Stine Ringholm Jørgensen

PGC-1alpha in exercise- and exercise training-induced metabolic adaptations

PhD thesis

Department of Biology

University of Copenhagen

2013
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Study I including co-authorship statement

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Acknowledgements

This PhD project has been conducted at the Molecular Integrative Physiology group, Department of Biology at the University of Copenhagen and it has involved many people who I owe a great thank. Especially I would like to thank the following people:

First of all I wish to thank my supervisor Henriette Pilegaard for excellent guidance, for always being there to help at any time and for supporting me during difficult times. For always being enthusiastic and passionate about science and Henriette is the one that I can thank for arousing my interest in molecular biology and exercise physiology during my bachelor and master thesis.

Furthermore, I would like to thank current coworkers from “HP lab”, Jesper Olesen, Rasmus Sjørup Biensø, Maja Munk Nielsen, Jakob Grunnet Knudsen, Christina Tingbjerg Brandt, Nina Brandt, Signe Larsson, Caroline Maag Kristensen, Anders Gudiksen, Karoline Barfoed and Jens Halling for great cooperation during experiments and fun in the lab. In addition, I wish to thank Tobias Nørresø Haase for help during study II.

I would like to thank the co-authors on the papers included in my thesis for great collaboration and essential feedback in the writing process, and Ylva Hellsten, Karina Olsen and Clara Prats for skilled technical assistance in Study IV. Last but not least I wish to thank all the subjects in Study I for signing up for the project.

Finally, I owe my family and especially Anders a great thank for their invaluable support and encouragement at all times. I would never have made it without you.

“Living systems are worn out by inactivity and developed by use”

Albert Szent-Györgyi (Physiologist, Nobel prize 1937)
Summary

The aim of the present thesis was to investigate the hypotheses that 1) bed rest reduces metabolic and angiogenic proteins and changes microRNA (miRNA) content as well as alters exercise-induced mRNA responses in human skeletal muscle, 2) Peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α is required for exercise-, exercise training- and fasting-induced mRNA and protein responses, respectively, of metabolic, angiogenic and gluconeogenic proteins in liver and adipose tissue in mice, 3) PGC-1α is required for both exercise training and resveratrol mediated prevention of age-associated decreases in oxidative and angiogenic proteins in mouse skeletal muscle.

Study I demonstrates that only 7 days of bed rest reduced leg muscle mass, mitochondrial enzyme activities, mitochondrial (mt)DNA/nuclear (n)DNA content, protein content of oxidative proteins and miRNA content in human skeletal muscle. Furthermore the physical inactivity abolished the exercise-induced mRNA response of PGC-1α and vascular endothelial growth factor (VEGF) in skeletal muscle that was present before bed rest. This indicates that just 7 days of physical inactivity reduces the metabolic capacity of human skeletal muscle and interferes with the exercise-induced adaptive response in human skeletal muscle.

Study II demonstrates that mouse liver glucose-6-phosphatase (G6Pase) mRNA content increased in recovery from acute exercise in both wildtype (WT) and PGC-1α knockout (KO) mice, while phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase mRNA content did not change in either genotype. Exercise training increased PEPCK protein content in both WT and PGC-1α KO mice. In addition, the mRNA and protein content of cytochrome (Cyt) c and cytochrome c oxidase (COX) subunit I increased in response to acute exercise and exercise training, respectively, in WT mice while there was no change in PGC-1α KO mice. Furthermore, fasting increased G6Pase and PEPCK mRNA content in both WT and PGC-1α KO mice. This implies that exercise- and exercise training-induced improvements in hepatic oxidative capacity, but not regulation of gluconeogenesis, requires PGC-1α, while fasting-induced regulation of gluconeogenic capacity does not require PGC-1α.

Study III demonstrates the time course of an exercise-induced uncoupling protein (UCP)1 mRNA response in WT mouse epididymal (eWAT) and inguinal (iWAT) white adipose tissue, and that these responses were absent in PGC-1α KO mice. Furthermore, while UCP1 protein content was undetectable in eWAT, exercise training increased UCP1 protein content in iWAT of WT, but not PGC-1α KO mice. This training response was associated with increased COX subunit IV and cluster of differentiation (CD)31 protein content in WT, but not in PGC-1α KO mice. This shows that exercise training increases UCP1, COXIV and CD31 protein in
mouse iWAT, likely as a cumulative effect of transient increases in mRNA expression after each exercise bout, and that PGC-1α is required for these adaptations.

Study IV demonstrates that citrate synthase (CS) activity and mtDNA/nDNA content decreased with age in skeletal muscle of WT mice. CS activity, mtDNA/nDNA content, pyruvate dehydrogenase-E1α and VEGF protein content increased with lifelong exercise training in WT mice but not in PGC-1α KO mice. In contrast, lifelong resveratrol supplementation had no significant effect on these proteins in either genotype. This indicates that lifelong exercise training, but not resveratrol supplementation alone increases the oxidative capacity of skeletal muscle and that combining resveratrol with exercise training does not elicit more marked improvement in oxidative capacity than exercise alone.

In conclusion, just 7 days of physical inactivity reduces metabolic capacity and abolished exercise-induced responsiveness of human skeletal muscle. PGC-1α is required for exercise-induced mRNA responses and exercise training-induced protein changes in oxidative and/or angiogenic proteins in mouse liver and adipose tissue, while fasting-induced regulation of gluconeogenic capacity does not. In addition, resveratrol supplementation seems to have minor effects on the content of oxidative and angiogenic proteins in mouse skeletal muscle compared with exercise training.
Formålet med denne afhandling var at undersøge hypoteserne 1) bed rest reducerer metaboliske og angiogenetiske proteiner og ændrer microRNA (miRNA) indholdet samt det arbejds-inducerede genrespons i human skeletmuskulatur, 2) peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α er nødvendig for arbejds-, trænings- og faste-induceret mRNA og proteinrespons på metaboliske, angiogenetiske og glukoneogenetiske proteiner i lever og fedtvæv, 3) PGC-1α er nødvendig for træning og resveratrol-medieret forhindring af alders-associeret fald i oxidative og angiogenetiske proteiner i skeletmuskulatur fra mus.

Studie I viser at bare 7 dages bed rest reducerer muskelmasse i benene, mitokondriel enzymaktivitet, mitokondriel (mt)DNA/nuklear (n)DNA ratio, proteinindhold af oxidative proteiner samt miRNA indhold i human skeletmuskulatur mens kapillarisering er uændret. Endvidere viste studie I, at fysisk inaktivitet fjernede det arbejds-inducerede mRNA respons af PGC-1α og vascular endothelial growth factor (VEGF), der sås før bed rest i skeletmuskulatur. Dette indikerer, at bare 7 dages fysisk inaktivitet reducerer den metaboliske kapacitet samt påvirker det arbejds-inducerede adaptive genrespons i human skeletmuskulatur.

Studie II viser, at glucose-6-phosphatase (G6Pase) mRNA indholdet i lever fra mus stiger i restitutionsperioden efter et akut arbejde både i vildtype (WT) og PGC-1α knockout (KO) mus, mens phosphoenolpyruvate carboxykinase (PEPCK) og pyruvate carboxylase mRNA indholdet ikke ændres i nogen af genotyperne. Træning øger PEPCK proteinindholdet i begge genotyper. Derudover øges mRNA og proteinindholdet af Cytochrome (Cyt) c og Cytochrome c oxidase (COX) subunit I som respons på henholdsvis akut arbejde og træning i WT mus, mens der ingen ændring er i PGC-1α KO mus. Endvidere øger faste G6Pase og PEPCK mRNA indholdet i både WT og PGC-1α KO mus. Dette indikerer at PGC-1α kræves for arbejds- og trænings-inducerede forbedring i leverens oxidative kapacitet, men ikke regulering af gluconeogenese-relaterede proteiner, hverken ved akut arbejde, træning eller faste.

Studie III viser tidsforløbet af arbejds-induceret uncoupling protein (UCP)1 mRNA respons i epididymalt (eWAT) og inguinalt (iWAT) hvidt fedtvæv i WT mus samt at dette respons ikke ses i PGC-1α KO mus, mens UCP1 proteinindholdet ikke detekteres i eWAT, øger træning UCP1 proteinindholdet i iWAT hos WT mus, men ikke i PGC-1α KO mus. Dette træningsrespons er assiciert med øget COX subunit IV og cluster of differentiation (CD)31 proteinindhold i WT, men ikke PGC-1α KO mus. Dette viser, at træning øger UCP1, COXIV og CD31 protein i iWAT fra mus, formentlig som en akkumulerende effekt af kortvarige stigninger i mRNA indholdet efter hvert enkelt arbejde, og at PGC-1α kræves for disse adaptationer.
Studie IV viser, at citrate synthase (CS) aktivitet og mtDNA/nDNA ratio falder med alderen i skeletmuskulaturen fra WT mus. CS aktivitet, mtDNA/nDNA ratio, pyruvate dehydrogenase-E1α og VEGF proteinindhold øges med livslang træning i WT, men ikke i PGC-1α KO mus. Derimod har livslang resveratroltilskud ingen signifikant effekt på disse proteiner i nogen af genotyperne. Dette indikerer, at livslang træning, men ikke resveratroltilskud alene øger skeletmuskulaturens oxidative kapacitet, og at kombination af resveratroltilskud med træning ikke giver ekstra forbedring af oxidativ kapacitet i forhold til træning alene.

Samlet kan det konkluderes, at bare 7 dages fysisk inaktivitet reducerer metabolisk kapacitet og fjerner det arbejdss-inducerede respons i human skeletmuskulatur. PGC-1α er nødvendig for arbejds-inducerede mRNA respons og trænings-inducerede ændringer i oxidative og/eller angiogenetiske proteiner i lever og fedtvæv fra mus, mens PGC-1α ikke kræves for faste-induceret regulering af gluconeogenetisk kapacitet. Derudover har resveratrol kun mindre effekter på indholdet af oxidative og angiogenetiske proteiner i skeletmuskulaturen fra raske aldrende mus i forhold til træning.
List of abbreviations

ACC  Acetyl-CoA carboxylase
AMPK  AMP-activated protein kinase
BAT  Brown adipose tissue
BCA  Bicinchoninic acid
BSA  Bovine serum albumin
CaMK  Ca\(^{2+}\)-calmodulin-dependent protein kinase
CD31  Cluster of differentiation 31
CoA  Coenzyme A
COX  Cytochrome c oxidase
CS  Citrate synthase
Cyt c  Cytochrome c
DEPC  Diethyl pyrocarbonate
DNA  Deoxyribonucleic acid
dNTP  Deoxyribonucleotide triphosphate
dUTP  Deoxyuridine triphosphate
EDL  Extensor digitorum longus
EDTA  Ethylenediamine tetraacetate
ERR\(\alpha\)  Estrogen-related receptor \(\alpha\)
FAM  6-carboxyfluorescein
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
Glu-6-P  Glucose-6-phosphate
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<td>Glucose transporter 4</td>
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<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
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<td>GT</td>
<td>Guanidinium thiocyanate</td>
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<td>HAD</td>
<td>β-hydroxyacyl-CoA dehydrogenase</td>
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<td>HK</td>
<td>Hexokinase</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Myosin heavy chain</td>
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<td>NRF</td>
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<td>PRC</td>
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<tr>
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<td>Reactive oxygen species</td>
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List of manuscripts

The present thesis is based on the following manuscripts referred to in the text as Study I-IV.

* Indicate shared authorship

Study


Each paper/manuscript is included in the Appendix.
Work was contributed to the following manuscripts not included in the present thesis.


Introduction

Through evolution the human genome has been selected for a physically active lifestyle and it is a dominating view that regular activation of certain genes is required to avoid a dysfunctional metabolism (Booth & Laye, 2010; Gollnick & Saltin, 1982). In accordance an escalating increase in lifestyle related metabolic diseases like type 2 diabetes is evident in part as a consequence of a more sedentary lifestyle (Booth & Laye, 2010; Booth & Lees, 2007). Many different tissues can potentially be affected by physical inactivity, which underlines the importance of a physically active lifestyle for maintaining a healthy and well functioning metabolism.

1 Skeletal muscle

Skeletal muscle is a highly plastic tissue that has the ability to both adjust to acute and adapt to chronic alterations in metabolic demands (Gollnick & Saltin, 1982). As it is the largest organ of the body (approximately 40% of total body weight in normal weight subjects) changes in skeletal muscle metabolism has great impact on whole body metabolism and hence on the risk of developing lifestyle related diseases. It is well established that endurance exercise training induces skeletal muscle adaptations leading to increased capacity of the muscles for aerobic metabolism (Booth & Baldwin, 1996; Booth & Laye, 2010; Gollnick & Saltin, 1982; Holloszy & Booth, 1976). Skeletal muscles are heterogeneous composed of muscle fibers with different properties including differences in respiratory capacity and endurance exercise training has been shown to change the composition of muscle fibers toward the oxidative slow-twitch red fibers (Holloszy & Booth, 1976) in both mice, rats and humans (Coggan et al., 1992; Gollnick et al., 1973; Holloszy, 1967; Röckl et al., 2007).

Endurance exercise training has been shown to shift metabolism towards a more pronounced fat oxidation. Thus exercise training increased fat utilization leading to muscle glycogen sparring while untrained individuals to a larger extent rely on carbohydrate as fuel (Henriksson, 1977). In addition, muscle glycogen stores of endurance trained individuals are larger than that of sedentary individuals (Hermansen et al., 1967). Conversely detraining results in a reduction in the glycogen concentration of skeletal muscle. Data on alterations in triglyceride (TG) stores in different fiber types with endurance exercise training are contradicting (Gollnick & Saltin, 1982).
Figure 1. Skeletal muscle substrate utilization during submaximal one-legged knee extensor exercise at the same relative intensity before and after endurance training (Gollnick & Saltin, 1982; Saltin & Astrand, 1993; Turcotte et al., 1992).

Substrate utilization during acute exercise is the result of acute regulation of metabolic pathways, and the altered substrate utilization pattern with endurance exercise training are due to training-induced adaptations in skeletal muscle enhancing the ability to utilize fat for oxidation at a given absolute and relative exercise intensity (Figure 1) (Gollnick & Saltin, 1982; Kiens et al., 1993; Saltin & Astrand, 1993; Turcotte et al., 1992). Physical activity therefore seems to have beneficial effects in maintaining normal metabolism in skeletal muscle.

1.1 Skeletal muscle oxidative capacity

1.1.1 Mitochondrial biogenesis

Activities of the oxidative enzymes cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) as well as hexokinase (HK)II and β-hydroxyacyldehydrogenase (HAD) were already more than 30 years ago found to be elevated in skeletal muscle of exercise trained subjects (Gollnick & Saltin, 1982). Several later training studies in humans and rodents have been shown to improve skeletal muscle mitochondrial capacity including enhanced citrate synthase (CS) and COX activity and increased basal mRNA content of cytochrome (Cyt) c, COX and glucose transporter (GLUT)4 (Figure 2) (Daugaard et al., 2000; Hussey et al.,
A close relationship between the percentage change in skeletal muscle oxidative enzyme activities and the concomitant change in whole body VO$_{2\text{max}}$ during the first weeks of exercise training was demonstrated almost 30 years ago (Henriksson & Reitman, 1977). After this the VO$_{2\text{max}}$ levels off whereas the activity of mitochondrial enzymes continues to increase (Figure 3). It appears that the concentration of mitochondrial enzymes is increased whenever there is an increased demand for high substrate oxidation by the muscle. A decrease in activity of mitochondrial enzymes with detraining is seen in both well-trained and sedentary individuals (Henriksson & Reitman, 1977). This decline in mitochondrial enzymes with detraining or physical inactivity/bed rest is faster than the decrease in VO$_{2\text{max}}$ (Henriksson & Reitman, 1977; Saltin et al., 1968).
reflecting the need for more frequent exercise stimulus to maintain the capacity of the oxidative proteins in skeletal muscle than VO$_{2\text{max}}$.

![Graph showing changes in two mitochondrial enzymes and VO$_{2\text{max}}$ during training and detraining (Henriksson & Reitman, 1977).](image)

1.1.2 Capillarization

The oxidative capacity of skeletal muscle is determined by both content and activity of oxidative enzymes but capillarization is also an important factor in oxidative capacity due to enhanced oxygen delivery and substrate exchange in the tissue (Romanul, 1965). Romanul (Romanul, 1965) demonstrated a close relationship between the oxidative metabolic capacity of a muscle fiber and the number of capillaries
surrounding the fiber. Endurance exercise training increases capillarization (Henriksson & Reitman, 1977; Jensen et al., 2004a; Saltin et al., 1968) and capillarization correspondingly decreases during detraining (Gollnick & Saltin, 1982; Henriksson & Reitman, 1977; Hudlicka et al., 1992). Furthermore, it is well known that red muscles have a high degree of capillarization compared with white muscles (Gollnick & Saltin, 1982), which indicates a higher capacity for substrate delivery in red muscles.

Enhanced physical activity is associated with an increase in the endothelial marker protein cluster of differentiation (CD)31 and proliferation of capillaries as a local response, i.e. it occurs only in the exercised muscle and only around fibers that are recruited during exercise (Hudlicka, 1998; Jensen et al., 2004a). The angiogenic factor vascular endothelial growth factor (VEGF) is widely accepted as a critical factor in the angiogenic process where VEGF stimulates vascular endothelial cell growth, survival and proliferation (Ferrara, 1999). Furthermore, increased capillarization after exercise training correlates with increased skeletal muscle VEGF expression (Amaral et al., 2001; Hudlicka et al., 1992) and exercise training has been shown to increase VEGF protein content in human skeletal muscle (Hoier et al., 2012). VEGF-deficient skeletal muscle has been demonstrated to induce capillary regression in mice (Tang et al., 2004) and exercise training-induced angiogenesis has been shown to be dependent on the availability of VEGF (Wagner et al., 2006).

In humans, increasing age is associated with a decrease in skeletal muscle capillarization and a concomitant decrease in VEGF protein and lower VEGF promoter activity (Croley et al., 2005; Rivard et al., 2000; Ryan et al., 2006). It has previously been demonstrated that lifelong endurance exercise trained subjects had higher skeletal muscle capillarization than age-matched sedentary subjects but also that sedentary elderly subjects maintained the ability to induce VEGF mRNA in response to a single exhaustive cycling exercise bout (Iversen et al., 2011).

2 White adipose tissue

White adipose tissue has traditionally been seen as an inert tissue with the function of storing excess energy in the form of TG, but it is now evident that it also functions as an endocrine organ playing an important role in whole body metabolism (Elmquist JK, 2012; Kusminski & Scherer, 2012). Adipose tissue located beneath the skin is denoted subcutaneous adipose tissue (SAT) and has been suggested to have a protective effect on metabolic diseases, while adipose tissue surrounding the internal organs is called visceral adipose tissue (VAT) and has been strongly associated with metabolic dysfunction (Fox et al., 2007; Terry et al., 1991; Visscher et al., 2001). SAT is often obtained from beneath the ligament inguinale (iWAT) while VAT is obtained from around epididymis (eWAT). As skeletal muscle, white adipose tissue has
previously been shown to adapt to endurance exercise much like skeletal muscle (Stallknecht et al., 1991; Stallknecht et al., 1993). Hence, COX activity (Stallknecht et al., 1991) and basal GLUT4 mRNA content increased in epididymal white adipose tissue after 10 weeks of endurance exercise training in rats (Stallknecht et al., 1993) and several other studies have shown exercise- and exercise training-induced increases in mRNA content of uncoupling protein (UCP)1 and protein content of COX IV in both eWAT and iWAT (Bostrom et al., 2012; Sutherland et al., 2009). Furthermore, inguinal- and epididymal-derived cell lines have been demonstrated to exhibit different responses to cAMP stimulation (Wu et al., 2012) and iWAT has recently been reported to contain beige precursor adipocytes (Petrovic et al., 2010; Wu et al., 2012) while eWAT does not, indicating a potential difference in responsiveness in the two adipose tissues.

2.1 Uncoupling protein 1
UCP1 was first identified as an important protein in the mitochondria of brown adipose tissue (BAT) playing an important role in adaptive thermogenesis (Nedergaard & Lindberg, 1982; Nicholls & Locke, 1984; Puigserver et al., 1998). UCP1 decreases the proton gradient generated in oxidative phosphorylation by increasing the permeability of the inner mitochondrial membrane allowing protons to return to the mitochondrial matrix (Figure 4) (Ricquier, 2005). More recently UCP1 has been located also in white adipose tissue (WAT) (Bostrom et al., 2012). UCP1 mRNA content has been shown to increase in iWAT but not in eWAT in response to cold-exposure (Waldén et al., 2012) and in both iWAT and eWAT, although with higher magnitude in iWAT, with three weeks of exercise training in mice (Bostrom et al., 2012). The different mRNA response patterns may indicate a difference in responsiveness of different adipose tissue depots and it has been suggested that iWAT comprises brown adipocytes (Waldén et al., 2012).
3 Liver

The liver is the major organ responsible for maintaining blood glucose levels during prolonged exercise and fasting. Like skeletal muscle and white adipose tissue, the liver has been demonstrated to respond to an acute exercise bout and exercise training (Aoi et al., 2004; da Silva et al., 2009; Légaré et al., 2001). The mRNA content and activity of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase (PC), as well as glucose-6-phosphatase (G6Pase), which plays an important role as the last step in gluconeogenesis and glycogenolysis, has already several decades ago been shown, to be upregulated in rat liver immediately after an acute exercise bout (Banzet et al., 2009; Dohm & Newsholme, 1983; Dohm et al., 1985; Hoene et al., 2009; Huston et al., 1975). During the initial phase of an exercise bout liver glycogenolysis is the main source of blood glucose and as the exercise proceeds hepatic gluconeogenesis increases and becomes the key source as the exercise duration continues (Wasserman & Cherrington, 1991).

4 Exercise-induced cellular adaptations

Exercise training-induced adaptations are thought at least in part to originate from cumulative effects of transient increases in gene expression in response to each single exercise bout as suggested for skeletal muscle (Figure 5) (Pilegaard et al., 2000; Pilegaard et al., 2003; Williams & Neufer, 1996). In accordance,
many studies have by now demonstrated an acute regulation of metabolic gene transcription and mRNA content in response to a single exercise bout (Neuffer & Dohm, 1993; Pilegaard et al., 2000; Pilegaard et al., 2003). In humans and rodents several metabolic proteins, including CS, HAD, GLUT4, HKII, pyruvate dehydrogenase kinase (PDK)4 and Cyt c has been shown to increase in mRNA content in recovery from acute exercise (Jorgensen et al., 2005; Kraniou et al., 2000; Leick et al., 2008; Leick et al., 2010b; Pilegaard et al., 2000; Pilegaard et al., 2002; Pilegaard et al., 2003).

Figure 5. Transient increases in mRNA content in recovery from an acute exercise bout and accumulation of mRNA if repeated bouts of exercise are performed. Adapted from (Saltin & Pilegaard, 2002).

An acute bout of exercise has also been demonstrated to induce an increase in VEGF mRNA and protein expression in skeletal muscle (Breen et al., 1996; Gustafsson et al., 2002; Gustafsson et al., 1999; Hiscock et al., 2003; Hoier et al., 2013; Richardson et al., 1999). In addition, an increase in VEGF mRNA expression in recovery from an acute exercise bout has been demonstrated in untrained subjects while the response was abolished in exercise trained subjects (Jensen et al., 2004b). In addition, exercise-induced mRNA responses of several metabolic and angiogenic proteins has been shown to decrease with repeated exercise bouts (Fischer et al., 2004; Jensen et al., 2004b; Nordsborg et al., 2010; Pilegaard et al., 2003). The observed change in mRNA content with acute exercise may both be due to increased transcription or decreased mRNA degradation (Figure 6). However, the reported exercise-induced increase in transcription of several
metabolic related proteins suggest that increased transcription is a major contributor to the increased mRNA content in recovery from exercise. However, it is clear that additional mechanisms are in play.

Figure 6. The central dogma of molecular biology describes the flow of genetic information within a biological system. Adapted from (Williams & Neufer, 1996).

4.1 Post-transcriptional modifications of mRNA

In addition to changes in transcription and concomitant changes in mRNA content, microRNAs (miRNAs) have been suggested to play an important role in regulation of protein expression. miRNAs are highly conserved small non-coding RNAs that are transcribed by RNA polymerase II as primary transcripts which are then processed in the nucleus by Drosha into pre-miRNA of ~70 nucleotides. The precursor miRNAs are then exported into the cytoplasm where they are processed into mature miRNAs (Figure 7). miRNAs have been suggested to be involved in post-transcriptional gene regulation by binding to the 3’ untranslated region (UTR) of target mRNAs to promote mRNA degradation or translation repression (van Rooij et al., 2008). Endurance exercise in mice has been reported to up-regulate muscle-specific miR-1, miR-29 and miR-133a which are important in the regulation of transcription factors and co-activators involved in myogenesis and muscle hypertrophy (Chen et al., 2006; Safdar et al., 2009; Wang et al., 2008). In addition, the study by Safdar et al. showed that acute exercise reduces the expression of miR-23 while peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α mRNA expression was increased, suggesting a regulatory role of miR-23 on PGC-1α (Safdar et al., 2009).
How the above mentioned adaptations to exercise training are induced and how the acute regulation of protein expression is affected by training status is still unclear.

5 Peroxisome proliferator-activated receptor-γ coactivator 1α

PGC-1α was first discovered in BAT as an important component of adaptive thermogenesis including mitochondrial biogenesis (Puigserver et al., 1998). PGC-1α has been shown to contain a LXXLL sequence which is a motif identified as a nuclear receptor-coactivator interaction motif (Puigserver et al., 1998). As a transcriptional coactivator, PGC-1α interacts with transcription factors to regulate transcription of specific genes (Puigserver & Spiegelman, 2003). PGC-1α is included in the PGC family together with PGC-1β (Lin et al., 2002) and PGC-related coactivator (PRC) (Andersson & Scarpulla, 2001). PGC-1α has been demonstrated to activate transcription factors regulating both nuclear and mitochondrial encoded genes including nuclear respiratory factor (NRF)-1 and -2 that binds to the promoter region of a broad range of nuclear genes encoding mitochondrial proteins, including Cyt c, COXIV and the mitochondrial transcription factor, mitochondrial transcription factor A (TFAM), which is thought to execute the effects of PGC-1α on gene regulation in the mitochondria (Evans & Scarpulla, 1990; Virbasius et al., 1993) as well as estrogen-related receptor (ERR)α (Mootha et al., 2004) and the PPARs (Puigserver et al., 1998; Vega et al., 2000).
Furthermore, it has been suggested that transcription of PGC-1α is regulated by PGC-1α itself in an autoregulatory loop (Handschin et al., 2003).

### 5.1 PGC-1α-mediated adaptations

After the initial demonstration that PGC-1α regulated adaptive thermogenesis in BAT, effects of PGC-1α in skeletal muscle became evident. Thus a previous study demonstrated that muscle-specific overexpression of PGC-1α in mouse skeletal muscle converted otherwise white glycolytic muscles to red oxidative muscles (figure 8) with a concomitant activation of genes involved in mitochondrial oxidative metabolism (Lin et al., 2002). In accordance, whole body PGC-1α knockout (KO) mice have been shown to have reduced expression of oxidative proteins in skeletal muscle (Leick et al., 2008; Lin et al., 2002) and this has likewise been shown in muscle-specific PGC-1α KO (Geng et al., 2010). This demonstrates the important role of PGC-1α in mitochondrial biogenesis in skeletal muscle. Furthermore, capillarization increased (Arany et al., 2008) and decreased (Leick et al., 2009) with overexpression and knockout of PGC-1α, respectively, indicating an overall role of PGC-1α in oxidative capacity of skeletal muscle. Furthermore, functional effects of muscle-specific overexpression of PGC-1α has been demonstrated by Calvo et al. with improvements in exercise performance and lowered respiratory exchange ratio (RER) during exercise reflecting increased fat oxidation (Calvo et al., 2008) as seen with exercise training (Henriksson, 1977; Kiens et al., 1993).

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Hindlimb and gastrocnemius/soleus (asterisk) muscle from wildtype (WT) and muscle-specific PGC-1α overexpression mouse strain (Tg) demonstrating the pronounced phenotypic change in color from white to red when PGC-1α is overexpressed (Lin et al., 2002).

### 5.2 Regulation of PGC-1α expression

#### 5.2.1 Acute exercise

In skeletal muscle PGC-1α was first demonstrated to be activated upon cold-exposure (Puigserver et al., 1998). In human skeletal muscle PGC-1α transcription and mRNA has been demonstrated to increase in
recovery from an acute exercise bout in both type I and II fibers (Leick et al., 2010b; Norrbom et al., 2004; Pilegaard et al., 2002). Studies in rodents have shown that PGC-1 mRNA increases with an acute exercise bout (Baar et al., 2002; Leick et al., 2008; Terada et al., 2002). Furthermore, PGC-1α protein has also been reported to increase in response to an acute exercise bout in humans and rats (Baar et al., 2002; Little et al., 2010a).

Sutherland et al. (Sutherland et al., 2009) has shown an increase in PGC-1α mRNA content in epididymal and retroperitoneal adipose tissue in response to an acute exercise bout and after exercise training in rats. Recently it has been demonstrated that lack of PGC-1α in adipose tissue reduces expression of mitochondrial proteins (Kleiner et al., 2012). In addition, PGC-1α mRNA content has been shown to increase in mouse liver immediately after an acute exercise bout although the fold change is very modest (Banzet et al., 2009; Hoene et al., 2009).

5.2.2 Exercise training
In human subjects 6 weeks of exercise training increases the basal mRNA content of PGC-1α in vastus lateralis muscle (Russell et al., 2003). In addition, 4 weeks of endurance exercise training enhances the exercise-induced response of PGC-1α transcription and mRNA while the exercise-induced response of other metabolic enzymes is reduced after exercise training as mentioned above (Pilegaard et al., 2003).

Furthermore, PGC-1α mRNA content has also been reported to be higher in rat epididymal and retroperitoneal adipose tissue after 4 weeks of exercise training (Sutherland et al., 2009).

5.2.3 Aging
Skeletal muscle PGC-1α mRNA expression is reduced in elderly compared with young subjects (Ling et al., 2004) and the age-associated decrease in skeletal muscle PGC-1α may therefore contribute to age-associated decrease in mitochondrial content. In addition, exercise training can counteract the age-associated decrease in mitochondrial proteins in WT mice but not in PGC-1α KO mice (Leick et al., 2010a) and transgenic PGC-1α mice overexpressing PGC-1α in skeletal muscle are largely protected against several mitochondrial myopathies and age-associated diseases (Wenz et al., 2009). In addition, a previous study has shown that although lifelong exercise trained subjects had higher level of mitochondrial enzyme activity, elderly untrained subjects demonstrated a marked exercise-induced PGC-1α mRNA response similar to young subjects (Iversen et al., 2011). Together this suggests that reduced physical activity with increasing age at least in part contributes to the observed decline in mitochondrial proteins in skeletal muscle with aging (Booth et al., 2000) and that this can be prevented by maintaining a physically active lifestyle.
5.2.4 Ca\(^{2+}\) and reactive oxygen species signaling

Exercise-induced regulation of PGC-1\(\alpha\) expression has been suggested to be mediated by calcium (Ca\(^{2+}\)) signaling (Lin et al., 2002). Hence, constitutively active Ca\(^{2+}\)-calmodulin-dependent protein kinase (CaMK) has been shown to regulate PGC-1\(\alpha\) in mice skeletal muscle (Wu et al., 2002). Furthermore, primary rat skeletal muscle cell culture treated with ionomycin (a calcium ionophore) demonstrated an up-regulation of PGC-1\(\alpha\) while this response was absent in the presence of inhibitors of calcium signaling (Kusuhara et al., 2007). In accordance, the induction of PGC-1\(\alpha\) with electrical stimulation of in vivo incubated rat extensor digitorum longus (EDL) muscle was abolished when the stimulation was performed with presence of Ca\(^{2+}\) signal inhibition (Kusuhara et al., 2007). It is well known that cytosolic Ca\(^{2+}\) increases during an acute exercise bout (Adhihetty et al., 2003; Booth & Baldwin, 1996; Williams & Neufer, 1996) and Ca\(^{2+}\) has been suggested as an important signal for exercise-induced adaptations. Experiments in mice expressing a constitutively active form of calcineurin in skeletal muscle has been demonstrated to have increased expression of genes involved in lipid metabolism and mitochondrial biogenesis (Long et al., 2007).

Reactive oxygen species (ROS) are formed during physiological processes (Radák et al., 1999) and during acute exercise (Gomez-Cabrera et al., 2008) and it has been suggested that the main ROS production during exercise takes place in the mitochondria (Adhihetty et al., 2003). There is increasing evidence that ROS not only are toxic but also play an important role in gene regulation and adaptation to exercise training. In agreement with this possibility, electrical stimulation of primary rat skeletal muscle cell culture elicited an increase in PGC-1\(\alpha\) mRNA 3 hours after the end of stimulation. But this PGC-1\(\alpha\) mRNA response was absent when the experiment was performed in the presence of a mixture of anti-oxidants indicating that ROS are required for contraction-induced increase in PGC-1\(\alpha\) transcription (Silveira et al., 2006).

5.3 Post-translational regulation of PGC-1\(\alpha\)

The PGC-1\(\alpha\) protein has been reported to be regulated by various post-translational mechanisms, including activation by AMP-activated protein kinase (AMPK)-mediated phosphorylation of Thr\(^{177}\) and Ser\(^{538}\) (Jager et al., 2007) and by sirtuin 1 (SIRT1)-mediated deacetylation of several lysine residues (figure 9) (Canto et al., 2009; Gerhart-Hines et al., 2007; Lagouge et al., 2006; Nemoto et al., 2005; Rodgers et al., 2005). As exercise increases AMPK phosphorylation and activity (Jorgensen et al., 2007), AMPK-mediated phosphorylation and activation of PGC-1\(\alpha\) has been suggested as an additional regulatory mechanism during exercise and PGC-1\(\alpha\) deacetylation has been reported in response to a single exercise bout (Canto et al., 2009). Furthermore, it has been suggested that AMPK primes PGC-1\(\alpha\) for SIRT1-mediated deacetylation (Canto et al., 2009), and this may increase the activity of PGC-1\(\alpha\) and thereby regulate transcription of mitochondrial proteins and in a positive feedback loop regulate its own transcription (Handschin et al., 2003).
Taken together PGC-1α seem to be an important factor in the regulation of exercise training-induced adaptations in oxidative protein expression, but similar adaptations has been shown with the synthetically produced compound resveratrol.

6 Resveratrol-induced adaptations

6.1 Resveratrol (3,5,4’-trihydroxy-trans-stilbene)

Resveratrol is a stilbenoid, a naturally occurring polyphenol that can act as a phytoalexin which is produced in plants, by the enzyme resveratrol synthase, when they are attacked by pathogens (Figure 10). Resveratrol is mainly found in the skin of red grapes, blueberries and peanuts.
Caloric restriction has in *Saccharomyces cerevisiae* been shown to extent lifespan and the effects is suggested to be mediated through activity of the gene SIR2 (Howitz et al., 2003). Like caloric restriction, resveratrol supplementation has been demonstrated to activate the mammalian homologue to SIR2, the NAD⁺-dependent deacetylase SIRT1 (Howitz et al., 2003). In addition, some of the first studies in mice have also shown beneficial effects of resveratrol on health and survival of mice on a high-fat diet (Barger et al., 2008; Baur et al., 2006; Lagouge et al., 2006). Resveratrol supplementation has been shown to improve endurance and mitochondrial function in mice gastrocnemius muscle (Lagouge et al., 2006; Menzies et al., 2013; Price et al., 2012; Um et al., 2010). These effects of resveratrol on lifespan, exercise performance and mitochondrial biogenesis in mice has been suggested to occur by activation of SIRT1 expression but muscle-specific knockout of SIRT1 does not inhibit exercise- or resveratrol-induced adaptations while the synergistic effect of exercise and resveratrol require SIRT1 (Menzies et al., 2013). In addition, metabolic effects of resveratrol on skeletal muscle and adipose tissue involve AMPK (Baur et al., 2006; Um et al., 2010).

Both resveratrol and exercise training have been shown to activate AMPK (Baur et al., 2006; Jorgensen et al., 2006) and NAD⁺-dependent SIRT1 (Lagouge et al., 2006; Little et al., 2010b).
Objectives of the thesis

The overall aims of the present thesis were:

- To test the hypothesis that physical inactivity changes exercise-induced mRNA and miRNA expression in human skeletal muscle (Study I)

- To test the hypothesis that PGC-1α is required for exercise- and exercise training-induced regulation of metabolic proteins in mouse liver (Study II)

- To test the hypothesis that PGC-1α is required for exercise-induced regulation of brown adipose tissue markers in mouse white adipose tissue (Study III)

- To test the hypothesis that lifelong exercise-training and/or resveratrol supplementation can counteract age-related changes in skeletal muscle oxidative capacity in mice and that these effects require PGC-1α (Study IV)
Methods

7 Human study

7.1 Subjects
Twelve healthy, physically active male subjects with an average (mean ±SD) age of 26.2 ±5.3 years, weight 75.5 ±11.3 kg, height 181.7 ±6.3 cm, and body mass index 22.8 ±2.7 kg·m\(^{-2}\) participated in study I. Six of these subjects participated in an exercise trial. The average age, weight, height and body mass index of these subjects was 28.7 ±5.3 years, 82.2 ±12.3 kg, 183.1 ±7.6 cm, and 24.4 ±2.2 kg·m\(^{-2}\), respectively.

In study I subjects were placed in hospital beds during the bed rest period and were allowed to sit up for 5 hours/day. In addition, they were at all times transported in wheelchairs. During the bed rest period subjects were served regular healthy food (10-20 % energy from protein, 50-60 % energy from carbohydrates, 25-35 % energy from fat) ad libitum from the hospital kitchen at Rigshospitalet, Copenhagen, Denmark.

7.2 Oral glucose tolerance test
An oral glucose tolerance test (OGTT) was performed on all 12 subjects before the onset of bed rest and 6 days into the bed rest period. Each subject consumed 1g glucose/kg body weight dissolved in 6.67 ml water per gram glucose. Blood samples were obtained from an arm vein before and after 30, 60 and 120 minutes after glucose intake and the samples were analyzed for plasma insulin and glucose (Department of Clinical Biochemistry, Rigshospitalet).

7.3 Body composition and performance tests
Prior to the bed rest period and immediately after, fat and fat-free mass were measured on all 12 subjects using a dual-energy X-ray absorptiometry (DEXA) scanner (Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA). Determination of body composition by DEXA absorptiometry is very accurate with deviations of 1-2 % (Madsen et al., 1997).

Maximal oxygen uptake (VO\(_{2\text{max}}\)) was determined by an incremental bicycle test and leg muscle endurance by a one-legged knee extensor exercise test using the same absolute intensity before and after bed rest on all 12 subjects, starting with 15 minutes at 75 % of Watt\(_{\text{max}}\) (before bed rest level) followed by 90 % Watt\(_{\text{max}}\) (before bed rest level) until exhaustion.
To determine workload to be used during the acute exercise experiment, the 6 subjects taking part in the acute exercise protocol performed a one-legged knee extensor exercise performance test one week prior to the experimental day. The workload was gradually increased every 2 minutes, and the highest load which could be sustained for 2 minutes was set as $\text{Watt}_{\text{max}}$.

### 7.4 Acute exercise protocol

The 6 subjects in the acute exercise protocol of study I completed an identical experimental trial 4-10 days before initiation of the bed rest as well as on the 7th day of bed rest.

The day prior to the experimental day before bed rest, the subjects refrained from intense and prolonged exercise. The subjects were physically inactive the day before the experimental day after bed rest, because this was within the bed rest period. The day before both experimental trials, subjects consumed a prepackaged dinner and evening snack, which was standardized based on the body weight of the subject (60 and 12 kJ/kg body weight, respectively).

On the experimental day before bed rest, the subjects arrived at the laboratory by minimum of physical activity, and on the experimental day after bed rest they were transported in wheelchairs. On the morning of both experimental trials, the subjects consumed a standardized breakfast regulated for body weight (30 kJ/kg body weight). A muscle biopsy was obtained from the middle portion of the vastus lateralis muscle, using the percutaneous needle biopsy technique (Bergström J., 1975) with suction 3.5 hours after breakfast (Pre). This was followed by 45 minutes of one-legged knee extensor exercise at ~60% of $\text{Watt}_{\text{max}}$ (before bed rest level and thus the same absolute intensity before and after bed rest), using a modified ergometer bicycle (Monark Ergomedic 839E, Monark Exercise). Three subjects worked with their dominant leg and three with their nondominant leg. Additional muscle biopsies were obtained from the exercised leg immediately after exercise (Post) and at 3 hours of recovery (3h rec). All muscle biopsies were taken through separate incisions, quickly frozen in liquid nitrogen (<15 sec), and stored at -80°C until they were analyzed. A small part of the Pre biopsies was mounted in embedding medium, frozen in isopentane precooled in liquid nitrogen and stored at -80°C. Furthermore, a catheter was placed in the femoral artery of one leg, and blood samples were obtained before (Pre), during (20 min), and immediately after (Post) exercise.
8 Mouse models

8.1 Phenotype of whole body PGC-1α KO mice

The PGC-1α whole body KO mouse strain was used in study II, III and IV. Whole body PGC-1α KO mice are born at the expected Mendelian ratio (Lin et al., 2004), however only half of the KO mice survive the early postnatal period and grow into adults (Lin et al., 2004). Furthermore, PGC-1α KO mice are infertile and have a body weight 15% lower than WT littermates at the start of the experiments. Furthermore, they exhibit behavioral characteristics and are quite easy to distinguish from WT and heterozygous mice. These phenotypic characteristics include exaggerated responses to unexpected stimuli and abnormal postures. Despite these differences PGC-1α KO mice seem to survive to similar age as WT mice.

8.2 Generation of whole body PGC-1α KO mice

To generate PGC-1α−/− mouse strain a homologous recombination method was used. A plasmid containing a loxP sites flanking exons 3-5 of the PGC-1α gene was constructed (Figure 11) and a cre recombinase was used to generate the PGC-1α knockout mouse strain (Lin et al., 2004) (study II, III and IV). The recombination results in a deletion of the LXXL L motif that mediates the interaction of PGC-1α with nuclear receptors. PGC-1α KO and WT mice were obtained by intercross breeding of heterozygous parents and homozygous offspring were used for experiments (Lin et al., 2004).

Animals were genotyped by extraction of genomic DNA followed by PCR using specific WT primers 5’ CTTCCATGTGTCAGTGAC 3’ and 5’ GGATGAGTAGTATCGTAC 3’ and specific KO primers 5’ TCCAGTAGCAGAGATTATGAC 3’ and 5’ CCAACTGTCTATAATTCCAGTC 3’. The amplified products for WT and PGC-1α KO alleles are 240 bp and 460 bp, respectively (Figure 12).
8.3 Acute exercise protocol/single exercise bout

In study II and III, male whole body PGC-1α KO and WT littermates of 3-4 month of age performed an acute exercise protocol.

Prior to the single exercise protocol, mice were acclimatized to treadmill exercise (TSE systems GmbH, Bad Homburg, Germany) two times 10 minutes a day on five consecutive days. Each 10 minute exercise period consisted of 2 minutes at 8 m/min, 2 minutes at 10 m/min, 4 minutes at 15 m/min and 2 minutes at 10 m/min, with a constant slope of 10%.

Whole body PGC-1α KO mice and WT littermates performed a single treadmill running bout, 48 hours after the end of adaptation to treadmill running, at 14 m/min at 10% slope for 1 hour. Mice were sacrificed by cervical dislocation immediately after (0h), 2 (2h), 6 (6h) and 10 (10h) hours after running, while mice not run acutely served as controls. Mice were euthanized by cervical dislocation 36-37 hours after the last exercise bout and skeletal muscles, liver and adipose tissue (inguinal and epididymal) was removed and quickly frozen in liquid nitrogen for later analyses.

8.4 Treadmill exercise training

In addition to the acute exercise bout in study II and III, a group of male whole body PGC-1α KO and WT littermates of 3 month of age were exercise trained on a treadmill for 1 hour 5 times/week for 5 weeks and had access to running wheels during the exercise period (Leick et al., 2007; Leick et al., 2009) with a control group not training. Running wheels of WT mice were blocked to ensure similar running duration and total running duration per day was 106 ±10 minutes in WT and 98 ±13 minutes PGC-1α KO mice per day.
8.5 Lifelong exercise training and resveratrol supplementation protocol

In study IV female whole body PGC-1α KO and WT littermate mice were from 3 month of age randomly placed in groups with access to running wheel, getting either standard chow or standard chow with a resveratrol supplement of 4 g/kg food (Orchid Chemicals & Pharmaceuticals Ltd., Chennai, India). WT running wheels had to be blocked once a day to obtain similar total running distance in PGC-1α KO and WT, with 98 ±13 and 106 ±10 minutes, respectively. In addition, PGC-1α KO and WT mice were placed in control groups not exercising while getting either standard chow or standard chow with resveratrol supplementation of 4 g/kg food and at 15 month of age (middle aged) mice were euthanized and quadriceps, triceps and EDL were quickly removed and frozen in liquid nitrogen for later analyses.

Running distance (Table 1) was noted every week while body weight and food intake was measured every second week.

Table 1. Study IV running distance and duration (km/week and hours/week):

<table>
<thead>
<tr>
<th>Month in experiment</th>
<th>WT Distance (km)</th>
<th>WT Time (hours)</th>
<th>PGC-1α KO Distance (km)</th>
<th>PGC-1α KO Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.45 ±1.11</td>
<td>3.11 ±0.02</td>
<td>6.98 ±0.64</td>
<td>4.52 ±0.01</td>
</tr>
<tr>
<td>2</td>
<td>6.78 ±0.45</td>
<td>3.12 ±0.01</td>
<td>6.07 ±0.14</td>
<td>4.40 ±0.00</td>
</tr>
<tr>
<td>3</td>
<td>7.00 ±0.67</td>
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<td>6.89 ±1.06</td>
<td>5.15 ±0.03</td>
</tr>
<tr>
<td>4</td>
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<td>7.33 ±1.40</td>
<td>5.45 ±0.02</td>
</tr>
<tr>
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<td>3.50 ±0.02</td>
<td>6.52 ±0.61</td>
<td>5.10 ±0.01</td>
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<tr>
<td>9</td>
<td>5.67 ±0.34</td>
<td>3.21 ±0.01</td>
<td>5.96 ±0.31</td>
<td>4.35 ±0.01</td>
</tr>
<tr>
<td>10</td>
<td>4.93 ±0.77</td>
<td>3.00 ±0.02</td>
<td>5.30 ±0.47</td>
<td>6.33 ±0.19</td>
</tr>
<tr>
<td>11</td>
<td>5.04 ±0.51</td>
<td>3.00 ±0.01</td>
<td>5.42 ±0.60</td>
<td>9.33 ±0.44</td>
</tr>
<tr>
<td>12</td>
<td>3.76 ±0.01</td>
<td>2.12 ±0.01</td>
<td>3.99 ±0.40</td>
<td>8.20 ±0.29</td>
</tr>
</tbody>
</table>

Running distance and duration (km/week and hours/week) from each month in experiment from wildtype (WT) and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α KO mice.

8.5.1 Performance test

Exercise capacity was in study IV determined by an incremental treadmill (TSE systems GmbH, Bad Homburg, Germany) running exercise test beginning at 12 m/min for 10 minutes with a slope of 10 % and increased with 2 m/min every 10 minutes until 20 m/min or less if mice repeatedly stopped running.
9 Analyses

9.1 Plasma adrenaline

In study I plasma adrenaline was measured using an adrenaline radioimmunoassay (RIA) kit (Millipore, Bedford, MA, USA). Plasma adrenaline was extracted by incubation of 10 µl standard, 10 µl control and 300 µl of the samples in assay buffer and extraction buffer, for 30 minutes at room temperature. This was followed by a washing step and incubation for 15 minutes in acylation buffer. After acylation, 150 µl hydrochloric acid (HCl) was added and the samples incubated for 10 minutes. After extraction and acylation, 100µl standard, control and samples were transferred to polystyrene tubes and 25 µl enzyme solution was added to each tube followed by incubation for 30 minutes at 37 °C. Radioactive adrenaline ($^{125}$I Adrenaline) was added to all standards, controls and samples and adrenaline antiserum was added to all tubes except for the blank sample and the samples were incubated overnight at 4 °C. One ml of precipitation reagent was added and all tubes incubated at 4 °C for 15 minutes followed by centrifugation for 15 minutes at 3000 g. The supernatant was discarded and each tube was left to dry for 2 minutes and gamma rays were counted in a gamma counter (Cobra Auto-gamma, Packard).

9.2 Immunostaining

9.2.1 Muscle fixation

In study I and IV vastus lateralis and EDL muscle biopsies, respectively, were mounted in Tissue-Tek (Sakura Finetek, Alphen aan den Rijn, The Netherlands), frozen in ice-cold isopentane and stored at -80 °C for fiber type composition and capillary staining.

In study IV one triceps muscle was removed and fixed 4 hours (30 minutes at room temperature and 3.5 hours at 4 °C) at immersion into 2 % paraformaldehyde supplemented with 0.15 % picric acid for single fiber immunostaining. Finally, the muscle sample was teased into bundles of 10-20 fibers and put into a vial with phosphate buffered saline (PBS) + glycerol (50:50) overnight at 4 °C. Fibers were stored at -20 °C for later analysis.

9.2.2 Fiber type composition

In study I mounted vastus lateralis biopsies were cut in serial size transverse sections, which were stained for myofibrillar adenosine triphosphatase to identify fibers as myosin heavy chain (MHC) type I, type IIa, or type IIx (Brooke & Kaiser, 1970) or with the amylase-p-aminosalicylic acid method to visualize capillaries (Andersen, 1975). Fiber type, fiber area, and capillary density were determined using a COMFAS image.
scanner (SBsysCOMFAS; Scan Beam, Hadsund, Denmark). This part of the analysis was performed by Thomas Bonne.

9.2.3 Immunostaining of capillaries

In study IV, the number of capillaries in the muscle samples was determined on 8 µm transverse sections of frozen samples of the EDL muscle. The sections were air-dried for 10 minutes and then fixed in -20 °C acetone for 30 seconds. The sections were then again air-dried and incubated for 1 hour in lectin (1:200; Vector B-1105, Vector Laboratories Inc., Burlingame, CA, USA). After washing the sections in PBS they were incubated in FITC-conjugated streptavidin (Dako, Glostrup, Denmark) for 1 hour in the dark. The sections were carefully washed before they were mounted in Vectashield H-1000 on a glass slide and images were obtained using a Zeiss Axioplan microscope.

For each muscle sample all analyses were performed on a minimum of three different areas for large samples and whole samples if small. The origin of all pictures was blinded for the observer before quantification of fibers and capillaries. The capillary-to-fiber ratios (C:F) and the number of muscle fibers and capillaries was counted according to Gundersen’s rule A estimating the number of objects per area unbiased by edge effect (Gundersen, 1978). C:F ratio was defined as the total number of capillaries per total number of fibers.

9.2.4 Immunostaining of single muscle fibers

In study IV single muscle fibers were stained for the mitochondrial marker COXIV. Before staining the triceps muscle fibers bundles were teased into single fibers with fine forceps and transferred to immuno buffer containing 50 mM glycine, 0.25 % bovine serum albumin (BSA), 0.03 % saponin and 0.05 % sodium azide in PBS.

Single fibers were then incubated over night at room temperature with primary anti-COX IV (#16056, Abcam, Cambridge, UK) antibody diluted in immuno buffer. After three washes of 15 minutes, the single fibers were incubated for 2 hours with Alexa-488 (Molecular Probes, Life Technologies, Nærum, Denmark) conjugated secondary antibody diluted in immuno buffer. Fibers were then washed 3 times 15 minutes with immuno buffer, 1 time 15 minutes with PBS and then mounted in Vectashield H-1000 on a glass slide. Images aquisition was performed with a Zeiss LSM 710 (Carl Zeiss, Jena, Germany; Zeiss Zen software version 2010) confocal microscope.
9.3 Glycogen

Before analyses human skeletal muscle tissue was freeze-dried and dissected free of blood, connective tissue and fat while mouse liver and skeletal muscle tissue was crushed to obtain homogeneity of samples.

9.3.1 Muscle and liver glycogen content

Muscle and liver glycogen content was determined as glycosyl units after acid hydrolysis (Lowry & Passonneau, 1972; Passonneau et al., 1967) using 400 µg of muscle homogenate protein or powdered muscle specimens and a fluoroscan (Thermo Labsystems, Bie & Berntsen, Rødovre, Denmark).

In study I muscle glycogen content was determined on muscle homogenate obtained by lysis of muscle specimens in TissueLyser II (Qiagen, Valencia, CA, USA), while liver glycogen in study II and muscle glycogen in study III-IV were determined directly from powdered muscle specimens. Homogenate or powdered liver and muscle specimens were boiled for 2 hours in hydrochloric acid (HCl) which degraded the muscle glycogen into glycosyl units. Sodium hydroxide (NaOH) was added to neutralize the acid and HEPES was added as a buffering agent. After centrifugation the liquid phase was transferred to an eppendorf tube and diluted x times for further analysis.

The measurements are based on the conversion of glucose-6-phosphate (G-6-P) to 6-phosphoglucono-δ-lactone (6-PG) and NADPH by glucose-6-phosphate dehydrogenase (glu-6-PDH) and that NADPH like NADH is naturally fluorescent.

The analytical reactions are:

\[ \text{glucose} + \text{ATP} \xrightarrow{HKII} \text{glucose} - 6 - \text{phosphate} + \text{ADP} + P_i \]

\[ \text{glucose} - 6 - \text{phosphate} + \text{NADPH} \xrightarrow{\text{glu-6-PDH}} \text{δ - phosphoglucono - δ - lactone} + \text{NADPH} + H^+ \]

Before adding hexokinase to the samples, a control measurement was obtained followed by the conversion of glucose into G-6-P. This step was followed by oxidation of G-6-P to 6-PG and NADPH by glu-6-PDH in the ratio 1:1. The fluorescence of NADPH was measured in the fluoroscan (Thermo Labsystems, Bie & Berntsen, Rødovre, Denmark) and the fluorescence measured is proportional to the glycogen content in each sample.

9.4 Plasma glucose

In study II and IV plasma glucose was determined using Contour glucose strips (Bayer Diabetes Care, Stockholm, Sweden).
9.5 Enzyme activity

In study I muscle tissue were freeze-dried and dissected free of blood, connective tissue and fat whereas in study IV wet weight muscle samples were used. Homogenate was produced in phosphate buffer and diluted 400 and 80 times, respectively, for CS activity measurements.

9.5.1 Citrate synthase

In study I maximal activity of CS was measured in vastus lateralis muscle sample while in study IV it was measured in quadriceps muscle by fluorometric analysis (Lowry & Passonneau, 1972).

CS catalyzes the reaction between acetyl-coenzyme (CoA) and oxaloacetate to form citric acid (citrate). Hydrolysis of the thioester of acetyl-CoA results in the formation of CoA-SH (CoA with a thiol group). The thiol group reacts with the DTNB in the reaction mixture to form the yellow product NTB, which is determined in the Multiscan (Thermo Labsystems, Bie & Berntsen, Rødovre, Denmark) by measuring absorbance at 412 nm.

Determination of CS activity is based on the following assay reaction:

\[ \text{CoA} - \text{SH} + \text{DTNB} (2 - \text{nitrobenzoic acid}) \rightarrow \text{CoA} - S - S - \text{TNB} (2 - \text{nitro} - S - \text{thiobenzoate}) \]

Activity is calculated based on the following equation (1):

\[
\text{Units (} \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \text{dw}) = \Delta \text{Abs} 412 \text{ nm} \cdot \text{min}^{-1} \cdot 0.2 \text{ ml} \cdot \text{dilution} / (13.6 \cdot 0.552 \text{ cm} \cdot V_{\text{enz ml}})
\]

Where 0.2 ml is the reaction volume, 13.6 is the extinction coefficient for TNB at 412 nm and 0.552 is the light path length of the well.

9.5.2 β-hydroxyacyl-CoA dehydrogenase

HAD catalyzes the reaction between (oxidation of) β-hydroxyacyl-CoA and NAD\(^+\) to form NADH, H\(^+\) and β-ketoacyl-CoA which can further be converted to acetyl-CoA and be metabolized in the citric acid cycle.

Determination of HAD activity is based on the following assay reaction:

\[ \text{S} - \text{acetoacetyl} - \text{CoA} + \text{NAD}^+ + \text{H}^+ \xrightarrow{\text{HAD}} \text{L} - 3 - \text{hydroxybutyryl} - \text{S} - \text{CoA} + \text{NAD}^+ \]

Activity is calculated based on the following equation (2):

\[
\text{Units (} \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \text{dw}) = \frac{\Delta \text{Emission} \cdot \text{min}^{-1} \cdot \text{sample dilution} \cdot \text{assay dilution}}{\text{std curve slope}}
\]
9.6 DNA isolation

In study I and IV, total DNA was isolated to determine mitochondrial DNA content. From ~10 mg of muscle tissue total DNA was extracted by use of the tris-saturated phenol:chloroform method (Pilegaard et al., 2000). After addition of phenol:chloroform and water phases were separated by centrifugation at 12000 g for 10 minutes at 4 °C and the upper phase (aqueous phase) was transferred to new tubes. Forty µl of 3M NaOAc as well as 800 µl 100 % ice cold ethanol (EtOH) was added and samples incubated at -20°C for at least 10 minutes. DNA was precipitated by centrifugation at 12000 g at 4 °C for 10 minutes and the pellet was washed in 1 ml ice-cold 75 % EtOH (in diethyl pyrocarbonate (DEPC) H₂O) twice followed by centrifugation at 12000 g for 5 minutes at 4 °C. DNA pellets were vacuum dried with a sterile pipette tip and resuspended in 25 µl distilled H₂O and stored at -80 °C until analyzed (Pilegaard et al., 2000).

9.7 RNA isolation

Total RNA for mRNA and microRNA analyses was isolated from ~20 mg of tissue by a modified guanidinium thiocyanate (GT)-phenol-chloroform extraction method adapted from Chomczynski & Sacchi (Chomczynski & Sacchi, 1987). The samples were placed in 2 ml of cold GT solution and homogenized for 2 minutes at 30 sec⁻¹ in a TissueLyser II (Qiagen, Valencia, CA, USA). Total RNA was extracted by addition of 700 µl DEPC-saturated phenol, 70 µl 2M NaOAc, pH 4.0 and 175 µl chloroform:isoamyl-OH (49:1), vigorously shaking and incubated on ice for 15 minutes followed by centrifugation at 12.000g for 20 minutes at 4 °C. Total RNA was precipitated from the aqueous phase by addition of an equal amount of isopropanol, incubation for 15 minutes at -20 °C, and centrifugation for 10 minutes at 12.000 g. The resulting pellets were rinsed two times in 75 % EtOH and resuspended in nuclease-free H₂O containing 0.1 mM ethylenediamine tetraacetate (EDTA). RNA was quantified using a NanoDrop 1000 (Thermo Scientific, Rockford, IL, USA), measuring absorbance at 260 nm and purity of the RNA samples was verified as the ratio between 260/280 nm and all samples was above 1.7.

9.7.1 Reverse transcription

9.7.1.1 mRNA

Reverse transcription of total RNA into cDNA was performed using Superscript II RNase H⁻ system and Oligo dT (Invitrogen, Carlsbad, CA, USA). Same amount of RNA was transferred to small eppendorf tubes and distilled H₂O was added to 11 µl followed by 1 µl of Oligo dT which was incubated at 65 °C for 5 minutes to denaturate the RNA. RNA samples was placed on ice and after quick spinning condensation down a mixture of first strand buffer, DTT and dNTP was added and samples incubated at 42 °C for 2 minutes to get the optimal temperature for the Superscript enzyme, which was added hereafter and samples were left at
42 °C for 50 minutes for the first strand reaction to occur. The reaction was heat-inactivated for 15 minutes at 70 °C. Before PCR analyses a standard dilution series was made and cDNA samples were diluted to 70 µl/µg cDNA for muscle and liver and 120 µl/µg cDNA for adipose samples.

9.7.1.2 miRNA
Before reverse transcription of miRNA, RNA samples were diluted to 2 ng RNA/µl. Each specific miRNA was then reverse transcribed to cDNA by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and miRNA-specific primers (Applied Biosystems, Foster City, CA, USA). The reaction was run in a thermal cycler (PTC-200; MJ Research, Waltham, MA, USA).

9.7.2 Real time PCR
9.7.2.1 DNA and mRNA
mRNA content of a given gene as well as mitochondrial (mt)DNA (represented by COX II in study I and COX I in study IV) and nuclear (n)DNA (represented by Cyt c) were determined in triplicates by real-time PCR using the fluorogenic 5’ nuclease assay with 1 µl of cDNA product in a total volume of 10 µl of reaction mixture with Universal mastermix, containing “hot start” DNA-polymerase AmpliTaq Gold, dNTPs, Mg²⁺ and Cl⁻ ions, AmpErase urasil N-glycolase (UNG) and ROX reference dye (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Rockford, IL, USA), forward and reverse primers and 5’-6-carboxyfluorescein (FAM)/3’-6-carboxy-N,N,N’,N’-tetramethylrhodamine (TAMRA) labeled TaqMan probes (Table 2).
Table 2. Primer and probe sequences used in real time PCR.

<table>
<thead>
<tr>
<th>Human genes</th>
<th>Primer and TaqMan probe sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA</strong></td>
<td><strong>Forward primer</strong></td>
</tr>
<tr>
<td>COX II</td>
<td>5’GATCCCTTACATCCATCAATC3’</td>
</tr>
<tr>
<td>Cyt c</td>
<td>5’GGTCTCTTTGGGCGGAAGAC3’</td>
</tr>
<tr>
<td>mRNA</td>
<td><strong>Forward primer</strong></td>
</tr>
<tr>
<td>CS</td>
<td>5’GACCTACATCTGGAACACTCAACTCA3’</td>
</tr>
<tr>
<td>Cyt c</td>
<td>5’GGTCTCTTTGGGCGGAAGAC3’</td>
</tr>
<tr>
<td>HAD</td>
<td>5’CATAGGCGACCAGCAAGATG3’</td>
</tr>
<tr>
<td>HKII</td>
<td>5’TGTGACGTGAAACCTGCCTCACTAT3’</td>
</tr>
<tr>
<td>PGC-1a</td>
<td>5’CAAGCCCAACCAACACATCTCTGCT3’</td>
</tr>
<tr>
<td>SIRT1</td>
<td>5’TAGAGGCTTCACATCAAGCTGCTT3’</td>
</tr>
<tr>
<td>VEGF</td>
<td>5’CTGAGCTCTTACCTCCACAGGCT3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse genes</th>
<th>Primer and TaqMan probe sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA</strong></td>
<td><strong>Forward primer</strong></td>
</tr>
<tr>
<td>COXI</td>
<td>5’TGCAAGCTTACCCCTGCTAATG3’</td>
</tr>
<tr>
<td>Cyt c</td>
<td>5’TGCGCAGTGGCCACACTGTT3’</td>
</tr>
<tr>
<td><strong>mRNA</strong></td>
<td><strong>Forward primer</strong></td>
</tr>
<tr>
<td>PRDM16</td>
<td>5’CGAGCAAGGTAAAGCCATTCC3’</td>
</tr>
<tr>
<td>UCP1</td>
<td>5’AGGGTACATCTGCGGCGGA3’</td>
</tr>
</tbody>
</table>

Forward and reverse primer and TaqMan probe sequences used in real time PCR for human and mouse genes. COX; cytochrome c oxidase, CS; citrate synthase, Cyt c; cytochrome c, HAD; β-hydroxacyl-CoA dehydrogenase, HKII; hexokinase, PRDM16; protein-containing PR (PRD1-BF-1-RIZ1 homologous) domain 16, SIRT1; sirtuin 1, UCP1; uncoupling protein1, VEGF; vascular endothelial growth factor.

PCR was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by use of the general cycle profile, 50 °C for 2 minutes (activation of AmpErase UNG and degradation of products containing dUTP), 95 °C for 10 minutes (inactivation of AmpErase UNG in addition to activation of “hot start” DNA polymerase) followed by 40 cycles of 95 °C for 15 seconds (denaturation of cDNA) and 60 °C for 1 minute (annealing of primers and probe followed by replication of the specific cDNA). The obtained cycle threshold (Ct) values reflecting the initial content of the specific transcript in the samples were converted to an arbitrary amount by using standard curves obtained from a serial dilution of a pooled sample made from all samples. The amount of a given mRNA was normalized to the ssDNA content of the cDNA sample, whereas DNA results were presented as mtDNA normalized to nDNA content (mtDNA/nDNA).
9.7.2.2 miRNA
The content of a given miRNA was determined by real-time PCR (as described above) using predeveloped miRNA assays containing specific primers and TaqMan probes labeled with 5’-6-carboxyfluorescein and minor groove binder quencher (non-fluorescent) (Applied Biosystems, Foster City, CA, USA). The obtained cycle threshold (Ct) values reflecting the initial content of the specific miRNA in the samples were converted to an arbitrary amount by using standard curves obtained from a serial dilution of a pooled sample made from all samples. The amount of a given miRNA was normalized to either the RNU6B content or the RNU48 content of the sample. RNU6B was unaffected by acute exercise but affected by bed rest in resting samples, whereas RNU48 was unaffected by bed rest in resting samples but changed by acute exercise. Therefore, RNU6B was used as endogenous control for the acute exercise samples and RNU48 for resting miRNA levels.

9.8 Lysate preparation
Muscle lysate was in study I obtained from freeze-dried muscle specimens that were dissected free of blood, fat and connective tissue under the microscope while in study II, III and IV muscle and adipose tissue lysate was prepared from wet muscle and adipose specimens. Tissue samples were homogenized in an ice-cold buffer (1:80 for freeze-dried muscle tissue and 1:20 for wet muscle tissue and 1:10 for adipose tissue) containing buffering agent, detergent, hydrolases, chelators and protein phosphatase and protease inhibitors for 2 minutes at 30 sec$^{-1}$ in TissueLyser II (Qiagen, Valencia, CA, USA), rotated end-over-end for 1 hour and centrifuged for 20 minutes at 16,000 g and 4 °C.

9.8.1 Protein determination
Protein content in lysates was determined by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA) that is based on the reaction of protein in the sample and Cu$^{2+}$ ions in the Pierce reagent which is converted to Cu$^{+}$ ions. Standards, samples and Pierce reagent (49/50 reagent A and 1/50 reagent B) was added to a microtiterplate and it incubated at 37 °C for 30 minutes for complex binding of Cu$^{+}$ ions and bicinchoninic acid (BCA). The absorbance of the Cu$^{+}$-BCA complex was measured at 550 nm in a Multiscan Emax Precision microtiterplate reader (Thermo Labsystems, Bie & Berntsen, Rødovre, Denmark).

Lysates were prepared with sample buffer containing sodium dodecyl sulfate (SDS) in a concentration of 2 µg protein/µl and boiled for 3 minutes at 96 °C and analyzed by SDS-PAGE and Western blotting.

9.9 SDS-PAGE and Western blotting
Protein phosphorylations and protein content as well as total proteins were measured by SDS-PAGE and Western blotting. After separating proteins using Tris-HCl gels (Bio-Rad Laboratories, CA, USA) or
homemade acrylamide gels, proteins were transferred by semidry transfer to PVDF-membranes (Immobilon Transfer Membrane, Millipore A/S, Denmark). PVDF membranes were blocked in Tris-buffered saline with Tween-20 (TBST) + 3% skimmed milk or fish gel and incubated with primary antibodies (Table 3) in 3% BSA or skimmed milk, followed by incubation with horseradish peroxidase (HRP)-conjugated polyclonal secondary antibody (Table 3) in TBST + 3% skimmed milk or fish gel. Acetyl CoA carboxylase (ACC) protein, which is a biotin-dependent enzyme, was detected by streptavidin-HRP which recognizes biotin side chains. Proteins were detected and quantified using Kodak Image Station (2000-2200MM, Kodak, Denmark) and Carestream Image Station and Carestream MI 5.0 SE software. Protein phosphorylation and content are expressed in units relative to control samples loaded on each side of each gel and by loading a standard dilution series it was ensured that the quantification was within the linear range for each protein.

### Table 3. Antibodies and dilutions used for Western blotting.

<table>
<thead>
<tr>
<th>Antibodies and dilutions used for Western blotting</th>
<th>Primary antibodies</th>
<th>Secondary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACC</strong>&lt;sub&gt;ser79&lt;/sub&gt; phos.</td>
<td>1:1000 (Upstate Biotech.)</td>
<td>1:3000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>ACC</td>
<td>Detected only by use of 1:2000 streptavidin-HRP-conjugated (Dako)</td>
<td></td>
</tr>
<tr>
<td>AMPK&lt;sub&gt;Thr172&lt;/sub&gt; phos.</td>
<td>1:1000 (Cell Signaling Tech.)</td>
<td>1:5000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>AMPKα2</td>
<td>1:10000 (kindly donated by D. G. Hardie, University of Dundee, UK)</td>
<td>1:5000, anti-sheep HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:2000 (Cell Signaling Tech.)</td>
<td>1:1000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>CD31/PECAM</td>
<td>1:500 (Santa Cruz Biotech.)</td>
<td>1:500, anti-goat HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>COX I</td>
<td>1:3000 (Invitrogen)</td>
<td>1:3000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>COX IV</td>
<td>1:1000 (Abcam)</td>
<td>1:4000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>Cyt c</td>
<td>1:10000 (BD Biosciences)</td>
<td>1:10000, anti-mouse HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>HKII</td>
<td>1:1000 (Cell Signaling Tech.)</td>
<td>1:500, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1:2000 (Cell Signaling Tech.)</td>
<td>1:10000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>PDH-E1α</td>
<td>1:1000 (kindly donated by D. G. Hardie, University of Dundee, UK)</td>
<td>1:5000, anti-sheep HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>SIRT1</td>
<td>1:1000 (Cell Signaling Tech.)</td>
<td>1:1000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>UCP1</td>
<td>1:2000 (Abcam)</td>
<td>1:2000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
<tr>
<td>VEGF</td>
<td>1:500 (Santa Cruz Biotech.)</td>
<td>1:1000, anti-rabbit HRP-conjugated (Dako)</td>
</tr>
</tbody>
</table>

Primary and secondary antibodies and dilutions used for Western blotting. ACC; acetyl-CoA carboxylase, AMPK; AMP-activated protein kinase, CD31; cluster of differentiation 31, COX; cytochrome c oxidase, Cyt c; cytochrome c, HKII; hexokinase II, GAPDH; glyceraldehydes 3-phosphate dehydrogenase, PDH-E1α; pyruvate dehydrogenase E1α, SIRT1; sirtuin 1, UCP1; uncoupling protein 1, VEGF; vascular endothelial growth factor.

### 9.10 Statistics and calculations

Values presented are means ±SE. Two-way analysis of variance (for repeated measures in study I) was applied to evaluate the effect of two factors (bed rest and time; genotype and time; genotype and training; genotype and intervention) on the measured parameters and the Student-Newman-keuls post hoc test was
applied to locate differences. A t-test (in study I a paired t-test) was used for evaluating intervention effect or genotype effect at the basal level. Differences were considered significant at $P \leq 0.05$, and a tendency is reported when $0.05 < P \leq 0.10$. Statistical calculations were performed in SigmaPlot version 11.0.
Results & discussion

10 Exercise-induced PGC-1α mRNA response

10.1 Skeletal muscle
In Study I it was demonstrated that the PGC-1α mRNA content in skeletal muscle from healthy human subjects increased in the recovery period from an acute exercise bout before bed rest, and this is in accordance with previous results (Nordsborg et al., 2010; Norrbom et al., 2004; Pilegaard et al., 2003) showing increased PGC-1α mRNA content in recovery from exercise in human skeletal muscle. In addition, an exercise-induced increase in PGC-1α mRNA is also observed in white gastrocnemius muscle from WT mice used in Study III (Figure 13), and this is in agreement with previous rodent studies (Baar et al., 2002; Leick et al., 2008; Pilegaard et al., 2003; Terada et al., 2002).

Of notice is that when PCR primers and TaqMan probe sequences located outside the deleted part in PGC-1α KO mice (Lin et al., 2004) are used to amplify a fragment of PGC-1α cDNA an exercise-induced increase in PGC-1α mRNA is evident in white gastrocnemius from PGC-1α KO mice (Figure 13). Furthermore, the observation that the PGC-1α mRNA response is higher in PGC-1α KO than in WT muscle may indicate that lack of functional PGC-1α protein in PGC-1α KO mice elicits an attempt to compensate. However, because the basal level is similar a likely alternative explanation is that the higher response is because the PGC-1α KO mice have exercised at a higher relative intensity than WT mice.
It is observed that the mRNA content of PGC-1α is peaking within the first couple of hours of the recovery period in mouse skeletal muscle and this is very similar to the exercise-induced response reported in human skeletal muscle (Pilegaard et al., 2003) and in rats the increase in PGC-1α mRNA was evident immediately after exercise (Terada et al., 2002). The mice in the present thesis were exercised by treadmill running whereas the reported rat study used swimming exercise the difference in time course between rats and mice may be due to the different exercise protocol. In addition, it has previously been suggested that swimming exercise may elicit a high adrenaline response compared to running exercise (Krüger et al., 2008).

The exercise-induced increase in PGC-1α mRNA content has been suggested to originate from increases in transcription rate (Pilegaard et al., 2003) or increased mRNA stability (Lai et al., 2010). Based on a previous study (Lai et al., 2010) showing that chronic contractile activity reduces PGC-1α mRNA stability the increased PGC-1α mRNA content is most likely due to increased transcription of the gene encoding PGC-1α.

### 10.2 Liver

In Study II PGC-1α mRNA content did not increase significantly in the liver in recovery from acute exercise, but it has previously been demonstrated to increase significantly in WT mice liver immediately after a 1 hour running exercise (Hoene et al., 2009). Furthermore, in Study II 24 hours of fasting was demonstrated to increase PGC-1α mRNA content in WT mice which is in accordance with previous studies (Handschin et
al., 2005; Herzig et al., 2001; Yoon et al., 2001) and in contrast to the previous observation that fasting reduced PGC-1α expression in mouse skeletal muscle (Wu et al., 1999). These data imply that fasting and acute exercise induce similar PGC-1α mRNA responses in the liver although most markedly with fasting while skeletal muscle only increases PGC-1α mRNA in response to exercise and not fasting (Pilegaard et al., 2003; Wu et al., 1999).

10.3 Adipose tissue
PGC-1α mRNA content has previously been shown to increase in rat white adipose tissue immediately after swimming exercise (Sutherland et al., 2009) and but not in mouse iWAT, at 5 hours of recovery from 1 hour swimming exercise (Bostrom et al., 2012). WT mice from Study III did not show up-regulation of PGC-1α mRNA in iWAT and eWAT in recovery from acute exercise (Figure 14). The reason for the distinct results in PGC-1α response in the previous study in rats (Sutherland et al., 2009) and mice in a previous study (Bostrom et al., 2012) and the present thesis may be related to species differences. Furthermore, swimming exercise has in mice been demonstrated to elicit a greater adrenaline response than running exercise (Krüger et al., 2008) and Sutherland et al. (Sutherland et al., 2009) has demonstrated that adrenaline and PGC-1α mRNA content increase proportionally in epididymal and retroperitoneal adipose tissue. This may indicate that a possible greater adrenaline response in rats elicit a PGC-1α mRNA response. Furthermore, the time course of the exercise-induced PGC-1α mRNA response in WAT could be peaking later than the measured time points, but in rats PGC-1α mRNA has been demonstrated to peak immediately after exercise and be absent 4 hours after the exercise bout (Sutherland et al., 2009). Based on these results it was expected that the time points in Figure 14 would be able to detect a possible peak in PGC-1α mRNA. As is the case in white gastrocnemius muscle, a higher PGC-1α mRNA content is evident in eWAT from PGC-1α KO mice including in resting mice than in WT mice. It may therefore be speculated that lack of functional PGC-1α protein has elicited a compensatory regulation of PGC-1α transcription.
Figure 14. a) Inguinal white adipose tissue (iWAT) and b) epididymal white adipose tissue (eWAT) peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α mRNA content relative to GAPDH mRNA content before (Pre), immediately after (0'), 2 (2h), 6 (6h) and 10 (10h) hours after an acute 1 hour treadmill exercise in wildtype (WT) and PGC-1α knockout (KO) mice. Amplified by use of primers binding outside the deleted fragment in PGC-1α KO mice (Lin et al., 2004). Values are mean ±SE, n=8.

*: Significantly different from Pre within given genotype, P≤0.05. (*): Tends to be significantly different from Pre within given genotype, 0.05<P≤0.10. #: Significantly different from WT within given time point, P≤0.05. (#): Tends to be significantly different from WT within given time point, 0.05<P≤0.10.

Together these data suggest a role of PGC-1α in exercise-induced adaptive gene response in several tissues by regulation of PGC-1α expression. In addition, PGC-1α activation by phosphorylation or deacetylation has previously been suggested (Gerhart-Hines et al., 2007; Jager et al., 2007) and cannot be excluded.

11 Exercise-induced mRNA response of oxidative and angiogenic proteins – role of PGC-1α

11.1 Skeletal muscle

Study I demonstrated that in addition to inducing PGC-1α mRNA, an acute exercise bout increased VEGF mRNA in human skeletal muscle, which is in line with previous studies (Hiscock et al., 2003; Jensen et al., 2004a). In addition, the exercise-induced VEGF mRNA response was in Study I similar to the PGC-1α mRNA response and therefore it is not obvious that PGC-1α could regulate the exercise-induced VEGF expression in human skeletal muscle as previously suggested in mice (Arany et al., 2008; Leick et al., 2009). Although increased PGC-1α activity may also regulate the expression of genes like VEGF as previously suggested (Olesen et al., 2010). It is also possible that PGC-1α regulates gene expression of metabolic proteins as previously suggested (Leick et al., 2008). The lack of changes in the mRNA content of oxidative proteins like
Cyt c in Study I (data not shown) may not necessarily be against this possibility. Hence, the mRNA of CS and HAD, has been shown to exhibit a late response (10-18 hours after exercise) to an acute exercise bout (Leick et al., 2010b) while the latest sampling point in Study I was 3 hours of recovery. The lack of changes in the mRNA content of metabolic proteins in Study I is in contrast to the previous findings in Nordsborg et al. (Nordsborg et al., 2010) showing that HKII and PDK4 mRNA content increased in recovery from an acute exercise bout and that exercise training lowered the exercise-induced response when working at the same absolute intensity. It may be speculated that the exercise intensity and/or duration in Study I was not sufficient to elicit an exercise response. The study by Leick et al. (Leick et al., 2008) reported exercise-induced increases in mRNA content of metabolic proteins in mouse skeletal muscle, and that PGC-1α was required for some (Cyt c) of the adaptive responses but not for others (HKII). In muscle samples from Study III acute exercise did not increase the content of HKII and Cyt c mRNA in either genotype (data not shown) which may seem surprising as the two studies used the same mouse strain, the same exercise protocol and the same time points. The use of different muscles may explain the different findings both with regard to muscle characteristics and how much it is recruited during the exercise. Thus Leick et al. (Leick et al., 2008) used soleus muscle and Study III used white gastrocnemius. Soleus represents a very red and oxidative muscle whereas the white part of the gastrocnemius muscle is glycolytic. While exercise-induced mRNA responses have been reported in soleus (Leick et al., 2008; Leick et al., 2010c), glycolytic skeletal muscle has previously been shown to exhibit marked exercise-induced transcriptional responses (Hildebrandt et al., 2003; Leick et al., 2008), which is in contrast to the findings in Study III. Therefore, the type of muscle does not seem to be the only explanation. Muscle glycogen content of white gastrocnemius was not reduced in response to the acute exercise bout in Study III, which is opposite of Leick et al. (Leick et al., 2008) and which may be indicate that the muscle was not recruited during the exercise bout. However, an increased phosphorylation of both AMPK and ACC support that the muscle was recruited during exercise. It has previously been demonstrated that muscle glycogen content affects exercise-induced gene transcription in human skeletal muscle (Pilegaard et al., 2002) and may suggest that the lack of exercise-induced mRNA responses in Study III can be due to increased muscle glycogen content. Furthermore, the increase in muscle glycogen content with bed rest in Study I may explain the lack of exercise-induced PGC-1α and VEGF mRNA responses after bed rest.

Study I examined the impact of physical activity level on acute exercise-induced adaptive responses in human skeletal muscle. It was anticipated that the observed exercise-induced increases in VEGF mRNA content before 7 days of bed rest would be elevated after bed rest, based on previous exercise training studies (Jensen et al., 2004b), while it was difficult to anticipate how bed rest would affect the exercise-induced mRNA response of PGC-1α because previous studies has shown both increased and reduced...
response after exercise training (Nordsborg et al., 2010; Pilegaard et al., 2003). The observation that both responses were abolished after bed rest was therefore unexpected and indicates that human skeletal muscle loses the ability to induce mRNA changes in response to an acute exercise bout after only 7 days of physical inactivity. As previously mentioned muscle glycogen content affects exercise-induced gene transcription in human skeletal muscle (Pilegaard et al., 2002) and it has previously been demonstrated that increased muscle glycogen content reduces AMPK activation (McBride et al., 2009). The observation in Study I that 7 days of bed rest result in an increase in muscle glycogen concentration could possibly have affected the exercise-induced mRNA response based on the previous studies. In addition, it has previously been demonstrated that bed rest induces methylation of the PGC-1α promoter (Alibegovic et al., 2010) and that this increased methylation is associated with a reduction in PGC-1α mRNA content (Alibegovic et al., 2010; Barres et al., 2009). This suggests that the lack of exercise-induced PGC-1α mRNA after bed rest was due to physical inactivity-induced hypermethylation of the PGC-1α promoter.

11.2 Liver

The observation in Study II that the mRNA content of the mitochondrial proteins Cyt c and COXI increased in response to acute exercise in WT but not PGC-1α KO mice indicates an important role of PGC-1α in exercise-induced mitochondrial biogenesis also in mouse liver. Furthermore, an acute exercise bout elicited an increase in the mRNA content of the gluconeogenic protein G6Pase and a main tendency to increase PEPCK is in accordance with the main function of the liver in maintaining plasma glucose levels during prolonged exercise and fasting and with a previous study (Hoene et al., 2009) showing an increase in both G6Pase and PEPCK mRNA content in WT mice after acute exercise. Study II of the present thesis demonstrates that PGC-1α is not required for the exercise-induced adaptive response in G6Pase to acute exercise. In addition, the findings in Study II that PEPCK and G6Pase mRNA increased in both WT and PGC-1α KO mice indicate that PGC-1α is not necessary for fasting-induced regulation of PEPCK and G6Pase expression either. This is in contrast to previous studies (Estall et al., 2009; Handschin et al., 2005; Lin et al., 2004) showing that PGC-1α regulates PEPCK and G6Pase expression. These differences may be due to use of different mice strains, where Estall et al. and Handschin et al. (Estall et al., 2009; Handschin et al., 2005) used a liver-specific PGC-1α KO strain while Study II uses whole body PGC-1α KO mice. Furthermore, different fasting duration was used in the previous studies (Estall et al., 2009; Handschin et al., 2005) than in Study II. This may suggest that the role of PGC-1α in regulating gluconeogenic proteins depends on the experimental settings. Furthermore, PGC-1α KO mice had lower liver glycogen content and plasma glucose than WT mice indicating that PGC-1α KO mice rely more on carbohydrates during exercise than WT mice, which is in accordance with a previous study (Calvo et al., 2008) reporting that muscle-specific PGC-1α overexpression mice have increased fat oxidation during exercise.
11.3 Adipose tissue
No exercise-induced response in the mRNA content of COXI was observed in Study III and PGC-1α did not seem to be required for the basal level of COXI mRNA. However, Study III demonstrates that acute exercise elicits a transient UCP1 mRNA increase in both iWAT and eWAT of WT but not PGC-1α KO mice. The lack of exercise-induced increases in UCP1 mRNA in both eWAT and iWAT of PGC-1α KO mice indicates that PGC-1α is required for the acute regulation of UCP1 mRNA in WAT. Both genotypes completed the 1 hour exercise bout and a similar reduction in muscle glycogen in WT and PGC-1α KO mice in response to exercise supports that the PGC-1α KO mice have been physically challenged as the WT and the lack of UCP1 mRNA response in PGC-1α KO mice is not due to lack of exercise stimulus. A role of PGC-1α in regulating UCP1 has previously been demonstrated in BAT (Cao et al., 2004;Puigserver et al., 1998), where PGC-1α has been shown to mediate cold-induced up-regulation of UCP1. In addition, a role of PGC-1α in regulating exercise-induced gene responses in WAT has also been suggested (Sutherland et al., 2009). Based on the observation that PGC-1α mRNA is increased in WAT in rats in response to exercise (Sutherland et al., 2009) the findings in Study III showing PGC-1α dependent increases in UCP1 mRNA support this proposal.

As no exercise-induced response was observed in PGC-1α mRNA content in Study III, this cannot explain the role of PGC-1α in regulation of UCP1 mRNA. But as previously suggested (Gerhart-Hines et al., 2007;Jager et al., 2007) post-translational modifications and thereby activation of PGC-1α may regulate UCP1 expression. Furthermore, the findings in Study III that UCP1 mRNA content peaks at different time points in recovery from exercise, with UCP1 mRNA peaking 6 hours into the recovery period in iWAT and immediately after exercise in eWAT. This may make an mRNA accumulation with repeated bouts of exercise more likely in iWAT than in eWAT.

12 Impact of physical activity level on regulation of oxidative and angiogenic proteins – role of PGC-1α
12.1 Skeletal muscle
The observations in Study I that 7 days of bed rest resulted in loss of leg muscle mass are in accordance with previous studies (Alibegovic et al., 2009;Mikines et al., 1991;Saltin et al., 1968). While intense exercise training has been shown to increase capillarization of human skeletal muscle (Jensen et al., 2004a), it was not expected to observe major changes in capillarization during the relatively short inactivity period in Study I based on a previous bed rest study by Mikines et al. (Mikines et al., 1991) showing no effect of 7 days of bed rest on capillarization.
Based on previous studies showing decreased activity of metabolic proteins in human skeletal muscle with detraining (Henriksson & Reitman, 1977) the present findings that HAD and CS activity as well as HKII and SIRT1 protein content were reduced after bed rest was expected. The observations that cytosolic HKII, nuclear SIRT1 and mitochondrial HAD and CS were all reduced indicate that bed rest affected several cellular compartments. Furthermore, the observed reduction in mtDNA/nDNA with bed rest provides evidens for a lower mitochondrial content in human skeletal muscle after only 7 days of total physical inactivity and this is accordance with the anticipation based on exercise training studies showing training-induced mitochondrial biogenesis (Gollnick & Saltin, 1982), although human studies in general report a lack of change in mtDNA/nDNA with exercise training (Jeppesen et al., 2006; Yeo et al., 2008). The mtDNA/nDNA ratio is often used as a reflection of mitochondrial number (Barres et al., 2009; Lagouge et al., 2006; Um et al., 2010) and mtDNA/nDNA is thus used as an indicator of increases in mitochondrial DNA content and thereby capacity to increase mitochondrial gene expression. The decrease in oxidative proteins indicate a high turnover rate of mitochondrial proteins, but the lack of change in Cyt c protein content, while HAD and CS activity is significantly reduced with bed rest in Study I may indicate a longer half-life of Cyt c than for mtDNA/nDNA content and other mitochondrial proteins including CS and HAD, but individual variation in the Cyt c response to bed rest is also a possibility. Based on the observed exercise-induced PGC-1α mRNA response before bed rest in Study I and the previous studies (Geng et al., 2010; Leick et al., 2008; Lin et al., 2002; Lin et al., 2004) demonstrating that PGC-1α is a key factor in mitochondrial biogenesis, the observed reductions in oxidative proteins with bed rest may be due to lack of regular activation of PGC-α.

Exercise training has been reported to elicit an increase in young and old skeletal muscle metabolic protein content in both humans and rodents (Leick et al., 2008; Russell et al., 2003; Short et al., 2003). In addition, lifelong exercise trained elderly subjects has been reported to have higher skeletal muscle metabolic protein content (Iversen et al., 2011). In accordance Study IV demonstrates that CS activity, mtDNA/nDNA content and pyruvate dehydrogenase-E1α and Cyt c protein content were higher in lifelong exercise trained WT mice than old sedentary WT mice. Furthermore, the protein content of the angiogenic markers VEGF and CD31 was also increased in skeletal muscle of WT mice with lifelong exercise training. Study IV moreover demonstrates that lifelong exercise training can prevent the observed age-associated decrease in CS activity, which is in accordance with a previous study (Leick et al., 2010a). Similarly exercise training prevented a decrease in mtDNA/nDNA ratio with age in WT mice which has not been demonstrated previously, but is in accordance with a human study showing higher mitochondrial protein content in lifelong exercise training compared with sedentary elderly (Iversen et al., 2011). These results indicate that elderly humans and aged mice retain the ability to improve mitochondrial capacity, which is in accordance
with a previous study (Ljubicic et al., 2009) that reported retained mitochondrial plasticity, but that the magnitude of the response to contractile activity was less in aged than young mice.

The findings in Study IV that lifelong exercise training increased the mitochondrial and angiogenic protein content in WT mouse skeletal muscle, but not in PGC-1α KO mice, show that PGC-1α is required for these changes. This is in line with a similar aging study from our group (Leick et al., 2010a) but in contrast to another study from our group showing that PGC-1α is not mandatory for exercise training-induced increases in Cyt c, COXI and IV protein content in young mice although VEGF adaptations were (Adhihetty et al., 2009; Leick et al., 2009). It may be speculated that these differences are related to the age of the mice and that aging is associated with loss of compensating mechanisms observed in young mice. However, another study has reported PGC-1α dependent exercise training adaptations of oxidative proteins and capillarization (Geng et al., 2010) suggesting that the differences are not solely related to age. Alternatively, training mode, intensity and/or duration may play a role. Hence, the studies reporting dependency of PGC-1α have all used running wheels (Geng et al., 2010; Leick et al., 2010a) which are characterized by intermittent exercise of self-chosen speed. The study by Leick et al. (Leick et al., 2008) used a combination of running wheel and treadmill exercise training 5 days per week for 5 weeks. Treadmill exercise is more continuous than running wheel and at a fixed similar speed. However, as the exercise capacity is reduced in PGC-1α KO mice (Leick et al., 2008; Lin et al., 2004) it is obvious that the PGC-1α KO mice exercise at a higher relative intensity than WT, and this may elicit a compensatory mechanism when PGC-1α is lacking. This idea needs to be further investigated. Of notice despite the absence of exercise training-induced adaptations in PGC-1α KO mice in Study IV, these mice improved running capacity indicating that other factors must contribute as well. Finally although PGC-1α is not necessarily required for exercise training-induced increases in mitochondrial protein content lack of PGC-1α reduces the basal level of mitochondrial proteins in skeletal muscle in previous studies and Study IV although muscle type specific differences are evident (Leick et al., 2008).

12.2 Liver
In liver tissue the findings of Study II demonstrate that exercise training increases the protein content of the gluconeogenic protein PEPCK and the two mitochondrial proteins Cyt c and COXI in WT mice. The observation that PEPCK, but not G6Pase and PC protein, increased with exercise training suggest that exercise training increases gluconeogenic capacity. The findings that exercise training elicited mitochondrial biogenesis in the liver is in accordance with a previous study showing increased activity and content of mitochondrial enzymes in aging mice (Navarro et al., 2004). In addition, Study II demonstrates that PGC-1α is required for the exercise training-induced increase in mitochondrial proteins while this was not the case.
for PEPCK protein content. This observation that PGC-1α is required for exercise training-induced adaptations in metabolic proteins in the liver is in accordance with previous studies (Estall et al., 2009; Leone et al., 2005). An increased level of oxidative proteins in the liver enhances the capacity for ATP production and hence improves liver energy metabolism. This may be an alternative mechanism to enhance gluconeogenesis without increasing gluconeogenic protein content if gluconeogenic enzymes are not limiting. The observed PGC-1α dependency therefore indicates that PGC-1α may play an important role in mitochondrial biogenesis in liver tissue as well as it was demonstrated in skeletal muscle.

12.3 Adipose tissue
The observation in Study III that exercise training increased the protein content of mitochondrial proteins in WAT of mice is in accordance with findings obtained already 20 years ago in rats (Stallknecht et al., 1991; Stallknecht et al., 1993). But the increased UCP1 protein in iWAT of WT mice with exercise training is novel and supports previous observations at the mRNA level (Bostrom et al., 2012). In addition, the combined transient increase in UCP1 mRNA to a single exercise bout and the increase in UCP1 protein with 5 weeks of training suggest that transient increases in mRNA content lead to accumulation of protein content (Study III). Whether exercise influences capillarization in WAT is still unknown but a recent study has shown that circulating VEGF was non-significantly up-regulated after a 12 week training period (Cullberg et al., 2012) which may support the findings in Study III that exercise training increased the endothelial marker CD31 protein content in WAT.

The increase in UCP1, COXIV and CD31 protein content in iWAT after exercise training observed in WT mice was absent in PGC-1α KO mice indicating a mandatory role of PGC-1α in the regulation of capillarization, oxidative proteins and UCP1 protein expression in iWAT in response to exercise training. However, the resting level of UCP1 protein content in iWAT did not differ between WT and PGC-1α KO mice which indicate that PGC-1α is not needed for the basal UCP1 levels in iWAT. Of notice is however, that the resting UCP1 protein level hardly is detectable and a genotype difference would likely be difficult to identify. In accordance with a previous study (Bostrom et al., 2012) indicating a browning potential of iWAT, but not eWAT. A browning of WAT may suggest that exercise training induces an increase in WAT metabolism and thereby may improve WAT quality. Although the level of UCP1 protein in WAT likely is very low even in exercise trained tissue and the functional relevance of such changes may be questionable, the fact that changes are observed does suggest that exercise training-induced UCP1 regulation can be of importance.
13 Resveratrol mediated effects on oxidative capacity – role of PGC-1α

The findings in Study IV indicate that lifelong resveratrol supplementation alone is not able to prevent an age-associated decrease in CS activity and mtDNA/nDNA content in mouse skeletal muscle which is in line with a recent mouse study (Menzies et al., 2013). But these findings are in contrast to previous studies (Lagouge et al., 2006; Um et al., 2010) where resveratrol supplementation increased CS activity, mitochondrial copy number and mRNA content of oxidative proteins in skeletal muscle of mice on high fat diet. Resveratrol dose and fabricant was the same in the present and the previous studies (Lagouge et al., 2006; Um et al., 2010) and can therefore not explain the difference in resveratrol-mediated effects on mitochondrial proteins. Of notice is that the previously mentioned studies showing resveratrol-mediated effects on mitochondrial protein content used mice fed a high-fat diet (Lagouge et al., 2006; Um et al., 2010), while Study IV used healthy aging mice. This notion is in part in accordance with recent human studies, where beneficial effects have only been reported in subjects with impaired glucose tolerance (Crandall et al., 2012), but not in healthy subjects (Poulsen et al., 2013; Timmers et al., 2011). This may imply that effects of resveratrol are not observed in healthy mice and humans.

14 miRNA regulation

miRNAs have been suggested to play an important role in the regulation of protein expression. Study I tested the hypothesis that acute exercise and physical activity level regulate the content of specific miRNAs. The finding that miR-23a content was decreased 3 hours into recovery from acute exercise after bed rest is in accordance with a previous study (Safdar et al., 2009) reporting a down-regulation of miR-23a content in mouse skeletal muscle after acute exercise. The subjects in Study I exercised at the same absolute workload before and after bed rest, and the relative exercise intensity is expected to have been higher after bed rest than before bed rest. The lower relative exercise intensity before bed rest is a likely explanation for the lack of exercise-induced changes in miR-23a expression before bed rest. Because only miR-23a was found to be regulated by an acute exercise bout and only after bed rest it may be speculated that the acute exercise bout was performed at an insufficient intensity to elicit an acute regulation of the investigated miRNAs. Therefore, it was tested whether exercise intensity affected the acute regulation of miRNAs. Vastus lateralis samples from Nordsborg et al. (Nordsborg et al., 2010) were used to test the effect of exercise intensity on the content of specific miRNAs. Male human untrained subjects performed 4 times 4 minutes (3 minutes rest between the 4 periods) of exhaustive intense intermittent cycling exercise at ~85 % of VO_{2max} and well trained subjects exercised at the same relative as well as the same absolute (corresponding to ~70 % of VO_{2max}) workload and miR-1, miR-23a and miR-133a content was determined before as well as 3 and 5 hours into the recovery period. Data suggest that exercise at 70 % and 85 % of VO_{2max} in trained subjects
does not affect miRNA expression in recovery from an acute exercise bout (Figure 15). But it cannot be excluded that the time course of changes in miRNA's is either faster or slower than the measured time points or that more prolonged exercise is required and this is the reason for the results obtained.

Figure 15. Expression of a) miR-1, b) miR-23a and c) miR-133a in vastus lateralis before (Pre), 3 hours after (3h) and 5 hours after (5h) 4 times 4 minutes of exhaustive intense intermittent cycling exercise in untrained subjects (UT) working at ~85 % of VO\textsuperscript{2max}, trained subject working at the same relative workload (T REL) and the trained subjects working at same absolute workload (T ABS) as untrained subjects (corresponding to ~70 % of VO\textsuperscript{2max}). Values are mean ±SE, n=5-9.

The observation in Study I that bed rest reduced miR-1 and miR-133a content indicates that training status affects expression of these miRNAs in skeletal muscle. However, no difference in expression of miR-1, miR-23a and miR-133a was observed in untrained and trained subjects in samples from the study by Nordsborg et al. (Nordsborg et al., 2010) (Figure 15). This may suggest that the total lack of muscle contraction for 7 days is a more dramatic change for the muscle than an increase due to regular exercise training. In addition, the finding that bed rest reduced vastus lateralis muscle miR-1 and miR-133a content is opposite of the expected based on previously reported reductions in miR-1 and miR-133a with exercise training (Nielsen et al., 2010), but it has been reported that miR-133a expression is reduced in T2D patients (Gallagher et al., 2010) and in mouse skeletal muscle in response to spaceflight (Allen et al., 2009). Both miR-1 and miR-133a have been reported to be involved in myogenensis (Chen et al., 2006; van Rooij et al., 2008), suggesting that bed rest reduces stability of mRNAs involved in muscle growth. Because the reduced miRNA level with bed rest suggests that activation level of skeletal muscle has an impact on miRNA expression the basal miRNA level was determined in a relatively active muscle, vastus lateralis, and a relatively inactive muscle, triceps of young male subjects. Interestingly, miR-1, 23a and 133a content did not differ between vastus lateralis and triceps muscle (Figure 16).
From these data miRNAs does not seem to be affected by acute exercise, exercise intensity or muscle type. But the basal miRNA content does seem to be regulated with changes in physical activity level. It has been suggested that miRNAs can both exert effects by marking mRNAs for degradation and by inhibiting translation (van Rooij et al., 2008). In either case miRNA mediated regulation seems to provide an additional physical activity dependent regulatory step in protein synthesis and this may indicate that stabilization of specific mRNAs are affected by physical activity.
Conclusion and future perspectives

The present thesis demonstrates that physical inactivity reduces the content of oxidative proteins and miRNAs involved in myogenesis in human skeletal muscle and abolishes exercise-induced mRNA responses of the transcriptional coactivator PGC-1α and the angiogenic marker VEGF in human skeletal muscle.

PGC-1α is shown to be required for exercise-induced mRNA responses and exercise training-induced protein adaptations in oxidative and/or metabolically related proteins in mouse skeletal muscle and liver, while fasting-induced regulation of gluconeogenic capacity does not require PGC-1α.

PGC-1α is shown to be required for exercise-induced up-regulation of UCP1 mRNA in mouse white adipose tissue.

Lifelong exercise training can prevent an age-associated decrease in CS and mtDNA content and increase the content of mitochondrial proteins in a PGC-1α dependent manner. However, lifelong resveratrol supplementation only exerts minor effects on the content of oxidative and angiogenic proteins and no additive effects of combining exercise training and resveratrol is evident.

Taken together the thesis also demonstrates that exercise- and exercise training-induced regulation of metabolic capacity in skeletal muscle, liver and white adipose tissue is complex involving multiple regulatory steps and likely depending on several factors including mode of exercise and intensity. It is clear that many unanswered questions remain regarding regulation of exercise- and exercise training-induced adaptations in skeletal muscle, liver and adipose tissue.

In the future it would be interesting to further investigate the lack of exercise-induced mRNA responses in human skeletal muscle after physical inactivity. Hypermethylation of the PGC-1α promoter has previously been suggested to reduce gene transcription and elucidating whether this was the case in Study I would add further knowledge to the mechanism behind exercise-induced mRNA responses. In addition, determining PGC-1α activity by measuring acetylation and phosphorylation of the PGC-1α protein in skeletal muscle would also contribute to the understanding of the potential role of PGC-1α in regulating exercise-induced responses.

Furthermore, an investigation of whether exercise training is sufficient to prevent age-associated and restore physical inactivity-induced epigenetic changes in skeletal muscle and adipose tissue and whether PGC-1α is necessary for this requires investigation. In addition, examining maintenance of healthy
mitochondria with exercise and exercise training, and a possible role of PGC-1α in regulation of mitophagy would be interesting to investigate.
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Bed rest reduces metabolic protein content and abolishes exercise-induced mRNA responses in human skeletal muscle

Stine Ringholm,1,2,3 Rasmus S. Bienso,1,2,3 Kristian Kiilerich,1,2,3 Amelia Guadalupe-Grau,4 Niels Jacob Aachmann-Andersen,1,5 Bengt Saltin,1,5 Peter Plomgaard,1,2,6 Carsten Lundby,1,5 Jørgen F. W. Wojtazsiewski,1,7 Jose A. Calbet,1,4 and Henriette Pilegaard1,2,3

1Copenhagen Muscle Research Centre, 2Centre of Inflammation and Metabolism, and 3Department of Biology, August Krogh Building, University of Copenhagen, Copenhagen, Denmark; 4Department of Physical Education, University of Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain; 5Rigshospitalet Section 7652, 6Rigshospitalet Section 7641, and 7Molecular Physiology Group, Department of Exercise and Sport Sciences, University of Copenhagen, Denmark

Submitted 10 May 2011; accepted in final form 7 July 2011

Ringholm S, Bienso RS, Kiilerich K, Guadalupe-Grau A, Aachmann-Andersen NJ, Saltin B, Plomgaard P, Lundby C, Wojtazsiewski JF, Calbet JA, Pilegaard H. Bed rest reduces metabolic protein content and abolishes exercise-induced mRNA responses in human skeletal muscle. Am J Physiol Endocrinol Metab 301: E649–E658, 2011. First published July 12, 2011; doi:10.1152/ajpendo.00230.2011.—The aim was to test the hypothesis that 7 days of bed rest reduces mitochondrial number and expression and activity of oxidative proteins in human skeletal muscle but that exercise-induced intracellular signaling as well as mRNA and microRNA (miR) responses are maintained after bed rest. Twelve young, healthy male subjects completed 7 days of bed rest with vastus lateralis muscle biopsies taken before and after bed rest. In addition, muscle biopsies were obtained from six of the subjects prior to, immediately after, and 3 h after 45 min of one-legged knee extensor exercise performed before and after bed rest. Maximal oxygen uptake decreased by 4%, and exercise endurance decreased nonsignificantly, by 11%, by bed rest. Bed rest reduced skeletal muscle mitochondrial DNA/nuclear DNA content 15%, hexokinase II and sirtuin 1 protein content ~45%, 3-hydroxyacyl-CoA dehydrogenase and citrate synthase activity ~8%, and miR-1 and miR-133a content ~10%. However, cytochrome c and vascular endothelial growth factor (VEGF) protein content as well as capillarization did not change significantly with bed rest. Acute exercise increased AMP-activated protein kinase phosphorylation, peroxisome proliferator activated receptor-γ coactivator-1α, and VEGF mRNA content in skeletal muscle before bed rest, but the responses were abolished after bed rest. The present findings indicate that only 7 days of physical inactivity reduces skeletal muscle metabolic capacity as well as abolishes exercise-induced adaptive gene responses, likely reflecting an interference with the ability of skeletal muscle to adapt to exercise.

Address for reprint requests and other correspondence: S. Ringholm, Dept. of Biology, August Krogh Bldg., Universitetsparken 13, 2100 Copenhagen, Denmark (e-mail: srjorgensen@bio.ku.dk).

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THE OXIDATIVE CAPACITY OF SKELETAL MUSCLE contributes to determining the ability of skeletal muscle to oxidize both carbohydrate and fat. Changes in skeletal muscle oxidative capacity can thus influence whole body metabolism (25). The plasticity of human skeletal muscle oxidative capacity is evident by the increased content and activity of oxidative enzymes/proteins and increased capillarization with exercise training and a corresponding decrease during detraining (16, 17, 19). Training-induced skeletal muscle protein adaptations are thought at least in part to originate from cumulative effects of transient increases in gene expression in response to each single exercise bout (46), as shown in human skeletal muscle for both metabolically related proteins (29, 40, 41, 44) and the angiogenic protein vascular endothelial growth factor (VEGF) (18, 21). Increased physical activity has been shown to reduce the exercise-induced responses of RNA’s encoding metabolic (41) and angiogenic (21) proteins, but an increased peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) mRNA response to an acute exercise bout was evident after 4 wk of one-legged knee extensor exercise training (41), potentially reflecting a sensitizing effect of training. Exercise training-induced changes in the protein content may also occur without any changes in transcription, for example, through regulation mediated by microRNAs (miRs), miR-1, miR-29b, and miR-133a have been shown to be implicated in myogenesis (45). Acute exercise has been reported to decrease and increase the miR-1 and miR-23 content, respectively, in mouse skeletal muscle (42) and to enhance miR-1 and miR-23 content in human skeletal muscle (39). In addition, miR-133a content has been demonstrated to be lowered in human skeletal muscle both with exercise training (39) and in type 2 diabetes (T2D) patients (14). Therefore, both mRNA and miR regulation are likely contributing mechanisms in skeletal muscle adaptations with repeated exercise.

The previous findings that overexpression of the transcriptional coactivator PGC-1α in mouse skeletal muscle converted otherwise white glycolytic muscles to red oxidative muscles (31) and that knockout of PGC-1α reduced expression of oxidative proteins (30) convincingly show the impact of PGC-1α on mitochondrial biogenesis in skeletal muscle. Similarly, the increased capillarization with PGC-1α overexpression (6) and decreased capillarization in PGC-1α-knockout mice (28), as well as the effect of PGC-1α on VEGF expression (6, 28), demonstrate a role of PGC-1α in angiogenesis. PGC-1α transcription and mRNA (41) as well as PGC-1α protein content (32) have been shown to be upregulated in human skeletal muscle in response to a single exercise bout. In addition, the PGC-1α protein has been reported to be regulated by various posttranslational mechanisms, including activation by AMP-activated protein kinase (AMPK)-mediated phosphorylation (20) and by sirtuin 1 (SIRT1)-mediated deacetylation (15). AMPK is known as an intracellular energy sensor, which is phosphorylated and thereby activated by a single exercise bout (23). Furthermore, the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) has
been shown to increase PGC-1α mRNA in mouse skeletal muscle (24) and repeated AICAR treatments to increase mitochondrial proteins in mouse skeletal muscle in a PGC-1α-dependent manner (28). Exercise training has also been shown to increase SIRT1 protein in humans (33), and PGC-1α deacetylation has been reported in mouse skeletal muscle in response to a single exercise bout (12). Together, this suggests that PGC-1α is a likely coordinator of exercise training-induced adaptations in skeletal muscle oxidative capacity, potentially involving both AMPK- and SIRT1-mediated regulation (12). Recent findings suggest that miR-29b may target PGC-1α mRNA (45), and such a mechanism could thus also play a role in PGC-1α-mediated gene regulation.

Although a physically inactive lifestyle is known to be an important risk factor in many diseases, and physical inactivity is an increasing problem in most parts of the world, less is known about the impact of physical inactivity on skeletal muscle oxidative capacity and on the ability of skeletal muscle to induce adaptive responses to an exercise bout. Therefore, the aim of the present study was to test the hypothesis that 7 days of bed rest will reduce expression and activity of oxidative proteins in skeletal muscle but not reduce the exercise-induced intracellular signaling, mRNA, and miR responses. This was addressed by placing young healthy subjects in bed for 7 days with a single exercise bout performed before and after the bed rest period.

METHODS

Ethical approval. Subjects were given both written and oral information about the experimental protocol and procedures and were informed about any discomfort that might be associated with the experiment before they gave their written consent. The study was performed according to the Declaration of Helsinki and approved by the Copenhagen and Frederiksberg Ethics Committee, Denmark (H-1-2008-0024).

Subjects. Twelve healthy, physically active male subjects with an average (means ± SD) age of 26.2 ± 5.3 yr, weight 75.5 ± 11.3 kg, height 181.7 ± 6.1 cm, and body mass index 22.8 ± 2.7 kg/m² participated in this study. Six of these subjects participated in an exercise trial. The average (means ± SD) age, weight, height, and body mass index of these subjects was 28.7 ± 5.3 yr, 82.2 ± 12.3 kg, 183.1 ± 7.6 cm, and 24.4 ± 2.2 kg/m², respectively.

Bed rest. The subjects were placed in hospital beds with manual head and leg elevation adjustments. During the bed rest period, the subjects were allowed to sit up for 5 h/day, and they were at all times transported in a wheelchair. During the bed rest period, subjects were served regular healthy food (10–20% energy from protein, 50–60% energy from carbohydrates, 25–35% energy from fat) ad libitum from the kitchen at Rigshospitalet, Copenhagen, Denmark.

Body composition. Six to 10 days before and immediately after the bed rest period, fat and fat-free tissue mass of the whole body, trunk, and extremities were measured on all 12 subjects, using a dual-energy X-ray absorptiometry (DEXA) scanner (Lunar Prodigy Advance; GE Healthcare, Madison, WI).

Oral glucose tolerance test. An oral glucose tolerance test (OGTT) was performed on all 12 subjects between 6 and 10 days before the onset of bed rest and 6 days into the bed rest. Each subject consumed 1 g/kg body wt glucose, with each gram of glucose dissolved in 6.67 ml of water. After consumption, blood was sampled from an arm vein after 30, 60, and 120 min, and the samples were analyzed for plasma insulin and glucose (Department of Clinical Biochemistry, Rigshospitalet).

Performance tests. Maximal oxygen uptake (VO2max) and muscle endurance were determined for all 12 subjects 6–10 days before the onset of bed rest and at the end of the bed rest period. VO2max was determined by an incremental bicycle test and leg muscle endurance by a one-legged knee extensor exercise test, using a modified ergometer bicycle (Monark Ergomedic 839E; Monark Exercise, Vansbro, Sweden). In the endurance test, the same absolute intensity was used before and after bed rest and starting with 15 min at 75% of maximal load (wattmax; before bed rest level) followed by 90% wattmax (before bed rest level) until exhaustion.

Each of the six subjects taking part in the acute exercise protocol performed an additional one-legged knee extensor exercise performance test 1 wk before the first experimental day to determine the workload to be used during the acute exercise experiments. The workload was gradually increased every 2 min, and the highest load, which could be sustained for 2 min, was set as the wattmax, as described previously (40).

Acute exercise experiment. The six subjects in the acute exercise protocol completed an identical experimental trial 4–10 days before initiation of the bed rest as well as on the 7th day of bed rest.

The day prior to the experimental day before bed rest, the subjects refrained from intense and prolonged exercise. The subjects were physically inactive the day before the experimental day after bed rest, because this was within the bed rest period. The day before both experimental trials, the subjects consumed a prepackaged dinner and evening snack, which was standardized based on the body weight of the subject (60 and 12 kJ/kg body wt, respectively).

On the experimental day before bed rest, the subjects arrived at the laboratory by minimum of physical activity, and on the experimental day after bed rest they were transported in wheelchairs. On the morning of both experimental trials, the subjects consumed a standardized breakfast regulated for body weight (30 kJ/kg body wt). A muscle biopsy was obtained from the middle portion of the vastus lateralis muscle, using the percutaneous needle biopsy technique (8) with suction 3.5 h after breakfast (Pre). This was followed by 45 min of one-legged knee extensor exercise at ~60% of wattmax (before bed rest level and thus the same absolute intensity before and after bed rest), using a modified ergometer bicycle (Monark Ergomedic 839E; Monark Exercise). Three of the subjects worked with their dominant leg and three with their nondominant leg. Additional muscle biopsies were obtained from the exercised leg immediately after exercise (Post) and at 3 h of recovery (3 h rec). All muscle biopsies were taken through separate incisions, quickly frozen in liquid nitrogen (<15 s), and stored at ~80°C until they were analyzed. A small part of the Pre biopsies was mounted in embedding medium frozen in isopentane precooled in liquid nitrogen and stored at ~80°C. Furthermore, a catheter was placed in the femoral artery of one leg, and blood samples were obtained before (Pre), during (20 min), and immediately after (Post) exercise. An article based on this experiment has recently been published, where body composition, OGTT, and performance data were reported for six of the subjects (26).

Plasma adrenaline. Plasma adrenaline was measured using an adrenaline RIA kit (Millipore, Bedford, MA).

Muscle glycogen. Muscle glycogen content was determined as glycoseyl units after acid hydrolysis (34) using 400 μg of muscle homogenate protein and an automatic spectrophotometer.

Fiber type and capillarization. The mounted biopsies were cut in serial transverse sections, which were stained for myofibrillar adenosine triphosphatase to identify fibers as myosin heavy chain (MHC) type I, type IIa, or type IIx (11) or with the amylase-p-aminosalicyclic acid method to visualize capillaries (5). Fiber type, fiber area, and capillary density were determined using a COMFAS image scanner (SBsysCOMFAS; Scan Beam, Hadsund, Denmark).

DNA isolation. Total DNA was isolated from ~10 mg of muscle tissue from Pre biopsies, as described previously (40). The DNA pellet was resuspended in 25 μl of distilled water. The isolated DNA was later used to determine the ratio between mtDNA and nuclear DNA content by real-time PCR.
RNA isolation, reverse transcription, and cDNA content. Total RNA for mRNA and miR was isolated from ~20 mg of muscle tissue by a modified guanidinium thiocyanate-phenol-chloroform extraction method adapted from Chomczynski and Sacchi (12a), as described previously (40), except that the tissue was homogenized for 2 min at 30 s⁻¹ in a TissueLyserII (Qiagen, Valencia, CA).

Superscript II RNase H⁻ system and Oligo dT (Invitrogen, Carlsbad, CA) were used to reverse transcribe the mRNA to cDNA, as described previously (40). The amount of single-stranded DNA (ssDNA) was determined in each cDNA sample by use of OliGreen reagent (Molecular Probes, Leiden, The Netherlands), as described previously (35).

Before reverse transcription of miR, RNA samples were diluted to 2 ng RNA/μL. Each specific miR was then reverse transcribed to cDNA by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and miR-specific primers (Applied Biosystems). The reaction was run in a thermal cycler (PTC-200; MJ Research, Waltham, MA).

Real-time PCR. The mRNA content of cytochrome c (cyt c), hexokinase II (HKII), PGC-1α, and SIRT1 as well as miRNA (represented by cyt c oxidase II) and nDNA (represented by cyt c) content were determined by real-time PCR using the fluorogenic 5′ nucleic assay with TaqMan probes and universal mastermix with UNG (ABI PRISM 7900 Sequence Detection System; Applied Biosystems), as described previously (35). All TaqMan probes were 5′-6-carboxyfluorescein and 3′-6-carboxy-N,N,N',N'-tetramethylrhodamine labeled (Table 1). The obtained cycle threshold (Ct) values reflecting the initial content of the specific transcript in the samples were converted to an arbitrary amount by using standard curves obtained from a serial dilution of a pooled sample made from all samples. The amount of a given mRNA was normalized to the ssDNA content of the cDNA sample, whereas DNA results were presented as miRNA normalized to nDNA content (miRNA/nDNA).

The content of miR-1, miR-23a, miR-29b, and miR-133a was determined by real-time PCR (as described above) using predesigned miR assays containing specific primers and TaqMan probe labeled with 5′-6-carboxyfluorescein and minor groove binder quencher (nonfluorescent) (Applied Biosystems). The obtained cycle threshold (Ct) values reflecting the initial content of the specific miR in the samples were converted to an arbitrary amount by using standard curves obtained from a serial dilution of a pooled sample made from all samples. The amount of a given miR was normalized to either the RNU6B content or the RNU48 content of the sample. RNU6B was unaffected by the acute exercise but affected by bed rest in resting samples, whereas RNU48 was unaffected by bed rest in resting samples but changed by acute exercise. Therefore, RNU6B was used as endogenous control for the acute exercise samples and RNU48 for resting miR levels.

Muscle lysate preparation. Freeze-dried muscle specimens were dissected free of blood, fat, and connective tissue under the microscope and homogenized in an ice-cold buffer as described previously (10), except that the tissue was homogenized for 2 min at 30 s⁻¹ in TissueLyserII (Qiagen). Protein content in lysates was measured by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). Lysates were prepared with sample buffer containing sodium dodecyl sulfate (SDS) and boiled for 3 min at 96°C and analyzed by SDS-PAGE and Western blottiing.

SDS-PAGE and Western blotting. AMPK and acetyl-CoA carboxylase (ACC) phosphorylation as well as AMPKα2, ACC, cyt c, HKII, SIRT1, VEGF, and β-actin protein content were measured in muscle lysates by SDS-PAGE (Tris-HCl, 5 and 10% gels; Bio-Rad) and Western blotting using PVDF membrane and semidyli transfer, as described previously (10). Protein content and phosphorylation are expressed in units relative to control samples loaded on each gel. Primary antibodies used were phospho-ACC (no. 07-303; Upstate Biotechnology, Lake Placid, NY), phospho-AMPK (no. 2535S; Cell Signaling Technology, Beverly, MA), AMPKα2 and AMPKα1 pro-
Statistical calculations were performed using SigmaStat version 3.1.

Table 2. Performance, body composition, fiber type, and capillarization

<table>
<thead>
<tr>
<th></th>
<th>Before Bed Rest</th>
<th>After Bed Rest</th>
</tr>
</thead>
<tbody>
<tr>
<td>V̇O₂max, l/min</td>
<td>3.9 ± 0.2</td>
<td>3.7 ± 0.2†</td>
</tr>
<tr>
<td>Time to exhaustion, min</td>
<td>28.66 ± 6.11</td>
<td>21.36 ± 3.01</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>75.2 ± 3.19</td>
<td>75.1 ± 3.29</td>
</tr>
<tr>
<td>Total muscle mass, kg</td>
<td>58.6 ± 2.42</td>
<td>58.3 ± 2.38</td>
</tr>
<tr>
<td>Leg muscle mass, kg</td>
<td>20.6 ± 0.99</td>
<td>20.0 ± 0.94†</td>
</tr>
<tr>
<td>Total fat mass, kg</td>
<td>13.3 ± 1.74</td>
<td>13.6 ± 1.77</td>
</tr>
<tr>
<td>Whole body fat, %</td>
<td>18.2 ± 2.11</td>
<td>18.5 ± 2.11</td>
</tr>
<tr>
<td>Type I fibers, %</td>
<td>54.0 ± 0.1</td>
<td>54.0 ± 0.1</td>
</tr>
<tr>
<td>Type IIa fibers, %</td>
<td>33.0 ± 0.0</td>
<td>30.0 ± 0.0</td>
</tr>
<tr>
<td>Type Ix fibers, %</td>
<td>12.0 ± 0.0</td>
<td>14.0 ± 0.0</td>
</tr>
<tr>
<td>Type I fiber size, μm²</td>
<td>4,962 ± 447</td>
<td>5,365 ± 525</td>
</tr>
<tr>
<td>Type IIa fiber size, μm²</td>
<td>5,888 ± 475</td>
<td>5,743 ± 577</td>
</tr>
<tr>
<td>Type Ix fiber size, μm²</td>
<td>5,735 ± 559</td>
<td>5,816 ± 994</td>
</tr>
<tr>
<td>Capillaries/fiber</td>
<td>3.6 ± 0.2</td>
<td>3.7 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12. V̇O₂max, pulmonary maximal oxygen uptake. Performance, body composition, myosin heavy-chain fiber type, and capillarization before and after 7 days of bed rest. †Significantly different from before bed rest, P ≤ 0.05.

RESULTS

Anthropometric parameters. Total body weight, total fat mass, and percent fat did not change in response to 7 days of bed rest, but leg muscle mass was on average reduced (P ≤ 0.05) ~600 g by bed rest (Table 2).

Performance. V̇O₂max was 4% lower (P ≤ 0.05) after bed rest, whereas time to exhaustion decreased only nonsignificantly (11%; decreased in 7 of the 12 subjects) in response to bed rest (Table 2).

Oral glucose tolerance test. Fasting plasma glucose concentration decreased (P ≤ 0.05) from 4.9 ± 0.1 to 4.5 ± 0.1 mM, and fasting insulin concentration increased (P ≤ 0.05) from 29.8 ± 4.3 to 45.7 ± 3.6 pmol/l as a result of the bed rest period. Two hours after the oral glucose intake, the plasma glucose concentration tended to be higher (0.05 < P ≤ 0.1) after bed rest (5.4 ± 0.3 mM) than before bed rest (4.9 ± 0.4 mM), and plasma insulin concentration was higher (P ≤ 0.05) after bed rest (259.5 ± 23.0 pmol/l) than before bed rest (106.2 ± 27.5 pmol/l). The area under curve (AUC) was calculated for plasma glucose and insulin in response to the OGTT before and after bed rest. The AUC for the plasma glucose response was unchanged, whereas AUC for plasma insulin was 1.4-fold larger (P ≤ 0.05) after bed rest than before bed rest.

MHC fiber type composition, fiber size, and capillarization. Despite the reduced muscle mass with bed rest, muscle fiber size did not change. In addition, percentage of type I, IIA, and IIX fibers and capillarization per fiber were similar before and after bed rest (Table 2).

Enzyme activity. Maximal activities of citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HAD) were measured by fluorometric methods, as described previously (34).

Statistics and calculations. Values presented are means ± SE. All 12 subjects contributed to the resting miR, mRNA and protein content, mtDNA/nDNA, and capillarization, and MHC fiber type composition, as well as the results from DEXA scanning and performance data. A paired t-test was applied to test the effect of bed rest on these parameters, with a one-tailed paired t-test used for resting mRNA and protein content, mtDNA/nDNA, and capillarization, and performance tests expecting a decrease with bed rest. Two-way analysis of variance for repeated measurements was applied to evaluate the effect of bed rest and exercise on muscle glycogen, mRNA, miR, protein phosphorylation, and protein content using the Student-Newman-Keuls post hoc test to locate differences. Differences were considered significant at P ≤ 0.05, and a tendency is reported when 0.05 < P ≤ 0.10. Statistical calculations were performed using SigmaStat version 3.1.

DNA content, enzyme activity, and protein content. mtDNA/nDNA tended to be 15% lower (0.05 < P ≤ 0.10) after bed rest than before bed rest (Fig. 1). A similar tendency for decreased (0.05 < P ≤ 0.10) mtDNA/nDNA with bed rest was evident when only the six subjects in the exercise protocol were included (data not shown).

The protein content of cyt c and VEGF was similar before and after bed rest, but HKII protein content was 50% lower (P ≤ 0.05), and SIRT1 protein content was 40% lower (P ≤ 0.05) after bed rest than before bed rest (Fig. 2B). Analysis of protein content only for the six subjects in the exercise protocol showed that SIRT1 protein content decreased (P ≤ 0.05) and HKII and cyt c protein content tended to decrease (0.05 < P ≤ 0.10), whereas VEGF protein content did not change significantly (data not shown).

Plasma adrenaline. The plasma adrenaline level was unaffected by bed rest, and plasma adrenaline during exercise did not change significantly either before or after bed rest (Fig. 3A).

Muscle glycogen. The muscle glycogen level tended to be higher (0.05 < P ≤ 0.10) after bed rest than before bed rest, but muscle glycogen breakdown during exercise was similar before (139 mmol/kg dry wt) and after bed rest (145 mmol/kg dry wt) (Fig. 2B). Muscle glycogen was lower (P ≤ 0.05) before bed rest.
immediately after exercise than Pre both before and after bed rest. In addition, the muscle glycogen level at 3 h of recovery tended to be lower (0.05 < \( P \leq 0.10 \)) than Pre before bed rest and was lower (\( P \leq 0.05 \)) than Pre after bed rest. These muscle glycogen data have been published recently (26).

**AMPK and ACC phosphorylation.** Bed rest had no effect on the resting AMPK\( \alpha \)2 protein or phosphorylation levels in skeletal muscle. AMPK phosphorylation/AMPK\( \alpha \)2 protein was 2.7-fold higher (\( P \leq 0.05 \)) immediately after exercise than Pre before bed rest but did not change with exercise after bed rest (Fig. 4A). A similar pattern was evident both for unnormalized AMPK phosphorylation and when normalized to AMPK\( \alpha \)1 protein content (data not shown). ACC phosphorylation/ACC protein increased (\( P \leq 0.05 \)) similarly, approximately fivefold, in response to exercise before and after bed rest (Fig. 4B).

**mRNA content.** Bed rest did not affect the resting levels of HKII, cytc, SIRT1, PGC-1\( \alpha \), or VEGF mRNA in skeletal muscle. Before bed rest, PGC-1\( \alpha \) and VEGF mRNA content increased (\( P \leq 0.05 \)) 3.3- and 2.5-fold, respectively, at 3 h of recovery from exercise relative to Pre but did not change with exercise after bed rest (Fig. 5, A and B). HKII, cytc, and SIRT1 mRNA content was not affected by the acute exercise.

**miR content.** The resting content of miR-133a was 8% lower (\( P \leq 0.05 \)), and miR-1 tended to be 9% lower (0.05 < \( P \leq 0.10 \)) after bed rest than before bed rest, whereas the resting levels of miR-23a and miR-29b were unaffected by bed rest (Fig. 6A).

Whereas the miR-23a content was unchanged in response to exercise before bed rest, miR-23a was 27% lower (\( P \leq 0.05 \)) at 3 h of recovery than Pre after bed rest (Fig. 6B). miR-1, miR-29b, and miR-133a content did not change in response to the exercise bout either before or after bed rest (data not shown).

**DISCUSSION**

The main findings of the present study are that only 7 days of bed rest abolished the exercise-induced PGC-1\( \alpha \) and VEGF mRNA responses and reduced the mtDNA/nDNA content, HAD and CS activity, and HKII and SIRT1 protein content as well as the miR-1 and miR-133a content in human skeletal muscle, but without significant changes in cytc and VEGF protein content or capillarization.

The present observations that bed rest resulted in loss of leg muscle mass and induced whole body glucose intolerance as well as reduced \( V_\text{O}_{2\text{max}} \) are in accord with previous bed rest (2, 37, 43) and physical inactivity studies (27). However, this is the first study to examine the impact of 7 days of bed rest on oxidative capacity and acute exercise-induced adaptive responses in skeletal muscle. Based on previous reports showing decreased activity of metabolic proteins in human skeletal muscle with detraining (17), it was expected that bed rest would induce similar changes. The present findings that HKII and SIRT1 protein content as well as HAD and CS activity were reduced by bed rest are thus in line with this anticipation and suggest that only 7 days of bed rest results in opposite changes of those previously shown to occur with training (16, 17, 48). The observed reductions in protein content/activity of the cytosolic HKII, the nuclear SIRT1, and mitochondrial HAD and CS indicate that proteins in several different cellular compartments were affected by bed rest. But the lack of significant change in cytc protein as such does not support a general decrease in all metabolic proteins with 7 days of bed rest. The decreased mt/nDNA ratio with bed rest in the present study provides evidence for a lower mitochondrial content in skeletal muscles after only 7 days of total physical inactivity and is in accord with training-induced mitochondrial biogene-
sis (16), although human studies in general report a lack of changes in mtDNA/nDNA with training (22, 47). In addition, this indication for decreased mitochondrial content and the observed lower HAD and CS activity suggests that the non-significant change in cyt c protein may be due to longer half-life of cyt c than the major part of mitochondrial proteins. But larger individual variation in the cyt c response to the bed rest period is also a possibility. The similar resting HKII, cyt c, PGC-1α, SIRT1, and VEGF mRNA levels before and after bed rest in the present study are in contrast to the reduction recently demonstrated for HKII, PGC-1α, and VEGF mRNA with 9 days of bed rest (1). This difference between the studies may be due to the duration of the bed rest and/or the number of subjects studied (12 subjects in the present study and 20 subjects in the previous study).

The lack of change in skeletal muscle capillarization after 7 days of bed rest in the current study is in accord with the results from a previous bed rest study (38). Moreover, the unaffected VEGF protein content is in accord with the unchanged capillarization and may support that regulation of capillarization is less sensitive to physical inactivity than at least some metabolic proteins. This suggestion is in line with previous findings.
showing that detraining had a more dramatic effect on metabolic enzyme activities than on capillarization (17). However, it may be noted that VEGF protein content was reduced nonsignificantly, ~50%, by bed rest, maybe indicating individual variation in the sensitivity to physical inactivity of this angiogenic regulator.

The unchanged capillarization despite decreased mitochondrial content may explain that the exercise endurance was not significantly reduced by bed rest in the present study and suggests that muscle endurance during such moderate-intensity exercise may not be that sensitive to short-term physical inactivity. An additional possibility is that some of the subjects are more sensitive to physical inactivity than others. However, the subjects showing no decrease in endurance with bed rest did exhibit reduced activity of CS and HAD as well as reduced mitochondrial DNA content in skeletal muscle, making this possibility less likely. Factors not related to muscle endurance, such as psychological factors, may also have influenced time to exhaustion for some subjects more than others and thereby contributed to the lack of significant effect of bed rest on endurance.

The observed exercise-induced increases in PGC-1α and VEGF mRNA in skeletal muscle before bed rest in the present study are in line with previous studies (18, 21, 41). Because the exercise-induced VEGF mRNA response has been shown to be reduced and the PGC-1α mRNA content to be increased in response to knee extensor exercise after a period of exercise training (21, 41), it was anticipated that the exercise-induced VEGF and PGC-1α mRNA responses would be elevated and reduced, respectively, after bed rest. Therefore, the findings that the exercise-induced PGC-1α and VEGF mRNA responses were totally blunted after bed rest were unexpected and, very interestingly, indicate that 7 days of total physical inactivity of the muscles abolishes the ability of the muscles to induce mRNA changes in response to an acute exercise bout.

**Fig. 5.** mRNA content. Peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α (A) and VEGF mRNA content in vastus lateralis (B) Pre, Post, 3 h rec from 45 min of one-legged knee extensor exercise BB and AB. The mRNA content is normalized to the single-stranded DNA content in the sample. Values are means ± SE; n = 6. *Significantly different from Pre in given trial, P ≤ 0.05; †significantly different from BB at given time point, P ≤ 0.05; (†) tendency to be significantly different from Pre in given trial, 0.05 < P ≤ 0.10.

**Fig. 6.** miR content. Resting microRNA (miR) content normalized to RNU48 in vastus lateralis BB and AB (A) and miR-23a content normalized to RNU6B in vastus lateralis (B) Pre, Post, and 3 h rec from 45 min of one-legged knee extensor exercise BB and AB. Values are means ± SE; n = 12 in A and n = 6 in B. *Significantly different from Pre in given trial, P ≤ 0.05; †significantly different from BB at given time point, P ≤ 0.05. (†) tendency to be significantly different from BB, 0.05 < P ≤ 0.10.
Because previous studies in mice indicate that AMPK regulates the expression of PGC-1α and VEGF in skeletal muscle (24, 28), the observation that exercise-induced AMPK phosphorylation was also present before and after bed rest may suggest that the lack of changes in AMPK phosphorylation after bed rest led to the absent PGC-1α and VEGF mRNA responses. In addition, the similar pattern of PGC-1α and VEGF mRNA content both before and after bed rest in the present study supports a possible AMPK/PGC-1α-mediated regulation of both PGC-1α and VEGF mRNA expression. On the other hand, other upstream factors may also be involved because it has been demonstrated that AMPKα2 is not required for exercise-induced PGC-1α mRNA increases in mouse skeletal muscle (24). Of notice is the similar exercise-induced phosphorylation of ACC at an assumed AMPK site (4) before and after bed rest in the present study. This may reflect that the in vivo AMPK activity was higher than indicated by the AMPK Thr172 phosphorylation due to allosteric regulation of AMPK (23) or that ACC was regulated by factors other than AMPK. Importantly, the maintained exercise-induced ACC phosphorylation in the present study and the recently shown exercise-induced pyruvate dehydrogenase regulation both before and after bed rest (26) together underline the fact that the muscles were indeed able to elicit some typical phosphorylation events in response to exercise after bed rest, indicating a specificity of the blunted AMPK phosphorylation after bed rest.

Because exercise-induced AMPK phosphorylation has been shown to be enhanced when muscle glycogen is reduced (36), it is possible that the elevated muscle glycogen level after bed rest in the present study contributed to the observed blunted AMPK phosphorylation and concomitantly the absent mRNA responses. However, it should be noted that muscle glycogen was increased only 20% by bed rest, indicating that this explanation may not be that likely, and the lack of mRNA responses may also be caused by an AMPK-independent mechanism. Hence, 9 days of bed rest has shown to increase PGC-1α promoter methylation (1) and increased methylation of the PGC-1α promoter to be associated with reduced PGC-1α mRNA expression (1, 7). In addition, the PGC-1α promoter has been reported to be hypermethylated in skeletal muscle of T2D patients (7), and a smaller exercise-induced PGC-1α mRNA response has also been demonstrated in T2D patients (13). Therefore, it is possible that the lack of exercise-induced PGC-1α mRNA increase after bed rest in the present study was due to physical inactivity-induced hypermethylation of the PGC-1α promoter.

The present findings that resting miR-133a and miR-1 levels were reduced after bed rest are as such opposite of the expected based on the previously reported decrease after exercise training (39) but in accord with the decrease in miR-133a in mouse skeletal muscle upon spaceflight (3) as well as the reduced miR-133a content in skeletal muscle of T2D patients (14). The miR-133a content has previously been suggested to correlate with fasting glucose levels in T2D patients, but no correlations were present between miR-133a and fasting plasma glucose in the present study. This difference between the previous finding in T2D patients and the present finding in healthy subjects even after bed rest likely reflects the ability of the subjects in the present study to compensate for a reduced ability to remove glucose in the periphery (9, 38) by increased insulin secretion (37). The decreased miR-23a content observed 3 h into recovery after bed rest is in accordance with the previous observation that an acute exercise bout downregulated miR-23a content in mouse skeletal muscle (42). Because the subjects in the present study exercised at the same absolute workload before and after bed rest, the relative exercise intensity is expected to have been higher after bed rest than before bed rest. Although there are to our knowledge no studies yet investigating the importance of exercise intensity on exercise-induced miR responses, a lower relative exercise intensity is a likely explanation for the lack of changes in miR-23a before bed rest.

In conclusion, the lack of exercise-induced PGC-1α and VEGF mRNA responses after 7 days of bed rest suggests that total physical inactivity abolishes exercise-induced gene responses and thus in part the ability of human skeletal muscle to adapt to acute exercise. In addition, the observed reduced miR-1 and miR-133a content in skeletal muscle after bed rest further implies that posttranscriptional regulation may also be influenced by physical inactivity. Finally, the findings that bed rest reduced mitochondrial DNA content and HAD and CS activity as well as HKII and SIRT1 protein content indicate that just 7 days of total physical inactivity lowers metabolic capacity and affects metabolic regulation in human skeletal muscle. Together, the present results provide evidence that a physically inactive lifestyle as well as short-term bed rest due to trauma or surgery induce unfavorable changes in skeletal muscle, potentially affecting not only functional capabilities but also the adaptability to exercise.

ACKNOWLEDGMENTS

We thank the subjects for the extraordinary effort and the whole Copenhagen Bed Rest 2008 Team for excellent collaboration. A. Guadalupe-Grau and J. A. Calbet each took part in the experiments while visiting Copenhagen Muscle Research Centre, Rigshospitalet, Denmark.

GRANTS

This study was supported by grants from the Lundbeck Foundation and The Danish Medical Research Council, Denmark. The Centre of Inflammation and Metabolism (CIM) is supported by a grant from the Danish National Research Foundation (no. 02-512-55). The Copenhagen Muscle Research Centre is supported by a grant from the Capital Region of Denmark. The CIM and the Molecular and Physiology Group, Department of Exercise and Sport Sciences, are part of the UNIK Project: Food, Fitness, and Pharma for Health and Disease, supported by the Danish Ministry of Science, Technology, and Innovation.

DISCLOSURES

The authors have nothing to declare.

REFERENCES


3A. Co-authorship statement

All papers/manuscripts with multiple authors enclosed as annexes to a PhD thesis synopsis should contain a co-author statement, stating the PhD student's contribution to the paper.

1. General information

<table>
<thead>
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<th>Name</th>
<th>Stine Ringholm Jørgensen</th>
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<td>030582-1586</td>
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<tr>
<td></td>
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<td><a href="mailto:srjorgensen@bio.ku.dk">srjorgensen@bio.ku.dk</a></td>
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2. Title of PhD thesis

PGC-1α in exercise- and exercise training-induced metabolic adaptations

3. This co-authorship declaration applies to the following paper

Bed rest reduces metabolic protein content and abolishes exercise-induced mRNA responses in human skeletal muscle


The extent of the PhD student's contribution to the article is assessed on the following scale

A. has contributed to the work (0-33%)
B. has made a substantial contribution (34-66%)
C. did the majority of the work independently (67-100%).

1/3

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4. Declaration on the individual elements

1. Formulation in the concept phase of the basic scientific problem on the basis of theoretical questions which require clarification, including a summary of the general questions which it is assumed will be answerable via analyses or concrete experiments/investigations.

2. Planning of experiments/analyses and formulation of investigative methodology in such a way that the questions asked under (1) can reasonably be expected to be answered, including choice of method and independent methodological development.

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- % [60]

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- % [40]

Please indicate which specific part(s) of the paper that has been produced as part of the PhD study:

All work has been done during my PhD except conduction of the experiment and some of protein analysis

6. Signatures of co-authors:

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<td>Kristian Kiilerich</td>
<td></td>
</tr>
<tr>
<td>04/03-2013</td>
<td>Amelia Guadalupe-Grau</td>
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Revised 29 January 2013
By signing the document, the PhD student hereby declares that the above information is correct.

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Role of PGC-1α in exercise and fasting-induced adaptations in mouse liver

Tobias Nørresø Haase,1* Stine Ringholm,1 Lotte Leick,1 Rasmus Sjorup Biense,1 Kristian Kührerich,1 Sune Johansen,1 Maja Munk Nielsen,1 Jørgen FP Wojtaszewski,2 Juan Hidalgo,3 Per Amstrup Pedersen,4 and Henriette Pilegaard1

1Centre of Inflammation and Metabolism and Copenhagen Muscle Research Centre, Section of Molecular and Integrative Physiology, Dept. of Biology, University of Copenhagen, Copenhagen, Denmark; 2Copenhagen Muscle Research Centre, Molecular Physiology Group, Section of Human Physiology, Department of Exercise and Sport Sciences, University of Copenhagen, Copenhagen, Denmark; 3Institute of Neurosciences and Department of Cellular Biology, Physiology and Immunology, Autonomous University of Barcelona, Barcelona, Spain; and 4Section of Molecular and Integrative Physiology, Department of Biology, University of Copenhagen, Copenhagen, Denmark

Submitted 29 November 2010; accepted in final form 9 August 2011

Haase TN, Ringholm S, Leick L, Biense RS, Kührerich K, Johansen S, Nielsen MM, Wojtaszewski JF, Hidalgo J, Pedersen PA, Pilegaard H. Role of PGC-1α in exercise and fasting-induced adaptations in mouse liver. Am J Physiol Regul Integr Comp Physiol 301: R1501–R1509, 2011. First published August 10, 2011; doi:10.1152/ajpregu.00775.2010.—The transcriptional coactivator peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α plays a role in regulation of several metabolic pathways. By use of whole body PGC-1α knockout (KO) mice, we investigated the role of PGC-1α in fasting, acute exercise and exercise training-induced regulation of key proteins in gluconeogenesis and metabolism in the liver. In both wild-type (WT) and PGC-1α KO mice, the mRNA content of the gluconeogenic proteins glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) was upregulated during fasting. Pyruvate carboxylase (PC) remained unchanged after fasting in WT mice, but it was upregulated in PGC-1α KO mice. In response to a single exercise bout, G6Pase mRNA was upregulated in both genotypes, whereas no significant changes were detected in PEPCK or PC mRNA. While G6Pase and PC protein remained unchanged, liver PEPCK protein content was higher in trained than untrained mice of both genotypes. The mRNA content of the mitochondrial proteins cytochrome c (Cyt c) and cytochrome oxidase (COX) subunit I was unchanged in response to fasting. The mRNA and protein content of Cyt c and COX1 increased in the liver in response to a single exercise bout and prolonged exercise training, respectively, in WT mice, but not in PGC-1α KO mice. Neither fasting nor exercise affected the mRNA expression of antioxidant enzymes in the liver, and knockout of PGC-1α had no effect. In conclusion, these results suggest that PGC-1α plays a pivotal role in regulation of Cyt c and COX1 expression in the liver in response to a single exercise bout and prolonged exercise training, which implies that exercise training-induced improvements in oxidative capacity of the liver is regulated by PGC-1α.

gluconeogenesis; oxidative proteins; antioxidant enzymes

THE LIVER IS THE MAJOR ORGAN responsible for maintaining plasma glucose levels during prolonged exercise and fasting, which is accomplished by increased hepatic glucose production through elevated hepatic glycogenolysis and gluconeogenesis. The key gluconeogenic proteins phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase (PC), as well as glucose-6-phosphatase (G6Pase), which plays an equally important role in gluconeogenesis and glycogenolysis are thought to be controlled at the transcriptional level. In accordance, the activity of PEPCK, PC, and G6Pase increases during exercise (11, 12) and fasting (23), and it has been demonstrated that the mRNA expression of PEPCK, PC, and G6Pase is upregulated in rodent liver during fasting and/or after a single bout of exercise (3, 9, 18, 20, 29). Several studies have investigated the effects of exercise training on hepatic mRNA and protein expression in rodents (1, 5, 6, 10, 24, 39). For example, endurance exercise training has been shown to increase hepatic glucose release from rat liver perfused in situ with glucagon (13), and this was suggested to be caused by an improved sensitivity to glucagon due to a higher hepatic glucagon receptor density after training (24). However, an increased capability for glucose production with exercise training could also originate from an exercise training-induced increase in gluconeogenic enzymes such as PEPCK, PC, and G6Pase, but this remains to be validated.

The transcriptional coactivator peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α potentially coordinates the expression of proteins that control diverse metabolic pathways in response to metabolic challenges (2, 28, 34). PGC-1α has been suggested to play a pivotal role in the control of gluconeogenesis in the liver (18, 35, 40), coactivating several transcription factors, including hepatocyte nuclear factor (HNF)-4α, forkhead box O1A, and the glucocorticoid receptor, to increase the expression of PEPCK and G6Pase (17, 35, 36). The mRNA expression of PGC-1α in mouse liver has also been demonstrated to be upregulated after a single bout of exercise (20) and with fasting (17, 18, 40), suggesting that PGC-1α mediates the previously reported exercise and/or fasting-induced changes in PEPCK, PC, and G6Pase expression (3, 9, 18, 20, 29). But whether PGC-1α is mandatory in exercise-induced gene responses of gluconeogenic proteins in the liver is not known.

In addition, exercise training has been demonstrated to increase the expression of rodent liver mitochondrial proteins (5) and antioxidant defense-related proteins, such as SOD 2 (6, 10), suggesting that the liver exhibits similar adaptations to regular physical activity as skeletal muscle (4, 15). However, the molecular mechanisms controlling the transcriptional responses of mitochondrial and antioxidant defense proteins in the liver in response to acute interventions, such as exercise and fasting, are still not fully understood. In skeletal muscle, PGC-1α seems to play a dispensable role in the regulation of

* T. N. Haase and S. Ringholm contributed equally to this work.
Address for reprint requests and other correspondence: H. Pilegaard, Dept. of Biology, Univ. of Copenhagen, Universitetsparken 13, 2100 Copenhagen, Denmark (e-mail: hpilegaard@bio.ku.dk).

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mitochondrial proteins, such as cytochrome c (Cyt c) and cytochrome oxidase (COX) subunit I, in response to exercise (26), because exercise raised the mRNA and protein levels of these proteins in both wild-type (WT) and PGC-1α knockout (KO) mice, although at a lower level in PGC-1α KO mice. Whether this is the case in the liver is not known.

The aim of the present study was to test the hypothesis that 1) fasting and a single bout of exercise induce the mRNA expression of gluconeogenesis-related proteins, as well as mitochondrial proteins in the liver through a PGC-1α-dependent mechanism and 2) PGC-1α is required for exercise training-induced regulation of gluconeogenic and mitochondrial proteins in mouse liver.

MATERIALS AND METHODS

Mice

Experiments for the present study were approved by the Danish Animal Experimental Inspectorate. The study used PGC-1α whole body KO mice (Mus musculus Linnaeus, 1758) and littermate WT mice produced by intercross breeding of heterozygous parents. This mouse strain has previously been described (28). During the experimental period, the mice were housed individually in cages with a 11:13-h light-dark cycle and with free access to standard chow (Altromin nr. 1324, Chr. Pedersen, Ringsted, Denmark) and water.

Experimental Protocols

The present study investigates the role of PGC-1α in both acute and long-term adaptations. It consists of three experimental protocols: two acute (fasting and a single exercise bout) and one prolonged intervention (exercise training). Results from acute exercise- and exercise training-induced regulation in skeletal muscle have previously been published from these experiments (26).

Fasting

Food was removed from PGC-1α KO and littermate WT mice (n = 8 in each group), and the animals were anesthetized after 24 h fasting by an intraperitoneal injection of pentobarbital sodium (6 mg of pentobarbital/100 g body wt). The liver was removed and quickly frozen in liquid nitrogen and later used for mRNA determinations, which is especially relevant for evaluating effects of such an acute intervention.

Adaptation to Exercise

Prior to both the single treadmill running bout and the treadmill exercise training period, all mice were acclimatized to treadmill exercise 10 min per day (Exer 4 treadmill; Columbus Instruments, Columbus, OH) on three consecutive days. Each 10-min exercise period consisted of 5 min at 8 m/min and 5 min at 14 m/min, with a constant slope of 10%. Running on the treadmill was encouraged by an air pistol when needed.

Single Exercise Bout

Forty-eight hours after the end of adaptation to exercise, PGC-1α KO and littermate WT mice performed a single treadmill running bout at 14 m/min at 10% slope for 1 h. The mice were killed by cervical dislocation immediately (0 h), 2 h or 6 h after running, while mice not run acutely served as controls (n = 8 in each group). Livers were quickly removed, frozen in liquid nitrogen, and later used for mRNA determinations, which is especially relevant for evaluating effects of acute interventions. The mice were killed between 4 and 6 PM, with food removed 2 h before sacrificing, except for the 2-h group, which because of the running procedure experienced 3 h without food before being killed. The exercise training protocol has previously been described (26).

Exercise Training

Another group of PGC-1α KO and littermate WT mice (n = 16 in each group) performed 5 wk of exercise training (treadmill + wheel running). Treadmill running was performed at 14 m/min and 10% slope for 1 h per day for 5 days each week, and the running distance on the running wheels was controlled [measured by a computer (Sigma Sport, Neustadt, Germany)], so that WT and PGC-1α KO mice completed a similar distance. The total wheel running duration per day was 106 ± 10 min for male WT, 98 ± 13 min for male KO, 92 ± 8 min for female WT, and 88 ± 13 min for female KO. The mice were killed by cervical dislocation 36 h after the last training bout to avoid acute effects of the last exercise bout, and with untrained mice serving as controls. The liver was quickly dissected out and frozen in liquid nitrogen for later determination of protein levels, which is especially relevant for evaluating effects of such a prolonged intervention. The mice were killed between 8 and 10 AM, and food was removed 2 h before. The exercise training protocol has previously been described (26).

Liver Glycogen and Plasma Glucose

Liver glycogen and plasma glucose in the exercise protocol were determined as previously described (31) by using a Fluoroscan (Thermo Scientific, Finland) with liver glycogen content determined as glycosyl units after acid hydrolysis. Plasma glucose in the fastin experiment was determined using Contour glucose strips (Bayer Diabetes Care, Stockholm, Sweden).

RNA Isolation and Reverse Transcription

RNA isolation was performed on 25–30 mg liver tissue by the phenol-chloroform extraction method (8) with modifications (32). The final pellet was resuspended in 0.1 mM EDTA in DEPC water (5 μl/mg liver tissue). A total of 3 μg RNA was reverse transcribed using the SuperScript II (Invitrogen, Carlsbad, CA) and oligo dT, as previously described (32).

Real-Time PCR

The amount of specific mRNAs was quantified by fluorescence-based real-time PCR (ABIT900; Applied Biosystems, Foster City, CA) using Taqman probes. Primers and Taqman probes to amplify a specific fragment of the mRNAs were designed using Primer Express (Applied Biosystems). Primer and Taqman probe sequences are given in Table 1. The primers and probes were optimized as previously described (33). The PCR was performed in triplicates, as previously described (30). The amount of specific mRNA was normalized to total cDNA content determined by Oligreen reagent (Molecular Probes, Leiden, The Netherlands), as previously described (30).

Western Blot Analysis

Extraction of protein from liver tissue was performed in cold lysis buffer [10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, 3 mM benzamidin (pH 7.5)]. The protein content of the lysate samples was determined by the bicinchoninic acid method ( Pierce, Rockford, IL).

Liver lysate proteins were separated using Tris-HCl gels (Bios-Rad, Stockholm, Sweden), and transferred (semi-dry) to PVDF membranes (Immobilon Transfer Membrane, Millipore A/S, Copenhagen, Denmark) and standard Western blotting procedures were used for detection of specific proteins. Following detection and quantification using a charge-coupled device-image sensor and 1D software (Kodak Image Station, 2000MM; Kodak, Brondby, Denmark), the protein content

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was expressed in arbitrary units relative to control samples loaded in duplicates on each separate gel. Primary antibodies used for Western blot analysis were Cyt c (no. 556433; BD Biosciences, San Jose, CA), COXI (no. 496900; Invitrogen), SOD2 (no. 06–984, Upstate, Lake Placid, NY), PEPC (no. 10004943; Cayman Chemical, Ann Arbor, MI), G6Pase [no. sc-27198 (c14), Santa Cruz Biotechnology, Santa Placid, NY), PEPCK (no. 10004943; Cayman Chemical, Ann Arbor, MI), and AMPK (Invitrogen). Recombinant G6Pase was produced in BL21 (DE3) and subsequently to the expression vector pET-DEST42 (Invitrogen). Recombinant G6Pase was produced in BL21 (DE3) (Invitrogen) grown in LB medium at 30°C after addition of 0.5 mM IPTG and 3% ethanol at OD450 = 0.5.

**Statistics**

The data are presented as means ± SE. The effect of fasting and genotype, acute exercise and genotype, as well as exercise training and genotype on mRNA and protein content, and of fasting and acute exercise on plasma glucose and liver glycogen was tested with the use of a two-way ANOVA test. The effect of acute exercise was also tested separately for each genotype by a one-way ANOVA test. When a main effect was detected, a Student Newman-Keuls post hoc test was performed to localize significant differences. P < 0.05 is used as a significance level, and a tendency is reported when 0.05 ≤ P ≤ 0.1 (actual P values are shown on graphs).

**RESULTS**

**Fasting**

_Liver glycogen and plasma glucose._ To confirm that animals were metabolically challenged, liver glycogen content and plasma glucose levels were measured. The liver glycogen content was reduced (P < 0.05) ~60% in WT mice and ~70% in PGC-1α KO mice in response to 24-h fasting relative to fed animals (Table 2). No genotype difference was evident.

Similarly plasma glucose was reduced (P < 0.05) ~45% in WT mice and ~33% in PGC-1α KO mice in response to 24-h fasting relative to fed animals (Table 2). In addition, the fasting plasma glucose concentration was 30% higher (P < 0.05) in PGC-1α KO mice than in WT.

PGC-1α. Fasting increased the liver PGC-1α mRNA content 2.5-fold (P < 0.05) in WT mice (Fig. 1A).

_G6Pase, PEPC, and PC._ Fasting induced a ~2-fold increase in liver G6Pase mRNA content in WT mice (P < 0.05) and tended to in PGC-1α KO mice (P = 0.095). No significant difference was found in G6Pase mRNA content between genotypes in either state (Fig. 1B).

_Liver mRNAs content._ The liver G6Pase mRNA content increased (P < 0.05) 5- to 6-fold with fasting in both genotypes, with no significant difference between genotypes (Fig. 1C). PC mRNA content was reduced in WT mice in response to fasting, whereas PC mRNA content in PGC-1α KO mice increased ~60% (P < 0.05) in response to fasting (Fig. 1D). There was a tendency for a main genotype difference (P = 0.052) in the PC mRNA content.

_Cyt c and COXI._ Fasting did not affect the liver Cyt c mRNA content in either genotype, and there was no genotype difference in the Cyt c mRNA content (Fig. 1E).

**Table 2. Liver glycogen content and plasma glucose levels in fed and 24-h fasted wild-type and PGC-1α knockout mice**

<table>
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<th>Plasma glucose, mmol/l</th>
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<td>WT</td>
<td>Fed 308.0 ± 20.6</td>
<td>6,333 ± 0.289</td>
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<tr>
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<td>Fast 122.4 ± 25.7*</td>
<td>4,514 ± 0.310*</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>Fed 343.6 ± 15.8</td>
<td>6,786 ± 0.350</td>
</tr>
<tr>
<td></td>
<td>Fast 110.8 ± 23.5*</td>
<td>4,514 ± 0.310*</td>
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Values are expressed as means ± SE; n = 7–8. WT, wild-type; KO, knockout. *Significantly different from Fed within given genotype, P < 0.05.

_Significantly different from WT at given time point, P < 0.05._
Similarly, fasting did not affect the liver COXI mRNA content in either genotype, and no genotype difference was observed (Fig. 1F).

Antioxidant enzymes. Fasting did not change the liver mRNA content of uncoupling protein (UCP) 2, SOD1, SOD2, catalase, glutathione peroxidase (Gpx) 1, and Gpx4 in either genotype, and no significant genotype difference was evident in these mRNAs (data not shown).

Single Exercise Bout

Liver glycogen and plasma glucose. To confirm that animals were metabolically challenged by the single exercise bout intervention, liver glycogen content and plasma glucose levels were measured. The liver glycogen content was reduced ($P < 0.05$) $\sim 14\%$ in WT mice and $\sim 40\%$ in PGC-1α KO mice in response to the single exercise bout relative to resting animals (Table 3). The liver glycogen content immediately after acute exercise was $\sim 40\%$ lower ($P < 0.05$) in PGC-1α KO mice than in WT.

The single exercise bout induced no statistically significant difference in plasma glucose immediately after exercise compared with before in either genotype (Table 3). However, the plasma glucose concentration was $\sim 20\%$ lower ($P < 0.05$) in PGC-1α KO mice relative to WT mice immediately after exercise.

AMPK phosphorylation. The level of AMPK phosphorylation (AMPK phosphorylation relative to total AMPKα2) was unchanged immediately after the single exercise bout in both WT mice (0.85 ± 0.09 before and 0.94 ± 0.06 after exercise) and PGC-1α KO mice (0.98 ± 0.07 before and 0.85 ± 0.02 after exercise). No genotype difference was found.

PGC-1α. A single exercise bout induced no statistically significant change in liver PGC-1α mRNA content (Fig. 2A).

G6Pase, PEPCK, and PC. The acute exercise bout increased ($P < 0.05$) liver G6Pase mRNA content 9.5 fold in WT mice and 4.5-fold in PGC-1α KO mice immediately after exercise.

Table 3. Liver glycogen content and plasma glucose levels in WT and PGC-1α KO mice at rest (pre) and immediately after exercise (0 h)

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<th>PGC-1α KO</th>
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<tr>
<td>pre</td>
<td>277.2 ± 29.3</td>
<td>248.0 ± 23.1</td>
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<tr>
<td>0 h</td>
<td>240.4 ± 18.3*</td>
<td>151.2 ± 28.0*#</td>
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<tr>
<td>Plasma glucose, mmol/l</td>
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</tr>
<tr>
<td>pre</td>
<td>7.64 ± 0.34</td>
<td>6.97 ± 0.4</td>
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<tr>
<td>0 h</td>
<td>8.67 ± 0.52</td>
<td>6.91 ± 0.78#</td>
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Values are expressed as means ± SE; $n = 7$ or 8. *Significantly different from pre within given genotype, $P < 0.05$. #Significantly different from WT at given time point, $P < 0.05$. 

Fig. 1. The mRNA content of PGC-1α (A), G6Pase (B), PEPCK (C), PC (D), Cyt c (E), and COXI (F) in liver from wild-type (WT) and PGC-1α knockout (KO) in fed (Fed) and 24-h fasted (Fast) mice. Target mRNA content is normalized to total cDNA. Values are expressed as means ± SE; $n = 6 – 8$. *Significantly different from Fed within given genotype, $P < 0.05$. The $P$ value shown in B indicates the difference from Fed within KO. Horizontal line indicates a tendency for a main genotype effect ($P$ value shown) in D.
However, no significant difference was evident in G6Pase mRNA content between genotypes. The G6Pase mRNA content returned to resting level at 2 h of recovery in both genotypes (Fig. 2B).

Application of a two-way ANOVA did not reveal any effect of acute exercise on the liver PEPCK mRNA content in either genotype. But a Student’s t-test revealed a difference (*P < 0.05) in PEPCK mRNA content between resting mice and immediately after exercise in WT mice. In addition, there was a tendency for a main genotype difference (#P < 0.089) in the PEPCK mRNA content (Fig. 2C).

PC mRNA content remained unchanged in response to a single exercise bout in both genotypes, and no genotype difference was observed at any time point (Fig. 2D).

Cyt c and COXI. The acute exercise bout induced a 40% increase (*P < 0.05) in liver Cyt c mRNA content immediately after exercise in WT mice, but not in PGC-1α KO mice, giving rise to a difference between genotypes (P < 0.05). In both genotypes, no differences were observed in liver Cyt c mRNA content at 2 h and 6 h of recovery relative to resting mice (Fig. 2E).

Liver COXI mRNA content increased (P < 0.05) 45% immediately after exercise in WT mice, but not in PGC-1α KO mice. This gave rise to a tendency for a difference between genotypes (P = 0.073) immediately after exercise. In both genotypes, no differences were observed in liver COXI mRNA content at 2 h and 6 h of recovery relative to resting mice (Fig. 2F).

**Antioxidant enzymes.** The acute exercise bout did not affect the liver UCP2, SOD1, SOD2, catalase, Gpx1, or Gpx4 mRNA content in either genotype, and no significant genotype difference was observed in these mRNAs (data not shown).

**Exercise Training**

**G6Pase, PEPCK, and PC.** G6Pase protein content in the liver remained unchanged with prolonged exercise training in both genotypes, and no genotype difference was evident in G6Pase protein content either in untrained or trained mice (Figs. 3A and 4A). Band specificity of G6Pase was verified by use of a recombinant G6Pase protein as a positive control (Fig. 4B). The two bands detected for G6Pase were analyzed together and used for protein quantification.

In response to exercise training, PEPCK protein content was ~40% higher (P < 0.05) in trained WT mice and tended to be 30% higher (P = 0.1) in PGC-1α KO mice than in the corresponding untrained mice. Furthermore PEPCK protein content was 45% higher (P < 0.05) in untrained PGC-1α KO mice than in untrained WT mice, and tended to be 20% higher (P = 0.058) in trained PGC-1α KO mice than in trained WT mice (Figs. 3B and 4A).
PC protein content in the liver remained unaffected by prolonged exercise training in both genotypes, and no genotype difference was evident in PC protein content either in untrained or trained mice (Figs. 3C and 4A).

Cyt c and COXI. Cyt c protein content increased \( (P < 0.05) \) 30% with exercise training in WT, but with no change in PGC-1α KO mice, resulting in 35% lower \( (P < 0.05) \) Cyt c protein content in trained PGC-1α KO mice than trained WT mice (Figs. 3D and 4A).

COXI protein content increased \( (P < 0.05) \) 25% with exercise training in WT, but with no change in PGC-1α KO mice. There was, however, no significant genotype difference in either untrained or trained mice (Figs. 3E and 4A).

Antioxidant enzymes. SOD2 protein content remained unchanged with prolonged exercise training in both genotypes, and no genotype difference was evident in the untrained or in the trained mice (Figs. 3F and 4A).

DISCUSSION

The main findings of the present study are that exercise training increased Cyt c, COXI, and PEPCK protein in mouse liver, and while the PEPCK response was independent of PGC-1α, the changes in Cyt c and COXI required PGC-1α. In accordance, acute exercise increased liver Cyt c and COXI mRNA content only in WT mice, while G6Pase mRNA increased in both genotypes. Together, this indicates that PGC-1α is required for exercise training-induced adaptations of mitochondrial oxidative proteins in mouse liver. In addition, PGC-1α KO mice had lower liver glycogen and plasma glucose content after acute exercise than WT mice, suggesting that the PGC-1α KO mice relied more on carbohydrates than WT mice during exercise.

One aim of the present study was to investigate the role of PGC-1α in fasting and exercise-induced mRNA and protein expression of metabolic proteins in the liver. The present observation that WT mice increased Cyt c and COXI protein content in the liver with exercise training is in accordance with a previous study showing that prolonged exercise training increased liver mitochondrial enzyme activities and proteins (5). But here, we show for the first time that PGC-1α is required for both acute exercise to induce an increase in Cyt c and COXI mRNA and for training to upregulate Cyt c and COXI protein content in the liver. A previous study from our laboratory revealed that exercise training increased Cyt c and COXI protein content in skeletal muscle of young mice at least partly independent of PGC-1α (26). However, the present findings show that PGC-1α is mandatory for the training-induced adaptations in Cyt c and COXI in the liver and hence
potentially, in general, for oxidative proteins in the liver. These observations support previous studies demonstrating the necessity of PGC-1α/H9251 for regulation of metabolism and energy homeostasis (14, 27).

Our results also show for the first time that prolonged exercise training increased PEPCK protein content, but not PC and G6Pase protein in the liver, while the finding that G6Pase mRNA increased much more potently than PEPCK in response to a single exercise bout is in accordance with previous studies (3, 9, 18, 20). Both PEPCK and G6Pase are important enzymes in gluconeogenesis, while G6Pase also is a key enzyme in glycogenolysis. These findings may therefore together suggest that acute exercise is associated with more marked induction of enzymes in glycogenolysis than of enzymes in gluconeogenesis, while prolonged exercise training improves gluconeogenic capacity.

PGC-1α has been suggested to function as a transcriptional coactivator for the expression of the gluconeogenic enzymes G6Pase and PEPCK (37, 40). However, the present observation that the G6Pase mRNA content in the liver increased after a single exercise bout, as well as after 24-h fasting in both WT and PGC-1α KO mice, shows that PGC-1α is not obligatory for the regulation of G6Pase mRNA in response to fasting and exercise. Similarly, the present finding of a similar exercise training-induced increase in PEPCK protein in WT and PGC-1α KO mice does not support that PGC-1α plays a role in the training adaptations in PEPCK. Likewise, the observed increase in hepatic PEPCK mRNA content in both WT and PGC-1α KO mice after 24 h of fasting demonstrates, that PGC-1α is not required for this response. This finding is somewhat in contrast to previous studies showing that whole body PGC-1α KO mice had elevated PEPCK mRNA in the fed state and no further change upon fasting (17, 28), but the reason for this difference between the previous and the current finding is not clear. Interestingly, the fasting-induced increase in PC mRNA content in PGC-1α KO mice but not in WT mice, as well as the higher PEPCK protein content in PGC-1α KO mice in the exercise training study, may indicate that lack of PGC-1α leads to a compensatory increase in the expression of PC and PEPCK and thus potentially an elevated hepatic glucose production. The observation that the fasting plasma glucose concentration was higher in PGC-1α KO mice than in WT mice is in accordance with this suggestion. A possible reason for an increased need for hepatic glucose output could be the reduced ability of skeletal muscle to oxidize fat. Hence, muscle-specific PGC-1α overexpression has been shown to elevate the mRNA expression of proteins important for fatty acid oxidation and transport in muscle (7, 38), while knockout of PGC-1α has been shown to reduce the mRNA expression of proteins in fat metabolism (16, 25). A reduced ability for fat oxidation in skeletal muscle of PGC-1α KO mice, therefore, lead to elevated carbohydrate use and a higher demand of glucose derived from the liver in these mice. In line with this possibility are the present findings that hepatic glycogen content in WT mice was reduced only ~14% in response to a single exercise bout, but ~40% in PGC-1α KO mice, and that plasma glucose was lower in PGC-1α KO mice than WT mice immediately after exercise. An increased demand of hepatic glucose could also explain the compensating increase in the gluconeogenetic protein PEPCK in the PGC-1α KO mice observed in the exercise study.

The combined fasting and exercise results in the present study suggest that more than one signaling pathway is implicated in fasting and exercise-induced PEPCK, PC, G6Pase, COXI, and Cyt c regulation in the liver. Hence, the present observations show that signaling pathways leading to regula-
tion of gluconeogenic proteins in the liver do not require PGC-1α, while PGC-1α is obligatory for exercise and exercise training-induced increases in Cyt c and COXI mRNA and protein content, respectively. Because exercise has been shown to increase AMPK phosphorylation in mouse liver (22), and in vitro studies have demonstrated that PGC-1α is a downstream target for AMPK (21), an AMPK-PGC-1α pathway could be a possible player in the observed exercise-induced increases in Cyt c and COXI mRNA in the liver in WT mice. However, the lack of significant changes in the level of AMPK phosphorylation after acute exercise in the present study does not support that AMPK was involved in mediating the observed responses to acute exercise.

No effect of exercise or fasting on antioxidant enzymes was detected in either genotype in this study, suggesting that these interventions do not affect antioxidant enzyme expression in the liver. In addition, the present finding that a lack of PGC-1α does not affect the basal mRNA and protein level of the examined antioxidant enzymes is different from previous findings in skeletal muscle (25), suggesting tissue-specific PGC-1α dependency in regulation of antioxidant enzyme expression.

The present findings also demonstrate that the exercise-induced mRNA responses in the liver are fast and peak immediately after a single exercise bout with resting levels restored already 2 h after the exercise stops. This rapid induction in hepatic mRNA expression was also observed in previous studies (3, 20); however, we add to these previous findings by providing a time course of hepatic mRNA expression after a single exercise bout. Thus, the kinetics of exercise-induced hepatic mRNA responses diverge from exercise-induced mRNA responses of metabolic proteins observed in muscle tissue, which have been shown typically to peak some hours into recovery (19, 26, 32).

In conclusion, the present study shows that PGC-1α is mandatory for acute exercise and exercise training-induced increases in Cyt c and COXI mRNA and protein expression in the liver. However, PGC-1α is not required for fasting, acute exercise, and/or exercise training-induced regulation of the gluconeogenic proteins PEPCK and PC or the gluconeogenic and glycogenolysis enzyme G6Pase. Furthermore, the divergent responses of the liver in response to exercise and fasting suggest that although similar adaptations are needed, different signaling pathways are involved.

**Perspectives and Significance**

The current finding that an exercise training-induced increase in mitochondrial oxidative protein content in the liver is impaired in PGC-1α KO mice, adds to the evidence that decreased level of this transcriptional coactivator could play a role in the mitochondrial impairment and decreased ability to cope with metabolic stress observed in obesity and type 2 diabetes patients. Understanding the actions and functions of PGC-1α may conceivably contribute to future treatment strategies of metabolic diseases.

**ACKNOWLEDGMENTS**

We would like to thank Professor B. Spiegelman for providing PGC-1α heterozygous mice initially to start breeding. We are grateful to Professor Graham Hardie, Dundee University, for donating an antibody essential for this study.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**


The study was supported by the Lundbeck Foundation, the Danish Medical Research Council and the EXGENESIS consortium of the European Commission (L5HM-CT-2004-005272). The Centre of Inflammation and Metabolism is supported by Danish National Research Foundation (Grant 02-512-555). Center of Inflammation and Metabolism is a part of the UNIK Project, Health & Pharma for Heath and Disease, supported by the Danish Ministry of Science, Technology and Innovation. The Copenhagen Muscle Research Centre is supported by a grant from the Capital Region of Denmark.


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2. Title of PhD thesis

PGC-1α in exercise- and exercise training-induced metabolic adaptations

3. This co-authorship declaration applies to the following paper

Role of PGC-1α in exercise and fasting-induced adaptations in mouse liver

The extent of the PhD student’s contribution to the article is assessed on the following scale

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<tr>
<td>06/03-2013</td>
<td>Tobias Norresø Haase</td>
<td>Tobias N. Haase</td>
</tr>
<tr>
<td>20/3-2013</td>
<td>Lotte Leick</td>
<td>Lotte Leick</td>
</tr>
<tr>
<td>4/3-2013</td>
<td>Rasmus S. Biensø</td>
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<td>5/3-2013</td>
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PGC-1α is required for exercise- and exercise training-induced UCP1 up-regulation in mouse white adipose tissue

Stine Ringholm, Jakob Grunnet Knudsen, Lotte Leick, Anders Lundgaard, Maja Munk Nielsen, Henriette Pilegaard

Centre of Inflammation and Metabolism, August Krogh Centre, Department of Biology, August Krogh Building, University of Copenhagen, Denmark

**Corresponding author:** Henriette Pilegaard, Department of Biology, August Krogh Building, Universitetsparken 13, 2100 Copenhagen, Denmark. Tel: +45 35321687. Fax: +45 35321567. Email: hpilegaard@bio.ku.dk

**Word count:** 4292

**Number of figures:** 4

**Conflict of interest:** The authors have nothing to declare

**Key words:** adipose tissue, exercise, exercise training, PGC-1α, UCP1

**Running title:** Exercise-induced regulation of UCP1 in WAT
ABSTRACT

Background: The aim of the present study was to test the hypotheses that 1) a single exercise bout increases UCP1 mRNA in both inguinal (i)WAT and epididymal (e)WAT, 2) UCP1 expression and responsiveness to exercise are different in iWAT and eWAT, 3) PGC-1α determines the basal levels of UCP1 and PRDM16 in WAT and 4) exercise and exercise training regulate UCP1 and PRDM16 expression in WAT in a PGC-1α-dependent manner. Methods: Whole body PGC-1α knockout (KO) and wildtype (WT) littermate mice performed a single treadmill exercise bout at 14 m/min and 10% slope for 1 hour. Mice were sacrificed and iWAT, eWAT and quadriceps muscle were removed immediately after, 2, 6 and 10 hours after running, and from sedentary mice that served as controls. In addition, PGC-1α KO mice and WT litterates were exercise trained for 5 weeks with sedentary mice as untrained controls. Thirty-six-37 hours after the last exercise bout iWAT was removed. Results: UCP1 mRNA content increased 19-fold in iWAT and 7.5-fold in eWAT peaking at 6h and 0’ of recovery, respectively, in WT but with no changes in PGC-1α KO mice. UCP1 protein was undetectable in eWAT and very low in iWAT of untrained mice but increased with exercise training to 4.4 (AU) in iWAT from WT mice without significant effects in PGC-1α KO mice. Conclusion: The present observations provide evidence that exercise training increases UCP1 protein in iWAT through PGC-1α, likely as a cumulative effect of transient increases in UCP1 expression after each exercise bout. Moreover, the results suggest that iWAT is more responsive than eWAT in exercise-induced regulation of UCP1. In addition, as PRDM16 mRNA content decreased in recovery from acute exercise, the present findings suggest that acute exercise elicits regulation of several brown adipose tissue genes in mouse WAT.
**Abbreviations:** CD31, cluster of differentiation 31; COX, cytochrome c oxidase; eWAT, epididymal white adipose tissue; GLUT4, glucose transporter 4; iWAT, inguinal white adipose tissue; PGC-1α, peroxisome proliferator-activated receptor γ co-activator 1α; PRDM16, protein-containing PR (PRD1-BF-1-RIZ1 homologous) domain 16; UCP1, uncoupling protein 1
INTRODUCTION

Life style related metabolic diseases are an increasing problem worldwide and is often associated with obesity and adipose tissue malfunction. Adipose tissue is an endocrine organ playing an important role in whole body metabolism. Several studies [1,2] indicate that inguinal white adipose tissue (iWAT), opposite of epididymal white adipose tissue (eWAT), has a protective effect on metabolic diseases. Therefore, the amount and distribution of adipose tissue seem important in development of metabolic diseases. Furthermore, inguinal- and epididymal-derived cell lines have been reported to exhibit different responsiveness to Forskolin/cAMP stimulation [3] and iWAT has recently been shown to contain beige precursor adipocytes [3,4] while eWAT does not. This indicates potential different responses in eWAT and iWAT to certain stimuli.

Stallknecht et al. [5,6] showed that WAT is able to adapt to endurance exercise training much like skeletal muscle. Hence Cytochrome c oxidase (COX) activity [5] and GLUT4 mRNA content [6] increased in rat eWAT after 10 weeks of endurance exercise training. In addition, an acute exercise bout has been shown to induce gene responses in adipose tissue from mice and rats [7,8] suggesting that cumulative effects of transient increases in mRNA lead to these adaptations. Furthermore, the uncoupling protein (UCP)1 mRNA content has been shown to increase in iWAT but not in eWAT in mice in response to cold-exposure [9] and in both adipose tissue depots with exercise training but most markedly in iWAT [10]. No changes were evident in iWAT UCP1 mRNA in response to acute exercise in mice in that study [10]. However, only one time point was measured (5h of recovery), and UCP1 mRNA was not measured in eWAT after a single exercise bout. In addition, exercise training-induced changes in UCP1 protein in WAT remains to be proven. Protein-containing PR (PRD1-BF-1-RIZ1 homologous) domain (PRDM)16 has also been identified as a regulator of the brown fat-like gene program and
thermogenesis in iWAT [11,12], but whether this protein is regulated in white adipose tissue in response to exercise is currently unresolved.

It is at present unknown which factors are regulating the exercise-induced UCP1 response in WAT. The transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α has previously been shown to drive the formation of brown fat gene program [13] in addition to playing a role in regulation of capillarization [14,15] and expression of oxidative proteins in skeletal muscle [15,16] and oxidative proteins in adipocytes [17]. Muscle PGC-1α has recently been suggested to influence UCP1 expression through PGC-1α mediated regulation of irisin release from skeletal muscle [10]. However, whether PGC-1α is required for exercise-mediated regulation of UCP1 expression in WAT is unresolved. Therefore, the aim of the present study was to test the hypotheses that 1) a single exercise bout induces UCP1 mRNA responses in both iWAT and eWAT, 2) PGC-1α determines the basal levels of UCP1 and PRDM16 in iWAT and eWAT, 3) exercise up-regulates UCP1 and PRDM16 mRNA in iWAT and eWAT in a PGC-1α-dependent manner and 4) exercise training up-regulates UCP1 protein in iWAT in a PGC-1α-dependent manner.
METHODS

Mice

The study used whole body PGC-1α knockout (KO) and wildtype (WT) littermate mice. PGC-1α KO and WT mice were obtained by intercross breeding of heterozygous parents [18] and homozygous offspring were used for experiments. During the experimental period, the mice were housed individually in cages with 12:12-h light-dark cycle and with free access to standard chow (Altromin, Brogården ApS, Lynge, Denmark) and water.

Experimental protocol

Acute exercise bout

Prior to the experimental day, mice were acclimatized to treadmill exercise (TSE systems GmbH, Bad Homburg, Germany) two times 10 min a day on five consecutive days. Each 10 min exercise period consisted of 2 min at 8m/min, 2 min at 10m/min, 4min at 15m/min and 2 min at 10m/min, with a constant slope of 10%.

Forty-eight hours after the end of adaptation to treadmill running, PGC-1α KO mice and WT littermates performed a single 1 hour treadmill exercise bout at 14 m/min with 10% slope and both genotypes completed the exercise bout, although PGC-1α KO mice exercised at a relatively higher intensity [19]. Mice were sacrificed by cervical dislocation immediately after (0h), 2 (2h), 6 (6h) or 10 (10h) hours after running, while mice not run acutely served as controls (Rest). Inguinal (iWAT), which are found anterior to the upper segment of the hind limb, and epididymal (eWAT), found underneath the abdomen skin, white adipose tissue and quadriceps muscle were quickly removed and frozen in liquid nitrogen for later analyses.
Exercise training

In addition to the acute exercise bout, a group of PGC-1α whole body KO and WT littermates were exercise trained for 1 hour 5 times/week for 5 weeks and had access to running wheels during the exercise period as previously described [15,19] with a control group not training. Running wheels were blocked occasionally in WT mice to ensure similar total running duration per day in WT and PGC-1α KO mice as previously published [19]. Mice were sacrificed by cervical dislocation 36-37 h after the last exercise bout and iWAT, was removed and quickly frozen in liquid nitrogen.

Analyses

Muscle glycogen

Muscle glycogen content was determined from 15 mg of muscle tissue as glycosyl units after acid hydrolysis [20] using a fluoroscan (Thermo Labsystems, Bie & Berntsen, Rødovre, Denmark).

RNA isolation and reverse transcription

Total RNA was isolated from ~30 mg of adipose tissue by a modified guanidinium thiocyanate-phenol-chloroform extraction method adapted from Chomczynski and Sacchi [21] as previously described [22] except that the tissue was homogenized for 2 min at 30 sec⁻¹ in a TissueLyserII (Qiagen, Valencia, CA, USA).

Superscript II RNase H⁻ system and Oligo dT (Invitrogen, Carlsbad, CA, USA) were used to reverse transcribe the mRNA to cDNA as previously described [22].

Real-time PCR
The mRNA content of UCP1 and PRDM16 were determined by real time PCR using the fluorogenic 5’ nuclease assay with TaqMan probes and universal mastermix with UNG (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, CA, USA) as previously described [23]. The sequences used to amplify a fragment of UCP1 were FP: 5’AAGCGTACCAAGCTGTGCGA3’, RP: 5’AGAAAGAAGGCCACAAACCCTT3’ and TaqMan probe: 5’CCATGTACACCAAGGAAGGACC3’ and to amplify a fragment of PRDM16 were FP: 5’CAGCACGGTGAAAGCCATTC3’, RP: 5’GGCGTGCATCCGCTTGT3’ and TaqMan probe: 5’ATGCGAGGTCTGCCACAAGTCCTAC3’. Both TaqMan probes were 5’-6-carboxyfluorescein (FAM) and 3’-6-carboxy-N,N,N’,N’-tetramethylrhodamine (TAMRA) labeled. The obtained cycle threshold (Ct) values reflecting the initial content of the specific transcript in the samples were converted to an arbitrary amount by using standard curves obtained from a serial dilution of a pooled sample made from all samples. The amount of a given mRNA was normalized to the ssDNA content of the cDNA sample determined by use of OliGreen as previously described [23].

**Lysate preparation**

Adipose tissue specimens were homogenized in an ice-cold buffer as previously described [24] except the tissue was homogenized for 2 min at 30 sec⁻¹ in TissueLyserII (Qiagen, Valencia, CA, USA). Protein content in lysates was measured by the bicinchoninic acid method (Pierce Biotechnology Inc., Rockford, IL, USA). Lysates were prepared with sample buffer containing Sodium dodecyl sulfate (SDS) and boiled for 3 min at 96°C and analyzed by SDS-PAGE and western blotting.

**SDS-PAGE and Western blotting**
Protein content was measured in adipose tissue samples by SDS-PAGE and western blotting using PVDF membrane and semi-dry transfer as previously described [24]. Protein content is expressed in units relative to control samples loaded on each gel. Primary UCP1 (ab10983 Abcam), COXIV (ab16056 Abcam) and CD31 (SC-1506 Santa Cruz) antibodies and polyclonal secondary antibodies (Dako, Glostrup, Denmark) were used.

Statistics and calculations

Values presented are means ± SE. Two-way analysis of variance was applied to test the effect of acute exercise and genotype on mRNA, protein content and muscle glycogen as well as the effect of exercise training and genotype on UCP1 protein content using the Student-Newman-Keuls post hoc test to locate differences. Differences were considered significant at $P \leq 0.05$. Statistical calculations were performed using SigmaPlot version 11.0.
RESULTS

Acute exercise

Muscle glycogen content

Resting muscle glycogen content was similar in WT and PGC-1α KO and muscle glycogen was reduced (P≤0.05) 25 and 65 % after the acute exercise bout in WT and PGC-1α KO, respectively, with no significant difference between genotypes (Table 1).

UCP1 mRNA content

The resting content of UCP1 mRNA was in both iWAT and eWAT similar in WT and PGC-1α KO (Figure 1A and B). The basal Ct level was on average ~31 and ~37 in iWAT and eWAT, respectively.

In WT, the mRNA content of UCP1 increased (P≤0.05) ~19-fold in iWAT (Figure 1A) at 6 hours of recovery relative to Rest and ~7-fold in eWAT (Figure 1B) immediately after the acute exercise bout relative to Rest, but with no changes in PGC-1α KO mice (Figure 1A and 1B).

PRDM16 mRNA content

The resting content of PRDM16 mRNA was in both iWAT and eWAT similar in WT and PGC-1α KO mice (Figure 2A and B).

In WT mice, the PRDM16 mRNA content decreased (P≤0.05) in both iWAT and eWAT at 10h of recovery from the acute exercise bout to ~20-30 % of the level in Rest, while in PGC-1α KO mice the PRDM16 mRNA content decreased (P≤0.05) at 0h, 2h and 6h of recovery from the acute exercise bout to ~30-60 % of the level in Rest, only in eWAT. In addition, the PRDM16
mRNA content in iWAT was at Rest, 2h and 6h of recovery 30-45 % lower (P≤0.05) in PGC-1α KO than in WT mice (Figure 2A and 2B).

**UCP1 protein content**

The resting content of UCP1 protein in iWAT was undetectable in 25 % of the resting samples from WT and PGC-1α KO mice taken together, while it was undetectable in all eWAT samples (Figure 3).

UCP1 protein content in iWAT did not change significantly during recovery from the acute exercise bout (Figure 3).

**Exercise training**

**UCP1 protein content**

The protein content of UCP1 in iWAT was undetectable in approximately 75 % of the untrained samples in WT and PGC-1α KO mice taken together and increased (P≤0.05) to 4.4 (A.U.) in trained WT, but did not change in PGC-1α KO (Figure 4).

**COXIV protein content**

The protein content of COXIV in iWAT was ~3-fold higher (P≤0.05) in untrained PGC-1α KO than in untrained WT mice. COXIV protein content in iWAT was ~2.5-fold higher (P≤0.05) in trained WT than in untrained WT mice, while there was no change in PGC-1α KO with exercise training (Table 2).

**CD31 protein content**
The protein content of CD31 in iWAT was ~2-fold higher (P≤0.05) in untrained PGC-1α KO than in untrained WT mice. CD31 protein content in iWAT was non-significantly ~1.6-fold higher (P=0.079) in trained WT than in untrained WT mice, while there was no change in PGC-1α KO with exercise training (Table 2).
DISCUSSION

The findings of the present study demonstrate transient exercise-induced UCP1 mRNA responses in mouse iWAT and eWAT, but with different time course of the response. Furthermore, UCP1 protein content increased with exercise training in iWAT. In addition, PGC-1α was required for both acute and exercise training-induced regulation of UCP1 in WAT.

The present study shows for the first time that exercise elicited a transient UCP1 mRNA increase in both iWAT and eWAT of WT mice in recovery from an acute exercise bout and the study demonstrates the time course of exercise-induced UCP1 mRNA responses in iWAT and eWAT. Furthermore, the novel observations that iWAT UCP1 protein content was higher in trained than in untrained WT mice in the present study add to the recent findings that the UCP1 mRNA content in iWAT and eWAT increased with exercise training in mice [10] and suggest that exercise training-induced UCP1 expression in iWAT may have functional significance. The demonstrated transient increase in UCP1 mRNA content in iWAT from WT mice makes it possible that the observed long term protein adaptations are accumulations from the repeated transient gene responses.

The lack of exercise-induced increases in UCP1 mRNA in both eWAT and iWAT of whole body PGC-1α KO mice indicates that PGC-1α is required for the acute exercise-induced regulation of UCP1 mRNA in WAT. The observed similar reduction in muscle glycogen content in WT and PGC-1α KO mice in response to the acute exercise bout supports that the PGC-1α KO mice have been physically challenged as the WT and that the lack of UCP1 response therefore is not due to lack of exercise stimulus. In addition, the long term UCP1 protein adaptations in iWAT with exercise training seem to require PGC-1α. However, the observation that the resting level of
UCP1 protein in iWAT did not differ between WT and PGC-1α KO mice indicates that PGC-1α is not needed for the basal UCP1 levels in WAT, although the very low basal UCP1 protein level makes this comparison difficult. A PGC-1α independent basal UCP1 level may also seem in contrast to the previous observation that muscle-specific PGC-1α overexpression mice had elevated iWAT UCP1 mRNA content [10]. However, as previous studies [16,19,25] have suggested that PGC-1α is involved but not necessarily required for exercise training-induced adaptations in mitochondrial proteins in skeletal muscle, muscle-specific PGC-1α overexpression mice may be seen as a model of exercise trained animals. The results may therefore indicate that basal UCP1 expression is independent of PGC-1α, while exercise-induced UCP1 regulation requires PGC-1α.

The present findings, that exercise training also increased the content of the oxidative protein, COXIV, and the capillarization marker, CD31, in iWAT in WT but not in PGC-1α KO mice, further suggest that PGC-1α exerts a concerted regulation of capillarization, oxidative proteins and UCP1 expression in iWAT with exercise training in mice. However, the higher basal COXIV and CD31 protein levels in iWAT of PGC-1α KO mice than WT is different from previous suggestions of PGC-1α mediated up-regulation of oxidative capacity in adipose tissue [7,26]. This may suggest that a compensatory mechanism is in play in iWAT of the PGC-1α KO mice leading to increased oxidative capacity of iWAT without clear effects on basal UCP1 expression. In addition, the different observations in Kleiner et al. [26], in adipose tissue-specific PGC-1α KO mice, and the present study in whole body PGC-1α KO mice, may be due to the different mouse models.

The current observations of PCR cycle threshold (Ct) levels for basal UCP1 mRNA around ~31 for iWAT and ~37 for eWAT, demonstrate that the UCP1 mRNA level is markedly higher in
iWAT than in eWAT with a hardly detectable level in eWAT. In addition, the present notion that UCP1 protein is undetectable in eWAT and in most samples also in iWAT is in accordance with a recent study by Wu et al. [3] showing that in the basal state UCP1 protein is only detectable in brown adipose tissue and not in iWAT and eWAT. In addition, the observed differences in fold change of UCP1 mRNA to acute exercise with 19-fold in iWAT and 7-fold in eWAT is in accordance with a recent study [10] showing that, after exercise training the relative mRNA content in iWAT is 22-fold higher than in eWAT, suggesting different responsiveness of the two adipose tissue depots to acute exercise. In addition, the present study identifies the time course of the exercise-induced UCP1 mRNA response in iWAT peaking at 6h while the UCP1 mRNA content in eWAT was peaking immediately after the acute exercise bout. The different time courses may contribute to different abilities for long term adaptations, because the longer lasting response in iWAT increases the chance for mRNA accumulation with repeated bouts of exercise [27].

The present finding that the PRDM16 mRNA content decreased in recovery from the acute exercise bout while UCP1 mRNA increased suggests that acute exercise elicited a response similar to cold-exposure with increased UCP1 and decreased PRDM16 expression [9]. In addition, the present observations does not suggest a role of PGC-1α in the regulation of PRDM16 mRNA content in recovery from the acute exercise bout, while PGC-1α seems at least in part involved in determining the basal PRDM16 mRNA content in iWAT.

In conclusion, the present results demonstrating that UCP1 mRNA in both iWAT and eWAT increases in response to a single exercise bout and that exercise training increased UCP1 protein in iWAT add to previous reports and support that exercise induces an up-regulation of UCP1 expression in WAT. The findings that basal UCP1 mRNA and/or protein in iWAT and eWAT
was similar in PGC-1α KO and WT mice indicate that PGC-1α is not required for basal UCP1 expression in WAT. However, the increase in UCP1 mRNA in iWAT and eWAT with acute exercise and UCP1 protein in iWAT of WT, but not PGC-1α KO provides evidence that PGC-1α is mandatory for exercise mediated regulation of UCP1 expression in iWAT. The functional role of such changes is unknown and additional studies are required to address this.

ACKNOWLEDGEMENT

We sincerely thank Professor B. Spiegelman for providing PGC-1α whole body knockout mice initially to start breeding.
Reference List


white fat and thermogenesis. Nature 481: 463-468. nature10777 [pii];10.1038/nature10777 [doi].


AUTHOR CONTRIBUTION

Stine Ringholm and Henriette Pilegaard designed the study. Henriette Pilegaard obtained funding for the experiments and analyses. All manuscript authors took part in various parts of the experiment and performed laboratory analyses. Stine Ringholm and Henriette Pilegaard wrote the manuscript and all manuscript authors commented on the paper.

FIGURE LEGENDS

Figure 1. UCP1 mRNA content in iWAT and eWAT in response to acute exercise.

Uncoupling protein (UCP) 1 mRNA content in iWAT (A) and eWAT (B) immediately after (0h), 2 (2h), 6 (6h) and 10 (10h) hours after an acute exercise bout and from rested (Rest) wildtype (WT) and whole body PGC-1α knockout (KO) mice. UCP1 mRNA is normalized to single stranded (ss) DNA. Values are means±SE; n=8. *: Significantly different from Rest within given genotype, P≤0.05. #: Significantly different from WT within given time point, P≤0.05.

Figure 2. PRDM16 mRNA content in iWAT and eWAT in response to acute exercise.

Protein-containing PR (PRD1-BF-1-RIZ1 homologous) domain (PRDM) 16 mRNA content in iWAT (A) and eWAT (B) immediately after (0h), 2 (2h), 6 (6h) and 10 (10h) hours after an acute exercise bout and from rested (Rest) wildtype (WT) and whole body knockout (KO) mice. PRDM16 mRNA is normalized to single stranded (ss) DNA. Values are means±SE; n=8. *: Significantly different from Rest within given genotype, P≤0.05. #: Significantly different from WT within given time point, P≤0.05.

Figure 3. UCP1 protein content in iWAT in response to acute exercise.
Uncoupling protein (UCP) 1 protein content in iWAT immediately after (0h), 2 (2h), 6 (6h) and 10 (10h) hours after an acute exercise bout and from rested (Rest) wildtype (WT) and whole body PGC-1α knockout (KO) mice given in arbitrary units (AU). Values are means±SE, n=8.

Figure 4. UCP1 protein content in iWAT in response to exercise training.

Uncoupling protein (UCP) 1 protein content in iWAT from untrained (UT) and trained (T) wildtype (WT) and whole body PGC-1α knockout (KO) mice given in arbitrary units (AU). Values are means±SE, n=8. *: Significantly different from UT within given genotype, P≤0.05.
Table 1. Muscle glycogen content at rest (Rest) and immediately after exercise (0h).

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<thead>
<tr>
<th></th>
<th>WT</th>
<th>PGC-1α KO</th>
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<tr>
<td>Rest</td>
<td>20.75 ±2.46</td>
<td>19.46 ±1.70</td>
</tr>
<tr>
<td>0h</td>
<td>15.59 ±1.32 *</td>
<td>6.53 ±1.01 *</td>
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Skeletal muscle glycogen content at rest (Rest) and immediately after (0h) an acute exercise bout from whole body PGC-1α knockout (KO) and wildtype (WT) littermate mice. Values are means±SE; n=8. *: Significantly different from Rest within given genotype, P≤0.05.

Table 2. COXIV and CD31 protein content in iWAT in response to exercise training.

<table>
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<tr>
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<th>Untrained</th>
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<tr>
<td></td>
<td>WT</td>
<td>PGC-1α KO</td>
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<tr>
<td>COXIV</td>
<td>0.3 ±0.1</td>
<td>0.8 ±0.2 #</td>
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<tr>
<td>CD31</td>
<td>0.4 ±0.1</td>
<td>0.9 ±0.1 #</td>
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COXIV and CD31 protein content (arbitrary units) in iWAT from untrained and trained whole body PGC-1α knockout (KO) and wildtype (WT) mice. Values are means±SE; n=8. *: Significantly different from untrained within given genotype, P≤0.05. #: Significantly different from WT within given time point, P≤0.05.
3A. Co-authorship statement

All papers/manuscripts with multiple authors enclosed as annexes to a PhD thesis synopsis should contain a co-author statement, stating the PhD student’s contribution to the paper.

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<tr>
<td>Name</td>
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<tr>
<td>Stine Ringholm Jørgensen</td>
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<tr>
<td>Civ.reg.no. (If not applicable, then birth date)</td>
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<tr>
<td>030582-1586</td>
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<tr>
<td>E-mail</td>
</tr>
<tr>
<td><a href="mailto:srjorgensen@bio.ku.dk">srjorgensen@bio.ku.dk</a></td>
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<td><strong>Principal supervisor</strong></td>
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<tr>
<td>Name</td>
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<tr>
<td>Henriette Pilegaard</td>
</tr>
<tr>
<td>E-mail</td>
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<tr>
<td><a href="mailto:hpilegaard@bio.ku.dk">hpilegaard@bio.ku.dk</a></td>
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2. Title of PhD thesis

PGC-1α in exercise- and exercise training-induced metabolic adaptations

3. This co-authorship declaration applies to the following paper

PGC-1α is required for exercise and exercise-training induced UCP1 up-regulation in mouse adipose tissue

*PLoS ONE* 2013, In pending review

The extent of the PhD student’s contribution to the article is assessed on the following scale

A. has contributed to the work (0-33%)
B. has made a substantial contribution (34-66%)
C. did the majority of the work independently (67-100%).
### 4. Declaration on the individual elements

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<td><strong>1.</strong> Formulation in the concept phase of the basic scientific problem on the basis of theoretical questions which require clarification, including a summary of the general questions which it is assumed will be answerable via analyses or concrete experiments/investigations.</td>
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<td><strong>2.</strong> Planning of experiments/analyses and formulation of investigative methodology in such a way that the questions asked under (1) can reasonably be expected to be answered, including choice of method and independent methodological development.</td>
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<td><strong>4.</strong> Presentation, interpretation and discussion of the results obtained in article form.</td>
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Please indicate which degree / thesis: PhD thesis by Lotte Leick

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Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

Conductance of training study (skeletal muscle tissue used before)

### 6. Signatures of co-authors:

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<td>7/3-2013</td>
<td>Anders Lundgaard</td>
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<td>20/3-2013</td>
<td>Lotte Leick</td>
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<tr>
<td>4/3-2013</td>
<td>Henriette Pilegaard</td>
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7. Signature

By signing the document, the PhD student hereby declares that the above information is correct.

PhD student: [Signature]  
Date: 25.03.2013  
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Effect of lifelong resveratrol supplementation and exercise training on skeletal muscle oxidative capacity in aging mice; impact of PGC-1α

Stine Ringholm1, Jesper Olesen1, Jesper Thorhauge Pedersen1, Christina Tingbjerg Brandt1, Jens Frey Halling1, Ylva Hellsten2, Clara Prats3, Henriette Pilegaard1

1Centre of Inflammation and Metabolism, August Krogh Centre, Department of Biology, August Krogh Building, University of Copenhagen, Denmark. 2Department of Nutrition, Exercise and Sports Sciences, August Krogh Building, University of Copenhagen, Denmark. 3Center for Healthy Aging, Department of Biomedical Sciences, University of Copenhagen, Denmark.

Corresponding author: Henriette Pilegaard, Department of Biology, August Krogh Building, Universitetsparken 13, 2100 Copenhagen, Denmark. Tel: +45 35321687. Fax: +45 35321567. Email: hpilegaard@bio.ku.dk

Word count: 6086

Number of figures: 5

Conflict of interest: The authors have nothing to declare

Running title: Effect of resveratrol and exercise training on mice skeletal muscle
Abstract

Background: The present study tested the hypothesis that lifelong resveratrol (RSV) supplementation counteracts an age-associated decrease in skeletal muscle oxidative capacity through peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and that RSV combined with lifelong exercise training (ET) exert additive effects through PGC-1α in mice. Methods: 3 month old PGC-1α whole body knockout (KO) and wildtype (WT) littermate mice were placed in cages with or without running wheel and fed either standard chow or standard chow with RSV supplementation (4g/kg food) for 12 month. Young (3 month of age), sedentary mice on standard chow served as control group. A graded running performance test and a glucose tolerance test were performed 2 and 1 weeks, before euthanization, respectively and, quadriceps, triceps and extensor digitorum longus (EDL) muscles were removed. Results: In PGC-1α KO mice, quadriceps citrate synthase (CS) activity, mtDNA/nDNA as well as pyruvate dehydrogenase (PDH)-E1α, Cytochrome (Cyt) c and vascular endothelial growth factor (VEGF) protein content was 20-75% lower and, EDL capillary-to-fiber (C:F) ratio was 15-30% lower than in WT mice. In EDL, RSV and/or ET had no effect on C:F ratio. CS activity tended (P=0.063) to decrease with age in WT mice, and CS activity, PDH-E1α protein and VEGF protein increased ~1.5-1.8-fold with lifelong ET in WT, but not in PGC-1α KO mice, while RSV alone had no significant effect on these proteins. Conclusion: Lifelong ET increased activity/content of oxidative proteins, mtDNA and angiogenic proteins in skeletal muscle through PGC-1α, while RSV supplementation alone had no effect. Combination of lifelong ET and RSV supplementation had no additive effect on skeletal muscle.

Keywords: resveratrol, exercise training, aging, oxidative capacity, peroxisome proliferator-activated receptor-γ coactivator-1α
Abbreviations: Cluster of differentiation, CD31; Citrate synthase, CS; Cytochrome c, Cyt c; Cytochrome c oxidase, COX; Extensor digitorum longus, EDL; Pyruvate dehydrogenase, PDH; Peroxisome proliferator-activated receptor-γ coactivator-1α, PGC-1α; Sirtuin 1, SIRT1; Vascular endothelial growth factor, VEGF;
1. Introduction

Aging is a significant risk factor for a range of diseases including cardiovascular disease and diabetes (Masoro, 2001). Changes in several tissues/organs may occur with age, and age-related metabolic changes in skeletal muscle can have important consequences for whole body metabolism (Nair, 2005). Hence, aging is associated with decreased skeletal muscle oxidative capacity due to decreased capillarization as well as a decrease in skeletal muscle mass and/or mitochondrial oxidative proteins (Conley et al., 2000; Essen-Gustavsson & Borges, 1986; Hollmann et al., 2007; Leick et al., 2010) with concomitant impact on whole body metabolism.

Endurance exercise training is known to enhance skeletal muscle oxidative capacity through increased capillarization and increased expression/activity of mitochondrial enzymes (Saltin & Rowell, 1980). Moreover, lifelong regular physical activity has been shown to counteract age-related changes in oxidative proteins and the angiogenic marker, vascular endothelial growth factor (VEGF) in mouse skeletal muscle (Hollmann et al., 2007; Leick et al., 2009; Leick et al., 2010). Daily intake of the naturally occurring polyphenol, resveratrol, has been shown to induce similar effects as exercise training, including enhanced exercise capacity as well as increased skeletal muscle citrate synthase (CS) activity in mice (Lagouge et al., 2006; Um et al., 2010), cytochrome (Cyt) c protein content and mitochondrial (mt) DNA content in gastrocnemius muscle of young mice (Um et al., 2010). But it is still unknown whether prolonged resveratrol intake can counteract an age-associated decrease in capillarization and mitochondrial proteins in skeletal muscle to a similar extent as seen with exercise training, and whether resveratrol and exercise training exert additive effects on oxidative proteins and capillarization in aging skeletal muscle.
The transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α has previously been shown to regulate metabolic pathways in skeletal muscle towards a more oxidative muscle type with increased content of oxidative proteins and capillarization (Arany et al., 2008; Lin et al., 2002; Lin et al., 2005; Olesen et al., 2010). Exercise-induced PGC-1α expression and potentially PGC-1α activation have been suggested to coordinate exercise training-induced adaptations of mitochondrial proteins in skeletal muscle (Olesen et al., 2010). In accordance, PGC-1α has been demonstrated to be required for the ability of exercise training to rescue age-associated decrease in VEGF protein and CS activity in mouse skeletal muscle (Leick et al., 2009; Leick et al., 2010) and overexpression of muscle PGC-1α has been shown to protect skeletal muscle from sarcopenia in aging mice (Wenz et al., 2009). Taken together this suggests that PGC-1α mediates some of the protective effects of physical activity on age-associated metabolic changes in skeletal muscle.

PGC-1α has also been suggested to mediate resveratrol-induced effects. Resveratrol regulates the deacetylase sirtuin (SIRT)1, which induces metabolic gene transcription in skeletal muscle by deacetylation and thereby activation of PGC-1α (Gerhart-Hines et al., 2007; Lagouge et al., 2006; Rodgers et al., 2005).

Therefore, the aim of the present study was to test the hypotheses that 1) resveratrol supplementation prevents an age-associated decrease in skeletal muscle oxidative capacity, 2) resveratrol supplementation and exercise training exert additive effects on skeletal muscle oxidative capacity, and 3) the effects of resveratrol on skeletal muscle oxidative capacity requires PGC-1α.
2. Methods

2.1 Mice

The study used female whole body PGC-1α KO and WT littermate mice produced by intercross breeding of heterozygous parents. During the experimental period the mice were housed individually in cages with 12:12-h light-dark cycle and with free access to food and water.

2.2 Experimental protocol

From 3 month of age until 15 month of age PGC-1α KO and WT mice were placed in groups either with or without access to a running wheel in the cage, getting either standard chow or standard chow with a resveratrol supplement of 4 g/kg (Altromin, Brogården, Lynge, Denmark). After addition of resveratrol to standard chow, the content of resveratrol was verified (3.7 g/kg). In addition, young WT and PGC-1α KO mice were from 2 month of age placed in individual cages and fed standard chow for 14 days before being euthanized at 3 month of age.

2.2.1 In vivo testing

Running performance of WT and PGC-1α KO mice was determined on a treadmill with an incremental exercise test up to 40 minutes (TSE systems GmbH, Bad Homburg, Germany). Glucose tolerance of WT and PGC-1α KO mice were determined after injecting with 2 g of glucose/kg mouse intraperitoneally and blood glucose was determined before and at 15, 30, 45, 60, 90 and 120 minutes after injection. Prior to the glucose tolerance test (GTT) mice were fasted for 18 hours.

At 15 month and 3 month of age, respectively, the mice were euthanized and quadriceps, triceps and extensor digitorum longus (EDL) muscles were quickly removed and frozen in liquid
nitrogen for later analyses. Furthermore, one triceps muscle was fixed 4 hours immersion into 2 
% paraformaldehyde supplemented with 0.15 % picric acid for single fiber immunostaining and 
one EDL muscle was embedded in Tissue-Tek (Sakura Finetek, Alphen aan den Rijn, The 
Netherlands) for histochemistry. Body weight and food intake was measured every second week 
and body composition was determined, one month prior to the end of the experiment, using MRI 
(EchoMRI, Echo Medical Systems, Houston, TX, USA) and fat mass was normalized to 
individual body weight.

2.3 Analyses

2.3.1 Immunohistochemical staining of capillaries

The number of capillaries was determined on 8 µm transverse sections of frozen samples of the 
EDL. The sections were air-dried for 10 min and then fixed in -20°C acetone for 30 sec. The 
sections were then again air-dried and incubated for 1 h in biotin conjugated Griffonia 
Simplifolica lectin (1:200). After washing the sections in phosphate-buffered saline they were 
incubated in FITC-conjugated streptavidin (Dako) for 1 h in the dark. The sections were 
carefully washed before they were mounted. Stained capillaries were examined and image 
acquisition performed with a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany).

For each muscle sample analyses were performed on a minimum of three different areas of a 
muscle section corresponding to a minimum of 200 muscle fibers. The origin of all pictures was 
blinded for the observer before quantification of fibers and capillaries. The capillary-to-fiber 
ratios (C:F) were determined according to Gundersen’s rule A (Gundersen, 1978). C:F ratio was 
defined as the total number of capillaries per total number of fibers.

2.3.2 Muscle fixation and Immunostaining of single muscle fibers
Muscle fibers from triceps muscle were teased into bundles of 1-3 fibers with fine forceps and transferred to immunobuffer. Single fibers were then incubated over night with primary anti-COX IV (#16056, Abcam, Cambridge, UK) antibodies diluted in immunobuffer (IB; 50 mM glycine, 0.25 % bovine serum albumin, 0.03 % saponin, and 0.05 % sodium azide in PBS). After three washes of 15 minutes with IB, single fibers were incubated for 2 h in Alexa-568 or Alexa-488 (Molecular Probes) conjugated secondary antibodies diluted in IB. Fibers were then washed 3 times 15 minutes with IB, 1 time 15 minutes with PBS and then mounted in Vectashield H-1000 on a glass slide. Images acquisition was performed with a Zeiss LSM 710 (Carl Zeiss, Jena, Germany) confocal microscope.

2.3.3 DNA isolation

Total DNA was isolated from ~10 mg of quadriceps muscle tissue as described previously (Pilegaard et al., 2000). The DNA pellet was resuspended in 25 µl of distilled water. The isolated DNA was later used to determine the ratio between mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) content by real-time PCR.

2.3.4 Real time PCR

The mtDNA (represented by COXI) and nDNA (represented by Cyt c) content were determined by real-time PCR using the fluorogenic 5’ nuclease assay with TaqMan probes and universal mastermix with UNG (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, CA, USA) as previously described (Lundby et al., 2005). The sequences used to amplify a fragment of COXI were FP: 5’ TGCAACCCTACACGGAGGTAATA 3’, RP: 5’ ATGTATCGTGAGCAGCAGGTCA 3’ and TaqMan probe: 5’ TCTAACCAGGATTTTATCCAA 3’ and to amplify a fragment of Cyt c were FP: 5’
TGCCCAGTGCCACACTGT 3’, RP: 5’ CTGTCTTCCGCAGCACGAACA 3’ and TaqMan probe: 5’
AGGCAAGCATAAGACTGGACCAAATCTCCA 3’. Both TaqMan probes were 5’-6-
carboxyfluorescein (FAM) and 3’-6-carboxy-N,N,N’,N’-tetramethylrhodamine (TAMRA)
labeled. The obtained cycle threshold (Ct) values reflecting the initial content of the specific
transcript in the samples were converted to an arbitrary amount by using standard curves
obtained from serial dilution of a pooled sample made from all samples. The DNA results were
presented as mtDNA normalized to nDNA content (mtDNA/nDNA).

2.3.5 Lysate preparation

Muscle tissue specimens were homogenized in an ice-cold buffer as previously described (Birk
& Wojtaszewski, 2006) except that the tissue was homogenized for 2 min at 30 sec–1 in
TissueLyserII (Qiagen, Valencia, CA, USA). Protein content in lysates was measured by the
bicinchoninic acid method (Pierce Biotechnology Inc., Rockford, IL, USA). Lysates were
prepared with sample buffer containing Sodium dodecyl sulfate (SDS) and boiled for 3 min at
96°C and analyzed by SDS-PAGE and western blotting.

2.3.6 SDS-PAGE and Western blotting

Protein content of specific proteins was measured in muscle tissue samples by SDS-PAGE and
western blotting using PVDF membrane and semi-dry transfer as previously described (Birk &
Wojtaszewski, 2006). Protein content is expressed in units relative to control samples loaded on
each gel. Primary Cyt c (#556433 BD Pharmigen, Franklin Lakes, NJ, USA), VEGF (#j806 A20
Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD31 (SC-1506 Santa Cruz Biotechnology,
Santa Cruz, CA, USA) and PDH-E1α (kindly donated by D. G. Hardie, University of Dundee,
Dundee, UK) antibodies and polyclonal secondary antibodies (Dako, Glostrup, Denmark) were used.

2.3.7 Statistics and calculations

Glucose stimulated glucose response was calculated by subtracting the basal value from the values obtained after glucose injection and the area under the curve was calculated. Values presented are means ± SE. Two-way analysis of variance (ANOVA) was applied to test the effect of genotype and intervention groups, and one-way ANOVA was used to test for differences between intervention groups separately within each genotype. Student-Newman-Keuls post hoc test was used to locate differences. Differences were considered significant at $P \leq 0.05$ and a tendency is reported when $0.05 < P \leq 0.10$. Statistical calculations were performed using SigmaPlot version 11.0.

3. Results

3.1 In vivo data

Running time in minutes was ~18-60 % lower ($P \leq 0.05$) in PGC-1α KO than in WT mice in all intervention groups and almost all WT mice completed the running protocol (Table 2). In PGC-1α KO mice running time was 24 % lower ($P \leq 0.05$) in 15mo UT C mice than in WT mice and ~1.6-fold higher ($P \leq 0.05$) in 15mo T C and 15mo T R than 15mo UT C, but with no effect of age, lifelong exercise training and/or resveratrol supplementation in WT mice (Table 1).

Area under the curve for the glucose stimulated glucose response was similar or lower in aged mice than in young, except for a higher value in 15mo UT R PGC-1α KO mice than in young
PGC-1α KO mice. In addition, the 2 hour blood glucose value was similar in all groups (data not shown). Together this indicates that aged mice did not have impaired glucose tolerance.

### 3.2 SIRT1 protein content

Quadriiceps muscle SIRT1 protein content did not change with age, lifelong exercise training and/or resveratrol supplementation in either WT or PGC-1α KO mice (data not shown).

### 3.3 Citrate synthase activity and mitochondrial DNA content

Quadriiceps muscle CS activity (µmol·min⁻¹·mg⁻¹ protein) was 20-45% lower (P≤0.05) in PGC-1α KO than in WT mice in all intervention groups (Figure 1a). Quadriiceps CS activity tended to be 20% lower (P=0.06) in 15mo UT C WT than in 3mo UT C WT mice and CS activity was ~1.5-fold higher (P≤0.05) in 15mo T C and 15mo T R WT mice than in 15mo UT C WT mice (Figure 1a).

Quadriiceps muscle mtDNA/nDNA content tended to be 30% lower (P=0.09) in 3mo UT C PGC-1α KO than in WT mice (Figure 1b). In addition, mtDNA/nDNA ratio in 15mo UT C, UT R and T R WT mice was 45-60% lower (P≤0.05) than in 3mo UT C and was higher (P≤0.05) in 15mo T C WT mice than in 15mo UT C WT mice (Figure 1b).

### 3.4 Oxidative protein content

Quadriiceps muscle PDH-E1α protein content was 45-70% lower (P≤0.05) in PGC-1α KO mice than in WT mice in all intervention groups (Figure 2a). In WT mice, PDH-E1α protein content was ~1.8-fold higher (P≤0.05) in 15mo T C mice and tended to be higher (P=0.07) in 15mo T R mice than both 3mo UT C and 15mo UT C, while there was no change in PGC-1α KO mice (Figure 2a).
Quadriceps muscle Cyt c protein content was 60-75 % lower (P≤0.05) in PGC-1α KO mice than in WT mice in all 15mo intervention groups and tended to be ~30 % lower (P=0.08) than WT in 3mo UT C (Figure 2b). Cyt c protein content was 60 % lower (P≤0.05) in 15mo UT R PGC-1α KO mice than in 3mo UT C PGC-1α KO mice. However, intervention tended to increase (P=0.08) Cyt c protein content ~1.4-fold in WT mice (Figure 2b).

3.5 Angiogenic protein content

Quadriceps muscle VEGF protein content was 70 % lower (P≤0.05) in PGC-1α KO mice than in WT mice in 15mo UT R, T C and T R intervention groups (Figure 3a). VEGF protein content was in WT mice ~4-fold higher (P≤0.05) in 15mo T C and 15mo T R than in 15mo UT C WT mice (Figure 3a).

Quadriceps muscle CD31 protein content was 35-60 % lower (P≤0.05) in PGC-1α KO mice than in WT mice in 15mo T C and 15mo UT C (Figure 3b). CD31 protein content was in WT ~2.1-fold higher (P≤0.05) in 15mo T C mice than in 3mo UT C, while CD31 protein content in quadriceps of PGC-1α KO mice only tended to be higher (P=0.07) in 15mo T R than in 3mo UT C and 15mo UT C mice, respectively (Figure 3b).

3.6 Capillarization

EDL muscle capillary-to-fiber (C:F) ratio was 15-30 % lower (P≤0.05) in PGC-1α KO mice than in WT mice in all intervention groups (Figure 5a+5b). EDL muscle C:F ratio did not change with age, lifelong exercise training and/or resveratrol supplementation in either WT or PGC-1α KO mice (Figure 5a) as also illustrated from capillary staining (Figure 5b).
4. Discussion

The main findings of the present study are that lifelong exercise training but not resveratrol supplementation alone increased the protein content of mitochondrial and angiogenic markers in aging mouse skeletal muscle, and combining exercise training and resveratrol did in general not enhance the effects relative to exercise training alone. In addition, the exercise training effects on mitochondrial and angiogenic markers required the presence of PGC-1α. Together this suggests that exercise training is more efficient in improving oxidative capacity of healthy aged skeletal muscle than resveratrol supplementation.

The present observations that lifelong resveratrol supplementation did not prevent the age-associated decrease in skeletal muscle mtDNA/nDNA and CS activity and, did not enhance the content or staining of oxidative enzymes were unexpected in light of previous reports. Resveratrol has previously been shown to increase mitochondrial copy number, CS activity and the mRNA content of oxidative proteins in skeletal muscle of mice on a high fat diet (HFD) (Lagouge et al., 2006;Price et al., 2012;Um et al., 2010). It may be noted that although the CS activity in the aged untrained resveratrol group in the present study did not differ significantly from old untrained mice, it was similar to young mice indicating that resveratrol did rescue the decline with aging. As the dose of resveratrol was similar in the present and the previous studies (Lagouge et al., 2006;Um et al., 2010), dose differences cannot explain the difference in resveratrol-mediated effects on mitochondrial proteins. Instead, the metabolic state of the control mice may have played a role, because the mice in the study by Lagouge et al. (Lagouge et al., 2006) and Um et al. (Um et al., 2010) were on HFD and showing impaired glucose tolerance, while the mice in the present study were 15 month old mice exhibiting normal glucose tolerance and only significant age-associated decreases in CS activity and mtDNA. This possibility is in
accordance with recent findings in humans, where resveratrol supplementation has been reported to exert beneficial effects in insulin resistant subjects (Brasnyo et al., 2011), but not in healthy subjects (Poulsen et al., 2013). Together this may suggest that resveratrol in general only improves metabolic parameters when metabolism initially is impaired. In addition, the observation that resveratrol supplementation did not affect VEGF and CD31 protein content or the C:F ratio significantly in aged muscle is in accordance with the findings for the oxidative proteins, but disagrees with the previously reported beneficial effects of resveratrol intake on vascular endothelium in aging mice (Pearson et al., 2008). Although the mice in the previous study (Pearson et al., 2008) were older than in the present study, these findings may suggest that resveratrol influences the vascular system by other means than increasing the number of capillaries.

The findings in the current study that lifelong exercise training, in contrast to resveratrol supplementation, enhanced VEGF, CD31, PDH-E1α and Cyt c protein, CS activity and mtDNA/nDNA in aging skeletal muscle are in accordance with previous studies (Iversen et al., 2011; Leick et al., 2009; Leick et al., 2010) showing that lifelong exercise training elevates mitochondrial and angiogenic proteins in human and mouse skeletal muscle. Although only CS activity and mtDNA/nDNA decreased significantly with age in the present study, the observed increase in oxidative and angiogenic proteins in skeletal muscle with exercise training shows that aging skeletal muscle benefits from regular physical activity and underlines the major impact regular physical activity has on the oxidative capacity of aging skeletal muscle. Of notice is that the distance run by the mice in the present study (on average 6 km/week), as well as in our previous study in which similar beneficial effects were observed (Leick et al., 2010), was short relative to previous reports with up to 10 km per night (Geng et al., 2010) for a mouse indicating
that beneficial effects on skeletal muscle oxidative capacity can indeed be obtained with a rather limited amount of exercise as long as it is performed regularly. Because previous studies have shown that both resveratrol and exercise training can increase the protein content/activity of oxidative proteins in mouse skeletal muscle (Lagouge et al., 2006; Leick et al., 2008; Leick et al., 2010; Um et al., 2010), it was hypothesized that combining lifelong exercise training and resveratrol supplementation would elicit additive effects on oxidative and angiogenic proteins in skeletal muscle as also recently suggested (Menzies et al., 2013). However, the observation that exercise training combined with resveratrol in general elicited similar effects as exercise training alone shows that this is not the case in the present study. The finding emphasizes the efficacy of exercise training over resveratrol supplementation in improving skeletal muscle oxidative capacity in healthy aged mice. A previous study on senescence-accelerated mice has reported that exercise training together with resveratrol intake increased the mRNA content of mitochondrial proteins in skeletal muscle relative to untrained mice on chow diet, but no comparison with exercise training alone was performed in that study (Murase et al., 2009).

PGC-1α has previously been demonstrated not to be mandatory for skeletal muscle mitochondrial adaptations in young mice in response to combined treadmill and running wheel exercise training (Leick et al., 2008), but also to be required for running wheel exercise-induced adaptations in VEGF and some mitochondrial proteins in both young (Geng et al., 2010) and aged mice (Leick et al., 2008; Leick et al., 2009). In accordance, the present findings that exercise training increased VEGF, CD31, PDH-E1α and Cyt c protein as well as CS activity and the mtDNA/nDNA ratio in skeletal muscle of WT, but not PGC-1α mice show that PGC-1α is required for these adaptations. Of note is that the PGC-1α KO mice improved running capacity despite absence of exercise training-induced adaptations in the investigated mitochondrial and
angiogenic proteins in skeletal muscle, indicating that additional factors must contribute. In addition, the lack of resveratrol-mediated effects on mitochondrial and angiogenic markers does not allow for conclusions on the potential role of PGC-1α, and additional studies will therefore be required to address this.

It cannot be excluded that the lack of effects of resveratrol supplementation alone in the present study was due to a too low or too high dose of resveratrol. This possibility does however not seem likely, as previous studies (Lagouge et al., 2006; Um et al., 2010) have obtained clear effects of resveratrol using the same dose as in the present study (4 g/kg food corresponding to approximately 400 mg/kg mouse per day) and even with resveratrol supplied by the same company as in the present study. In addition, while resveratrol and exercise training alone only reduced adipose tissue mass non-significantly, combining exercise training and resveratrol intake in the current study reduced adipose tissue mass indicating that resveratrol did indeed exert some effects in the present study (unpublished data, Olesen et al. 2013). It might therefore be argued that resveratrol potentially did not reach or did not signal in skeletal muscle in the present study, which as such is supported by the unaffected SIRT1 protein level in skeletal muscle in the present study. However, recent data from our group obtained from the same mice as in the present study demonstrate that resveratrol supplementation alone prevented an age-associated increase in protein carbonylation in skeletal muscle (unpublished data, Olesen et al. 2013) showing that resveratrol did induce adaptations in skeletal muscle of the mice used in the present study. Together this supports that resveratrol did reach skeletal muscle in the present study, but that resveratrol did not influence the oxidative capacity of skeletal muscle likely because of no major impairments to adjust for in the healthy aging mice.
In conclusion, the present study demonstrate that lifelong exercise training increased the content/activity of oxidative proteins, mtDNA and angiogenic proteins in mouse skeletal muscle through PGC-1α, whereas resveratrol supplementation had no effect on mitochondrial markers or capillarization in healthy aging mice. In addition, combination of lifelong exercise training and resveratrol supplementation did in general not affect oxidative proteins or capillarization. Together this suggests that exercise training but not resveratrol effectively improves skeletal muscle oxidative capacity in healthy aging mice.

Acknowledgements

We sincerely thank Professor B. Spiegelman for providing PGC-1α KO mice initially to start breeding, and we are grateful for the skillful technical assistance of Karina Olsen. The Core Facility for Integrated Microscopy (CFIM) was used for image acquisition.
Reference List


Figure legends

**Figure 1. Citrate synthase activity and mitochondrial DNA content in quadriceps muscle.**

a) Citrate synthase (CS) activity ($\mu$mol·min$^{-1}$·mg$^{-1}$ protein) and b) mtDNA/nDNA ratio in quadriceps muscle from wildtype (WT) and whole body peroxisome proliferator-activated receptor-$\gamma$ coactivator (PGC)-1$\alpha$ knockout (KO) mice, either 3 month old untrained mice on standard chow (3mo UT C), 15 month old untrained mice on standard chow (15mo UT C), 15 month old untrained mice on standard chow with resveratrol supplementation (15mo UT R), 15 month old trained mice on standard chow (15mo T C), 15 month old trained mice on standard chow with resveratrol supplementation (15mo T R). Values are means ±SE; n=8-10. †: Significantly different from 3mo UT C within given genotype, $P \leq 0.05$. *: Significantly different from 15mo UT C within given genotype, $P \leq 0.05$. #: Significantly different from WT within given intervention group, $P \leq 0.05$. (†): Tends to be significantly different from 3mo UT C within given genotype, $0.05 < P \leq 0.10$. (#): Tends to be significantly different from WT within given intervention group, $0.05 < P \leq 0.10$. (T): Tends to be significantly different from WT within 3mo UT C obtained with a t-test, $0.05 < P \leq 0.10$.

**Figure 2. Oxidative protein content in quadriceps muscle.**

a) Pyruvate dehydrogenase (PDH)-E1$\alpha$ protein content and b) cytochrome (Cyt) c protein content relative to GAPDH protein content in quadriceps muscle from wildtype (WT) and whole body peroxisome proliferator-activated receptor-$\gamma$ coactivator (PGC)-1$\alpha$ knockout (KO) mice, either 3 month old untrained mice on standard chow (3mo UT C), 15 month old untrained mice on standard chow (15mo UT C), 15 month old untrained mice on standard chow (15mo UT C), 15 month old untrained mice on standard chow with resveratrol supplementation (15mo UT R), 15 month old trained mice on standard chow (15mo T C), 15
month old trained mice on standard chow with resveratrol supplementation (15mo T R). Values are means ±SE; n=8-10. †: Significantly different from 3mo UT C within given genotype, P≤0.05. *: Significantly different from 15mo UT C within given genotype, P≤0.05. #: Significantly different from WT within given intervention group, P≤0.05. (†): Tends to be significantly different from 3mo UT C within given genotype, 0.05<P≤0.10. (*): Tends to be significantly different from 15mo UT C within given genotype, 0.05<P≤0.10. (#): Tends to be significantly different from WT within given intervention group, 0.05<P≤0.10.

**Figure 3. Angiogenic protein content in quadriceps muscle.**

a) Vascular endothelial growth factor (VEGF) and b) cluster of differentiation (CD) 31 protein content relative to GAPDH protein content in quadriceps muscle from wildtype (WT) and whole body peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α knockout (KO) mice 3 month old untrained mice on standard chow (3mo UT C), 15 month old untrained mice on standard chow (15mo UT C), 15 month old untrained mice on standard chow with resveratrol supplementation (15mo UT R), 15 month old trained mice on standard chow (15mo T C), 15 month old trained mice on standard chow with resveratrol supplementation (15mo T R). Values are means ±SE; n=8-10. †: Significantly different from 3mo UT C within given genotype, P≤0.05. *: Significantly different from 15mo UT C within given genotype, P≤0.05. #: Significantly different from WT within given intervention group, P≤0.05. (†): Tends to be significantly different from 3mo UT C within given genotype, 0.05<P≤0.10. (*): Tends to be significantly different from 15mo UT C within given genotype, 0.05<P≤0.10.
Figure 4. Oxidative protein content in triceps muscle.

Representative images of the intracellular distribution of Cytochrome c oxidase (COX) subunit IV in single muscle fibers from triceps muscle from wildtype (WT) and whole body peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α knockout (KO) mice, either 3 month old untrained mice on standard chow (3mo UT C), 15 month old untrained mice on standard chow (15mo UT C), 15 month old untrained mice on standard chow with resveratrol supplementation (15mo UT R), 15 month old trained mice on standard chow (15mo T C), 15 month old trained mice on standard chow with resveratrol supplementation (15mo T R). Values are means ±SE; n=8-10.

Figure 5. Capillarization of EDL muscle.

a) Capillary-to-fiber (C:F) ratio and b) representative capillary staining of a cross section of extensor digitorum longus (EDL) muscle from wildtype (WT) and whole body peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α knockout (KO) mice, either 3 month old untrained mice on standard chow (3mo UT C), 15 month old untrained mice on standard chow (15mo UT C), 15 month old untrained mice on standard chow with resveratrol supplementation (15mo UT R), 15 month old trained mice on standard chow (15mo T C), 15 month old trained mice on standard chow with resveratrol supplementation (15mo T R). Values are means ±SE; n=8-10. #: Significantly different from WT within given intervention group, P≤0.05.
Table 1. Running performance

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Running duration in minutes for wildtype (WT) and whole body peroxisome proliferator-activated receptor-γ coactivator-1α knockout (PGC-1α KO) mice. Of notice is that the test was stopped after 40 minutes and 10 out of 10 3mo UT C WT, 6 out of 7 15mo UT C WT and 7 out of 8 15mo T C WT mice completed the 40 minutes. Values are means ±SE, n=6-10. †: Significantly different from 3mo UT C within given genotype, P ≤ 0.05. *: Significantly different from 15mo UT C within given genotype, P ≤ 0.05. #: Significantly different from WT within given intervention group, P ≤ 0.05. (†): Tends to be significantly different from 3mo UT C within given genotype, 0.05 < P ≤ 0.10.
3A. Co-authorship statement

All papers/manuscripts with multiple authors enclosed as annexes to a PhD thesis synopsis should contain a co-author statement, stating the PhD student's contribution to the paper.

1. General information

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<tr>
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<th>Name</th>
<th>Stine Ringholm Jørgensen</th>
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<tr>
<td></td>
<td>Civ.reg.no. (If not applicable, then birth date)</td>
<td>030582-1586</td>
</tr>
<tr>
<td></td>
<td>E-mail</td>
<td><a href="mailto:srjorgensen@bio.ku.dk">srjorgensen@bio.ku.dk</a></td>
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<td></td>
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<td><a href="mailto:hpilegaard@bio.ku.dk">hpilegaard@bio.ku.dk</a></td>
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2. Title of PhD thesis

PGC-1α in exercise- and exercise training-induced metabolic adaptations

3. This co-authorship declaration applies to the following paper

Effect of lifelong resveratrol supplementation and exercise training on skeletal muscle oxidative capacity in aging mice; impact of PGC-1α
Submitted to Exp Geront, 2013.

The extent of the PhD student's contribution to the article is assessed on the following scale

A. has contributed to the work (0-33%)
B. has made a substantial contribution (34-66%)
C. did the majority of the work independently (67-100%).

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