Studies on endurance exercise training adaptation and endurance performance in mice under different pharmacological, physiological, and dietary conditions

Mark Christian Manio

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Endurance training leads to changes in whole-body metabolism, phenotype and endurance improvements through the activation of contraction-induced signals. Downstream to these signals are transcription factors that control genes that regulate cellular metabolism and overall adaptation to increased energy demand brought about by exercise. As these transcription factors are also activated by non-contraction signals e.g. pharmacological metabolic activators, and nutrients such as fatty acids and carbohydrates, it goes to say that endurance training together with pharmacological or dietary manipulation could influence downstream adaptations. Likewise, genetic background which could influence the chemical milieu of cells, particularly the muscle could also affect training-induced adaptations. In this doctoral thesis, adaptations that occur in training with pharmacological, genetic or physiological, and dietary environment was investigated.

Pharmacological agents that activate the AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor delta (PPARδ) have been considered as exercise mimetics. Their individual use causes changes on muscle physiology and endurance performance that are somewhat similar to that of exercise training. However, their simultaneous administration with endurance exercise training have not been investigated. The author determined the effects of their combined pharmacological activation with endurance exercise training in this chapter. Four (4) weeks of daily administration of the AMPK activator AICAR and PPARδ selective agonist GW0742 with training on alternate days greatly improved performance than training alone. This could be attributed to increased mRNA expression of the substrate utilization switch pyruvate dehydrogenase kinase 4 (PDK4), preference for fat oxidation during exercise and muscle glycogen accumulation. AMPK activation with training also improved endurance but modestly relative to training. Increased citrate synthase activity was increased indicating mitochondrial biogenesis. Likewise, muscle glycogen was spared with AICAR administration. These imply that while pharmacological activation of AMPK can potentiate endurance, greater potentiation could be achieved with combined pharmacological activation of AMPK and PPARδ.

Abstract

The uptake of long-chain fatty acids (LCFAs) in cells are mediated by different fatty acid transporters on cell membranes. The major fatty acid transporter in the muscle is CD36. While it has been general knowledge that LCFAs are utilized for energy production, their significant role in fatty-acid mediated signaling by the PPAR family have been recognized in the past decades. Because fatty acid uptake is increased by muscle contraction during training, this could also influence the exercise-training adaptations by influencing PPAR-mediated signaling. In this chapter, the author determined how CD36 influences exercise training adaptations and endurance in chronically trained mice. Exercise training failed to ameliorate endurance in CD36 knock-out (KO) mice despite having similar adaptations in mitochondrial biogenesis and intact albeit lower glycogen accumulation with training as wild-type (WT) mice. Likewise, exercise training-induced whole-body metabolism changes at rest and during exercise, as well as transcriptional increases in PPARα and PPAR target genes observed in WT mice were absent in KO mice. These findings show the importance of CD36 not only in endurance but also in exercise training-induced adaptive changes in substrate metabolism and PPAR-related transcriptional regulation.

Different fat sources contain varying composition of fatty acids. Fatty acids classified broadly on chain length have been demonstrated to influence metabolism differently. Medium-chain fatty acids (MCFAs) are easily metabolized in cells attributed to their lack of dependence on fatty acid transport proteins unlike LCFAs. Increased endurance in swimming exercise in mice fed with diet supplemented with purified MCFAs were observed in comparison to LCFAs. Also, MCFAs increase mitochondrial biogenesis in the muscle. However, the influence of MCFAs on training adaptations on a treadmill particularly on endurance, whole-body metabolism, substrate handling and transcriptional changes in the muscle and liver remained unexplored. Also, the use of coconut oil as a source of MCFAs and its comparison to diets with different fat content has yet to be studied. In this chapter, the author compared the effects of low-fat diet and medium-fat diets containing coconut oil and soybean oil on exercise training adaptations. Coconut oil modestly improved endurance attributed to increased fatty acid oxidation markers in the muscle. With training, all diet groups increased endurance likely through increased mitochondrial functions, and exercise efficiency. Despite this, coconut oil showed inhibition of training-induced increase in transcription of PPARδ and its targets in the muscle. In the liver, coconut oil increased fatty

acid oxidation markers likely through PPARα activation. Furthermore, while glycogen accumulation was inhibited by coconut oil with training, compensatory mechanisms by glycogen sparing through ketogenesis and ketolysis possibly prevented impairment of endurance in this group. These finding show that training improves endurance by generally improving muscle mitochondrial biogenesis however, transcriptional and metabolic adaptations in the muscle and liver are diet-dependent.

Overall, this thesis explored the complex interaction of non-contraction stimuli and contraction with exercise on different training adaptations in endurance, whole-body metabolism and local adaptations in the muscle and liver. Results presented in this thesis show that in a healthy and genetically intact state, training primarily determines the endurance performance of mice. Furthermore, pharmacological compounds that alter metabolism as well as natural compounds commonly found in food can variably impact adaptations that occur during endurance exercise training. Data from this thesis may suggest a potential use of nutritional supplementation or alteration in the diet for the improvement of endurance of genetically compromised individuals predisposed with impaired metabolism.

General introduction

Exercise as a form of physical activity has been shown to improve the quality of life of different individuals. According to the World Health Organization (WHO, 2017), lack of sufficient physical activity is associated with high number of mortality, approximately 3.2 million, each year. Also, as comprehensively reviewed by Pedersen and Saltin (2015), there is evidence to show that different exercise training regimens improve diseases ranging from psychiatric illnesses to cancer underscoring the significance of exercise as a part of lifestyle. It is to these reasons that endurance exercise has been extensively studied in the past century up to the present.

Exercise is classified into aerobic (endurance) and anaerobic (resistance) exercise. Aerobic exercise is characterized by repetitive motion accompanying a light load while resistance exercise utilizes heavy load but with less repetitions. Because the level of muscle stimulation and physical demands are different with different exercise modalities, adaptations and health benefits accompanying different exercise modalities vary as summarized by Egan and Zierath (2013). Muscle contraction is involved in both exercise modalities. However, adaptations are dependent on the intensity and frequency that is to say acute or chronic training (Egan and Zierath, 2013). In the muscle, adaptations are caused by contraction-induced downstream activation of different signaling proteins. These proteins include the hypoxia-inducible factor (HIF) (Formenti et al., 2010; Mason et al., 2004; Vogt et al., 2001), cadmodulin-dependent protein kinase (CaMK) and AMP-Activated Protein Kinase (AMPK) (Bergeron et al., 2001; Chin, 2010; Dolmetsch et al., 1997; Egan et al., 2010; Ventura-Clapier et al., 2008), mitogen activated protein kinase (MAPK) (Akimoto et al., 2005; Coffey et al., 2006; Yu et al., 2003), the sirtuin family (SIRT) (Cantó et al., 2009) and phospholipase D (Fang et al., 2001; O'Neil et al., 2009). Activation of these proteins trigger subsequent activation of different transcription factors leading to diverging adaptations particular to an exercise modality (Egan and Zierath, 2013). On top of these, changes in the chemical milieu of the cells due to contraction such as increase in fatty acid uptake can activate the peroxisome proliferator-activated receptor (PPAR) transcription factor family (Fan and Evans, 2015; Narkar et al., 2008). Because these adaptations are not exclusively induced by

exercise, non-contraction stimuli may influence their basal or exercise-induced levels. In the three chapters of this thesis, the influences of pharmacological agents, genetic or intrinsic physiological factors, and diet composition on endurance exercise training adaptations were investigated.

Pharmacological agents that modify or activate metabolism are being used not only for their originally intended purpose as therapeutic agents for metabolic diseases but as supplementation to boost physical performance as in competitive sports. These drugs include anabolic agents, hormones and their mimetics, and metabolic modulators, all of which are prohibited under the World Anti-Doping Agency (WADA) (Van Thuyne et al., 2006). The latter of the group, metabolic modulators, have well-documented effects on endurance performance in animals but not all have finished human clinical trials as some of these have unwanted side-effects or low-oral bioavailability (e.g. GW501516, GW0742, and AICAR) which limit their applicability for practical and safe use (Narkar et al., 2008; Wall et al., 2016). The above-exemplified drugs, which activate AMPK and PPARδ termed as "exercise mimetics," have gained popularity because of their purported effects which mimic the effect of exercise in several adaptations particularly in the muscle. While banned for competitive athletics, these "exercise mimetics" could be beneficial for leisure sports or health maintenance with exercise and therefore, validation of their efficacy side-by-side safety is of necessity. In chapter 1 of this thesis, the effect of pharmacological activation of AMPK and PPARδ with training on endurance improvement and whole-body metabolism was investigated.

While training improves athletic performance, genes contribute significantly to human athletic performance (Guth and Roth, 2013; Heck et al., 2004; Lippi et al., 2010). Some genes are associated with basal endurance performance (e.g. ACTN3, PPARδ, PGC1α, etc.) (Guth and Roth, 2013; Lippi et al., 2010) while some are upregulated with exercise which further improve performance (e.g. CD36, LPL, etc.) (Egan and Zierath, 2013; Heck et al., 2004). Adaptations in the muscle with acute and chronic exercise have been extensively studied in mice as reviewed by Egan and Zierath (2013). However, because certain genes regulate signaling pathways involved in energy production or substrate utilization, they are potentially capable of modifying training adaptations. Among these are fatty acid transport

proteins. Fatty acid transport proteins play a critical role in the control of metabolism as fatty acids not only participate as substrates in oxidative metabolism and ATP synthesis but also as signaling molecules of the PPAR family which control fatty acid utilization in different oxidative organs such as the muscle (Drover and Abumrad, 2005; Goldberg et al., 2009; McFarlan et al., 2012). Among these fatty acid transport proteins, CD36 has been shown to control fatty acid uptake as well as its oxidation in the muscle due to its localization on both the cell membrane and outer membrane of the mitochondria (Holloway et al., 2009). Therefore, it could regulate the influx of fatty acids in cells for energy or transcriptional control. In chapter 2 of this thesis, the role of the fatty acid transporter CD36 in endurance exercise training adaptations was explored.

In athletes, physiological aspects, psychological aspects, and social influences affect food choices (Birkenhead and Slater, 2015). Regardless of influences in food choices however, there is constant interest in dietary management in competitive sports where little gains could decide between a win or a loss. This manifests in the number of studies on different diets relating to endurance performance specifically those which compare the effects of different percentages of macronutrients rather than the component of macronutrient, i.e., relative percentages of fats, carbohydrates and proteins. Because different fats and their fatty acid composition influence the rate of energy provision on one hand and downstream adaptive regulation through transcription factor signaling on the other hand (Bach et al., 1996; Berning, 1996; Mizunoya et al., 2013; Montgomery et al., 2013), studies on specific fat sources must be conducted for adoptability in athletic or health dietary management. Furthermore, purified fatty acids may not be practical for dietary management which necessitates their replacement by more practical and readily sourced fats (e.g. coconut oil instead of purified medium-chain triglyceride preparations). Moreover, while medium-chain triglycerides particularly (octanoic and decanoic acids) on endurance performance have been studied (Fushiki et al., 1995), the influence of coconut oil (mostly composed of lauric acid) on endurance and metabolism has not been evaluated. In chapter 3 of this thesis, low-fat diet, and medium fat diets containing soybean oil or coconut oil were assessed on their effects on sedentary and endurance exercise-trained animals.

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Chapter 1
Combined pharmacological activation of AMPK and PPARδ potentiates the effects of exercise in trained mice

Introduction

AMPK is a cellular energy sensor activated during conditions of stress via upstream kinases. It is sensitive to the ratio of AMP to ATP during periods of energy deprivation such as in exercise, hypoxia, and starvation (Shackelford and Shaw, 2009). Reactive oxygen species produced in the mitochondria during hypoxia and influx of calcium during muscle contraction or nerve stimulation in other organs also activate AMPK (Hawley et al., 2005; Jensen et al., 2007; Mungai et al., 2011). The phosphorylation of AMPK at its α subunit leads to a cascade of events involving direct immediate control of metabolism by promoting energy production while inhibiting energy expensive anabolic processes (Horman et al., 2002; Kurth-Kraczek et al., 1999; Winder and Hardie, 1996). It also functions indirectly in energy homeostasis by regulating gene expression through activation or repression of transcription (Chen et al., 2010; Jäger et al., 2007; Li et al., 2011; Yang et al., 2009). Its beneficial role has been widely recognized because of its central role in metabolism particularly in improving fat oxidation and glucose uptake in the muscle, and improvement of lipid handling and oxidation in both fat depots and liver, thereby ameliorating metabolic disorders.

PPARs are nuclear transcription factors which function as lipid sensors leading to transcriptional programming within cells. The PPAR isotypes are present in most tissues but vary in abundance and function as defined by their target genes. PPARγ is abundantly expressed in the adipose tissues orchestrating adipogenic differentiation, lipogenesis and insulin sensitivity (Ahmadian et al., 2013; Desvergne and Wahli, 1999). PPARα is highly

expressed in the liver and other oxidative tissues such as the heart and skeletal muscle where it regulates oxidative metabolism of fat (Desvergne and Wahli, 1999; Lefebvre et al., 2006). PPARδ, highly abundant in the skeletal muscle and present in other metabolic organs albeit in moderate expression, has been shown to regulate the change to an oxidative phenotype characterized by increased fatty acid oxidation, preferential use of fatty acids as substrate, but with improved glucose uptake (Krämer et al., 2007; Reilly and Lee, 2008). The potential of PPARδ in the management of metabolic disorders has been recognized by many researchers owing to its positive role in both fat and glucose utilization. Moreover, its role in the improvement of exercise performance has been demonstrated both by genetic manipulation and by pharmacological activation (Gan et al., 2011; Narkar et al., 2008; Wang et al., 2004).

The interaction of AMPK and PPARδ has been investigated in different contexts. For example, despite the lack of exercise training, mice that underwent 4 weeks of treatment with AICAR together with PPARδ selective agonist GW501516 had increased expression of genes related to endurance training thus termed “exercise mimetics” (Narkar et al., 2008). In another study, the mouse model of muscle dystrophy *Mdx* mouse showed improved muscle functional performance with exercise and the above mentioned drugs in some tests albeit not improving in the running test (Bueno Júnior et al., 2012). It has been demonstrated that AMPK and PPARδ but not PPARα physically interact leading to an increase in glucose oxidation via the upregulation of the lactate dehydrogenase B (LDHB) gene associated with improved exercise performance (Gan et al., 2011; Narkar et al., 2008). Much work is still needed to reveal the complexity of AMPK and PPARδ interaction as well as interaction with other PPAR isotypes.

The combined pharmacological activation of AMPK and PPARδ raised a question whether their activators could further improve endurance above that brought about by an exercise training regimen in healthy individuals. In connection to this, considering the consistent demand for ergogenic aids and recovery supplements (Maughan, 1999), the use of these chemicals as doping substances being reported was not surprising despite insufficient safety and efficacy studies in humans. To be able to identify food components and natural compounds with similar benefits, we initially determined the effects of combined AMPK and

PPARδ activation in trained mice. We show that considerable improvements in endurance could be attained with combined pharmacological activation in healthy exercise-trained mice.

Materials and methods

Animals and drugs

Male 7 wk old Balb/c mice (Shimizu Laboratory Supplies Co. Ltd., Japan) were utilized in the study. The animals were divided into 5 groups and acclimatized to the housing environment 7 d before the experimental treatments while receiving daily handling and i.p. injection of saline (5 mL·kg BW^{-1}) to eliminate the effect of stress at the start of the treatments. All animals were housed in a room maintained at 22±0.5 °C, 50 % humidity, and a light-dark cycle of 12 h (6:00 lights on; 18:00 lights off). Mice had free access to standard diet for mature rodents (D10012m, AIN-93M; Research Diets, NJ) and water. Mice in one group received the vehicle DMSO (Wako Pure Chemicals, Japan) at 2.5 % concentration in physiological saline and were not exercise-trained (hereby termed SED). The other 4 groups received treatment together with exercise training. The vehicle group (hereby termed V) received the vehicle. The PPARδ group (hereby termed G) received the potent and selective agonist GW0742 (5 mg·kg BW^{-1}, s.c.; Sta. Cruz Biotechnology, CA). The AMPK group (hereby termed A) received the activator AICAR (500 mg·kg BW^{-1}, i.p.; Wako Pure Chemicals, Japan) and the combined pharmacological activation group (hereby termed A+G) received both GW0742 and AICAR at the same dosage as groups G and A. The administered dose of AICAR was similar to Narkar et al. (2008). GW0742 dosage used was similar to the dosage of another PPARδ selective and potent agonist GW501516 used in the same paper. All administered solutions were prepared to provide a target dosage in a volume of 5 mL·kg BW^{-1}. Injections and/or exercise training were conducted daily between 8:00-12:00 for 4 wk. Mice assigned to sedentary indirect calorimetry were placed in metabolic chambers from day 26 to acclimatize as described in the succeeding section. Mice assigned to exercise-to-exhaustion test with indirect calorimetry were left undisturbed for 3 d. Animal experiments were in accordance to the Kyoto University Guidelines for the Ethical Treatment of Laboratory Animals.

Exercise training protocol

The experiment schedule is depicted in Fig. 1A. In brief, mice in the exercise-trained groups (V, G, A and A+G) were accustomed to a rodent treadmill (MK-680; Muromachi, Tokyo,

Japan) by walking for 15 min on 2 alternate days before commencing with the actual training program. Training was conducted on alternate days for 4 wk with the last 2 training sets done on a rodent treadmill enclosed in a metabolic chamber (Columbus Instruments, Columbus, OH) coupled to a mass spectrometer (ARCO-2000; Arco System, Tokyo, Japan). A weekly increasing intensity protocol was followed (Fig. 1B). Briefly, warm up was conducted at the following program: 6 $m \cdot min^{-1}$ for 1 min, 8 $m \cdot min^{-1}$ for 2 min followed by 10 $m \cdot min^{-1}$ for 2 min. Following warm-up, the intensity was set to 12 $m \cdot min^{-1}$ for 20 min. On the second week, the warm up program included 12 $m \cdot min^{-1}$ for 2 min and followed by the intensity of 15 $m \cdot min^{-1}$ for 20 min. On the third week, the warm up program included 15 $m \cdot min^{-1}$ for 2 min and followed by the intensity of 18 $m \cdot min^{-1}$ for 25 min. Finally, on the fourth week, the warm up program included 18$m \cdot min^{-1}$ for 2min and followed by the intensity of 21 $m \cdot min^{-1}$ for 25 min. An electric stimulus of 0.5 V was employed to force the mice to run. All mice in the exercise-trained groups were able to fully comply with the training protocol. Mice in the SED group were acclimatized on the rodent treadmill only on the last two training days (Fig. 1A).

Sedentary indirect calorimetry

Mice were placed in metabolic chambers on day 26 to acclimatize as well as eliminate the effect of environmental stress (Fig. 1A). After the final exercise training bout and drug treatment, sedentary indirect calorimetry was conducted on mice designated to the before-exercise group. In brief, mice were kept in metabolic chambers and indirect calorimetry was conducted on day 29 for 48h. Respiratory gases (O_2 and CO_2) from each chamber were measured and Respiratory Quotient (RQ), oxygen consumption, carbohydrate oxidation and fat oxidation were calculated relative to the body weight of mice. The room was maintained at 25±2°C at a relative humidity of 40-90%. The same diet and water were supplied *ad libitum* during measurement. The apparatus had 16 lanes each connected to a metabolic chamber (internal dimension: 10 x 16 x 8 cm). Mass spectrometer (ARCO-2000; Arco System, Tokyo, Japan), and air-lane switching system sampler (ARCO-2000-GS-16; Arco System, Tokyo, Japan) were used to measure oxygen consumption and RQ. An activity meter on top of each chamber counted movement. Fat oxidation and carbohydrate oxidation was automatically calculated from the amount of oxygen consumed and respiratory gas exchange ratio using the ARCO-2000 software coupled to the equipment

according to the formula by Frayn (1983) while energy expenditure was calculated using the formula by (Lusk, 1924). Data during the last 24 h were analyzed and presented.
Two (2) hours prior to sacrifice, food was removed from each chamber. Mice were sacrificed by decapitation and blood was collected. Blood was centrifuged and the collected serum was stored at -80 °C for subsequent analyses. Gastrocnemius, and a piece of the liver were clamp frozen in liquid nitrogen and stored at -80 °C for metabolite quantification. Data from these samples were referred in-text as before exercise or pre-exercise test data. Weights of right gastrocnemius, right epididymal fat, and whole liver were recorded.

Exercise-to-exhaustion test with indirect calorimetry

Three (3) days after the final exercise training bout and drug treatment, exercise-to-exhaustion test was conducted (Fig. 1C). In brief, mice were weighed and placed in an airtight indirect calorimetry treadmill chamber undisturbed for 1.5 h. The treadmill was set at an inclination of 3 ° and running was commenced at an initial intensity of 6 $m \cdot min^{-1}$ gradually increasing by 1 $m \cdot min^{-1}$ every 30 sec until the intensity of 12 $m \cdot min^{-1}$ was reached totaling to 3 min. This intensity was maintained for 27 min. The inclination was increased to 20 ° followed by increasing the intensity by 2 $m \cdot min^{-1}$ every min until the intensity of 24 $m \cdot min^{-1}$ was reached. An electric stimulus of 0.5 V was employed to force the mice to run. This was kept constant until the mice were deemed unable to continue running. Exhaustion was defined as remaining on the shocker plate for 10 sec in spite of momentary increases in electrical stimulus together with tapping on the chamber walls as an auditory stimulus. Indirect calorimetry was conducted before and during exercise using the same system in the sedentary-state indirect calorimetry section.
At the point of exhaustion, mice were immediately sacrificed and samples were collected similar to mice in the sedentary state. In addition, quadriceps and a piece of the liver were stored in RNAlater RNA Stabilization Reagent (Qiagen, Germany) according to manufacturer's instructions until total RNA extraction. Data from these samples were referred in-text as after exhaustion or post-exercise test data.

Serum glucose, triglycerides and non-esterified fatty acids

Serum glucose concentration was measured using an enzymatic colorimetric test kit (Glucose C I Test Wako; Wako Pure Chemicals Industries, Japan) on a 96 multi-well plate

reader. Serum triglycerides (TG) and non-esterified fatty acids (NEFA) were measured using the Triglyceride E and NEFA C Test Kits, respectively (Wako Pure Chemicals Industries, Japan).

Muscle and liver glycogen

Frozen gastrocnemii and liver samples were powdered in liquid nitrogen-submerged mortar and pestle. Glycogen was extracted using the following method in our laboratory. In brief, weighed samples approximately 100 mg was digested in 0.3 mL 30 % KOH solution in a heating block set at 100 °C for 30 min. The resulting digest was cooled on ice followed by the addition of 50 µL saturated Na_2SO_4 and 0.5 mL ice-cold ethanol. The mixture was vortexed and spun down at 5,000 rpm for 5 min. The glycogen precipitate was dissolved in 200 µL distilled water followed by 250 µL ice-cold ethanol precipitation. The mixture was centrifuged to pellet the glycogen and the supernatant discarded. To convert the glycogen to glucose units, 0.6 mL of 0.6 M HCl was added and heated on a heating block set at 100 °C for 2 h. Measurement of glucose was similar to that of serum glucose. Glucose was converted to glycogen by factoring the water eliminated if glucose units were polymerized.

Muscle and liver non-esterified fatty acids

From the powdered tissue samples, approximately 50 mg were weighed. One (1) mL of Folch reagent (chloroform:methanol, 2:1) was added to the sample then vortexed. The mixture was incubated at 4 °C for 16 h. To obtain the lipid containing fraction, 0.2 mL 4 mM $MgCl_2$ was added then vortexed. The mixture was centrifuged at 3,500 rpm for 1 h at 4 °C. From the lower chloroform layer containing the extracted lipids, 0.2 mL was collected and the solvent evaporated. The desolvated lipids were resuspended in 0.1 mL 10 % triton-X in isopropanol. NEFA was measured using the NEFA C Test kit.

Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR)

Pre-exercise clamp-frozen gastrocnemius samples and post-exercise quadriceps and liver stored in RNAlater were powdered in liquid nitrogen. Total RNA was extracted using a combination of TriPure Isolation Reagent (Roche, Germany) and RNeasy Mini Kit (Qiagen, Germany). In brief, approximately 100 mg of the sample was mixed with 1 mL TriPure reagent followed by brief sonication. The mixture was clarified by centrifugation and 0.2 mL

chloroform was added to the supernatant. The mixture was vortexed followed by centrifugation at 12,000 g for 15 min at 4 °C. Equal parts of 70 % ethanol and supernatant were combined, mixed, and then transferred to an RNeasy spin column. The succeeding steps were according to manufacturer's instructions with DNase I (Qiagen, Germany) digestion. Total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega, WI) and RNase Inhibitor (Toyobo, Osaka, Japan). Messenger RNA expression levels were measured from signals from Universal ProbeLibrary probes (Roche, Germany).
Intron spanning oligonucleotide primer sets of mouse genes were designed using the program at the website of Roche Universal ProbeLibrary Assay Design Center and the predicted amplicons were validated using the program at the BLAST website of the National Institutes of Health. Primer sequences are as follows: hypoxanthine-guanine phosphoribosyltransferase (HPRT) as an internal control (Fwd: 5'-tcctcctcagaccgctttt-3'; Rev: 5'-cctggttcatcatcgctaatc-3'), peroxisome proliferator-activated receptor 1-alpha transcript variant 1 (PCG1a /PPARGC1a) (Fwd: 5'- tgtggaactctctggaactgc-3'; Rev: 5'-agggttatcttggttggcttta-3'), pyruvate dehydrogenase kinase 4 (PDK4) (Fwd: 5'-cgcttagtgaacactccttcg-3'; Rev: 5'- cttctgggctcttctcatgg-3'), lipoprotein lipase (LPL) (Fwd: 5'-gctcatgatgaagcttaagtgga-3'; Rev: 5'- tccctagcacagaagatgacc-3'), fatty acid translocase (FAT/CD36) (Fwd: 5'- ttgtacctatactgtggctaaatgaga -3'; Rev: 5'- cttgtgttttgaacatttctgctt-3'), muscle carnitine palmitoyltransferase 1 (CPT1b) (Fwd: 5'- gcccatgtgctcctacca-3'; Rev: 5'-ctctgagaggtgctgtagcaag-3'), uncoupling protein 3 (UCP3) (Fwd: 5'- tgctggagtctcacctgtttac-3'; Rev: 5'- cgggtctttaccacatccac-3'), vascular endothelial growth factor A (VEGFa) (Fwd: 5'-actggaccctggctttactg-3'; Rev: 5'- tctgctctccttctgtcgtg-3'), hormone sensitive lipase (LIPE/HSL) (Fwd: 5'- agcgctggaggagtgtttt-3'; Rev: 5'- ccgctctccagttgaacc-3'), citrate synthase (CS) (Fwd: 5'- ctgcctgagggcttattttg-3'; Rev: 5'- cattctcgtgagagccaagac-3'), PPARδ (PPARD) (Fwd: 5'-gaagtggccatgggtgac-3'; Rev: 5'- gaggaaggggaggaattctg-3'), glucose transporter type 4 (GLUT4) (Fwd: 5'- gacggacactccatctgttg-3'; Rev: 5'- gccacgatggagacatagc-3'), adipose triglyceride lipase (ATGL) (Fwd: 5'- cgggtagcatctgccagta-3'; Rev: 5'- cagttccacctgctcagaca-3'), 3-oxoacid CoA-transferase (OXCT1) (Fwd: 5'- aggcctgactgttgatgaca-3'; Rev: 5'-ctgcattggcatgaggttt-3'), muscle glycogen phosphorylase (PGYM) (Fwd: 5'-agtggaggacgtggaaagg-3'; Rev: 5'- gctcaggaattcggtcgtag-3'), muscle glycogen synthase (GSY1) (Fwd: 5'- ggggtcttcccctcctacta-3'; Rev: 5'- ctccataaagcagccaaagc-3'), and carbohydrate-responsive element-binding protein (CHREBP) (Fwd: 5'- cttcagcagtgggatcctg-3'; Rev: 5'-

atccaagggtccagagca-3'). Messenger RNA expression results were normalized to *Hprt* expression and presented as a ratio relative to SED. HPRT was used as the reference gene because it is stable in the context of exercise (Cappelli et al., 2008).

Mitochondrial DNA copy number

Total DNA was isolated from powdered gastrocnemius samples using the QIAamp DNA Mini Kit (Qiagen). Independent reactions were performed for mitochondrial DNA cytochrome c oxidase subunit I (COI) and nuclear 18S rDNA using the primers as detailed by Tal et al. (2009) with the LightCycler Carousel-Based System (Roche). Primers used to amplify mouse DNA are as follows: 18S rDNA forward, 5'-TAGAGGGACAAGTGGCGTTC-3'; 18S rDNA reverse, 5'-CGCTGAAGCCAGTCAGTGT-3'; COI forward, 5'-GCCCCAGTATAGCATTCCC-3'; and COI reverse, 5'-GTTCATCCTGTTCCTGCTCC-3'. In brief, 5ng of total DNA was used with the SYBR green I reaction system (Roche) according manufacturer's instructions. Temperature cycling program as detailed by Brown and Clayton (2002) with some modifications are as follows: initial denaturation a 95 °C for 30 s followed by 40 cycles at 95 °C for 3 s, 55 °C for 6 s then 72 °C for 10 s each cycle with ramping rate set at 20 °C/s. Data was acquired at the end of the extensions at 72 °C. At the end of the program, the homogeneity of the amplicons was confirmed by a melting curve analysis. Relative copy number are expressed as the ratio of mtDNA COI over 18S rDNA then rationalized to SED.

Immunoblotting

Approximately 10 mg gastrocnemius samples were homogenized in 500 µl cold RIPA lysis buffer with EDTA supplemented with protease and phosphatase inhibitor cocktail tablets (Roche). Insoluble matter was precipitated by centrifugation at 10000 *g*, 4 °C for 20 min and the lysate collected and protein content measured using the Coomassie Brilliant Blue solution (Nacalai Tesque, Japan). Lysate was adjusted with lysis buffer to achieve similar concentrations among samples followed by addition of 4x Laemmili buffer containing β-mercaptoethanol. Samples (33 µg protein) were subjected to electrophoresis followed by transfer to a nitrocellulose membrane. Membranes were incubated in Ponceau S stain then rinsed with excess water to remove unbound stain. These were then sandwiched in glass plates then photographed and digitized (LAS-3000, Fujifilm, Japan). After digitization, membranes were washed in TBS-T buffer to remove bound stain then followed by blocking

in 2% BSA in TBS-T. Membranes were cut at previously identified locations to separate PGC-1α and α-Tubulin containing regions. Respective primary antibody incubation was done with anti-α-Tubulin antibody and anti-PGC-1α antibody (Santa Cruz Biotechnology) overnight. Membranes were sufficiently washed and incubated with respective secondary antibodies linked to horseradish peroxidase (Dako). Immunodetection by chemiluminescence (Western Lightning Plus ECL, PerkinElmer) was conducted according to manufacturer's directions followed by visualization using the LAS-3000 equipment. Quantification of total signals from Ponceau S, and protein-specific chemiluminescent signals were performed using the coupled software (MultiGauge V.3.2, Fujifilm, Japan). Relative protein expression was obtained by comparing protein-specific signals to total Ponceau S signal.

Citrate synthase activity

Muscle lysates were diluted with the lysis buffer to achieve a similar protein concentration of 2 µg/µl. The principle of the assay is based on the paper by Srere (1969). In a 96-well plate, 4 µl of adjusted lysate (8 µg protein) was mixed with acetyl-CoA (Roche) dissolved in 0.1M Tris-HCl buffer at pH 8.0 and DTNB (Sigma) dissolved in ethanol. After thorough mixing, deacetylase activity was measured at 412 nm on a kinetic program of a 96-well plate reader for 2.5 min with 50 sec interval between each reading. Citrate synthase reaction was initiated by the addition of oxaloacetate (Sigma) dissolved in the same buffer. Final concentrations of reactants were as follows: 0.3 mM acetyl-CoA, 0.1 mM DTNB and 0.1 mM oxaloacetate. The plate was immediately shaken and absorbance at 412 nm was read on a kinetic program with the duration of 10 min and an interval of 50 sec between each reading. Beyond this time, absorbance readings deviated from linearity. The change in absorbance before the addition of oxaloacetate was subtracted from the change in absorbance after the addition of oxaloacetate. Citrate synthase activity is expressed relative to protein loaded.

Statistical Analysis

Statistical analysis was performed using Prism 5.0 (Graphpad Software, CA). Time course data values of indirect calorimetry are presented as means. Average and cumulative values in different experiments are presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test was applied to determine the presence of significant differences among groups. Student t-test was applied to within-group pre and

post-exercise data. Significance level was set at α=0.05 and *p* values lower than α were deemed significant.

Results

Body weight and sedentary-state indirect calorimetry

The effects of combined pharmacological activation on body weight, organ weight, RQ, oxygen consumption, carbohydrate and fat oxidation, energy expenditure and spontaneous motor activity were measured. No significant effect on body weight, gastrocnemius and epididymal fat weights were observed (Table 1). However, liver weight was significantly increased in G (p<0.05) relative to SED, and in A+G (p<0.001) relative to all groups. No significant differences were observed in RQ, total oxygen consumption, carbohydrate and fat oxidation, energy expenditure, and spontaneous motor activity among groups (Fig. 2A-F).

Running Endurance

To determine the effect of the activators on endurance in trained mice, mice were subjected to an exercise-to-exhaustion test. As expected, mice in the SED group were not able to run at the same time as the exercise-trained groups (Fig. 3A and B). All the trained groups were able to run more than twice (p<0.001) the time to exhaustion of SED. Among them, only A+G was able to run longer (p<0.001) than V. A tendency for Group A to run longer was observed. The total distance covered until exhaustion was also calculated from the running time (Fig. 3C). However, work may be a more appropriate measure as it takes into account body weight. Indeed, calculating for work showed that A did significantly more (p<0.05) than both V and G however, A+G was still significantly higher (p<0.001) even to A (Fig. 3D). These results show that AICAR could potentiate endurance in trained mice. Furthermore, the combination of AICAR and GW0742 in A+G could further potentiate endurance than that imparted solely by AICAR.

Exercise-to-exhaustion indirect calorimetry

The effects of combined pharmacological activation on whole body metabolism were examined by indirect calorimetry before and during the running test. Only values obtained during the first 45 min of the run wherein SED was capable of completing were calculated to facilitate statistical comparison of all groups. No considerable differences were observed at each time point in the time course curves (Fig. 4A to E; curve) similar to the observation in

sedentary-state indirect calorimetry. Calculating for the average RQ, and total oxygen consumption, carbohydrate and fat oxidation before running, no significant differences were observed among the groups regardless of training status (Fig. 4A to E; rest bar). However, during the run, the exercise-trained groups had significantly lower (p<0.05) average RQ compared to SED (Fig. 4A; run bar). Total oxygen consumption, fat oxidation, and energy expenditure did not show any significant differences (Fig. 4B, D and E; run bar). Only A had significantly decreased carbohydrate oxidation (p<0.05) relative to SED although G and A+G had a tendency to have lower total carbohydrate oxidation (Fig. 4C; run bar). To visualize the changes in substrate oxidation before reaching exhaustion, the same calculation as above was applied to the last 10 time points corresponding to the last 30 min before exhaustion of each mice (Fig. 4F to J; curve). The average RQ was further decreased in all exercise-trained groups with the highest decrease in A+G (Fig. 4F, bar). Total oxygen consumption was not different among groups (Fig. 4G; bar). Interestingly, total carbohydrate oxidation of all exercise-trained groups showed a decrease (p<0.01-0.001) >50% that of SED (Fig. 4H; bar). Also, A+G had a further drop about 50% that of V, G or A. Fat oxidation, on the other hand, was elevated (p<0.05) in A+G relative to SED in contrast to the lack of difference during the 45 min run (Fig. 4I; bar). This suggests an enhanced shift in fuel source during prolonged exercise especially near exhaustion as energy expenditure among all groups was not significantly different (Fig. 4J; bar).

Serum glucose

Serum glucose before exercise, and after exhaustion were measured because hypoglycemia during exercise is implicated as one of the factors that lead to exercise cessation (Williams et al., 2013). Serum glucose before exercise was significantly increased in A+G relative to all the other groups. At the point of exhaustion, serum glucose was decreased (p<0.01-0.001) at about similar levels in all exercise-trained groups irrespective of treatment compared to SED (Fig. 5A). And this decrease was significant (p<0.001) relative to the before-exercise glucose levels. The SED group on the other hand did not have a significant decrease even at the point exhaustion.

Serum TG and NEFA

Serum TG and NEFA are sources of fatty acids as energy source during exercise. Serum TG before exercise was similar among groups with A+G having a non-significant increase (Fig. 5B). No inter-group differences were observed at the point of exhaustion. Comparison of pre- and post-exercise values showed a significant decrease (p<0.05-0.01) in all groups except V.

Pre-exercise serum NEFA was similar among the groups (Fig. 5C). At the point of exhaustion, serum NEFA significantly increased (p<0.001) relative to before exercise despite an apparent lower concentration in G. This low concentration was only significantly different (p<0.05) with A+G.

Muscle and liver glycogen

Both muscle and hepatic glycogen were measured because glycogen plays a role in the maintenance of glucose homeostasis in the blood (Baldwin et al., 1973). Before exercise, the basal level of muscle glycogen was significantly elevated (p<0.05-0.01) in A+G relative to all groups. At the point of exhaustion, muscle glycogen in A and A+G remained elevated (p<0.01-0.001) compared to the other groups (Fig. 5D). Furthermore, A+G was significantly higher (p<0.05) than A. Comparison of pre- and post-exercise muscle glycogen showed that all groups had decreased. However, the decrease in A and A+G was not statistically significant compared with the other groups (P<0.001).

In the liver, pre-exercise hepatic glycogen had a tendency to be lower in exercise-trained groups (Fig. 5E). Because of increased liver weight in G and A+G (Table 1), calculating for absolute liver glycogen showed that SED and A+G had almost similar total glycogen while the other groups had lower content (Fig. 5F). At the point of exhaustion, all exercise-trained groups had similar depleted glycogen stores about 15x less (p<0.001) than SED (Fig. 5E). All groups had decreased glycogen post-exercise (P<0.001) but the decrease in SED did not drop to the same extent as in the exercise trained.

Muscle and liver NEFA

Because NEFA, albeit measured in plasma, delay the depletion of glycogen during exercise (Rennie et al., 1976), NEFA within the tissues were measured as no significant differences were observed in serum NEFA at least relative to SED at the point of exhaustion. Pre-exercise muscle NEFA were similar in all groups except in V where a significant decrease (p<0.05-01) relative to SED, A and A+G was observed (Fig. 5G). However, at the point of exhaustion, muscle NEFA was increased (p<0.01) in A+G relative to SED. Moreover, A+G had 25 % more NEFA in the gastrocnemius than other exercise-trained groups. Muscle NEFA decreased after exercise however, in V where pre-exercise NEFA was lower, the observed decrease was not statistically significant.

Liver NEFA, on the other hand, is the source of circulating NEFA and ketone bodies. Pre-exercise values were similar among groups however, at exhaustion, exercise-trained groups significantly increased (p<0.001) relative to SED (Fig. 5H). An increase (p<0.01-0.001) in all groups relative to pre-exercise values were observed but the level in SED was only about 50 % that of the exercise-trained groups.

Gene expression in the muscle

The transcriptional coactivator PGC-1α has been associated with phenotypic changes leading to improved exercise. The effects of combined pharmacological activation on the gene expression of this coactivator were determined. It should be noted that the primer used in the study amplify the full-length, well-characterized transcript variant 1. Before the exercise test in the gastrocnemius, PGC-1α mRNA expression was not different among groups regardless of training status (Fig. 6A). In A+G, *Pdk4* but not *Lpl* had significant upregulation (p<0.05). This elevation was not significant relative to A. Protein expression, on the other hand, tended to remain elevated after 72 h in the sedentary state in G and A+G but not in other exercise groups (Fig. 6B and C). Comparison was made against total Ponceau S signal instead of commonly used loading controls. α-Tubulin expression seemed to be influenced by exercise as its signal relative to total Ponceau S stain tended to be lower in exercise-trained groups relative to SED and was further lowered by AICAR treatment (not shown). Likewise β-actin expression was variable despite similar amounts protein loaded among samples as quantified (not shown).

After the exercise test in the quadriceps, exercise training significantly increased (p<0.05-0.001) PGC-1α mRNA expression relative to SED (Fig. 6D). The lack of significant differences among V, G and A suggests that individual drug administration does not increase *Pgc1a* expression above that of exercise training at least at the time point measured. Interestingly, combined pharmacological activation in A+G further increased (p<0.05) the *Pgc1a* levels than V, G and A. Expression of mRNA of genes involved in fatty acid and glucose availability, mitochondrial genes as well as PPARδ and the angiogenic factor VEGFa were measured. Genes related to fatty acid availability were all highly expressed in A+G (Fig. 6E). In particular, AICAR treatment in A and A+G rescued the decrease of *Hsl* and *Atgl* compared to V. *Lpl* increased with AICAR treatment and A+G had the most robust upregulation (p<0.01). *Cd36* was decreased in V but agonist treatment in G and A rescued it. Furthermore, it was further increased in A+G relative to G and A (p<0.05). Glucose availability-related genes (*Glut4*, *Gsy1* and *Pygm*) did not change. However, a tendency to increase with GW0742 treatment in *Gsy1* and with either agonist treatment in *Pygm* could be observed (Fig. 6F). Messenger RNA expression of several mitochondrial genes was also measured at exhaustion (Fig. 6G). *Pdk4* was elevated by exercise and GW0742 in G induced a significant increase (p<0.05). The combination of the activators in A+G significantly elevated *Pdk4* (p<0.05-0.001) relative to all groups and almost 150% more than SED. *Cpt1b* was equally increased (p<0.05) in G and A+G, indicating no apparent significant effect of AMPK activation. *Cs* was increased (p<0.05-0.001) only in the drug treated groups although a tendency for V to increase could be observed. *Oxct1* did not increase with exercise or drug treatment. *Ucp3* increased in G and A+G but did not attain significance. The pattern of PGC-1α protein expression in the exercise-trained groups in particular was clearly reflected in the above-mentioned genes. Exercise training increased the mRNA expression of PPARδ (Fig. 6H). However, only G had the highest expression (p<0.01) relative to SED. Messenger RNA expression of VEGFA, another PGC-1α target, was increased (p<0.05-0.001) by exercise training with G having the highest expression.

Gene expression in the liver

The maintenance of euglycemia during exercise is an important function of the liver. Transcription-related factors known to influence the synthesis and storage of glucose in this organ were measured. At exhaustion, *Pgc1α* increased in all drug treated groups however, only A+G was significantly different (p<0.05) relative to both SED and V (Fig. 6). The lipogenic transcription factor CHREBP mRNA was significantly decreased (p<0.01-0.001) only in A+G. This was not observed even in the other exercise-trained or drug treated groups. Both of these suggest increased synthesis and storage of glycogen with repression of glycolytic and lipogenic pathways in A+G (Burgess et al., 2006; Iizuka and Horikawa, 2008).

Mitochondrial density

PGC-1α is important in the stimulation mitochondrial biogenesis (Austin and St-Pierre, 2012). Despite increased quadriceps *Pgc1α* (Fig. 6A) in all exercise-trained groups, and elevated gastrocnemius PGC-1α protein expression in G and A+G (Fig. 6B), mtDNA copy number was not increased relative to SED (Fig. 8A). However, several studies emphasized that citrate synthase may be a better marker of mitochondrial biogenesis rather than mtDNA copy number (Kim et al., 2008; Larsen et al., 2012). Indeed, a tendency to have increased citrate synthase activity could be observed in all exercise-trained groups (Fig. 8B). A moderate but non-significant increase was observed in V and G which was shadowed by the robust increase in citrate synthase activity (p<0.05-0.01) in AICAR-treated groups.

Discussion

This study demonstrated that combined pharmacological activation of AMPK and PPARδ potentiates endurance in exercise-trained mice. Although a small but significant potentiation was observed with AMPK activation, in contrast to Narkar et al. (2008), no potentiation was observed with PPARδ activation. AMPK is a cellular sensor of energy status while PPARδ is a transcription factor known to induce a change towards an oxidative phenotype in metabolic tissues (Barish et al., 2006; Liu et al., 2011; Mihaylova and Shaw, 2011). The lack of potentiation by PPARδ agonism in 2 independent trials in contrast with Narkar et al. (2008) could be attributed to the differences in agonists, mice strain, and training and exhaustion test protocol. Despite this, our data are in agreement with Bueno Júnior et al. (2012) that combined activation of AMPK and PPARδ with exercise training elicits phenotypic changes related to physical performance probably arise from the physical interaction of AMPK and PPARδ (Gan et al., 2011; Narkar et al., 2008).

Whole body metabolism, both during sedentary and early phase of exercise, were not different among groups. However, in A+G, shift in fuel utilization from carbohydrate to fat was observed towards exhaustion without a change in energy expenditure. Shift in whole body fuel utilization has been attributed to PDK4, a PGC-1α target gene and mitochondrial protein responsible for the preferential β-oxidation of fatty acids with a decline in glycolysis (Calvo et al., 2008; Wende et al., 2007, 2005). Indeed, despite an absence of changes in PGC-1α mRNA transcription and an increase, albeit not significant, protein expression in the gastrocnemius of A+G before the exercise test, a small but significant elevation in *Pdk4* could observed. Furthermore, at exhaustion, *Pdk4* expression was increased significantly compared to all groups supporting the shift in fuel utilization towards exhaustion in A+G. In addition, PDK4 and PGC-1α mRNA expression at exhaustion resembles that of fat oxidation towards exhaustion.

Combined pharmacological activation of AMPK and PPARδ with exercise training increases substrate availability. Serum glucose, muscle glycogen and hepatic glycogen were increased in A+G. With increased total available glucose in the form of free glucose, skeletal muscle

and hepatic glycogen, the onset of hypoglycemia during exercise could be delayed. Increased basal glycogen in the muscle is associated with increased preference to fatty acid oxidation (Wong et al., 2014) while glycogen sparing during exercise both in muscle and liver is influenced by increased NEFA and substrate shift to fat by PDK4 thereby contributing to improved endurance (Hickson et al., 1977; Rennie et al., 1976; Pilegaard and Neufer, 2004). AICAR has been shown to increase muscle glycogen synthesis (Holmes et al., 1999) however, at least in our experimental design, our data suggests that increased PDK4 through synergism with PPARδ may be necessary to cause significant glycogen accumulation which explains the lack of increase in basal glycogen in A relative to A+G. Increased intramuscular NEFA at exhaustion in A+G is associated with elevated mRNA transcription of genes related to fatty acid uptake in this group as observed with significant elevation in *Hsl*, *Atgl*, *Lpl* and *Cd36* permitting increased lipolysis from circulating triglycerides as well as uptake of fatty acids in the muscle. Taken together, the increase in *Pdk4* at basal and during exercise, transcription of fatty acid availability related genes, and consequently increased fatty acid availability during exercise would possibly delay the depletion of glucose and rapid onset of hypoglycemia consequently delaying fatigue and cessation of physical activity (Williams et al., 2013).

The liver not only stores energy in the form of glycogen but also maintains energy homeostasis by maintaining normal blood glucose concentration through glycogenolysis and gluconeogenesis as well as ketone body synthesis during periods of energy deficit (Cotter et al., 2013; Mitra and Metcalf, 2009; Radziuk and Pye, 2001). The non-significant increase in total hepatic glycogen in A+G relative to other exercise-trained groups may be influenced by the elevated expression of *Pgc1a* and *Chrebp*. Increased hepatic PGC-1α leads to an improvement in oxidative metabolism, gluconeogenesis and glycogenesis (Burgess et al., 2006; Finck and Kelly, 2006) while CHREBP downregulation or silencing leads to decreased glycolysis and lipogenesis resulting in increased glycogen synthesis (Iizuka and Horikawa, 2008). Moreover, elevated hepatic NEFA may suggest increased ketone body production as well as supply circulating fatty acids as observed in elevated serum NEFA, both contributing not only to muscle energy production but sparing of circulating glucose and glycogen. Mice in the exercise-trained groups attained hypoglycemia at the point of exhaustion with concomitant exhaustion of liver glycogen not observed in SED. In SED however, the absence

of hypoglycemia despite reaching exhaustion suggests that other factors other than hypoglycemia prevented these mice from running longer (Piña et al., 2003), such as complete hepatic glycogen utilization, as well as other factors possibly developed or enhanced with training. Also, our observations indicate that muscle glycogen is dispensable in agreement with Pederson et al. (2005) using MGSKO mouse, a mouse model lacking muscle glycogen, that it is dispensable for exercise performance and concluded that muscle glycogen could limit exercise capacity only if the level of hepatic glycogen is low coinciding with our observations and Baldwin et al. (1973).

Exercise induces metabolic changes through the upregulation of transcription factors. As a major exercise-inducible transcription factor co-activator, PGC-1α has been shown to improve endurance through mitochondrial biogenesis, gluconeogenesis, triglyceride metabolism, glucose homeostasis, and muscle capilliarization (Arany et al., 2008; Calvo et al., 2008; Lira et al., 2010; Narkar et al., 2011; Ryan and Hoogenraad, 2007). In skeletal muscle, PPARδ together with retinoid X receptor (RXR) activation controls its transcription while AMPK phosphorylates the PGC-1α protein causing its self-induced upregulation demonstrating the complex direct and indirect interaction of these two proteins (Gan et al., 2011; Hondares et al., 2007; Jäger et al., 2007; Narkar et al., 2008; Schuler et al., 2006). Contrary to expectation, *Pgc1a* was not elevated in the pre-exercise gastrocnemius at the time of sampling. However, this is not surprising as reversion to basal levels towards 24h post-exercise in skeletal muscle following elevation immediately after exercise has been reported (Mathai et al., 2008). Because *Pgc1a* is induced by exercise, the length of time to exhaustion could influence the transcription levels in each group, with A+G running longer, thus having the highest expression level. This is also reflected by the mRNA expression of some of its target genes such as PDK4, LPL and CD36 which are especially highest in A+G. *Oxct1*, a key enzyme in ketone body catabolism that is induced by exercise (Askew et al., 1975), tended to be highest in A+G. Furthermore, together with increased gene expression not exclusive to A+G (*Hsl, Atgl, Cpt1b, Cs, Vegfa,* and *Ppard*) would support the observed elevated intramuscular NEFA level at the point of exhaustion and the shift to whole body substrate oxidation to fat towards exhaustion in this group. Collectively, these findings suggest that combined pharmacological activation of AMPK and PPARδ with exercise training results in a unique gene signature similar to that observed in oxidative muscle

capable of improved delivery, uptake and oxidation of fat and its preferential utilization. On one hand, because pre-exercise test values likely reverted to baseline as seen in the lack of elevation in *Pgc1a* and *Lpl* and significant but modest elevation in *Pdk4* in A+G, protein expression rather than mRNA expression may reveal the participation of these genes in the observed endurance potentiation. On the other hand, post-exercise data presented here may suggest that transcription induction of these genes during running had an additive or synergistic effect on endurance especially in A+G.

Exercise training through PGC-1α promotes mitochondrial biogenesis consequently influencing oxidative metabolism and energy production (Austin and St-Pierre, 2012). The lack of mtDNA increase was not indicative of increased mitochondrial biogenesis as in muscle, CS activity is a more reliable marker (Larsen et al., 2012). Indeed, exercise-trained groups had higher CS activity but interestingly, AICAR-treated groups were significantly increased. Like PGC-1α mRNA expression, CS activity presented may already be towards decline beyond 24h similar to that observed by Leek et al. (2001). The observed small but significant elevation in work in A may be explained by the increase in CS activity. That of A+G however, can be attributed to synergism of substrate availability, utilization shift and increased mitochondrial density. Although not measured, metabolism of lactate and ketone bodies in A and A+G may coincide with increased mitochondrial density (Baldwin et al., 1978; Cotter et al., 2013).

While pharmacological activation of AMPK potentiates endurance in trained and untrained mice as shown here and many studies (Murase et al., 2006, 2005; Narkar et al., 2008), combined activation with PPARδ greatly potentiates endurance through the orchestration of transcriptional programs in the muscle and liver, leading to increased substrate availability, substrate shift to fat, and mitochondrial density.

Figures

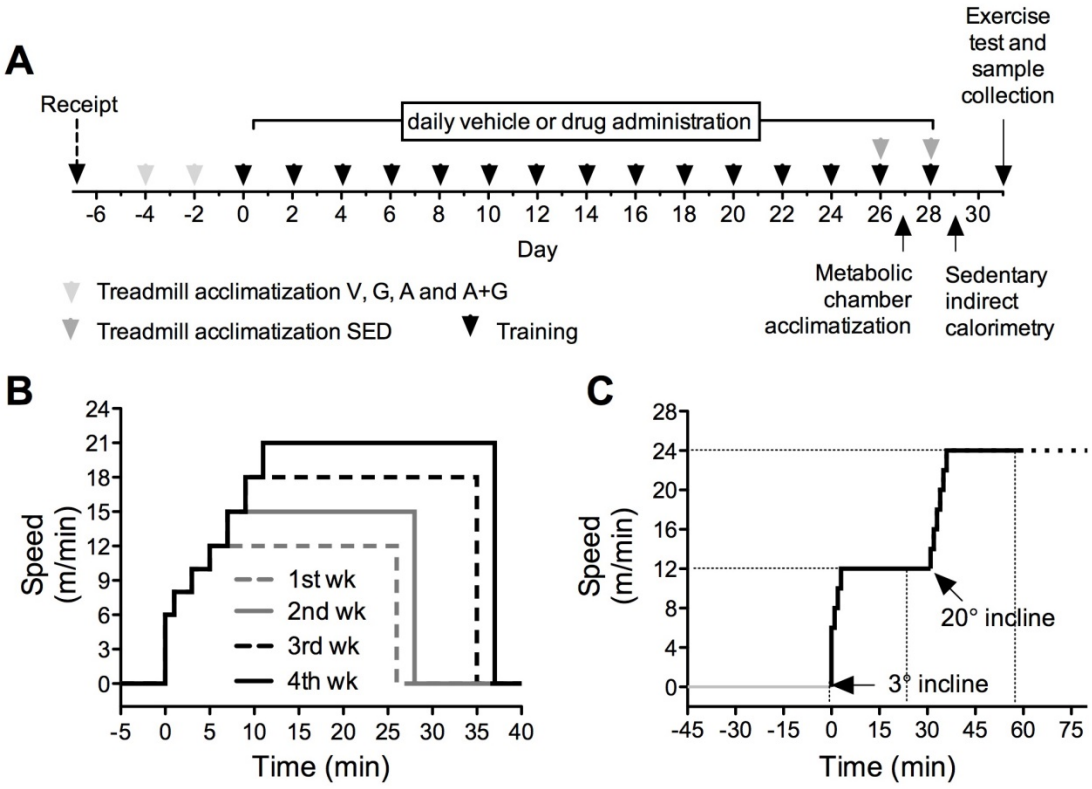

Figure 1. Schedule and exercise protocols. A) Experiment schedule, B) training protocol on the treadmill, C) exercise-to-exhaustion test with indirect calorimetry protocol.

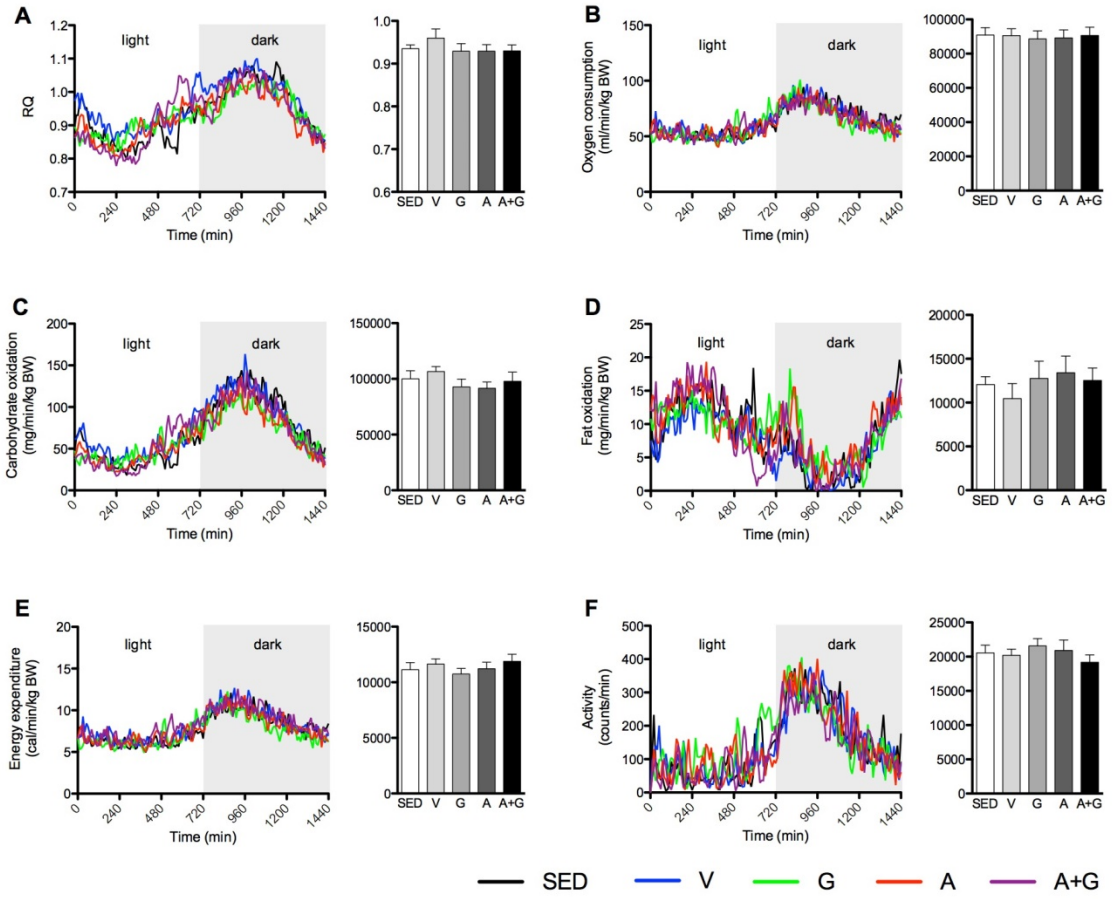

Figure 2. Combined pharmacological AMPK and PPARδ activation does not influence basal metabolism. Indirect calorimetry with activity measurement was conducted in the sedentary state. A) Respiratory quotient (RQ), B) oxygen consumption, C) carbohydrate oxidation, D) fat oxidation, E) energy expenditure, and F) spontaneous motor activity. The graph on the left depicts values at each time point while that on the right shows the average (only for RQ) or cumulative values within 24 h. Data are expressed as means ± SEM (*n* = 8-9). No significant differences were observed as analyzed by one-way ANOVA.

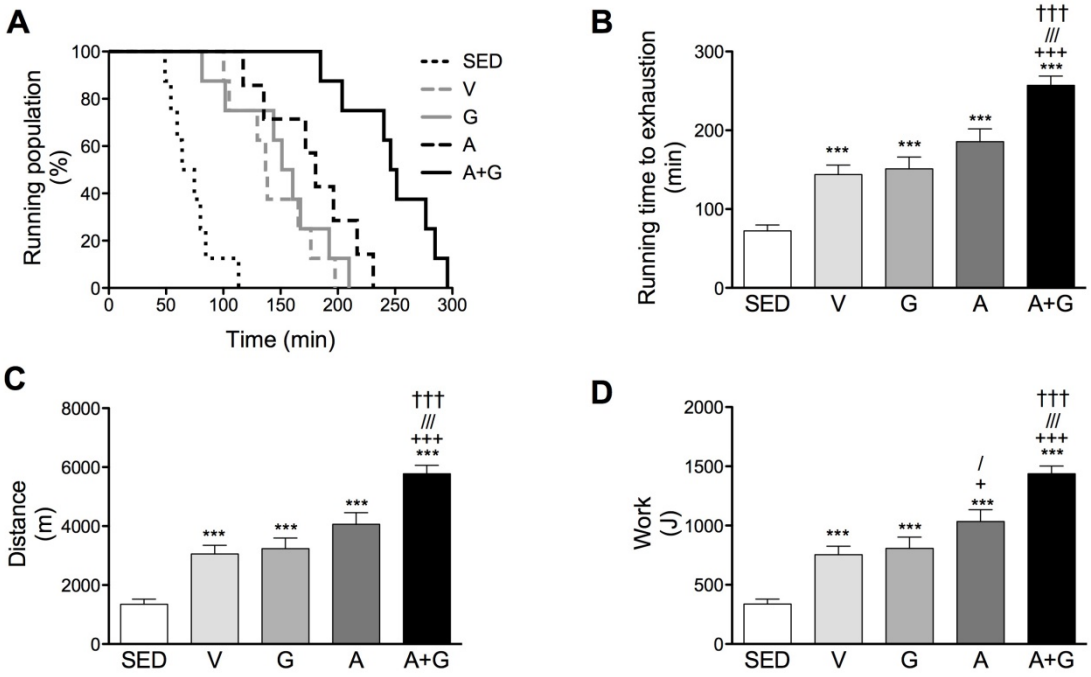

Figure 3. Combined pharmacological AMPK and PPARδ activation improves endurance in trained mice. Mice were subjected to exercise-to-exhaustion test. A) Percentage of running population on the treadmill, B) total running time to exhaustion, C) total distance covered, and D) total work done. Data are expressed as means ± SEM (*n* = 7-8). Significant differences were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Asterisk (*), plus sign (+), slash (/) and dagger (†) represent significant difference relative to SED, V, G and A, respectively. Single symbols indicate $p<0.05$ while triple symbols indicate $p<0.001$.

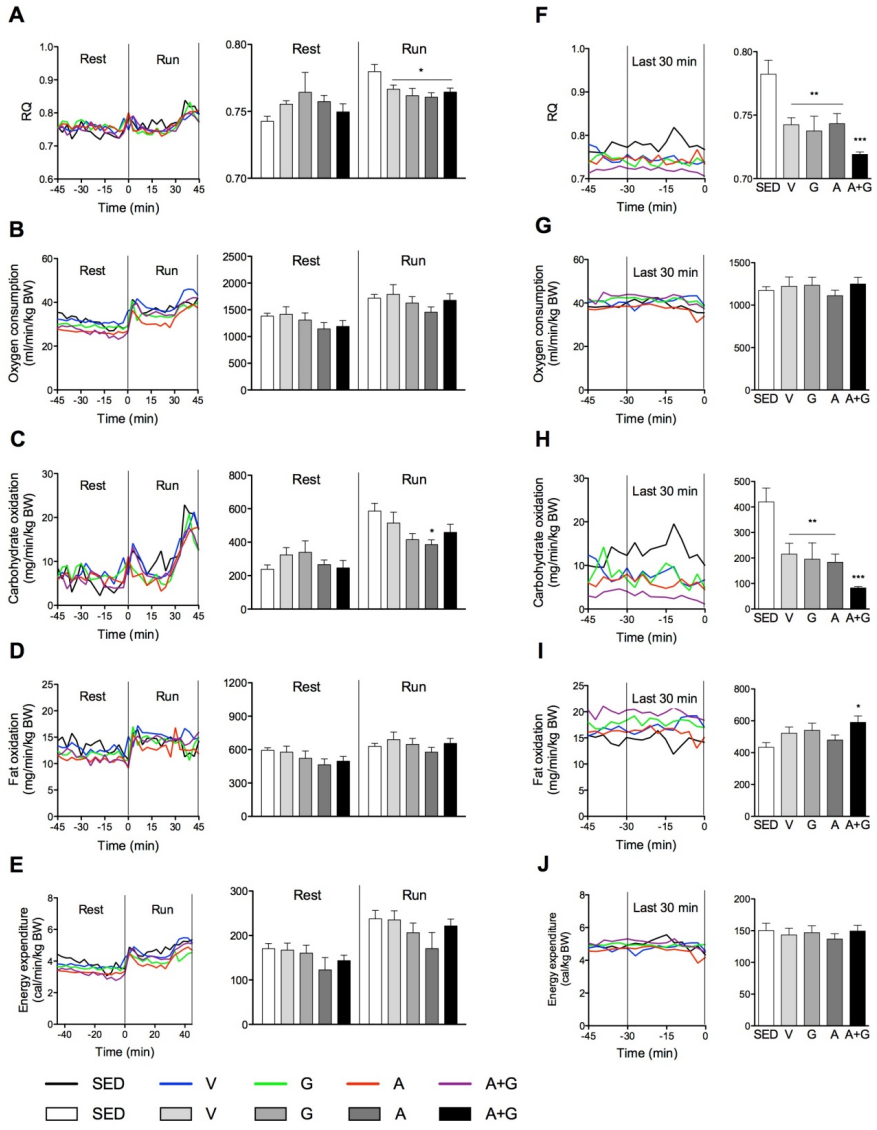

Figure 4. Combined pharmacological AMPK and PPARδ activation promotes enhanced substrate shift during prolonged exercise in trained mice. Indirect calorimetry was conducted before and during the exercise-to-exhaustion test. A) Respiratory quotient (RQ), B) oxygen consumption, C) carbohydrate oxidation, D) fat oxidation, and E) energy expenditure. The same parameters were measured during the last 30 min until exhaustion: F) RQ, G) oxygen consumption, H) carbohydrate oxidation, I) fat oxidation, and J) energy expenditure. Time course data are expressed as means while average RQ and cumulative values are expressed as means ± SEM (n = 6). Significant differences were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. *, p<0.05; **, p<0.01; and ***, p<0.001 relative to SED.

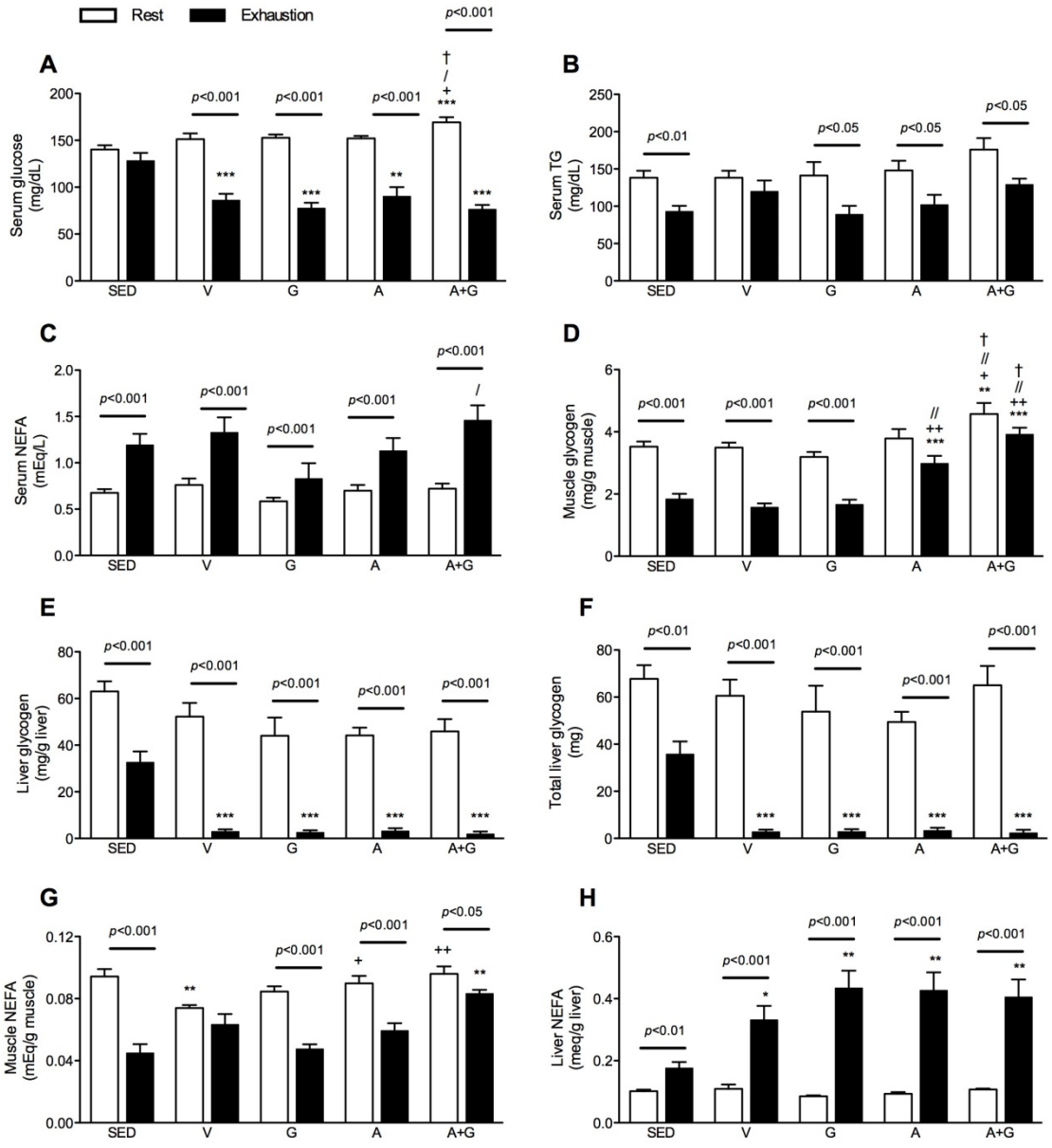

Figure 5. Combined pharmacological AMPK and PPARδ activation increases available substrates and muscle glycogen sparing in trained mice. Blood, gastrocnemius and liver were collected at rest and at the point of exhaustion. A) Serum glucose, B) serum TG, C) serum NEFA, D) muscle glycogen, E) liver glycogen, F) total liver glycogen, G) muscle NEFA and H) liver NEFA were measured. Data are expressed as means ± SEM (*n* = 8-9, at rest; 7-8, at exhaustion). Inter-group significant differences were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test while intra-group differences were analyzed by unpaired Student's t-test. Asterisk (*), plus sign (+), slash (/) and dagger (†) represent significant difference relative to SED, V, G and A, respectively. Single symbols, $p<0.05$; double symbols, $p<0.05$ while triple symbols, $p<0.001$.

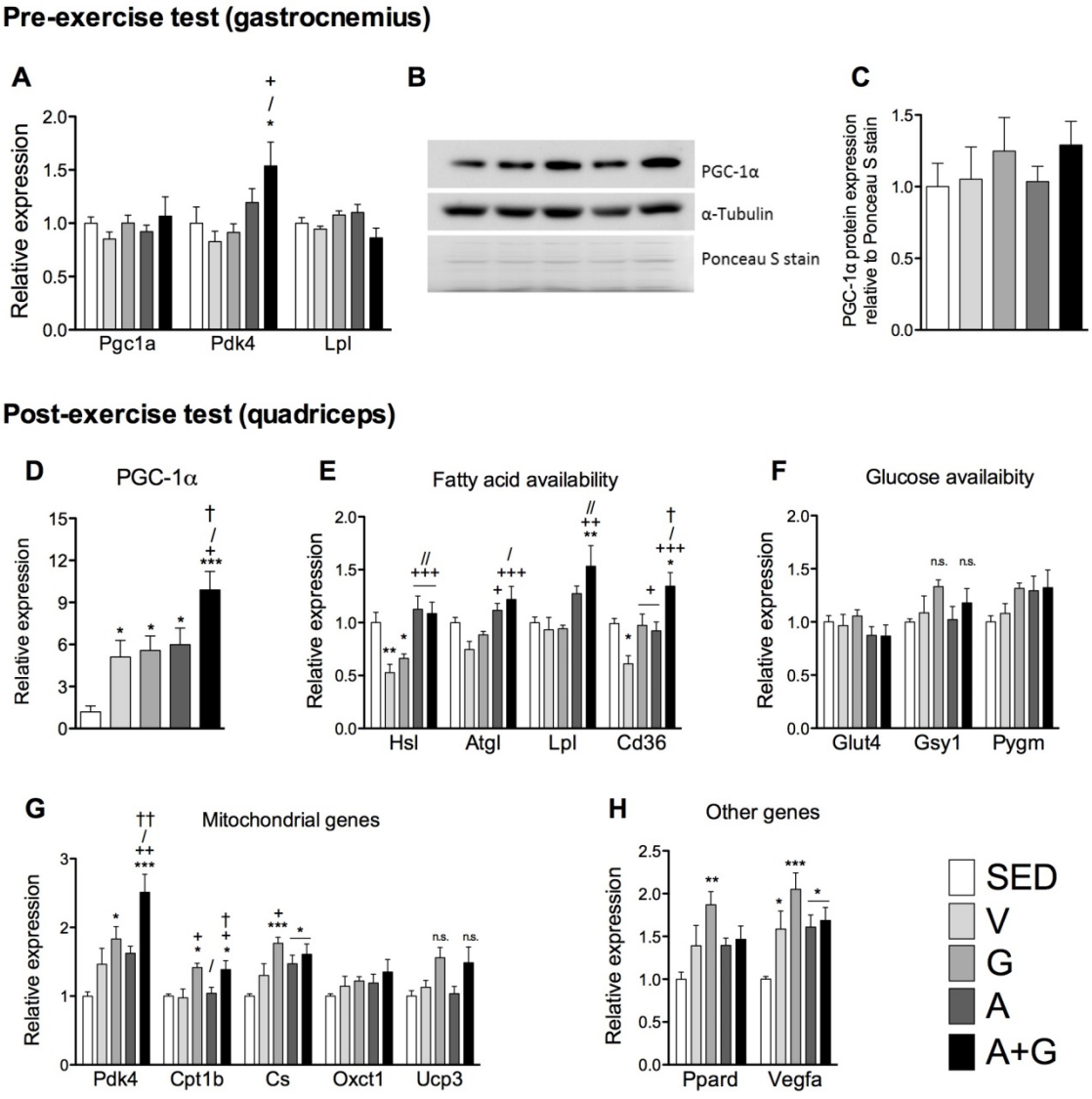

Figure 6. Combined pharmacological AMPK and PPARδ activation influences gene expression of substrate utilization related genes in skeletal muscle in trained mice. Pre-exercise test A) *Pgc1α*, *Pdk4* and *Lpl* expression and, B) representative immunoblot image of PGC-1α and α-Tubulin, and Ponceau S digitized image, and C) relative PGC-1α protein expression in gastrocnemius muscle. Post-exercise test messenger RNA expression of D) PGC-1α and genes related to E) fatty acid availability, F) glucose availability, G) mitochondrial oxidative metabolism, and H) *Ppard* and *Vegfa* were measured. For mRNA expression, data were normalized to *Hprt* expression while for protein expression, data were normalized to total Ponceau S signal. Data were rationalized to SED, and expressed as means ± SEM (pre-exercise test *n* =6, post-exercise test *n*=7-9). Significant differences were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Asterisk (*), plus sign (+), slash (/) and dagger (†) represent significant difference relative to SED, V, G and A, respectively. Single symbols, $p<0.05$; double symbols, $p<0.01$; while triple symbols, $p<0.001$.

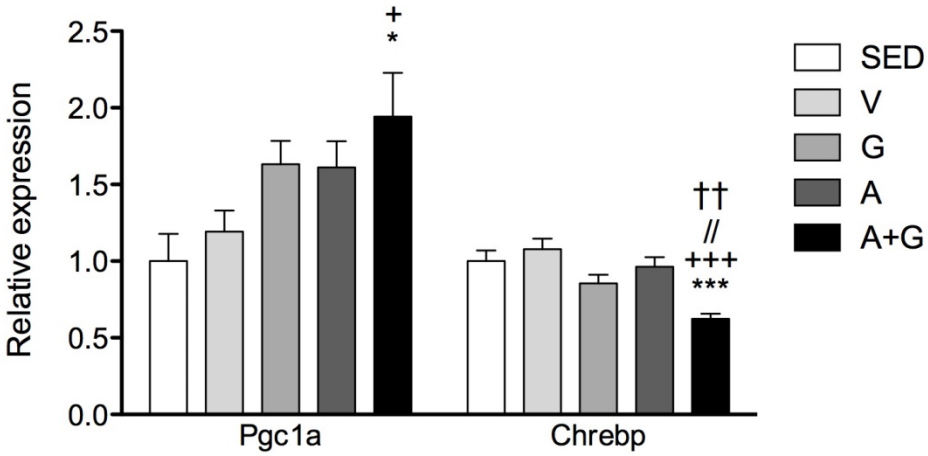

Figure 7. Combined pharmacological AMPK and PPARδ activation modifies hepatic mRNA expression of PGC-1α and CHREBP in trained mice. After exhaustion, liver was analyzed for *Pgc1a*, and *Chrebp* expression. Data were normalized to *Hprt* expression, rationalized to SED, and expressed as means ± SEM (*n* = 7-8). Significant differences were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Asterisk (*), plus sign (+), slash (/) and dagger (†) represent significant difference relative to SED, V, G and A, respectively. Single symbols, $p<0.05$ while triple symbols, $p<0.001$.

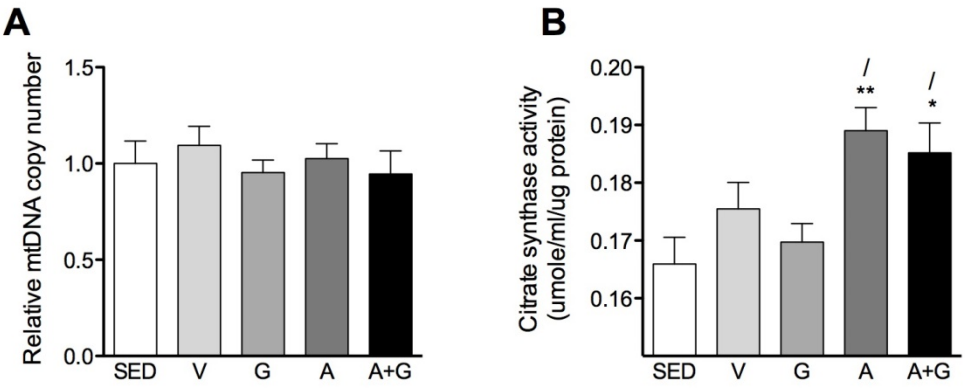

Figure 8. Pharmacological activation of AMPK but not PPARδ improved skeletal muscle mitochondrial density in trained mice. A) Relative mtDNA copy number was measured by comparing the copy number of mitochondrial cytochrome oxidase I (COI) DNA with the copy number of nuclear 18S rDNA. B) Citrate synthase activity was measured from muscle lysates. Mitochondrial DNA copy number data were rationalized to SED. Data are expressed as means ± SEM (*n* = 8-9). Significant differences were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Asterisk (*), and slash (/) represent significant difference relative to SED, and G, respectively. Single symbols, $p<0.05$ while double symbols, $p<0.01$.

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Chapter 2

CD36 is essential for endurance improvement, changes in whole-body metabolism and efficient PPAR-related transcriptional responses in the muscle with exercise training

Introduction

Fatty acids provide energy during metabolic challenges such as fasting and exercise. Uptake of fatty acids in cells occurs primarily by active transport facilitated by several fatty acid transporters such as fatty acid transport proteins (FATPs), fatty acid binding proteins (FABPs), and fatty acid translocase CD36 on the cell membrane which exhibit ligand binding preference [for review see Glatz et al. (2010)]. Expression of these proteins varies by cell type and their significance in organ function and whole-body physiology are still being elucidated to date.

CD36 regulates different physiological functions in many metabolic organs such as the heart, adipose tissue and muscle as well as in non-metabolic tissues such as nasal mucosa and these functions range from inflammation, nutrient absorption, metabolism, fatty acid sensing and signal transduction with various endogenous ligands (Lee et al., 2015; Silverstein and Febbraio, 2009). In the skeletal muscle, CD36 is the predominant fatty acid transporter that regulates fatty acid transport and consequently fatty acid oxidation (McFarlan et al., 2012; Nickerson et al., 2009) as this transporter localizes to the plasma membrane and mitochondrial outer membrane under different metabolic conditions (Holloway et al., 2009; Jeppesen et al., 2011; Luiken et al., 2002; Smith et al., 2011). As such, it is likely that this transporter also influences fatty acid-mediated signaling in the organs it is expressed on.

Regular exercise training is an efficient stimulus for metabolic remodeling of the muscle [for review see Egan and Zierath (2013)]. Increased fatty acid catabolism, glycogen storage, shifting of substrate utilization from glucose to fat, oxidation of ketone bodies, muscle fiber transition to an oxidative phenotype and mitochondrial biogenesis and function have been observed in exercise training (Egan and Zierath, 2013). These may be considered as adaptation mechanisms in the muscle to sustain energy production possibly to conserve glucose reserves for the brain (Nybo, 2003) with consistent elevated energy demand. Transcription factors responsible for these changes in the muscle are the members of the peroxisome-proliferator activated receptor (PPAR) family particularly PPARα and PPARβ/δ as well as the estrogen-related receptor (ERR) family (Gan et al., 2013; Hondares et al., 2007; Huss et al., 2004; Narkar et al., 2011; Rangwala et al., 2010; Schreiber et al., 2004; Wang et al., 2004). Common to these transcription factors is their activation by PPARγ-coactivator 1 α (PGC1α), which is upregulated and highly responsive to both acute and chronic exercise (Lira et al., 2010; Pilegaard et al., 2003). Although it has been established that fatty acids are ligands of PPARs and exercise training induces PPAR targets in the muscle, the role of CD36 in transcriptional adaptations with exercise has yet to be determined.

By employing whole-body CD36 knockout mice, the role of CD36 in untrained mice on exercise performance and substrate utilization has been demonstrated (Fujitani et al., 2014; McFarlan et al., 2012). Exercise training in these mice upregulated mitochondrial proteins (McFarlan et al., 2012) but the consequence of this on endurance was not evaluated. In addition, several training-induced adaptations in the muscle that could potentially compensate for the lack of CD36, that is to say impaired uptake and oxidation of fatty acids, during exhaustive exercise have yet to be investigated. In this study, we supplement these above reports and show that CD36 is essential not only for basal endurance performance but also for its training-induced improvement. Also, we show that efficient transcriptional activation of exercise-responsive genes in the muscle and downstream phenotypic manifestations in whole-body metabolism are, in part, dependent on CD36.

Materials and methods

Animals

Whole-body CD36 knock-out mice (KO) (hereby denoted as K) and wild-type (WT) (hereby denoted as W) littermates were housed in a room maintained at 22±0.5°C, 50% humidity and a 12h light-dark cycle (6:00 lights on; 18:00 lights off). These mice were received by our laboratory as a gift from Dr. Mason W. Freeman of Harvard Medical School (Moore et al., 2002) and was maintained on a C57BL6/J background. Mice were bred in-house and from 4 wk old, mice were weaned, genotyped and had free access to a regular chow diet (Oriental Yeast Co., Tokyo, Japan) and water. Only male mice were used in this study. A purified diet containing 30 % kcal from fat (soybean oil), 20 % kcal from protein, and 50 % kcal from carbohydrates (Research Diets, NJ, USA) and water was provided ad libitum at 8 wk old when experiments were commenced. Animal experiments as detailed in the following sections were conducted according to the Kyoto University Guidelines for the Ethical Treatment of Laboratory Animals as approved by the committee (No. 28-28).

Exercise training

W and K at 8 wk old were randomly assigned to either untrained (U) or exercised (E) groups and 30 d of training was commenced. On the first 15 d, mice ran at a constant speed of 15 m/min for 50 min on a motorized treadmill (MK-680; Muromachi, Tokyo, Japan) kept an angle of 3°. On the last 15 d, time was increased to 75 min while other parameters were kept constant. Mice were stimulated to run by poking with a metal rod. All mice were responsive to stimulation and completed the training protocol. Training was done within the first 3 h of the light phase. Mice were subdivided into groups intended for basal indirect calorimetry, fixed-time exercise, exercise-to-exhaustion test with indirect calorimetry, and exogenous glucose oxidation during exercise.

Whole-body metabolism at rest

Basal indirect calorimetry was performed according to Manio et al. (2016). Mice were placed individually in acrylic chambers from the 3rd day before the last training session. Mice were left undisturbed for 48 h after the last training session. Mice had ad libitum access to food and water while in the chambers. Measurement was commenced from the acclimatization period with sampling every 10 min. However only data from the final 24 h corresponding to a full light and dark cycle were analyzed to represent the chronic effect of training on basal whole-body metabolism. Mice were sacrificed after measurements and time between the first and last mouse was kept within 2 h to avoid the effects of time differences in metabolite concentration and gene expression.

Fixed-time exercise

Fixed-time exercise was performed 48 h after the last training session. Mice were placed on a moving treadmill set at 10 m/min. After 2 min of warm-up, mice were immediately transferred to a moving treadmill set at 15 m/min at an inclination of 10°. Mice were made to run for 1 h with an electrical stimulus of 0.2 mA. Mice were sacrificed after the run.

Whole-body metabolism during exercise and exercise-to-exhaustion

Exercise-to-exhaustion test with indirect calorimetry was performed 48 h after the last training session. Mice were individually placed in airtight treadmill at an incline of 10° (Mousebelt-200; Arco System, Tokyo, Japan) and were left undisturbed for 1.5 h. After a short warm-up, mice were made to run for 3 h at 15 m/min followed by 1 h at 17 m/min. Intensity was increased to 19 m/min and kept herein until mice reached exhaustion. Indirect calorimetry was commenced upon assignment of mice to the treadmill. Similar measurement parameters were employed as in the basal indirect calorimetry and only the time interval of sampling was changed to 2 min. Mice were forced to run by mild electrical stimulus of 0.2 mA. At the point of exhaustion, mice were immediately sacrificed. Mice were declared exhausted after failing to respond or sustain running for 20 sec despite poking and

tapping on chamber walls with occasional elevation of electrical stimulus. Exhaustion was confirmed by serum glucose, muscle and liver glycogen depletion (not shown).

Single bout of exercise

W and K at 8 wk old were fed the same purified diet for 32 d. At the onset of the light phase, mice were assigned into no run (N) or 1x run (R) groups and made to run for 50 min at 15 m/min, 3° incline similar to the training protocol. Mice were returned to cages and allowed to recover normally with access to food and water. Mice were sacrificed after 8 h.

Exogenous glucose oxidation during exercise

Exogenous glucose oxidation using ^{13}C-labelled glucose was based on Fujitani et al. (2014). Forty-eight (48) h after the last training session, mice were placed individually in treadmill chambers for indirect calorimetry. One (1) h after acclimatization, ^{13}C-labelled D-glucose (Cambridge Isotope Laboratories, Inc., MA, USA) in water was administered orally at a dose of 36 mg/kg BW (10 mL/kg BW) and mice were returned to chambers. After 8 min, the run was commenced starting with a brief warm-up at 10 m/min then increased to 15 m/min for a total of 30 min. Expired CO_2 expressed as $\%^{13}C/^{12}C$ as calculated by the accompanying software was plotted against time.

Sample collection

Mice were sacrificed by decapitation. Blood was collected and an aliquot was deproteinized by immediately mixing (1:1) with 0.8 M perchloric acid. After 5 min, the mixture was centrifuged at 1.5x1000 *g* for 5 min at 4 °C. The resulting supernatant was aliquoted and flash frozen in liquid nitrogen. Remaining whole blood was left to coagulate for 30 min to 1 h and serum was collected after centrifugation at 1.5x1000 *g* for 10 min at 4 °C. Gastrocnemius and liver samples were clamp frozen in liquid nitrogen. All samples were transferred to a -70 °C freezer until processing. Clamp frozen gastrocnemius and liver were powdered using a liquid nitrogen-cooled mortar and pestle, aliquoted and weighted for

glycogen, lipid, protein, total RNA and acid-soluble compounds extraction. Samples were returned to the same storage conditions.

Blood chemistry

Kits were used to analyze blood chemistry. Serum was analyzed for glucose, non-esterified fatty acids (NEFA), triglycerides (TG), and β-hydroxybutyrate (β-HB) (Wako, Osaka, Japan) while deproteinized blood was analyzed for lactate (Kyowa Medex, Tokyo, Japan) according to manufacturers' instructions.

Glycogen

Glycogen measurement was based on Good et al. (1933) and Sahyun and Alsberg (1931) with some modifications. In brief, approximately 25 mg powdered muscle and liver samples were digested in alkali with 300 µL 30 % KOH at 100 °C for 30 min. Precipitation of glycogen was carried out by addition of 50 µL saturated Na_2SO_4 and 500 µL cold ethanol followed by centrifugation at room temperature (RT) for 2.3x1000 *g* for 5 min. The pellet was washed with 500 µL distilled water, vortexed and precipitated with 625 µL ethanol followed by centrifugation. The pellet was dehydrated to completeness at 50-70 °C for 1.5-2 h. The pellet was suspended in 500 µL 0.6 M HCl. An aliquot was digested for 3 h at 99 °C using a thermal cycler with a heated lid to prevent evaporation. Liberated glucose was measured similar to serum glucose and weight of glycogen was calculated from this value using a formula that assumes all glucose units are linearly polymerized into one molecule of glycogen.

Muscle lipids

Lipids were extracted from powdered muscle samples as described in Manio et al. (2016) with some modifications. Approximately 30 mg samples were mixed thoroughly with 1 mL Folch reagent. After more than 16 h incubation at 4 °C in a revolving mixer, 200 µL of 4 mM $MgCl_2$ was added and vortexed. Following 10 min incubation at 4 °C, samples were centrifuged at 1.2x1000 *g* for 1 h. From the bottom chloroform layer, 500 µL was collected

and evaporated to dryness at 40 °C for about 2 h. The desolvated lipids from muscle samples were suspended in 50 µL of 10 % Triton-X in isopropanol. Measurement of TG and NEFA was similar to serum samples.

Muscle lactate and β-HB

Lactate and β-HB were extracted from muscle samples based on Ohtsu et al. (2003) with some modifications. In brief tissues were incubated in 3 volumes of 1 M PCA with occasional shaking for 1 h at 4 °C. After centrifugation at 10x1000 *g* for 2 min, the supernatant was collected. For every 50 µL of PCA extract, 21.5 µL of 1 M K_2CO_3 was added to neutralize the solution between pH 6.5-7.5. The precipitate was spun down and the supernatant was aliquoted and stored at -70 °C until analysis. Measurement of lactate and β-HB was similar to blood chemistry samples.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from approximately 20 mg powdered gastrocnemius samples with Tripure Isolation Reagent (Roche, Mannheim, Germany) followed by purification using Genelute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, MO, USA). In brief, frozen samples were sonicated in the isolation reagent to ensure complete lysis followed by addition of chloroform according to the reagent's protocol. After centrifugation, supernatant was transferred to the blue column of the kit to shred potential protein and DNA contaminants. From here, the procedure was according to the manufacturer's instructions. Total RNA (1.8 µg) was reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) according to manufacturer's instructions. Messenger RNA (mRNA) levels were amplified with Premix ExTaq for probe qPCR (Takara Bio, Shiga, Japan) using the primers that span intronic regions for mRNA specificity and corresponding Universal Library Probes (Roche, Mannheim, Germany) as listed in Table 1. Values were rationalized to *Hprt* expression (Cappelli et al., 2008).

Table 1. RT-qPCR primers and probes

mRNA primers		Sequence (5' to 3')	Universal Probe No.	Accession no.
Pgc1a	Forward	tgtggaactctctggaactgc	63	NM_008904.2
	Reverse	agggttatcttggttggcttta		
Ppara	Forward	ccgagggctctgtcatca	11	NM_011144.6
	Reverse	gggcagctgactgaggaa		
Ppard	Forward	atgggggaccagaacacac	11	NM_011145.3
	Reverse	ggaggaattctgggagaggt		
Cycs	Forward	aacgttcgtggtgttgacc	104	NM_007808.4
	Reverse	ttatgcttgcctccctttc		
Pdk4	Forward	cgcttagtgaacactccttcg	22	NM_013743.2
	Reverse	cttctgggctcttctcatgg		
Oxct1	Forward	caggcaatgtgattttcagg	22	NM_024188.6
	Reverse	gcaaatgagccaatgtctacaa		
Cd36	Forward	tttcctctgacatttgcaggt	11	NM_001159558.1
	Reverse	gattctggaggggtgatgc		
Fatp1	Forward	cttcctaaggctgccattgt	49	NM_011977.3
	Reverse	ggcagtcatagagcacatcg		
Cpt1b	Forward	ccatcattgggcacctct	104	NM_009948.2
	Reverse	gtctccgtgtagcccaggt		
Lpl	Forward	tggataagcgactcctacttcag	22	NM_008509.2
	Reverse	tccctagcacagaagatgacc		
Myh7	Forward	tgcattgacctcatcgagaa	63	NM_080728.2
	Reverse	gtcatgtctgtggccttgg		
Myh4	Forward	tgcttacgtcagtcaaggtga	104	NM_010855.2
	Reverse	aatcccaggatatcaacagca		
Myh2	Forward	tcttctctggggcacaaact	22	NM_001039545.2
	Reverse	cccttcttcttggcaccttt		
Glut4	Forward	tcgtcattggcattctggt	104	NM_009204.2

	Reverse	agcagtggccacagggta		
Vegfa	Forward	actggaccctggctttactg	22	NM_001025250.3
	Reverse	tctgctctccttctgtcgtg		
Mct1	Forward	atgctgccctgtcctcct	49	NM_009196.3
	Reverse	ccacaagcccagtacgtgtat		
Mct4	Forward	ctcatagatctttatgactggacagg	11	NM_146136.1
	Reverse	tgtttgattggctgtggatg		
Hprt	Forward	cctcctcagaccgcttttt	95	NM_013556.2
	Reverse	aacctggttcatcatcgctaa		

Protein extraction

Powdered muscle samples were lysed in a buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM mercaptoethanol and 1 % NP-40 adjusted to pH 8.0 and supplemented with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany) according to manufacturer's instructions. Insoluble matter was precipitated by centrifugation at 10x1000 *g* for 20 min at 4 °C. Supernatant was collected and transferred to a clean tube. The concentration was measured by Bradford protein assay and an aliquot was adjusted with lysis buffer to 2 µg/µL. This was further aliquoted for enzyme activity measurements and immunoblot experiments. The concentration of this adjusted aliquot was measured to assure accurate loading of samples in succeeding experiments. The original lysate and adjusted lysate were snap frozen in liquid nitrogen before storing at -70 °C.

Enzyme activities

Beta-hydroxyacyl-CoA dehydrogenase (β-HAD) activity was measured based on Holloway et al. (2007) with some modifications. Four (4) µL of adjusted lysate containing approximately 8 µg protein was pipetted to a 96-well plate. All solutions were warmed to 37 °C prior to addition. To wells, 100 µL of a solution containing 0.5 mM NADH in 50 mM Tris and 2 mM EDTA at pH 7.0 was pipetted. The plate was briefly shaken followed by 5 min incubation at

37 °C. After incubation, 100 µL of a solution containing 0.2 mM acetoacetyl-CoA in the same buffer was pipetted, and the plate briefly shaken before reading at 340 nm for 10 min at 37 °C on a kinetic program.

The forward reaction of succinyl-CoA: 3-oxoacid CoA-transferase (SCOT) activity was measured based on Williamson et al. (1971) with some modifications. A general buffer containing 50 mM Tris, 10 mM $MgCl_2$ and 5 mM iodoacetamide at pH 8.5 was prepared. To an aliquot, 50 mM sodium succinate was prepared. To another aliquot, 0.1 mM acetoacetyl-CoA was prepared. Four (4) µL of adjusted lysate containing approximately 8 µg protein was pipetted to a 96-well plate. To wells, 190 µL of succinate solution was added and incubated for 5 min at RT followed by the addition of 10 µL of acetoacetyl-CoA solution. The plate was briefly shaken before reading at 303 nm for 10 min at RT on a kinetic program.

The activity of pyruvate-to-lactate reaction of lactate dehydrogenase (LDH) was measured according to Crabtree and Newsholme (1972) and Howell et al. (1979) with some modifications. A reaction buffer containing 0.1 mM pyruvate and 0.15 mM NADH in 50 mM potassium phosphate buffer at pH 7.4 was prepared and incubated at 37 °C prior to reaction. To 5 µL of adjusted lysate containing approximately 10 µg protein, 200 µL of the reaction buffer was pipetted, briefly shaken and read at 340 nM for 10 min at 37 °C on a kinetic program.

Citrate synthase (CS) activity was measured based on Manio et al. (2016) and Srere (1969). Protein concentration of the final adjusted lysate was used in all the calculations. In all enzyme activity assays, blank wells contained the lysis buffer.

Immunoblotting

Muscle lysates were adjusted with lysis buffer and 4x Laemilli buffer to achieve a concentration of 1.25 µg/µL. Forty (40) µg of protein was subjected to electrophoresis in a 10 % polyacrylamide gel. Transfer to PVDF membranes was performed in transfer buffer containing 20% methanol for 1 hr. Membranes were incubated with shaking for 2 min in 0.5% Ponceau S stain. After 3 washings in distilled water for 1 min each with shaking,

membranes were sandwiched in a plastic film, visualized and digitized (LAS-3000; Fujifilm, Tokyo, Japan). Membranes were cut into 2 pre-determined sections known to contain CD36 and PDK4 protein. Membranes were blocked in Block Ace Powder (DS Pharma Biomedical, Osaka, Japan) in Tris-buffered saline with 0.1 % Tween-20 (TBS-T) buffer according to manufacturer's instructions followed by incubation overnight at 4 °C in anti-CD36 primary antibody (1:2000; AF2519, R&D Systems, MN, USA) or anti-PDK4 primary antibody (1:1000; SC-14495, Santa Cruz Biotechnology, CA, USA) diluted in blocking solution. Membranes were washed in 3 replacements of 20 mL TBS-T buffer for 5 min each with shaking before incubation in horseradish peroxidase-labelled anti-goat IgG secondary antibody (1:1000; P0449, Dako, Tokyo, Japan) for 3 h at 4 °C. Membranes were washed with TBS-T buffer thrice and reacted for chemiluminescence detection with Western Lightning Plus ECL (Perkin Elmer, MA, USA) for 1 min. Detection and visualization using the LAS-3000 system was performed every 10 sec for 3.5 and 8.5 min for CD36 and PDK4, respectively. Band intensity at 88 kDa and 47 kDa corresponding to CD36 and PDK4, respectively and Ponceau S stain signals were quantified using the software MultiGauge V3.2 (Fujifilm, Tokyo, Japan) with automatic background detection.

Statistical analyses

Statistical analyses were performed on the software Prism 5.0 (Graphpad Software, CA, USA). Indirect calorimetry time-course data are presented as means. All other data are presented as mean ± SEM. To determine genotype and treatment interaction effect, and group x time differences in exogenous glucose oxidation, two-way Analysis of Variance (ANOVA) was performed wherever appropriate. Between genotype (same treatment), between treatment (same genotype) and metabolite concentration (between pre- and post-exercise) comparisons were performed using unpaired Student's *t*-test at a significance level of α=0.05.

Results

To determine the effect of training on endurance, mice were subjected to exercise-to-exhaustion test. Training improved endurance in W (46.7%; $p<0.001$). As previously reported (Fujitani et al., 2014; McFarlan et al., 2012), K were exercise intolerant relative to W counterparts ($p<0.001$) (Fig. 1A and B). Despite completing the same training program, exercise intolerance in KU was not ameliorated in KE. Furthermore, KU and KE were not able to sustain running and most reached exhaustion before the increase in intensity to 17 m/min and 19 m/min in contrast to their W counterparts (Fig. 1B).

To assess basal whole-body metabolism, mice were left undisturbed after the last bout of exercise for 48 h. Respiratory gases were measured during this period and data from the last 24 h was analyzed. The first 24 h was not used as it reflected whole-body metabolism immediately after exercise towards recovery. In contrast to K, significant increase in average respiratory exchange ratio (RER) in the light phase in WE relative to WU ($p<0.05$) and a non-significant decrease ($p=0.0913$) in the dark phase (Fig. 2A and D) were observed. Also, KU was lower ($p=0.0797$) relative to WU in the dark phase. Total oxygen consumption was significantly increased ($p<0.05$) in WE relative to WU which reflected the increases in both light and dark phases ($p<0.01$ and $p=0.0565$, respectively) (Fig. 2B and E). In K, oxygen consumption was significantly higher ($p<0.05$) in KE relative to KU only in the light phase. These changes in whole-body metabolism were not because of changes in spontaneous motor activity (Fig. 2C and F). Also, body weight was not different between genotypes and this was not affected by the training protocol (not shown). While no significant increase in food intake because of training was observed, both K groups had higher food intake relative to W counterparts (not shown).

Whole-body metabolism during exercise was analyzed during the first 60 min where all mice were able to run without additional stimulation. Thus, this reflected whole-body metabolism purely due to exercise and not because of external stimulation. K had higher RER ($p<0.001$) than W counterparts (Fig. 2G). Lower RER ($p<0.05$) was observed in WE relative to WU. In contrast, this decrease was not observed in KE relative to KU. Oxygen

consumption was not affected in the W while lower values were observed in KE relative to KU (p<0.01) (Fig. 2H).

To assess exogenous glucose oxidation during exercise, ^{13}C-labelled glucose was administered to mice and running was commenced. Values of expired ^{13}C-labelled CO_2 expressed as $\%^{13}C/^{12}C$ were significantly lower (p<0.05) in WE relative to WU (Fig. 3A). Significant differences (p<0.05) in were observed between W and K in both training states (Fig. 3BD) while significant difference was not observed between KU and KE (Fig. 3C). Significant differences in oxygen consumption were not observed between genotypes and training states during the test (not shown). No exercise effect was observed in glucose disposal by IPGTT after an 8 h fast and after an exercise bout although K had generally better glucose disposal than W counterparts (not shown).

Table 2. Blood, muscle and liver metabolites at rest and immediately post-exercise

Parameter	Time	Untrained		Exercised	
		WT	KO	WT	KO
Serum glucose (mg/dL)	Basal	144.69 ± 7.64	150.19 ± 9.64	156.50 ± 7.87	137.02 ± 2.88*
	1 h run	206.79 ± 7.49†	158.46 ± 5.61+	190.24 ± 12.29†	162.89 ± 3.57†
Muscle glycogen (mg/g)	Basal	1.269 ± 0.103	1.759 ± 0.185+	2.382 ± 0.099*	2.395 ± 0.164*
	1 h run	0.469 ± 0.073†	0.544 ± 0.112†	0.320 ± 0.039†	0.335 ± 0.067†
Liver glycogen (mg/g)	Basal	57.91 ± 3.10	31.38 ± 5.30+	39.50 ± 4.47*	43.75 ± 5.07
	1 h run	32.01 ± 4.54†	23.67 ± 3.70	30.44 ± 5.58	30.79 ± 3.84
Blood lactate (mg/dL)	Basal	21.87 ± 1.60	20.61 ± 1.72	23.50 ± 1.01	21.29 ± 0.91
	1 h run	19.40 ± 1.28	17.60 ± 1.01	18.79 ± 0.80†	18.24 ± 1.20
Muscle lactate (mg/g)	Basal	1.276 ± 0.100	1.067 ± 0.066	1.133 ± 0.060	1.373 ± 0.086*+
	1 h run	1.349 ± 0.050	1.161 ± 0.087	1.435 ± 0.045†	1.555 ± 0.090*
Serum NEFA (mEq/L)	Basal	0.694 ± 0.049	0.913 ± 0.050+	0.426 ± 0.032*	0.814 ± 0.037+
	1 h run	0.787 ± 0.028	1.031 ± 0.052+	0.762 ± 0.039†	1.009 ± 0.035+†

Serum TG (mg/dL)	**Basal**	101.28 ± 15.87	65.50 ± 5.39	73.00 ± 11.09	60.22 ± 5.04
	1 h run	105.08 ± 13.56	52.17 ± 2.38+†	91.13 ± 11.24	58.78 ± 3.54+
Serum β-HB (μM)	**Basal**	54.50 ± 9.57	75.07 ± 13.77	46.93 ± 4.20	71.17 ± 15.07
	1 h run	274.38 ± 37.09†	638.96 ± 60.24+†	250.11 ± 28.88†	503.22 ± 52.97+†
Muscle NEFA (mEq/g)	**Basal**	0.0127 ± 0.0011	0.0157 ± 0.0018	0.0097 ± 0.0010	0.0094 ± 0.0009*
	1 h run	0.0039 ± 0.0005†	0.0040 ± 0.0003†	0.0042 ± 0.0002†	0.0049 ± 0.0003†
Muscle TG (mg/g)	**Basal**	10.918 ± 1.552	12.627 ± 1.564	7.911 ± 0.926	7.290 ± 1.809*
	1 h run	1.876 ± 0.459†	1.510 ± 0.325†	1.732 ± 0.147†	1.225 ± 0.108+†
Muscle β-HB (μmol/g)	**Basal**	0.1027 ± 0.0341	0.1452 ± 0.0506	0.0882 ± 0.0093	0.1638 ± 0.0542
	1 h run	0.0691 ± 0.0109	0.1611 ± 0.0164+	0.0620 ± 0.0073†	0.1457 ± 0.0146+

Table 2. Blood, muscle and liver metabolites at rest and immediately post-exercise.
Metabolites in the blood, muscle and liver were measured at the basal state and after 1 h exercise. Data are expressed as mean ± SEM (n = 7-9). No significant interaction effect was observed as assessed by 2-way ANOVA. Between group differences were assessed by Student's unpaired *t*-test where symbols represent *p* values less than 0.05-0.001. Asterisk (*) represents significant difference between exercise status of the same genotype, plus (+) represents significant difference between genotypes of the same exercise status while cross (†) represents significant difference between basal and 1 h run data.

Metabolites in the blood, muscle and liver at basal and post-exercise states are summarized in Table 2. Basal serum glucose was lower ($p<0.05$) in the KE relative to KU. Post-exercise glucose was higher in WU, WE and KE ($p<0.0001$-0.05) but not in KU. The increase in KE was not significantly different with KU. Both K groups had lower serum glucose than W counterparts post-exercise ($p<0.0001$ and $p=0.0507$ for KU and KE, respectively). Basal muscle glycogen was higher in KU relative to WU ($p<0.05$). Training increased muscle glycogen in WE and KE ($p<0.0001$-0.05) but the degree of elevation in KE was 12% less (79.4% increase relative to KU) than WE (91.4% increase relative to WU). After exercise, muscle glycogen content significantly decreased and was similar in all groups ($p<0.0001$).

Basal blood lactate was not significantly different in all groups. WE and KE had lower blood lactate after exercise (p<0.01 and p=0.0593, respectively). Basal muscle lactate, on the other hand, was significantly higher in KE relative to KU and WE (p<0.05). After exercise, no significant change was observed in WU and KU as well as KE that remained significantly elevated relative to KU. WE however, had significantly increased muscle lactate compared to basal values. Basal liver glycogen was lower in KU relative to WU (p<0.001). Training decreased liver glycogen in WE (p<0.01) while not significantly affecting KE. After exercise, significant decrease in WU and a non-significant decrease in KE (p<0.001 and p=0.0645, respectively) was observed. At exhaustion, muscle and liver glycogen were depleted and serum glucose decreased to similar levels in all groups (not shown).

Lipid metabolites in the blood and muscle were also measured. Basal and post-exercise serum NEFA was higher (p<0.0001-0.01) in the K groups relative to W counterparts. Training lowered basal serum NEFA in WE (p<0.001) but not in KE. After exercise, WE and KE had significantly higher serum NEFA relative to basal level (p<0.0001 and p<0.001, respectively) with WE attaining similar values as WU. Basal serum TG was lower but not significant in untrained groups relative to trained counterparts in both W and K. KU had significantly lower serum TG from basal (p<0.05) albeit a small change after exercise. No change was observed in the other groups compared to basal values. However, at this period, serum TG were significantly lower in K groups relative to W counterparts (p<0.01-0.05). Basal serum β-HB was not significantly different between groups. After exercise, all groups had significant increases relative to basal values (p<0.0001) and values in K were significantly higher relative to W counterparts (p<0.0001-0.001). Basal intramuscular NEFA was lower in WE and KE relative to untrained counterparts (p=0.0679 and p<0.01, respectively). After exercise, values significantly decreased in all groups (p<0.0001-0.001). Basal muscle TG was lower in trained groups but significantly different only between KE and KU (p<0.05). After exercise, all groups had decreased muscle TG (p<0.0001-0.01). KE, however, was significantly lower than WE (p<0.05). Basal intramuscular β-HB appeared to be higher but not significantly different in K groups relative to W counterparts. After exercise, a small but significant decrease from basal value was observed in WE (p<0.05) but not in other groups. K at this state was significantly higher than W counterparts (p<0.0001-0.001).

Sedentary W and K mice ran on a treadmill to observe the influence of CD36 on early response gene transcription after a single bout of exercise. Eight (8) h during recovery, *Pgc1a* but not *Pdk4* mRNA expression was significantly upregulated in the muscle of WR and KR relative to WN and KN, respectively (p<0.0001-0.001) (Fig. 4AB). Interestingly, the increase in transcription of this coactivator was significantly higher in WR relative to KR (p<0.05; interaction effect, p<0.05). CS activity did not change with a single bout of exercise in both genotypes (Fig. 4C).

We determined the influence of CD36 on mRNA expression of exercise adaptation genes after training. *Pgc1a* was significantly increased with training in both genotypes (p<0.01-05) (Fig. 5A). No difference between genotypes was observed in contrast to adaptation after a single bout of exercise (Fig. 4A). Transcription factors *Ppara* and *Ppard* were not influenced by training in KE while *Ppara* but not *Ppard* was significantly upregulated (p<0.05) in WE (Fig. 5A). Interestingly, levels of both transcription factors were significantly higher in KU than WU (p<0.05). In WE, non-significant increases in *Cycs* and *Pdk4* and a significant increase (p<0.05) in *Oxct1* was observed relative to WU while in KE, significant increase in *Cycs* (p<0.0001) and a non-significant increase in *Oxct1* was observed relative to KU (Fig. 5B) suggesting increased mitochondrial gene transcription.

Fat utilization genes (*Cd36*, *Fatp1*, and *Lpl*) were significantly upregulated by training in WE (p<0.01-0.05) (Fig. 5C). *Lpl* and *Fatp1* were also increased with training in KE but these were not significantly different with KU. Intriguingly, *Fatp1* was significantly higher in KU relative to WU (p<0.05). Non-significant increases were observed in the gene corresponding to myosin heavy chain type IIb (*Myh4*), *Vegfa* and *Mct1* in trained groups relative to untrained counterparts of both genotypes (Fig. 5DE).

In the liver, CS activity was not influenced by training nor the absence of CD36 (not shown). In the muscle, however, activities of mitochondrial enzymes in the citric acid cycle (CS; p<0.0001-01), fatty acid β-oxidation (β-HAD; p<0.01) and ketone body oxidation (SCOT; p=0.0561 and p<0.05) were all increased by exercise training (Fig. 6A-C) independent of CD36. The activity of LDH, a cytosolic enzyme participating in anaerobic glycolysis, was neither influenced by training nor CD36 (Fig. 6D).

Training led to a significant increase (21%; $p<0.05$) in total CD36 protein expression in the gastrocnemius of WE (Fig. 7AB). As expected, no signal was detected in K. On the other hand, training did not significantly influence the PDK4 content in the gastrocnemius (Fig. 7AC).

Discussion

The properties of exercised skeletal muscle on fatty acid uptake, oxidation and mitochondrial respiration in CD36 KO muscle have been characterized in an ex-vivo experimental system (McFarlan et al., 2012). However, the contribution in endurance of circulating fatty acids, glucose homeostatic systems and other factors external to the muscle could not be accounted for in this type of set-up. In this study, we measured whole-body metabolism at rest and during exercise, exogenous glucose oxidation and changes in metabolites in exercise, gene expression in the muscle, and enzyme activities after training to supplement previous observations. Furthermore, we demonstrate that CD36 is essential not only for basal endurance but also for its training-induced improvement in whole animals.

Exercise training adaptations in mitochondrial machinery (e.g. increase in electron transport chain and oxidative proteins, and enzyme activity) are associated with improvements in endurance (Cartoni et al., 2005; Holloszy and Coyle, 1984; Rangwala et al., 2010). However, the balance between fatty acid supply and catabolism in the mitochondria appears to be more important during exercise as demonstrated in mice lacking HSL, ATGL, and CD36 but having intact mitochondrial function (Fernandez et al., 2008; Fujitani et al., 2014; Huijsman et al., 2009; McFarlan et al., 2012). Above the observed impaired endurance in the untrained state, we show the absence of its amelioration with training despite unaltered adaptations in mitochondrial density and function (Larsen et al., 2012) in CD36 KO mice. This is in stark contrast to improved endurance in WT mice. Exercise capacity as measured by maximal oxygen consumption, and oxygen consumption at submaximal exercise intensity are similar between genotypes (McFarlan et al., 2012). Because we did not measure maximal oxygen consumption, whether our training protocol improved exercise capacity in WT and CD36 KO mice remains to be determined. However, we show that changes in metabolite handling as an adaptation to training contributed to the observed improved endurance in WT mice during our exhaustion-to-exercise test as discussed in the succeeding sections.

Changes in the skeletal muscle with training can manifest in whole-body metabolism both at rest and during exercise. At rest, training induced a photophasic shift in RER and an overall increase in oxygen consumption in WT mice. While circadian effects on activity were not observed, our data suggests the involvement of CD36 in the effects of training on the peripheral clocks (Yasumoto et al., 2015), and whole-body metabolic activity without accompanying muscle PDK4 protein increase. During exercise, CD36 KO mice had elevated RER and exogenous glucose oxidation indicating carbohydrate as the preferred substrate (Fujitani et al., 2014; McFarlan et al., 2012). We show that training caused a decrease in RER associated with reduced exogenous glucose utilization without changes in oxygen consumption in WT mice suggesting the occurrence of substrate switch to fat during exercise and this adaptation is impaired in the absence of CD36. As glucose handling nor *Glut4* expression were unchanged with training, the contribution of elevated fatty acid uptake with increased CD36 protein and its sarcolemmal localization with contraction could contribute to substrate selection (Jeppesen et al., 2011; Rennie et al., 1976). These data demonstrate the influence of CD36-mediated fatty acid uptake on the control of mitochondrial substrate selection and oxidation (Holloway et al., 2009; McFarlan et al., 2012) and show that this phenomenon is observable in whole animals both at rest and during exercise. To compensate for decreased intramuscular lipid energy substrates in trained CD36 KO mice, elevated glycogenolysis, and anaerobic glycolysis likely occurred as suggested by lower oxygen consumption, increased muscle lactate and glycogen depletion (Rogatzki et al., 2015). Accumulated lactate might be locally oxidized because blood lactate was not increased basally and during exercise (Rogatzki et al., 2015).

Training increases muscle glycogen and this improves endurance by retarding the depletion of circulating glucose and hepatic glycogen (Baldwin et al., 1973; Manabe et al., 2013; Pederson et al., 2005). The absence of CD36 did not impair nor improve skeletal muscle glycogen accumulation with training. Conversely, the observed elevated muscle glycogen in untrained CD36 KO mice might be caused by promotion of glycogenesis with decreased fatty acid availability (Cazzolli et al., 2002). In the case of liver glycogen, the diet used in the study and 48 h recovery time may not be sufficient to fully recover hepatic glycogen as diets high in fat delay its replenishment after exercise (Conlee et al., 1990; Taylor et al., 2006) in

trained WT mice. In relation to this, constant elevated circulating NEFA in CD36 KO mice may cause low basal hepatic glycogen. However, the level of glycogen is not a determinant of endurance per se as its utilization is influenced by the sparing effect of fatty acids (Hickson et al., 1977; Rennie et al., 1976). Indeed, the elevation of serum NEFA and uptake through CD36 during exercise, and elevated basal muscle glycogen in conjunction with suppressed exogenous glucose oxidation resulted in hepatic glycogen sparing in trained WT mice during exercise which likely influenced endurance improvement despite a relatively lower basal hepatic glycogen than its untrained counterpart. In CD36 KO mice, on the other hand, unaltered high demand for glucose without improvement in fatty acid uptake and substrate switch despite increased muscle glycogen led to early onset of hypoglycemia and fatigue (Newsholme and Blomstrand, 2006; Nybo, 2003). Overall, our data demonstrate that training-induced muscle glycogen adaptation is intact in CD36 KO mice but this insufficient to ameliorate exercise intolerance and substrate selection during exercise. This also suggests that the role muscle glycogen in endurance is auxiliary to fatty acid uptake and oxidation.

Molecular adaptations related to lipid handling and mitochondrial biogenesis induced by training in the muscle involves the PPARs (α and β/δ) and ERRs (α and β/γ) in transcriptional activation cascade through the co-activator PGC1α (Fan and Evans, 2015; Muoio et al., 2002; Rangwala et al., 2010; Schreiber et al., 2004; Tanaka et al., 2003). Decreased fatty acid availability in CD36 KO muscle during a single bout of exercise and through recovery likely impaired effective upregulation of *Pgc1a* via decreased fatty acid activation of PPARβ/δ (Hondares et al., 2007; Mottillo et al., 2012) despite having increased basal *Ppard* (Drover and Abumrad, 2005) and not because of impaired AMPK activation as it is not different between WT and CD36 KO after exercise (McFarlan et al., 2012). In chronic exercise however, adaptations involving calcium handling with increased motor activity (Olson and Williams, 2000) could lead to the autoregulatory feed-forward loop of PGC1α participated by calcineurinA, calcium/cadmodulin dependent protein kinase IV (CaMKIV) and myocyte enhancer factor 2 (MEF2) (Handschin et al., 2003) and potentially other transcription factors (Jäger et al., 2007; Kleiner et al., 2009; Schreiber et al., 2004) which may explain the difference in expression after a single bout and chronic exercise. On the other hand, *Ppara*

but not *Ppard* was increased in the WT with training. ERRα, an early PGC1α response gene that is upregulated with exercise, controls the transcription of PPARα (Cartoni et al., 2005; Huss et al., 2004; Mootha et al., 2004). Increased fatty acid uptake with elevated CD36 protein expression may upregulate *Ppara* in trained WT muscle as PPARα positively regulates its own transcription (Pineda Torra et al., 2002). In CD36 KO muscle, it appears that elevated basal *Ppara* and *Ppard* are likely controlled by other mechanisms (Drover and Abumrad, 2005).

As PGC1α is increased, PPARs, ERRs, and other transcription factors are activated and downstream targets are induced. ERRs particularly ERRβ/γ are constitutively active and binding of PGC1α potentiates its activity (Greschik et al., 2002; Willy et al., 2004). Indeed, ERR-dependent mitochondrial biogenesis-related gene expression (*Pgc1a*, *Cycs* and *Oxct1*) and enzyme function were not dependent on CD36 as previously reported (McFarlan et al., 2012; Rangwala et al., 2010; Svensson et al., 2016). However, unlike the ERRs, PPARs require fatty acids or other lipid ligands for transcriptional activity (Armstrong et al., 2014; Kliewer et al., 1997). Coinciding with increased CD36 protein and *Ppara* mRNA expression, PPAR targets such as fat utilization genes (*Cd36*, *Fatp1*, *Lpl*) were significantly increased (Ehrenborg and Krook, 2009; Kleiner et al., 2009) with training in WT but not in CD36 KO muscle. In brown adipocytes and cardiac muscle, cell types that show genetic similarity with skeletal muscle, fatty acid availability through increased lipolysis is required for maximal induction of PPARα and PPARβ/δ controlled oxidative genes (Haemmerle et al., 2011; Mottillo et al., 2012). Therefore, it is likely that the absence of CD36, that is to say impaired fatty acid availability, prevented significant increases in gene transcription of fat utilization genes through the PPAR pathway.

Intriguingly, apparent but non-significant increase in transcription of some PPAR target genes (e.g. *Lpl*, *Fatp1*, *Myh2*, *Vegfa*, and *Mct1*) (Ehrenborg and Krook, 2009; Gan et al., 2013; Kleiner et al., 2009; König et al., 2008) was also observed in trained CD36 KO muscle. Assuming corresponding increase in protein expression, this suggests that elevated basal *Ppara*, *Fatp1*, and training-induced *Pgc1a* in CD36 KO muscle (possibly other fatty acid transporters as well) could induce the same PPAR targets but not with similar efficiency as

afforded in the presence of CD36 (Hostetler et al., 2009; Nickerson et al., 2009). Likewise, transcriptional overlap or cooperativity of PPARs and ERRs could not be discounted (Huss et al., 2004; Rangwala et al., 2010; Wang et al., 2004). While contribution of some fatty acid transporters to PPAR signaling have been demonstrated in previous studies [e.g. FABP5 to PPARδ (Armstrong et al., 2014), FABP1 to PPARα (Hostetler et al., 2009) and FABP4 to PPARγ (Tan et al., 2002)], their independent and cooperative function require further investigation.

In summary, we report that CD36 is necessary for basal endurance and its improvement or amelioration with training independent of elevation in mitochondrial function, and muscle glycogen accumulation. In addition, we show that training effects on substrate switch at rest and during exercise is impaired in the absence of CD36. These changes, at least in WT mice, possibly involve CD36 in efficient upregulation of exercise-responsive genes controlled by PPARs.

Humans deficient in CD36 have decreased aerobic exercise capacity because of limited fatty acid uptake in muscle and heart (Hames et al., 2014; Yanai et al., 2007). Because training could not improve endurance performance in the absence of CD36, frequent glucose supplementation may benefit human athletes with CD36 deficiency during prolonged exercise bouts.

Figures

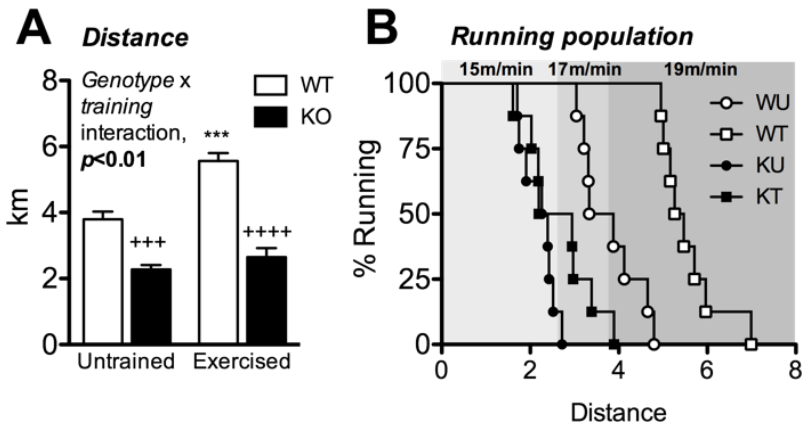

Figure 1. CD36 is necessary for basal endurance and its training-induced improvement. (A) Distance covered until attaining exhaustion under a forced running exercise-to-exhaustion protocol and (B) percentage of mice running at a specific intensity and distance. Data in (A) are expressed as mean ± SEM (n=7-8). Interaction effect was assessed by 2-way ANOVA. Between group differences were assessed by Student's unpaired *t*-test where triple and quadruple symbols represent *p* values less than 0.001 and 0.0001, respectively. Asterisk (*) represents significant difference between exercise status of the same genotype while plus (+) represents significant difference between genotypes of the same exercise status. Each data point in (B) represents an individual mouse plotted against the distance covered by the said mouse at exhaustion.

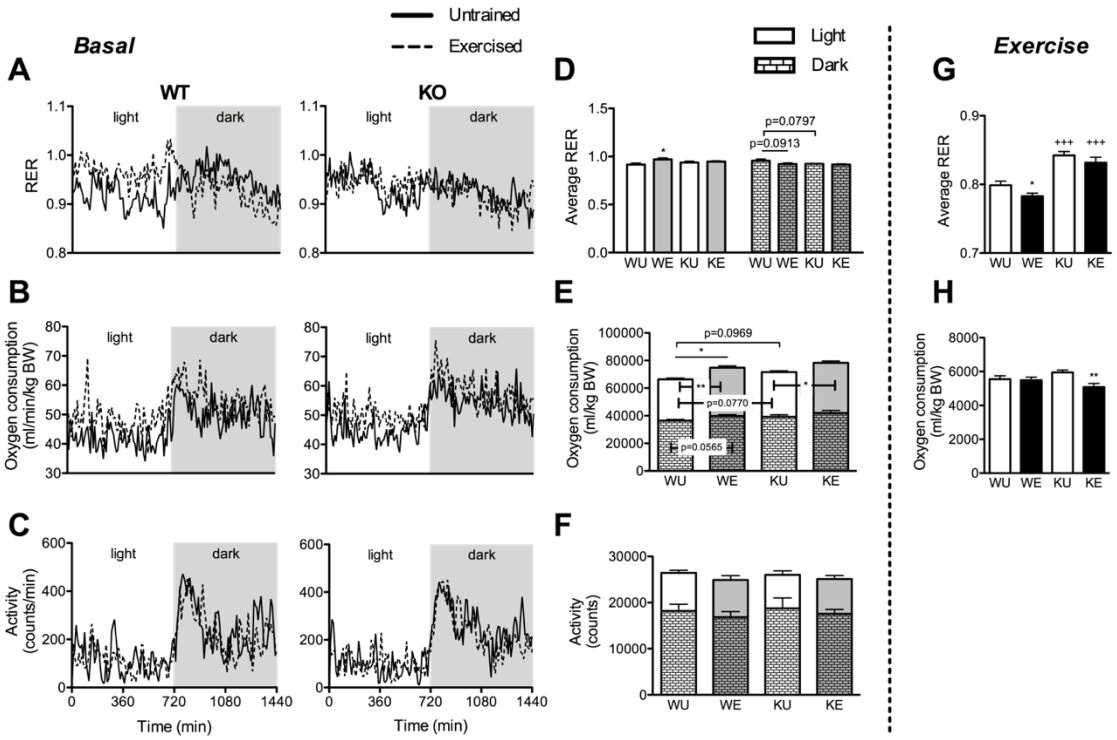

Figure 2. Changes in whole-body metabolism at rest (basal) and during exercise. (A-C) Basal time-course changes in RER, oxygen consumption, and activity of WT (left) and CD36 KO (right) mice for 24 h. (D-F) Corresponding average RER, and cumulative oxygen consumption and activity counts in total and relative contributions during light and dark phases. (G-H) Average RER and cumulative oxygen consumption of mice during a 60 min run. Each point in the time course data are expressed as means while average and cumulative data are expressed as mean ± SEM (n=7-9). No significant interaction effect was observed as assessed by 2-way ANOVA. Between group differences were assessed by Student's unpaired *t*-test where single, double and triple symbols represent *p* values less than 0.05, 0.01 and 0.001, respectively. Asterisk (*) represents significant difference between exercise status of the same genotype while plus (+) represents significant difference between genotypes of the same exercise status. For basal whole-body metabolism, annotations within the bars represent differences within the photophase while above the bars represent 24 h differences.

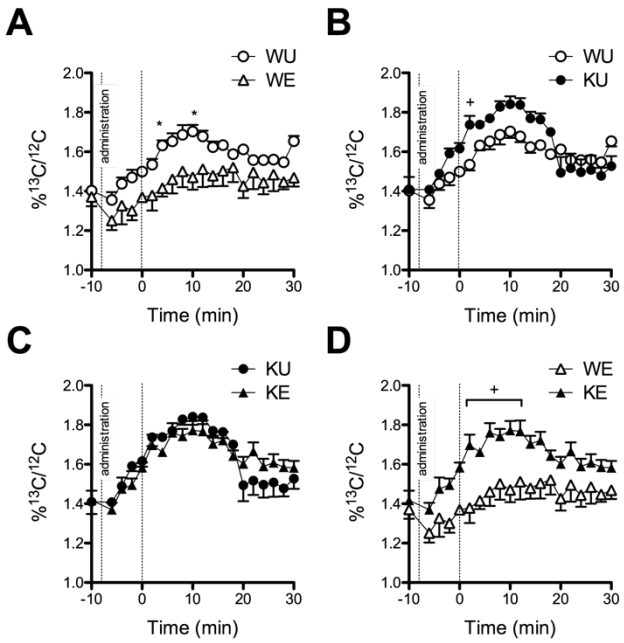

Figure 3. Training-induced changes in whole-body exogenous glucose oxidation is absent in CD36 KO mice. (A-D) Exogenous glucose oxidation using ^{13}C-labelled glucose in untrained and exercised mice during a 30 min run. Data are expressed as mean ± SEM (n=5-7). Differences (p<0.05) at certain time points were observed as assessed by 2-way ANOVA followed by Bonferroni's post-hoc test.

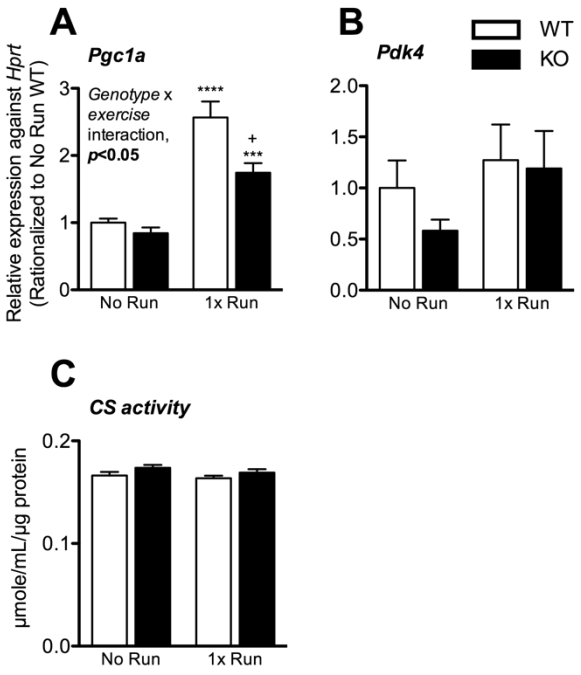

Figure 4. CD36 is required for efficient transcriptional response of *Pgc1a* during recovery after a single bout of exercise. (A) *Pgc1a* and (B) *Pdk4* mRNA expression, and (C) CS activity in gastrocnemius of WT and CD36 KO mice 8 h after a single bout of treadmill running. Data are expressed as mean ± SEM (n = 7-10). Interaction effect was assessed by 2-way ANOVA. Between group differences were assessed by Student's unpaired *t*-test where single, triple and quadruple symbols represent *p* values less than 0.05, 0.001 and 0.0001, respectively. Asterisk (*) represents significant difference between exercise status of the same genotype while plus (+) represents significant difference between genotypes of the same exercise status.

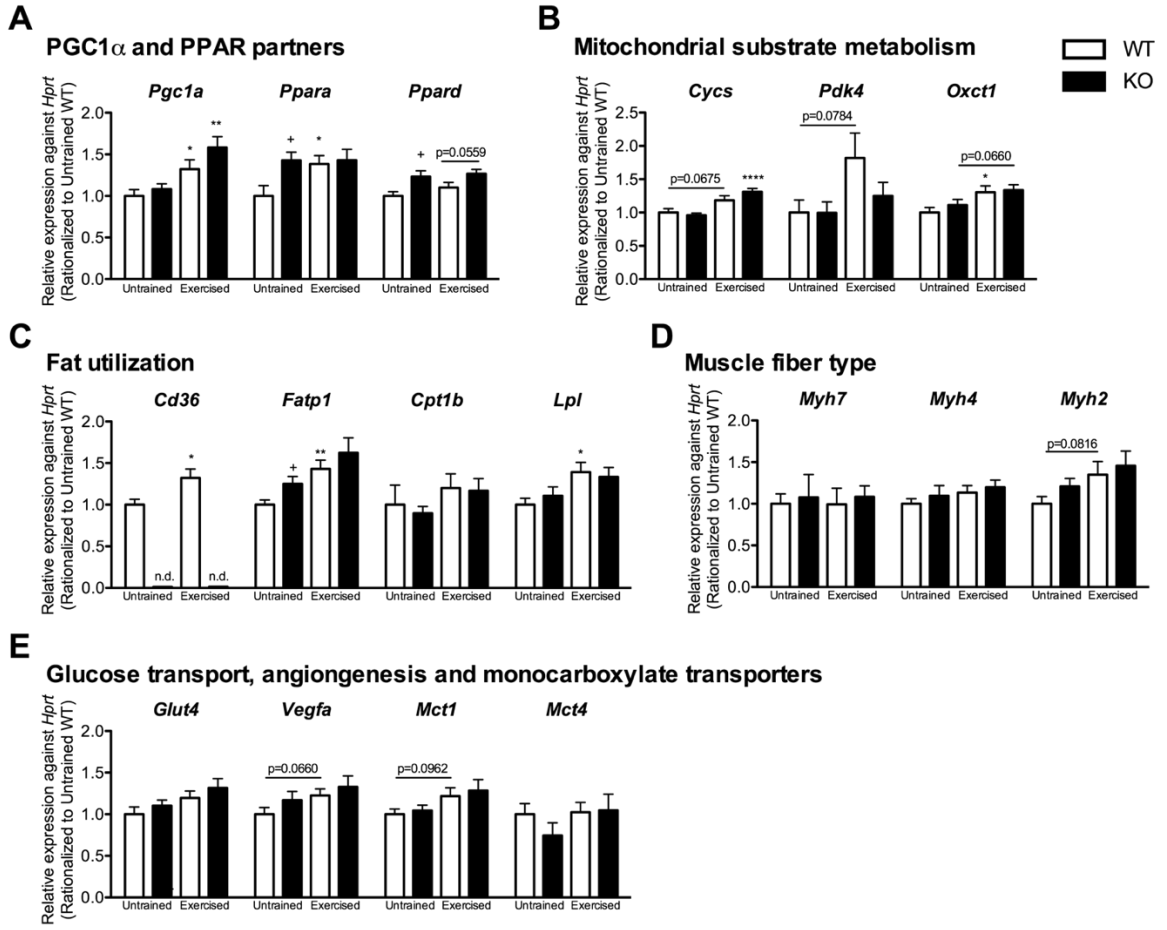

Figure 5. Changes in messenger RNA expression after training in WT and CD36 KO mice. Messenger RNA expression levels of (A) PGC1a and PPARs, and genes related to (B) mitochondrial substrate metabolism, (C) fat utilization, (D) muscle fiber type and (E) glucose transport, angiogenesis and monocarboxylate transporters in the gastrocnemius of WT and CD36 KO mice after 30 d of treadmill training. Data are expressed as mean ± SEM (n=7-9). No significant interaction effect was observed as assessed by 2-way ANOVA. Between group differences were assessed by Student's unpaired *t*-test where single, and double symbols represent *p* values less than 0.05, and 0.01, respectively. Asterisk (*) represents significant difference between exercise status of the same genotype while plus (+) represents significant difference between genotypes of the same exercise status.

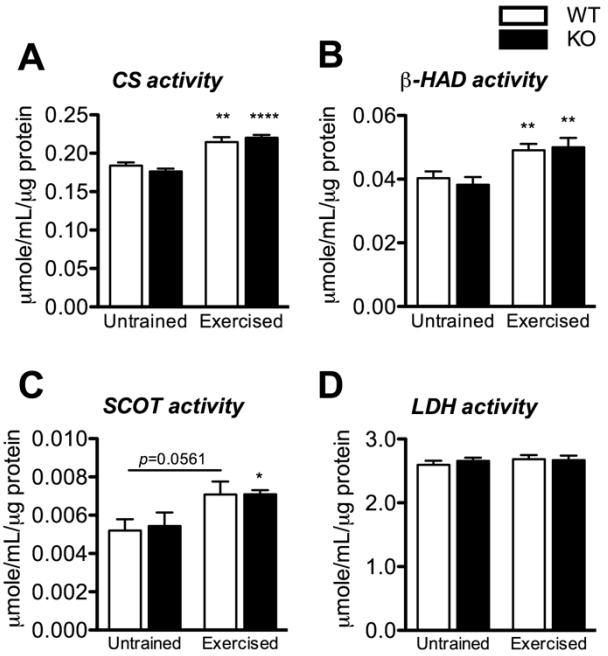

Figure 6. Training-induced improvements in muscle mitochondrial enzyme activities involved in substrate oxidation are not dependent on CD36. (A) CS, (B) β-HAD, (C) SCOT and (D) LDH activity after 30 d of treadmill exercise in the muscle of WT and CD36 KO mice. Data are expressed as mean ± SEM (n=7-9). No interaction effect was observed as assessed by 2-way ANOVA. Differences between training status of the same genotype were assessed by Student's unpaired *t*-test where single, double and quadruple asterisks (*) represent *p* values less than 0.05, 0.01 and 0.0001, respectively.

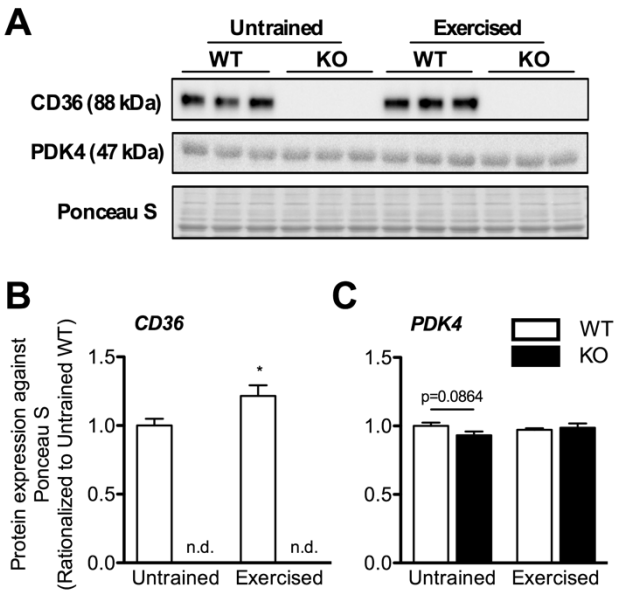

Figure 7. Protein expression of CD36 and PDK4 in WT and CD36 KO mice after training. (A) Representative immunoblot images of CD36, PDK4 and digitized image of Ponceau S stained membrane. Quantification of signals corresponding to (B) CD36 and (C) PDK4 bands rationalized to Ponceau S signal. Values are presented as mean ± SEM (n=7-9) of data from three separate membranes. Membranes were processed in parallel and all groups were represented in each membrane. No significant interaction effects were observed as assessed by 2-way ANOVA. Significant difference (*, $p<0.05$) was observed in CD36 expression between exercise status of WT mice as assessed by Student's unpaired *t*-test. Signal corresponding to CD36 was not observed in CD36 KO mice.

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Chapter 3

Low-fat diet, and medium-fat diets containing coconut oil and soybean oil exert different metabolic effects in untrained and treadmill-trained mice

Introduction

Oils from different plant sources differ in fatty acid composition. Coconut oil is rich in lauric acid (C12:0), comprising about 45-53% of the total fatty acid composition, while having very low content of fatty acids above C14 (Amri, 2011; Dayrit, 2015; Wang et al., 2015). Soybean oil, on the other hand, is rich in polyunsaturated and monounsaturated fatty acids particularly linolenic acid (C18:3), linoleic acid (C18:2) and oleic acid (C18:1) occupying 7.8%, 53.2% and 23.4%, respectively of its total fatty acid composition while almost absent of C14 fatty acids and below (Wang, 2011; Wang et al., 2015). These differences not only lead to diverging physico-chemical properties but also metabolic fates. Medium chain fatty acids (MCFAs; C8-C14) can enter cells without requiring fatty acid transporters unlike long chain fatty acids (LCFAs; C16-C22). However, both types require carnitine activation in the muscle mitochondria (Groot and Hülsmann, 1973). Moreover, most MCFAs are absorbed and metabolized in the liver for conversion to ketone bodies or incorporation to liver triglycerides (TG) especially with prolonged feeding (Crozier, 1988; Pégorier et al., 1988). These imply that MCFAs function as direct or precursor energy substrates for non-hepatic organs.

Training leads to several adaptations in whole body and organs. In the muscle, prolonged training increases glycogen storage, fatty acid uptake and utilization, and mitochondrial biogenesis among others (Egan and Zierath, 2013). Trained muscle is then able to produce energy to sustain endurance. These adaptations are brought about by contraction- and high AMP/ATP ratio-induced AMP-activated protein kinase (AMPK) activation accompanying

prolonged training (Egan and Zierath, 2013). The peroxisome proliferator-activated receptors (PPARs) family and the estrogen-related receptors (ERRs) family are involved in these transcriptional adaptations with the coactivator PPARγ-coactivator 1α (PGC1A) synchronizing signals from AMPK and therefore of exercise (Fan and Evans, 2015).

Fats with varying fatty acid composition differentially affect PPAR isotypes. PPARα and PPARβ/δ are highly expressed in peripheral tissues such as the muscle and control genes for oxidative metabolism while PPARγ is present mainly in adipose tissues orchestrating adipogenesis (Georgiadi and Kersten, 2012). LCFAs activate PPARs and the intensity of activation being influenced by the type of fatty acid and the PPAR (Kliewer et al., 1997; Xu et al., 1999). MCFAs, particularly C10 and C12 strongly activate PPARγ while C10 and C14 activate PPARα and PPARβ/δ to a certain extent (Kliewer et al., 1997; Liberato et al., 2012; Malapaka et al., 2012; Xu et al., 1999). Also, MCFAs upregulate mitochondrial biogenesis better than LCFAs (Montgomery et al., 2013). Therefore, MCFAs and LCFAs function as direct or indirect signaling molecules for mitochondrial oxidative capacity possibly through PPARα and/or PPARβ/δ (Fan and Evans, 2015; Lim et al., 2013; Montgomery et al., 2013; Turner et al., 2009a). Unlike PPARs, no endogenous ligands for ERRs have been identified. However, ERRs are constitutively active and the binding of PGC1A potentiates its activity (Greschik et al., 2002; Willy et al., 2004). Therefore, downstream effects of training on transcriptional adaptations are also influenced not only by the expression of these transcription factors but also by available fatty acids for activation.

The effects of short- and long-term feeding of MCFAs and LCFAs with or without training have been investigated. In our laboratory, MCFAs (coconut oil) improved swimming capacity in trained mice and increased mitochondrial enzyme activities relative to LCFAs (soybean oil) (Fushiki et al., 1995). With training, MCFAs (C8 and C10) increased energy expenditure in rats relative to LCFAs (rapeseed oil) (Ooyama et al., 2008). In untrained mice, increased mitochondrial markers with MCFAs (coconut oil) relative to LCFAs (lard) was associated with whole body and localized muscle oxygen consumption as assessed with in vitro, ex vivo and in vivo experiments (Montgomery et al., 2013). In rats, while MCFAs (C8 to C12) did not affect endurance performance, LCFAs (primarily C16) decreased endurance associated with

increased cardiac mitochondrial uncoupling (Murray et al., 2011). In contrast, LCFAs (soybean oil) with training did not impair but rather improved endurance on a treadmill in wild-type C57BL/6J mice (Manio et al., 2017). Literature is scarce on the comparative effects of MCFAs and LCFAs on training adaptations. Furthermore, absence of comparison with high-carbohydrate, low-fat diet may limit the adaptability of these diets in athletic dietary management.

The objective of the study was to update current knowledge of physiological adaptations occurring during exercise training with low-fat diet, and medium-fat diets containing coconut oil or soybean oil specifically on whole-body metabolism at rest and during exercise, substrate metabolism, mitochondrial functions, and genetic adaptive responses in the muscle and liver under the treadmill exercise modality.

Materials and methods

Animals

Seven (7)-week old male C57BL/6N mice were purchased from Shimizu Laboratory Supplies (Hamamatsu, Shizuoka, Japan). Mice were housed in an animal room at 22±0.5°C and 50% humidity with a 12h light-dark cycle (lights on and off at 6:00 and 18:00, respectively). Mice were acclimatized to this environment with ad libitum access to a standard chow diet (Oriental Yeast Co., Tokyo, Japan) and water for 7 to 10d before changing to assigned diets. Mice were randomly assigned to the following purified diets: low-fat diet (L; 20% kcal from casein, 70% kcal from cornstarch and 10% kcal from soybean oil); coconut oil diet (C; 20% kcal from casein, 50% kcal from cornstarch, 10% kcal from soybean oil and 20% kcal from coconut oil); and soybean oil diet (S; 20% kcal from casein, 50% kcal from cornstarch and 30% kcal from soybean oil). All diets were prepared by Research Diets (NJ, USA) adapted from the D12450K formulation. Training of mice was commenced following diet assignment. Dietary groups were divided into untrained (U) and trained (T) groups. Animal experiments were conducted according to the Kyoto University Guidelines for the Ethical Treatment of Laboratory Animals as approved by the Kyoto University Animal Experimentation Committee with the number (29-39).

Treadmill training

Training was conducted daily for 30d from 6:00 using a treadmill for rodents (MK-680; Muromachi, Tokyo, Japan). On the first 15d, mice ran for 1h at 15m/min at 3° incline. From the 16th day, intensity was increased to 18m/min. Mice were forced to run by poking. All mice completed the training program.

Basal indirect calorimetry

Basal indirect calorimetry using the ARCO-2000 system (Tokyo, Japan) under ad libitum feeding and resting conditions as described in Manio et al. (2016) was performed on a

subset of mice. Mice were assigned to calorimetry chambers on the 28th day of training to facilitate acclimatization. Actual measurement was performed 24h to 48h post-training which represents a full light-dark cycle devoid of acute exercise effects. Mice were sacrificed after measurements. Sample collection was based on Manio et al. (2017).

Exercise indirect calorimetry and endurance test

Indirect calorimetry during exercise with treadmill endurance test was performed on a subset of mice. About 1.5-2h before the run (6:00), mice were placed in sealed treadmill chambers (Mousebelt-200; Arco System, Tokyo, Japan) at 10° incline to acclimatize. After warming-up for 2min, intensity was increased to 15m/min. After 30min, the intensity was increased to 18m/min and maintained for 30min. Then, the intensity was increased to 21m/min and kept herein until exhaustion. Mice were stimulated with electrical stimulus (0.2mA) and occasional noise and poking. Exhaustion was ruled if mice remained on electrodes or could not sustain running for 20sec despite additional stimulation.

Fixed time run

Fixed time run was performed on a subset of mice. Food was removed 2h before running. Mice were individually placed on a moving treadmill set at an intensity of 10 m/min. After 2 min of warm-up, mice were transferred to a moving treadmill set at 15 m/min, 10° incline. After exactly 30 min, mice were removed from the treadmill and immediately sacrificed. Experiments were performed in the same conditions as exercise indirect calorimetry.

Blood chemistry and tissue metabolites

Serum was measured for glucose, triglycerides (TG), non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (β-HB). Glycogen and TG were measured in organs (Folch et al., 1957; Good et al., 1933; Sahyun and Alsberg, 1931). Measurements are detailed in Manio et al. (2017).

Protein extraction and enzyme activities

Muscle and liver were lysed in a 1% NP-40 buffer as detailed in Manio et al. (2017). Beta-hydroxyacyl-CoA dehydrogenase (β-HAD) activity was measured according to a procedure by Holloway et al. (2007). Succinyl-CoA:3-oxoacid CoA-transferase (SCOT) activity was measured based on Williamson et al. (1971). Citrate synthase (CS) activity was measured according to Srere (1969). Cytochrome c oxidase of the mitochondrial electron transport chain also known and hereby referred to as Complex IV was measured based on MacArthur et al. (2008) and Spinazzi et al. (2012). Acetoacetyl-CoA thiolase (AACT) and deacylase (AACD) activities were measured in liver lysates based on Williamson et al. (1968). All enzyme activity measurements were modified to adapt to a 96-well plate system as detailed in Manio et al. (2017).

Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted with Tripure Isolation Reagent (Roche, Mannheim, Germany) and GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, MO, USA) as detailed in Manio et al. (2017). Total RNA (1.8µg and 1.5µg for muscle and liver, respectively) was reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) and mRNA expression levels were quantified using intron-spanning primers and corresponding Universal Library Probes (Roche, Mannheim, Germany) listed in **Table 1**. Values were rationalized to *Hprt* expression (Cappelli et al., 2008).

Table 1. Primers and probes in RT-qPCR

Gene name	Sequence (5' to 3')	Universal Probe No.	Accession No.
PPARγ Coactivator 1α (Pgc1a)	F: tgtggaactctctggaactgc R: agggttatcttggttggcttta	63	NM_008904.2
PPARα (Ppara)	F: ccgagggctctgtcatca R: gggcagctgactgaggaa	11	NM_011144.6

PPARβ/δ (Pparb/d)	F: atgggggaccagaacacac R: ggaggaattctgggagaggt	11	NM_011145.3
ERRα (Erra)	F: gtgggcatgctcaaggag R: ggaaaggcaaagggtcca	29	NM_007953.2
ERRβ (Errb)	F: ggcgttcttcaagagaacca R: tccgtttggtgatctcacatt	49	NM_011934.4
ERRγ (Errg)	F: aagtgggcatgctgaaagaa R: cagcatctattctgcgcttg	29	NM_011935.3
Lipoprotein lipase (Lpl)	F: tggataagcgactcctacttcag R: tccctagcacagaagatgacc	22	NM_008509.2
Carnitine palmitoyltransferase 1B (Cpt1b)	F: ccatcattgggcacctct R: gtctccgtgtagcccaggt	104	NM_009948.2
Glucose transporter 4 (Glut4)	F: tcgtcattggcattctggt R: agcagtggccacagggta	104	NM_009204.2
Fatty acid transport protein 1 (Fatp1)	F: cttcctaaggctgccattgt R: ggcagtcatagagcacatcg	49	NM_011977.3
Myosin heavy chain 2a (Myh2)	F: tcttctctggggcacaaact R: cccttcttcttggcaccttt	22	NM_001039545.2
Carnitine palmitoyltransferase 1A (Cpt1a)	F: aaagcaccagcacctgtacc R: aacctccatggctcagacag	34	NM_013495.2
3-Hydroxy-3-methylglutaryl-CoA synthase 2 (Hmgcs2)	F: ctgtggcaatgctgatcg R: tccatgtgagttcccctca	93	NM_008256.4
Hypoxanthine-guanine phosphoribosyl transferase (Hprt)	F: cctcctcagaccgcttttt R: aacctggttcatcatcgctaa	95	NM_013556.2

F: forward

R: reverse

Immunoblotting

Protein concentration of lysates was adjusted with lysis buffer and 4x Laemilli buffer containing 20% mercaptoethanol. Samples were loaded on 8% polyacrylamide gels. Semi-

dry transfer to PVDF membranes was performed in a transfer buffer containing 20% methanol. After transfer, Ponceau S staining was performed. Excess stain was removed in distilled water. Membranes were visualized and digitized (LAS-3000; Fujifilm, Tokyo, Japan). Membranes were blocked in 5% skim milk powder in Tris-buffered saline with 0.1% Tween-20 buffer (TBST) containing 0.05% ProClin 300 (Sigma Aldrich, MO, USA). Membranes were cut at sections corresponding to regions of molecular weight previously identified to contain the protein of interest. Membranes were incubated in goat anti-fatty acid translocase/cluster of differentiation 36 (CD36) antibody (1:2000; AF2519, R&D Systems, MN, USA), goat anti-pyruvate dehydrogenase kinase 4 (PDK4) antibody (1:1000; C-16, Santa Cruz Biotechnology, CA, USA), rabbit anti-PGC1A antibody (1:1000; H-300, Santa Cruz Biotechnology, CA, USA) or rabbit anti-glucose transporter 4 (GLUT4) antibody (1:2000, AB1346, Chemicon International, CA, USA) at 4°C for 20h. Membranes were washed 3x in TBST to remove excess primary antibody. Horseradish peroxidase-labelled secondary antibody incubation was performed in anti-goat IgG (1:1000; P0449; Dako, Tokyo, Japan) or anti-rabbit IgG (1:1000; P0399; Dako, Tokyo, Japan) for 3h at 4°C. After washing 3x in TBST to remove excess secondary antibody, membranes were visualized by chemiluminescent detection (Western Lightning Plus ECL; Perkin Elmer, MA, USA). Signals corresponding to PGC1A (91kDa), CD36 (88kDa), GLUT4 (58kDa), PDK4 (47kDa) and Ponceau S signals were quantified using the software MultiGauge V3.2 (Fujifilm, Tokyo, Japan) with automatic background detection.

Statistical analyses

Statistical analyses were performed using the Prism 5.0 software (Graphpad Software, CA, USA). Points in exercise indirect calorimetry time-course data are presented as means ± SEM. Each time point was analyzed by one-way analysis of variance (ANOVA) to compare groups of different diets and by Student's unpaired t-test to compare groups of different training states receiving the same diet. Other data are presented as means ± SEM and were analyzed accordingly using one-way ANOVA followed by Newman-Keuls post-hoc test and Student's t-test. Significance level (α) was set at 0.05.

Results

Table 2. Body and organ weights, and food intake

Training		Untrained			Trained		
Diets		Low-fat	Coconut oil	Soybean oil	Low-fat	Coconut oil	Soybean oil
Food intake, g (n = 5-9)		91.26 ± 0.8612[a]	84.66 ± 0.9539[b]	84.0 ± 1.724[b]	96.81 ± 2.659[a]*	86.82 ± 1.732[b]	82.97 ± 1.342[b]
Calorie intake, kcal (n = 5-9)		351.4 ± 3.316	364.1 ± 4.102	361.2 ± 7.412	371.7 ± 10.24*	373.3 ± 7.447	356.8 ± 5.771
Body weight (n = 24-25)	D1, g	22.93 ± 0.2239	23.02 ± 0.2694	22.94 ± 0.2482	23.01 ± 0.2307	23.02 ± 0.1949	22.84 ± 0.27
	D32, g	25.92 ± 0.3709	26.86 ± 0.4547	26.12 ± 0.3233	26.99 ± 0.3762	27.42 ± 0.3689	26.47 ± 0.3991
	%Δ	13.04 ± 1.177	16.84 ± 1.877	13.97 ± 1.35	17.56 ± 2.08	19.35 ± 2.034	15.64 ± 2.172
Gastrocnemii, g (n = 8)		0.247 ± 0.0052	0.243 ± 0.0060	0.244 ± 0.0056	0.254 ± 0.0082	0.2515 ± .0076	0.2446 ± 0.0065
Liver, g (n = 8)		1.289 ± 0.0692	1.22 ± 0.0702	1.104 ± 0.0274	1.491 ± 0.0596*	1.306 ± 0.0469	1.322 ± 0.0613*
Epididymal fat, g (n = 8)		0.6666 ± 0.0558	0.7541 ± 0.0578	0.5898 ± 0.04942	0.5869 ± 0.0320	0.6601 ± 0.03855	0.6855 ± 0.03806

D1: day 1

D32: day 32

%Δ: %change at day 32 from day 1

a,b: dissimilar alphabets indicate significant difference ($p < 0.05$) among groups of the same training status as assessed by one-way ANOVA followed by Newman-Keuls post-hoc test

*: $p < 0.05$ vs untrained counterpart as assessed by Student's t-test

Training increased calorie intake in low-fat diet but did not affect body weight

Food intake was measured every third day. Total food and calorie intake for 30 days were calculated (**Table 2**). Body weight and weight of some organs were measured. Total food intake was lower in C and S relative to L in either training state ($p < 0.05$). Training did not increase food intake in C and S but significant increase was observed in L ($p < 0.05$). Total

calorie intake showed no difference among diet groups in either training state. Training increased calorie intake in L ($p < 0.05$). Despite this, diets nor training did not cause significant body weight change at the end of the experimental period relative to day 1. While no significant differences were observed in muscle and fat weight among diet groups of either training state, training significantly increased liver weight in L and S ($p < 0.05$) but not in C. Therefore, the type of diet combined with training affected calorie intake and liver weight but not body weight.

Coconut and soybean oil decreased basal respiratory exchange ratio (RER) without affecting oxygen consumption (VO_2) in untrained and trained states

Indirect calorimetry at rest 24h after the last training session was performed to determine the effects of diet and training on whole-body metabolism. Basal RER was significantly higher in L relative to C and S in either training state ($p < 0.05$) **(Fig. 1A)**. Furthermore, training increased RER in all diet groups ($p < 0.05$). Basal VO_2 and energy expenditure (not shown) were not significantly affected by diet nor training **(Fig. 1B)**. L in either training state had higher carbohydrate oxidation (CHO) than C and S ($p < 0.05$) **(Fig. 1C)**. Furthermore, training increased CHO in all diet groups ($p < 0.05$). Fat oxidation (FAT) in L was significantly lower than C and S in either training state ($p < 0.05$) **(Fig. 1D)**. Training decreased FAT in all diet groups but significant decrease ($p < 0.05$) was observed only in L and S but not in C ($p = 0.0726$). Therefore, at rest in ad libitum fed state, training shifted substrate utilization favoring CHO beyond the effects of diet. Also, coconut oil and soybean oil slightly differed in FAT with training.

Training but not diet strongly influenced whole-body metabolism during exercise

Energy metabolism was measured during the first hour of endurance test. Diet groups had similar RER in either training state **(Fig. 2AB)**. Training decreased RER in several time points during the run especially at the onset of running ($p < 0.05$). Diet groups had similar VO_2 in either training state **(Fig. 2CD)**. Training lowered VO_2 at several points during the run ($p < 0.05$). However, cumulative data showed that S with training was not significantly different to its untrained counterpart ($p = 0.0845$). Diet groups had similar CHO in either training

state **(Fig. 2EF)**. However, trained groups had consistently lower CHO during the run ($p < 0.05$). Drastic elevation in CHO at the onset of running in untrained groups was absent with training. Cumulative CHO was lower with training but significant only in L ($p < 0.05$, $p = 0.0746$ and $p = 0.0571$ in L, C and S, respectively). Diet groups had similar FAT in either training state **(Fig. 2GH)**. FAT was higher in the trained groups particularly in the first few time points of the run ($p < 0.05$). A reversal was observed as the run progressed with untrained groups having higher FAT than trained groups except in S. In all parameters, no significant differences at any time point were observed among diet groups in either training state. Therefore, diet composition did not affect whole-body metabolism among groups of the same training status during exercise. On the other hand, training strongly affected exercise metabolism causing a slight shift to FAT if relative contribution to energy expenditure was considered as reflected in the mild but observable lowering of RER.

Training overwhelmed the treadmill endurance improvement effect of coconut oil in the untrained state

All mice were able to run past 60min and exceed the 18m/min intensity **(Fig. 3A)**. Diet groups had similar time-to-exhaustion and distance in either training state **(Fig. 3BC)**. Training led to nearly twice the treadmill endurance of untrained counterparts ($p < 0.05$) regardless of diet. Calculating for work revealed that C had higher treadmill endurance than L and S in the untrained but not in the trained state ($p < 0.05$) **(Fig. 3D)**. Therefore, coconut oil slightly but significantly improved endurance in the untrained state but did not potentiate the endurance improvement observed with training.

Table 3. Pre- and post-exercise metabolites

Training		Untrained (n = 8/group)			Trained (n = 8/group)		
Diets		Low-fat	Coconut oil	Soybean oil	Low-fat	Coconut oil	Soybean oil
Serum glucose mg/dL	Pre-	152.9 ± 12.7	156.1 ± 8.32	156.0 ± 5.06	131.1 ± 5.80	142.7 ± 6.58	146.1 ± 5.51
	Post-	203.1 ± 6.02†	219.6 ± 9.96†	219.6 ± 8.25†	177.9 ± 8.07^{b}†	194.5 ± 5.01^{ab}†	202.6 ± 3.38^{a}†
	%Δ	32.83	40.68	40.77	35.70	36.30	38.67
	Pre-	1.548 ± 00.199	1.518 ± 0.193	1.606 ± 0.199	2.154 ± 0.175*	1.703 ± 0.208	2.294 ± 0.173*

Muscle glycogen mg/g	Post-	0.932 ± 0.156†	0.942 ± 0.125†	1.083 ± 0.173[1]	0.764 ± 0.080[b]†	1.335 ± 0.147[a]	1.182 ± 0.229[ab]†
	%Δ	-39.80	-37.98	-32.57	-64.54	-21.61	-48.47
Liver glycogen mg/g	Pre-	71.06 ± 3.87	59.93 ± 4.78	64.28 ± 9.09	63.13 ± 2.67[a]	46.42 ± 5.09[b2]	54.73 ± 5.15[ab]
	Post-	79.87 ± 5.74[a]	56.25 ± 4.41[b]	73.61 ± 3.08[a]	70.57 ± 5.78	56.43 ± 5.73	56.76 ± 2.56
	%Δ	12.40	-6.14	14.51	11.79	21.56	3.71
Serum TG mg/dL	Pre-	118.0 ± 10.17	116.3 ± 10.73	107.3 ± 8.48	109.4 ± 10.36	115.4 ± 6.53	115.4 ± 16.08
	Post-	101.8 ± 9.01[a]	93.3 ± 5.35[ab]	76.3 ± 4.67[b]†	87.5.76	102.7 ± 14.66	71.0 ± 4.04†
	%Δ	-13.73	-19.77	-28.91	-19.71	-11.01	-38.44
Serum NEFA mEq/L	Pre-	0.8783 ± 0.0781	0.8174 ± 0.0741	0.6600 ± 0.0297	0.6516 ± 0.0716*	0.7938 ± 0.0613	0.6739 ± 0.0543
	Post-	0.7384 ± 0.0399[a]	0.6748 ± 0.096[a1]	0.5502 ± 0.0141[b]†	0.6460 ± 0.0367	0.6564 ± 0.0363[1]	0.5514 ± 0.0206[1]
	%Δ	-15.93	-17.45	-16.64	-0.86	-17.31	-18.18
Serum β-HB µmol/L	Pre-	24.22 ± 4.35	20.56 ± 2.59	24.06 ± 6.73	12.73 ± 2.10*	17.87 ± 2.93	15.75 ± 2.18
	Post-	170.10 ± 34.82†	257.50 ± 22.18†	178.60 ± 29.39†	96.90 ± 18.08[b]†	204.50 ± 24.61[a]†	135.60 ± 19.10[b]†
	%Δ	602.31	1152.43	642.31	661.19	1044.38	760.95
Muscle TG mg/g	Pre-	3.379 ± 1.002	3.133 ± 0.507	2.759 ± 0.602	2.320 ± 0.339	2.537 ± 0.677	2.894 ± 0.472
	Post-	1.527 ± 0.175	1.982 ± 0.317	2.111 ± 0.303	1.323 ± 0.324[1]	0.918 ± 0.190†	1.345 ± 0.236†
	%Δ	-54.81	-36.74	-23.49	-42.97	-63.81	-53.52

Pre-: pre-exercise (basal)

Post-: post-exercise (after 30min run)

%Δ: % difference between pre- and post- values

a,b: dissimilar alphabets indicate significant difference ($p < 0.05$) among groups of the same training status as assessed by one-way ANOVA followed by Newman-Keuls post-hoc test

*: $p < 0.05$ vs untrained counterpart as assessed by Student's t-test

†: $p < 0.05$ vs pre- value as assessed by Student's t-test

1: $p < 0.1$ vs pre- value as assessed by Student's t-test

2: $p < 0.1$ vs untrained counterpart as assessed by Student's t-test

Coconut oil negatively affected glycogen accumulation with training but promoted sparing of muscle glycogen during exercise

Pre- (basal) and post-exercise (30min run) metabolites were measured and presented in **Table 3**. Pre-exercise serum glucose was similar among diet groups in either training state. Training tended to lower serum glucose. At post-exercise, all groups had significantly increased serum glucose ($p < 0.05$). L tended to have lower serum glucose than C and S and this was highlighted in trained groups ($p < 0.05$). Pre-exercise muscle glycogen was similar among diet groups in either training state. However, training increased this in L and S but not in C ($p < 0.05$). At post-exercise in the untrained state, it decreased ($p < 0.05$) relative to pre-exercise values in L and C while a non-significant decrease ($p = 0.0672$) was observed in S. With training, muscle glycogen also significantly decreased in L and S ($p < 0.05$) but not in C. Pre-exercise liver glycogen was similar among diet groups in the untrained state. It was significantly lower in C than L in the trained state ($p < 0.05$). Also, a non-significant decrease ($p = 0.0735$) was observed in C with training relative to its untrained counterpart. However, a longer run might be required to observe utilization of glycogen in the liver. Therefore, coconut oil prevented glycogen accumulation in the muscle and liver with training but preserved it in the muscle during exercise.

Coconut oil but not soybean oil promoted ketosis during exercise

Pre-exercise serum TG and NEFA was similar among diet groups in either training state. However, training caused significant decrease in NEFA in L ($p < 0.05$). At post-exercise, TG significantly decreased in S in both training states relative to pre-exercise values ($p < 0.05$). Post-exercise NEFA also decreased significantly in S without training ($p < 0.05$) and non-significantly in other C and S groups regardless of training ($p < 0.1$). Pre-exercise serum β-HB was similar among diet groups in either training state. However, it generally decreased with training and was significant in L ($p < 0.05$). At post-exercise, drastic elevation was observed in C in both training states and was emphasized by training relative to L and S ($p < 0.05$). Similarly, training relatively lowered β-HB levels post-exercise in all diet groups. Pre-exercise muscle TG was not significantly affected by diets nor training. Significant decrease was not observed in any untrained groups at post-exercise. However with training, muscle TG non-

significantly decreased ($p = 0.0522$) in L and significantly decreased ($p < 0.05$) in C and S relative to pre-exercise values. Therefore, coconut oil caused ketosis during exercise but not at rest. On the other hand, training promoted intramuscular TG utilization during exercise.

Mitochondrial enzyme activities varied with diets but were generally amplified by exercise

The effects of diets and exercise on mitochondrial enzyme activities of the muscle were assessed. CS catalyzes the formation of citrate from oxaloacetate and acetyl-CoA and is commonly used as a measure of mitochondrial density in muscle (Larsen et al., 2012). In either training state, CS activity increased in C and S relative to L ($p < 0.05$) **(Fig. 4A)**. Training significantly increased its activity in all diet groups ($p < 0.05$). β-HAD activity reflects the capacity of cells to oxidize fatty acid (Holloway et al., 2007). In the untrained state, C had a non-significant increase in β-HAD relative to L and S. Its activity was similar among diet groups in the trained state **(Fig. 4B)**. Training significantly increased it in L and S ($p < 0.05$). Elevation was also observed in C but this was not statistically significant. SCOT is an enzyme involved in ketolysis in the muscle (Fukao et al., 2004). In the untrained state, SCOT activity increased in C and S relative to L ($p < 0.05$) **(Fig. 4C)**. No difference among diet groups was observed with training. Training generally increased its activity in all groups but significant only in L and C ($p < 0.05$). Complex IV catalyzes the last step of the electron transport chain in the mitochondria and also regulates oxidative phosphorylation (Li et al., 2006). Similar activity was observed among diet groups in either training state **(Fig. 4D)** and training increased its activity ($p < 0.05$). Overall, coconut oil and soybean oil increased some muscle mitochondrial enzyme activities in the untrained state. However, training increased and normalized them across the diet groups.

Coconut oil inhibited training-induced increases in mRNA expression of PPARβ/δ and some target genes in the muscle

Changes in mitochondrial enzyme activities and whole-body metabolism are influenced by transcriptional adaptations in the muscle. We measured known transcription factors affecting the said processes. Diet and training failed to significantly influence *Pgc1a* **(Fig. 5A)**. *Ppara* non-significantly increased in C and S relative to L **(Fig. 5B)**. While training failed

to increase its expression in C and S, 40% increase albeit not significant (p = 0.0702) was observed in L. *Pparb/d* expression was not affected in untrained diet groups **(Fig. 5C)**. Training increased its expression in L and S but not in C ($p < 0.05$). ERRs showed relative suppression of expression in C and S in the untrained state **(Fig. 5D-F)**. Significant decrease in expression was only observed in *Errg* ($p < 0.05$). Training increased expression of *Erra* and *Errb* in all groups but significant only in S ($p < 0.05$). On the other hand, *Errg* decreased with training in L while significant increase was not observed in C and S ($p < 0.05$).

We also determined the expression of some PPAR targets. In the untrained state, *Lpl* expression was significantly increased in C and S relative to L ($p < 0.05$) **(Fig. 5G)**. With training, the intergroup differences were abolished attributed to the non-significant 20% increase in L. In the untrained state, *Cpt1b* was significantly higher in C relative to L but not to S ($p < 0.05$) **(Fig. 5H)**. With training, *Cpt1b* did not increase in C in contrast to considerable and significant (>50%; $p < 0.05$) increases in L and S. *Glut4* was significantly decreased in C and S relative to L ($p < 0.05$) **(Fig. 5I)**. Training did not change its expression in L and C. In the S however, significant increase similar to the level of L was observed ($p < 0.05$). *Fatp1* expression was similar among diet groups in either training state. Training led to a non-significant increase (p = 0.0567) in S but not in L and C **(Fig. 5J)**. *Myh2* non-significantly decreased in S in the untrained state **(Fig. 5K)**. Training significantly increased its expression and normalized its expression with L and C ($p < 0.05$). Lastly, no significant differences in *Cd36* and *Pdk4* were observed because of diet nor training (not shown).

In summary, the amount of fat but not the type significantly affected the expression of PPARs and ERRs in the untrained state. With training however, the responses of these genes and their targets varied with PPARs being influenced by the type of fat while the ERRs with the exception of ERRγ generally being unaffected by diet. In particular, coconut oil inhibited increases in PPARβ/δ mRNA expression as well as some of its targets.

Coconut oil inhibited training-induced significant increases in GLUT4 protein

Protein expression of glucose and fatty acid metabolism-related genes in the muscle were measured by immunoblotting. GLUT4 expression was non-significantly decreased in S in the

untrained state **(Fig. 6AB)**. Training generally increased its expression in all diet groups but significant only in L and S ($p < 0.05$) but not in C ($p > 0.1$). PGC1A, CD36, and PDK4 expression were not significantly influenced by diet nor training **(Fig. 6A; quantification not shown)**. Therefore, training increased GLUT4 but coconut oil prevented the same degree of induction as in other diets.

Coconut oil and soybean oil differed in liver mitochondrial enzyme activation with training

The effects of diets and training on mitochondrial functions of the liver were assessed. AACT and AACD catalyze the reversible conversion of acetyl-CoA to acetoacetyl-CoA and the non-reversible conversion of acetoacetyl-CoA to acetoacetate (Williamson et al., 1968), respectively was significantly different among diet groups with training but not in the untrained state **(Fig. 7AB)**. AACT was significantly higher in S than L and C ($p < 0.05$). AACD, on the other hand, was significantly higher in S than C and was significantly higher in C than L ($p < 0.05$). Similar to AACT, training only significantly increased AACD activity in C and S but not in L ($p < 0.05$). Complex IV activity was not significantly affected by diet nor training but a trend towards increased activity could be observed in C and S in both training states **(Fig. 7C)**. β-HAD was similar among diet groups in either training state **(Fig. 7D)**. However, there was a robust but non-significant ($p = 0.0579$) increase in β-HAD activity with training in C. Therefore, coconut oil tended to improve β-oxidation with moderate ketogenic enzyme activation while soybean oil strongly activated ketogenic enzymes but not β-oxidation in the liver with training. These were not accompanied by increased mitochondrial density as universal changes in mitochondrial functions were not observed.

Coconut oil but not soybean oil promoted PPARα mRNA expression in the liver with training

PPARα influences hepatic ketogenesis and β-oxidation (Georgiadi and Kersten, 2012). *Ppara* in the liver was similar among diet groups in the untrained state **(Fig. 8A)**. Training significantly increased its expression in C relative to L and S, and to its untrained counterpart ($p < 0.05$). *Cpt1a* non-significantly increased with training in C **(Fig. 8B)** while *Hmgcs2* was

not significantly affected by diet nor training **(not shown)**. Therefore, in the untrained state, the type of fat did not affect PPARα gene expression while coconut oil but not soybean oil induced its expression with training.

Discussion

Fat as a bioactive compound influences metabolism by inducing muscle and liver phenotypic remodeling through transcriptional activation of PPARs (Badman et al., 2007; Mizunoya et al., 2013; Montgomery et al., 2013; Patsouris et al., 2006; Turner et al., 2009b). We hypothesized that diets varying in fat source and proportion together with training would lead to different adaptations in the muscle and liver consequently affecting whole-body metabolism and endurance. Comparative studies on the effects of different diets with training have been conducted (Fushiki et al., 1995; Hill et al., 2007; Simi et al., 1991) however, data on energy expenditure at rest and during exercise, substrate utilization, and gene transcription are scarce. Coconut oil is a good source of MCFAs which are rapidly metabolized relative to LCFAs (Bach et al., 1996). MCFAs with training improve endurance in swimming (Fushiki et al., 1995) but other aspects of adaptation require further investigation. We aimed to update the current knowledge on the effects of MCFAs and conduct a comparative study on the physiological effects of low-fat diet, and diets containing coconut oil and soybean oil with treadmill training.

Medium-fat diets increased FAT regardless of fat type and training generally promoted CHO at rest without affecting energy expenditure. Utilizing a lower intensity training protocol with the same soybean oil diet, increased CHO was observed but it accompanied increased energy expenditure without affecting FAT (Manio et al., 2017) suggesting that different training intensities differentially affect resting metabolism (Hildebrandt et al., 2003; Pilegaard et al., 2000). Our data in relation to Montgomery et al. (2013) suggest that higher absolute MCFAs content may increase VO_2 even without training relative to LCFAs.

Whole-body metabolism during exercise under different diets is associated with changes in VO_2max (Simi et al., 1991). Unfortunately, we could not measure VO_2max because of technical limitations. During exercise under slight food deprivation, training but not diet influenced whole-body metabolism suggesting that at rest with ad libitum feeding, energy metabolites in the diet determined differences in resting energy metabolism while general effects were due to training. Training lowered VO_2 (and energy expenditure) implying that

exercise economy increased in trained groups during exercise (Barnes and Kilding, 2015). In our previous study using a lower training intensity, decreased RER without changes in VO_2 was observed suggesting higher fat utilization (Manio et al., 2017). This further indicate that changes in energy metabolism at rest or during exercise is influenced by training intensity. It is important to note that inaccurate calculations in CHO and FAT, especially in C with ketosis during exercise, may exist because the complete oxidation of acetoacetate and β-HB give respiratory quotient values of 1.0 and 0.89, respectively (Frayn, 1983).

Our data and others show that diets high in fat induce muscle mitochondrial biogenesis and also imply that MCFAs promote mitochondrial biogenesis better than LCFAs at the same absolute concentration (Montgomery et al., 2013; Turner et al., 2009b). β-HAD activity increased in C without training. Also, in C2C12 muscle cells, C10 and C12 fatty acids increased succinate dehydrogenase activity (Montgomery et al., 2013). However, other enzymes may not be entirely dependent on fatty acid species but on the amount as seen with SCOT and CS. We also showed that training universally increased mitochondrial function suggesting mitochondrial biogenesis. Therefore, elevated oxidative capacity in C, albeit small, likely improved endurance in the untrained state while robust increase in mitochondria improved endurance in trained mice regardless of diet (Hood, 2009; Rangwala et al., 2010). With the swimming modality however, MCFAs increased swimming time attributed to increased CS and SCOT relative to LCFAs (Fushiki et al., 1995) underscoring the notion that different training modalities variably influence adaptation, and potentially, endurance.

Exercise increases PGC1A and this co-activates or potentiates the PPARs and ERRs in the control of mitochondrial biogenesis (Fan and Evans, 2015). We did not observe changes in PGC1A mRNA and protein despite increased mitochondrial function suggesting differential effects of training intensity on their half-lives (Lai et al., 2010; Manio et al., 2017; Pilegaard et al., 2003). However, we show that diets influenced basal and training-induced changes in mRNA expression of PPARs and ERRs. ERRs had decreased expression in medium-fat diets. This did not negatively affect mitochondrial enzyme activities, *Pgc1a* or *Erra* in the untrained state suggesting that at the basal level, homeostatic control and/or other ERR isotypes likely compensated for decreased mRNA expression of ERRγ (Alaynick et al., 2007;

Liu et al., 2005). Training generally increased *Erra* and *Errb,* which could explain the increased mitochondrial biogenesis in the muscle (Rangwala et al., 2010).

High-fat diets increases *Ppara* but not other isotypes in rats (Kannisto et al., 2006). We did not observe significant elevations in *Ppara* in the untrained state possibly due to lower fat content of our diets. On the other hand, only *Pparb/d* increased with training in contrast to increased *Ppara* and unchanged *Pparb/d* when trained at a lower intensity even with the same soybean oil diet (Manio et al., 2017) suggesting that PPARs respond differently with training intensity. Interestingly, coconut oil impaired the training-induced upregulation of *Pparb/d*. Consistent with increased *Pparb/d* with training, target genes related to glucose and fat utilization, and fiber type remodelling (*Glut4*, *Cpt1b*, *Fatp1* and *Myh2*) responded similarly especially in S (Burkart et al., 2007; Kleiner et al., 2009; Tanaka et al., 2003; Yuan et al., 2013). Because exercise increases fatty acid uptake by CD36 translocation to the sarcolemma, not only increased expression of PPARβ/δ but also increased fatty acid-induced activation and availability could upregulate these targets (Manio et al., 2017; McFarlan et al., 2012) which may explain some of the differences between S and L with training. On the other hand, these changes could not be attributed to increased CD36 as its mRNA and protein did not respond as expected (Manio et al., 2017) indicating that training intensity affects genetic adaptations.

The expression of *Cpt1b* and *Lpl* expression in medium-fat diets in the untrained state is probably related to PPARα as this isotype also controls its transcription in the muscle (Muoio et al., 2002). Because functions of PPAR isotypes overlap in some of these genes, we could not discount the contribution of PPARγ. Although PPARγ is abundant in adipose tissues, it is also present in skeletal muscle and MCFAs and LCFAs strongly activate PPARγ (Kliewer et al., 1997; Liberato et al., 2012; Malapaka et al., 2012; Schoonjans et al., 1996; Xu et al., 1999).

The minimum amount of consumed soybean oil was similar among diet groups suggesting that coconut oil inhibited training-induced upregulation of PPARβ/δ and some downstream targets. Whether MCFAs inhibited LCFAs by competitive binding in PPARβ/δ activation

requires further research. While competitive binding assays between fatty acids and synthetic agonists have been performed (Kliewer et al., 1997; Malapaka et al., 2012; Xu et al., 1999), competitive binding assay among fatty acids to PPARs has yet to be undertaken. Overall, PPAR-related gene transcription as a training adaptation was influenced by the type of fat in the diet. Also, these adaptations reflect the route of catabolism of energy metabolites within these diets during exercise.

In the untrained state, diets did not affect pre-exercise muscle glycogen possibly due to similar circulating lipids or serum β-HB as these influence glycogen storage (Auclair et al., 1988; Pinckaers et al., 2017; Saitoh et al., 1993; Satabin et al., 1989). Training increases muscle glycogen however ketone bodies, particularly acetoacetate, inhibit insulin-stimulated glucose uptake that occurs during feeding after training (Maizels et al., 1977). This may explain the inhibited glycogen accumulation in C. Furthermore, while GLUT4 is not essential for glycogen repletion per se it could influence glycogen accumulation with insulin post-exercise by increasing the rate of glucose transport (Kuo et al., 1999; Ren et al., 1994; Ryder et al., 1999). Liver glycogen accumulation was also impaired in C and to a lesser extent in S which was emphasized by training. This could partly be explained by exhaustion of hepatic glycogen reserves with MCFAs and the glycogen replenishing effect carbohydrates (Kerly and Ottaway, 1954; Sanbar et al., 1965).

Increased muscle glycogen and glycogen sparing improves endurance by slowing the utilization of circulating glucose and liver glycogen (Baldwin et al., 1973; Pederson et al., 2005). Muscle glycogen is spared by improved utilization of fatty acids and ketone bodies (Goodman et al., 1974; Hickson et al., 1977; Rennie et al., 1976; Ruderman et al., 1971) linking glycogen sparing with high serum β-HB, decreased serum NEFA and intramuscular TG especially in C with training post-exercise. These suggest the existence of compensatory mechanisms to preserve endurance despite low pre-exercise muscle glycogen in this group. Nevertheless, muscle glycogen availability and utilization together with increased oxidative and mitochondrial functions likely promoted robust endurance improvement in all trained groups.

In the liver, unlike LCFAs, MCFAs can bypass fatty acid transport proteins to enter the cell and mitochondria for oxidation and these undergo β-oxidation for complete oxidation or ketogenesis (Doege et al., 2006; Frost and Wells, 1981; Fukao et al., 2004; McFarlan et al., 2012; Smith et al., 2011; Zammit, 1980). Improved oxidative capacity with increased upstream β-HAD activity suggested increased capacity to produce ketones particularly in C (Mannaerts et al., 1978) despite higher downstream ketogenic enzyme activities in S than C. Furthermore, because MCFAs are undetectable in the serum at rest, the overwhelming increase in β-HB during exercise in C suggests that liver and adipose tissues stored MCFAs, released them to circulation during exercise and were immediately catabolized (Crozier, 1988; Frost and Wells, 1981; Fushiki et al., 1995; Lhuillery et al., 1988; Maragoudakis et al., 1975; Scheig and Klatskin, 1968).

Fatty acid oxidation and ketogenesis in the liver is controlled by PPARα (Georgiadi and Kersten, 2012). Diets did not influence *Ppara* but training increased its expression in C. Fibroblast growth factor 21 (FGF21) is induced by acetoacetate via an upstream regulator and this upregulates PPARα (Badman et al., 2007; Vilà-Brau et al., 2011) thus connecting the link between training, coconut oil, increased ketone bodies during training and increased *Ppara*. While non-significant, increased *Cpt1a* and β-HAD activity in this group suggest PPARα activation in the liver (Mannaerts et al., 1978). *Hmgcs2*, the gene that encodes the first enzyme of ketogenesis (Fukao et al., 2004), was unaffected by diet nor training suggesting that high β-HB observed post-exercise in C was primarily caused by increased supply of ketogenic precursors for β-oxidation in C rather than changes in ketogenic activity. On the other hand, relatively lower β-HB with training at post-exercise is likely because of increased muscular utilization accompanying increased SCOT activity. Whether increased oxidative capacity prevented increase of liver weight in C with training was not investigated. Overall, coconut oil with training promoted liver remodeling to an oxidative phenotype without influencing mitochondrial biogenesis.

Conclusion

Our study provides evidence that prolonged feeding of coconut oil, and indirectly MCFAs, can improve endurance even in the untrained state. More importantly, training dictates endurance in wild-type mice (Manio et al., 2017). Moreover, while training increases mitochondrial functions in the muscle, training-induced transcriptional adaptations in the muscle and liver are differentially influenced by diet. Our data suggest that with training coconut oil inhibits PPARβ/δ in the muscle while activating PPARα in the liver. This study and that of Fushiki et al. (1995) and Manio et al. (2017) highlight the importance of the modality used in training and endurance testing as adaptations are clearly influenced by it. Also, we show that results of exercise studies depend on diet composition and this has to be carefully considered in data interpretation. Unlike most studies investigating the effects of fat, the caloric composition of our medium-fat diets is lower and does not greatly differ from the diets of athletes (Brouns and van der Vusse, 1998) which may explain why observed changes were relatively modest. Although these adaptations benefit endurance exercise, diets rich in coconut oil may be detrimental to activities that require high glycogen stores.

Figures

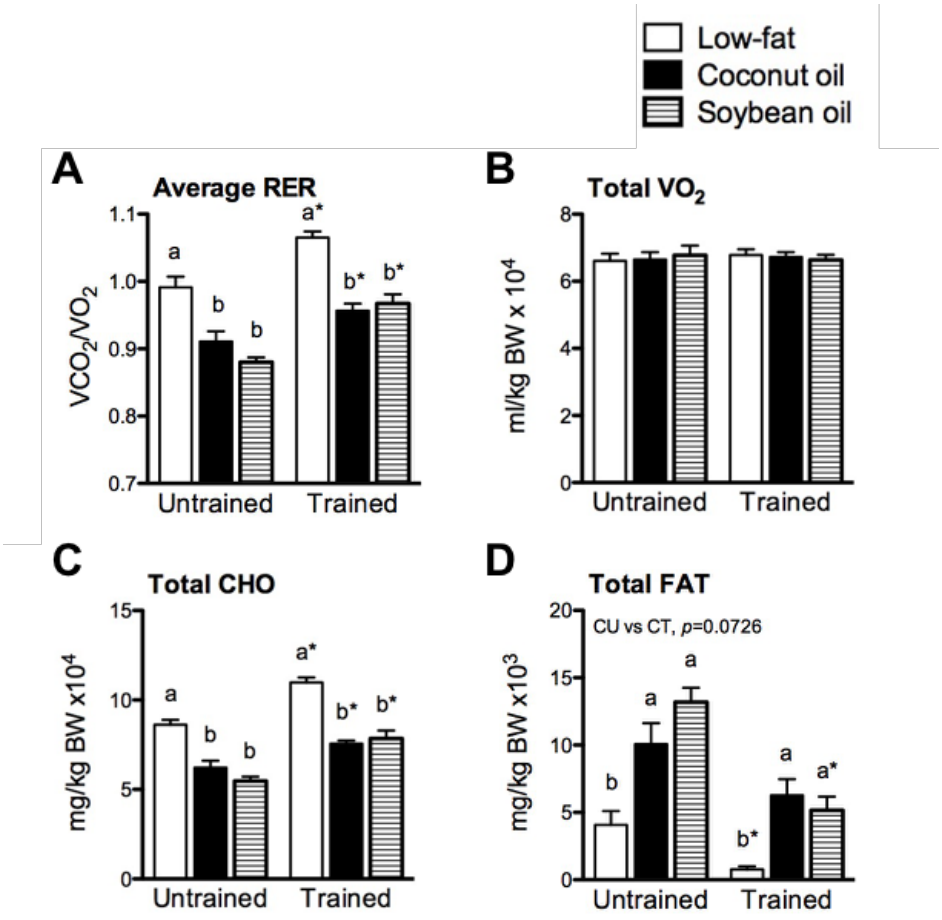

Figure 1. Basal indirect calorimetry. (A) Average RER, (B) total VO_2, (C) total CHO, and (D) total FAT of mice at rest during a 24h light-dark cycle. Values are presented as means ± SEM (n = 8). Significant differences among groups of different diets were determined by one-way ANOVA followed by Newman-Keuls post-hoc test. Dissimilar alphabets indicate significant differences ($p < 0.05$). Significant differences between groups of the same diet having different training states were determined by Student's t-test (*, $p < 0.05$).

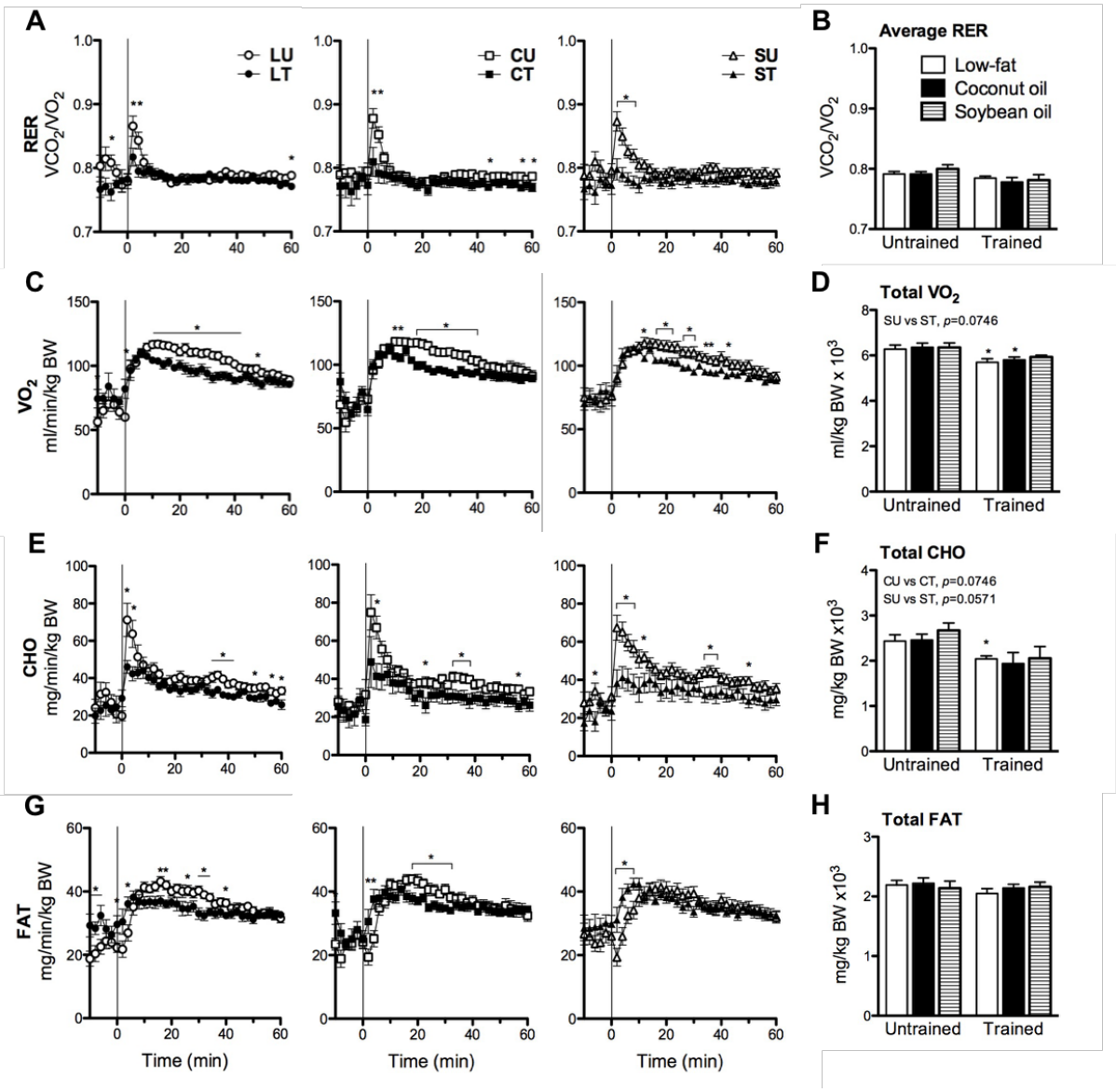

Figure 2. Exercise indirect calorimetry. (A,B) RER, (C,D) VO_2, (E,F) CHO, and (G,H) FAT of mice during a 60min treadmill run presented as time-course changes (A,C,E,G) and average and total values (B,D,F,H). No significant difference in each time point and average and cumulative values was observed among groups of different diets as assessed by one-way ANOVA while significant differences were observed between groups of the same diet having different training status as assessed by Student's unpaired t-test (*, $p < 0.05$). Values in both types of graph are presented as means ± SEM (n = 9-13).

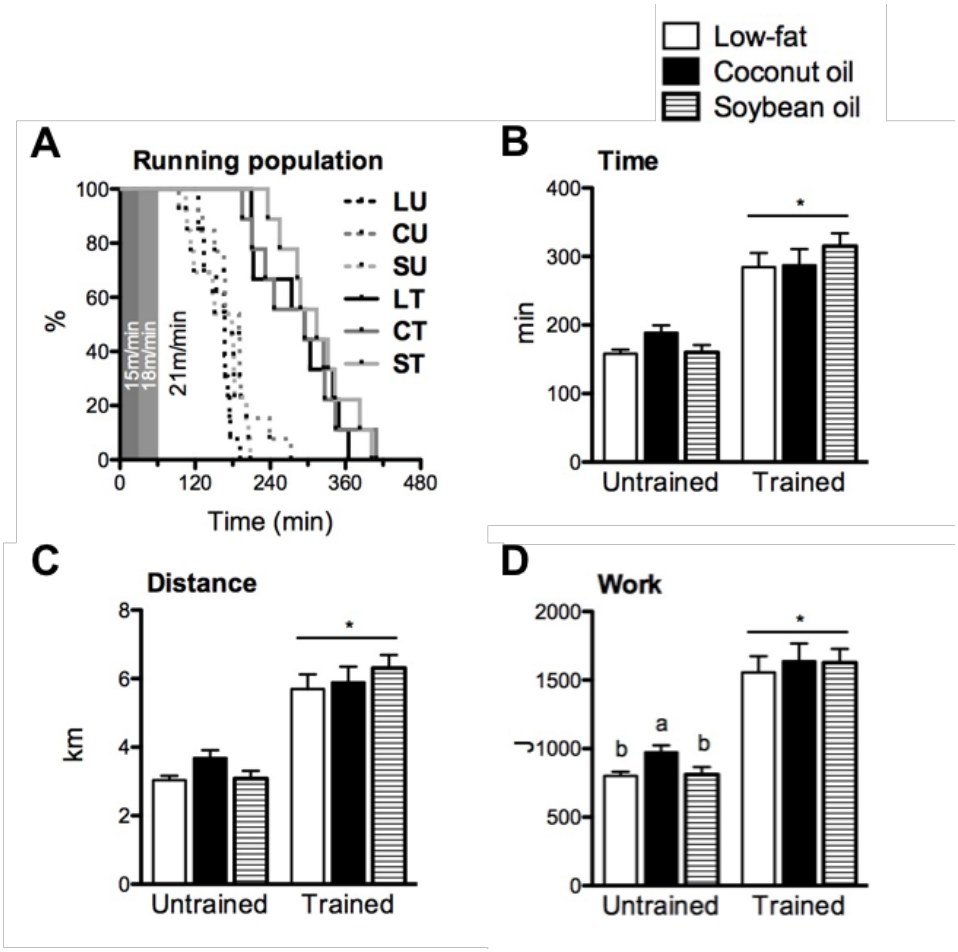

Figure 3. Treadmill endurance performance. (A) Running population plotted against time, (B) time-to-exhaustion, (C) distance and (D) work. Each point in A represents an individual mouse. For the bar graphs, data are presented as means ± SEM (n = 9-13). Significant differences among groups of different diets were determined by one-way ANOVA followed by Newman-Keuls post-hoc test. Dissimilar alphabets indicate significant differences ($p < 0.05$). Significant differences between groups of the same diet having different training states were determined by Student's t-test (*, $p < 0.05$).

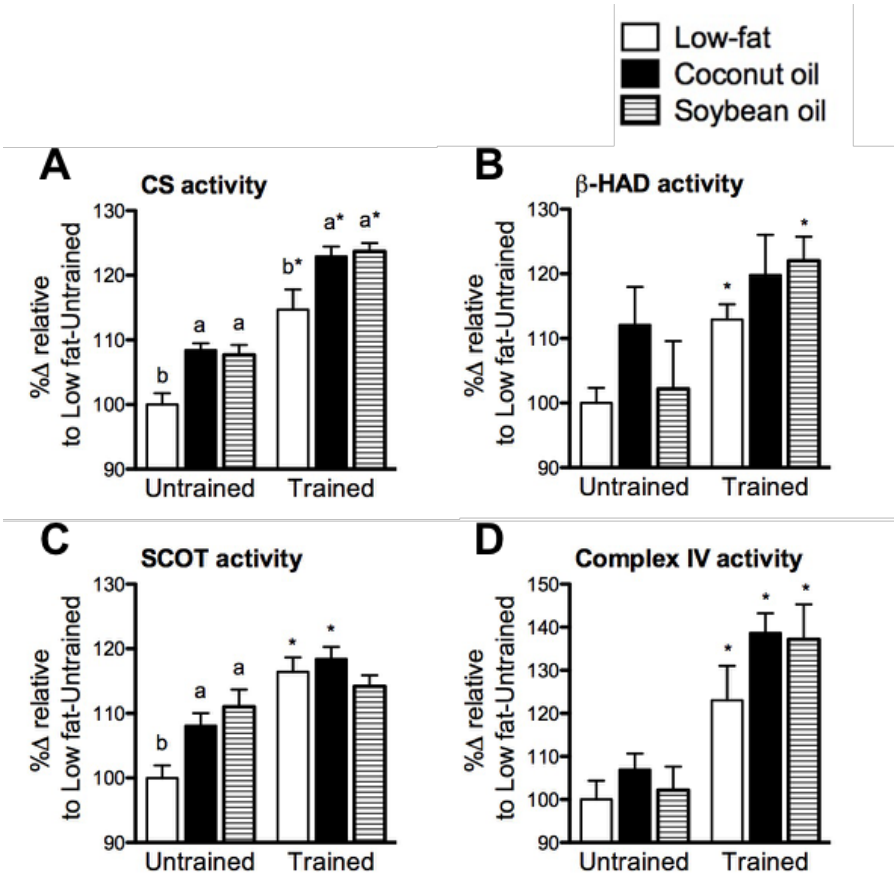

Figure 4. Muscle mitochondrial enzyme activities. (A) CS, (B) β-HAD, (C) SCOT, and (D) mitochondrial Complex IV activities of gastrocnemius from untrained and trained mice. Data are presented as means ± SEM (n = 8). Significant differences among groups of different diets were determined by one-way ANOVA followed by Newman-Keuls post-hoc test. Dissimilar alphabets indicate significant differences ($p < 0.05$). Significant differences between groups of the same diet having different training states were determined by Student's t-test (*, $p < 0.05$).

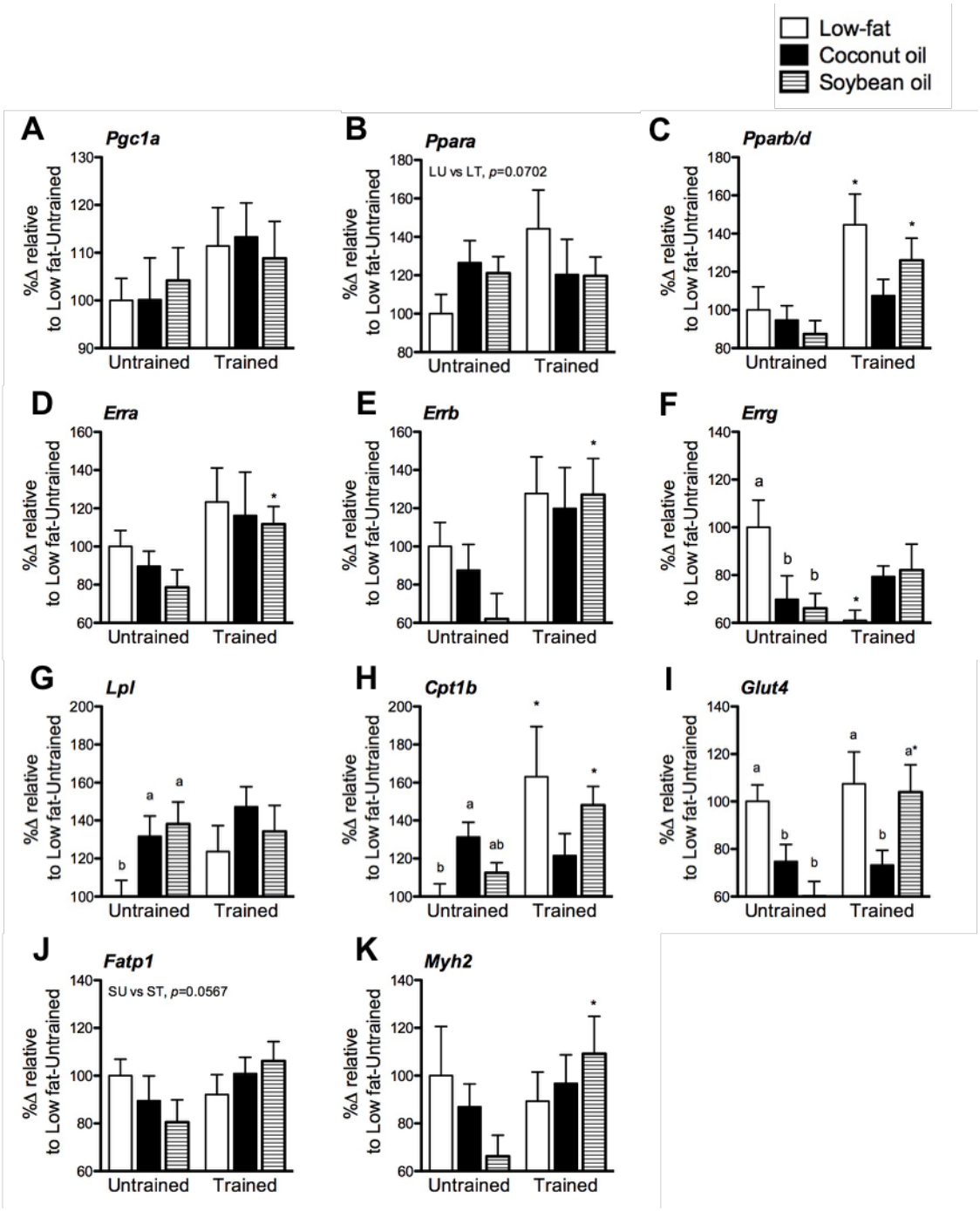

Figure 5. Muscle mRNA expression. (A) *Pgc1a*, (B) *Ppara*, (C) *Pparb/d*, (D) *Erra,* (E) *Errb,* (F) *Errg,* (G) *Lpl,* (H) *Cpt1b,* (I) *Glut4,* (J) *Fatp1,* and (K) *Myh2* expression in gastrocnemius from untrained and trained mice. Data are presented as means ± SEM (n = 8). Significant differences among groups of different diets were determined by one-way ANOVA followed by Newman-Keuls post-hoc test. Dissimilar alphabets indicate significant differences ($p < 0.05$). Significant differences between groups of the same diet having different training states were determined by Student's t-test (*, $p < 0.05$).

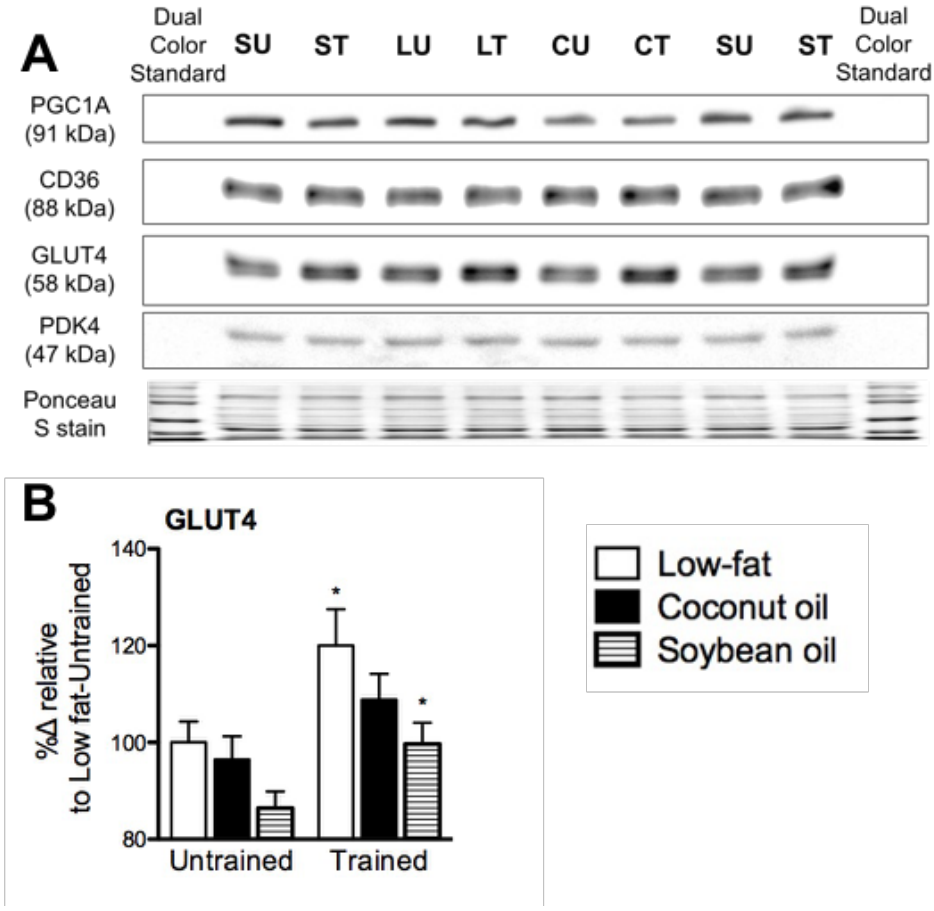

Figure 6. Muscle protein expression. (A) Representative blots and Ponceau S stained membrane, and (B) quantified GLUT4 protein expression in gastrocnemius from untrained and trained mice. Signals from proteins of interest were compared to total Ponceau S signal. Data are presented as means ± SEM (n = 8). Significant differences among groups of different diets were determined by one-way ANOVA followed by Newman-Keuls post-hoc test. Dissimilar alphabets indicate significant differences ($p < 0.05$). Significant differences between groups of the same diet having different training states were determined by Student's t-test (*, $p < 0.05$). Samples were processed simultaneously and each group was represented in each gel. Location and molecular weight of proteins were confirmed by colored protein standards. Results were data pooled from 6 gels.

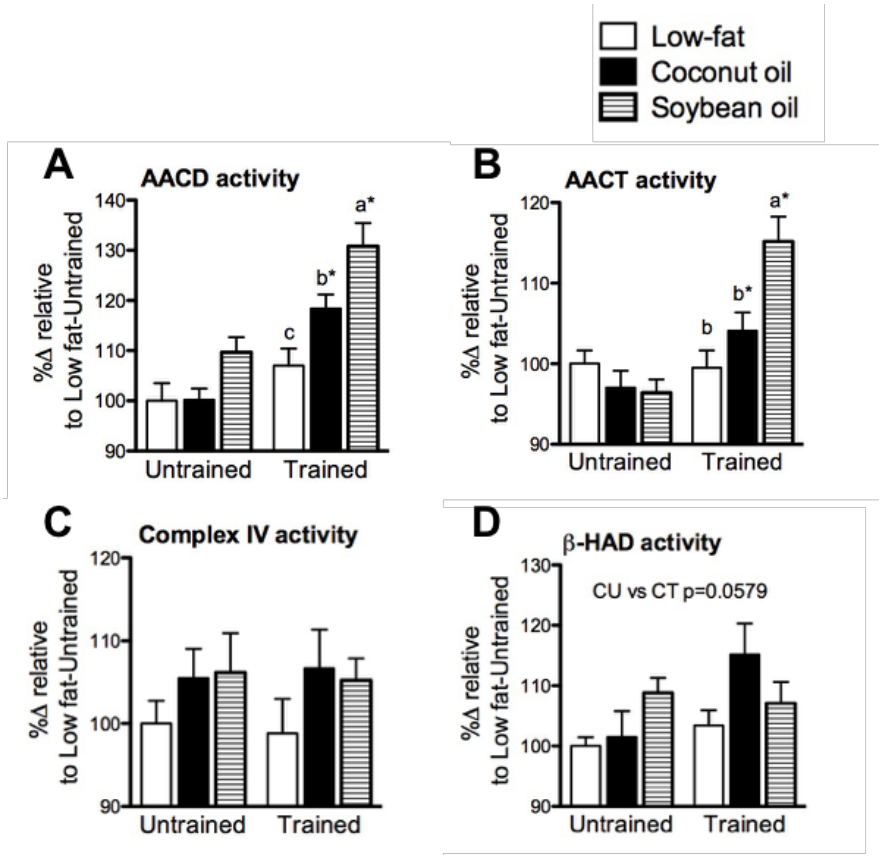

Figure 7. Liver mitochondrial enzyme activities. (A) AACD, (B) AACT, (C) Complex IV and (D) β-HAD activities of liver from untrained and trained mice. Data are presented as means ± SEM (n = 8). Significant differences among groups of different diets were determined by one-way ANOVA followed by Newman-Keuls post-hoc test. Dissimilar alphabets indicate significant differences ($p < 0.05$). Significant differences between groups of the same diet having different training states were determined by Student's t-test (*, $p < 0.05$).

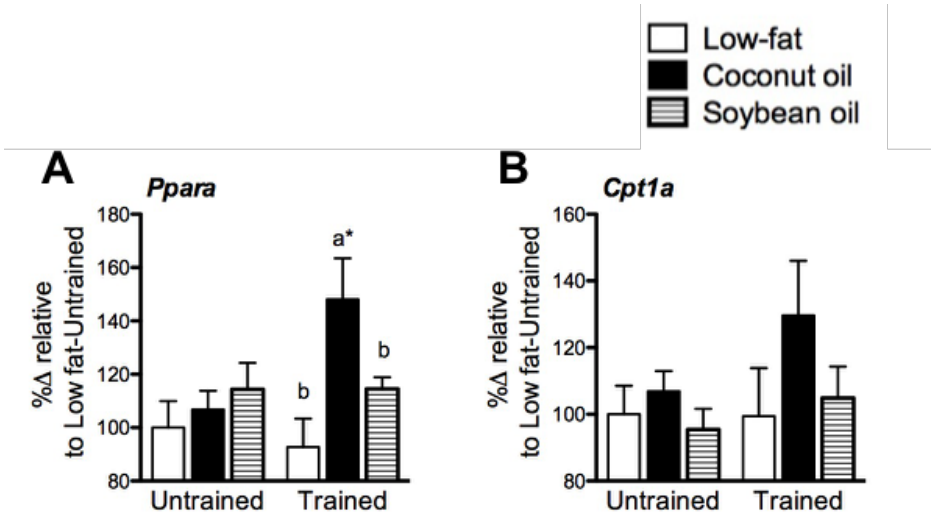

Figure 8. Liver mRNA expression. (A) *Ppara*, and (B) *Cpt1a* expression in liver from untrained and trained mice. Data are presented as means ± SEM (n = 8). Significant differences among groups of different diets were determined by one-way ANOVA followed by Newman-Keuls post-hoc test. Dissimilar alphabets indicate significant differences ($p < 0.05$). Significant differences between groups of the same diet having different training states were determined by Student's t-test (*, $p < 0.05$).

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Summary

AMPK and PPARδ improve endurance and muscle function by mimicking or simulating the effects of exercise training. However, whether or not they potentiate the effects of exercise training remained unexplored. The author presented in Chapter 1 the result of combined activation of AMPK and PPARδ together with exercise training on a treadmill. Significant and remarkable improvement in endurance performance was observed as measured in all running parameters with combined activation but not in sole activation of either proteins. However, AMPK activation by AICAR modestly but significantly improved work relative to other groups suggesting that AMPK activation to some extent potentiated endurance exercise training effects. Combined activation of AMPK and PPARδ with training resulted in significant increases in muscle glycogen and its sparing despite running the longest period. This was attributed to increased basal PDK4 mRNA expression and mitochondrial density resulting in pronounced whole-body fat oxidation and carbohydrate sparing. Improved endurance with AMPK activation together with training however could be attributed to similar increases in mitochondrial density. While significant changes in gene expression and mitochondrial markers were not observed, training generally promoted lower RER at rest and towards the end of the run without changes in energy expenditure suggesting increased fat utilization relative to sedentary mice.

Fatty acids are ligands to the PPARs above that of their function as energy substrates. Because PPARs are involved in the adaptations brought about by exercise training, fatty acid uptake therefore could influence these adaptations. CD36, being the primary fatty acid transporter on the muscle, is critical in controlling fatty acid uptake and oxidation, and basal endurance performance. However, its role in exercise training adaptations have not been investigated. The author presented in Chapter 2 the effects of whole-body ablation of CD36 on endurance exercise training adaptations. While basal endurance performance was impaired in CD36 KO mice, training-induced improvement was absent. This was unexpected as muscle glycogen accumulation and mitochondrial biogenesis with training was intact. Moreover, training-induced changes in whole body metabolism at rest and during exercise in WT mice was absent in CD36 KO mice suggesting that upstream fatty acid uptake by CD36

primarily controls metabolism and not glycogen nor mitochondrial functions. In addition, inhibited exogenous glucose oxidation in trained WT mice during exercise was observed despite unchanged glucose clearance and this was absent in CD36 KO mice reflecting the downstream effect of improved fat utilization by training. Furthermore, upregulation of exercise-induced mRNA expression related to PPARs were absent in CD36 KO muscle but not mitochondrial genes suggesting intact ERR and other mitochondrial biogenesis-related signaling. These results highlight the importance of intact fatty acid uptake by CD36 for efficient training adaptations likely mediated by PPARs.

Fats in diets can influence metabolism by the type of fatty acids in them. Long-chain fatty acids are more commonly found in plant oils however medium-chain fatty acids are also abundant in certain oils in specific regions particularly in tropical countries. Several types of plant oils have been studied however limited studies have been conducted on their effects on endurance exercise training adaptations. As presented in Chapter 2, fatty acids influence exercise adaptation through PPARs. Therefore, the type of fatty acid may also influence exercise adaptation through this and other mechanisms. The author presented in Chapter 3 the influence of fat type and content on adaptations with training. Training but not diet significantly affected whole-body metabolism during exercise. Likewise, only training promoted exercise efficiency. Endurance however was increased with coconut oil in the untrained state but no potentiation by diets was observed with training. Furthermore, mitochondrial biogenesis with training was not potentiated by diets despite increased oxidative enzyme activities by coconut oil or soybean oil diets. Interestingly, coconut oil prevented training-induced increase in muscle glycogen but preserved it during exercise probably due to ketogenesis which was accompanied by improved ketolysis. In terms of PPAR activation, PPARβ/δ was inhibited in the muscle but PPARα was activated in the liver by coconut oil with training. Therefore, with intact fatty acid uptake machinery, exercise but not diet controls endurance and differential training adaptations with diets preserve endurance by promoting unique sets of compensatory systems to accommodate energy substrates within these diets.

Overall, exercise training adaptations not only differ with the modality of exercise, and the intensity and frequency of exercise training but also by the internal physiological

environment of the muscles and organs. While it is difficult to establish standardized training protocols, these studies show that in healthy and genetically intact state, training improves endurance performance. Data from this thesis may suggest the potential use of pharmacological compounds and diet or nutritional supplementation to improve basal endurance or potentiate performance possibly even in a genetically compromised state.

List of main publications

1. Combined pharmacological activation of AMPK and PPARδ potentiates the effects of exercise in trained mice
   MC Manio, K Inoue, M Fujitani, S Matsumura, T Fushiki
   Physiological Reports, 4:5, e12625, March 2016

2. CD36 is essential for endurance improvement, changes in whole-body metabolism and efficient PPAR-related transcriptional responses in the muscle with exercise training
   MC Manio, S Matsumura, D Masuda, K Inoue
   Physiological Reports, 5:10, e13282, May 2017

3. Low-fat diet, and medium-fat diets containing coconut oil and soybean oil exert different metabolic effects in untrained and treadmill-trained mice
   MC Manio, S Matsumura, K Inoue
   Under submission

Other publications

1. Hepatocyte β-Klotho regulates lipid homeostasis but not body weight in mice
   *K Kobayashi, T Tanaka, S Okada, Y Morimoto, S Matsumura, **MC Manio**, K Inoue, K Kimura, T Yagi, Y Saito, T Fushiki, H Inoue, M Matsumoto, Y Nabeshima*
   FASEB Journal, 30:2, pp849-862, October 2015

2. Deletion of the transcriptional coactivator PGC1α in skeletal muscles is associated with reduced expression of genes related to oxidative muscle function
   *Y Hatazawa, K Minami, R Yoshimura, T Onishi, **MC Manio**, N Sawada, O Suzuki, S Miura, Y Kamei*
   Biochemical and biophysical research communications, 481:3-4, pp251-258, November 2016

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