were similar between genotypes at the 10 week time point. Exercise alone elicited comparable improvements in glucose clearance in NT and MCK-PGC1α mice (Figure 3B), and the combination of Ex/CR further enhanced glucose tolerance in both groups (Figure 3B). In both the NT and MCK-PGC1α mice the Ex/CR intervention resulted in a dramatic 80% decline in fasting plasma insulin levels as compared to the sedentary groups, indicative of profound improvements in whole body insulin sensitivity (Figure 3C). The Ex/CR regimen decreased muscle glycogen levels in both genotypes (Figure 3D), consistent with negative energy balance. Muscle insulin sensitivity per se was evaluated by measuring insulin-stimulated glycogen synthesis in isolated soleus and
extensor digitorum longus (EDL) muscles, which are comprised predominantly of red/oxidative and white/glycolytic fiber types, respectively. When mice were fed a HF diet and remained sedentary, basal rates of glycogen synthesis were similar in both genotypes, but PGC-1α overexpression decreased rates of insulin-stimulated glycogen synthesis in soleus muscle by 35% (Figure 3E). A similar trend in rates of insulin-stimulated glycogen synthesis was evident in the EDL (Figure 3F). In both genotypes, Ex/CR improved insulin-responsiveness in soleus muscles, from approximately 1.3-fold in the sedentary state to 4-fold over the basal condition in the intervention groups. By contrast, insulin sensitivity in isolated EDL muscles, which are only minimally recruited during wheel running, was not improved in either group (Figure 3D).

Using a targeted metabolomics approach we proceeded to evaluate a comprehensive set of lipid molecules and metabolic intermediates that have been linked to the pathogenesis of insulin resistance. Intramuscular triacylglycerol (IMTG) levels were similar between genotypes in sedentary mice, but decreased 60% and 45% in response to Ex/CR in NT and MCK-PGC1α mice, respectively (Figures 4A&B). Total diacylglycerol (DAG) levels and most individual DAG species (Figure 4C&D) were modestly elevated in MCK-PGC1α compared to NT mice. Whereas Ex/CR tended to decrease the 18:1 and 18:2 DAG species in NT mice, the opposite response was observed in MCK-PGC1α mice. Total muscle ceramide content was unaffected by genotype and the Ex/CR intervention, whereas specific species (C16 and C20) were decreased in response to Ex/CR, but only in the NT mice (Figure 4E&F).
Acylcarnitine metabolites have emerged as strong biomarkers of mitochondrial stress and/or nutrient load. These metabolites are generated by a family of mitochondrial localized acyltransferase enzymes that convert acyl-CoA intermediates of glucose, fatty acid and amino acid catabolism to their cognate carnitine esters (23,28). Evaluation of muscles from HFD fed, sedentary mice revealed robust increases in several carnitine and CoA species as a result of PGC-1α overexpression (Figure 5A-D), including free carnitine, free CoA and multiple even chain acyl moieties. Most even chain acyl groups represent partially oxidized intermediates of fatty acid β-oxidation; thus, this metabolite profile aligns with the mitochondrial phenotype presented in Figure 1. In the MCK-PGC1α group, the Ex/CR intervention lowered several fatty acid-derived mitochondrial intermediates, implying that excessive nutrient load as well as increased mitochondrial mass contributed to the foregoing accumulation of metabolites. By contrast, in the NT control group, the same intermediates were largely unchanged or modestly increased by Ex/CR (Figure 5B-D).

In the sedentary, HFD condition, muscle concentrations of the tricarboxylic acid cycle intermediates, succinate, fumarate and malate, were elevated in MCK-PGC1α compared to NT mice, whereas levels of pyruvate, the end product of glycolysis, were lower. With the exception of alpha-ketoglutarate, which decreased uniformly with Ex/CR, the effects of the intervention differed by genotype. Most notably, succinate increased by 75% in NT but decreased by 40% in MCK-PGC1α mice. We also examined a cluster of amino acids that have been identified as strong predictors of diabetes risk in humans, including the branched chain amino acids (BCAA; leucine,
isoleucine, valine) as well as tyrosine and phenylalanine (30). PGC-1α overexpression did not affect muscle levels of these amino acids, but did cause dramatic increases in glutamate/glutamine (Glx) and arginine, along with a marked reduction in glycine levels. In general, the Ex/CR intervention tended to lower most amino acids in both genotypes. Catabolism of amino acids gives rise to the odd chain species of acylcarnitines and acyl-CoAs. With the exception of isovaleryl carnitine (C5), these metabolites were elevated in sedentary MCK-PGC1α mice (Figure 6C&D). Only succinyl-CoA declined upon Ex/CR in the transgenic animals. By contrast, the intervention increased most of these metabolites in NT mice, with the notable exception of isovaleryl carnitine (C5), which declined in both genotypes (Figure 6C).

The conversion of acyl-CoAs to their membrane permeant carnitine esters permits acyl group efflux from the mitochondrial matrix to the general circulation, which typically occurs when substrate provision exceeds flux. Accordingly, the plasma acylcarnitine profile provides a systemic view of mitochondrial metabolism. Compared to the NT control group, C2-C5 plasma carnitine species were elevated in sedentary MCK-PGC1α mice and decreased in response to EX/CR in both genotypes (Figure 6E&F). Principal components analysis (PCA) was employed as an unbiased data reduction strategy to examine the relationship between muscle metabolites and several physiologic outcomes (Figure 7). Factor 1 emerged as a large group of mitochondrial-derived intermediates (Figure 7A). This factor was affected by genotype but unrelated to changes in body weight and insulin action (Figure 7B). Factor 2, representing a large cluster of ceramide metabolites, was not affected by genotype or the CR/Ex treatment and was unrelated to glucose tolerance. Factor 4, consisting of muscle glycogen,
several IMTG species and the 18:1 and 18:2 DAG species, was affected by the Ex/CR intervention and correlated with body weight, running distance fasting insulin and insulin-stimulated glycogen synthesis in soleus muscle. Factor 5, which was also robustly influenced by Ex/CR, emerged as the most compelling correlate of glucose control evidenced by strong associations with multiple measures of muscle and whole body insulin action (Figure 7B). Interestingly, this factor was heavily weighted by an eclectic group of metabolites that included specific species of ceramides, acylcarnitines, amino acids and TCA cycle intermediates. Factor 7, consisting of a sizable cluster of amino acids, was the only factor influenced by an interaction between genotype and treatment and was modestly associated with fasting insulin and whole body glucose tolerance.

**Discussion**

This study was designed to test the idea that elevated mitochondrial mass and oxidative potential in skeletal muscle affords protection against metabolic disease, and/or facilitates metabolic improvements in response to lifestyle modification. The results offer two important outcomes regarding the relationship between mitochondrial function, intramuscular lipid balance and whole body energy homeostasis. First, PGC-1α-mediated enhancement of oxidative potential did not prove beneficial for preventing or treating obesity and glucose intolerance. Secondly, comprehensive metabolic profiling of several leading candidate mediators of muscle insulin resistance failed to reveal a compelling frontrunner that tracked with changes in glucose tolerance irrespective of genotype. Instead, PCA identified a chemically diverse cluster of
metabolites (Factor 5) that correlated with multiple measures of muscle and whole body insulin responsiveness. Together, these findings show that the interplay between mitochondrial performance, lipotoxicity and insulin action is more complex than previously proposed (31).

In the current study, as well as a previous report (16), diet-induced weight gain was similar between controls and MCK-PGC1α mice despite markedly augmented capacity for lipid oxidation in the latter group. These findings argue against the possibility that increasing muscle mitochondrial density raises resting energy expenditure by elevating uncoupled respiration due to inherent mitochondrial leak. Because PGC-1α transgenic mice had a lower RER during treadmill running, we questioned whether this exercise-induced augmentation of fat oxidation would result in diminished weight gain or enhanced weight loss when animals were given access to running wheels. As anticipated, exercise and caloric restriction improved metabolic homeostasis in both groups. Interestingly, in two separate experiments, running distance had a stronger influence on body weight and glucose homeostasis than genotype. In aggregate, these findings suggest that changes in adiposity depended on the energetic costs of muscle contraction rather than muscle mitochondrial content or substrate selection. By contrast, an earlier report found that MCK-PGC-1α transgenic mice had enhanced weight loss and glucose metabolism in response to an exercise intervention comprised of graded high intensity treadmill running performed three times weekly for the final 3.5 weeks of a 6-week high fat diet (32). Although these results imply that increased mitochondrial biogenesis might benefit weight loss during a high
intensity exercise intervention, the lower intensity running wheel model better represents lifestyle modification programs that are likely to be maintained in human populations.

When animals remained sedentary, diet-induced glucose intolerance was actually more severe in the MCK-PGC1\(\alpha\) mice. This phenotype was originally reported by (16) in a study that confirmed muscle insulin resistance by hyperinsulinemic-euglycemic clamp after three weeks of HF feeding. Investigators attributed the diabetic nature of MCK-PGC1\(\alpha\) mice to elevated intramuscular TAG and DAG, and consequent activation of serine kinases that interfere with insulin signaling. In the current study, muscle levels of total TAG, DAG and ceramide were similar between genotypes after a prolonged (30 week) HFD, whereas a subset of specific DAG species was modestly increased in the MCK-PGC1\(\alpha\) mice. These results are similar to a study wherein intramuscular DAG species were elevated in trained athletes (33). The metabolite clusters that best discriminated the MCK-PGC1\(\alpha\) from NT mice were those of mitochondrial origin, including acetyl-CoA, acetylcarnitine, long chain acylcarntines, long chain acyl-CoAs, succinate and succinyl-CoA. Thus, raising mitochondrial content in the context of caloric excess and physical inactivity elevated muscle production and/or accumulation of mitochondrial intermediates without changing energy expenditure. A strong indication that nutrient load contributed to this signature comes from the observation that muscle concentrations of many of these metabolites decreased in MCK-PGC1\(\alpha\) mice in response to the Ex/CR intervention. Whether this form of nutrient-induced mitochondrial stress contributed to adverse physiological outcomes remains uncertain at this stage.
The current study afforded the opportunity to query muscle tissues for metabolic signatures that associate with improved glucose tolerance in response to a weight loss intervention. We interrogated a comprehensive array of lipids and other metabolites previously implicated as chief culprits of insulin resistance. These included multiple species of IMTG, DAG, ceramides, acylcarnitines, acyl-CoAs, as well as the BCAAs. The most consistent response to the weight loss intervention was a marked decline in muscle energy reserves. Thus, in both the NT and MCK-PGC1α groups the Ex/CR intervention lowered muscle levels of glycogen and IMTG, likely reflecting a local shift from positive to negative energy balance. In general, these macromolecular fuel reservoirs are considered innocuous, whereas the foregoing metabolic intermediates harness more potential as bioactive signaling molecules. Remarkably however, few of the over 200 metabolic intermediates measured changed uniformly in the NT and transgenic mice when comparing the sedentary to the Ex/CR condition. For example, Ex/CR caused marked reductions in muscle concentrations of several mitochondrial-derived intermediates in MCK-PGC1α mice, but these same metabolites were either unchanged or modestly increased by the intervention in the control group. Likewise, gross changes in muscle levels of DAGs, ceramides and BCAAs failed to explain disparities in glucose tolerance across genotypes and treatments. Interestingly, Ex/CR lowered circulating levels of short chain acylcarnitines in both geneotypes. Although circulating metabolites originate from multiple tissues, the observation that several serum acylcarnitines were increased in MCK-PGC1α compared to NT mice suggests that the systemic pool of these analytes can be derived from and report on muscle energy metabolism. Thus, the foregoing reduction in serum short chain acylcarnitines
could be reflecting diminished nutrient load on mitochondria residing in skeletal muscle as well as other tissues.

Interestingly, an unbiased PCA identified a single factor (Factor 5) that correlated with changes in energy balance and running distance as well as multiple measures of muscle insulin sensitivity and whole body glucose tolerance. This factor was heavily weighted by a diverse group of metabolites comprised of specific species of ceramides, acylcarnitines, amino acids and organic acids. The simplest interpretation of these data is that intermediates arising from multiple nutrient sources and metabolic pathways influence insulin action. Alternatively, flux of these metabolites might be coupled to the generation of common regulatory molecules that were not evaluated. Also possible is that metabolite concentrations measured in whole tissues do not reflect the specific pool(s) that interact with the insulin signaling pathway.

Lastly, it is important to consider the utility and caveats of the MCK-PGC1α transgenic mouse model. We selected this model because when fed a standard chow diet, the transgenic mice phenocopy an exercise trained state in nearly every physiological parameter examined. They outperform their NT counterparts during an exercise challenge, have improved metabolic and cognitive function upon aging, and they live longer (15,17,32,34-36). Although it could be argued that the ~2.5-fold induction of mitochondrial content measured in PGC-1α transgenic muscles lacks physiological relevance, this is true only when considering the adaptive impact of an exercise intervention in a given individual. Alternatively, when comparing mitochondrial content in muscles of individuals at far ends of the aging, disease and/or fitness spectrums, a 3-fold difference does fall within a physiological range (37-39). A caveat
of the model is that PGC-1α targets other metabolic processes in addition to mitochondrial biogenesis and oxidative capacity, including nutrient delivery and storage (40). Nonetheless, these same processes are upregulated by exercise training (41,42). Additionally, both exercise and PGC-1α overexpression have been shown to regulate muscle production and secretion of myokines that act both centrally and peripherally to regulate energy metabolism (43). Thus, intermittent versus constitutive production of these factors could yield different outcomes. Three other genetically engineered mouse models of increased mitochondrial biogenesis in skeletal muscle have been described (20,44-46). Skeletal muscle-specific overexpression of either ERRγ (44) or PPARδ (20,45) failed to confer protection against diet-induced metabolic derangement, whereas mice overexpressing constitutively active PPARδ resisted both diet-induced obesity and glucose intolerance. Notably, overexpression of constitutively active PPARδ caused a dramatic increase in whole body energy expenditure that coincided with improvements in glucose control (20), further underscoring the tight connection between glucose homeostasis and energy balance.

In summary, exercise-induced mitochondrial adaptations in skeletal muscle are known to play a key role in mediating improvements in fitness and athletic performance; however, their presumed role in combating metabolic disease is based largely on circumstantial evidence. The MCK-PGC1α transgenic mouse model provides a tractable experimental tool for proof-of-concept studies aimed at understanding the health benefits of increased mitochondrial biogenesis in the absence of exercise training. The findings reported here raise doubt that pharmacological exercise mimetics
that increase oxidative capacity have high potential as anti-obesity and/or anti-diabetic agents. Instead, the evidence suggests that the salutary metabolic effects of habitual physical activity depend on the energetic costs of muscle contraction, and that lifestyle factors such as diet and exercise induce local shifts in intramyocellular energy balance, which in turn impacts a broad network of metabolic intermediates involved in nutrient sensing and insulin action.

Acknowledgements.

Funding. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grants R01-DK089312 (DMM), 2P01-DK058398 (DMM), 1F32DK094573 (KEW), R01-DK082803 (CLK)

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

Author contributions. K.E.W. conducted research, designed experiments and wrote the manuscript. C.R.M. performed statistical, analysis data interpretation and reviewed and edited the manuscript. D.H.S., S.E.S., and K.L.D., executed metabolic experiments. O.R.I. K.I.C. performed mass spectrometry measurements. K.I.C. measured muscle lipid content with gas chromatography. M.K. performed the proteomic analysis. C.L.K. reviewed and edited the manuscript and conducted research. R.D.S. performed mass spectrometry analysis and reviewed the manuscript. D.M.M. designed experiments and wrote the manuscript. Dr. Muoio is the guarantor of
this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

References


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**Antioxidant**

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**Table 1. Skeletal muscle mitochondrial proteomics.** Mitochondria were isolated from the quadriceps muscles of MCK-PGC1α and NT mice fed a high fat diet for 6 weeks. Protein abundance was analyzed by a triple quadrupole mass spectrometer using a targeted assay and normalized to a BSA standard. VDAC was measured as a housekeeping protein and the range of p values from four separate assays is provided. *Expression levels of VDAC was significant (p<0.05) in only one of the four assays performed. Data are expressed as a ratio of protein abundance measured in MCK-PGC1α relative to NT mice. Statistical significance was analyzed by Student's t-test. n=3-4 per group.
Figure 1. **Muscle-specific overexpression of PGC-1α increased mitochondrial preference for lipid substrate.** MCK-PGC1α transgenic mice and non-transgenic littermates (NT) were fed a high fat diet for six weeks prior to experiments. Mitochondria isolated from gastrocnemius muscles were used to assess oxidation of 200 µM $[^{14}\text{C}]$palmitate to (A) CO$_2$ and (B) acid soluble metabolite (ASM), measured in the absence (FA) or presence (FA+Pyr) of 1 mM pyruvate. Whole body energy metabolism and exercise performance were assessed during a graded treadmill test during which (C) oxygen consumption, (D) distance to exhaustion, (E) respiratory exchange ratio and (F) average RER were evaluated. Values are mean ± S.E. for 6-9 mice per group. *indicates p<0.05 between genotypes. **indicates p<0.005 between genotypes.

Figure 2. **Muscle-specific overexpression of PGC-1α does not defend against diet-induced obesity or promote weight loss in response to exercise and caloric restriction.** (A) Non-transgenic (NT) and MCK-PGC1α mice were fed a 45% high fat diet (HFD) for ten weeks prior to ten weeks of voluntary wheel running (HFD+Ex) followed by an additional ten weeks of wheel running combined with 25% caloric restriction (Ex/CR) with continued HF feeding. (B) Body weight measurements taken throughout the course of this study for both sedentary (Sed) and Ex/CR groups. Rates of weight change per week during the three phases of the study for the Sed (C) and Ex/CR (D) group. (E) Caloric intake measured every two days during the HFD+Ex phase. Food efficiency, an estimate of how much food ingested is converted to body mass (weight gain (g) per week/food ingested (g) per week), during the HFD+Ex phase...
of the study. (G) Average daily running distance during HFD+Ex and Ex/Cr. The relationships between running distance and (H) weight gain or (I) weight loss during HFD+Ex and Ex/CR, respectively. Values are mean ± S.E. for 6-8 mice per group. *indicates p<0.05 between genotypes.

Figure 3. Muscle-specific overexpression of PGC-1α does not defend against glucose intolerance or enhance glucose control in response to exercise and caloric restriction. Mice were fed a high fat diet (HFD) for 26 weeks and measures of insulin action were made at the designated time points. Intraperitoneal glucose tolerance tests (GTT; 1.5 mg/kg lean body weight) were performed on age-matched cohorts of non-transgenic (NT) and MCK-PGC1α mice that (A) remained Sed for the duration of the HFD or (B) were given access to running wheels (HFD+Ex) during weeks 11-20, followed by an additional 10 weeks of Ex combined with 25% caloric restriction (Ex/CR). Blood and tissues were harvested at 30 weeks and used for analysis of (C) fasting insulin levels and (D) glycogen content in gastrocnemius muscles. Insulin-stimulated glycogen synthesis, expressed as fold change relative to basal rates assessed in the contralateral muscles, was measured in (E) isolated soleus and (F) isolated EDL. Data were analyzed by two-way ANOVA. A main effect of Ex/CR on insulin, glycogen and insulin-stimulated glycogen synthesis in the soleus were detected, but symbols were excluded for simplicity. Data represent means ± S.E. for 6-8 mice per group. *indicates p<0.05 between genotypes. # indicates p<0.05 within a genotype between treatment conditions (Sed versus Ex/CR).
Figure 4. Effects of exercise and caloric restriction on muscle content of triacylglycerol, diacylglycerol and ceramides in NT and MCK-PGC1α mice fed a high fat diet. Lipid metabolites were measured in gastrocnemius muscles of non-transgenic and MCK-PGC1α mice following 30 weeks of high fat feeding without or with exercise and caloric restriction (Ex/CR). (A) Total triacylglycerol (TAG) and (B) individual TAG species. (C) Total diacylglycerol (DAG) and (D) individual DAG species. (E) Total ceramides and (F) individual ceramide species. 2-Way ANOVA revealed an interaction between genotype and TAG and DAG levels. Data represent means ± S.E. for 6-8 mice per group. Statistical differences were analyzed by two-way ANOVA. *indicates p˂0.05 between genotypes, # indicates p˂0.05 within a genotype between treatment conditions (Sed versus Ex/CR).

Figure 5. Effects of exercise and caloric restriction on lipid-derived acylcarnitine and acyl-CoA metabolites in NT and MCK-PGC1α mice fed a high fat diet.

Lipid metabolites were measured by tandem mass spectrometry using gastrocnemius muscles from NT and MCK-PGC1α mice, following 30 weeks of high fat feeding without or with exercise and caloric restriction (Ex/CR). (A) Free carnitine and acetylcarnitine. (B) Even-chain acylcarnitines. (C) Free CoA and acetyl-CoA. (D) Even chain acyl-CoAs. Data represent means ± S.E. for 6-8 mice per group. Statistical differences were analyzed by two-way ANOVA. There was an effect of treatment group on free carnitine, acylcarnitine, and acyl-CoA levels. *indicates p˂0.05 between genotypes, # indicates p˂0.05 within a genotype between treatment conditions (Sed versus Ex/CR).
Figure 6. Effects of exercise and caloric restriction on muscle organic and amino acid and plasma acylcarnitine metabolites in NT and MCK-PGC1α mice fed a high fat diet.

Metabolites were measured by tandem mass spectrometry using gastrocnemius muscles from non-transgenic and MCK-PGC1α mice, following 30 weeks of high fat feeding without or with exercise and caloric restriction (Ex/CR). (A) Organic acids. (B) Amino acids. (C) Acylcarnitine and (D) acyl-CoA intermediates of amino acid catabolism. (E) Plasma acetylcarnitine. (F) Plasma short chain acylcarnitines. Data represent means ± S.E. for 6-8 mice per group. Statistical differences were analyzed by two-way ANOVA. There was an effect of treatment group on organic acid, amino acid, and their acylcarnitine and acyl-CoA metabolites. *indicates p<0.05 between genotypes, # indicates p<0.05 within a genotype between treatment conditions (Sed versus Ex/CR).

Figure 7. Principal components analysis and factor associations with functional outcomes. Principal components analysis (PCA) was used as a data reduction strategy for exploratory purposes. Factors comprised of strongly corrected metabolites were surveyed for potential relationships with measures of energy and glucose homeostasis (see Methods Section). (A) Key metabolites in PCA Factors 1-7 and the effect of genotype and treatment (Ex/CR) and on each Factor. (B) Heat map illustrating the positive (red) and negative (blue) associations between Factors 1-7 and physiologic outcome measures. GTT (26 weeks); blood glucose levels measured 120 minutes after
an intraperitoneal glucose injection; ISGS- insulin stimulated glycogen synthesis; EDL extensor digitorum longus muscle.
Figure 1

A

Palmitate oxidation to CO₂ (nmol/mg protein/h)

- NT
- MCK-PGC1α

FA FA+Pyr

B

Palmitate oxidation to ASM (nmol/mg protein/h)

- NT
- MCK-PGC1α

FA FA+Pyr

C

VO₂ (ml/kg/h)

- NT
- MCK-PGC1α

Time (sec)

p<0.0001

D

Distance (m)

- NT
- MCK-PGC1α

E

RER

- NT
- MCK-PGC1α

Time (sec)

F

Average RER

- NT
- MCK-PGC1α

* **
Figure 4

A

Total TAG (µg/mg tissue)

B

TAG (µg/mg tissue)

C

Total DAG (µg/mg tissue)

D

DAG (µg/mg tissue)

E

Ceramide (pmol/mg tissue)

F

Ceramide (pmol/mg tissue)
**Figure 7**

<table>
<thead>
<tr>
<th>Factor</th>
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<th>Treatment</th>
<th>Genotype and treatment</th>
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<td>Long, medium and short chain acylcarnitines, long, medium, and short chain acyl CoAs, Gly</td>
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<tr>
<td>3</td>
<td>Carnitine, free CoA, short chain acylcarnitines (C3-DC, C5:1, C5-OH), acyl-CoAs (succinyl, oleoyl, linoleoyl) ceramides (C23, C22) Asx, Glx, Arg</td>
<td>&lt;0.001*</td>
<td>0.196</td>
<td>0.642</td>
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<td>0.289</td>
<td>0.153</td>
<td>0.006*</td>
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Key metabolites within each retained factor (i.e. metabolites with factor load ≤|0.4|) and an overall description of each factor are presented. Underlined metabolites had a negative load score. *p > 0.05, OH-hydroxyl, DC-dicarboxylic, TAG-triacylglycerol, DAG-diacylglycerol.
Supplemental Figure 1. **Metabolic responses to an exercise intervention initiated concurrently with high fat feeding were similar between genotypes.** NT and MCK-PGC1α mice were housed individually with running wheels at the onset of a high fat diet for ten weeks. (A) Body weight and (B) food intake were similar between groups over time. (C) Glucose levels were monitored over time, showing no significant difference between groups. (D) Fasting insulin levels were significantly higher in the MCK-PGC1α group compared to NT. (E) Distance traveled per day was measured and showed no significant difference between groups. (F) % weight change per week was plotted against distance traveled, with a significant correlation observed (r² = 0.5367, p = 0.0029).
the course of the experiment. (C) Glucose tolerance examined by i.p. glucose injections at the beginning (week 0) and end (week 10) of the study was comparable between genotypes. (D) Fasting plasma insulin levels measured at week 10 were similar between genotypes. (E) Voluntary running distance was similar between NT and MCK-PGC1α mice. (F) Weight gain during the 10 week high fat diet correlated negatively with running distance. N=6-7 per group.