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Published in Am J Physiol Endocrinol Metab. 2017 312(5): E437-46. PMID: 28223292. doi: 10.1152/ajpendo.00361.2016

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Running title: Muscle PGC-1α and ketogenic diets

Keywords: Skeletal muscle; ketogenic diet; PGC-1α; exercise; ketone bodies

ABSTRACT

Low carbohydrate/high-fat (LCHF) diets are increasingly popular dietary interventions for body weight control and as treatment for different pathological conditions. However, the mechanisms of action are still poorly understood, in particular in long-term administration. Besides liver, brain and heart, skeletal muscle is one of the major organs involved in the regulation of physiological and pathophysiological ketosis. We now assessed the role of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) in skeletal muscle of male wild type control (CTRL) and PGC-1α muscle-specific knockout (PGC-1α mKO) mice upon 12 weeks of LCHF diet feeding. Interestingly, LCHF diet administration increased oxygen consumption in a muscle PGC-1α-dependent manner concomitant with a blunted transcriptional induction of genes involved in fatty acid oxidation and impairment in exercise performance. These data reveal a new role for muscle PGC-1α in regulating the physiological adaptation to long-term LCHF diet administration.

Abbreviations: Acadl, Acyl-CoA dehydrogenase long chain; Acadvl, Acyl-CoA dehydrogenase very long chain; Acat1, Acetyl-CoA acetyltransferase 1; ALAT, Alanine transaminase; ASAT, Aspartate transaminase; Atp5a, ATP synthase 5 alpha; Bdh1, 3-hydroxybutyrate dehydrogenase type 1; β-OHB, β-hydroxybutyrate; CD36, Cluster of differentiation 36; Cpt1b, Carnitine palmitoyltransferase 1b; Cs, Citrate synthase; ERRα, Estrogen-related receptor α; Glut 4, Glucose transporter 4; HKII, Hexokinase II; LCHF, Low carbohydrate/high-fat; Mct1, Monocarboxylate transporter 1; Ndufb8, mitochondrial NADH dehydrogenase 1 beta subcomplex subunit 8; NEFA, non-esterified fatty acids; Oxct1, Succinyl-CoA:3-ketoacid-coenzyme A transferase 1; Pdk4, Pyruvate dehydrogenase lipoamide kinase isozyme 4; Pfkm, Phosphofructokinase; PGC-1α, Peroxisome proliferator-activated receptor γ coactivator 1α; PGC-1β, Peroxisome proliferator-activated receptor γ coactivator 1β; Pkm1, Pyruvate kinase muscle 1; PPARα, Peroxisome proliferator-activated receptor α; PPARδ, Peroxisome proliferator-activated receptor δ; RER, Respiratory exchange ratio; Sdhb, Mitochondrial succinate dehydrogenase iron-sulfur subunit; Uqcrc2, Mitochondrial cytochrome b-c1 complex subunit 2

INTRODUCTION

In recent years, ketogenic diets have emerged as potent therapeutic strategies for numerous diseases (27). In contrast to classical high fat diets, ketogenic diets are characterized by a lower content of carbohydrates and proteins and will promote a dietary state reminiscent of fasting, diametrically opposite of the fed-like phenotype evoked by high fat diets. Historically, low carbohydrate/high-fat (LCHF) diets have been developed for and successfully used in the treatment of epilepsy, in particular to reduce seizures in children who are non-responders to pharmacological interventions (19). Increasing evidence has expanded the usage of LCHF diets to metabolic disorders like obesity, cardiovascular diseases or type 2 diabetes, but also certain types of cancer (6, 7, 9, 12, 30, 37). LCHF diets induce a state known as ketosis, which also occurs physiologically after prolonged fasting periods, exercise or other contexts of low carbohydrate availability (20). Ketosis is characterized by the increased production of ketone bodies like β-hydroxybutyrate (β-OHB) and acetoacetate in a process called ketogenesis in the liver (14). Circulating ketone bodies are then used by extrahepatic tissues as energy substrates in the Krebs cycle and oxidative phosphorylation (OXPHOS), in particular in the brain, skeletal and heart muscles. The exact mechanisms by which LCHF diets exert their actions are still poorly understood. However, increased fatty acid oxidation (25, 34), mitochondrial biogenesis and ATP production (8) have been proposed to be important pathways mediating the positive effects of ketogenic diets.

The peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) functions as an essential transcriptional coactivator for target genes in all of these metabolic processes (4). Furthermore, PGC-1α regulates ketolytic gene expression in skeletal muscle and thereby potently affects systemic ketosis (33). Strikingly, high muscle PGC-1α reduced post-exercise ketosis in mice as previously observed in trained vs. untrained individuals (1, 33) and thus constitutes a major regulator of ketone body homeostasis in exercise. Moreover, skeletal muscle emerges as the key tissue to actively and voluntarily modulate ketone body homeostasis. Importantly, the beneficial and detrimental effects of long term administration of LCHF diets are still debated and the compatibility with exercise training is unclear. Therefore, we now tested whether muscle PGC-1α, the regulatory nexus in endurance

85 training, also contributes to the local and systemic effects of long-term LCHF diet feeding and thus
86 evaluated whole body homeostasis and skeletal muscle metabolism in wild type control (CTRL) and
87 PGC-1α muscle-specific knockout (PGC-1α mKO) mice fed a LCHF diet for 12 weeks. Indeed, we
88 demonstrate that PGC-1α in skeletal muscle is not only essential for basal ketolytic gene expression,
89 but also affects exercise performance and whole body oxygen consumption upon LCHF diet feeding.
90 These findings reveal a new role for PGC-1α in systemic ketone body metabolism and shed new light
91 onto the mechanisms through which LCHF diets exert their effects.
92

MATERIAL & METHODS

Mice and diets

Male mice at the age of 15 weeks were housed in a conventional facility with a 12 h light/12 h dark cycle with free access to food and water. Experiments were performed in accordance with Swiss federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt. The C57BL/6 PGC-1α muscle-specific knockout (mKO) mice used in this study were generated as described in (33). A chow diet (AIN-93G; 7% fat, 58.5% carbohydrates, and 18% protein) and a ketogenic diet (XL75:XP10; 74.4% fat, 3% carbohydrates, and 9.9% protein) were purchased from Provimi Kliba AG (Kaiseraugst, Switzerland). After 12 weeks of chow or LCHF diet feeding *ad libitum*, mice were not fed for 2h in the morning, euthanized by CO_2 inhalation and tissue samples collected.

Body composition and indirect calorimetry

Body weight was monitored weekly and body composition was determined using an EchoMRI-100™ analyzer (EchoMRI Medical Systems) at the end of the treatment period.

Mice were placed in a CLAMS system (Columbus Instruments) to assess VO_2 consumption, VCO_2 production, the respiratory exchange ratio (RER) as well as food intake and spontaneous locomotion (number of breaks of infrared beams in XYZ dimensions).

Exercise tests

Animals were acclimatized to an open treadmill (Columbus Instruments) for 2 d before the start of the experiment, for 5 min at 0 m/min followed by 5 min at 8 m/min and 5 min at 10 m/min, with an incline of 5°. The endurance exercise trial started at 5 m/min for 5 min with a 5° incline, followed by 8 m/min for 10 min. The speed of the treadmill was subsequently increased by 2 m/min every 15 min until exhaustion. Basal blood glucose and lactate levels were assessed in tail vein blood before and after exercise. For indirect calorimetry assessments, mice were acclimatized to treadmill running as described above. Mice were placed in a closed treadmill (Columbus Instruments) where they first sat

for 5 min at 0 m/min at a 5° incline. Subsequently, the test started at 8 m/min for 5 min and the speed was increased every 5 min for 2 m/min until exhaustion.

Blood analysis

Blood glucose, lactate and β-OHB were measured in tail vein blood with a glucose meter (Accu-Chek; Roche, Mannheim, Germany), a lactate plus meter (Nova Biomedical; LSF, Menziken, Switzerland) or a β-OHB meter (Precision Xtra; Abbott Laboratories, Chicago, IL, USA). For plasma analysis, whole tail vein blood was collected in microvette tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 2000 g for 5 min. Total cholesterol, ASAT and ALAT levels were analyzed with a Cobas c 111 system (Roche Diagnostics AG, Rotkreuz, Switzerland). NEFA were measured in plasma using a NEFA-Kit according to the manufacturer's instructions (Wako Diagnostics, Richmond, VA, USA).

Glycogen measurement

10 mg of frozen tissue were homogenized in 200 µl of water using a motorized pestle. To inactivate enzymes samples were boiled at 95°C in a water bath for 10 min before centrifugation at 18000 g. Supernatant was assayed for glycogen using a glycogen assay kit according to the manufacturer's instructions (Abcam, Cambridge, UK).

RNA extraction and qRT-PCR

Frozen tissue was homogenized and total RNA was extracted with Trizol reagent (Thermo Scientific-Invitrogen, Zug, Switzerland) according to the manufacturer's protocol. cDNA synthesis was done using 1 µg of total RNA. Semi-quantitative real-time PCR analysis was performed with Fast SYBR Green Master Mix on a StepOnePlus Real-Time PCR System (both from Thermo Scientific-Applied Biosystems, Foster City, CA, USA). Relative expression levels for each gene of interest were calculated with the ΔΔ*Ct* method, using 18S rRNA as the normalization control. The primer sequences are listed in Table 1.

Immunoblot analysis

Tissues were homogenized in RIPA buffer, and equal amounts of proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Whatman; Sigma-Aldrich). The proteins of interest were detected with the following antibodies: OXCT1 (ab105320; Abcam), ACAT1 (HPA004428; Sigma-Aldrich), eEF2 (2332; Cell Signaling Technology), mitoprofile (MS604; MitoSciences) and polyclonal swine anti-rabbit immunoglobulins/horseradish peroxidase or polyclonal rabbit anti-mouse immunoglobulins/horseradish peroxidase, respectively (P0399 and P0260, Dako, Kyoto, Japan). Densitometric analysis of immunoblots was performed on 6 individual samples with ImageJ software (National Institutes of Health, Bethesda, MD, USA); a representative selection from this group is presented in the respective figures.

Seahorse assay

Total mitochondria were isolated from fresh *Quadriceps* muscle using gradual centrifugation. Minced muscle was homogenized with a motorized pestle and centrifuged at 700 g for 10 min. Supernatant was re-centrifuged at 10500 g for 10 min to obtain crude mitochondrial pellet. Equal amounts of protein were plated on a 96-well Seahorse plate and mitochondrial respiration was measured using the Seahorse XF cell mito stress test kit (103015-100, Seahorse Bioscience) on a XF^{e}96 extracellular flux analyzer (Seahorse Bioscience). The assay buffer was supplemented with either 10 mM malate / 10 mM pyruvate or 20 mM succinate / 2 µM rotenone, respectively, to assess complex I or complex II activity. The amount of ADP used was 4 mM and ATP production was estimated by subtracting ADP-induced OCR values from Oligomycin-induced OCR values.

Statistical analysis

Data are presented as means ± SEM. The unpaired 2-tailed Student's *t* test was used to determine differences between groups. Significance was set at $p < 0.05$ and significant differences between the genotypes (CTRL CHOW vs. mKO CHOW and CTRL LCHF vs. mKO LCHF) were marked with an asterisks (*) while significant differences between the conditions (CTRL CHOW vs. CTRL LCHF and mKO CHOW vs. mKO LCHF) were marked with a hashtag (#).

RESULTS

PGC-1α mKO mice fail to increase oxygen consumption on a LCHF diet

PGC-1α mKO and control mice were fed a normal chow-diet or a LCHF diet for 12 weeks. Both LCHF-fed control and PGC-1α mKO mice showed a reduction in body weight after 1 week compared to the chow-fed cohorts (Fig. 1A). After 12 weeks, only the LCHF-fed control mice were significantly lighter than their chow-fed counterparts (Fig. 1A). At the end of the 12 weeks of LCHF diet feeding, control and PGC-1α mKO mice displayed a significant increase in fat mass (Fig. 1B) as well as reduced lean mass (Fig. 1C) compared to the chow-fed cohorts. This was further reflected in the relative decrease in heart weight (Fig. 1D) in LCHF-fed compared to chow-fed mice. LCHF diet feeding resulted in reduced food intake by weight (Fig. 1E), but importantly not by caloric content (Fig. 1F). LCHF-fed mice showed a significant decrease in respiratory exchange ratio (RER) compared to chow-fed mice (Fig. 1G), which reflected the high fat content of the LCHF diet. Interestingly, LCHF diet feeding increased the oxygen consumption rate (VO_2) only in control mice, whereas PGC-1α mKO mice displayed no increase with LCHF diet feeding (Fig. 1H). LCHF-fed PGC-1α mKO mice also showed a significantly reduced ambulatory activity compared to LCHF-fed control mice (Fig. 1I). These findings indicate that PGC-1α mKO mice exhibit a blunted adaptation to long-term LCHF diet feeding.

PGC-1α mKO mice show a reduced induction of genes encoding proteins involved in fatty acid metabolism in skeletal muscle

LCHF diets affect both glucose and cholesterol metabolism (6, 7, 9, 12, 30, 37). In our study, LCHF diet feeding led to reduced circulating glucose levels and increased muscle glycogen content in control and PGC-1α mKO mice (Fig. 2A and 2B) compared to the chow-fed counterparts. Circulating cholesterol levels were increased in both genotypes (Fig. 2C). However, blood cholesterol was significantly lower in LCHF-fed PGC-1α mKO mice compared to LCHF-fed control mice (Fig. 2C). Circulating non-esterified fatty acids (NEFA) were not different between the groups (Fig. 2D). In line with previous studies (15, 17), significantly increased circulating levels of aspartate transaminase (ASAT) and alanine

transaminase (ALAT) were observed in LCHF-fed control and PGC-1α mKO mice (Fig. 2E and 2F), indicative of liver stress caused by LCHF diet feeding. Furthermore, LCHF diet feeding elevated circulating β-hydroxybutyrate (β-OHB) levels in both cohorts, even though PGC-1α mKO mice depicted a significant hyperketonemia in comparison to control mice (Fig. 2G), similar to our previous observations (33). Next, we assessed the impact of LCHF diet feeding on metabolic pathways in skeletal muscle of control and PGC-1α mKO mice. In line with the reduced circulating glucose levels with LCHF diet feeding, there was a significant reduction in the expression of genes involved in glucose uptake (glucose transporter 4, *Glut4*) and glycolysis (hexokinase 2, *Hk2*; muscle phosphofructokinase, *Pfkm;* pyruvate kinase muscle 1, *Pkm1*) in skeletal muscle from LCHF-fed control and PGC-1α mKO mice (Fig. 2H). Surprisingly, the transcription of ketolytic genes (3-hydroxybutyrate dehydrogenase type 1, *Bdh1* and succinyl-CoA:3-ketoacid-coenzyme A transferase 1, *Oxct1*) was significantly reduced upon LCHF diet feeding (Fig. 2I). In stark contrast, protein levels of OXCT1 and acetyl-CoA acetyltransferase 1 (ACAT1) were significantly increased (Fig. 2J and 2K). The transcript levels of *Glut4*, *Pfkm,* monocarboxylat-transporter 1 *Mct1, Bdh1, Oxct1* and *Acat1* (Fig. 2H and 2I) were lower in PGC-1α mKO mice, even when compared to LCHF-fed control animals. The increased levels of pyruvate dehydrogenase lipoamide kinase isozyme 4 (*Pdk4*) with LCHF diet feeding (Fig. 2L) and various genes encoding proteins involved in fatty acid uptake (cluster of differentiation 36, *Cd36*) and oxidation (carnitine palmitoyltransferase 1b, *Cpt1b*; acyl-CoA dehydrogenase long chain, *Acadl*; acyl-CoA dehydrogenase very long chain, *Acadvl*) indicate a substrate shift towards fatty acid metabolism in control mice (Fig. 2M). Importantly, the induction of these genes was blunted in PGC-1α mKO mice (Fig. 2M). Interestingly, despite the central role of PGC-1α and peroxisome proliferator-activated receptor α (*PPARα)* for the transcriptional control of fatty acid metabolism in skeletal muscle (35), gene expression of both of these regulators was reduced in muscle with LCHF diet feeding (Fig. 2N). Furthermore, the expression levels of PGC-1β, PPARδ and estrogen-related receptor α (*ERRα*) were not changed upon LCHF diet feeding but transcript levels of PPARδ and ERRα were significantly reduced in PGC-1α mKO mice (Fig. 2N).

LCHF diet feeding leads to impaired exercise performance specifically in PGC1α mKO mice

Since LCHF-fed PGC-1α mKO mice showed a blunted induction of fatty acid metabolism in skeletal muscle, we were interested if this would affect exercise performance and substrate utilization during endurance exercise. In line with previous findings (16), PGC-1α mKO mice displayed reduced endurance exercise performance compared to control mice (Fig. 3A). LCHF diet feeding did not affect the endurance capacity of control mice (Fig. 3A). Strikingly however, this diet specifically impaired the exercise performance of PGC-1α mKO mice (Fig. 3A). This phenotype was not associated with any impairment in the ability of PGC-1α mKO mice to increase circulating glucose levels with exercise (Fig. 3B). Moreover, while PGC-1α mKO mice showed elevated blood lactate levels upon exhaustion, as previously published (32), this effect was comparable between chow-fed and LCHF-fed PGC-1α mKO mice (Fig. 3C). In closed treadmills, LCHF-fed mice displayed elevated oxygen consumption during the exercise compared to chow-fed mice (Fig. 3D). Control mice were able to maintain this elevated oxygen consumption during the entire exercise period, except for the last time point of measurement (Fig. 3D). In contrast, VO_2 levels rapidly dropped in PGC-1α mKO animals as exercise intensity increased (Fig. 3D). Similarly, PGC-1α mKO animals could not maintain the low RER observed in LCHF-fed control mice, and displayed an earlier shift to carbohydrate metabolism indicated by the sharp increase in RER (Fig. 3E). These differences were however diet-independent since chow-fed mKO mice also performed significantly worse than their control littermates. Collectively, these findings suggest that LCHF-fed PGC-1α mKO mice have difficulties to keep up with the increased energy demand in endurance exercise and are unable to properly cope with the metabolic changes elicited by LCHF feeding, in particular in exercise.

LCHF diet feeding does not lead to increased mitochondrial biogenesis or ATP levels in skeletal muscle

Ketogenic diet feeding has been proposed to increase mitochondrial biogenesis and ATP levels in the context of neurological diseases (8). Thus, to test whether LCHF diet feeding also leads to an induction of mitochondrial biogenesis in skeletal muscle, we measured the levels of mitochondrial gene expression (mitochondrial succinate dehydrogenase iron-sulfur subunit, *Sdhb*; citrate synthase, *Cs*;

255 mitochondrial cytochrome b-c1 complex subunit 2, *Uqcrc2*) as well as mitochondrial proteins (ATP
256 synthase 5 alpha, ATP5A; UQCRC2; mitochondrial NADH dehydrogenase 1 beta subcomplex subunit 8,
257 NDUFB8) (Fig. 4A and B). As expected, PGC-1α mKO mice exhibited reduced mitochondrial gene
258 expression and protein content (22, 23). However, in contrast to studies in neurological tissues (8),
259 LCHF diet feeding did not lead to increased mitochondrial transcript or protein levels in skeletal muscle
260 (Fig. 4A and B). Furthermore, mitochondria isolated from *Quadriceps* muscles of LCHF-fed mice showed
261 a drop in ADP-induced complex I respiration and concomitant complex I ATP production (Fig. 4 C and
262 D) while complex II respiration was not affected by LCHF diet feeding (Fig. 4E and 4F).
263

DISCUSSION

Besides physical activity, dietary interventions are a mainstay of prevention and therapy of many diseases. LCHF diets have been increasingly studied in the past decades due to their therapeutic potential, not only in the treatment of epilepsy and other brain-related disorders, but also other pathologies that are associated with peripheral organs (27). Endogenous ketone body levels are in part controlled by hepatic ketogenesis. Dietary ketosis is however largely determined by ketone body metabolism in brain, heart and skeletal muscle. Of these three main consumers, only skeletal muscle can be directly and voluntarily affected and indeed, training can reduce post-exercise ketosis (1). Moreover, we have previously demonstrated that muscle PGC-1α can modulate systemic ketosis in numerous acute physiological and pathophysiological contexts (33). Here, we show that muscle PGC-1α likewise contributes to the local and systemic adaptations of long term LCHF diet feeding. In particular, LCHF diet-induced oxygen consumption was severely blunted in PGC-1α mKO mice. Even more dramatic, LCHF-fed PGC-1α mKO mice displayed a marked impairment in running performance already at moderate exercise intensities and the initial increased oxygen consumption rate quickly dropped to the same level as of chow-fed PGC-1α mKO mice. In contrast, LCHF-fed control mice were able to run the same amount of time as their chow-fed counterparts despite their reduced lean mass assuming that the efficiency of consuming energy from fats is higher upon LCHF diet feeding as suggested by the study of Paoli et al. (25). The analysis of skeletal muscle samples revealed that transcript levels of genes involved in fatty acid uptake and oxidation were elevated in LCHF-fed control mice, while the upregulation of these genes in PGC-1α mKO animals was blunted. It is conceivable that the difference in oxygen consumption rates between LCHF-fed PGC-1α mKO and control mice is in part due to this reduced induction of the respective genes in skeletal muscle in PGC-1α mKO animals. Thus, PGC-1α seems to participate in the LCHF diet-controlled metabolic switch from glucose to ketone body and fatty acid utilization. Furthermore, the decrease in activity levels in LCHF-fed mKO mice could also contribute to the reduced oxygen consumption. Thus, muscle PGC-1α might thereby influence whole body metabolism in LCHF diet feeding.

290 Given the important role of PGC-1α in systemic ketone body metabolism (33) and exercise (28), these
291 findings raise questions about the compatibility of LCHF diets and training. Studies so far have been
292 inconclusive whether LCHF diets improve or hinder training adaptations (24). For example, in the
293 recent study of Zajac et al. (38), VO_{2max} and the lactate threshold were significantly increased in off-
294 road cyclists treated with a LCHF diet. In competitive gymnasts, LCHF diets do not negatively impact
295 explosive and strength performance only when an adequate amount of protein is provided (26). Thus,
296 administration of LCHF diets might differ in endurance compared to resistance training since LCHF diet
297 feeding induces a "fasting-like" state that could hinder the buildup of muscle mass. The inherent
298 problems of the LCHF diet could be circumvented by direct administration of ketone bodies, e.g. in the
299 form of transesterified β-OHB precursor metabolites without the massive acid/salt load associated
300 with intake of β-OHB in acid or salt form (11). The nutritional ketosis elicited by such metabolites
301 promoted an improvement in endurance performance in cyclists even in the presence of normal
302 muscle glycogen, elevated insulin levels or co-administrated carbohydrates (11).
303 The "Atkins diet", a particular form of LCHF diet, has popularized LCHF interventions for weight loss
304 (3). However, despite the widespread use of the Atkins and related diets, the molecular mechanisms
305 and potential detrimental effects are still largely unknown. Indeed, in our study, LCHF diet fed mice
306 displayed some negative effects on whole body metabolism. Even though LCHF diet feeding led to an
307 initial weight loss after one week of treatment, which has also been shown in other rodent studies (5,
308 17, 18), the difference in body weight after 12 weeks of LCHF diet feeding was only minor. Second,
309 LCHF fed mice displayed an increase in fat mass and a concomitant decrease in lean mass (10, 36).
310 Even more alarmingly, LCHF-fed animals showed increased circulating levels of cholesterol, ASAT and
311 ALAT indicative of dyslipidemia and a certain degree of liver stress in line with other studies in mice
312 and humans (13, 21, 31, 39). In fact, long term administration of LCHF diets in rodents in most cases
313 leads to the development of hepatic steatosis and non-alcoholic fatty liver disease (29). Thus, even
314 though the effect of such diets on hepatic lipid levels in humans is less clear, caution is advised in
315 particular in patients with non-alcoholic fatty liver disease (2). It is possible that administration of
316 transesterified ketone body precursor metabolites could act therapeutically without the potential side-

effects of a LCHF diet (11). Furthermore, while the reason for the reduced cholesterol levels in LCHF fed mKO compared to CTRL mice is unclear, this change might be a consequence of the hyperketonemia in mKO animals. Thus, our previous (33) and present findings would suggest that physical activity, and thereby elevation of muscle PGC-1α, is an important adjuvant intervention to manage the pathological consequences of ketosis.

In the brain, the therapeutic effect of LCHF diets on seizures and other pathologies have been linked to increased mitochondrial biogenesis or ATP levels (8). Surprisingly, even though the elevated oxygen consumption and the lower RER values of LCHF-fed mice indicate an overall increase in oxidative metabolism, we did not find any change in mitochondrial gene expression and protein levels in skeletal muscle. Intriguingly, ATP production in isolated mitochondria from LCHF-fed mice was even lower than in chow-fed mice. Thus, the observed increase in oxidative metabolism upon LCHF diet feeding is most likely due to the availability of energy substrates, which are mainly ketone bodies and other kind of fats. Furthermore, these data indicate that LCHF diet feeding predominantly acts on fatty acid oxidation rather than on mitochondrial biogenesis or ATP production in skeletal muscle. Moreover, a recent study in mitochondrial myopathy patients showed short-term adverse and long-term beneficial effects of LCHF diet feeding on skeletal muscle health. Acute treatment of patients with a modified Atkins diet resulted in muscle damage, especially in ragged-red fibers, indicating that nutrition can modify mitochondrial disease progression (1). Surprisingly, in the 2.5 years follow up study patients showed improvements in muscle strength suggesting that the initial fiber degeneration promoted subsequent fiber regeneration resulting in increased muscle force. Thus, care must be taken when administering LCHF diets to patients with mitochondrial-associated diseases and in evaluating responses to short-term treatment.

Taken together, our results clearly demonstrate that PGC-1α in skeletal muscle is essential for maintaining sufficient energy levels during prolonged muscle contractions, especially when carbohydrate availability is low, with important implications for whole body metabolism and energy homeostasis. Finally, it is important to note that even though a LCHF diet induces beneficial health effects by increasing systemic oxidative metabolism, such interventions also exert potentially

detrimental effects, including increasing total blood cholesterol levels, a known risk factor for cardiovascular diseases, or impaired liver function. Therefore, future studies should aim at elucidating the potential of non-LCHF diet-based interventions to modulate ketone body levels such as nutritional ketosis. Alternatively, physiological, e.g. by adjuvant physical activity, or pharmacological modulation of muscle PGC-1α should be considered to mitigate the unwanted side effects of such interventions.

Author contributions: S.S., K.S. and C.H. wrote the manuscript; S.S., K.S. and B.C. designed and performed research and analyzed data.

Acknowledgements:

This project was funded by the Swiss National Science Foundation, the European Research Council (ERC) Consolidator grant 616830-MUSCLE_NET, Swiss Cancer Research grant KFS-3733-08-2015, the Swiss Society for Research on Muscle Diseases (SSEM), SystemsX.ch, the "Novartis Stiftung für Medizinisch-Biologische Forschung" and the University of Basel.

Competing financial interests

The authors declare that they have no conflict of interests.

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FIGURE LEGENDS

Figure 1. *LCHF diet feeding increases fat mass and oxygen consumption while lowering the respiratory exchange ratio.*

A) Body weight curve of mice with an initial weight of 28 g fed a chow or a LCHF diet for 1 or 12 weeks (n=13-16). B) Fat mass in percent of total body weight measured by EchoMRI in mice fed a chow or LCHF diet for 12 weeks (n=7-8). C) Lean mass in percent of total body weight measured by EchoMRI in mice fed a chow or LCHF diet for 12 weeks (n=7-8). D) Relative heart weight of mice fed a chow or LCHF diet for 12 weeks (n=7-8). E) Average food intake measured over a 48 h period in mice fed a chow or LCHF diet for 8 weeks (n=6-8). F-I) Average calorie intake (F), respiratory exchange ratio (RER) (G), oxygen consumption rate (H) and total ambulatory activity (I) measured by indirect calorimetry over a 48 h period in mice fed a chow or LCHF diet for 8 weeks (n=7-8). Error bars represent SEM, and significant differences between chow-fed CTRL and mKO mice and LCHF-fed CTRL and mKO mice ($p < 0.05$), respectively, are indicated by an asterisk (*). Significant differences between chow and LCHF-fed CTRL and chow and LCHF-fed mKO mice ($p < 0.05$), respectively, are indicated by a hashtag (#).

Figure 2. *LCHF-fed mice show a PGC-1α dependent switch from glucose to fatty acid oxidation in skeletal muscle.*

A) Plasma glucose levels of mice fed a chow or LCHF diet for 12 weeks (n=7-8). B) Relative glycogen levels in *Gastrocnemius* muscle of mice fed a chow or LCHF diet for 12 weeks (n=6-8). C-G) Plasma total cholesterol (C), non-esterified fatty acids (NEFA) (D), ASAT (E), ALAT (F) and β-hydroxybutyrate (β-OHB) (G) levels of mice fed a chow or LCHF diet for 12 weeks (n=7-9). H-I) Gene expression in *Gastrocnemius* muscle relative to 18S of genes involved in glucose metabolism (H) and ketolysis (I) (n=6-8). J-K) Representative immunoblots (J) and protein levels of OXCT1 and ACAT1 (K) in *Gastrocnemius* muscle relative to eukaryotic elongation factor 2 (eEF2) (n=6). L-N) Gene expression in *Gastrocnemius* muscle relative to 18S of PDK4 (L) and genes involved in fatty acid uptake and oxidation (M) and transcriptional regulation (N) (n=6-8). Error bars represent SEM, and significant differences

between chow-fed CTRL and mKO mice and LCHF-fed CTRL and mKO mice ($p < 0.05$), respectively, are indicated by an asterisk (*). Significant differences between chow and LCHF-fed CTRL and chow and LCHF-fed mKO mice ($p < 0.05$), respectively, are indicated by a hashtag (#).

Figure 3. *PGC-1α in skeletal muscle is essential to maintain adequate energy levels during exercise upon LCHF diet feeding.*

A) Endurance exercise test of mice fed a chow or LCHF diet for 10 weeks (n=7-8). B-C) Blood glucose (B) and lactate (C) levels before and after exhaustive endurance exercise test of mice fed a chow or LCHF diet for 10 weeks (n=7-8). D-E) Average oxygen consumption rate and respiratory exchange ratio (RER) (E) measured by indirect calorimetry in a closed treadmill of mice fed a chow or LCHF diet for 11 weeks and corresponding bar graphs (n=6-8). Error bars represent SEM, and significant differences between chow-fed CTRL and mKO mice and LCHF-fed CTRL and mKO mice ($p < 0.05$), respectively, are indicated by an asterisk (*). Significant differences between chow and LCHF-fed CTRL and chow and LCHF-fed mKO mice ($p < 0.05$), respectively, are indicated by a hashtag (#).

Figure 4. *LCHF diet feeding does not affect mitochondrial biogenesis and lowers ATP production in skeletal muscle.*

A) Gene expression in *Gastrocnemius* muscle relative to 18S of genes involved in mitochondrial homeostasis (n=6-8). B) Protein levels of different mitochondrial chain complexes in *Gastrocnemius* muscle relative to eukaryotic elongation factor 2 (eEF2) and representative immunoblots (n=6). C-D) Complex I induced oxygen consumption rate (C) and estimated ATP production (D) of isolated mitochondria from *Quadriceps* muscle (n=4-6). E-F) Complex II induced oxygen consumption rate (E) and estimated ATP production (F) of isolated mitochondria from *Quadriceps* muscle (n=4-6). Error bars represent SEM, and significant differences between chow-fed CTRL and mKO mice and LCHF-fed CTRL and mKO mice ($p < 0.05$), respectively, are indicated by an asterisk (*). Significant differences between chow and LCHF-fed CTRL and chow and LCHF-fed mKO mice ($p < 0.05$), respectively, are indicated by a hashtag (#).

528 **Table 1.** *qPCR primer list.*

Gene Name	Forward primer	Reverse primer
18S	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
Acadl	CCAGCTAATGCCTTACTTGGAGA	GCAATTAAGAGCCTTTCCTGTGG
Acadvl	GTAGCCTCCATCCGAAGCTC	CAGGCCCCCATTACTGATCC
Acat1	GTGAAGGAAGTCTACATGGGCA	TGTGGTGCATGGAGTGGAAATA
Bdh1	TTTGCTGGCTGTTTGATGAAGG	TTGAGCTGGATGGTTCTCAGTC
CD36	GGCAAAGAACAGCAGCAAAAT	TGGCTAGATAACGAACTCTGTATGTGT
Cpt1b	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
Cs	CCCAGGATACGGTCATGCA	GCAAACTCTCGCTGACAGGAA
ERRα	ACTGCAGAGTGTGTGGATGG	GCCCCCTCTTCATCTAGGAC
Glut 4	GATGAGAAACGGAAGTTGGAGAGA	GCACCACTGCGATGATCAGA
HKII	AAAACCAAGTGCAGAAGGTTGAC	GAACCGCCTAGAAATCTCCAGAA
Mct1	TGCAACGACCAGTGAAGTATCA	ACAACCACCAGCGATCATTACT
Oxct1	CCCATACCCACTGAAAGACGAA	CTGGAGAAGAAAGAGGCTCCTG
Pdk4	AAA ATTTCCAGGCCAACCAA	CGAAGAGCATGTGGTGAAGGT
Pfkm	GGGGATCACCAATCTGTGTGT	ATCATTCAGCAAGTCGCTCCA
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
PGC-1β	CCATGCTGTTGATGTTCCAC	GACGACTGACAGCACTTGGA
Pkm1	CATTATCGTGCTCACCAAGTCTG	GATTTCGAGTCACGGCAATGATA
PPARα	ACAAGGCCTCAGGGTACCA	GCCGAAAGAAGCCCTTACAG
PPARδ	GCAAGCCCTTCAGTGACATCA	CCAGCGCATTGAACTTGACA
Sdhb	TGACGTCAGGAGCCAAAATGG	CCTCGACAGGCCTGAAACTG
Uqcrc2	CCCATCTTGCTTTGCTGTCTG	AATAAAATCTCGAGAAGGACCCG

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Figure 1

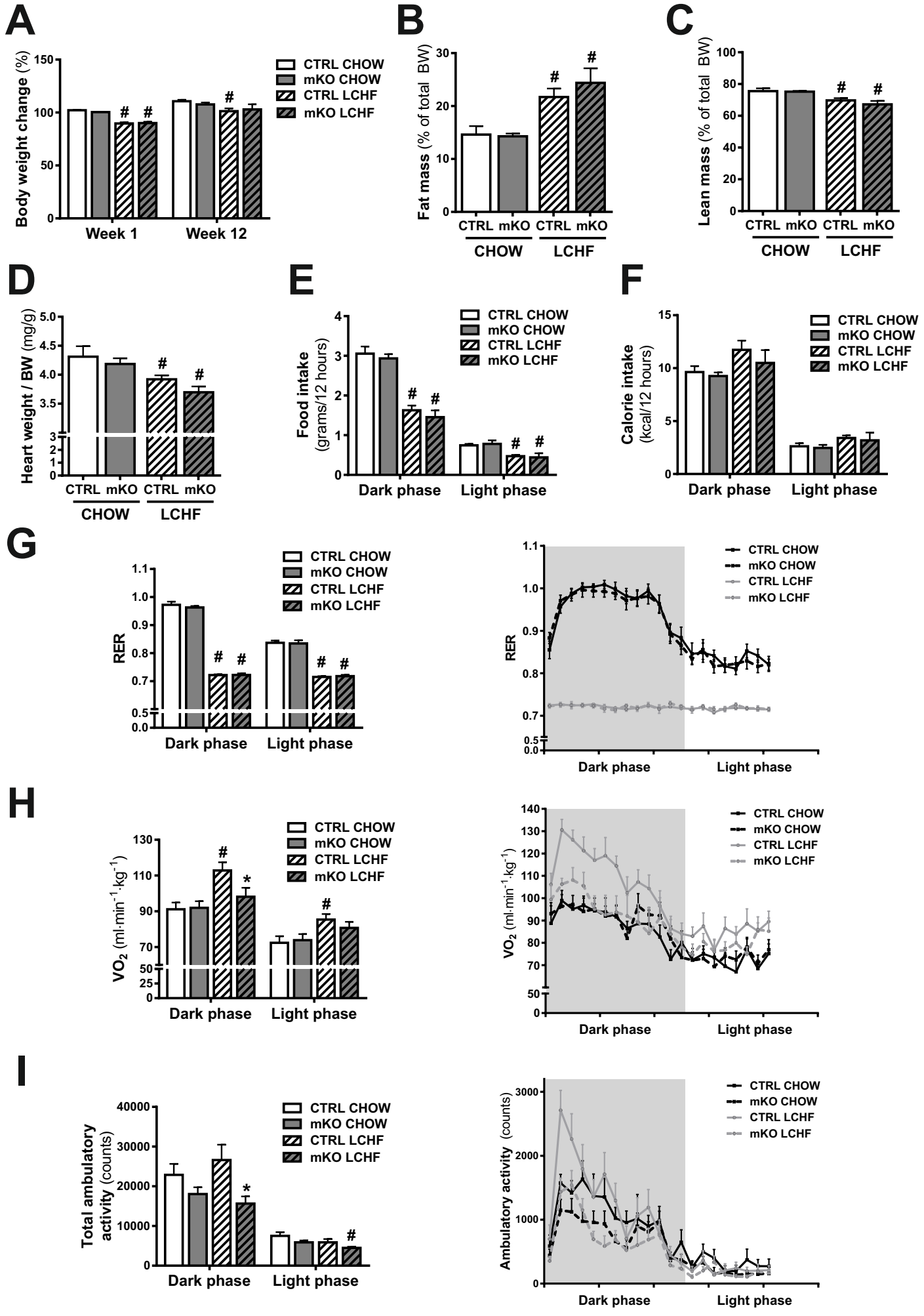

A
Body weight change (%)
Week 1
Week 12
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
B
Fat mass (% of total BW)
CTRL mKO CTRL mKO
CHOW LCHF
C
Lean mass (% of total BW)
CTRL mKO CTRL mKO
CHOW LCHF
D
Heart weight / BW (mg/g)
CTRL mKO CTRL mKO
CHOW LCHF
E
Food intake (grams/12 hours)
Dark phase
Light phase
F
Calorie intake (kcal/12 hours)
Dark phase
Light phase
G
RER
Dark phase
Light phase
H
VO2 (ml·min-1·kg-1)
Dark phase
Light phase
I
Total ambulatory activity (counts)
Dark phase
Light phase
Ambulatory activity (counts)
Dark phase
Light phase

Figure 2

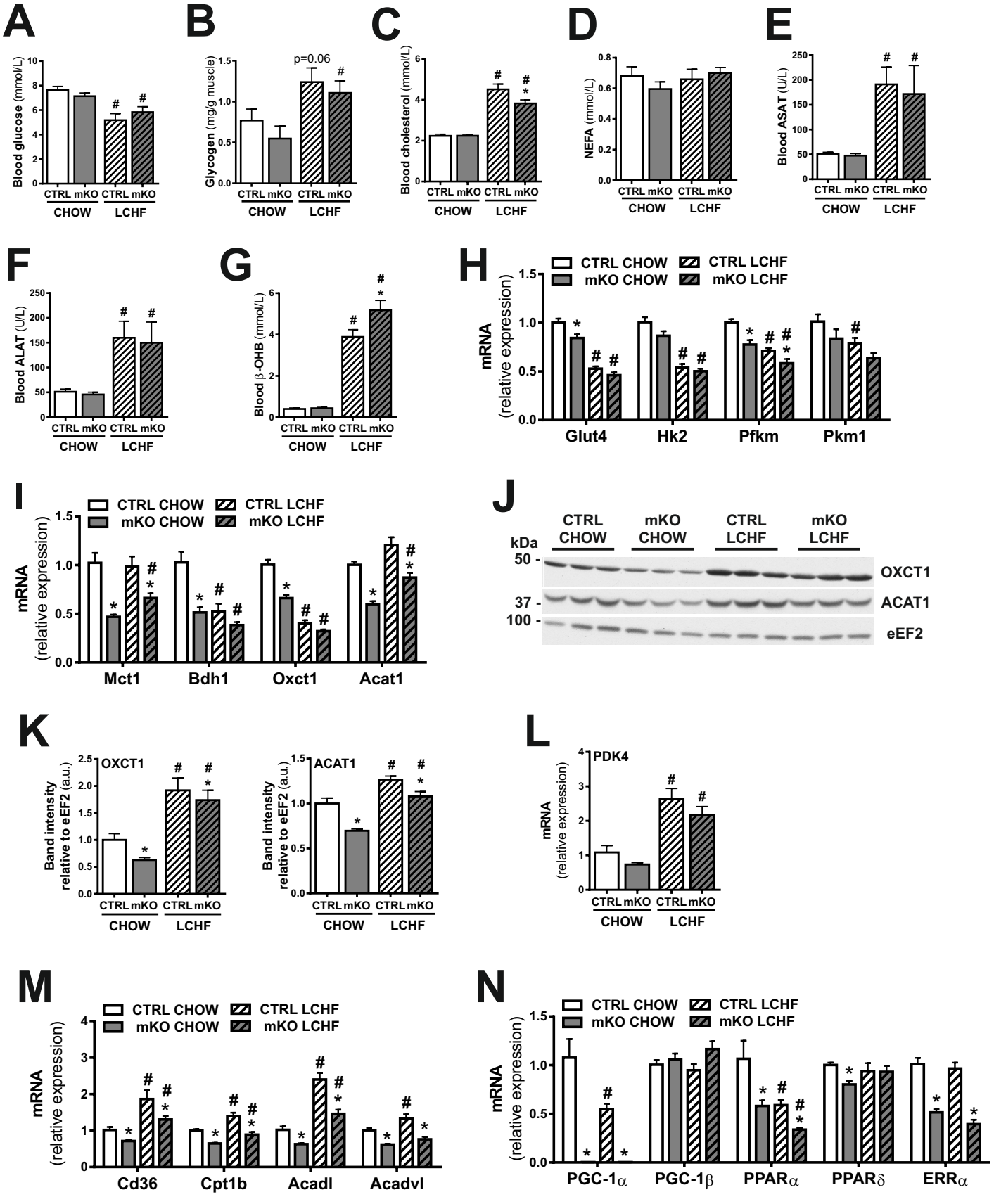

A
Blood glucose (mmol/L)
B
Glycogen (mg/g muscle)
p=0.06
C
Blood cholesterol (mmol/L)
D
NEFA (mmol/L)
E
Blood ASAT (U/L)
F
Blood ALAT (U/L)
G
Blood β-OHB (mmol/L)
CTRL mKO CTRL mKO
CHOW LCHF
H
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
mRNA (relative expression)
Glut4
Hk2
Pfkm
Pkm1
I
Mct1
Bdh1
Oxct1
Acat1
J
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
kDa
50
37
100
OXCT1
ACAT1
eEF2
K
OXCT1
ACAT1
Band intensity relative to eEF2 (a.u.)
L
PDK4
M
Cd36
Cpt1b
Acadl
Acadvl
N
PGC-1α
PGC-1β
PPARα
PPARδ
ERRα

Figure 3

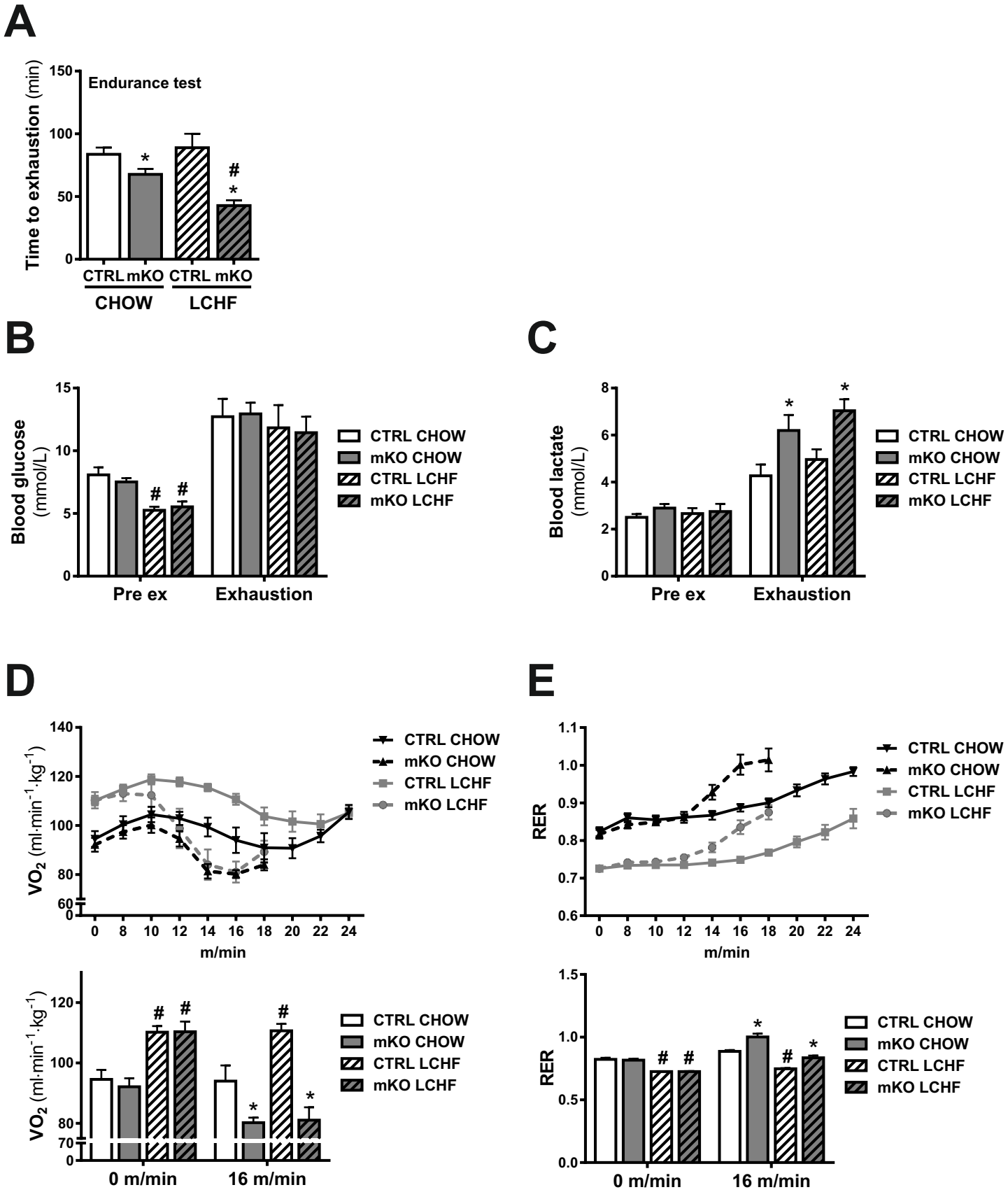
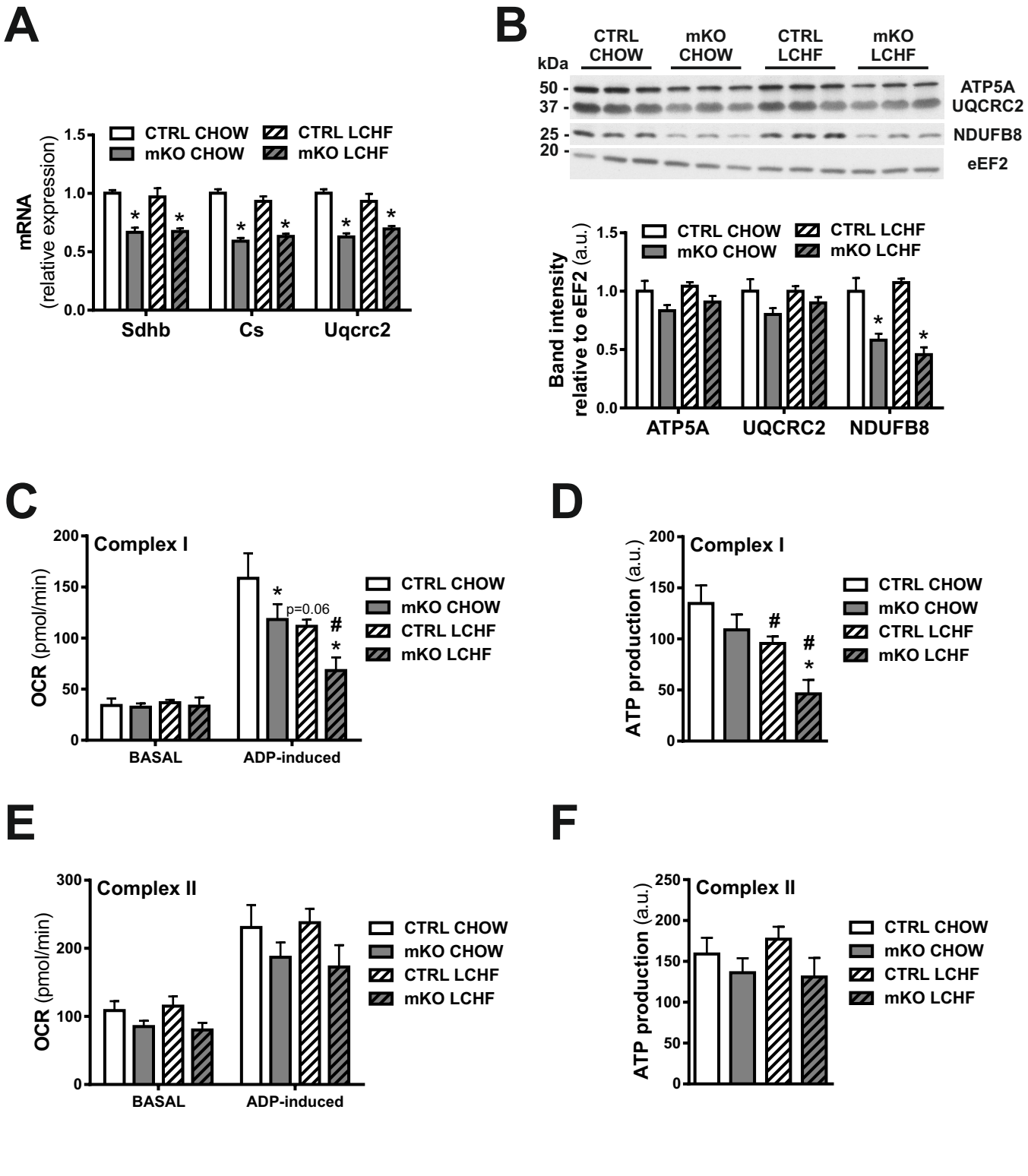

A
Endurance test
Time to exhaustion (min)
150
100
50
0
CTRL mKO CTRL mKO
CHOW LCHF
B
Blood glucose (mmol/L)
15
10
5
0
Pre ex
Exhaustion
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
C
Blood lactate (mmol/L)
8
6
4
2
0
Pre ex
Exhaustion
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
D
VO2 (ml·min-1·kg-1)
140
120
100
80
60
0
0 8 10 12 14 16 18 20 22 24
m/min
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
VO2 (ml·min-1·kg-1)
120
100
80
70
0
0 m/min
16 m/min
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
E
RER
1.1
1.0
0.9
0.8
0.7
0.6
0 8 10 12 14 16 18 20 22 24
m/min
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
RER
1.5
1.0
0.5
0.0
0 m/min
16 m/min
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF

Figure 4


A
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
mRNA (relative expression)
1.5
1.0
0.5
0.0
Sdhb
Cs
Uqcrc2
B
CTRL CHOW
mKO CHOW
CTRL LCHF
mKO LCHF
kDa
50
37
25
20
ATP5A
UQCRC2
NDUFB8
eEF2
Band intensity relative to eEF2 (a.u.)
ATP5A
UQCRC2
NDUFB8
C
Complex I
OCR (pmol/min)
200
150
100
50
0
p=0.06
BASAL
ADP-induced
D
Complex I
ATP production (a.u.)
E
Complex II
OCR (pmol/min)
300
200
100
0
F
Complex II
ATP production (a.u.)
250