Fasting- and exercise-induced PDH regulation in skeletal muscle

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Summary

Pyruvate dehydrogenase PDH constitutes the only mammalian pathway for irreversible conversion of pyruvate to acetyl-CoA thus providing the vital link between glycolytic energy production, the TCA cycle, and oxidative phosphorylation. Because the PDC controls the conversion of pyruvate it occupies a central position in relation to the control of mitochondrial energy production and cellular substrate metabolism. Suppression and activation of PDH becomes essential in situations where glucose availability and/or use changes with swift and appropriate regulation of the complex to maintain energy homeostasis in response to varying nutritional and metabolically challenging states.

The aim of the present thesis was to investigate the fasting- and exercise induced regulation of PDH to test the hypotheses that 1) Skeletal muscle IL-6 contributes to the regulation of PDH in mouse skeletal muscle at rest as well as during prolonged exercise and that this is associated with IL-6 mediated regulation of AMPK. 2) Skeletal muscle IL-6 affects short-term and prolonged fasting-induced PDH regulation and substrate utilization in mice. 3) Lack of muscle PGC-1α affects the time course of the switch in substrate utilization during the transition from the fed to the fasted state affecting regulation of fasting-induced changes in PDHa activity, PDH phosphorylation and PDH acetylation in skeletal muscle. 4) Differences in substrate utilization between trained and untrained individuals are associated with differences in adaptive changes and regulation of PDH in skeletal muscle.

Study I demonstrated that lack of skeletal muscle IL-6 led to elevated PDHa activity, both at rest and during exercise, without significant changes in PDHa activity during prolonged exercise. IL-6 MKO mice had an overall higher RER during exercise than controls, but maintained the ability to reduce RER during prolonged exercise. Lack of muscle IL-6 did not affect AMPK activation during exercise. Together this indicates that muscle IL-6 influences substrate utilization in skeletal muscle through effects on PDH, but is not required for the shift in substrate use during prolonged exercise.

Study II demonstrated that lack of skeletal muscle IL-6 resulted in elevated resting RER in the fed state, while the RER in the fasted state was similar in IL-6 MKO and Control. Furthermore, skeletal muscle PDHa activity was higher and PDH phosphorylation lower in IL-6 MKO than Control mice in the fasted state, but knockout of muscle IL-6 did not prevent the ability to regulate skeletal muscle PDH in response to fasting. Taken together this indicates that skeletal muscle IL-6 regulates substrate utilization at rest potentially through effects on
skeletal muscle PDH, whereas muscle IL-6 is not required for fasting-induced substrate switch and skeletal muscle PDH regulation in mice.

Study III demonstrated that lack of muscle PGC-1α did not affect the switch from carbohydrate to predominant fat utilization in the transition from the fed to the fasted state. Fasting-induced down-regulation of PDHa activity in skeletal muscle of control mice was associated with increased phosphorylation of all four known sites in PDH-E1α as well as with increased PDK4 and SIRT3 protein without changes in total acetylation of PDH-E1α. Lack of muscle PGC-1α reduced PDH-E1α, PDK1, 2, 4, PDP1, and SIRT3 protein content as well as increased total lysine PDH-E1α acetylation in the fed state. Knockout of muscle PGC-1α did not influence the fasting-induced increase in PDH-E1α phosphorylation, but prevented the fasting-induced increase in SIRT3 protein.

Study IV demonstrated that exercising at the same relative intensity at steady state and with a short-term increase in intensity, elicited similar PDH activation in skeletal muscle from untrained and trained subjects. Skeletal muscle PDHa activity was higher in trained than untrained humans at exhaustion providing a contributing mechanism for the augmented capacity for carbohydrate oxidation in the trained state. This reflects a well-controlled tight regulation of PDH together with the ability to rely on fat oxidation at a higher exercise intensity in the trained than untrained state. This metabolic response was associated with higher PDH-E1α content, PDH phosphorylation and PDH acetylation as well as protein content of PDH regulators.

In conclusion, IL-6 appears to exert dampening effects on skeletal muscle PDHa activity, both during fasting and prolonged exercise. Furthermore, both IL-6 and PGC-1α are dispensable for maintaining short-term metabolic flexibility, as muscle IL-6 was not necessary for exercise-induced switches in substrate utilization and neither lack of skeletal muscle IL-6 or PGC-1α affected fasting–induced switch to fat oxidation. Lack of muscle PGC-1α did however blunt the fasting-induced increase in selected mitochondrial proteins. Lastly, increased oxidative capacity leads to exercise-induced skeletal muscle PDH activation that is closely matched to the relative exercise intensity at submaximal exercise, while reaching a higher level at maximal exercise in trained individuals. These responses are associated with increased PDH phosphorylation, acetylation and content of covalent regulators.
**Resumé (danish summary)**

Pyruvat dehydrogenase (PDH) står for irreversibel omdannelse af pyruvat til acetyl-CoA i pattedyr, hvilket dermed giver den en vitale forbindelse mellem glykolytisk flux, Krebs cyklussen og oxidativ fosforylering. Fordi PDH styrer omdannelsen af pyruvat indtager enzymet en central position i forhold til kontrollen med mitokondriel energiproduktion og cellulær substrat metabolisme. Inaktivering og aktivering af PDH er afgørende i situationer, hvor glukosetilgængelighed er enten begrænset eller glukose oxidation er nødvendig under metabolisk udfordrende tilstande.

Formålet med denne afhandling var at undersøge reguleringen af pyruvat dehydrogenase (PDH) under indflydelse af IL-6, PGC-1α og træningstillstand under henholdsvis faste og arbejde. Herunder blev følgende hypoteser testet: 1) Skeletmuskulatur IL-6 bidrager til reguleringen af PDH i skeletmuskulatur hos mus i hvile samt under langvarigt arbejde og er forbundet med IL-6 medieret regulering af AMPK. 2) IL-6 påvirker kortvarig og længerevarende faste-induceret PDH regulering og substratvalg i skeletmuskulatur hos mus. 3) Fravær af PGC-1α i skeletmuskulatur påvirker tidsforløbet for substratskiftet fastende tilstand og har betydning for reguleringen af fastende-inducerede ændringer i PDHa aktivitet, PDH fosforylering og PDH acetylering i skeletmuskulatur. 4) Forskelle i substratvalg mellem trænede og utrænede personer er forbundet med forskelle i adaptive metaboliske ændringer og post-translationel regulering af PDH i skeletmuskulatur.

Studie I viste, at fravær af IL-6 i skeletmuskulatur førte til forhøjet PDHa aktivitet, ikke kun under arbejde, men også i hvile og uden signifikante ændringer i PDHa aktivitet under langvarigt arbejde. IL-6 MKO mus havde en generelt højere respiratorisk udvekslingskvotient (RER) under arbejde end kontrolmus, men opretholdt evnen til at reducere RER under langvarigt arbejde. Sammen indikerer dette, at muskel IL-6 påvirker substratudnyttelse i skeletmuskulatur via indflydelse på PDH, men ikke er påkrævet for skiftet til fortrinsvis fedtoxidation under langvarigt arbejde i mus.

Studie II viste, at fravær af IL-6 i skeletmuskulatur resulterede i forhøjet hvile RER på fuld mave, mens RER i fastende tilstand var ens i IL-6 MKO og Control. Desuden skeletmuskulatur PDHa aktivitet var højere og PDH fosforylering lavere i IL-6 MKO end Control i fastende, men knockout af muskel IL-6 hæmmede ikke evnen til at regulere PDH i skeletmuskulatur under faste. Tilsammen betyder dette, at muskel IL-6 ikke er nødvendig for faste-induceret substratskifte til fortrinsvis fedtoxidation og regulering af PDH i skeletmuskulatur i mus.

Studie III viste, at fravær af muskel PGC-1α ikke påvirkede skiftet fra overvejende kulhydrat til fedtoxidation i overgangen fra fodret til fastende tilstand, men var forbundet med højere kulhydratforbrug i fastende tilstand.
Faste-induceret nedregulering af PDHa aktivitet i skeletmuskulatur i kontrolmus var forbundet med øget fosforylering af alle fire sites på PDH-E1α samt med øget PDK4 og SIRT3 protein uden ændringer i den totale acetylering af PDH-E1α. Manglende muskel PGC-1α medførte reduceret PDH-E1α, PDK1, 2, 4, PDP1, og SIRT3 proteinindhold samt PDH-E1α hyperacetylering i fodret tilstand. Knockout af muskel PGC-1α havde ingen indflydelse på den faste-inducerede stigning i PDH-E1α fosforylering, men forhindrede en faste-induceret stigning i SIRT3 protein.

Studie IV viste, at arbejde ved samme relative intensitet og med stigende intensitet, fremkaldte samme PDH aktivering i skeletmuskulatur hos utrænede og trænede personer. Ydermere var PDHa aktivitet højere i trænede end utrænede personer lige efter udmattende arbejde, indikativt for øget kapacitet for kulhydrat oxidation i trænede. Dette afspejler en øget regulering af PDH sammen med øget tilbøjelighed til fedt oxidation ved en højere arbejdsintensitet i trænede end utrænede personer. Dette metaboliske respons blev forbundet med højere PDH-E1α indhold, øget PDH fosforylering og PDH acetylering samt øget indhold af kovalente PDH regulatorer.

Samlet kan det konkluderes, at IL-6 synes at udøve en dæmpende effekt på PDHa aktivitet i skeletmuskulatur, både under faste og langvarigt arbejde. desuden er både IL-6 og PGC-1α kan undværlig for opretholdelse af metaboliske fleksibilitet, da muskel IL-6 ikke var nødvendigt for substratskifte under arbejde, og hverken fravær af IL-6 eller PGC-1α påvirkede et faste-induceret skifte til fedtoxidation. Dog synes mangel på muskel PGC-1α at hæmme den faste-inducerede stigning i udvalgte mitokondrielle proteiner. Endelig, viste det sig at arbejde ved samme relative intensitet og med stigende intensitet, fremkaldte samme PDH aktivering i skeletmuskulatur hos utrænede og trænede personer, afspejlende en øget kontrol af PDH og forbedret substratregulering ved øget oxidativ kapacitet.
List of studies

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


Work was contributed to the following manuscripts not included in the present thesis.


# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>CaMK</td>
<td>Ca$^{2+}$-calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetate</td>
</tr>
<tr>
<td>ERRα</td>
<td>Estrogen-related receptor α</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>GOT</td>
<td>Glutamic oxaloacetic transaminase</td>
</tr>
<tr>
<td>HAD</td>
<td>β-hydroxyacyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>NRF</td>
<td>Nuclear respiratory factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>PDC</td>
<td>Pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>PDH-E1α</td>
<td>Pyruvate dehydrogenase-E1α</td>
</tr>
<tr>
<td>PDK1-4</td>
<td>Pyruvate dehydrogenase kinase 1-4</td>
</tr>
<tr>
<td>PDP1-2</td>
<td>Pyruvate dehydrogenase phosphatase 1-2</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor-γ coactivator-1α</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SIRT3</td>
<td>Sirtuin 3</td>
</tr>
<tr>
<td>SOCS3</td>
<td>suppressor of cytokine signaling 3</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline Tween-20</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>MKO</td>
<td>Muscle specific knockout</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotine adenine dinucleotide</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>p38</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
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<td>WT</td>
<td>wild type</td>
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Introduction

Metabolism

In their seminal work from 1963, Randle and coworkers proposed a glucose fatty acid cycle, naming it the Randle cycle, describing the dynamics of fuel interaction and selection in mammalian tissue (Randle et al., 1963). The Randle cycle draws attention to the competitive interaction occurring between carbohydrate and fatty acids for oxidation in both muscle and adipose tissue and Randle et al. succeeded in demonstrating that oxidative utilization of one macronutrient could directly inhibit the use of other independent of hormonal influence (Randle et al., 1963; Randle, 1964; Randle et al., 1988). The impairment of carbohydrate metabolism by fatty acid oxidation was shown to be mediated by a transient inhibition of a number of steps in glucose metabolism, specifically at the level of glucose transport and phosphorylation, phosphofructokinase, and lastly pyruvate dehydrogenase (PDH) (Randle et al., 1988). As proposed, elevated β-oxidation initiates a sequence of inhibitory steps emanating from the accumulation of mitochondrial acetyl-CoA and NADH suppressing PDH activity. Acetyl-CoA-derived accumulation of cytosolic citrate consequently leads to accumulated glucose-6-phosphate halting hexokinase activity and possible lowering of glucose uptake (GARLAND et al., 1963). This correlates well with metabolic events in the fasted state, where this inhibitory effect of prevailing fatty acid metabolism on glucose oxidation in skeletal muscle allows for glucose to be spared ensuring that the obligatory requirements of the brain and blood cells are sustained for extended survival under substrate deprived circumstances. Likewise, with pronounced glucose oxidation at the onset and early phase of exercise, closely correlated with workout intensity, a reciprocal shift in fuel selection towards fatty oxidation occurs with prolonged exercise. This was established as early as 1939 by Hohwü Christensen and Hansen demonstrating that the respiratory exchange ratio (RER; by the authors presented as RQ) gradually declined during a 4 hour steady state exercise bout on a cycle ergometer reflecting the increased reliance fatty acid oxidation with exercise duration (Christensen & Hansen, 1939).
Skeletal muscle an metabolic flexibility

Skeletal muscle is one of the largest organs in the body contributing to around 40-50% of the average lean individual (Hollmann & Hettinger, 1990) and by proportion alone, constitutes the main determinant for whole-body metabolism both at rest and during physical activity (Zurlo et al., 1990). A defining trait for skeletal muscle tissue is a remarkable plasticity in response to changes in physical activity level including both structural and metabolic adaptations. However, skeletal muscle also possesses an extraordinary ability to regulate fuel selection when energy demands call for a sudden change in metabolic turnover and when substrate availability changes. The ability of healthy lean muscle to adjust oxidative preference for macronutrients according to transitory demands has been defined as metabolic flexibility (Corpeleijn et al., 2008; Galgani et al., 2008; Kelley & Mandarino, 2000; Storlien et al., 2004). First coined by Kelley and Mandarino (Kelley & Mandarino, 2000), it was described how skeletal muscle in lean, healthy individuals could aptly switch from predominant fat oxidation and low carbohydrate use in the fasted state to suppression of fat oxidation and elevation in glucose oxidation with insulin stimulation. Furthermore, they demonstrated how oxidative fuel selection was compromised in individuals with lifestyle-related metabolic disorders such as obesity and type 2 diabetes evident by their diminished ability to modulate fatty acid and carbohydrate oxidation making them metabolically inflexible, as illustrated in figure 2.

Figur 1. Respiratory quotient (RQ) during 240 minutes of cycle ergometer exercise when on a CHO rich diet (K.-h.), protein rich diet, and high fat diet (F) ((Christensen & Hansen, 1939; Christensen & Hansen, 1939))
Figure 2. Illustration of metabolic flexibility in a lean and obese individual. Differences in glucose and fat oxidation in skeletal muscle during fasting (A,B) and during insulin-stimulated conditions (C,D) in a lean, aerobically fit individual (A,C) and an obese, sedentary individual (B,D). Skeletal muscle of the lean subject has a high reliance upon fat oxidation during fasting conditions (A) and insulin strongly suppresses fat oxidation and induces a high reliance upon glucose oxidation (C), while in an obese subject there is less reliance on fat and a greater reliance on glucose oxidation during fasting (D) and less stimulation of glucose oxidation by insulin and a blunted suppression of fat oxidation (Kelley, 2005).

AMPK and ACC

The mechanistic link for the mediation of the substrate transition observed with exercise may in part be explained by the cellular energy sensor AMP-activated protein kinase (AMPK), which is highly responsive to metabolic stress in an attempt to reestablish energy homeostasis (Hardie, 2011). The highly conserved AMPK kinase, first discovered in 1987 (Carling et al., 1987) consists of catalytic α and two regulatory β and γ subunits all of which are found in multiple isoforms and is activated with changes in cellular energy status, reflected by an increase in cytosolic [AMP/ATP] (Carling et al., 1987) and [Ca²⁺] as observed with muscle contractions (Hawley et al., 2005). Numerous studies have shown that exercise increases AMPK activity in both human, rat and mouse skeletal muscle (Fujii et al., 2000; Winder et al., 1997; Wojtaszewski et al., 2000) and that AMPKThr172
phosphorylation reflects increased AMPK activity (Sakamoto et al., 2004). Furthermore, the exercise-induced AMPK phosphorylation in human skeletal muscle has been shown to be both intensity and duration dependent (Rasmussen & Winder, 1997; Wojtaszewski et al., 2000). AMPK activation turns on catalytic ATP-generating processes, while inhibiting anabolic processes such as lipid and protein synthesis. Furthermore, AMPK also prompts recruitment of GLUT4 transporters to the plasma membrane during exercise allowing for a marked increase in insulin independent glucose uptake in skeletal muscle (Kurth-Kraczek et al., 1999). At the same time AMPK also allows for an increase in mitochondrial fatty acid uptake and oxidation by phosphorylating and inactivating its downstream target acetyl-CoA carboxylase (ACC)2 (Winder et al., 1997). Upon its inactivation, ACC2 ceases to constitutively produce malonyl-CoA that inhibits the mitochondrial carnitine–palmitoyltransferase (CPT) 1, which controls the entry of fatty acids into the mitochondria allowing ample substrate for β-oxidation. In accordance, endurance exercise increases skeletal muscle ACC2Ser212/ACC2Ser221 phosphorylation in mouse and human skeletal muscle (Hardie, 2011). This induction occurs typically in parallel with an increased AMPK phosphorylation in line with the potential AMPK-mediated regulation of ACC, although dissociation between exercise-induced regulation of AMPK and ACC phosphorylation has been demonstrated in human skeletal muscle during prolonged exercise (Wojtaszewski et al., 2002). However, strong evidence also points to a central role of PDH in the regulation of skeletal muscle substrate utilization during metabolic challenges.

**Pyruvate dehydrogenase**

The pyruvate dehydrogenase complex (PDC), with its 11 MDa sized structure, is one of the largest multifunctional enzymes in the mammalian cell (Milne et al., 2006). Located within the mitochondrial matrix, the PDC constitutes the only mammalian pathway for irreversible conversion of pyruvate to acetyl-CoA thus providing the vital link between glycolytic energy production, the TCA cycle, and oxidative phosphorylation. Structural characterization techniques have demonstrated features of both octahedral and icosahedral core symmetry of PDC, but the complex is essentially assembled from numerous copies of four key constituent subunit enzymes required for its full catalytic cycle: the thiamine-dependent tetrameric α2β2 pyruvate decarboxylase (E1), also simply known as pyruvate dehydrogenase (PDH), the dihydrolipoyl acetyltransferase (E2), the flavoprotein dihydrolipoyl dehydrogenase (E3), and lastly, unique to the mammalian PDC, the E3-binding protein (E3BP) (Lengyel et al., 2008; Hezaveh et al., 2016; Perham, 2000).
The E2 and E3BP are arranged to form the inner core bridging the E1 and E3 via a unique E3BP based swinging arm able to visit active sites on all of the other enzymes. The functional significance of this is that it enables the E1 and E2 to coordinately transfer a formed acetyl group, catalyzed by E1 from the oxidative decarboxylation of pyruvate, into CoA via reduction of the dihydrolipoyl group of the E2 domain. For the enzymatic process to come full circle, the E3 then reconstitutes the lipoyl domain to its oxidized state (Yang et al., 1998; Milne et al., 2002). The net PDH reaction is then pyruvate + CoA + NAD+ → acetyl-CoA + CO2 + NADH, with the end-product, acetyl-CoA, entering the TCA cycle for oxidation. Worth noting is that the E1 enzyme, which is composed of 2 alpha and 2 beta subunits, is considered the rate-limiting enzyme (Danson et al., 1978) and by far outnumbers E2 and E3 in the native complex (Perham, 1991; Reed & Hackert, 1990).

Pyruvate dehydrogenase regulation

Because the PDC controls the conversion of pyruvate it occupies a central position in relation to the control of mitochondrial energy production and cellular substrate metabolism. Suppression and activation of PDC becomes essential in situations where glucose availability and/or use changes with swift and appropriate regulation of the complex to maintain energy homeostasis in response to varying nutritional and metabolically challenging states. In effect, being dependent on the activity of PDH (E1) the activity of the PDC as a whole is reflected by, the activity of PDH-E1α in the unphosphorylated active form PDHa activity (Harris et al., 2002). The regulation of PDH is exerted by four mitochondrially located PDH kinase (PDK) isozymes, PDK1-4, able to
phosphorylate four known serine residues on the E1α subunits of PDH, site 1: Ser293; site 2: Ser300; site 3: Ser232, and site 4: Ser295 (Kiilerich et al., 2010a; Korotchkina & Patel, 2001; Patel & Korotchikina, 2001; Yeaman et al., 1978). In vitro studies have shown that phosphorylation of any one of the first three sites is sufficient to inactivate PDH (Davis et al., 1977; Sugden & Randle, 1978), while the impact of site 4 phosphorylation remains to be determined. The four PDKs are distributed in a tissue-specific manner (Bowker-Kinley et al., 1998). PDK1 has been reported mainly to be present in heart and pancreatic islet, PDK2 to be ubiquitously expressed and PDK4 especially expressed in oxidative tissues such as heart, skeletal muscle and liver, while PDK3 is rather limited to gonadal tissue. Additionally, two known PDH phosphatase (PDP) isozymes dephosphorylate the E1α subunits causing an increase in catalytic activity. PDP1 is primarily expressed in heart, brain and skeletal muscle, while PDP2 is found mainly in liver, adipose tissue and kidney (Huang et al., 1998; Huang et al., 2003).

In addition, the PDK isoforms have different site specificities for the various phosphorylation sites on the PDH-E1α subunit (Korotchikina, Patel 2001), thus the hierarchical affinity for PDH Ser 293 (site1) is in the order PDK2>PDK4>PDK1>PDK3, PDH Ser300 (site2) in the order PDK3>PDK4>PDK2>PDK1, and PDH Ser232 (site3), which has been reported to be solely phosphorylated by PDK1 (Korotchikina & Patel, 2001; Patel & Korotchikina, 2001), while the affinity for PDH Ser295 is yet to be examined. Different kinetic properties of the PDKs have also been established adding a further level of complexity to the covalent regulation of the PDH phosphorylation dynamics (Korotchikina & Patel, 1995; Korotchikina et al., 1995) PDK activity is regulated by substrates and products of the PDH overall reaction. All PDKs are activated by an increase in the ratio of mitochondrial acetyl CoA/CoA and NADH/NAD+ content (Pettit et al., 1975), which imposes end-product inhibition of the PDC. Mechanistically this inhibition is thought to be mediated through changes in acetylation and redox state of the lipoyl groups on the E2 domain precipitating a negative feedback mechanism on E1 (Ravindran et al., 1996). Pyruvate, however, reduces PDK activity in a more isoform- specific manner with PDK2 shown to be far more sensitive to inhibition than PDK4 (Bowker-Kinley et al., 1998), although an effect of this specific allosteric regulation of PDK on PDHa activity remains to be demonstrated (Constantin-Teodosiu et al., 2004).
Figure 4: Pyruvate dehydrogenase regulation. Pyruvate dehydrogenase (PDH) is interconverted between its active (PDHa) and inactive (PDHb) forms by the PDH kinase/phosphatase system. Metabolic factors that regulate the kinases (PDK1-4) and phosphatases (PDP1-2) are indicated. In addition, active PDHa is inhibited by increased concentrations of its products, acetyl CoA and NADH, relative to its substrates free coenzyme A (CoA) and NAD⁺ (modified from Sugden et al., 2002).

PDPs are similarly allosterically regulated by both hormonal release and PDH related metabolites. While both isoforms require Mg²⁺ for activation, only PDP1 activity is stimulated increased in Ca²⁺ (Huang et al., 1998), while insulin has been reported to stimulate both PDP1 and PDP2 activity via PKC and subsequent translocation to the mitochondria (Caruso et al., 2001; Huang et al., 1998).

With the dawn of proteomics and the discovery of in the excess of thousands of protein acetylation sites (Choudhary et al., 2009; Lundby et al., 2012) the acetylome has gained increasing attention in recent years. An additional posttranslational modification in the form of acetylation has recently been proposed to regulate PDH activity mediated through the mitochondrial NAD⁺-dependent deacetylase sirtuin (SIRT) 3 (Fan et al., 2014; Jing et al., 2013; Ozden et al., 2014). SIRT3, being the only member of the mitochondrial sirtuins with robust deacetylase activity (Lombard et al., 2007; Verdin et al., 2010), is highly expressed in metabolically active tissue like heart, liver, brown adipose tissue, and skeletal muscle (Lombard et al., 2007; Ahn et al., 2008). PDH-E1α in mitochondrial lysates from skeletal muscle of SIRT knockout mice has been reported to be hyperacetylated on specific lysine target K336 leading to increased phosphorylation of PDH-E1α, although this site did not affect PDH activity in C2C12 myoblast (Jing et al., 2013), suggesting that additional lysine targets
are also necessary for PDH regulation. Another study confirmed SIRT3-mediated deacetylation of PDH-E1\(\alpha\) in mutant cancer cells and attributed lysine K321 to be another specific target (Ozden et al., 2014). Additionally, SIRT3-mediated deacetylation of PDP1 in cancer cells has also been reported to regulate catalytic activity of PDH-E1\(\alpha\) (Fan, 2013). Taken together, this underlines a definite, likely complex, mechanistic role for acetylome-dependent regulation of PDH that needs further investigation under different metabolic conditions to be fully understood.

**Exercise-induced PDH regulation**

In resting skeletal muscle, energy homeostasis is maintained at relatively high ATP/ADP, NADH/NAD and Acetyl-CoA/CoA ratios ensuring high PDK activity. At the onset of exercise cellular \([\text{Ca}^{2+}]\) increases leading to stimulation of PDP activity and a rise in glycolytic flux prompts inhibition of PDK activity, all in all contributing to the activation of PDH. Ward et al. were the first to measure total PDH activity and demonstrate that PDHa activity increased in human skeletal muscle in response to exhaustive exercise (Ward et al., 1982). This finding has been established in many studies since then (Putman, 1995; Pilegaard 2006; Watt 2001; more). The potential rapid activation of the PDH enzyme was first shown in human skeletal muscle already after 1 minute into an acute bout exercise by Howlett et al. (Howlett et al., 1998). In a later study examining the time course of PDH activation high intensity sprints on an isokinetic cycle ergometer displayed rapid activation of PDH after as little as 6 seconds with maximal activation already reached after 15 seconds (Parolin et al., 1999a). The flux through the PDH has been estimated to correlate closely with PDHa activity in skeletal muscle in response to various exercise modes and intensities (Howlett et al., 1998; Putman et al., 1993; Gibala et al., 1998), although this has later been challenged. Thus, PDH activation was not inhibited during short term exercise bouts at intensities around 75% VO\(2_{\text{max}}\) (Constantin-Teodosiu et al., 2004) despite depleted glycogen stores and consequently reduced pyruvate availability and lower flux through the PDC furthermore downplaying the role pyruvate as a key allosteric regulator in muscle PDH activation. Several studies have also demonstrated dephosphorylation of PDH site 1 and 2 in human (Bienso et al., 2015b; Kiilerich et al., 2008; Kiilerich et al., 2010b; Pilegaard et al., 2006) and mouse (Klein et al., 2007) skeletal muscle in response to exercise in line with the increase in PDHa activity. This supports that regulation of PDH phosphorylation plays a central role in PDH regulation during exercise.
With prolonged exercise, the glucose sparing switch towards fat oxidation seems to involve changes in PDHa activity. This has been demonstrated in several studies with different approaches. Prolonged cycling and two-legged knee extensor exercise protocols at moderate exercise (~45-57% VO2max) have shown that PDHa activity increased in human skeletal muscle and remained elevated for several hours followed by a decrease towards the resting level with a corresponding transient decrease in PDH site 1 and site 2 phosphorylation (Pilegaard et al., 2006). This swift in PDHa activity during prolonged exercise was associated with a reciprocal shift in carbohydrate and fat utilization supporting/suggesting a central role of PDH in this regulation (Mourtzakis et al., 2006; Pilegaard et al., 2006; Watt et al., 2002).

The regulation of PDK expression during prolonged exercise has been examined in several studies and while neither PDK1, 2, or 3 seem to change during exercise it has been shown that a single bout of prolonged exercise increased transcription and mRNA content of PDK4 in human skeletal muscle (Mourtzakis et al., 2006; Pilegaard et al., 2002; Pilegaard & Neufer, 2004) with responses that were by far most pronounced into exercise recovery (Pilegaard et al., 2000; Pilegaard et al., 2002; Pilegaard & Neufer, 2004). In accordance, PDK4 protein content was unchanged in human skeletal muscle during 3 h of exercise (Watt et al., 2004) supporting that PDK expression does not seem to explain the down-regulation in PDHa activity during prolonged exercise. However, the finding that PDK activity increased at 3h of exercise concomitant with lowering of PDHa activity (Watt et al., 2004) may suggest a PDK mediated regulation of PDHa activity independent of PDK expression during prolonged exercise.

Exercise training is well known to increase skeletal muscle oxidative capacity increasing the reliance on fatty acid oxidation and consequently an improved metabolic control of ATP supply and demand (Perry et al., 2008). In accordance, a previous study has shown that 7 weeks of aerobic exercise training decreased pyruvate production and attenuated PDH activation in human skeletal muscle during submaximal exercise at the same absolute workload as the untrained state (LeBlanc et al., 2004a). Several studies have reported increases in PDH-E1α protein with exercise training in both humans and rodents (Bienso et al., 2015a; Kiilerich et al., 2010a; LeBlanc et al., 2004b). Leblanc et al. reported that 8 weeks of aerobic training increased protein levels of PDH-E1α and PDK2, as well as increased total PDH and PDK activity in skeletal muscle. This led the authors to conclude that increased metabolic control was achieved through larger contribution to total PDK activity of the pyruvate sensitive PDK2 isoform (LeBlanc et al., 2004b). Little attention has been given to PDP dynamics in relation to exercise training. One study, however, reported positive correlation between increased CS activity,
PDK2, as well as both PDP1 activity and protein levels in isolated mitochondria from human m. gastrocnemius with the caveat that neither insulin sensitivity nor fiber type distribution in the biopsies were accounted for, possibly influencing the outcome of the study (Love et al., 2011).

SIRT3 expression in human and mouse skeletal muscle has also been reported to be regulated with both acute exercise and exercise training. In mice, Brandauer et al. found that, six and a half weeks of voluntary wheel running led to an increase in SIRT3 protein and related this finding to be AMPK-activation dependent, as AMPKa2 knockdown had an almost completely blunted SIRT3 response to training (Brandauer et al., 2015). In accordance, another study showed that six weeks of voluntary wheel led to increases in skeletal muscle SIRT3 protein in mice (Palacios et al., 2009). Acute exercise, as little as one hour of moderately intense exercise (18m/min), have also been shown to elicit increases SIRT3 protein in both exercise trained and untrained mice ((Brandauer et al., 2015). However, exercise studies have presented more or less ambiguous results in relation to SIRT3 expression in humans. While SIRT3 been reported to increase with exercise training in overweight adolescents (Vargas-Ortiz et al., 2015), 3 weeks of knee extensor endurance exercise training conducted in humans elicited no significant changes in SIRT 3 protein (Brandauer et al., 2015). In fact, a recent study reported a drop in SIRT3 mRNA following an acute bout of 55% VO2peak ergometer cycling, although it did not correlate to any change in SIRT3 protein levels (Edgett et al., 2016).

Knockdown of SIRT3 in mice does not seem to reduce the total mitochondrial density as measured by abundance of OXPHOS complexes. However, SIRT3 knockdown does seem to markedly reduce skeletal muscle fatty acid oxidation by around 50% in mice (Hirschey et al., 2010) establishing it a potential influential candidate in controlling the substrate switch and hence metabolic flexibility during exercise, possibly by both direct enzymatic deacetylation of PDH and indirect on covalent effectors of PDH (Fan et al., 2014;Jing et al., 2013).

Fasting-induced PDH regulation

Food deprivation has a huge impact on whole body carbohydrate and fatty acid oxidation and consequently also PDH regulation and activity. Studies have demonstrated that fasting markedly reduced PDHa activity in skeletal muscle after a 40 hour fast in both men and women (Spriet et al., 2004), and in mitochondrial extracts from skeletal muscle in Wistar rats after 48 hours of fasting (Sugden et al., 2000). In accordance, increases in skeletal muscle PDH$^{\text{Ser293}}$ and PDH$^{\text{Ser300}}$ and PDH$^{\text{Ser295}}$ phosphorylation (Bienso et al., 2014a;Kiilerich et al., 2010a).
as well as increase in PDH-E1α acetylation (Jing et al., 2013) have been reported after 18-24 hours of fasting in mice indicating that these posttranslational effectors may play crucial roles in the fasting-induced regulation of PDH. In accordance, SIRT3 mRNA and/or protein content has been shown to decrease in mouse and human skeletal muscle with fasting (Edgett et al., 2016; Jing et al., 2013). Furthermore, while PDK1, 2 and 3 have been reported to be relative unaffected by fasting interventions (Spriet et al., 2004) PDK4 seems to be a driving regulatory force in the down-regulation of PDHa activity as human and rodent studies have demonstrated increased PDK4 transcription (Pilegaard et al., 2003a), PDK4 mRNA (Pilegaard et al., 2003a; Sugden et al., 2000; Wu et al., 1999a) and protein (Kiilerich et al., 2010a; Spriet et al., 2004; Sugden et al., 2000) in skeletal muscle with fasting. To that effect, total PDK activity has also been shown to be increased with 48 hours of fasting in rats (Fuller & Randle, 1984; Sugden et al., 2000). PDP dynamics with fasting have gained little attention in the past, but one study has reported decreased PDP1 mRNA in mouse skeletal muscle with 18 hours of fasting (Bienso et al., 2014a), while another study reported a decrease in both PDP1 and PDP2 protein in rat kidney, but only a decrease in PDP2 protein in rat cardiac muscle (Huang et al., 2003). However, as PDP2 is very modestly expressed in skeletal muscle, the impact of such regulation of PDP2 in fasting-induced PDH regulation in skeletal muscle may only be minor. Although the regulation of PDHa activity through post-translational modifications is well established, the presented studies reveal missing pieces of the puzzle in signaling pathways inducing these modifications.

**Substrate availability and PDH regulation**

Previous studies investigating the impact of substrate availability on PDH regulation suggest that circulating free fatty acid (FFA) levels influence the exercise-induced PDH regulation in human skeletal muscle. Thus, infusion a lipid emulsion 4 hours up to and during 3 hours of two-legged knee extensor exercise elevated phosphorylation of both sites PDH^{Ser293} and PDH^{Ser30}, but exercise partially overrode this effect suggesting that additional regulatory events triggered by exercise can bypass the influence of high levels of FFA to avoid compromising PDH activation with prolonged exercise (Pilegaard et al., 2006). Conversely, administering doses of anti-lipolytic nicotinic acid, reducing FFA pre-exercise, led to increases in PDHa activity during a 40 minute bout of moderate exercise (Stellingwerff et al., 2003). In support, a high fat diet intervention has been shown to reduce the exercise-induced increase in PDHa activity in human skeletal muscle likely through elevated phosphorylation (Kiilerich et al., 2010b) underlining a role for FFA in modulating PDH regulation. In addition,
prior glycogen reduction in muscle glycogen, reduced the exercise-induced PDH activation and PDH dephosphorylation, but with no additive effect when combined with diet-induced elevation in circulating FFA (Kiilerich et al., 2010b). Similarly, increased levels of circulating fatty acids have been demonstrated to contribute to the regulation of PDK4 in rodent skeletal muscle with fasting. Hence a previous study has reported that feeding rats the PPARalpha agonist WY-14,643 increased PDK4 mRNA and protein content markedly in skeletal muscle (Wu et al., 1999b). Because long-chain fatty acids are activators of PPARalpha (Wu et al., 2001) which supports that fasting induced-increases in circulating FFA induce PDK4 expression with concomitant PDH phosphorylation and decreased PDH activity in skeletal muscle. Together this suggests that substrate availability contributes to PDH regulation through effects on PDH phosphorylation.

The potential impact of hormonal influence on PDH regulation in skeletal muscle has also been investigated. For instance adrenaline infusion during moderate exercise has been reported to synergistically increase PDH activity (Watt et al., 2001) without any obvious mechanistic explanation. Muscle derived factors may well play a role as previous studies also suggest that the cytokine interleukin (IL) 6 produced and released from skeletal muscle influence the regulation of substrate utilization potentially via effects on PDH.

**Interleukin 6**

Originally discovered in fibroblasts and defined for its role in the acute immune phase response (Bendtzen, 1988; Kishimoto & Hirano, 1988), IL-6 has since proven to be produced in numerous cell types and be implicated in many biological functions (Pedersen & Febbraio, 2008a). Of particular interest is the establishment of adipose tissue and skeletal muscle as major sources of IL-6 production (Jonsdottir et al., 2000; Mohamed-Ali et al., 1997; Steensberg et al., 2000). Furthermore, exercise has been shown to increase circulating IL-6 concentration in both humans and rodent (Drenth et al., 1995; Lundby & Steensberg, 2004; Nielsen et al., 1996; Ostrowski et al., 1998). The observation that electrical stimulation of rodent skeletal muscle increased muscle IL-6 mRNA (Jonsdottir et al., 2000) and the finding that one-legged knee extensor exercise resulted in a negative arterio-venous difference of IL-6 across the working but not resting muscle suggested that circulating IL-6 derived from skeletal muscle (Keller et al., 2001; Steensberg et al., 2000). Such IL-6 secretion from skeletal muscle was further supported by other studies in humans and rodents (Febbraio et al., 2004; Lauritzen et al., 2013; Steensberg et al., 2000; Whitham et al., 2012) and very elegantly demonstrated in a study using GFP-transfected mouse skeletal muscle displaying IL-6 containing vesicles being reduced in
numbers during muscle contraction, clearly suggesting secretion from working skeletal muscle (Lauritzen et al., 2013). The increase in circulating IL-6 has since been shown to be both intensity and duration dependent (Ostrowski et al., 2000; Steensberg et al., 2000). For instance, 5 hours of low-intensity one-legged knee-extensor exercise measured a 2000% increase in arterial IL-6 (Steensberg et al., 2000) with far the majority of the increase occurring in the latter 2 hours of exercise. A similar observation in plasma IL-6 increase has been reported with only 2 hours of two-legged knee-extensor exercise but at higher intensity (Keller, 2001) and in mice with 120 minutes of treadmill exercise (Ellingsgaard et al., 2011). A glucose dependent aspect of IL-6 expression, production and release has been suggested as depletion of glycogen prior to exercise resulted in both larger increase in plasma IL-6 and transcription in human skeletal muscle with exercise (Keller et al., 2001). Conversely, infusion of glucose prior to exercise led to a reduced IL-6 release with exercise in humans (Febbraio et al., 2003) suggesting that IL-6 release from skeletal muscle signals to the liver to recruit glucose and ensure delivery to the working muscle. This is further supported by the fact that the glucose rate of appearance has been shown to increase with IL-6 infusions during low intensity exercise in humans (Febbraio et al., 2004). Of note is that IL-6 has also more recently been reported to increase in the blood to a rather high level (2000 pg/ml) during 24 hours of fasting in mice (Wueest et al., 2014a). A concomitant increase in skeletal muscle IL-6 mRNA (Wueest et al., 2014a) indicated that the circulating IL-6 might be derived from skeletal muscle also in response to fasting. IL-6 exerts effects in the target tissues by binding to a receptor an eliciting an intracellular signaling cascade. Belonging to the gp130 family, a subclass of class 1 cytokine receptors (Yamauchi-Takahara & Kishimoto, 2000), IL-6 signals through the IL-6 receptor system comprised by

![Figure 5: IL-6 mediated signal transduction through classical (A) and trans-signalling (B) pathways. In both pathways induction is engaged through membrane-bound gp130 (Dayer et al. 2009).](image)


the ligand-binding IL-6 receptor α (IL-6Rα) and a homodimer of the signal transducing β-subunit, gp130, also functioning as a scaffold (Murakami et al., 1993). Existing in both a membrane bound and a soluble form (sIL-6R), the IL-6 receptor can in the absence of membrane receptors form free complexes with IL-6 and translocate to bind to gp130 at the cell surface (Narazaki et al., 1993; Yasukawa et al., 1992) thus enabling IL-6 to act through both classical and trans-receptor signaling. Upon activation of the receptor, IL-6 signals through the Janus kinase (JAK) 2 that initially auto-phosphorylates activating a cytoplasmic domain on gp130 allowing for recruitment of signal transducer and activator of transcription (STAT) 3 which is subsequently phosphorylated and activated by JAK. Upon activation STAT3 dimerizes and then relocates to the nucleus activating a number of transcription factors and genes (Wunderlich et al., 2013).

IL-6 has been reported to influence both carbohydrate and lipid metabolism in different models. Infusion of IL-6 in fasted individuals has been shown to induce lipolysis and fat oxidation indicated by an increase in plasma free fatty acid and palmitate clearance independently of insulin, epinephrine and glucagon levels (van et al., 2003; Wolsk et al., 2010). This is supported by similar findings in IL-6 incubated muscle cells (Al-Khalili et al., 2006) and isolated rat skeletal muscle (Kelly et al., 2009). A potential role of fasting-induced increases in circulating IL-6 in augmenting lipolysis is supported by the findings that IL-6 KO mice had no fasting-induced increases in circulating FFA leading the authors to conclude that IL-6 is necessary for fasting-induced increase in lipolysis (Wueest et al., 2014a) and other studies have reported increased fat depots in IL-6 KO mice in support of this (Matthews et al., 2010; Wallenius et al., 2002). Likewise, both glucose uptake and oxidation have been reported to improve in muscle cells (Al-Khalili et al., 2006) and humans (Carey et al., 2006) with IL-6 infusion. A more controversial subject, however, is whether exercise-mediated IL-6 release actually improves glucose uptake. Although several studies have shown beneficial effects of IL-6 on glucose metabolism and uptake (Carey et al., 2006; Febbraio et al., 2004; Helge et al., 2003) other studies in humans and mouse knockout models have contested this notion (Helge et al., 2011; O'Neill et al., 2013).

A metabolic link between IL-6 and AMPK activation has been proposed and would make sense in the light of aforementioned effects on both glucose and lipid metabolism. Studies have reported an IL-6 associated increase in muscle AMPK phosphorylation (Al-Khalili et al., 2006; Bienso et al., 2014a; Kelly et al., 2004; Wueest et al., 2014a) potentially contributing to the observed shifts in substrate oxidation. However, IL-6-mediated AMPK regulation does not always seem to be a consistent finding (Bienso et al., 2014a; O'Neill et al., 2013).
Interestingly, injections with physiologically relevant doses of recombinant IL-6 have been shown to lead to a decrease in PDHα activity, and activation of AMPK, in mouse skeletal muscle, but only during fasted and not fed conditions (Bienso et al., 2014a) suggesting that fuel status may likely play an important role. Furthermore, a lack of AMPKα2 has been shown to amplify the increase PDHα activity in mouse skeletal muscle during exercise (Klein, 2007). Taken together this may suggest an inhibitory effect of AMPK on PDH in skeletal muscle possibly mediated by IL-6 during both exercise and fasting.

**PGC-1α**

First discovered and identified as a PPARγ-binding protein in brown adipose tissue (BAT) (Puigserver et al., 1998), the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α has been shown to be expressed in numerous tissues with highest levels in oxidative tissues such as heart, liver, kidney, skeletal muscle, and BAT. By specific binding to a myriad of transcription factors and binding partners, PGC-1α coordinates the transcription of a large number of genes making the co-activator a hub in metabolic regulation. PGC-1α is induced by exercise in both human and rodent skeletal muscle and has been reported to be regulated both transcriptionally and post-translationally. Thus, a single bout of exercise has been shown to potently upregulate PGC-1α transcription (Pilegaard et al 2003; Jørgensen et al 2005) mRNA (Baar et al., 2002; Jorgensen et al., 2005; Leick et al., 2008; Pilegaard et al., 2003b; Terada et al., 2002) and protein (Baar et al., 2002; Perry et al., 2010; Wright et al., 2007b). However, due to a very short half-life because of an inherent susceptibility to proteosomal degradation of the PGC-1α proteins (Adamovich et al., 2013) the phasic response of PGC-1α is rather short-lived with a reversion back to basal levels within hours. Several intracellular factors and inducing pathways have been shown to confer the PGC-1α gene response. Contraction-induced Ca2+ signaling likely induce both PGC-1α transcription and activity as incubations with ionomycin and caffeine has shown upregulation of PGC-1α mRNA in isolated rat muscle (Wright et al., 2007a), an effect that was abrogated with calcineurin and CamK inhibitors. Accordingly, transgenic mice expressing constitutively active calcineurin in skeletal muscle had increased mitochondrial biogenesis (Long et al., 2007).

The muscle stress induced p38 mitogen-activated protein kinase (p38 MAPK) offers stabilization of existing PGC-1α protein by direct phosphorylation of three different serine residues, shown in C2C12 cells (Puigserver et al., 2001), increasing the activity of the co-acativator (Akimoto et al., 2005; Puigserver et al., 2001). Moreover, p38 MAPK phosphorylates activating transcription factor 2 (ATF2) also promoting PGC-1α transcription (Cao et al., 2004). AMPK has also been demonstrated to regulate PGC-1α. Hence, direct
phosphorylation and activation of PGC-1α by AMPK on two residues has been reported based on in vitro experiments (Jager et al., 2007), although exercise-induced upregulation does not seem to require the prevalent AMPKα2 isoform in skeletal muscle (Jorgensen et al., 2005). Furthermore, pharmacological activation of AMPK with the AMP analogue, AICAR, has been shown to upregulate PGC-1α mRNA in rat and mouse skeletal muscle (Jorgensen et al., 2005; Terada et al., 2002). PGC-1α has also been demonstrated to be regulated by acetylation and of special interest in terms of acetylation is the interaction of the sirtuins (SIRT) 1 and 3 with PGC-1α. SIRT1 has been demonstrated to directly deacetylate and activate PGC-1α possibly in an AMPK dependent manner in C2C12 (Canto et al., 2010a), while SIRT3 has been attributed to have similar effects on PGC-1α with both exercise and diet (Brandauer et al., 2015; Palacios et al., 2009). Additionally, SIRT3 and PGC-1α may have a mutual influence on each other’s expression levels through a reported positive feedback mechanism through deacetylation of liver kinase B (LKB) 1, AMPK activation and increased CREB transcription (Pillai et al., 2010; Thomson et al., 2008).

PGC-1α is known to have tissue-specific roles, but collectively is most renowned as a master regulator of mitochondrial biogenesis through the co-activation of nuclear respiratory factor (NRF) 1 and 2 (Wu et al., 1999c) and estrogen-related receptor (ERR) α (Huss et al., 2002; Olesen et al., 2010; Perez-Schindler et al., 2012; Puigserver et al., 1998; Wu et al., 1999c). Thus, PGC-1α has repeatedly been shown to regulate the expression of mitochondrial, oxidative proteins (Lin et al., 2002; Wu et al., 1999c). Transgenic muscle specific overexpression of PGC-1α and inducible overexpression in skeletal muscle increased the content of OXPHOS and other oxidative proteins in skeletal muscle (Calvo et al., 2008; Lin et al., 2002; Wende et al., 2007; Wu et al., 1999c), whereas whole body KO and muscle specific KO of PGC-1α mice have been demonstrated to have lowered content of oxidative proteins (Geng et al., 2010; Handschin et al., 2007; Leick et al., 2008; Lin et al., 2004a). In addition, PGC-1α has been shown to positively affect the mRNA level of CD36, CPT1 and MCAD (Calvo et al., 2008) potentially influencing fatty acid transport and availability for β-oxidation and in relation to glucose metabolism, PGC-1α has been shown to regulate both GLUT4 mRNA and protein content in skeletal muscle (Leick et al., 2010a).

In accordance with the effects of PGC-1α overexpression and knockout in skeletal muscle (Geng et al., 2010; Leick et al., 2008; Lin et al., 2002), studies in both humans and rodents suggest that exercise training-induced adaptations in skeletal muscle oxidative capacity are mediated by PGC-1α (Baar et al., 2002; Geng et al., 2010; Leick et al., 2010a; Leick et al., 2010b; Olesen et al., 2010; Perry et al., 2010; Pilegaard et al., 2010).
2003b; Russell et al., 2003), although PGC-1α also has been shown not to be required for exercise training induced adaptations in oxidative proteins in mouse skeletal muscle (Leick et al., 2008). Moreover, PGC-1α mRNA and protein levels have been established to be higher in skeletal muscle from trained than untrained individuals (Perry et al., 2010; Russell et al., 2003; Short et al., 2003; Wright et al., 2007) further supporting a major role of PGC-1α in metabolic adaptations with exercise training. In addition, muscle specific PGC-1α overexpression mice have been shown to exhibit lower RER during treadmill running (Calvo et al., 2008; Wong et al., 2015), which suggests that PGC-1α also influences regulation of substrate utilization and metabolic flexibility. In accordance, the level of skeletal muscle PDH-E1α protein content has been shown to follow differences in the levels of muscle PGC-1α in mice (Kiilerich et al., 2010a) and PGC-1α to regulate PDK4 expression in mouse skeletal muscle (Kiilerich et al., 2010a; Calvo et al., 2008; Wende et al., 2005). Furthermore, whole body PGC-1α knockout mice were unable to downregulate PDHα activity in skeletal muscle in response to fasting (Kiilerich et al., 2010). In support, PGC-1α protein increases have been reported with 24 hours of fasting in mice (Palacios et al., 2009) together suggesting that the level of muscle PGC-1α influences fasting-induced metabolic flexibility.

![Figure 6](image)

**Figure 6.** Schematic illustration of the suggested peroxisome proliferator-activated receptor-γ coactivator 1α (PGC)-1α-mediated upregulation of pyruvate dehydrogenase kinase (PDK) 4 leading to increased fat oxidation in skeletal muscle (from Olesen et al., 2010). Oestrogen-related receptor α (ERRα). Pyruvate dehydrogenase complex (PDC).

Taken together, these findings emphasize PGC-1α as an important regulator of substrate utilization. However, the impact of muscle specific knockout of PGC-1α on fasting-induced substrate switch and skeletal muscle PDH regulation remains to be determined.
Objectives

The objectives of the present thesis was to investigate new aspects of regulation of PDH in acute exercise, exercise training, and fasting in mouse and human skeletal muscle with specific focus the role of IL-6 on PDH regulation in fasting and acute prolonged exercise and PGC-1α in fasting.

The following hypotheses were addressed:

- Skeletal muscle IL-6 contributes to the regulation of PDH in mouse skeletal muscle at rest as well as during prolonged exercise and that this is associated with IL-6 mediated regulation of AMPK.

- Skeletal muscle IL-6 affects short-term and prolonged fasting-induced PDH regulation and substrate utilization in mice.

- Lack of muscle PGC-1α affects the time course of the switch in substrate utilization during the transition from the fed to the fasted state and affects regulation of fasting-induced changes in PDHa activity, PDH phosphorylation and PDH acetylation in skeletal muscle.

Methods

Mouse experiments

Mice

Mice were kept on a 12:12 light-dark cycle at 22°C with ad libitum access to water and chow diet (Altromin 1314F, Brogaarden, Lynge, Denmark) until the intervention of the experiment. All experiments were approved by the Danish Animal Experimental expectorate and complied to the European Convention for the protection of vertebrate animals used for experiments and other scientific purposes (Council of Europe no. 123. Strasbourg, France 1985).

For studies I and II skeletal muscle-specific IL-6 knockout (MKO) mice were used and in study III PGC-1α MKO mice were used. Floxed littermate controls were used to standardize for any potential metabolic effects of the inserted loxP sites. Generation of muscle-specific IL-6 knockout mice was performed by establishing floxed IL-6\textsuperscript{lox/lox} mice from the 129X1/SvJ background with insertion of LoxP sites flanking exon 2 of the IL-6 gene and backcrossing for 10 generations with a C57BL/6 background as previously described (Quintana et al., 2013). Exon 2 has been shown to code for a sequence essential for biological activity and secretion of IL-6 (Kopf et al., 1994; Rose-John et al., 1993). The floxed IL-6\textsuperscript{lox/lox} mice were subsequently crossed with C57BL/6 mice expressing Cre-recombinase under the control of the skeletal muscle-specific myogenin promoter.
Muscle-specific IL-6 knockout mice

Figure 7. Schematic illustration showing the generation of skeletal muscle-specific IL-6 knockout (MKO) mice. Exon 2 of the IL-6 gene is flanked by LoxP sites on either side of Exon 2 (Floxed). Exon 1 and 2 is therefore present in both wildtype (WT) and Floxed mice and loxP sites in the Floxed mice. Additional presence of the myogenin Cre recombinase will result in recombination at the loxP sites resulting in deletion in Exon 2 in skeletal muscle cells giving rise to IL-6 MKO mice.

The mice for study I and II were obtained by crossing parent mice both homozygous for the loxP inserts and one parent heterozygous for the myogenin (myo)-Cre. This resulted in all mice being homozygous for the loxP insert and approximately half carrying the MyoCre and expected to be IL-6 MKO. The mice were at the age of 3-4 weeks genotyped for the presence of the LoxP site and the myoCre on DNA isolated from tail or ear samples using PCR (Method described on page 36). However, due to the MyoCre being inefficient, not all mice carrying the myoCre became IL-6 MKO. Therefore, additional genotyping was performed on DNA isolated from quadriceps muscle tissue after euthanization of the mice (Method described on Page 36, as shown in Figure 8). When recombination had taken place this was exclusively in skeletal muscle as also recently verified in another study using the same mouse strain (Knudsen et al., 2015).
**Muscle-specific PGC-1α knockout mice**

![Schematic illustration showing the generation of the skeletal muscle-specific PGC-1α knockout (MKO) mice. Exon 3 and 5 of the PGC-1α gene is flanked by a LoxP site (Floxed). All exons are therefore present in both wildtype (WT) and Floxed mice and loxP sites in the Floxed mice. Additional presence of the myogenin Cre recombinance will result in recombination at the loxP sites resulting in deletion of Exon 3,4 and 5 in skeletal muscle cells giving rise to PGC-1α MKO mice.](image)

**Figur 8.** Schematic illustration showing the generation of the skeletal muscle-specific PGC-1α knockout (MKO) mice. Exon 3 and 5 of the PGC-1α gene is flanked by a LoxP site (Floxed). All exons are therefore present in both wildtype (WT) and Floxed mice and loxP sites in the Floxed mice. Additional presence of the myogenin Cre recombinase will result in recombination at the loxP sites resulting in deletion of Exon 3,4 and 5 in skeletal muscle cells giving rise to PGC-1α MKO mice.

The original generation of the PGC-1α muscle-specific knockouts (MKO) mouse strain exploited the construct for the whole body PGC-1α knockout strain, in which a targeting plasmid was inserted flanking exons 3 to 5 of the PGC-1α gene with two loxP sites, with the exception of the neomycin-cassette being absent in the PGC-1α MKO mice (Lin et al., 2004b). The exons, 3, 4 and 5, encode a highly conserved region in PGC-1α, including the functionally essential LXXLL motif that facilitates its interaction with nuclear receptors (Lin et al., 2004b; Nolte et al., 1998). Mice for study III were obtained by crossing homozygous floxed PGC-1α<sup>lox/lox</sup> mice with mice heterozygous for cre recombinase under the control of the myogenin promoter generating PGC-1α<sup>lox/lox, myo-cre+/-</sup> MKO mice. This resulted in all mice being homozygous for the loxP insert and approximately half carrying the MyoCre and expected to be PGC-1α MKO. The mice were at the age of 3-4 weeks genotyped for the presence of the LoxP site and the myoCre on DNA isolated from tail or ear samples using PCR (Method described on page 37). As for the IL-6 MKO above, the additional genotyping was performed by PGC-1α mRNA determination on quadriceps muscle tissue after euthanization of the mice to verify that PGC-1α was knocked out in skeletal muscle (Method described on Page 40).
Mouse acute exercise protocol
In study I male IL-6 MKO and littermate floxed mice (n=10) were adapted to treadmill running (303401 series, TSE Systems, Bad Hamburg, Germany) starting 6 days before experimental procedure; 2x10 minutes a day using an incremental program from 9m/min to 14m/min at a 10° incline for 5 consecutive days with a day of rest prior to the day of the experimental intervention. The acute exercise protocol consisted of a single running bout at a constant speed of 14m/min and 10° incline initiated at 8AM with mice being euthanized at 10min, 1h, or 2h of running. Rested mice served as controls and were euthanized at the same time of day as the running mice to avoid confounding effects from metabolic circadian rhythm. Quadriceps muscle were quickly obtained and frozen in liquid nitrogen and trunk blood was collected and plasma obtained by centrifugation.

Mouse fasting protocols
Male IL-6 MKO and female PGC-1a with littermate Floxed control mice (n=11) were at the age of 3 month used in study II and III, respectively. The mice were individually housed for 3 days and allocated to either a fed group (FED), which maintained ad libitum access to both food and water or a fasted group (FAST) with access to only water for the duration of the given fasting period. In study II the food was removed at 2am and mice were fasted for either 6 or 18 hours and in study III the food was removed at 6am and the mice were fasted for 24 hours. To account for variations in circadian metabolism FED mice were sacrificed at the same time points as FAST mice to serve as controls. FED and FAST mice were euthanized by cervical dislocation at the end of the fasting interventions. Quadriceps muscles were quickly removed and snap frozen in liquid nitrogen. Trunk blood was collected and plasma obtained by centrifugation.

Metabolic chambers
Mice in study II (n=11) and III (n=8-10) were subjected to single housing in climate controlled metabolic chambers (Phenomaster, TSE Systems, Bad Homburg, Germany) with a constant setting of 22°C, 30% humidity and a 12:12 light-dark cycle for determination of activity level and indirect calorimetry measurements. The mice were acclimatized to the chambers for minimum 3 days followed by data collection (activity level, respiratory exchange ratio (RER) for 24h with ad libitum access to food and water followed by 18h and 24h of fasting in study II and III, respectively.
**Metabolic treadmill cage protocol**

In study I the respiratory exchange ratio (RER) was determined during running (n=5-7) on metabolic treadmills (Calosys, TSE systems, Bad Homburg, Germany) connected to a Phenomaster unit (TSE Systems, Bad Homburg, Germany) at 14 m/min with a 10° incline for 120 min. On the day of the main experiment, mice were prompted to run at 14 m/min with a 10° incline (n=10) for 10 min, 60 min, or 120 min between 8:00 and 10:30 AM before being euthanized by cervical dislocation. Quadriceps muscles were swiftly removed and snap frozen in liquid nitrogen. Trunk blood was obtained in EDTA containing tubes and plasma was obtained after centrifugation. Both muscle and plasma were stored at -80°C.

**Human experiments**

**Human subjects**

Nineteen healthy male subjects between the ages of 20-35 years were recruited for study IV. Based on training status, determined by an incremental maximal oxygen uptake (VO2max) test as inclusion criteria performed on a magnetized cycle ergometer, they were divided into 2 groups with trained individuals having a VO2max above 55 ml · min⁻¹ kg⁻¹, and untrained individuals a VO2max below 45 ml · min⁻¹ kg⁻¹. Ethical approval was obtained and subjects were informed about the experimental protocol as well as risks and discomforts that might occur in association with the intervention.

**VO2max protocol**

Human subjects were tested for determination of the maximal oxygen uptake (VO2max). VO2max was determined by an incremental bicycle test. For untrained individuals, the test started with 5 minutes at 85 watts followed by 5 minutes at 140 watts to attain steady state and a subsequent increase of 20 watts each minute until exhaustion. For trained individuals the test started with 5 minutes at 125 watts followed by 5 minutes at 200 watts and a subsequent increase of 25 watts each minute until exhaustion. If subjects fell within the thresholds of the inclusion criteria, incremental peak power output (IPPO) was calculated based on their completed VO2max test.
**Human acute exercise protocol**

Nine untrained (UT) and ten trained (T) young male subjects performed an incremental exercise bout on a magnetically braked cycle ergometer: 40 min at 50% VO$_2$max, 10 min at 65% VO$_2$max, and finally 80% VO$_2$max until exhaustion. Muscle biopsies were taken from m. vastus lateralis prior to exercise, after 30 min, 41 min, and at exhaustion. RER was measured in 5 min intervals leading up to each biopsy. Blood samples were obtained regularly throughout the exercise bout.

**Figure 1:** Schematic of the human exercise protocol in study IV.

**Muscle biopsies**

In study IV muscle biopsies were acquired from the vastus lateralis muscle. Prior to the muscle biopsies procedure, the incision area was wiped with chlorhexidine and anesthetized (lidocain) and a small pre-incision was made using a surgical blade. Muscle biopsies were obtained using the percutaneous needle biopsy technique (Bergstrom, 1975) modified with suction (Evans *et al.*, 1982). A part of the biopsy was directly frozen in liquid nitrogen, while the remaining quickly was dissected free from connective tissue and visual blood if necessary, frozen in liquid nitrogen and stored at -80°C.
Analyses

Genotyping
Genomic DNA was isolated using an Extracta kit (Quanta Biosciences, MA, USA) to confirm presence of Cre recombinase at the muscle specific myogenin promotor as well as presence of loxP sites flanking exon 2, in the case of IL-6 MKO mouse strain, and exons 3 to 5, in the case of the PGC-1α mouse strain. Extraction buffer was added to ear punches and placed on a heating plate for 30 minutes at 95°C, then cooled to room temperature and mixed with stabilization buffer. DNA was amplified and run on a 3% agarose gel. Additional confirmation of Cre-recombination was made on quadriceps muscle samples post intervention. In the case of IL-6 MKO and floxed control mice 10 mg of muscle was treated with digestion buffer and proteinase K (Sigma-Aldrich, MO, USA) overnight at 50°C and proteins precipitated with a Phenol:chloroform:isoamyl-OH solution. DNA was precipitated, amplified with primers flanking exon 2 of the IL-6 gene, and run on a 3% agarose gel (see figure 7).

Figure 9. Representative PCR gel of products generated with primers surrounding exon 2 of the IL-6 gene in quadriceps muscle from floxed and IL-6 skeletal muscle-specific knockout mice. A reduction in band size from 1,000 bp to 260 bp in is equal to the loss of exon 2 of the IL-6 gene. WT Ctrl: Wild-type control, Flox Ctrl: Floxed control, MKO Control: Skeletal muscle-specific knockout Control.

Table 1. Primer sequences for mouse genotyping.

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<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-Cre</td>
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<td>5’ GTGAAACGACTTGCTGACTTT 3’</td>
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<tr>
<td>IL-6 LOX</td>
<td>5’CCCACCAAGAGATGTCA 3’</td>
<td>5’ GGTATCCCTGTGAAGTCCCTC 3’</td>
</tr>
<tr>
<td>IL-6 delta</td>
<td>5’CCCACCAAGAGATGTCA 3’</td>
<td>5’ ATGCCAGCCTAATCTAGGT 3’</td>
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<tr>
<td>PGC-1α LOX</td>
<td>5’ TCCAGTAGGCGAGATTATGAC 3’</td>
<td>5’ TGTCTGTTTGTGACAATCTGCTAGGT 3’</td>
</tr>
</tbody>
</table>

Myogenin Cre recombinase (Myo-Cre), Interleukin (IL); Peroxisome proliferator activated receptor Y coactivator (PGC), loxP insert (LOX)
**Freeze drying**

In study IV frozen muscle pieces for PDHa activity and mRNA measurements were cut off while 70-80 mg of the remaining tissue was freeze-dried. The samples were placed in -25°C and 0.1 bar pressure for at least 48 hours. Afterwards the samples were moved to room temperature for at least 1 hour at 0.1 bars to prepare for dissection. Prior to the dissection, a water calibration curve was made to normalize the exact dry weight of the samples. A random sample was weighed every tenth second from 10th – 120th second and 1 hour after depressurization. The dissected muscle samples were weighed out in tubes for western blotting analyses, glycogen content, citrate synthase enzyme activity, and perchloric acid (PCA) extract.

**Muscle glycogen, G-6-P, and lactate**

In studies I, II and III muscle glycogen content was determined from 10-15mg wet weight, and in study IV from ~0.8-1.5 mg dry weight quadriceps muscle as previously described (Passonneau & Lauderdale, 1974). The tissue was boiled for 2 hours in 1 M HCl, hydrolyzing the glycogen into glycosyl units. The samples were quick spun and supernatants were transferred to new tubes. The samples were then added to white microtiter plates along with a reaction mix containing Tris buffer, H2O, ATP, MgCl, DTT, and NADP+ and G-6-PD. After addition of Hexokinase the samples were measured spectrofluorometrically.

\[
\text{Glycogen} \rightarrow \text{boiling for 2 hours} \rightarrow \text{Glucose}
\]

\[
\text{G-6-P + NADP}^+ \rightarrow \text{G-6-PDH} \rightarrow 6PG + \text{NADPH} + \text{H}^+
\]

\[
\text{Glucose} + \text{ATP} \rightarrow \text{HK} \rightarrow \text{G-6-P} + \text{ADP}
\]

A standard row was loaded together with the samples on the plate to calculate final concentrations. In studies I, II and III, muscle glucose 6 phosphate (G-6-P) content was determined from 10-15mg wet weight, and in study IV from ~1-1.2 mg dry weight quadriceps muscle as previously described (Bergmeyer & Moelling, 1965) after extraction with 0.6 M PCA for 30 minutes at 4°C. G-6-P concentrations in the muscle samples were
determined similarly to above description in the glycogen assay. Muscle lactate was measured in study I from the same wet weight PCA extract, and in study IV from the same dry weight PCA extract in a reaction mix containing glycy1-glycine, H2O, NAD+ and Glutamic acid (Lowry & Passonneau, 1972).

\[
\text{L-Lactat} + \text{NAD}^+ \rightarrow \text{LDH} \rightarrow \text{Pyruvat} + \text{NADH} + \text{H}^+
\]

\[
\text{Pyruvat} + \text{Lglutamat} \rightarrow \text{GPT} \rightarrow \text{Lalanine} + \alpha\text{-Oxoglutarate}
\]

**Muscle creatine**

Creatine content was determined in the homogenates used for determining PDHa activity to account for the fraction of pure muscle tissue in the individual samples that could then be normalized to the PDHa activity. The creatine extraction process used incubation with 0.6 M PCA for 1 hour at room temperature, neutralization with 1M KHCO3 and centrifugation. The creatine content of the supernatants was added to a reaction mix containing Hepes, MgCl2, phosphoenol pyruvate, NADH, pentaphosphate pentasodium, ATP, pyruvate kinase (PK) and lactate dehydrogenase (LDH) and determined spectrofluorometrically.

\[
\text{ADP} + \text{P-Pyruvate} \rightarrow \text{PK} \rightarrow \text{ATP} + \text{Pyruvate}
\]

\[
\text{Pyruvate} + \text{NADH} + \text{H} \rightarrow \text{LDH} \rightarrow \text{Lactate} + \text{ADP}
\]

\[
\text{Creatine} + \text{ATP} \rightarrow \text{CK} \rightarrow \text{P-Creatine} + \text{ADP}
\]
RNA, RT and PCR

Total RNA for mRNA analyses was isolated from ~20-25 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 oscillations per second in a TissueLyser II (Qiagen, Valencia, CA, USA) followed by addition of 700μl of DEPC-saturated phenol, 70 μl of 2M NaOAc, and 175 μl of chloroform:isoamyl-OH (49:1) and vigorous shaking for 15 seconds. The samples were incubated on ice for 15 minutes and subsequent centrifuged 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 tetra-acetate (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 were above 1.8.

Reverse transcription (RT) of total RNA into cDNA was performed using Superscript II RNase and Oligo dT (Invitrogen, Carlsbad, CA, USA). The same amount of total RNA was transferred to 0.5 ml 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 were placed on ice and, after quick spinning condensation down, a mixture of first strand buffer, DTT and dNTP was added. The Superscript enzyme was added and samples were left at 42 °C for 50 minutes for the first strand reaction to occur followed by heat-inactivated for 15 minutes at 70 °C. 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, 95 °C for 10 minutes, followed by 40 or 50 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. 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 in each study. The target mRNA content was normalized to βb-actin mRNA content in Study I and to single stranded (SS) DNA determined by Oligreen reagent (Molecular Probe/Thermo Scientifics) as previously described in Study II.
Table 2. Primer and TaqMan probe sequences used in real time PCR.

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<th>Primer (5’-3’)</th>
<th>TaqMan probe (5’-3’)</th>
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<td></td>
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<td>5’ TTCCTCCTCGGTTCGATTTCTCAT 3’</td>
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<td>IL-6</td>
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<td>5’ GCCGGAGAAGTTGATGAC 3’</td>
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<td>5’ GCCACTGGAGCCTCCAGAGAAA 3’</td>
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Peroxisome proliferator activated receptor γ coactivator (PGC), Interleukin (IL), pyruvate dehydrogenase kinase (PDK), pyruvate dehydrogenase phosphatase (PDP), sirtuin (SIRT) and suppressor of cytokine signalling (SOCS).

SDS-page and Immunoblotting

In study I, II, and III muscle lysate was made from ~25-30 mg crushed mouse quadriceps muscle, and in study IV from ~10-15 mg freeze-dried human muscle biopsies (dissected free of connective tissue, blood and visible fat). The tissue was homogenized in a ratio of 1:20 for wet weight (WW) and 1:80 for dry weight (DW) samples in lysis buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 20 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM Na3VO4, 3 mM benzamidine, and deacetylase inhibitors (nicotinamide (1mM) and sodium butyrate (5mM), pH 7.5) using a Tissue Lyser II (Qiagen, Germany). Protein concentration in each of the samples was determined using the bicinchoninic acid method (Thermo Fischer Scientific, USA) and protein concentration was adjusted with sample buffer to a concentration of 1µg/µl. Phosphorylation and protein content were determined by SDS-PAGE using hand casted gels and western blotting. PVDF membranes were incubated in primary antibody overnight at 4°C. Species-specific horseradish peroxidase conjugated immunoglobulin secondary antibodies (DAKO, Denmark) were used for incubation the following day. ACC2 protein was detected using streptavidin (Dako, Glostrup, Denmark). Protein bands were subsequently visualized using an ImageQuant LAS 4000 imaging system and quantified with ImageQuant TL 8.1 software (GE Healthcare, Freiburg, Germany).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer/lot number</th>
<th>MW (kDa)</th>
<th>Blocking solution</th>
<th>Primary antibody [C] in 5% BSA</th>
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<td>3% FG</td>
<td>1:1.000</td>
<td>1:2.000 anti-rabbit</td>
</tr>
<tr>
<td>PDK2</td>
<td>Calbiochem/ST1643</td>
<td>43</td>
<td>3% FG</td>
<td>1:1.000</td>
<td>1:3.000 anti-mouse</td>
</tr>
<tr>
<td>PDK4 (mouse)</td>
<td>Hardie</td>
<td>46</td>
<td>3% FG</td>
<td>1:4.000</td>
<td>1:10.000 anti-sheep</td>
</tr>
<tr>
<td>PDK4 (human)</td>
<td>Boster/AA-91-125</td>
<td>43</td>
<td>3% FG</td>
<td>1:1.000</td>
<td>1:3.000 anti-rabbit</td>
</tr>
<tr>
<td>PDP1</td>
<td>Sigma/SAB4301009</td>
<td>61</td>
<td>3% FG</td>
<td>1:1.000</td>
<td>1:3.000 anti-rabbit</td>
</tr>
<tr>
<td>SIRT3</td>
<td>CS/#5490</td>
<td>28</td>
<td>3% FG</td>
<td>1:1.000</td>
<td>1:3.000 anti-rabbit</td>
</tr>
<tr>
<td>STAT3</td>
<td>CS/#9139S</td>
<td>79, 86</td>
<td>3% FG</td>
<td>1:1.000</td>
<td>1:3.000 anti-mouse</td>
</tr>
<tr>
<td>STAT3 Tyr705</td>
<td>CS/#9145</td>
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<td>1:2.000 anti-rabbit</td>
</tr>
<tr>
<td>TBC1D4</td>
<td>Abcam/ab24469</td>
<td>160</td>
<td>3% FG</td>
<td>1:20.000</td>
<td>1:2.000 anti-rabbit</td>
</tr>
</tbody>
</table>

Table 3. List of antibodies used in studies I-IV. AMP-activated protein kinase (AMPK), Acetyl-CoA carboxylase (ACC), hexokinase (HK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38), pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase (PDK), pyruvate dehydrogenase phosphatase (PDP), sirtuin (SIRT), signal transducer and activator of transcription factor (STAT), TBC1 domain family member 4 (TBC1D4).
**Immunoprecipitation**

A total of 200 µg protein from lysate was immuno-precipitated for the determination of PDH-E1α acetylation. Briefly, the lysate was added to PBS-rinsed protein G agarose beads (EMD Millipore, Bedford, USA) in a 50:50 solution with PBS buffer containing 0.5% Triton X with 2µg of PDH-E1α antibody. The samples were rotated end over end at 4°C overnight and on the subsequent day the beads were washed, sample buffer was added and the samples were heated at 96°C for 3 minutes. The beads were spun down to avoid transfer and lysate loaded on a hand-casted gel for SDS-page and western blotting as described above. For each sample acetylated protein was normalized to the amount of precipitated PDH-E1α protein content determined by western blotting.

**Enzyme activities**

**PDHa activity**

The activity of PDH in the active form (PDHa) is determined indirectly measuring 14C-citrate based on the rate of acetyl-CoA formation from a known amount of added pyruvate using a modified radioisotopic assay (Cederblad *et al.*, 1990; Constantin-Teodosiu *et al.*, 1991). Approx. 8-15 mg of frozen wet weight muscle sample was added to ice-cold homogenizing buffer containing sucrose, KCl, MgCl2, EGTA, Tris HCl, NaF, DCA, and Triton X-100 in a final volume of 30 times the weight of the muscle sample and homogenized in a quartz glass tube (Sigma, USA) kept on ice for ~50 sec using a motor-driven homogenizer attached to a glass pestle and immediately snap frozen in liquid nitrogen. This process is assessed to be the gentlest method for homogenizing tissue without compromising the integrity mitochondria within the sample. Muscle homogenate was transferred to an assay buffer solution containing Tris-base, EDTA and MgCl2 and kept at 37°C. Pyruvate was added, and after 45 s, 90 s and 135 s the reaction was halted by transferring part of the sample to a new tube containing PCA. Samples were run in duplicates along with blanks made with H2O instead of pyruvate. Samples were then neutralized after 5 min incubation by addition of 1M K2CO3 and the samples were centrifuged at 10000 g for 3 min. The following determination of acetyl-CoA was then attained by the reaction principles described in figure 4. The supernatant containing acetyl-CoA were incubated in a reaction mix containing DTT, H2O, and CuSO4 for 30 minutes at room temperature before EDTA was added to inhibit
endogenous processes. NEM was then added to bind later formed citrate and drive the equilibrium to the far right, preventing product inhibition of the reaction.

\[
\text{\textsuperscript{14}C-aspartate} + 2\text{-oxoglutarate} \xrightarrow{\text{GOT}} \text{glutamate} + \text{\textsuperscript{14}C-oxaloacetate}
\]

\[
\text{Acetyl-CoA} + \text{\textsuperscript{14}C-oxaloacetate} \xrightarrow{\text{CS}} \text{\textsuperscript{14}C-citrate} + \text{NEM}
\]

\[
\text{\textsuperscript{14}C-aspartate} + \text{Dowex}
\]

**Figure 2:** Flowchart describing the chemical reaction principles of the PDHa activity protocol. Acetyl CoA determination part of the PDH activity assay. Non-commercially available \textsuperscript{14}C-oxaloacetate was formed by transamination of \textsuperscript{14}C-aspartate and 2-oxoglutarate catalyzed by glutamic oxaloacetic transaminase (GOT) and added to the samples along with CS enzyme to form \textsuperscript{14}C-citrate. Positively charged excess \textsuperscript{14}C-aspartate was removed from the aqueous solution by addition of Dowex cation exchange resin leaving only \textsuperscript{14}C-citrate. N-ethylmaleimide (NEM) catalyzes the formation of citrate and drags the equilibrium far to the right.

Non-commercially available \textsuperscript{14}C-oxaloacetate was formed by transamination of \textsuperscript{14}C-aspartate and 2-oxoglutarate catalyzed by glutamic oxaloacetic transaminase (GOT) and added to the samples along with CS enzyme to form \textsuperscript{14}C-citrate. Positively charged excess \textsuperscript{14}C-aspartate was removed from the aqueous solution by addition of Dowex cation exchange resin leaving only \textsuperscript{14}C-citrate. Supernatants were transferred into scintillation vials and mixed with Ultima Gold scintillation fluid (Perkin Elmer, Skovlunde, Denmark) and \textsuperscript{14}C-citrate \(\beta\)-radiation was measured in a scintillation counter (Tri-Carb, Perkin Elmer) and compared with an acetyl-CoA standard curve run in parallel with samples in the assay. Each sample was normalized to the total creatine content in the sample, to adjust for the presence of non-muscle tissue in the samples (St Amand et al. 2000). In study I and IV acetyl CoA measurements were made from a subset of neutralized PCA treated muscle extracts prepared as previously described. Optimized amounts of extract and neutralized PCA standard curves
were run in parallel with muscle samples through the PDHa activity assay for determination of acetyl-CoA content as described by Cederblad et al. (Cederblad et al., 1990).

**CS activity**

Citrate synthase (CS) catalyzes the reaction between acetyl-CoA and oxaloacetate leading to formation of citrate in the TCA cycle. CS activity was determined in study IV (Lowry & Passonneau, 1972). Hydrolysis of the thioester of acetyl-CoA results in formation of CoA-SH. The thiol group reacts with DTNB in the reaction mix to form NTB, which can be determined colorimetrically at 460 nm at baseline and after addition of oxaloacetate. The absorbance was measured every 10 second over 90 second giving rise to a rate of NTB formation reflecting the CS activity. CS activity was normalized to protein content measured from sample homogenate.

**HAD activity**

3-hydroxyacyl-CoA dehydrogenase (HAD) catalyzes the oxidation of $\beta$-hydroxyacyl-CoA and NAD+ to form NADH, H+ and $\beta$-ketoacyl-CoA in the fatty acid oxidation pathway. In study I, HAD activity was kinetically determined at 460 nm (Lowry & Passonneau, 1972). After addition of acetoacetyl-CoA, the delta emission was converted to activity and HAD activity was normalized to protein content measured in the respective samples.

**Plasma glucose and lactate**

In study I plasma lactate was measured fluorometrically on plasma samples using the same principle as described for muscle lactate. In studies I, II, and III plasma glucose was measured fluorometrically on plasma samples using the same principle as described for glycogen. In study IV plasma glucose and lactate was determined in venous arm blood using an ABL800 FLEX (Radiometer, Denmark).

**Plasma IL-6**

Plasma IL-6 was determined in studies I and II using MSD single and multiplex immunoassay kits (Meso Scale Discovery, Rockville, MD, USA). Spots in each well of the kits were coated with IL-6 capture antibody. Samples were added to each well and washed in sulfo-tagged labeled antibody. The samples were then read in a MESO Quickplex SQ 120 (Meso Scale Discovery), which by applying voltage to the plate electrodes excited the
captured sulfo-tags thus emitting light. The emission intensity could then be related to a standard curve loaded along with the samples to quantify IL-6 amounts present in the plasma.

**Plasma NEFA**

In all studies Plasma NEFA was determined using a Wako NEFA HR (2) kit with microtiter procedure protocol. The primary reagent of the kit converts non-esterified fatty acids to acetyl-CoA catalyzed by acetyl-CoA synthase (ACS).

\[
RCOOH + ATP + CoA \rightleftharpoons ACS \rightleftharpoons \text{acetyl-CoA} + \text{AMP} + P\\text{Pi}
\]

The secondary reagent oxidizes acetyl-CoA catalyzed by acetyl-CoA oxidase (ACOD) yielding hydrogen peroxide (H\(_2\)O\(_2\)) as a byproduct.

\[
\text{Acetyl-CoA} + O_2 \rightleftharpoons ACOD \rightleftharpoons 2,3\text{-trans-enoyl-CoA} + H_2O_2
\]

The H\(_2\)O\(_2\) formed drives the oxidative condensation of 3-methyl-N-ethyl-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple pigment colorimetrically measurable at 550nm and related to a supplied standard curve run in parallel on each plate to determine NEFA plasma concentrations.

**Statistics**

In all studies the values were presented as means ± standard error. In studies I, II and III A two-way ANOVA was applied to test the effects of genotype and/or intervention. In study II, isolated two way ANOVA’S were performed separately on 6 hour and 18 hour interventions as well as between 6 hour and 18 hour fed groups to obtain detailed analyses on potential effects of fasting and genotype. When a main effect was detected, a Student-Newman-Keuls test was applied as a post-hoc test to locate differences. For single grouped data, a
student’s t-test was used to test if a difference was present. When a main effect was detected, a Student-Newman-Keuls test was applied as a post-hoc test to locate differences. In study IV, a two-way ANOVA with repeated measures was applied to test the effects of training status and time on parameters within the acute exercise bout. When a main effect was observed, a Student-Newman-Keuls post hoc test was used to locate differences between untrained and trained subjects. A student paired t-test was used to test basal differences between groups in pre exercise biopsies. Significance was accepted at P values less than 0.05 and a tendency was reported for 0.05≤P≤0.1. The statistical tests were performed using Sigmaplot 13.0 (Systat, USA).
Results and discussion

In the following chapter the findings obtained in studies I-IV will be discussed. Moreover, unpublished data and experiments that have been performed during the course of this PHD will be incorporated into the discussion to elaborate on the results from the four manuscripts.

Effects of muscle IL-6 on exercise-induced PDH regulation

Muscle IL-6 and exercise-induced PDH regulation in mice

Previous findings have reported no perturbations in glucose tolerance, insulin tolerance, or alterations in body weight, and as such no evidence of significantly altered metabolic phenotype with the loss of skeletal muscle IL-6 in the mouse strain used in study I and II (Knudsen et al., 2015). However, study I demonstrated that the lack of skeletal muscle IL-6 led to elevated PDHa activity both at rest and during exercise, and blunted exercise-induced changes in PDHa activity during prolonged exercise as observed in floxed control mice and in previous human studies (Mourtzakis et al., 2006; Odland et al., 1998; Watt et al., 2004). This was supported by the novel observation that IL-6 MKO mice had moderately higher RER during exercise than controls. Although the findings could not directly be attributed to differences in covalent regulation, it is indicative of the fact that muscle IL-6 influences substrate utilization in skeletal muscle through effects on PDH. However the finding that IL-6 MKO mice preserved the ability to reduce RER during prolonged exercise reflects a maintained metabolic flexibility during prolonged exercise showing that muscle IL-6 does not seem to be required for the shift in substrate use during prolonged exercise. In addition, the observation indicates that flux through PDH may not always be reflected by PDHa activity measured in vitro, as previously stated by others (Constantin-Teodosiu et al., 2004). The observed effect of muscle-specific IL-6 knockout on PDHa activity, in both study I and II, suggests that muscle IL-6 normally exerts an inhibitory effect on skeletal muscle PDH with a concomitant reduction in carbohydrate oxidation. Although differences in PDHa activity do not necessarily relate to flux and may therefore not be linked to the difference in RER between IL-6 MKO and control mice, the observed higher RER in IL-6 MKO mice than controls during the exercise bout does support that IL-6 reduces carbohydrate oxidation. Furthermore, the findings in study I, that exercise-induced AMPK activation was similar in 3 months old IL-6
MKO and floxed control mice does not support the suggestion that IL-6 mediates its effects on PDH through AMPK (Bienso et al., 2014b) (Kelly et al., 2004). It may be speculated that the relatively high PDHa activity observed in IL-6 MKO mice in study I and II were near full activation already at rest and that a change therefore was not detected during exercise in study I. As such it cannot be excluded that the measured levels were close to maximal PDH activation for the specific IL-6 strain used in study I. However, previous observations of higher levels of PDHa activity in WT and PGC-1α whole body KO mice than the IL-6 MKO and controls in Study I, when running at similar speed and treadmill slope (figure 9), suggest that the maximal level was not obtained in the IL-6 MKO mice in Study I. In addition, high intensity exercise in humans can elicit higher PDHa activity level in skeletal muscle as shown in study IV, and as previously reported (Kiilerich et al., 2010b).

![Figure 10](image)

**Figure 10:** PDHa activity measured in quadriceps of PGC-1α KO and WT mice after performing a single 1-h bout at 14 m/min and a 10˚ incline. Values presented as means ± SE (n=8), (unpublished data).

To further investigate the effects of IL-6 on PDH regulation and skeletal muscle metabolism, groups of control and IL-6 MKO mice were exposed to 2 hours of treadmill running as described in study I, and euthanized 6 and 10 hours into recovery along with resting mice serving as controls. The data from these mice further support the findings of study I, that lack of IL-6 affects the regulation of PDH not only at rest, and during exercise, but also into recovery from prolonged exercise (figure 10).
Figure 11. PDHa activity measured in quadriceps from floxed Control and IL-6 MKO mice, non-exercised (Rest), 6 hours (6h), and 10 hours (10h) after 2 hours of treadmill exercise at ~14 m/min at a slope of 10°. Values presented as means ± SE. (n=9-10), (unpublished data).

Muscle IL-6 and exercise-induced PDH phosphorylation and acetylation in mice
The similar resting and exercise-induced changes in PDHSer300 and PDHSer232 and similar lack of change in site PDHSer293 and PDHSer295 phosphorylation in IL-6 MKO and control mice indicate that diverse regulation of PDH phosphorylation is not the main underlying mechanism for the observed genotype differences in PDHa activity in study I. In addition, the lack of difference in PDK1, PDK2, PDK4 and PDP1 protein during exercise in either genotype suggests that protein content does not account for the observed PDH phosphorylation response. The observation that total lysine acetylation of immunoprecipitated PDH-E1α was similar in IL-6 MKO and control mice in Study I does not support the possibility that acetylation is regulated by skeletal muscle IL-6.

Muscle IL-6 and exercise-induced plasma IL-6 response in mice
The finding that plasma IL-6 concentrations were significantly elevated after 2 hours of running in control mice is in accordance with previous murine studies (Castellani et al., 2015; Colbert et al., 2001; Fritsche et al., 2010). Of notice is however that plasma IL-6 concentrations in the control mice reached rather modest levels of ~15 pg/ml indicating that the loxP inserts in the floxed control mice possibly affect either protein expression or IL-6 from skeletal muscle cells. Hence, other studies have reported increases in circulating plasma IL-6 to levels between 40-70 pg/ml with treadmill exercise (Castellani et al., 2015; O’Neill et al., 2013), although the use of
different commercially available kits may contribute to this difference. In addition, based on the confirmation of IL-6 being knocked out at the level of DNA in study I, it can be concluded that the exercise-induced increase in circulating IL-6 in the IL-6 MKO mice did not originate from skeletal muscle fibers. Another source of circulating IL-6 may be macrophages as part of an exercise-induced inflammatory response (Pedersen & Febbraio, 2008b) or as previously suggested the liver and/or subcutaneous adipose tissue (Mohamed-Ali et al., 1997; Reilly et al., 2015; Sopasakis et al., 2004). However, arterio-venous IL-6 measurements across the subcutaneous abdominal tissue bed may suggest that adipose tissue contributes to exercise-induced circulating IL-6 only into recovery from exercise (Lyngso et al., 2002) indicating that the potential release of IL-6 from adipose tissue in response to exercise still remains to be elucidated.

**Muscle IL-6 and exercise-induced PDH regulation in humans**

An important question is whether the findings in mice in study I, that IL-6 suppresses PDHa activity, are transferrable to humans. Previous studies have demonstrated that PDHa activity in human skeletal muscle during prolonged exercise follows a pattern with an initial increase followed by a decline towards resting level after approximately 2 hours of exercise (Mourtzakis et al., 2006; Pilegaard et al., 2006; Watt et al., 2004). Other human studies have shown that plasma IL-6 increases gradually during prolonged exercise of moderate intensity (Keller et al., 2001). Together this does support the possibility that an exercise-induced increase in circulating IL-6 contributes to downregulation of PDHa activity in human skeletal muscle during prolonged exercise as suggested from Study I, although the role of the change in PDH activity over time on the switch in substrate utilization remains to be clarified.

Human studies have also indicated that the training state influences the exercise-induced IL-6 response (Fischer et al., 2004; Ronsen et al., 2001) Hence, two human studies have reported an attenuation of the exercise-induced increase in plasma IL-6 with exercise training (Fischer et al., 2004; Ronsen et al., 2001). However, in contrast, study IV did not support this notion as plasma IL-6 was only different between trained and untrained at rest with lower level in trained than untrained, at rather minute levels, ~0.1 pg/ml,. However, it must be added, that the trained subjects exercised for longer to reach exhaustion, even at the same relative exercise intensity, and the protocol used in study IV could mask a potential lower release in trained than untrained, possibly evident at the same absolute workload. Accordingly, the observation that contraction-induced p38 MAPK phosphorylation, suggested to mediate IL-6 release with muscle contractions (Chan et al., 2004), was similar between the two groups supports this notion. Furthermore, the observation that STAT3
phosphorylation, a marker of IL-signaling (Ernst & Jenkins, 2004), was distinctly higher in untrained than trained subjects at exhaustion in Study IV may indicate higher autocrine IL-6 signaling within untrained than trained muscle tissue. The observation that PDHa activity was higher in trained than untrained at exhaustion in Study IV therefore does support the possibility that muscle IL-6 inhibits PDHa activity in human skeletal muscle. Thus, it cannot be excluded that the dampening effects of IL-6 on PDH seen in mice in study I and II may also apply to human skeletal muscle and merits further studies.

Effects of muscle IL-6 on fasting-induced PDH regulation

Muscle IL-6 and fasting-induced PDH regulation in mice
during fasting skeletal muscle is a key target for attenuation of PDHa activity and carbohydrate oxidation and the major determinant of resting metabolic rate (Zurlo et al., 1990). The finding that lack of muscle IL-6 was associated with elevated RER in the fed and early fasting state in Study II reflecting higher carbohydrate oxidation in IL-6 MKO than Control mice is novel. The observation is however in accordance with findings in study I that RER was higher in IL-6 MKO than Control mice during exercise and previous findings showing that IL-6 KO mice exhibited higher RER both during rest and treadmill running (Faldt et al., 2004). This further indicates that skeletal muscle IL-6 elicits an inhibitory influence on carbohydrate oxidation, while increasing fat oxidation both at rest and during prolonged running as shown in Study I, and in accordance with previous findings (van et al., 2003).

Furthermore, the observation that skeletal muscle PDHa activity was higher in IL-6 MKO than control in the fed state in Study II is in accordance with study I and the observed RER results in Study II. This suggests that skeletal muscle IL-6 modulates substrate utilization at rest potentially through effects on skeletal muscle PDH. In addition, the observation that skeletal muscle PDHa activity was higher and PDH phosphorylation lower in IL-6 MKO than Control mice at 18h of fasting in Study II, is in part in accordance with the observations in the fed state in Study II, findings in study I as well as previous findings in mice (Bienso et al., 2014a). However, the impact of muscle IL-6 on PDHa activity in the fasted state seems to be overruled by other factors leading to similar substrate utilization despite an elevated PDHa activity in IL-6 MKO mice as determined from the RER results. It may be speculated that reduced availability of pyruvate as indicated by the reduced muscle glycogen and G-6-P at 18h of fasting relative to fed has contributed to this.
The finding that lack of muscle IL-6 did not impede the ability to switch substrate and down-regulate skeletal muscle PDH in response to fasting indicates that metabolic flexibility was maintained when skeletal muscle IL-6 was absent. The reduction in RER with fasting independently of muscle IL-6 in study II is supported by the observations that the changes in plasma FFA and glucose as well as reduction in muscle glucose and glycogen occurred similarly in IL-6 MKO and Control mice. However, this does not correlate with the previous finding that whole body IL-6 KO mice had a blunted fasting-induced increase in plasma FFA (Wueest et al., 2014a) possibly indicating that this effect may, as proposed in study I, have been mediated by IL-6 derived from several other tissues than skeletal muscle (Nybo et al., 2002; Reilly et al., 2015).

In addition, the finding that lack of skeletal muscle IL-6 only affected PDHa activity at 18h and not 6h conflicts with the findings in study I. Circadian-dependent fluctuations in PDHa activity and several metabolic proteins over the course of a day (Dyar et al., 2014) as observed in study II, as well as differentiated feeding patterns prior to fasting may have obscured important metabolic differences between control and IL-6 MKO mice.

**Muscle IL-6 and fasting-induced PDH phosphorylation and acetylation in mice**

In contrast to findings in study I, results in study II associate the genotype difference in PDHa activity with lowered PDH phosphorylation at PDHSer293 and PDHSer300 as well as lower levels of PDK4 protein in IL-6 MKO than Control mice providing a mechanistic basis for the IL-6 mediated regulation of PDHa activity in skeletal muscle.

**Muscle IL-6 and fasting-induced plasma IL-6 response**

The finding in study II that quadriceps IL-6 mRNA increased in control mice after both 6h and 18h of fasting is in accordance with a previous mouse study (Wueest et al., 2014a) and supports the notion that fasting induces skeletal muscle IL-6 expression with potential effects on metabolic regulation during fasting. Yet, the observation that plasma IL-6 in study II did not increase in response to either 6h or 18h of fasting is in stark contrast to the extremely high transient increase previously reported (Wueest et al., 2014b). However, this may be due to a reduced release of IL-6 specific to the mouse strain, as also speculated in study I, or a phasic IL-6 response that could conceivably rise and fall in between the measured time points in study II.

In order to investigate potential fasting-induced metabolic regulation in humans, untrained and trained human subjects fasted for 36h with blood samples every 12h. Plasma IL-6 showed only a subtle increase in untrained
individuals and no effects in trained subjects (figure 11), indicating that the fasting–induced regulation of PDH may be thoroughly independent of IL-6, as shown in mice in study II, although an autocrine effect may be present.

![Figure 12](image)

Figure 12. Plasma IL-6 in trained and untrained human subjects (as defined in study IV) during 36H of fasting. Values are given as means ± SE, (unpublished data).

**Impact of PGC-1α on fasting and exercise-induced PDH regulation**

**Muscle PGC-1α and fasting-induced PDH regulation in mice**

The finding in Study III that lack of muscle PGC-1α resulted in lower RER in the fed state was not as expected based on the lower oxidative capacity of the PGC-1α MKO mice than the controls (Geng et al., 2010; Leick et al., 2008; Lin et al., 2004a). The similar plasma glucose and NEFA concentrations as well as similar muscle glycogen in fed PGC-1α MKO and control mice in Study III indicates that the RER differences in the fed state are not due to differences in substrate availability between the genotypes. However the observed higher RER in PGC-1α MKO mice than controls in the fasted state suggest that reduced oxidative capacity first is reflected on resting
substrate utilization when metabolically challenged. In accordance, PGC-1α MKO mice has higher RER during treadmill running than Controls (unpublished results; will be discussed later). In addition, there was a larger absolute decrease in mean muscle glycogen in PGC-1α MKO than control mice with fasting indicating a higher dependence on CHO as fuel in the PGC-1α MKO than the control mice in accordance with the metabolic phenotype that lack of PGC-1α presents. Of notice is that skeletal muscle PGC-1α was dispensable for the switch from carbohydrate to fat utilization with PGC-1α MKO mice retaining short term metabolic flexibility during the transition from the fed to the fasted state in Study III in concurrence with previous findings (Finley et al., 2012). However, the increased CHO use in the absence of muscle PGC-1α, may compromise the ability of PGC-1α MKO mice to endure starvation beyond the duration of 24 hours.

Furthermore, the downregulation of PDHa activity with fasting in Study III is in line with the reduction of PDHa activity with fasting in the IL6 MKO strain in Study II. However, the fasting-induced reduction in PDHa activity in PGC-1α MKO mice is different from the previously observed blunted fasting-induced effect in PGC-1α KO (Kiilerich et al., 2010a). This may suggest that lack of PGC-1α in additional tissues contributed to the previous observations.

Muscle PGC-1α and fasting-induced PDH phosphorylation and acetylation in mice
In study III, fasting for 24 hours led to increases in phosphorylation of all four known sites in PDH-E1α, including PDHSer232 which is novel, as well as an increase in PDK4 protein as in agreement with other studies (Kiilerich et al., 2010a; Pilegaard et al., 2003a; Wu et al., 1999a; Wu et al., 2001). The fasting-induced increase in skeletal muscle PDH^Ser232 phosphorylation in study III is in accordance with the similar findings in study II. The observed increase in PDH-E1α protein with fasting in study III, in both genotypes, is surprising and indicates that PGC-1α is partly dispensable for fasting-induced increases in PDH-E1α protein.

In agreement with the findings in study III, previous studies have reported that SIRT3 protein increased with fasting in mice (Caton et al., 2011; Hirschey et al., 2010; Palacios et al., 2009) indicating an increased potential for mitochondrial acetylation. Furthermore, lack of muscle PGC-1α led to reduced PDH-E1α, PDK1, 2, 4, PDP1, and SIRT3 protein content as well as hyperacetylation of PDH-E1α in the fed state while, as a novel finding, it appears that knockout of muscle PGC-1α blunted the fasting-induced increase in SIRT3 protein. Furthermore, the higher lysine acetylation level of PDH-E1α in PGC-1α MKO mice than controls in the fed state is in agreement with a previous study using myocytes (Jing et al., 2013) and with the present genotype difference in SIRT3 protein content. The lack of a genotype difference in PDH-E1α acetylation in the fasted state indicates on the other hand, that PGC-1α deficient mouse skeletal muscle is capable of maintaining the PDH acetylation
state equal to control muscle despite reduced SIRT3 protein level. As muscle NAD$^+$ levels have been shown to increase with fasting (Canto et al., 2010b) this may upregulate SIRT3 activity (Schwer et al., 2002) sufficiently in PGC-1α MKO mice to obtain an acetylation state as in control mice.

**Impact of oxidative capacity on PDH regulation**

**Oxidative capacity and fasting-induced PDH regulation in humans**

![Graph](image)

**Figure 13** A) PDHa activity (n=5-7) and B) respiratory exchange ratio (RER) (n=7-8) in trained and untrained human subjects fasting for 36H. Subjects ingested a standardized meal and had biopsies taken at rest 2H, 12 H, 24H, and finally 36H after. RER was measured at rest for 15 min prior to the biopsies. Values are given as means ± SE, (unpublished data).

Fasting for 24hours may be considered a severe metabolic challenge in mice due to a much higher basic metabolic rate in mice than in humans. However, fasting for only 9 hours has been observed to be sufficient to elicit a significant rise in PDK4 and a reduction in PDHa activity in human skeletal muscle, similar to an overnight fast (unpublished results; Kiilerich et al). In contrast, another study has reported no significant decreases in PDHa activity after 15 hours fasting in humans (Spriet et al., 2004). Further investigation of the
fasting-induced PDH regulation in humans has been conducted in an on-going project investigating the impact of skeletal muscle oxidative capacity on the time course of fasting–induced PDH regulation in humans. The preliminary findings show that PDHa activity did not change significantly within the first 24 hours in either untrained or trained subjects (Figure 12A), which was supported by insignificant decreases in resting RER within the same time frame (Figure 12B). Fasting for 36h did however downregulated the PDHa activity in skeletal muscle similarly in untrained and trained. These preliminary findings suggest that fasting-induced PDH regulation in human skeletal muscle is independent of training status and hence skeletal muscle oxidative capacity.

**Oxidative capacity and exercise-induced PDH regulation in humans**

The main findings of study IV were that exercising at the same relative intensity at steady state and with a graded increase in intensity, elicited similar skeletal muscle PDH activation in untrained and trained subjects. The similar PDHa activity at the submaximal exercise intensities may indicate similar carbohydrate oxidation, although the trained subjects exercised at a higher absolute work load. In addition, the finding that RER was lower in trained than untrained in Study IV, reflecting a higher contribution from fat utilization in the trained than untrained at the same relative exercise intensity, is in line with numerous studies showing an increased ability to oxidize fat at a higher exercise intensity in trained than untrained (Holloszy, 1967;Kiens *et al.*, 1993). This ensures the additional ATP needed when the trained subjects exercise at the higher absolute work load than the untrained subjects. The observation in Study IV that plasma and muscle lactate accumulation was lower in trained than untrained subjects at the submaximal exercise intensities further supports that a larger fraction of the used glycogen was used for oxidation in the trained than the untrained in study IV.

Additionally trained subjects were able to reach higher levels of PDHa activity at exhaustion likewise underlining an increased capacity for carbohydrate oxidation in the trained state when the metabolic need arises as previously reported (Jansson & Kaijser, 1987;Perry *et al.*, 2008). This response was associated with higher PDH-E1a content, PDH phosphorylation and PDH acetylation as well as protein content of PDH regulators in accordance with previous studies (Consitt *et al.*, 2016;LeBlanc *et al.*, 2004b). Muscle Acetyl CoA concentration also was similar in trained and untrained subjects at 50% and 65% IPPO, but higher in trained than untrained at exhaustion indicating a flux through PDH closely correlated to PDHa activity (Parolin *et al.*, 1999b;Putman *et al.*, 1993).
Oxidative capacity and exercise-induced PDH regulation in mice

To further investigate the impact of different skeletal muscle oxidative capacity in the regulation of PDH, indirect calorimetry measurements were made on PGC-1α MKO and floxed control mice performing an incremental exercise bout on metabolic treadmills (unpublished data). The observation that PGC-1α MKO mice revealed a higher RER than floxed control mice throughout the exercise bout (figure 13), indicating

Figure 14: Respiratory exchange ratio measured in PGC-1α MKO and littermate floxed mice after performing an acute incremental bout of exercise at 14m/min, 17m/min and ending at 20 m/min at 10˚ incline. Values are presented as means of continuous 1 min measurements. (n=8-11)

higher glucose oxidation in the MKO mice is in accordance with previous findings showing that PGC-1α overexpression mice exhibited lower RER during treadmill running (Calvo et al., 2008; Wong et al., 2015) and the observations in the human subjects in Study IV. In addition, in accordance with the higher PDHα activity in trained than untrained at exhaustion in Study IV, the PGC-1α MKO mice had lower PDHα activity than control mice at the end of exercise (Figure 14), which appeared close to exhaustion for the PGC-1α MKO mice. Of notice is that the higher PDHα activity in trained than untrained subjects was obtained at a higher absolute work load and therefore most likely higher CHO utilization for the trained than the untrained subjects. The
lower PDHa activity in PGC-1α MKO mice than Controls was associated with higher RER also reflecting higher CHO utilization in the PGC-1α MKO mice. However, the mice exercised at the same absolute intensity, while the human subjects exercised at the same relative intensity, and the control mice were therefore not exhausted at the end of the exercise bout, where the PDHa activity was measured. Hence it is expected that the control mice would obtain a higher CHO utilization - and likely also higher PDHa activity if they were exercised to exhaustion.

**Figure 15:** PDHa activity in quadriceps of PGC-1α MKO and floxed littermate mice after performing an acute incremental bout of exercise at 14m/min, 17m/min and ending at 20 m/min at 10° incline. Values are presented as means ± SE (n=8), (unpublished data).

**Oxidative capacity and exercise-induced PDH phosphorylation and acetylation in humans**

The findings that exercise dephosphorylated PDHSer293 and PDHSer300 in human skeletal muscle is in accordance with several previous studies (Kiilerich *et al.*, 2010b;Mourtzakis *et al.*, 2006;Pilegaard *et al.*, 2006). However the observed exercise-induced dephosphorylation of PDHSer232 in human skeletal muscle apparently independent of exercise intensity is novel. Because PDHSer232 is thought to be regulated mainly by PDK1 (Korotchkina & Patel, 2001), the present observations support the possibility that PDK1 contribution plays a larger role in PDH regulation in human skeletal muscle than previously assumed. Taken together, these findings demonstrate site-specific regulation in response to exercise. Furthermore, the observations that PDH-E1α lysine acetylation was higher in skeletal muscle of trained subjects than untrained and increased in response to
exercise in trained are novel findings and suggest that acetylation may contribute to differences in PDH regulation in trained and untrained individuals.

**AMPK and ACC-mediated PDH regulation**

Several studies have reported that IL-6 induces AMPK phosphorylation in skeletal muscle (Bienso *et al.*, 2014a;Kelly *et al.*, 2004;Kelly *et al.*, 2009). Skeletal muscle AMPK phosphorylation has been reported to increase in rodents with shorter fasting protocols (Canto *et al.*, 2010b;de *et al.*, 2006;Frier *et al.*, 2011). However, in study I the similar exercise-induced AMPK phosphorylation in IL-6 MKO and control mice did not support IL-6 mediated regulation of AMPK as previously reported, although it might be speculated that IL-6-induced regulation of AMPK was overshadowed by stronger effectors in an acute exercise setting. In study II, the fasting-induced increase in AMPK and ACC phosphorylation at 18h was not dependent on the presence of muscle IL-6 indicating that skeletal muscle IL-6 is not needed for fasting-induced AMPK signaling in mouse skeletal muscle. On the other hand, the similar fasting-induced increase in AMPK and ACC in parallel with downregulation of PDHa activity in both IL-6 MKO and Control mice in Study II advocates for a role of AMPK in fasting-induced PDH regulation in skeletal muscle as previously suggested (Bienso *et al.*, 2014a;Klein *et al.*, 2007). Such fasting induced metabolic regulation may be mediated by factors like adrenaline and cortisol, but additional studies are required in order to establish this signaling axis. However, the present finding of no change in AMPK phosphorylation with 24 hours fasting in Study III, as in study II, indicates that AMPK does not exhibit increased phosphorylation levels after 24 hours of fasting in contrast to previously reports in both mice and humans (Gonzalez *et al.*, 2004;Wijngaarden *et al.*, 2014).

The fact that AMPK phosphorylation was attenuated in trained compared with untrained subjects during exercise in Study IV is in line with previous findings that even short-term exercise training reduces AMPK activation during exercise (McConell *et al.*, 2005) possibly due to adaptive alterations in AMPK subunit isoform expression (Durante *et al.*, 2002). Moreover, it is also conceivable that the generally higher content of glycogen storages, and less use during submaximal exercise in trained individuals as supported by study IV, may keep AMPK subdued as the β-subunit has been found to contain a glycogen interaction domain (Derave *et al.*, 2000;Hardie, 2011).
Conclusions and perspectives

The results presented in this thesis indicate that skeletal muscle IL-6 inhibits skeletal muscle PDHa activity, both at rest, during fasting and during prolonged exercise. In support, IL-6 MKO mice had an overall higher RER during exercise than controls indicating increased CHO oxidation. Likewise, lack of skeletal muscle IL-6 resulted in elevated resting RER in the fed state, while the RER in the fasted state was similar in IL-6 MKO and Control. Moreover, the higher skeletal muscle PDHa activity in IL-6 MKO than Control mice in the fasted state was associated with lower PDH phosphorylation in IL-6 MKO than Control. However, both IL-6 and PGC-1α are dispensable for maintaining short-term metabolic flexibility, as muscle IL-6 was not necessary for exercise-induced switches in substrate utilization and neither lack of skeletal muscle IL-6 or PGC-1α affected fasting-induced switch to fat oxidation. Lack of muscle PGC-1α did however blunt the fasting-induced increase in particular mitochondrial proteins.

Exercise-induced skeletal muscle PDH activation was closely matched to the relative exercise intensity at submaximal exercise, while reaching a higher level at maximal exercise in endurance trained than untrained individuals. These responses were associated with increased PDH phosphorylation, acetylation and content of covalent regulators in trained subjects. PDHa activity and PDH dephosphorylation were correspondingly well adjusted to the relative exercise intensity during submaximal exercise, while the higher PDH activity in trained than untrained at exhaustion seemed related to differences in glycogen utilization rather than differences in PDH phosphorylation and acetylation state, although site-specific contributions cannot be ruled out.

While acetylation of PDH-E1α in quadriceps mouse muscle during prolonged treadmill exercise in study I did not elicit an increase in lysine acetylation, study IV clearly showed a higher PDH-E1α acetylation level in skeletal muscle of trained than untrained subjects, a novel finding, while the regulation of acetylation during exercise was less marked. Fasting for 6 and 18 hours in study II led to an overall lowering of PDH-E1α acetylation in mouse skeletal muscle, while 24 hours fasting in study III elicited no differences in acetylated muscle PDH-E1α, although muscle lacking PGC-1α appeared to exhibit hyperacetylated PDH-E1α in the fed state. Together, this indicates a role for acetylation in the regulation of PDH. However, the exact mechanism and metabolic effects are still not fully known.
It may be that specific acetylation sites on PDH hold more influential positions than others in acute and long term regulation of PDH, and it would be interesting to pursue important specific lysine sites through the use of targeted proteomic analyses. Furthermore, since the NAD⁺-dependent deacetylase SIRT3 has been proposed to directly deacetylate PDH-E1α, establishing NADH/NAD⁺ ratios during fasting and exercise to evaluate SIRT3 activity in both mouse and human skeletal muscle is important.

To elucidate on the mechanism of IL-6-mediated regulation of skeletal muscle PDH, the use inducible IL-6 muscle knockout models could prove useful, or alternatively, electroporation of IL-6 vectors into mouse muscle to transiently overexpress IL-6 could contribute to the understanding of the potential role of IL-6 and its autocrine and paracrine effects on PDH regulation in skeletal muscle.
References

Reference List


Ref Type: Generic


STUDY I
Lack of Skeletal Muscle IL-6 Affects Pyruvate Dehydrogenase Activity at Rest and during Prolonged Exercise


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Abstract

Pyruvate dehydrogenase (PDH) plays a key role in the regulation of skeletal muscle substrate utilization. IL-6 is produced in skeletal muscle during exercise in a duration dependent manner and has been reported to increase whole body fatty acid oxidation, muscle glucose uptake and decrease PDHa activity in skeletal muscle of fed mice. The aim of the present study was to examine whether muscle IL-6 contributes to exercise-induced PDH regulation in skeletal muscle. Skeletal muscle-specific IL-6 knockout (IL-6 MKO) mice and floxed littermate controls (control) completed a single bout of treadmill exercise for 10, 60 or 120 min, with rested mice of each genotype serving as basal controls. The respiratory exchange ratio (RER) was overall higher (P<0.05) in IL-6 MKO than control mice during the 120 min of treadmill exercise, while RER decreased during exercise independent of genotype. AMPK and ACC phosphorylation also increased with exercise independent of genotype. PDHa activity was in control mice higher (P<0.05) at 10 and 60 min of exercise than at rest but remained unchanged in IL-6 MKO mice. In addition, PDHa activity was higher (P<0.05) in IL-6 MKO than control mice at rest and 60 min of exercise. Neither PDH phosphorylation nor acetylation could explain the genotype differences in PDHa activity. Together, this provides evidence that skeletal muscle IL-6 contributes to the regulation of PDH at rest and during prolonged exercise and suggests that muscle IL-6 normally dampens carbohydrate utilization during prolonged exercise via effects on PDH.

Introduction

Skeletal muscle possesses a remarkable ability to regulate substrate use with changing substrate availability and energy demands [1,2]. As the Randle cycle originally proposed [3], lipids and carbohydrates (CHO) play competitive but equally essential roles as substrate in energy production in muscle. The coordinated dynamic switch between these substrates is vital to sustaining ATP production during prolonged metabolic challenges such as exercise. The demand for energy supply increases many fold over resting state requirements at the onset of exercise and
simultaneous induction of numerous metabolic pathways are initiated across tissues in order to increase both fat and carbohydrate availability and oxidation [4,5]. During prolonged low to moderate intensity exercise, a reciprocal shift from CHO to lipid oxidation occurs in skeletal muscle in order to spare muscle glycogen stores and hence prolong the ability for the muscle to contract [6,7]. However, the molecular mechanisms behind this remain to be elucidated.

The pyruvate dehydrogenase complex (PDC) represents the only point of entry for CHO derived fuel into the mitochondria for complete oxidation [8,9] and is therefore seen as a metabolic gatekeeper. Located within the mitochondrial matrix, the PDC exerts its role by catalyzing the rate-limiting and irreversible decarboxylation of pyruvate thereby connecting glycolysis with the Krebs cycle. The PDC is composed of multiple copies of the three enzymatic subunits E1, E2, and E3, where the tetrameric (2α/2β) E1 enzyme, also termed pyruvate dehydrogenase (PDH), is the initial catalyst in the decarboxylation step (Harris, 2001). Covalent modifications by means of phosphorylation of at least four different serine sites (site 1: Ser293; site 2: Ser300; site 3: Ser232, and site 4: Ser295) on the E1 enzyme have so far been thought to be the main regulatory mechanism controlling the activity of the PDC, although allosteric regulation by the substrates, pyruvate and NAD⁺, and the products, acetyl-CoA and NADH, as positive and negative allosteric effectors, respectively, may also contribute [10–12]. The activity of PDH in its active form (PDHa activity) is inhibited by phosphorylation catalyzed by 4 isoforms of PDH kinases (PDK) and stimulated by dephosphorylation catalyzed by 2 isoforms of PDH phosphatases (PDP), of which PDK2 and PDK4 and the Ca²⁺-sensitive PDP1 have been suggested to be the most highly expressed isoforms in skeletal muscle [13,14]. PDHa activity is rapidly increased within the first minutes of exercise strongly correlated with exercise intensity [15–17]. In addition, PDHa activity has been shown to decrease after 2h of exercise in humans [12,18] reflecting a dominant reliance on CHO at the onset of exercise which gradually decreases over time as FFA available and lipid oxidation increase [7,18,19]. Furthermore, the exercise-induced regulation of PDHa activity has been shown to be associated with opposite changes in PDH phosphorylation in human skeletal muscle [19–21] indicating phosphorylation as an important regulatory mechanism in the regulation of PDH. Moreover, recent studies have provided evidence for acetylation of PDH-E1α, with the NAD⁺-dependent deacetylase sirtuin 3 (SIRT3) shown to target PDH-E1α, possibly playing an important role in maintaining the tight control of the complex [22,23].

Although the regulation of PDHa activity through post-translational modifications is well established, the signaling pathways inducing these modifications remain to be fully investigated. Previous studies suggest that interleukin (IL) 6 may play a role. Thus, human studies have shown that IL-6 is produced in and released from skeletal muscle during exercise in a duration and intensity dependent manner [24,25]. Furthermore, IL-6 infusion in humans has been shown to increase skeletal muscle fat oxidation [26], and some studies link IL-6 to augmented glucose uptake during exercise in humans [27,28], although others report no correlation between plasma IL-6 concentrations and glucose uptake in human [29] and mouse muscle [30]. In addition, injections with physiologically relevant doses of recombinant IL-6 have been shown to decrease PDHa activity in mouse skeletal muscle during fed conditions [31] and rodent studies have indicated that IL-6 activates AMP activated protein kinase (AMPK) [27,32]. Moreover, because lack of AMPKα2 has been shown to enhance PDHa activity in mouse skeletal muscle [33], a regulatory metabolic axis may exist between IL-6, AMPK, and PDH during exercise. Taken together, IL-6 seems to be a potential candidate for regulating skeletal muscle PDH during prolonged exercise.

The purpose of this present study was to test the hypothesis that skeletal muscle IL-6 contributes to the regulation of PDH in mouse skeletal muscle at rest as well as during prolonged exercise and that this is associated with IL-6 mediated regulation of AMPK.
Methods

Animals

Male C57BL/6 mice carrying loxP inserts that flanked exon 2 of the IL-6 gene as previously described [34] were crossbred with C57BL/6 mice carrying the cre recombinase gene under control of the myogenin promoter generating mice carrying the loxP inserts (floxed), serving as control mice, and skeletal muscle-specific IL-6 knockouts (IL-6 MKO) as previously shown [35]. Animals were maintained on a 12:12 light-dark cycle at 22°C with ad libitum access to water and chow diet (Altromin 1314F, Brogaarden, Lyngby, Denmark) and were therefore in a fed state before the exercise intervention. All experiments were approved by the Danish Animal Experimental expectorate and complied to the European Convention for the protection of vertebrate animals used for experiments and other scientific purposes (Council of Europe no. 123. Strasbourg, France 1985).

Endurance test protocol

Mice also included in another study (Knudsen et al., 2015) were used to estimate relative endurance capacity. Mice performed a graded treadmill test (TSE systems, Bad Hamburg, Germany) at 10 degree incline and an initial speed of 11 m/min for 2 min. This was followed by gradually increasing the velocity every 12th min to 14, 16, 18, 20, and 22 m/min, after which the incline was increased by 2 degrees every 12th min until exhaustion. Exhaustion was characterized as the point where mice could not be encouraged to run further despite being subjected to gentle electrical shock and stimulation with compressed air.

Acute exercise procedure

At 3 months of age, mice were housed individually and acclimatized to treadmill running (TSE Systems, Bad Hamburg, Germany) 10 min, 2 times a day for 5 days with 1 day of rest prior to the experimental day.

The respiratory exchange ratio (RER) was determined during running (n = 5–7) on treadmills connected to a Phenomaster unit (TSE Systems, Bad Hamburg, Germany) at 14 m/min with a 10° incline for 120 min.

On the day of the main experiment, mice were prompted to run at 14 m/min with a 10° incline (n = 10) for 10 min, 60 min, or 120 min between 8:00 and 10:30 AM before being euthanized by cervical dislocation. Quadriceps muscles were swiftly removed and snap frozen in liquid nitrogen. Trunk blood was obtained in EDTA containing tubes and plasma was obtained after centrifugation. Both muscle and plasma were stored at -80°C.

Plasma analyses

Plasma NEFA concentrations were measured colorimetrically using a NEFA-HR (2) kit according to the manufacturer’s guidelines (WAKO Diagnostics GmbH, Germany). Plasma IL-6 was measured using a mesoscale v-plex kit according to the manufacturer’s guidelines (MSD, Rockville, MD, USA). Plasma glucose and lactate were measured fluorometrically as previously described [36].

Muscle analyses

Muscle glucose, lactate, glucose-6 phosphate and glycogen. Whole quadriceps muscles were crushed in liquid nitrogen to achieve tissue homogeneity. For measurements of muscle glucose, lactate, and glucose-6-phosphate (G-6-P) 10-15mg of crushed muscle tissue was extracted in perchloric acid (PCA) and neutralized to a pH of 7–8.
Muscle glycogen was determined fluorometrically as glycosyl units after hydrolyzing 10–15 mg wet weight muscle samples by boiling for 2h in HCl (1M) as previously described [36].

HAD and CS activity

The maximal activity of citrate synthase (CS) was measured using a citrate synthase Assay Kit (Sigma-Aldrich) according to manufacturer’s guidelines and maximal β-hydroxyacyl-CoA dehydrogenase (HAD) activity was measured as previously described [37].

Immunoblotting

Crushed muscle samples (25-35g) were homogenized in lysis buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, 3 mM benzamidine, pH 7.5) using a Tissue Lyser II (Qiagen, Germany). Samples for immunoprecipitation had deacetylase inhibitors (nicotinamide (1mM) and sodium butyrate (5mM)) added to the lysis buffer. Content in each of the samples was determined using the bicinchoninic acid method (Thermo Fisher Scientific, USA) and content was adjusted with sample buffer to a concentration of 2μg/μl for STAT3Tyr705 and 1μg/μl for the remaining proteins. Protein phosphorylation and protein levels were determined by SDS-PAGE using hand casted gels and western blotting. Membranes were incubated in primary antibody for determination of AMPKα2, PDK4 and PDH-E1α protein, PDH-E1αSer293, PDH-E1αSer300, and PDH-E1αSer295 phosphorylation (all kindly provided by Professor Graham Hardie, University of Dundee, Scotland), P38 protein, P38Thr180/Tyr182 phosphorylation, Hexokinase (HK) II protein, AMPKThr172 phosphorylation, signal transducer and activator of transcription (STAT3) protein, STAT3Tyr705 phosphorylation (#9212, #4511, #2867, #2535, #9139, #9138, and #9441, respectively, Cell Signaling Technologies, Danvers, MA, USA), acetyl CoA carboxylase (ACC)-Ser212 phosphorylation and PDH-E1αSer232 phosphorylation (07-303 and #AP1063, respectively, EMD Millipore, Bedford, USA), PDK1 and OXPHOS proteins (ab90444 and ab110413, respectively, Abcam, Cambridge, U.K.), PDK2 protein (ST1643, CalBioChem, Bedford, USA), PDP1 protein (Sigma-Aldrich, St. Louis, USA) and GLUT4 protein (PAI-1065, ABR, Connecticut, USA). Species-specific horseradish peroxidase conjugated immunoglobulin secondary antibodies (DAKO, Denmark) were used for incubation the following day (ACC2 was incubated with streptavidin as primary antibody) and protein bands were subsequently visualized using an ImageQuant LAS 4000 imaging system and quantified with ImageQuant TL 8.1 software (GE Healthcare, Freiburg, Germany).

Immunoprecipitation for protein acetylation

A total of 100 μg of protein from lysate was immunoprecipitated for the determination of PDH acetylation state. Briefly the lysate was added to washed protein G agarose beads (EMD Millipore, Bedford, USA) in a 50:50 solution with PBS buffer containing 10% Triton X with 2μg of PDH-E1α antibody. The samples were rotated end over end at 4°C overnight and on the subsequent day the beads were washed, sample buffer was added and the samples were shortly heated to 96°C for 3 minutes. The beads were spun down and lysate loaded on a hand-casted gel for SDS-page and western blotting as described above and incubated with total lysine acetylation antibody (#9441, Cell Signaling Technologies, Danvers, MA, USA). Acetylated protein was normalized to the amount of precipitated PDH-E1α protein content for each sample.
RNA isolation, reverse transcription, and Real-time PCR

RNA was isolated using a modified guanidium-thiocyanate-chloroform protocol as previously described [38–40]. Reverse transcription was performed on 3μg of RNA from quadriceps muscle using the Superscript II RNAsase H⁻ system and oligodT (Life Technologies, Nærum, Denmark). To determine mRNA content, real-time PCR was performed using the ABI-7900 Sequence Detection System (Applied biosystems, Foster City, CA, USA). A fragment of cDNA was amplified using the following oligo sequences for IL-6: forward 5´GCTTAATTACACATGTTCTCTGGGAAA3´, reverse 5´CAAGTGCATCGTTGTTCATAC3´ and Taqman probe 5´ATCAGAATTGCATTGCACACTCTTTTCTCAT3´, and for SOCS3: forward 5´GCCACCTGGACTCCTATGAGAA3´, reverse 5´GAGCATCATACTGATCCAGGAACTC3´ and Taqman probe 5´TGACCCAGCTGCCTGGACCCATT3´. Samples were run in triplicates together with a serial dilution made from a pool of aforementioned samples. The serial dilution was used to create a standard curve for quantification of the specific amount of mRNA from obtained sample Ct values. For each sample, the target mRNA content was normalized to β-actin mRNA, which was not affected by either exercise or genotype.

PDHa activity

PDHa activity (activity of PDH in the active form) was determined after homogenizing 10-15mg of wet weight muscle tissue and snap-freezing the homogenate in liquid nitrogen as previously described [15,19,41,42] and normalized to creatine content in each muscle sample.

Statistics

All values are expressed as means ± SE. A two-way ANOVA was used to test the effects of genotype and exercise. When a main effect was detected a Student-Newmann-Keuls test was applied as a post-hoc test to locate differences. For single group data, a student’s t-test was used to test if a difference was present. Significance was accepted at P<0.05 and a tendency for 0.05<P<0.1. Results were analyzed using Sigmaplot 13.0 (Systat, USA).

Results

Endurance Test

Running duration was shorter (P<0.05) for IL-6 MKO mice than for control mice (Fig 1A).

HK II, GLUT 4, OXPHOS complexes, HAD and CS activity. There were no differences in basal protein content of HKII and GLUT4 (Fig 1B) or OXPHOS complexes in skeletal muscle of control and IL-6 MKO mice (Fig 1C). Similarly, there were no genotype differences in basal CS or HAD activity in skeletal muscle (Fig 1D).

Plasma IL-6

Plasma IL-6 was non-detectable at rest and at 10 min of exercise in both control and IL-6 MKO mice, but increased (P<0.05) in both genotypes after 60 min and 120 min of exercise. IL-6 MKO had higher (P<0.05) plasma IL-6 than control mice after 120 min of exercise (Fig 2A).

IL-6 and SOCS3 mRNA. There was an overall difference (P<0.05) in IL-6 mRNA content between control and IL-6 MKO mice with ~300% higher (P<0.05) IL-6 mRNA content in control than IL-6 MKO mice at rest and after 10 min and 60 min of exercise, and ~140% higher (P<0.05) in control than IL-6 MKO mice after 120 min of exercise (Fig 2B). There was no effect of exercise on muscle IL-6 mRNA at 10 min and 60 min of exercise, while IL-6 mRNA content was lower (P<0.05) at 120 min of exercise than rest in control mice. The residual IL-6 mRNA measured in the IL-6 MKO mice is assumed to originate from non-muscle tissue.
SOCS3 mRNA was lower (P < 0.05) at 60 min and 120 min of exercise than at rest in both genotypes with no difference between genotypes at any of the time points (Fig 2C).

**STAT3.**  STAT3<sup>Tyr705</sup> phosphorylation in muscle was higher (P < 0.05) than at rest after 60 min of exercise in control mice and after 120 min in both genotypes (Fig 2D). There was no difference in STAT3<sup>Tyr705</sup> phosphorylation and STAT3 protein between the genotypes.

**Plasma glucose, NEFA, and lactate.** The plasma glucose concentration was higher (P < 0.05) in IL-6 MKO than in control mice at rest. The plasma glucose level was higher (P < 0.05) at 10 min and 60 min of exercise than at rest in control mice, whereas plasma glucose was lower (P < 0.05) after 120 min of exercise than at rest in IL-6 MKO mice (Table 1). While plasma NEFA concentrations did not change significantly during exercise in control mice, the plasma NEFA level in IL-6 MKO mice was lower (P < 0.05) after 10 and 60 min of exercise and higher (P < 0.05) after 120 min of exercise than at rest. The plasma NEFA was lower (P < 0.05)
in IL-6 MKO than control mice at 10 min of exercise and tended to be higher (0.05 < P < 0.1) in IL-6 MKO than control mice at 120 min of exercise (Table 1). The plasma lactate concentration was lower (P < 0.05) after 120 min of exercise than at rest in both genotypes. Furthermore, the plasma lactate concentration was lower (P < 0.05) in IL-6 MKO than in control mice at rest and after 10 min of exercise (Table 1).

Muscle glucose, G-6-P, glycogen, lactate and acetyl CoA

Muscle glucose concentration in control mice was higher (P < 0.05) after 10 min and tended to be higher (0.05 < P < 0.1) after 60 min of treadmill exercise than at rest. Furthermore, muscle

Table 1. Plasma concentrations of glucose, NEFA, and lactate. Plasma glucose, non-esterified fatty acids (NEFA), and lactate in skeletal muscle specific IL-6 knockout (IL-6 MKO) and littermate floxed controls (Control) mice at rest and after 10, 60 or 120 min of exercise. Values are given as mean ± SE; n = 9–10.

<table>
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<th></th>
<th>Rest</th>
<th>10 min</th>
<th>60 min</th>
<th>120 min</th>
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<tr>
<td></td>
<td>Control</td>
<td>IL6-MKO</td>
<td>Control</td>
<td>IL6-MKO</td>
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<tr>
<td>Glucose (mmol/L)</td>
<td>7.29 ±0.25</td>
<td>8.26 ±0.31*</td>
<td>8.45 ±0.20*</td>
<td>8.38 ±0.42</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.86 ±0.09</td>
<td>0.76 ±0.05</td>
<td>0.75 ±0.05</td>
<td>0.57 ±0.03*</td>
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<tr>
<td>Lactate (mmol/L)</td>
<td>6.76 ±0.68</td>
<td>4.64 ±0.36*</td>
<td>6.70 ±0.70</td>
<td>4.21 ±0.29*</td>
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*: significantly different from rest within given genotype, P < 0.05.

#: significantly different from control within given time point, P < 0.05. (*): Tendency to be significantly different from rest within given genotype, 0.05 < P < 0.01. (#): Tendency to be significantly different from control within given time point, P < 0.05.

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glucose was higher (P<0.05) in control than in IL-6 MKO mice at both these time points (Table 2). Muscle G-6-P tended to be lower (0.05<P<0.1) after 10 and 60 min of exercise than at rest in both genotypes. G-6-P in IL-6 MKO mice was lower (P<0.05) after 60 min than at rest and in both genotypes lower (P<0.05) after 120 minutes of exercise than at rest (Table 2). Muscle glycogen gradually decreased with exercise (P<0.05) and was different from rest at every time point without any significant differences between control and IL-6 mice (Table 2). It may be noted that the difference in muscle glycogen from Rest to 120 min of exercise was 50% less in IL-6 MKO than Control mice, but this difference was not significant. Furthermore, the muscle lactate concentration was lower (P<0.05) after 120 min of exercise than at rest in both genotypes (Table 2). Muscle acetyl CoA was not affected by exercise or genotype.

**AMPK and ACC**

AMPK<sup>Thr172</sup> phosphorylation in IL-6 MKO mice was higher (P<0.05) after 10, 60, and 120 min of exercise than at rest and in control mice higher (P<0.05) after 60 and 120 min than at rest (Fig 3A). There were no genotype differences in skeletal muscle AMPK<sup>Thr172</sup> phosphorylation and AMPKα2 protein (Fig 3A). ACC<sup>Ser211</sup> phosphorylation after 10, 60, and 120 min of

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**Table 2. Skeletal muscle glucose, G-6-P, glycogen, lactate, and acetyl CoA content.** Skeletal muscle glucose, glucose -6 phosphate (G-6-P), glycogen, lactate and acetyl CoA in skeletal muscle specific IL-6 knockout (IL-6 MKO) and littermate floxed controls (Control) mice at rest and after 10, 60 or 120 min of exercise. Values are given as mean ± SE; n = 9–10.

<table>
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<th>Rest</th>
<th>10 min</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IL6-MKO</td>
<td>Control</td>
<td>IL6-MKO</td>
</tr>
<tr>
<td>Glucose (mmol/kg)</td>
<td>0.41 ± 0.04</td>
<td>0.2 ± 0.04</td>
<td>0.66 ± 0.08*</td>
<td>0.46 ± 0.05*</td>
</tr>
<tr>
<td>G-6-P (mmol/kg)</td>
<td>5.85 ± 0.14</td>
<td>5.86 ± 0.19</td>
<td>5.64 ± 0.21</td>
<td>5.39 ± 0.16*</td>
</tr>
<tr>
<td>Glycogen (mmol/kg)</td>
<td>16.25 ± 1.1</td>
<td>18.41 ± 1.49</td>
<td>12.14 ± 0.39*</td>
<td>12.35 ± 1.14*</td>
</tr>
<tr>
<td>Lactate (mmol/kg)</td>
<td>3.83 ± 0.29</td>
<td>4.37 ± 0.54</td>
<td>3.97 ± 0.55</td>
<td>3.88 ± 0.19</td>
</tr>
<tr>
<td>Acetyl CoA (mmol/kg)</td>
<td>0.95 ± 0.06</td>
<td>0.85 ± 0.05</td>
<td>0.821 ± 0.03</td>
<td>0.865 ± 0.04</td>
</tr>
</tbody>
</table>

*: significantly different from rest within given genotype, P<0.05.

#: significantly different from control within given time point, P<0.05. (*): Tendency to be significantly different from rest within given genotype, 0.05<P<0.01. (#): Tendency to be significantly different from control within given time point, P<0.05.
exercise was higher ($P < 0.05$), apart from MKO mice having a tendency to be higher ($0.5 < P \leq 0.1$) at 60 min ($P = 0.078$), than at rest in both genotypes (Fig 3B). There were no genotype differences in skeletal muscle ACCSer212phosphorylation and ACC2 protein (Fig 3B).

**PDH-E1α phosphorylation, PDK and PDP protein**

While skeletal muscle PDH-E1αSer293 and PDH-E1αSer295 phosphorylation (site 1 and 4) did not change during the 120 min of treadmill exercise, PDH-E1αSer300 and PDH-E1αSer232 phosphorylation (site 2 and 3) was lower ($P < 0.05$) after 10 and 60 min of exercise than at rest in both control and IL-MKO mice (Fig 4A and 4B). There were no differences in skeletal muscle PDK1, PDK2, PDK4 or PDP1 protein content between time points or genotypes (Fig 4D).

**PDH acetylation and SIRT3**

There were no differences in skeletal muscle SIRT3 or PDH-E1α acetylation (Fig 4C).

**PDHa activity**

PDHa activity was higher ($P < 0.05$) after 10 and 60 min of exercise than at rest in control mice, while there was no effect of exercise on PDHa activity in IL-6 MKO mice. The PDHa activity was higher ($P < 0.05$) in IL-6 MKO than control mice at rest and 60 min of exercise (Fig 5). There were no differences in skeletal muscle PDH-E1α protein.

**Indirect calorimetry**

RER was lower ($P < 0.05$) after 120 min than after 10 min of exercise in both genotypes. Furthermore, IL-6 MKO mice displayed an overall higher ($P < 0.05$) RER than control mice during the 120 min of treadmill exercise (Fig 6).
Fig 5. PDHa activity in skeletal muscle from skeletal muscle specific IL-6 knockout (IL-6 MKO) and littermate floxed controls (Control) at rest and after 10, 60 or 120 min of exercise. Values are given as mean ± SE; n = 10. *: significantly different from rest within given genotype, P < 0.05. #: significantly different from control within given time point, P < 0.05.

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Fig 6. Respiratory Exchange Ratio (RER) in skeletal muscle specific IL-6 knockout (IL-6 MKO) and littermate floxed controls (Control) during 120 min of metabolic treadmill exercise. Values are given as mean ± SE for every 10 minutes of continuous measurements; n = 5–7. *: significantly different from 10 min within given genotype, P < 0.05. #: significantly different from control within given time point, P < 0.05.

doi:10.1371/journal.pone.0156460.g006
Western blots

For all representative western blots consult Fig 7.

Discussion

The main findings of the present study are that lack of skeletal muscle IL-6 led to elevated PDHa activity at rest and during exercise without changes in PDHa activity during prolonged exercise as observed in control mice. In addition, IL-6 MKO mice had an overall higher RER during exercise than controls, but maintained the ability to reduce RER during prolonged exercise. Together this indicates that muscle IL-6 influences substrate utilization in skeletal muscle through effects on PDH, but is not required for the shift in substrate use during prolonged exercise.

The observations that the exercise bout in the present study was associated with a gradual decrease in RER and a transient increase in skeletal muscle PDHa activity in control mice as previously reported in humans [7,18,19]), suggest that the experimental setup provides the basis for studying the impact of muscle IL-6 on exercise-induced PDH regulation in skeletal muscle and substrate utilization.

The present findings that IL-6 MKO mice had higher skeletal muscle PDHa activity at rest and after 1h of exercise and did not exhibit a significant change in PDHa activity during exercise show for the first time that lack of skeletal muscle IL-6 affects the regulation of PDH in skeletal muscle. It should be noted that this suggestion is possible although PDHa activity was not significantly different between the genotypes at 2 h of exercise, because the plasma IL-6 levels were increased in both genotypes at 2 h of exercise. Thus, PDHa activity may have been influenced by IL-6 derived from other tissues than skeletal muscle late during exercise. It cannot be excluded that the lack of change in PDHa activity in response to exercise in IL-6 MKO mice is due to PDHa activity being close to full activation already at rest and that a change therefore was not detected during exercise in the present study. However, we have previously observed ~25% higher levels of PDHa activity in mice running at a higher speed and treadmill slope than in the present study (unpublished results). In addition, the observed muscle IL-6 dependent regulation of PDHa activity is in accordance with a previous mouse study showing that a single injection of recombinant IL-6 altered PDHa activity in skeletal muscle.
muscle, although the change was dependent on the nutritional state as IL-6 lowered PDHa activity in the fed state and increased PDHa activity in the fasted state [31]. Furthermore, the observed impact of muscle-specific IL-6 knockout on PDHa activity suggests that muscle IL-6 normally exerts an inhibitory effect on skeletal muscle PDH with a concomitant decrease in carbohydrate oxidation. Although differences in PDHa activity do not necessarily relate to flux and may therefore not be linked to the difference in RER between IL-6 MKO and control mice, the observed higher RER in IL-6 MKO mice than controls during the exercise bout does support that IL-6 reduces carbohydrate oxidation. On the other hand, the maintained ability of the IL-6 MKO mice to reduce RER during the exercise bout indicates opposite of the hypothesis that muscle IL-6 is not required for a switch towards fat oxidation during prolonged exercise in mice. However, the increase in circulating IL-6 in IL-6 MKO mice is in accordance with a potential effect of non-muscle-derived IL-6 on substrate utilization and hence RER during prolonged exercise. In addition, the higher PDHa activity in IL-6 MKO than control mice not only during exercise, but also at rest emphasizes that muscle IL-6 may not only function as a contraction-induced myokine, but also regulates basal skeletal muscle metabolism.

To elucidate the mechanistic regulatory pattern behind the different regulation of PDHa activity when muscle IL-6 is lacking, the four known PDH phosphorylation sites, site 1: Ser293; site 2: Ser300; site 3: Ser232, and site 4: Ser295, were examined. The observation that phosphorylation of Both sites 2 and 3 followed an overall inverse pattern of the PDHa activity in control mice with robust decreases in absolute phosphorylation during exercise is in accordance with previous findings for site 1 and 2 in humans [19–21]. Moreover, the similar resting and exercise-induced changes in site 2 and 3 and similar lack of change in site 1 and 4 phosphorylation in IL-6 MKO and control mice indicates that diverse regulation of PDH phosphorylation is not the main underlying mechanism for the observed genotype differences in PDHa activity in the present study. In addition, the lack of difference in PDK1, PDK 2, PDK4 and PDP1 protein during exercise in either genotype suggests that protein content does not account for the observed PDH phosphorylation response. Due to the complex site-specific affinity patterns of the various PDKs [43] measuring the activities of all PDK isoforms may likely be needed to fully understand the exercise-induced regulation of PDH phosphorylation and PDHa activity. As it recently has been reported that the mitochondrial NAD+ dependent deacetylase, SIRT3, targets the PDH-E1α subunit in C2C12 cells [22], the acetylation state in skeletal muscle at rest and during exercise might reveal the mechanism explaining the observed changes in PDHa activity with lack of skeletal muscle IL-6. However, the observation that total lysine acetylation of immunoprecipitated PDH-E1α was similar in IL-6 MKO and control mice indicates that diverse regulation of PDH phosphorylation is not the main underlying mechanism for the observed genotype differences in PDHa activity in the present study. In addition, the lack of difference in PDK1, PDK 2, PDK4 and PDP1 protein during exercise in either genotype suggests that protein content does not account for the observed PDH phosphorylation response. Due to the complex site-specific affinity patterns of the various PDKs [43] measuring the activities of all PDK isoforms may likely be needed to fully understand the exercise-induced regulation of PDH phosphorylation and PDHa activity. As it recently has been reported that the mitochondrial NAD+ dependent deacetylase, SIRT3, targets the PDH-E1α subunit in C2C12 cells [22], the acetylation state in skeletal muscle at rest and during exercise might reveal the mechanism explaining the observed changes in PDHa activity with lack of skeletal muscle IL-6. However, the observation that total lysine acetylation of immunoprecipitated PDH-E1α was similar in IL-6 MKO and control mice does not support that acetylation is regulated by skeletal muscle IL-6, although it cannot be excluded that the total acetylation pattern disguises more subtle differences in acetylation that may be more significant to regulation, but this remains to be determined.

As IL-6 has previously been reported to increase AMPK activity both in human [27] and mouse tissue [31,32] and AMPK has been suggested to regulate PDH [33,44], it was hypothesized that AMPK mediates the effects of IL-6 on PDHa activity and substrate utilization. However, the similar exercise-induced AMPK phosphorylation in IL-6 MKO and control mice does not support IL-6 mediated regulation of AMPK as previously reported, although it might be speculated that IL-6-induced regulation of AMPK is overshadowed by stronger effectors in an acute exercise setting.

The finding that plasma glucose at rest was higher in the IL-6 MKO than control mice and the plasma glucose did not drop significantly until the latter stages of the exercise bout might reflect lower glucose removal by skeletal muscle in IL-6 MKO mice or be due to higher hepatic glucose output in IL-6 MKO than in control mice. Furthermore, the observation that basal
hexokinase and GLUT4 protein content as well as G-6-P concentrations in skeletal muscle were similar in the two genotypes, while muscle glucose was lower in the IL-6 MKO than control mice may advocate more for a higher glycolytic flux and carbohydrate utilization in mice lacking skeletal muscle IL-6 rather than a lower glucose uptake. In addition, the genotype difference in running endurance during an incremental exercise running test may suggest that the IL-MKO mice were running at a higher relative intensity than the control mice in the current exercise study. However, the IL-6 MKO mice did not require more motivation than the controls during the 2 hour moderate intensity exercise protocol and completed the protocol as the controls in the present study. Furthermore, as a previous human study has reported intensity dependent AMPK regulation [45], the similar exercise-induced AMPK and ACC phosphorylation in the two genotypes in the present study suggests that the relative exercise intensity was equal in the IL-6 MKO and control mice during the exercise bout, which is further supported by the identical decrease in muscle glycogen in the two genotypes during exercise.

The observation that the plasma IL-6 concentrations were significantly elevated after 2 hours of running in control mice is in accordance with previous murine studies [46,47]. The finding that plasma IL-6 concentrations in the control mice reached rather modest levels of ~15 pg/ml points towards the possibility that the loxP inserts in the floxed control mice might affect expression or release of IL-6. Hence, other studies with various treadmill protocols have reported increases in circulating plasma IL-6 to levels between 40–70 pg/ml [30,48]. The observation that skeletal muscle IL-6 mRNA did not increase with the 2 hours of treadmill exercise and even decreased at the end of the exercise bout, opposes previous findings [49], and suggests that exercise did not induce IL-6 transcription in skeletal muscle of the floxed IL-6 mice. It cannot be excluded that exercise may have increased IL-6 mRNA in other muscles than the investigated quadriceps, but regardless, the skeletal muscle IL-6 mRNA data show that IL-6 was knocked out, which was also confirmed at the level of DNA (S1 Fig). Therefore, the exercise-induced increase in circulating IL-6 levels in the IL-6 MKO mice cannot be originating from skeletal muscle fibers and based on previous findings [50] suggests that IL-6 derived from adipose tissue may be responsible for the increase in plasma IL-6 during exercise in the present experiment. This may indicate that the observed skeletal muscle IL-6 dependent effects on skeletal muscle PDHa activity are mediated by autocrine/paracrine effects of IL-6. However, the identical exercise-induced STAT3 phosphorylation and identical decline in SOCS3 mRNA in the two genotypes suggest that signaling pathways traditionally accredited to be induced by IL-6 were affected similarly by exercise in the two genotypes. Thus, the observed metabolic differences must be exerted through other pathways. In addition, a variety of other cytokines and hormones have been reported to trigger the STAT3 signaling pathway [51,52] and any of these, or non-muscle derived IL-6 may be responsible for the observed STAT3 pathway inductions and explain why this response was also present in IL-6 MKO mice.

The current observation that IL-6 MKO mice displayed a lower exercise capacity than the control mice when submitted to an incremental endurance exercise test is consistent with some [53], but not all previous observations in IL-6 whole body knockout mice [30,54]. The findings that skeletal muscle CS and HAD activities as well as the content of OXPHOS proteins were similar in the two genotypes indicate that skeletal muscle oxidative capacity was unaffected by the lack of skeletal muscle IL-6. Together with the observed higher RER value during exercise in IL-6 MKO mice than controls in the present study this may suggest that the reduced exercise endurance in the IL-6 MKO mice is due to increased carbohydrate/reduced fat utilization relative to controls rather than reduced skeletal muscle oxidative capacity.

In conclusion, the present findings show, for the first time, that lack of skeletal muscle IL-6 leads to elevated PDHa activity in skeletal muscle both at rest and during exercise, and
prevented significant changes in PDHa activity during prolonged exercise. Differences in PDH phosphorylation did not seem to explain these genotype differences in PDHa activity and the mechanisms behind are still elusive. In addition, lack of skeletal muscle IL-6 resulted in higher RER during prolonged exercise suggesting that IL-6 normally reduce carbohydrate oxidation during exercise via effects on skeletal muscle PDH.

Supporting Information
S1 Fig. Representative PCR gel of products generated with primers surrounding exon 2 of the IL-6 gene in quadriceps muscle from floxed and IL-6 skeletal muscle-specific knockout mice. A reduction in band size from 1,000 bp to 260 bp in is equal to the loss of exon 2 of the IL-6 gene. WT Ctrl: Wild-type control, Flox Ctrl: Floxed control, MKO Control: Skeletal muscle-specific knockout Control. (TIF)

Author Contributions
Conceived and designed the experiments: AG JGK HP. Performed the experiments: AG CS LB EJ JGK HP. Analyzed the data: AG CS LB EJ JGK. Contributed reagents/materials/analysis tools: HP. Wrote the paper: AG CS LB EJ JGK HP.

References


STUDY II
Muscle interleukin 6 and fasting-induced PDH regulation in mouse skeletal muscle

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Keywords
IL-6, PDH, skeletal muscle, metabolism, fasting, substrate utilization

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Abstract

Fasting prompts a metabolic shift in substrate utilization from carbohydrate to predominant fat oxidation in skeletal muscle and pyruvate dehydrogenase (PDH) is seen as a controlling link between the competitive oxidation of carbohydrate and fat during metabolic challenges like fasting. Interleukin (IL)-6 has been proposed to be released from muscle with concomitant effects on both glucose and fat utilization. The aim was to test the hypothesis that IL-6 has a regulatory impact on fasting-induced suppression of skeletal muscle PDH. Skeletal muscle-specific IL-6 knockout (IL-6 MKO) mice and floxed littermate controls (Control) were either fed or fasted for 6 or 18 hours. Lack of muscle IL-6 elevated the respiratory exchange ratio (RER) in the fed and early fasting state, but not with prolonged fasting. PDHα activity was higher in fed and fasted IL-6 MKO than Control mice, while lack of muscle IL-6 did not prevent down-regulation of PDHα activity in skeletal muscle or changes in plasma and muscle substrate levels in response to 18h of fasting. Phosphorylation of 3 out of 4 sites on PDH-E1α increased with 18h of fasting, but was lower in IL-6 MKO mice than Control. In addition, both PDK4 mRNA and protein increased with 6h and 18h of fasting in both genotypes, but PDK4 protein was lower in IL-6 MKO than Control. In conclusion, muscle IL-6 seems to regulate resting substrate utilization potentially through effects on skeletal muscle PDH regulation, but is not required for maintaining metabolic flexibility in response to fasting.
Introduction

Skeletal muscle has the ability to efficiently adjust fuel preference from carbohydrate to lipid use depending on substrate availability and demand. This metabolic switch is evident in response to fasting where fat oxidation is markedly increased to spare carbohydrates and proteins (7; 37; 38)

The pyruvate dehydrogenase (PDH) complex, catalyzing the irreversible conversion of pyruvate to acetyl CoA, serves as the only entry for carbohydrate into the mitochondria for oxidation (13). PDH has been suggested to be a key factor in the interaction between fat and carbohydrate oxidation during metabolic challenges like fasting (31). In accordance, fasting has been shown to downregulate the activity of PDH in the active form (PDHa activity) in both rodent and human skeletal muscle (2; 18; 34; 37). Although PDHa activity is affected by a range of allosteric effectors, the main regulatory mechanism is thought to be covalent modifications. Hence, the activity of the catalytic subunit PDH-E1α is regulated by phosphorylation and dephosphorylation of four serine sites (site 1: Ser293; site 2: Ser300; site 3: Ser232, and site 4: Ser295) catalyzed by four PDH kinases, designated PDK1, PDK2, PDK3, and PDK4, and two PDH phosphatases, PDP1 and PDP2, respectively. Phosphorylation of PDH-E1α results in inhibition of activity, while dephosphorylation increases PDHa activity (13; 34). Furthermore, acetylation state has recently been suggested to play an important role in the control of PDH as the NAD+-dependent mitochondrial deacetylase, SIRT3, may be responsible for deacetylating regulators of PDHa activity and/or PDH-E1α directly (9; 14; 22). Previous studies in mice have demonstrated increased skeletal muscle PDH-E1α site 1,2 and 4 phosphorylation with 18h (2) and 24h (18) of fasting and increased PDH-E1α acetylation after 24h of fasting (14). This supports that PDH regulation by phosphorylation and acetylation is important in the fasting-induced reduction in PDHa activity. In addition, fasting has been shown to increase PDK4 mRNA and protein and decrease PDP1 mRNA in rodent and human skeletal muscle (2; 18; 34; 37) in line with the observed changes in PDH phosphorylation. While increased levels of circulating fatty acids have been
demonstrated to contribute to the regulation of PDK4 in rodent skeletal muscle with fasting (37), hormones and muscle derived factors may as well play a role (10; 15; 32; 33).

The cytokine Interleukin (IL)-6 has been shown to be produced and released from human skeletal muscle during exercise with suggested autocrine, paracrine and endocrine effects on metabolism (25). In addition, a recent mouse study showed that skeletal muscle IL-6 mRNA and plasma IL-6 increased at 6 and 12 hours of fasting and that the fasting-induced switch to fat oxidation was blunted in whole-body IL-6 knockout mice (39). This suggests that muscle IL-6 contributes in substrate regulation during fasting, but this remains to be clarified.

Previous studies have shown that injection of recombinant IL-6 downregulated PDHa activity in mouse skeletal muscle (2) and knockout of muscle IL-6 conversely resulted in elevated skeletal muscle PDHa activity suggesting that muscle IL-6 contributes in the regulation of skeletal muscle PDH both at rest and during exercise (12). Furthermore, IL-6 has been reported to increase phosphorylation of the intracellular energy sensor AMPK in rat skeletal muscle (16) and AMPK has been suggested to contribute in the regulation of PDH (2; 20). In addition, IL-6 has previously been shown to increase p38 MAPK phosphorylation in mouse (39) and human skeletal muscle (11) potentially influencing PDH (29). Together with findings indicating that IL-6 increases fat oxidation (26; 36), this suggests that muscle-derived IL-6 mediates fasting-induced regulation of PDH in skeletal muscle potentially involving AMPK and/or p38 MAPK signaling with concomitant switch in substrate utilization from carbohydrates to fat. However, this remains to be elucidated.

The aim of the present study was therefore to examine the impact of skeletal muscle IL-6 on fasting-induced PDH regulation in mouse skeletal muscle. This includes examining the effect of muscle specific IL-6 knockout on substrate utilization at the whole body level and on skeletal muscle PDHa activity, PDH phosphorylation, PDH acetylation and regulators of PDH in response to short-term and prolonged fasting in mice.
Methods

Animals

Three month old male muscle-specific IL-6 knockout (MKO) mice lacking exon 2 of the IL-6 gene and littermate controls only carrying the loxP inserts flanking exon 2 of the IL-6 gene (floxed) were used in the present study. Generation of IL-6 MKO mice has previously been described (12; 30). Mice were at 3 to 4 weeks of age genotyped based on DNA isolated from ear pieces. In addition, the genotype was verified on DNA isolated from quadriceps muscle tissue after euthanization of the mice. Animals were maintained on a 12h:12h light-dark cycle at 22°C before and during the experimental protocol and all mice had ad libitum access to water and chow diet (Altromin 1314F, Brogaarden, Lynge, Denmark) until initiation of the intervention. All experiments were approved by the Danish Animal Experimental expectorate and complied to the European Convention for the protection of vertebrate animals used for experiments and other scientific purposes (Council of Europe no. 123. Strasbourg, France 1985).

Experimental procedure

IL-6 MKO and Control mice were individually housed for 3 days and allocated to either a fed group (FED), which maintained ad libitum access to both food and water or a fasted group (FAST) with access to only water for the duration of the given fasting period. Food was removed at 2am and mice were fasted for either 6 or 18 hours. To account for potential variations due to circadian rhythm (8), a group of FED mice was euthanized at the same time as both the 6h and 18h fasted mice (8am and 8pm, respectively) to serve as controls for each of the fasting groups. The fed and fasted mice were euthanized by cervical dislocation at the end of the fasting interventions. Quadriceps muscles were quickly removed and snap frozen in liquid nitrogen. Trunk blood was collected and plasma obtained by centrifugation.
A subset of mice were allocated to metabolic chambers (Phenomaster, TSE Systems, Bad Homburg, Germany) and monitored for indirect calorimetry. Mice were acclimatized to the ambient conditions in the metabolic chambers for minimum 2 days prior to data collection. Respiratory exchange ratio (RER) and locomotor activity were measured over a continuous period of at least 32 hours with an initial fed period followed by 18h of fasting. Chamber cabinets were maintained at a constant computer monitored climate of ~22°C, ~30% humidity and a 12h:12h light-dark cycle.

**Plasma analyses**

Plasma glucose was measured fluorometrically as previously described (1; 21; 24)

Plasma NEFA concentrations were measured colorimetrically using a NEFA-HR 2 kit according to the manufacturer’s guidelines (WAKO Diagnostics GmbH, Germany).

Plasma IL-6 was measured using a mesoscale v-plex kit (K152QXD) according to the manufacturer’s guidelines (MSD, Rockville, MD, USA). In order to attain sufficient plasma for performing duplicate measurements, plasma samples from 2 mice were pooled resulting in n=5 for this analysis.

**Muscle analyses**

Whole quadriceps muscles were crushed in liquid nitrogen for tissue homogeneity. Muscle glycogen, G-6-P, and glucose were measured fluorometrically as previously described (1; 21; 24)

**RNA isolation RT and Real-time PCR**

RNA was isolated from ~20-25 mg crushed quadriceps muscle by homogenization in a Tissuelyser II (Qiagen, Hilden, Germany) and using a modified guanidium thiocyanate phenol-chloroform method as previously
described (4; 28). Reverse transcription was performed using superscript II (Thermo Fisher Scientific, USA) and Oligo dT as previously described (28).

Specific mRNA content was determined by real time polymerase chain reaction (PCR) on either an ABI PRISM 7900HT or a Quantstudio 7 FLEX detection system (Applied Biosystems, Foster City, CA, USA). The analyses were performed in triplicates with 10µl reaction volume using Taqman probes and Universal Mastermix (Applied Biosystems, Foster City, CA, USA). For primers and Taqman probes see table 1. Cycle threshold (Ct) values were converted to an arbitrary amount of mRNA by use of a standard curve constructed from the Ct results of a serial dilution made from a pool of representative samples run in parallel with the study samples. The target mRNA content was normalized to the single stranded (ss) DNA content in the cDNA samples determined by Oligreen reagent (Thermo Fisher Scientific, USA).

**Immunoblotting**

Lysate from 25-30 mg of quadriceps muscle was homogenized for 2 min at 30 seconds in a Tissuelyser II (Qiagen, Hilden, Germany) in an ice-cold buffer consisting of 10% glycerol, 20mM Na-pyrophosphate, 150mM NaCl, 50mM HEPES, 1% NP-40, 20mM β-glycerophosphate, 10mM NaF, 1mM EDTA, 1mM EGTA, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 2mM Na₃VO₄, 3 mM benzamidine, and deacetylase inhibitors (nicotinamide (1mM) and sodium butyrate (5mM)). Protein content in individual samples was determined using the bicinchoninic acid method (Thermo Fisher Scientific, USA) and content was adjusted with sample buffer to a concentration of 1µg/µl. Protein phosphorylation and protein levels were determined by SDS-PAGE using hand casted gels and western blotting. Membranes were incubated in primary antibody for determination of AMPKα2, PDK4 and PDH-E1α protein, PDH-E1α Ser293, PDH-E1α Ser300, and PDH-E1α Ser295 phosphorylation (all kindly provided by Professor Grahame Hardie, University of Dundee, Scotland), p38 MAPK Thr180/Tyr182 phosphorylation, AMPK Thr172 phosphorylation, stress-activated protein kinase/c-Jun-amino-terminal kinase (SAPK/JNK) isoform 1 and 2, JNK Thr183/Tyr185 phosphorylation, sirtuin (SIRT)3 and lysine
acetylation antibody (#4511, #2535, #9252, #9251 #D22A3, #9441, respectively, Cell Signaling Technologies, Danvers, MA, USA), acetyl CoA carboxylase (ACC$^{\text{Ser212}}$ phosphorylation and PDH-E1$^\alpha$$^{\text{Ser232}}$ phosphorylation (07-303 and #AP1063, respectively, EMD Millipore, Bedford, USA), PDK1 (ab90444, Abcam, Cambridge, U.K.) and PDK2 protein (ST1643, CalBioChem, Bedford, USA), PDP1 protein (Sigma-Aldrich, St. Louis, USA). Species-specific horseradish peroxidase conjugated immunoglobulin secondary antibodies (DAKO, Glostrup, Denmark) were used for incubation the following day. ACC2 protein content was determined by incubation with streptavidin (DAKO, Glostrup, Denmark). Protein bands were subsequently visualized using an ImageQuant LAS 4000 imaging system and quantified with ImageQuant TL 8.1 software (GE Healthcare, Freiburg, Germany).

**Immunoprecipitation for protein acetylation**

A total of 200 µg of protein from lysate was immunoprecipitated for the determination of PDH acetylation state. Briefly the lysate was added to washed protein G agarose beads (EMD Millipore, Bedford, USA) in a 50:50 solution with PBS buffer containing 10% Triton X with 2µg of PDH-E1$^\alpha$ antibody. The samples were rotated end over end at 4°C overnight and on the subsequent day the beads were washed, sample buffer was added and the samples were shortly heated to 96°C for 3 minutes. The beads were spun down and lysate loaded on a hand-casted gel for SDS-page and western blotting as described above. Acetylated protein was normalized to the individual amount of precipitated PDH-E1$^\alpha$ protein content for each sample.

**PDH$^\alpha$ activity**

PDH$^\alpha$ activity was determined after homogenizing 10-15mg of wet weight muscle tissue on ice for 50 s using a micro glass homogenizer (Kontes, USA) and quick-freezing the samples (<15 s) in liquid nitrogen as
previously described (3; 5; 27). PDHa activity was normalized to the creatine content in each muscle sample as previously described (35).

Statistics

All values are expressed as means ± SE. A two-way ANOVA was applied separately on 6h and 18h interventions as well as 6h FED and 18h FED interventions to determine effects of circadian time effects. When a main effect was detected a Student-Newmann-Keuls test was applied as a post-hoc test to locate differences. For single group data, a student’s t-test was applied to test if a difference was present. Significance was accepted at $P<0.05$ and a tendency reported for $0.05 ≤ P ≤ 0.1$. Results were analyzed using Sigmaplot 13.0 (Systat, USA).
Results

Indirect calorimetry
RER was lower (P<0.05) after 18h of fasting than in the fed state in both IL-6 MKO and Control mice. Furthermore, RER was overall higher (P<0.05) in IL-6 MKO than Control in the dark phase and fed state (Figure 1; Dark phase FED) and during the switch from the fed to the fasted state (Figure 1; Dark phase early FAST). There was no difference in RER between genotypes at 18h of fasting (Figure 1; Dark phase late FAST). There were no overall differences in locomotor activity between genotypes.

Plasma glucose and NEFA
The plasma glucose concentration was lower (P<0.05) at both 6h FAST and 18h FAST than the corresponding FED groups in both IL-6 MKO and Control mice. The plasma non-esterified fatty acid (NEFA)/free fatty acid (FFA) concentration was higher (P<0.05) in FAST than FED at both 6h and 18h and lower (P<0.05) in 18h FED than 6h FED in both IL-6 MKO and Control mice. There were no differences in either plasma NEFA/FFA or glucose levels between the genotypes (Table 2).

Muscle metabolites
Quadriceps glucose-6-phosphate (G-6-P) and glycogen concentrations were lower (P<0.05) in FAST than FED at both 6h and 18h and the muscle glucose concentration was lower (P<0.05) at 6h FAST than 6h FED in both IL-6 MKO and Control mice. The muscle G-6-P concentration was lower (P<0.05) in IL-6 MKO than Control mice in both 6h FED and 6h FAST, while there was no genotype difference in muscle glucose or glycogen concentrations (Table 3).
IL-6 content and signaling

There were no significant effects of fasting or genotype on plasma IL-6 levels (Figure 2A). Within Control mice, quadriceps IL-6 mRNA content was higher (P<0.05) in FAST than FED at both 6h and 18h (Figure 2B). The SOCS3 mRNA content was lower (P<0.05) in FAST than FED at 18h within IL-6 MKO mice and higher (P<0.05) in 18h FED than 6h FED within IL-6 MKO mice (Figure 2C).

P38 MAPK\textsuperscript{Thr180/Tyr182} phosphorylation in quadriceps was higher (P<0.05) at 18h FAST than 18h FED in both genotypes and was higher (P<0.05) in IL-6 MKO than Controls at 18h FED (Figure 2D). There were no differences in JNK1/2 protein content or JNK1/2\textsuperscript{Thr183/Tyr185} phosphorylation between groups or genotypes (Table 4).
AMPK and ACC phosphorylation

Quadriceps AMPK\textsuperscript{Thr172} phosphorylation overall tended to be higher (0.05≤P≤0.1) in 18h FAST than 18h FED, and lower (P<0.05) at 18h FED than 6h FED within Control mice (Figure 3A). ACC\textsuperscript{Ser212} phosphorylation was higher (P<0.05) at 18h FAST than 18h FED and higher (P<0.05) in 18h FED than 6h FED in both genotypes (Figure 3B).

AMPK\textsubscript{α1} and α\textsubscript{2} protein content in quadriceps was lower (P<0.05) in 18h FED than 6h FED within both IL-6 MKO and Controls (Table 4), and similarly ACC\textsubscript{2} protein content tended to be lower (0.05≤P≤0.1) and was lower (P<0.05) at 18h FED than 6h FED in IL-6 MKO and Controls, respectively (Table 4).

PDH phosphorylation and PDHa activity

PDHa activity in quadriceps was lower (P<0.05) at 18h FAST than 18h FED and higher (P<0.05) in 18h FED than 6h FED in both IL-6 MKO and Controls. Furthermore, PDHa activity was higher (P<0.05) in IL-6 MKO than Control at 18h FED and 18h FAST (Figure 4A). There were no differences in PDH-E1α protein content between groups or genotypes (Table 4).

PDH-E1α total lysine acetylation in quadriceps overall tended to be higher (0.05≤P≤0.1) in 18h FAST than 18h FED and was in IL-6 MKO mice lower (P<0.05) in 18h FED than 6h FED (Figure 4B).

Quadriceps PDH\textsuperscript{Ser293} and PDH\textsuperscript{Ser300} phosphorylation was higher (P<0.05) in 18h FAST than 18h FED in both IL-6 and Control mice and PDH\textsuperscript{Ser300} phosphorylation was also higher (P<0.05) in 6h FAST than 6h FED in IL-6 MKO. Furthermore, PDH\textsuperscript{Ser293} and PDH\textsuperscript{Ser300} phosphorylation was at 18h FAST lower (P<0.05) in IL-6 MKO than Control mice and PDH\textsuperscript{Ser293} phosphorylation overall tended to be higher (0.05≤P≤0.1) in IL-6 MKO than Control at 6h (Figure 4C and D). In addition, PDH\textsuperscript{Ser232} phosphorylation was in IL-6 MKO mice higher (P<0.05) in FAST than FED at both 6h and 18h and lower (P<0.05) in 18h FED than 6h FED. Similarly,
PDH$_{\text{Ser}232}$ phosphorylation in Control mice tended to be higher (0.05≤P≤0.1) and was higher (P<0.05) in 6h FAST and 18h FAST, respectively, than the corresponding FED mice (Figure 4E). PDH$_{\text{Ser}295}$ phosphorylation was higher (P<0.05) in 6h FAST than 6h FED only in IL-6 MKO mice (Figure 4F).

**PDK, PDP and SIRT3 mRNA and protein content**

There were no effects of fasting or genotype on quadriceps PDK1 and PDK2 mRNA or protein content. PDK3 mRNA was lower (P<0.05) in 18h FAST than 18h FED only in IL-6 MKO mice (Table 4 and 5). PDP2 mRNA content was higher (P<0.05) in FAST than FED at 6h and 18h in both IL-6 MKO and Control mice (Table 4). There were no effects of fasting or genotype on SIRT3 mRNA content (Table 5).

Quadriceps PDK4 mRNA was higher (P<0.05) in FAST than FED at both 6h and 18h and lower (P<0.05) at 18h FED than 6h FED in both IL-6 MKO and Control mice (Figure 5A). PDK4 protein content in quadriceps tended to be higher (0.05≤P<0.1) and was higher (P<0.05) in FAST than FED at 6h in Control and IL-6 MKO, respectively, and was higher (P<0.05) in FAST than FED at 18h in both genotypes. PDK4 protein also tended to be higher (0.05≤P<0.1) in 18h FED than 6h FED within IL-6 MKO. Furthermore, PDK4 protein was higher (P<0.05) in IL-6 MKO than Control in both 18h FED and 18h FAST (Figure 5B). Quadriceps PDP1 mRNA was lower (P<0.05) in 6h FAST than 6h FED in both genotypes (Figure 5C) and PDP1 protein was higher (P<0.05) in 18h FAST than 18h FED in Controls only (Figure 5D).
Discussion

The main findings of the present study are that lack of skeletal muscle IL-6 resulted in elevated resting RER in the fed state, while the RER in the fasted state was similar in IL-6 MKO and Control. Furthermore, skeletal muscle PDHa activity was higher and PDH phosphorylation lower in IL-6 MKO than Control in the fasted state, but knockout of muscle IL-6 did not prevent the ability to regulate skeletal muscle PDH in response to fasting. Taken together this indicates that skeletal muscle IL-6 regulates substrate utilization at rest potentially through effects on skeletal muscle PDH, whereas muscle IL-6 is not required for fasting-induced substrate switch and skeletal muscle PDH regulation in mice.

The fasting-induced changes in plasma glucose and FFA, reduction in whole body RER, reduced PDHa activity, elevated PDH phosphorylation and changed PDK4 and PDP1 protein content in skeletal muscle of control mice are in accordance with previous findings (18; 34). The present experimental set-up therefore provides the basis for examining the impact of skeletal muscle IL-6 on fasting induced PDH regulation.

The present observation that lack of muscle IL-6 was associated with elevated RER in the fed and early fasting state indicating higher carbohydrate oxidation in IL-6 MKO than Control mice is novel, but in accordance with our previous finding that RER was higher in IL-6 MKO mice than Control during treadmill running (12). The finding that the activity level overall was similar in the two genotypes indicates that differences in activity level cannot explain the higher RER value in IL-6 MKO mice than Control. Together this suggests that skeletal muscle IL-6 exerts an inhibitory effect on carbohydrate oxidation with concomitant higher fat oxidation both in the resting fed state and during running. This is in accordance with previous studies reporting that IL-6 infusion in humans increased fat oxidation (36). However, the observation that RER was reduced to a similar level in IL-6 MKO and Control mice from approximately 8h of fasting shows that muscle IL-6 is not required for the fasting-induced metabolic switch towards fat oxidation in mice. This is in line with our previous observations that although IL-6 MKO mice exercised with higher RER than Control mice, muscle IL-6 was not mandatory for the shift in substrate utilization during
prolonged exercise as evidenced by a similar reduction in RER during 2h of running (12). Furthermore, the reduction in RER with fasting independent of muscle IL-6 in the present study is supported by the observations that the changes in plasma FFA and glucose as well as reduction in muscle glucose and glycogen occurred similarly in IL-6 MKO and Control mice. On the other hand, this genotype independent fasting response in plasma FFA is not in accordance with the previous finding that whole body IL-6 KO mice had a blunted fasting-induced increase in plasma FFA (39) indicating that this effect may have been mediated by IL-6 from other tissues than skeletal muscle. Taken together this suggests that muscle IL-6 affects the level of carbohydrate oxidation both at rest and during exercise, but is not required for the switch in substrate utilization towards increased fat oxidation during fasting and prolonged exercise.

The finding that IL-6 MKO mice had elevated quadriceps PDHa activity at 18h in both the fed and the fasted state relative to Control is in accordance with our previous finding in IL-6 MKO mice at rest and during exercise (12). This provides further evidence that muscle IL-6 regulates PDHa activity in mouse skeletal muscle. However, the similar reduction in PDHa activity in response to fasting in IL-6 MKO and Control mice shows that muscle IL-6 is not required for this regulation of PDH. This is further in line with the previous observation that IL-6 MKO mice downregulated PDHa activity during prolonged exercise as seen in Control mice (12). The observed genotype difference in PDHa activity in fed mice at 18h (8pm) is in accordance with the higher RER in IL-6 MKO than Control mice suggesting a PDH-mediated regulation of substrate utilization. On the other hand, the findings that the same genotype difference in PDHa activity was present at 18h of fasting without any genotype difference in RER indicate that the in vitro measured PDHa activity does not necessarily reflect the in vivo substrate flux through PDH as previously pointed out (6). In addition, the finding that lack of skeletal muscle IL-6 only affected PDHa activity at 18h and not 6h is not in accordance with our previous study, where PDHa activity was higher in IL-6 MKO mice than Control at rest in the fed state (12). It is however possible that the exact time of the day influences the level of PDHa activity and therefore potentially the impact of fasting and skeletal muscle IL-6 on PDHa activity. Hence, a previous study has shown that muscle PDHa activity fluctuates over the day (8), which is supported by the
present observation that PDHa activity was higher in mice euthanized at 8pm than at 8am. The observation that the genotype difference in PDHa activity at 18h of fasting was associated with lower PDH phosphorylation at Ser 293 and Ser 300 as well as lower PDK4 protein in IL-6 MKO than Control mice provides a potential underlying mechanism for the IL-6 mediated regulation of PDHa activity in skeletal muscle. Previous studies have reported that an elevated plasma fatty acid concentration increased PDK4 expression in rat skeletal muscle (37) suggesting that fatty acid-mediated upregulation of PDK4 contributes in fasting-induced downregulation of PDHa activity in skeletal muscle. However, the present finding that fasting was associated with a similar increase in circulating FFA in IL-6 MKO and Control does not support that differences in fatty acid availability mediated the observed genotype difference in PDK4 protein although intracellular differences in fatty acid concentration may be present. Furthermore, reduced muscle glycogen has previously been shown to be associated with more marked exercise-induced PDH dephosphorylation and PDHa activity increase in humans (19), but the similar levels of muscle glycogen both in the fed and fasted state do not support that the effects of muscle IL-6 on PDH regulation were through differences in muscle glycogen. On the other hand, the observed lower muscle G-6-P in IL-6 MKO than Control at 6h independent of food availability may indicate a different glycolytic flux when IL-6 is lacking. However, as this genotype difference in G-6-P only was evident at 6h, where PDHa activity was similar, a potential relationship between glycolytic flux, PDHa activity and muscle IL-6 in not directly evident. In addition, the finding that the associated genotype differences in PDH phosphorylation and PDK4 protein content were not present in the fed state at 18h in the current study or a previous study (12), where a genotype difference in PDHa activity was evident, indicates that additional molecular mechanisms are involved in the IL-6 mediated regulation of PDH in the fed state. In accordance, an impact of acetylation in PDH regulation is supported by the present observation that PDH-E1α acetylation tended to increase with 18h of fasting, similar to the observations in a previous study (14). However, the lack of a genotype difference in the level of PDH acetylation and the similar fasting-induced increase in PDH-E1α acetylation in
IL-6 MKO and Control mice in the present study do not support that IL-6 exerts effects on PDH regulation via PDH-E1α acetylation, although the level appears lower in IL-6 MKO than Control mice.

The present finding that quadriceps IL-6 mRNA increased in control mice after both 6h and 18h of fasting is in accordance with a previous mouse study (39) together supporting that fasting is indeed regulating skeletal muscle IL-6 expression with potential effects on metabolic regulation during fasting. However, the unchanged plasma IL-6 in the present study in response to fasting is in contrast to the marked increase reported in the previous study (39). The lack of change in plasma IL-6 in the current study together with the similar plasma IL-6 level in IL-6 MKO and Controls suggests that the observed genotype differences in skeletal muscle PDH regulation and whole body RER are not mediated directly by circulating IL-6. Furthermore, while previous studies have indicated that IL-6 induces AMPK phosphorylation in skeletal muscle (2; 16; 17), the present observation that the fasting-induced increase in AMPK and ACC phosphorylation at 18h was unaffected by knockout of muscle IL-6 provides evidence that skeletal muscle IL-6 is not needed for fasting-induced AMPK signaling in mouse skeletal muscle. In addition, the similar level of the typical IL-6 signaling markers (23), JNK and p38 phosphorylation as well as SOCS mRNA, in skeletal muscle from IL-6 MKO and Control mice further indicates that muscle IL-6 either signals through other intracellular signaling pathways or exerts effects in skeletal muscle indirectly as also previously suggested (12).

The observations that the decline in RER with 18h of fasting in the present study was associated with increased AMPK, ACC and p38 phosphorylation in skeletal muscle suggest that these metabolic regulators may contribute to the metabolic switch in substrate utilization during prolonged fasting. Furthermore, the similar fasting-induced increase in AMPK, ACC and p38 phosphorylation as well as downregulation in PDHa activity in IL-6 MKO and Control mice in the present study supports that AMPK and/or p38 signaling potentially could play a role in the fasting-induced regulation of PDHa activity in skeletal muscle as previously suggested (2; 20), although additional studies are needed to clarify this. Furthermore, the
changes in plasma and muscle substrates clearly reflected that food was not available at both 6h and 18h. This was however only associated with fasting-induced regulation of AMPK and ACC phosphorylation as well as PDHa activity at 18h of fasting demonstrating dissociation between metabolic changes and changes in these molecular markers in the early period of fasting. This may suggest that more prolonged metabolic changes than 6h are required to induce the molecular adjustments. The already reduced RER value at 6h of fasting, although not as marked as at 18h of fasting, may therefore indicate that the changes in substrate delivery is sufficient to drive an initial switch towards fat oxidation without molecular adjustments in key metabolic enzymes.

In conclusion, lack of muscle IL-6 elevated resting whole body RER in the fed state but not during fasting. Furthermore, IL-6 MKO mice had elevated skeletal muscle PDHa activity both in the fed and fasted state. This suggests that muscle IL-6 regulates resting substrate utilization in the fed state via effects on skeletal muscle PDH while additional factors contribute during fasting. In addition, IL-6 MKO mice maintained the ability to elicit fasting-induced substrate switch and down-regulation of skeletal muscle PDHa activity, showing that muscle IL-6 is not required for metabolic flexibility during fasting.


Ref Type: Generic


Acknowledgements

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Table and figure legends

Table 1

Primers and Taqman probe sequences used for PCR. Interleukin (IL), pyruvate dehydrogenase kinase (PDK), pyruvate dehydrogenase phosphatase (PDP), sirtuin (SIRT) and suppressor of cytokine signalling (SOCS).

Table 2

Plasma glucose and non-esterified fatty acids (NEFA) at 6h fed (6h FED), 6h fasting (6h FAST), 18h fed (18h FAST), and 18h fasting (18h FAST) in skeletal muscle-specific IL-6 knockout (MKO) and littermate floxed control (Control) mice. Values are given as mean ± SE; (n=10-11). *: Significantly different from FED at same time point within given genotype, P<0.05. #: Significantly different from 6h FED within given genotype, P<0.05.

Table 3

Quadriceps muscle glucose, glucose-6-phosphate (G-6-P), and glycogen concentrations at 6h fed (6h FED), 6h fasting (6h FAST), 18h fed (18h FAST), and 18h fasting (18h FAST) in skeletal muscle-specific IL-6 knockout (MKO) and littermate floxed control (Control) mice. Values are given as mean ± SE; (n=10-11). *: significantly different from FED at same time point within given genotype, P<0.05. #: Significantly different from Control within given time point, P<0.05.

Table 4
Quadriiceps protein content and phosphorylation level in muscle specific IL-6 (MKO) and littermate floxed control (Control) mice at 6h fed (6h FED), 6h fasting (6h FAST), 18h fed (18h FED) and 18h fasting (18h FAST). Pyruvate dehydrogenase (PDH)-E1a, PDH kinase (PDK), sirtuin (SIRT), AMP activated protein kinase (AMPK), acetyl CoA carboxylase (ACC), stress-activated protein kinase/c-Jun-amino-terminal kinase(SAPK/JNK) Thr183/Tyr185 phosphorylation (JNK), p38 mitogen activated protein kinase (p38 MAPK).

Protein content and phosphorylation level are given in arbitrary units (AU). Values are given as mean±SE; n=10-11. *: significantly different from FED at same time point within given genotype, P<0.05. (*) : tends to be significantly different from FED at same time point within given genotype, 0.05≤P<0.1. ¤: significantly different from 6h FED within given genotype, P<0.05; (¤): tends to be significantly different from 6h FED within given genotype, 0.05≤P<0.1.

Table 5

Quadriiceps mRNA content in muscle specific IL-6 (MKO) and littermate floxed controls (Control) at 6h fed (6h FED), 6h fasting (6h FAST), 18h fed (18h FED), and 18h fasting (18h FAST). Pyruvate dehydrogenase kinase (PDK), pyruvate dehydrogenase phosphatase (PDP), sirtuin (SIRT). The mRNA content is normalized to single stranded (ss) DNA content in the samples. Values are given as mean ± SE; (n=10-11). *: significantly different from FED at same time point within given genotype, P<0.05. (*) : tends to be significantly different from FED at same time point within given genotype, 0.05≤P<0.1.

Figure 1

Indirect calorimetry and locomotor activity: A) 26 hours of continuous respiratory exchange ratio (RER) and locomotor activity B) average RER divided into light and dark phases in the fed and the fasted state in skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed control (Control) mice. Values are
given as mean ± SE; (n=8-10). *: Significantly different from FED at same time point within given genotype, P<0.05. #: Significantly different from Control within given time point, P<0.05. Yellow line (2am) marks beginning of fast.

**Figure 2**

IL-6 content and signaling pathways: **A)** Plasma IL-6 and quadriceps muscle **B)** IL-6 mRNA, **C)** SOCS3 mRNA **D)** P38 MAPK Thr180/Tyr182 phosphorylation at 6h fed (6h FED), 6 h fasting (6h FAST), 18h fed (18h FAST), and 18h fasting (18h FAST) in skeletal muscle-specific IL-6 knockout (MKO) and littermate floxed control (Control) mice. The mRNA content is normalized to singlestranded (ss) DNA content in the samples. The phosphorylation level is given in arbitrary units (AU). Values are given as mean ± SE; n=10. *: significantly different from FED at same time point within given genotype, P<0.05. #: significantly different from Control within given time point, P<0.05.

**Figure 3**

**A)** AMP-activated protein kinase (AMPK) Thr172 phosphorylation and **B)** Acetyl-CoA carboxylase 2 (ACC2) phosphorylation at 6h fed (6h FED), 6 h fasting (6h FAST), 18h fed (18h FAST), and 18h fasting (18h FAST) in skeletal muscle-specific IL-6 knockout (MKO) and littermate floxed control (Control) mice. Values are given as mean ± SE; n=9-10. Phosphorylation levels are given in arbitrary units (AU). *: significantly different from FED at same time point within given genotype, P<0.05. (*): Tendency to be significantly different from FED at same time point within given genotype, 0.05<P<0.1. #: significantly different from Control within given time point, P<0.05.
Figure 4

**A)** PDHα activity **B)** total lysine acetylation of immuno-precipitated PDH-E1α protein **C)** PDH<sup>Ser293</sup> phosphorylation **D)** PDH<sup>Ser300</sup> phosphorylation **E)** PDH<sup>Ser232</sup> phosphorylation **F)** PDH<sup>Ser295</sup> phosphorylation at 6h fed (6h FED), 6 h fasted (6h FAST), 18h fed (18h FAST), and 18h fasted (18h FAST) in skeletal muscle-specific IL-6 knockout (MKO) and littermate floxed control (Control) mice. Values are given as means ± SE; (n=10-11). Protein and phosphorylation (phos) levels are given in arbitrary units (AU). *: significantly different from FED at same time point within given time point, P<0.05. (*): tends to be significantly different from FED at same time point within given genotype, 0.05≤P<0.1. ¤: significantly different from 6h FED within given genotype, P<0.05; (¤): tends to be significantly different from 6h FED within given genotype, 0.05≤P<0.1.

Figure 5

Covalent regulators of PDH: **A)** PDK4 mRNA **B)** PDK4 protein **C)** PDP1 mRNA and **D)** PDP1 protein at 6h fed (6h FED), 6 h fasting (6h FAST), 18h fed (18h FAST), and 18h fasting (18h FAST) in skeletal muscle-specific IL-6 knockout (MKO) and littermate floxed control (Control) mice. The mRNA content is normalized to single stranded (ss) DNA content in the samples. Protein levels are given in arbitrary units (AU). *: significantly different from FED at same time point within given genotype, P<0.05. (*): tends to be significantly different from FED at same time point within given genotype, 0.05≤P<0.1. ¤: significantly different from 6h FED within given genotype, P<0.05; (¤): tends to be significantly different from 6h FED within given genotype, 0.05≤P<0.1.
Representative blots of PDH-E1α, PDK1, PDK2, SIRT3, AMPKα1, AMPKα2, ACC2, lysine acetylated pdh-E1α, JNK1/2 protein content and JNK1/2 Thr183/Tyr185 phosphorylation at 6h fed (6h FED), 6 h fasted (6h FAST), 18h fed (18h FAST), and 18h fasted (18h FAST) in skeletal muscle-specific IL-6 knockout (MKO) and littermate floxed control (Ctrl) mice.
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<th>TaqMan probe</th>
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Table 1: Primer and TaqMan probe sequences used in real time PCR. Interleukin (IL), pyruvate dehydrogenase kinase (PDK), pyruvate dehydrogenase phosphatase (PDP), sirtuin (SIRT) and suppressor of cytokine signalling (SOCS).
Figure 1

Respiratory exchange ratio

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*Significant difference compared to control
Table 2. Plasma glucose and NEFA concentrations.

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Table 3. Muscle glucose, glycogen and G-6-P concentrations
Table 4: Quadriceps protein content

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Figure 2

A

![Plasma IL-6 (pg/ml)]

B

![IL-6 mRNA/ssDNA](Control)

C

![SOCS3 mRNA/ssDNA](Control)

D

![P38 MAPK Thr180/Tyr182 phosphorylation (AU)](Control)
Figure 4

A

![Bar graph showing PDH activity (mmol/min/kg) across different conditions.]

B

![Western blot showing acPDH-E1α protein expression.]

C

![Bar graph showing PDH-Ser293 phosphorylation (AU) across different conditions.]

D

![Bar graph showing PDH-Ser300 phosphorylation (AU) across different conditions.]

E

![Western blot showing PDH-Ser232 phosphorylation.]

F

![Bar graph showing PDH-Ser295 phosphorylation (AU) across different conditions.]

Legend:
- Ctrl: Control
- IL-6 MKO: IL-6 Mouse Knockout
- 6h FED: 6 hours of Fasting
- 18h FED: 18 hours of Fasting
- 6h FAST: 6 hours of Feeding
- 18h FAST: 18 hours of Feeding

Note: The figure includes various panels showing the effects of different treatments on PDH activity and protein expression. The graphs and blots illustrate changes in phosphorylation levels and protein expression across different conditions, with statistical significance indicated by symbols.* and #.
Figure 5

A

![Graph A: PDK4 mRNA/ssDNA](image)

B

![Graph B: PDK4 protein (AU)](image)

C

![Graph C: PDP1 mRNA/ssDNA](image)

D

![Graph D: PDP1 protein (AU)](image)
Figure 6
STUDY III
Title: PGC-1α and fasting-induced PDH regulation in mouse skeletal muscle

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Keywords
PGC-1α, PDH, skeletal muscle, metabolism, fasting, substrate utilization

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**Introduction**

Skeletal muscle plays a major role in lipid and carbohydrate (CHO) utilization and is instrumental in maintaining metabolic flexibility with various metabolic challenges. Specifically fasting robustly prompts the protection of CHO stores through a coordinated decrease in skeletal muscle glucose oxidation and a switch towards fat oxidation. The pyruvate dehydrogenase complex (PDC) is thought to be a key element in such regulation of substrate utilization [38]. Hence, PDC controls the access of CHO to the TCA cycle by irreversibly converting pyruvate to acetyl CoA thereby bridging glycolysis and oxidative substrate utilization [16]. However, the detailed regulation of PDH during energy deprivation is not fully understood and merits further investigation.

The main regulation of PDH activity is thought to be through inhibitory phosphorylation by pyruvate dehydrogenase kinases (PDKs) and activating dephosphorylation by pyruvate dehydrogenase phosphatases (PDPs) at four known serine residues on the catalytic subunit PDH-E1α (PDH) [16;21;30;39]. In addition, recent studies point towards other important post translational modifications as the mitochondrially located deacetylase Sirtuin 3 (SIRT3) has been reported to regulate the acetylation state of PDH-E1α and thus the activity of PDH [10;19;28]. Previous studies have demonstrated that fasting reduces the activity of PDH in the active form (PDHa activity) [41;42], increases PDH$^{\text{Ser293}}$ and PDH$^{\text{Ser300}}$ phosphorylation [21], increases PDH-E1α acetylation as well as PDK4 mRNA [32;42;47] and protein [21;41;42] in rodent skeletal muscle. Furthermore, fasting has been reported to elicit a muscle type dependent regulation of PDK
activity and PDK4 expression in rat skeletal muscle (Sugden et al 2000). However, the factors determining this are not resolved.

PGC-1α, first discovered and identified as a PPARγ-binding protein in brown adipose tissue [33], is a transcriptional coactivator that has been established to be an important regulator of mitochondrial biogenesis. Hence, muscle-specific PGC-1α overexpression mice have been shown to have increased and conversely whole-body PGC-1α knockout and muscle specific PGC-1α knockout mice to have lowered content of oxidative proteins in skeletal muscle [13;23;25]. Furthermore, muscle specific PGC-1α overexpression mice have been shown to exhibit lower RER during treadmill running [3;46]. This may be due to the enhanced skeletal muscle oxidative capacity of these mice [24], but may also be related to metabolic flexibility and regulation of substrate use. In accordance, the level of skeletal muscle PDH-E1α protein content has been shown to follow differences in the level of muscle PGC-1α in mice [21] and PGC-1α to regulate PDK4 expression in mouse skeletal muscle [3;21;43]. In addition, the fasting-induced down-regulation of PDHα activity was blunted in PGC-1α KO mice [21] supporting that PGC-1α plays a role in PDH mediated metabolic regulation during fasting. However, the impact of PGC-1α on the switch from CHO to fat utilization during the transition from the fed to the fasted state, and on the level of fat utilization in the fasted state and the associated regulation of PDH in skeletal muscle is not fully resolved.

Therefore the aim of the present study was to examine whether lack of muscle PGC-1α 1) affects the time course of the switch in substrate utilization during the transition from a fed to fasted state, 2) affects regulation of fasting-induced changes in PDHα activity, PDH phosphorylation and PDH acetylation in skeletal muscle.
Methods

Animals

Generation of PGC-1α MKO mice used in the present study was carried out as described previously [13;25]. The mice were genotyped using PCR-based muscle and tail genotyping as previously described [23] and the genotype was confirmed based on determination of PGC-1α mRNA in muscle tissue after euthanization. Animals were kept on a 12:12 light-dark cycle at 22°C with ad libitum access to water and chow diet (Altromin 1314F, Brogaarden, Lynge, Denmark) until the intervention of the experiment. All experiments were approved by the Danish Animal Experimental expectorate and complied to the European Convention for the protection of vertebrate animals used for experiments and other scientific purposes (Council of Europe no. 123. Strasbourg, France 1985).

Fasting procedure

At 3 months of age, female mice were individually housed for 3 days and subsequently allocated to either a fed group (FED), which maintained ad libitum access to both food and water or a fasted group (FAST) for which the food was removed at 6am and only water was available for the following 24 hours. Fed and fasted mice were euthanized by cervical dislocation at the end of the 24 hours intervention period and quadriceps muscles were rapidly excised and snap frozen in liquid nitrogen. Trunk blood was obtained in EDTA containing tubes and plasma was obtained after centrifugation. Both muscle and plasma were stored at -80°C. A subset of mice (individually housed) was acclimatized to the environment of the cabinets in a Phenomaster unit (TSE Systems, Bad Hamburg, Germany) for 3 days. The respiratory exchange ratio (RER) and cage activity were
then continuously measured for 24 hours with ad libitum access to food and water followed by 24 hours of fasting with only access to water.

**Plasma analyses**

Plasma free fatty acid concentrations were measured using a NEFA-HR kit according to the manufacturer’s guidelines (WAKO Diagnostics GmbH, Germany). Plasma glucose and lactate were measured fluorometrically as previously described [27].

**Muscle analyses**

Whole quadriceps muscles were crushed in liquid nitrogen to achieve tissue homogeneity. For measurements of muscle glucose, lactate, and glucose-6-phosphate (G-6-P), 10-15mg of crushed muscle tissue was extracted in perchloric acid (PCA) and neutralized to a pH of 7-8. Muscle glycogen was determined fluorometrically as glycosyl units after hydrolyzing 10-15 mg wet weight muscle samples by boiling for 2 hours in 1M HCl as previously described [27].

**Immunoblotting**

Crushed muscle samples (25-30mg) were homogenized in lysis buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na₃VO₄, 3 mM benzamidine, and deacetylase inhibitors (nicotinamide (1mM) and sodium butyrate (5mM), pH 7.5) using a Tissue Lyser II (Qiagen, Germany). Protein concentration in each of the samples was
determined using the bicinchoninic acid method (Thermo Fischer Scientific, USA) and protein concentration was adjusted with sample buffer to a concentration of 1µg/µl. Protein phosphorylation and protein content were determined by SDS-PAGE using hand casted gels and western blotting. PVDF membranes were incubated in primary antibody overnight at 4°C for determination of AMPKα2, PDK4 and PDH-E1α protein, PDH-E1α$^{\text{Ser293}}$, PDH-E1α$^{\text{Ser300}}$, and PDH-E1α$^{\text{Ser295}}$ phosphorylation (all kindly provided by Professor Grahame Hardie, University of Dundee, Scotland), Hexokinase (HK) II protein, AMPK$^{\text{Thr172}}$ phosphorylation, total lysine acetylation (#2867, #2535 and #9441, respectively, Cell Signaling Technologies, Danvers, MA, USA), acetyl CoA carboxylase (ACC)$^{\text{Ser212}}$ phosphorylation and PDH-E1α$^{\text{Ser232}}$ phosphorylation (07-303 and #AP1063, respectively, EMD Millipore, Bedford, USA), TBC1D4, PDK1 and OXPHOS proteins (ab24469, ab90444 and ab110413, respectively, Abcam, Cambridge, U.K.), PDK2 protein (ST1643, CalBioChem, Bedford, USA), PDP1 protein (Sigma-Aldrich, St. Louis, USA) and GLUT4 protein (PAI-1065, ABR, Connecticut, USA). Species-specific horseradish peroxidase conjugated immunoglobulin secondary antibodies (DAKO, Denmark) were used for incubation the following day. ACC2 protein was detected using streptavidin (Dako, Glostrup, Denmark). Protein bands were subsequently visualized using an ImageQuant LAS 4000 imaging system and quantified with ImageQuant TL 8.1 software (GE Healthcare, Freiburg, Germany).

**Immunoprecipitation and PDH-E1α acetylation**

A total of 200 µg protein from lysate was immuno-precipitated for the determination of global PDH-E1α acetylation. Briefly, the lysate was added to PBS-rinsed protein G agarose beads (EMD Millipore, Bedford, USA) in a 50:50 solution with PBS buffer containing 0.5% Triton X with 2µg of
PDH-E1α antibody. The samples were rotated end over end at 4°C overnight and on the subsequent day the beads were washed, sample buffer was added and the samples were heated at 96°C for 3 minutes. The beads were spun down to avoid transfer and lysate loaded on a hand-casted gel for SDS-page and western blotting as described above. For each sample acetylated protein was normalized to the amount of precipitated PDH-E1α protein content determined by western blotting.

**PDHa activity**

PDHa activity was determined after homogenizing 10-15mg of wet weight muscle tissue and snap-freezing the homogenate in liquid nitrogen as previously described [6;7;31;34] and normalized to creatine content in each muscle sample.

**Statistics**

All values are expressed as means ± standard error. A two-way ANOVA was applied to test the effects of genotype and intervention. When a main effect was detected, a Student-Newman-Keuls test was applied as a post-hoc test to locate differences. For single grouped data, a student’s t-test was used to test if a difference was present. Significance was accepted at P values less than 0.05 and a tendency for 0.05≤P≤0.1. The statistical tests were performed using Sigmaplot 13.0 (Systat, USA).
Results

Metabolic markers

OXPHOS and GLUT4 basal protein levels were lower (P<0.05) in PGC-1α MKO than control mice. There were no differences in basal HKII and TBC1D4 protein between genotypes (Fig. 1).

Indirect calorimetry and locomotor activity

RER was overall lower (P<0.05) and activity higher (P<0.05) in PGC-1α MKO than control mice at night in the fed state, while RER was overall higher (P<0.05) and activity lower (P<0.05) in PGC-1α MKO than control mice at night in the fasted state (Fig. 2a and b). In the final hour of the dark, fed period (5-6 am), RER was lower (P<0.05) and in the last hour of dark, fasting period higher (P<0.05) in PGC-1α MKO than control mice (Fig. 2c).

Plasma glucose and NEFA

The plasma free fatty acid concentration increased (P<0.05) and the plasma glucose concentration decreased (P<0.05) in both PGC-1α MKO and control mice with fasting. There were no differences in either NEFA or glucose plasma levels between genotypes (Table 1).
**Muscle metabolites**

Muscle glucose and glycogen concentrations decreased (P<0.05) in both PGC-1α MKO and control mice in response to fasting with no difference between genotypes. G-6-P decreased (P<0.05) with fasting in PGC-1α MKO and tended to be lower (0.1≤P≤0.05) in PGC-1α MKO mice than control mice (Table 2).

**AMPK and ACC**

There were no differences in either absolute (Fig.3a) or normalized AMPK phosphorylation or in AMPKα2 protein in skeletal muscle with fasting or between genotypes (Fig.3b). There was a tendency for an increase (0.05≤P≤0.1) in absolute ACC phosphorylation (Fig.3c) with fasting in control mice, while there was no difference in normalized ACC phosphorylation with fasting in control mice (Fig.3d). Fasting increased (P<0.05) both absolute (Fig.3c) and normalized ACC phosphorylation (Fig.3d) in skeletal muscle of PGC-1α MKO mice, while ACC2 protein was lower (P<0.05) in PGC-1α MKO than control mice in the fed state.

**PDHa activity and PDH-E1α protein**

PDHa activity decreased (P<0.05) markedly in skeletal muscle with 24 hours of fasting in both PGC-1α MKO and control mice with no difference between genotypes (Fig.4a). PDH-E1α protein content increased (P<0.05) in both genotypes after 24 hours of fasting relative to fed and PDH-E1α protein was lower (P<0.05) in PGC-1α MKO than control mice in both the fed and fasted state (Fig.4b).
PDH phosphorylation

PDH-E1α PDH$^{\text{Ser293}}$, PDH$^{\text{Ser300}}$, PDH$^{\text{Ser232}}$, and PDH$^{\text{Ser295}}$ phosphorylation increased (P<0.05) with 24 hours of fasting, regardless of genotype. PDH$^{\text{Ser293}}$ and PDH$^{\text{Ser300}}$ phosphorylation (P<0.05) was lower (P<0.05) in PGC-1α MKO than control mice in the fasted state (Fig.5a, c, e, g). When normalized to PDH-E1α protein, phosphorylation sites PDH$^{\text{Ser293}}$ and PDH$^{\text{Ser300}}$ increased (P<0.05) to a similar level with fasting in PGC-1α MKO and control mice, while PDH$^{\text{Ser232}}$ overall was higher (P<0.05) in PGC-1α MKO than control mice and PDH$^{\text{Ser295}}$ tended to be higher (0.05≤P≤0.1) in PGC-1α MKO than control mice both in the fed state and after 24 hours of fasting (Fig.5b, d, f, h).

PDK1, 2, 4 and PDP1

There was an overall tendency for lower (P<0.05) skeletal muscle PDK1 protein in PGC-1α MKO than control mice, while PDP1 protein and PDK2 protein were lower (P<0.05) in PGC-1α MKO than control mice with no effect of the fasting intervention (Fig.6a-c). PDK4 protein increased (P<0.05) in both genotypes after 24 hours of fasting and was lower (P<0.05) in PGC-1α MKO than control mice both in the fed and the fasted state (Fig.6d).

SIRT3 and PDH acetylation

Skeletal muscle SIRT3 protein was lower (P<0.05) in PGC-1α MKO than control mice in the fed state and increased (P<0.05) after 24 hours of fasting only in control mice (Fig.7a). PDH-E1α
acetylation was higher (P<0.05) in PGC-1α MKO than control mice in the fed state, but was not different between genotypes after 24 hours of fasting (Fig. 7b).
Discussion

The main findings of the present study are that lack of muscle PGC-1α did not affect the switch from CHO to fat utilization in the transition from the fed to the fasted state, but was associated with higher CHO use in the fasted state potentially influencing the ability to endure prolonged starvation. Fasting-induced down-regulation of PDHα activity in skeletal muscle of control mice was associated with increased phosphorylation of all four known sites in PDH-E1α as well as with increased PDK4 and SIRT3 protein without changes in total acetylation of PDH-E1α. Lack of muscle PGC-1α reduced PDH-E1α, PDK1, 2, 4, PDP1, and SIRT3 protein content as well as increased total lysine PDH-E1α acetylation in the fed state. Knockout of muscle PGC-1α did not influence the fasting-induced increase in PDH-E1α phosphorylation, but prevented the fasting-induced increase in SIRT3 protein, while additional factors seem to override PGC-1α mediated regulation of PDH-E1α acetylation during fasting.

The observed switch in substrate utilization from CHO to fat and the lower RER value during fasting than in the fed state in the control mice are in accordance with numerous previous studies [12;18;20;49] demonstrating an experimental foundation for examining the impact of skeletal muscle PGC-1α on the ability to switch substrate during fasting. The present finding that lack of skeletal muscle PGC-1α had no clear impact on the time course of the change in RER during the switch from the fed to the fasted state suggests that skeletal muscle PGC-1α is not required for metabolic flexibility during this transition. This is as such in accordance with the previous observation that lack of muscle PGC-1α did not influence the fluctuations in RER over the course of 24 hours with access to food [11].
On the other hand, the present observation that the RER in the fed state was lower, indicating elevated fat oxidation, in PGC-1α MKO than control mice, is different from the previous reports that lack of muscle PGC-1α [11] as well as PGC-1α overexpression [3] did not affect RER in the fed state. Because the activity level was higher in PGC-1α MKO than control mice during the dark period in the fed state, and increased activity is expected to enhance carbohydrate utilization, the genotype difference in substrate oxidation in the fed state does not seem to be related to activity differences. Moreover, the similar plasma glucose and NEFA concentrations as well as similar muscle glycogen in fed PGC-1α MKO and control mice indicate that the RER differences in the fed state are not due to differences in substrate availability between the genotypes.

The observation that PGC-1α MKO mice had higher RER in the fasted state than the control mice indicates that lack of skeletal muscle PGC-1α reduced fat oxidation in the fasted state. The finding that the PGC-1α MKO mice overall were less physically active than the controls during the last 12 hours of fasting shows that the higher carbohydrate oxidation was not due to increased activity level in the PGC-1α MKO mice relative to control. Furthermore, the similar plasma glucose and NEFA concentrations as well as muscle glycogen level in the two genotypes suggests that differences in substrate availability did not elicit the difference in substrate utilization. However, while the decrease in plasma glucose and the increase in plasma NEFA concentration were independent of genotype, the finding that the absolute decrease in mean muscle glycogen was 44% greater in PGC-1α MKO than control mice supports the higher reliance on carbohydrates in the PGC-1α MKO than the control mice. Based on the previous observation that muscle specific PGC-1α overexpression resulted in reduced glycogen phosphorylase content and phosphorylation [44] with concomitantly reduced glycogen use during exercise, it may be suggested that the higher glycogen use in PGC-1α MKO mice in the present study is caused by an elevated glycogen
phosphorylase activity. In addition, the reduced fat oxidation in PGC-1α MKO mice relative to control mice in the fasted state may imply that low skeletal muscle PGC-1α content is associated with impaired carbohydrate sparing during food deprivation.

The present finding that SIRT3 protein increased with 24 hours of fasting in control mice is in agreement with previous findings [5;17;29]. ROS has been shown to increase in mouse skeletal muscle with fasting [35;37] and as SIRT3 has been shown to play a role in ROS scavenging [36;50] increasing SIRT3 protein may be an adaptation to handling a prolonged period of increased ROS production. SIRT3 protein has previously been shown to increase with fasting and caloric restriction in wild-type mice (Palacios et al., 2009) and to increase with both repeated AICAR injections and exercise training in a PGC-1α dependent manner using PGC-1α KO mice [2]. The present observation that skeletal muscle SIRT3 protein was lower in PGC-1α MKO mice than controls is therefore in accordance with previous studies. However, the present finding that SIRT3 protein content was completely unresponsive to 24 hours of fasting in PGC-1α MKO mice is novel.

Furthermore, the higher lysine acetylation level of PDH-E1α in PGC-1α MKO mice than controls in the fed state is in agreement with a previous study using myocytes [19] and with the present genotype difference in SIRT3 protein content. The lack of a genotype difference in PDH-E1α acetylation in the fasted state indicates on the other hand, that PGC-1α deficient mouse skeletal muscle is capable of maintaining the PDH acetylation state equal to control muscle despite reduced SIRT3 protein level. As muscle NAD+ levels have been shown to increase with fasting [4] this may upregulate SIRT3 activity [40] sufficiently in PGC-1α MKO mice to obtain an acetylation state as in control mice.
The present observation that 24 hours of fasting led to robust increases in absolute phosphorylation of all the four PDH phosphorylation sites, PDH\textsuperscript{Ser}\textsuperscript{293}, PDH\textsuperscript{Ser}\textsuperscript{300}, PDH\textsuperscript{Ser}\textsuperscript{232}, and PDH\textsuperscript{Ser}\textsuperscript{295}, in both genotypes is in line with the downregulation of PDH\textalpha activity and the previously reported fasting effects in PGC-1a KO and PGC-1a MCK mice [21]. On the other hand, the fasting-induced increase in skeletal muscle PDH\textsuperscript{Ser}\textsuperscript{232} phosphorylation has not been reported previously. The observed increase in PDH-E1\textalpha protein with fasting was unexpected, but might be explained by a possible increase in PGC-1\textalpha and thus mitochondrial content as previously reported in liver [15] and skeletal muscle [4;29]. However, the lack of a similar increase in OXPHOS protein with fasting in the present study (data not shown) does not support this notion. Regardless, it appears that PGC-1\textalpha is partly dispensable for fasting-induced increases in PDH-E1\textalpha protein.

PDH phosphorylation levels were also normalized to PDH-E1\textalpha protein content due to the impact of both genotype and intervention on PDH-E1\textalpha protein content. The observation that normalization of PDH phosphorylation to PDH-E1\textalpha protein resulted in similar or even higher levels of PDH phosphorylation in PGC-1\textalpha MKO than control mice indicates a maintained capability to regulate PDH relative to the amount of PDH-E1\textalpha protein despite lack of PGC-1\textalpha. Furthermore, the small genotype differences in both site PDH\textsuperscript{Ser}\textsuperscript{232} and PDH\textsuperscript{Ser}\textsuperscript{295} when normalized to PDH-E1\textalpha protein may be due to the site specific affinity of the different PDK isoforms as previously reported [22;30].

The finding that skeletal muscle PDK4 protein was markedly increased with fasting is in agreement with numerous previous studies in humans, rats, and mice [21;32;41;47;48;51]. Although PDK4 protein levels were lower in PGC-1a MKO than control mice both in the fed and the fasted state, the same absolute increase in PDK4 protein was evident in both genotypes underlining that
skeletal muscle PGC-1α plays a role in basal PDK4 expression, but is not necessary for the observed fasting-induced upregulation of PDK4 protein content. The finding that the protein content of PDP1, PDK1, and PDK2 were unaffected by fasting is in accordance with previous studies [1;41], but the lower protein levels of PDP1, PDK1, and PDK2 in PGC-1α MKO mice than controls has not been reported previously. This observation, in conjunction with reduced skeletal muscle OXPHOS and PDH-E1α content in PGC-1α MKO mice, as also previously shown in PGC-1α KO mice [21] indicates a reduced capacity for regulation of glucose oxidation. However, the present observation that the PDHa activity did not differ between genotypes suggests that additional factors influence PDHa activity. In addition, the observation that the genotype differences in RER were not reflected in differences in skeletal muscle in vitro PDHa activity supports the previous findings that there can be a dissociation between PDHa activity and actual flux through the PDC [8].

Skeletal muscle AMPK phosphorylation has been reported to increase in rodents with shorter fasting protocols [4;9;12]. However, the present finding of no change in AMPK phosphorylation indicates that AMPK does not exhibit increased phosphorylation levels after 24 hours of fasting as reported previously in mice [14] and humans [45]. It cannot be excluded that the mice in the present study had transiently increased AMPK activation during the fasting intervention in the hours prior to euthanization, and that this activation differed between genotypes, especially with differing locomotor activity in the light phase of both the fed and fasting intervention. The observed increase in phosphorylation of ACC with fasting likely contributing to the enhanced fatty acid oxidation (FAO) might reflect a preceding, transient activation of AMPK as previously observed in rat skeletal muscle after 12 hours of fasting, but not 48 hours of fasting [9]. On the other hand, ACC deactivation may also occur through a proposed fasting-induced β-adrenergic epinephrine/PKA axis [12]. The finding that ACC phosphorylation was higher in PGC-1α MKO mice
than controls during fasting when normalized to protein content may indicate fewer active ACC proteins and hence less malonyl CoA to inhibit lipid uptake into the mitochondria in PGC-1α MKO than control mice. Furthermore, the observed similar FFA plasma levels in the two genotypes suggests a similar delivery of fatty acids to the muscles in the two genotypes. These observations are therefore not in accordance with the higher RER values indicating elevated CHO oxidation in PGC-1α MKO mice than controls. However, both CD36 mRNA and CPT1 mRNA content have been shown to increase with fasting [12;26] and to be PGC-1α dependent [3] suggesting that the mitochondrial fatty acid transport step may limit fat oxidation during fasting in PGC-1α deficient muscle and hence potentially explain the reduced fat oxidation during fasting when PGC-1α is lacking.

In summary, the present study suggests that skeletal muscle PGC-1α plays an important role in skeletal muscle mitochondrial protein adaptations to prolonged fasting, as seen with PDH-E1α and SIRT3 is, but is dispensable for maintaining metabolic flexibility in the transition from the fed to the fasted state. Furthermore, lack of skeletal muscle PGC-1α reduces the content of PDH regulatory proteins and PDH-E1α protein as well as alters PDH phosphorylation and the acetylation pattern in skeletal muscle. In addition, while both fed and fasting-induced PDHa activities appear independent of PGC-1a, PGC-1a is required for fasting-induced SIRT3 protein upregulation in skeletal muscle.


**Table 1**) Plasma glucose and non-esterified fatty acids (NEFA) in fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed control (Control) mice. Values are given as mean ± SE; (n=8-10). *: Significantly different from FED within given genotype, P<0.05.

**Table 2**) Concentrations of skeletal muscle glucose, glucose-6-phosphate (G-6-P), and glycogen in fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed control (Control) mice. Values are given as mean ± SE; (n=11). *: significantly different from FED within given genotype, P<0.05. #: Significantly different from Control within given group, P<0.05. (#): Tendency to be significantly different from Control within given group, 0.05<P<0.01.

**Fig. 1 a**) Skeletal muscle total OXPHOS, GLUT4, HKII, and TBC1D4 protein content and b) Representative western blots from fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed control (Control) mice. Values are given as mean ± SE; (n=11). Protein levels are given in arbitrary units (AU). *: Significantly different from FED within given genotype, P<0.05. #: Significantly different from Control within given group, P<0.05. (#): Tendency to be significantly different from Control within given group, 0.05<P<0.01.

**Fig. 2 a**) 48 hours of continuous respiratory exchange ratio (RER) and locomotor activity b) average RER divided into light and dark phases of both the 24h fed and 24h fasting intervention c) average RER in the last hour of the fed and the fasted state in skeletal muscle-specific PGC-1α knockout
(MKO) and littermate floxed control (Control) mice. Values are given as mean ± SE; (n=8-10). *: Significantly different from FED within given genotype, P<0.05. #: Significantly different from Control within given group, P<0.05.

Fig. 3 a) AMP-activated protein kinase (AMPK) Thr172 phosphorylation b) AMPK Thr172 phosphorylation normalized to AMPKα2 protein content c) Acetyl-CoA carboxylase 2 (ACC2) Ser212 phosphorylation d) ACC Ser212 phosphorylation normalized to ACC2 protein content in quadriceps from fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed controls (Control) mice. Values are given as mean ± SE; (n=11). Protein and phosphorylation (phos) levels are given in arbitrary units (AU). *: Significantly different from FED within given genotype, P<0.05. #: Significantly different from Control within given group, P<0.05. (*): Tendency to be significantly different from FED within given genotype, 0.05<P<0.01.

Fig. 4 a) PDHα activity and b) total PDH-E1α protein in quadriceps muscle from fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed controls (Control) mice. Values are given as mean ± SE; (n=11). Protein levels are given in arbitrary units (AU). *: Significantly different from FED within given genotype, P<0.05. #: Significantly different from Control within given group P<0.05.
Fig. 5 a) PDH Ser293 phosphorylation  b) PDH Ser293 phosphorylation normalized to PDH-E1α protein content  c) PDH Ser300 phosphorylation  d) PDH Ser300 phosphorylation normalized to PDH-E1α protein content  e) PDH Ser232 phosphorylation  f) PDH Ser232 phosphorylation normalized to PDH-E1α protein content  g) PDH Ser295 phosphorylation  h) PDH Ser295 phosphorylation normalized to PDH-E1α protein content in quadriceps muscle from fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed controls (Control) mice. Values are given as mean ± SE; (n=11). Protein and phosphorylation (phos) levels are given in arbitrary units (AU). *: Significantly different from FED within given genotype, P<0.05. #: Significantly different from Control within given group, P<0.05. #: Tendency to be significantly different from Control within given group, 0.05<P<0.01.

Fig. 6 a) PDP1 protein content  b) PDK1 protein content  c) PDK2 protein content  d) PDK4 protein content in quadriceps muscle from fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed controls (Control) mice. Values are given as mean ± SE; (n=11). Protein levels are given in arbitrary units (AU). *: Significantly different from FED within given genotype, P<0.05. #: Significantly different from Control within given group, P<0.05. #: Tendency to be significantly different from Control within given group, 0.05<P<0.01.

Fig. 7 a) SIRT3 protein content  b) total lysine acetylation of immuno-precipitated PDH-E1α protein in quadriceps muscle from fed (FED) and 24h fasted (FAST) skeletal muscle-specific PGC-1α knockout (MKO) and littermate floxed controls (Control) mice. Values are given as mean ± SE; (n=11). Protein and acetylation levels are given in arbitrary units (AU). *: significantly different
from FED within given genotype, $P<0.05$. #: significantly different from Control within given group, $P<0.05$. 
Table 1. Plasma glucose and NEFA concentrations.

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<tr>
<td></td>
<td>Control</td>
<td>MKO</td>
<td>Control</td>
<td>MKO</td>
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<tr>
<td>Glucose (mmol/L)</td>
<td>6.11±0.2</td>
<td>6.03±0.2</td>
<td>4.03±0.17*</td>
<td>4.24±0.22*</td>
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<tr>
<td>NEFA (mmol/L)</td>
<td>0.15±0.01</td>
<td>0.13±0.01</td>
<td>0.23±0.01*</td>
<td>0.25±0.02*</td>
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Table 2. Muscle glucose, glycogen and G-6-P concentrations

<table>
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<td>Control</td>
<td>MKO</td>
<td>Control</td>
<td>MKO</td>
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<tr>
<td>Glucose (mmol/kg)</td>
<td>0.84±0.06</td>
<td>0.77±0.06</td>
<td>0.47±0.04*</td>
<td>0.48±0.03*</td>
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<tr>
<td>G-6-P (mmol/kg)</td>
<td>2.37±0.17</td>
<td>2.20±0.11</td>
<td>1.97±0.35</td>
<td>1.40±0.18*[#]</td>
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<tr>
<td>Glycogen (mmol/kg)</td>
<td>23.9±2.3</td>
<td>24.5±1.6</td>
<td>15.9±1.6*</td>
<td>13.0±1.5*</td>
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Figure 2

a) Light FED

b) 0-12h FED Light

RER (VCO2/VO2)

RER Control
RER MKO

Activity Control
Activity MKO

Figure 2b

Table: RER (VCO2/VO2) with significance levels:

- *: p < 0.05
- #: p < 0.01

Figure 2c

Table: RER (VCO2/VO2) with significance levels:

- *: p < 0.05
- #: p < 0.01

Legend:
- Control
- MKO
Figure 3

(a) AMPK Thr172 Phosphorylation (AU)
(b) AMPKa2 Phosphorylation (AU)
(c) ACC Ser212 Phosphorylation (AU)
(d) ACC2 Phosphorylation (AU)

Control
MKO
FAST

(*) P=0.063

(+) 75 kDa

50 kDa

250 kDa
Figure 4

(a) Graph showing PDH activity (mmol·min\(^{-1}\)·kg\(^{-1}\)) for Control and MKO groups under FED and FAST conditions. The bars indicate mean values with error bars representing standard deviations. Significant differences are denoted by asterisks (*).

(b) Western blot analysis of PDH-E1α protein expression under FED and FAST conditions for Control and MKO groups. The blots show bands at 50 kDa and 37 kDa, with asterisks (*) and hashes (#) indicating significant differences between groups.
Figure 5
Figure 7

(a) SIRT3 Protein (AU)

(b) Acetylation/PDH-E1α Protein (AU)
STUDY IV
The effect of training status on PDH regulation in human skeletal muscle during incremental exercise

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Key words: pyruvate dehydrogenase, skeletal muscle, acetylation, phosphorylation, exercise training, metabolic flexibility

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Key point summary

- The ability to fluently transition between fat and carbohydrate as fuel source is a key feature of exercising skeletal muscle and pyruvate dehydrogenase (PDH) has been suggested to be pivotal in substrate utilization and the switch between fuels.

- Exercise training increases the maximal capacity for substrate oxidation as well as fat utilization during submaximal exercise.

- This study shows that exercise-induced skeletal muscle PDH activation is closely matched to the relative exercise intensity at submaximal exercise, while reaching a higher level at maximal exercise in trained individuals. These responses are associated with increased PDH phosphorylation, acetylation and content of covalent regulators.

- The present findings provide knowledge to a better understanding of the physiology behind control of PDH regulation and improved substrate utilization occurring with increased training state.
Abstract

The ability to transition between lipid and carbohydrate (CHO), termed metabolic flexibility, is key to maintaining energy homeostasis and deliver proper fuel supply to the working muscle. Pyruvate dehydrogenase (PDH) is the gateway enzyme for CHO derived intermediates into the TCA cycle. PDH may play a central role in regulating substrate shifts during exercise, but the influence of training state on PDH regulation and metabolic flexibility during exercise is not fully elucidated. The purpose of this study was to investigate the impact of training state on metabolic flexibility and post-translational regulation of PDHa activity during incremental exercise. Eight untrained and nine endurance exercise trained healthy male subjects performed incremental exercise on a cycle ergometer: 40 min at 50% incremental peak power output (IPPO), 10 min at 65% (IPPO), followed by 80% (IPPO) until exhaustion. Exercising at the same relative intensity led to similar muscle PDH activation in untrained and trained subjects, whereas PDHa activity at exhaustion was higher (P<0.05) in trained than untrained. Trained subjects had higher (P<0.05) PDH-E1α, PDK2, PDK4, and PDP1 protein content as well as PDH phosphorylation and PDH acetylation. Furthermore, PDH acetylation was increased (P<0.05) in response to exercise only in trained subjects. In conclusion, PDHa activity and PDH dephosphorylation were correspondingly well adjusted to the relative exercise intensity during submaximal exercise, while the higher PDH activity in trained than untrained at exhaustion seems related to differences in glycogen utilization rather than differences in PDH phosphorylation and acetylation state, although site-specific contributions cannot be ruled out.
Introduction

The ability to switch between carbohydrate and fat utilization during changes in energy demand and substrate delivery is a trademark feature of skeletal muscle (Randle, 1964), termed metabolic flexibility (Kelley & Mandarino, 2000). Such adjustments are evident with enhanced carbohydrate utilization during increasing intensity of exercise, and increased fat utilization during prolonged exercise (Henriksson, 1977; Holloszy & Booth, 1976; Kelley, 2005; Watt et al., 2004). Endurance exercise training is known to increase the reliance on β-oxidation during submaximal exercise (Burgomaster et al., 2008) concomitant with increased levels of circulating free fatty acids (Klein et al., 1994) providing a glucose sparing effect (Hermansen et al., 1967; Jansson & Kaijser, 1987). At the same time, exercise trained individuals possess the ability to obtain a higher carbohydrate oxidation at maximal exercise than untrained (Holloszy, 1967; LeBlanc et al., 2004a) together reflecting performance enhancing metabolic adjustments with endurance exercise training. The Randle cycle originally proposed that the interaction between fat and carbohydrate utilization in skeletal muscle occurs through an inhibitory effect of increased fat availability on glucose utilization and suggested that this took place at the level of pyruvate dehydrogenase (PDH) (Randle, 1998). In accordance, numerous studies have since then indicated a role of PDH in relation to the interplay between fat and carbohydrate oxidation in skeletal muscle during exercise (Mourtzakis et al., 2006; Ward et al., 1982; Watt et al., 2004).

The PDH complex catalyzes the irreversible decarboxylation of pyruvate to acetyl CoA and hence controls the entry of carbohydrate-derived fuel into the mitochondria for complete oxidation (Harris et al., 2002). Furthermore, inactivation of PDH enforces fat utilization (Spriet et al., 2004; Wu et al., 1999) and PDH therefore has the potential to mediate an interaction between fat and carbohydrate utilization. The activity of PDH in the active form (PDHa activity) is covalently regulated by the phosphorylation state of four sites on the PDH-E1alpha subunit, PDHSer293, PDHSer295, PDHSer300, PDHSer232 (Kiilerich et al., 2010a; Korotchkina et al., 1995) determined by the activity of PDH kinases (PDK), which phosphorylate and
inactivate, and PDH phosphatases (PDP), which dephosphorylate and activate PDH (Sugden & Holness, 2006). The prevalent PDK/PDP isoforms in skeletal muscle are PDK2, PDK4 and PDP1 (Bowker-Kinley, 1998) with different sensitivities to metabolic challenges (Sugden & Holness, 2003), but PDK1 may play a role in skeletal muscle through phosphorylation of PDHser232, although present at low level in skeletal muscle (Bowker-Kinley et al., 1998; Korotchkina & Patel, 2001). Moreover, posttranslational modification in the form of acetylation has recently been proposed to regulate PDH activity mediated through the mitochondrial NAD⁺-dependent deacetylase sirtuin (SIRT) 3 (Fan et al., 2014; Jing et al., 2013; Ozden et al., 2014).

Skeletal muscle PDHa activity has been shown to increase rapidly at the onset of exercise in an intensity dependent manner (Gibala et al., 1998; Howlett et al., 1998; Parolin et al., 1999; Putman et al., 1993) and to decrease gradually towards the resting level when the exercise is prolonged (Pilegaard et al., 2006; Watt et al., 2002; Watt et al., 2004). In accordance, exercise-induced dephosphorylation of PDHser293 and PDHser300 has been demonstrated in human skeletal muscle in response to moderate intensity, prolonged exercise (Pilegaard et al., 2006) as well as high-intensity exercise (Kiilerich et al., 2008; Kiilerich et al., 2010c), while PDHser295 seems less susceptible to exercise-mediated regulation (Bienso et al., 2015b; Kiilerich et al., 2010c). Although previous observations most often reveal tight regulation between PDH phosphorylation and activation, discrepancies have been reported (Kiilerich et al., 2010c; Pilegaard et al., 2006) suggesting additional post-translational modifications as a contributing factor. In accordance, the mitochondrial redox state may change with exercise as the NADH/NAD⁺ ratio may be altered (Graham & Saltin, 1989), and an acetylome-dependent regulation of PDH with exercise at different intensities may therefore exist. Moreover, no studies have examined PDH acetylation in human skeletal muscle in response to acute exercise. Taken together, the exercise-induced post-translational regulation of PDH-E1α in human skeletal muscle is yet not fully elucidated.

The well-known endurance exercise training-induced increase in fat oxidation during submaximal exercise (Kiens et al., 1993) may involve adaptations and adjustments in PDH regulation. Hence, a previous study
has shown that 7 weeks of aerobic exercise training decreased pyruvate production and attenuated PDH activation in human skeletal muscle during submaximal exercise at the same absolute workload as the untrained state (LeBlanc et al., 2004a). Furthermore, several studies have reported increased skeletal muscle PDH-E1α protein content with exercise training in both humans and rodents (Bienso et al., 2015a; Kiilerich et al., 2010b; LeBlanc et al., 2004b; Ringholm et al., 2013). In addition, 8 weeks of endurance exercise training increased PDK2, but not PDK4 protein levels, as well as increased total PDH and PDK activity in human skeletal muscle (LeBlanc et al., 2004b). Together these findings indicate that adaptations in PDH regulatory factors contribute to exercise training-induced changes in metabolic flexibility during exercise. However, the impact of the adaptive changes associated with increased skeletal muscle oxidative capacity on post translational modifications of PDH during steady state and incremental exercise remains to be thoroughly studied in human skeletal muscle. In addition, no studies have examined the impact of exercise training on PDK1 and PDP1 protein in human muscle.

Interleukin (IL-) 6 is released from muscle during exercise and has been proposed to affect skeletal muscle PDH activation (Bienso et al., 2014; Gudiksen et al., 2016). Several studies have reported an attenuation of IL-6 release from skeletal muscle with increased training state (Fischer et al., 2004; Ronsen et al., 2001), which may influence exercise-induced PDH regulation in skeletal muscle. The intracellular energy sensor AMPK is activated in skeletal muscle by exercise (Winder & Hardie, 1996) and has been suggested to be regulated by IL-6 (Kelly et al., 2004) and potentially influence PDH regulation (Klein et al., 2007). However, whether these factors and intracellular signaling pathways contribute to the exercise-induced PDH regulation in human skeletal muscle is not known.

The aim in the present study was therefore to investigate the impact of training state on post-translational regulation of PDHa activity and the associated metabolic changes in human skeletal muscle during steady state and incremental exercise.
Methods

Subjects

Nineteen healthy male subjects were recruited based on training state. The initial inclusion criteria was a VO$_2$max below 45 ml min$^{-1}$ kg$^{-1}$ (untrained) or VO$_2$max above 55 ml min$^{-1}$ kg$^{-1}$ (trained). This was followed by an additional inclusion criteria based on muscle oxidative capacity. Hence, CS activity and OXPHOS complex protein content were measured in skeletal muscle biopsies as markers of oxidative capacity after the intervention to establish two non-overlapping populations of trained and untrained individuals. This led to the exclusion of two subjects and resulting in nine trained and eight untrained individuals in the two groups, age 27±2 and 28±4, height 184±5 and 184±6, body weight 88±12 and 79±9 (mean±SD) for untrained and trained subjects, respectively. The study was approved by the ethics Committee of Copenhagen and Frederiksberg communities (H-15010768) and was conducted in accordance with the guidelines of The Declaration of Helsinki. The subjects were informed about the experimental protocol, the risks and discomforts that might occur in association with the intervention and provided written informed consent before the initiation of the study.

VO$_2$max protocol

Initially subjects performed an incremental test on an electromagnetic brake cycle ergometer (Ergomedic 839E, Monark, Denmark) to measure maximal oxygen uptake (VO$_2$max) on a metabolic ergospirometry system (Oxycon Pro, Jaeger, Hoechberg, Germany). For subjects who signed up to the experiment as untrained, the test was performed with 5 minutes at 85 watts followed by 5 minutes at 140 watts to reach steady state and a subsequent increase of 20 watts each minute until exhaustion. For trained individuals, the test started with 5 minutes at 125 watts followed by 5 minutes at 200 watts and a subsequent increase of 25 watts each minute until exhaustion. If subjects fell within the thresholds of the inclusion criteria,
incremental peak power output (IPPO) was calculated \( (W_{\text{peak}} \cdot x \text{ seconds} / 60 \text{ seconds} \times 25 \text{W}) \) based on their completed VO\textsubscript{2}max test for use on the experimental day.

**Nutrition and activity standardization**

For 24 hours prior to the experimental day, subjects were prompted to refrain from vigorous physical activity and consumption of alcohol. Furthermore, subjects were instructed to eat their normal breakfast and lunch. Subjects received a standardized meal (12% CHO; 3.6% protein; 6% fat per 100 grams) for dinner on the night before and high carbohydrate breakfast, consisting of oatmeal, raisins, and bananas, based on their estimated caloric requirements (Trumbo *et al.*, 2002) Breakfast was instructed consumed 2 hours before arriving at the laboratory.

**Acute exercise protocol**

The untrained (UT) and trained (T) subjects performed an incremental exercise bout on a magnetically braked cycle ergometer: 40 min at 50% IPPO, 10 min at 65% IPPO, and finally 80% IPPO until exhaustion. Muscle biopsies were obtained from m. vastus lateralis and venous blood samples from an arm vein prior to exercise (Pre), after 30 min, 41 min, and at exhaustion (Exh). The respiratory exchange ratio (RER) was measured from 20-25 min and 45-50 min of exercise. Additionally, abdominal adipose tissue biopsies were obtained before exercise and at 30 min point for other research purposes; the reason for the 3 min break at the 30 min time point (Figure 1).

**Blood and biopsy sampling**

Muscle biopsies were obtained using the percutaneous needle biopsy technique (Bergstrom, 1975) with suction (Evans *et al.*, 1982). The muscle biopsies were obtained through separate incisions, which all were
prepared before initiation of the exercise using lidocain as anesthesia. The exercise muscle biopsies were obtained with the subjects still on the bike leaning back to ensure fast muscle sampling during exercise. The muscle biopsies were instantly frozen in liquid nitrogen with less than 15 seconds for the part used for PDHa activity and stored at -80°C. Venous blood samples were drawn from a catheter inserted into the antecubital vein and transferred to vials containing EDTA (final concentration 0.21 mM) and centrifuged at 2600 g for 15 minutes to isolate plasma, aliquoted and stored at -80°C. Frozen muscle pieces for PDHa activity were cut off, while the remaining muscle tissue was freeze-dried for at least 48h and dissected free of visual blood and connective tissue under the microscope.

**Blood analyses**

Plasma glucose and lactate was determined using an ABL800 FLEX (Radiometer, Denmark). Plasma NEFA was determined using a Wako NEFA HR (2) kit with microtiter procedure protocol. Plasma IL-6 was measured using a mesoscale v-plex human IL-6 kit (K151QXD-1) according to the manufacturer’s guidelines (MSD, Rockville, MD, USA). Plasma adrenaline and noradrenaline were measured using a 2-CAT plasma ELISA kit (BA E-4500) according to the manufacturer’s guidelines (LDN, Nordhorn, Germany).

**Muscle metabolites and glycogen**

Muscle glycogen content was determined on 0.8-1.5 mg freeze-dried, dissected muscle tissue as previously described (Passonneau & Lauderdale, 1974) by boiling samples for 2 hours in 1 M HCl and determining glycogen as glycosyl units. Muscle glucose, glucose 6 phosphate (G-6-P) and lactate concentrations were determined in PCA extracts made from 1.0-1.2 mg freeze-dried, dissected muscle tissue as previously described (Bergmeyer & Moellerling, 1965) (Lowry & Passonneau, 1972a). Acetyl-CoA concentrations were
measured from dry weight muscle PCA extract using previously described principles (Constantin-Teodosiu et al., 1991a).

**SDS-page and immunoblotting**

Lysate was made from 4-12 mg freeze-dried muscle biopsies (dissected free of connective tissue, blood and visible fat). The tissue was homogenized for 2 min at 30 second\(^{-1}\) in a Tissuelyser II (Qiagen, Hilden, Germany) in a 1:80 weight to volume ratio in ice-cold buffer consisting of 10% glycerol, 20mM Na-pyrophosphate, 150mM NaCl, 50mM HEPES, 1% NP-40, 20mM β-glycerophosphate, 10mM NaF, 1mM EDTA, 1mM EGTA, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 2mM Na\(_3\)VO\(_4\), 3 mM benzamidine, and deacetylase inhibitors (nicotinamide (1mM) and sodium butyrate (5mM)). Protein content in individual samples was determined using the bicinchoninic acid method (Thermo Fischer Scientific, USA) and content was adjusted with sample buffer to a concentration of 1μg/μl. Protein phosphorylation and protein levels were determined by SDS-PAGE using hand casted gels and western blotting. Membranes were incubated in primary antibody for determination of AMPKα2 and PDH-E1α protein, PDH-E1α\(^{\text{Ser293}}\), PDH-E1α\(^{\text{Ser300}}\), and PDH-E1α\(^{\text{Ser295}}\) phosphorylation (all kindly provided by Professor Grahame Hardie, University of Dundee, Scotland), AMPK\(^{\text{Thr172}}\) phosphorylation, p38 protein p38 MAPK\(^{\text{Thr180/Tyr182}}\) phosphorylation, sirtuin (SIRT)3 protein, signal transducer and activator of transcription (STAT)3 protein, STAT3\(^{\text{Tyr705}}\) phosphorylation and lysine acetylation antibody (#2535, #9212, #4511, #D22A3, #9139, #9138, #9441, respectively, Cell Signaling Technologies, Danvers, MA, USA), acetyl CoA carboxylase (ACC)\(^{\text{Ser212}}\) phosphorylation and PDH-E1α\(^{\text{Ser232}}\) phosphorylation (07-303 and #AP1063, respectively, EMD Millipore, Bedford, USA), PDK1 (ab90444, Abcam, Cambridge, U.K.), PDK2 protein (ST1643, CalBioChem, Bedford, USA), PDK4 protein (AA 91-125, Boster Biological technology, CA, USA), and PDP1 protein (Sigma-Aldrich, St. Louis, USA). Species-specific horseradish peroxidase conjugated immunoglobulin secondary antibodies (DAKO, Glostrup, Denmark) were used for incubation the following day. ACC2 protein content was determined by incubation with
streptavidin (DAKO, Glostrup, Denmark). Protein bands were subsequently visualized using an ImageQuant LAS 4000 imaging system and quantified with ImageQuant TL 8.1 software (GE Healthcare, Freiburg, Germany).

**Immunoprecipitation for protein acetylation**

A total of 200 µg of protein from lysate was immunoprecipitated for the determination of PDH-E1α acetylation state. The lysate was added to washed protein G agarose beads (EMD Millipore, Bedford, USA) in a 50:50 solution with PBS buffer containing 0.5% Triton X with 2µg of PDH-E1α antibody. The samples were rotated end over end at 4°C overnight and on the subsequent day the beads were washed, sample buffer was added and the samples were heated to 96°C for 3 minutes to release precipitated protein from the beads. The beads were spun down and lysate loaded on a hand-casted gel for SDS-page and western blotting as described above. Acetylated protein was normalized to the individual amount of precipitated PDH-E1α protein content for each sample.

**CS activity**

Freeze-dried, dissected muscle samples were homogenized for 2 min at 30 second¹ in phosphate buffer with added albumin (1:400 weight:volume) using a Tissuelyser II (Qiagen, Hilden, Germany). Citrate synthase (CS) activity was determined colorimetrically on the homogenate at baseline and after addition of oxaloacetate (Lowry & Passonneau, 1972b) and normalized to protein content measured in the homogenate samples using the bicinchoninic acid method (Thermo Fischer Scientific, USA).
**PDHa activity**

PDHa activity was determined after homogenizing 10-15mg wet weight muscle tissue in ice-cold buffer containing sucrose, KCl, MgCl₂, EGTA, Tris HCl, NaF, DCA, and Triton X-100 for ~50 sec using a motor-driven glass homogenizer and pestle (Kimble-Kontes, NJ, USA) and immediately snap frozen in liquid nitrogen as previously described (Cederblad et al., 1990; Constantin-Teodosiu et al., 1991b; Pilegaard et al., 2006). PDHa activity was normalized to creatine content to account for non-muscle tissue in the samples (St Amand et al., 2000).

**Statistics**

Values are presented as means ± standard error. A two-way ANOVA with repeated measures was applied to test the effects of training status and time on the various parameters. When a main effect was observed, a Student-Newman-Keuls post hoc test was used to locate differences. A student paired t-test was used to test the effect of training status (untrained versus trained subjects) on basal parameters in the pre exercise biopsies. Significance is accepted at P<0.05. To minimize chances of committing type 2 errors, results within 0.05≤P≤0.1 are referred to as tendencies. The statistical tests were performed using Sigmaplot 13.0 (Systat, USA).
Results

Markers of training state

Trained subject had higher (P<0.05) VO$_2$\textsubscript{max} as well as higher (P<0.05) skeletal muscle CS activity and OXPHOS than untrained subjects (Table 1).

Indirect calorimetry

RER was higher (P<0.05) at 45-50 min of exercise than at 20-25 min of exercise in both untrained and trained subjects, and RER was lower (P<0.05) in trained than untrained in both periods (Table 2).

Blood substrates and metabolites

The venous plasma glucose concentration was higher (P<0.05) at Exh than Pre in both untrained and trained. Moreover, the plasma glucose concentration was higher (P<0.05) in the trained than the untrained subjects at 50% IPPO, 65% IPPO and at Exh (Table 3).

The venous plasma lactate concentration was in the untrained subjects higher (P<0.05) at 50% IPPO, 65% IPPO and Exh than Pre and in the trained subjects higher (P<0.05) at Exh than at Pre. Furthermore, the plasma lactate concentration was lower (P<0.05) in the trained than the untrained subjects at 50% IPPO and at 65% IPPO (Table 3).

The venous plasma NEFA concentration in the trained subjects was higher (P<0.05) and tended to be higher (0.05≤P<0.1) at 50% IPPO and 65% IPPO, respectively, than at Pre, while there was no effect of exercise in
the untrained subjects. Furthermore, the plasma NEFA concentration was higher (P<0.05) in the trained than untrained subjects at 50% IPPO (Table 3).

**Blood adrenaline and IL-6**

Plasma IL-6 was higher (P<0.05) at 65% IPPO and Exh than Pre in both untrained and trained subjects. Furthermore, plasma IL-6 was higher (P<0.05) in untrained than trained subjects at Pre and tended to be higher (0.05≤P<0.1) at 50% IPPO (Table 3). The plasma adrenaline concentration was higher (P<0.05) at 41 min and Exh than Pre in both untrained and trained subjects and tended to be higher (0.05≤P<0.1) at 30 min than Pre in trained, while the plasma noradrenaline concentration was higher (P<0.05) at Exh than Pre in both untrained and trained subjects (Table 3).

**Muscle metabolites**

The muscle glucose concentration was higher (P<0.05) at Exh than Pre in both the untrained and the trained subjects and was higher (P<0.05) in trained than untrained subjects at Exh (Table 4).

The muscle G-6-P concentration in the untrained subjects tended to be higher (0.05≤P<0.1) at 50% IPPO and 65% IPPO and was higher (P<0.05) at Exh than at Pre, and was in the trained subjects higher (P<0.05) at 65% IPPO and Exh than before exercise. In addition, the muscle G-6-P concentration tended to be lower (0.05≤P<0.1) in trained than untrained at 50% IPPO (Table 4).

The muscle glycogen concentration was lower (P<0.05) at 50% IPPO, 65% IPPO and Exh than at Pre in both untrained and trained subjects and the muscle glycogen level was higher (P<0.05) in trained than untrained at 50% IPPO and at 65% IPPO (Figure 2a). The decline in muscle glycogen from Pre to 30 min of exercise was 50% lower (P<0.05) in trained than untrained and the utilization of glycogen from 41 min of exercise to Exh (the 3rd to the 4th biopsy) was 2.5 fold higher (P<0.05) in trained than untrained.
The muscle lactate concentration was in the untrained subjects higher (P<0.05) at 50% IPPO, 65% IPPO and Exh than Pre and in trained subjects higher (P<0.05) at Exh than Pre. In addition, muscle lactate was lower (P<0.05) in trained than in untrained at all three time points during exercise (Figure 2b).

The muscle acetyl CoA concentration was higher (P<0.05) at all time points during exercise than Pre in both untrained and trained subjects and was higher (P<0.05) in trained than untrained at Exh (Figure 2c).

**PDHa activity and PDH-E1α protein**

Muscle PDHa activity was higher (P<0.05) at all time points during exercise than Pre in both untrained and trained subjects. In addition, PDHa activity was higher (P<0.05) in trained than untrained at Exh (Figure 2a). PDH-E1α protein was unaffected by exercise and was higher (P<0.05) in trained than untrained subjects at all time points (Figure 2b).

**p38 and STAT3**

Phosphorylation of the exercise responsive p38 MAPK was determined to compare the metabolic challenge imposed by the exercise in the trained and untrained subjects. Muscle p38\(^{\text{Thr180/182}}\) phosphorylation was higher (P<0.05) at Exh than Pre in both untrained and trained subjects with no difference between untrained and trained (Figure 3c). p38 protein content was unaffected by exercise and similar in untrained and trained subjects.

STAT3 phosphorylation was determined to evaluate the potential differences in exercise-induced IL-6 signaling in the trained and untrained subjects. Muscle STAT3\(^{\text{Tyr705}}\) phosphorylation in untrained subjects tended to be higher (0.05≤P<0.1) and was higher (P<0.05) at 65% IPPO and at Exh, respectively, than Pre, while there was no effect of exercise in trained subjects. STAT3\(^{\text{Tyr705}}\) phosphorylation was lower (P<0.05) in
trained than untrained at 65% IPPO and Exh (Figure 3d). STAT3 protein was unaffected by exercise and similar in untrained and trained subjects.

**PDH phosphorylation**

Absolute PDH$^{\text{Ser293}}$ phosphorylation in muscle was lower ($P<0.05$) at all time points during exercise than Pre in both untrained and trained subjects. In addition, absolute PDH$^{\text{Ser293}}$ phosphorylation was higher ($P<0.05$) in trained than untrained subjects at 50% IPPO and 65% IPPO (Figure 4a). Muscle PDH$^{\text{Ser293}}$ phosphorylation normalized to PDH-E1α protein was also lower ($P<0.05$) at all time points during exercise than Pre and was lower ($P<0.05$) in trained than untrained at Pre (Figure 4b).

PDH$^{\text{Ser300}}$ phosphorylation in muscle was lower ($P<0.05$) at all time points during exercise than Pre in both untrained and trained subjects (except non-detectable level in untrained at Exh) and was higher ($P<0.05$) in trained than untrained at all time points (Figure 4c). Muscle PDH$^{\text{Ser300}}$ phosphorylation/PDH-E1α protein was also lower ($P<0.05$) at all time points during exercise than at Pre in both untrained and trained subjects, and there was an overall difference in PDH$^{\text{Ser300}}$ phosphorylation/PDH-E1α protein between untrained and trained subjects (Figure 4d).

PDH$^{\text{Ser232}}$ phosphorylation in muscle was lower ($P<0.05$) at all time points during exercise than at Pre in both untrained and trained subjects. Moreover, PDH$^{\text{Ser232}}$ phosphorylation was higher ($P<0.05$) in trained than untrained at Pre, 50% IPPO and Exh and tended to be higher ($0.05 \leq P < 0.1$) at 65% IPPO (Figure 4e). Furthermore, muscle PDH$^{\text{Ser232}}$ phosphorylation/PDH-E1α protein was also lower ($P<0.05$) at all time points during exercise than at Pre in both untrained and trained subjects. In addition, PDH$^{\text{Ser232}}$ phosphorylation/PDH-E1α protein was lower ($P<0.05$) in trained than untrained subjects at Pre, 65% IPPO and Exh and tended to be lower ($0.05 \leq P < 0.1$) at 50% IPPO (Figure 4f).
PDH$^{\text{Ser295}}$ phosphorylation was unaffected by exercise and was higher (P<0.05) in trained than untrained subjects at 50% and 65% IPPO (Figure 4g). PDH$^{\text{Ser295}}$ phosphorylation/PDH-E1α protein was lower (P<0.05) in trained than untrained subjects at Pre, 50% IPPO and 65% IPPO (Figure 4h).

**PDK1, 2, 4 and PDP1**

PDK1, PDK2 and PDK4 protein content in muscle was unaffected by exercise in both untrained and trained subjects. PDK2 protein was higher (P<0.05) in trained than untrained at all time points and PDK4 protein was higher (P<0.05) in trained than untrained at 50% IPPO, 65% IPPO and at Exh (Figure 5a-c). Furthermore, PDP1 protein content was higher (P<0.05) at all time points during exercise than at Pre. In addition, PDP1 protein content was higher (P<0.05) in trained than untrained at all time points during exercise and tended to be higher (0.05≤P<0.1) at Pre (Figure 5d).

**SIRT3 and PDH acetylation**

Muscle SIRT3 protein was higher (P<0.05) in trained than untrained subjects at all time points (Figure 6a). In addition, PDH-E1α acetylation in muscle was in trained subjects higher (P<0.05) at 50% IPPO and Exh than Pre, and was higher (P<0.05) in trained than untrained subjects at all time points (Figure 6b).

**AMPK and ACC**

Muscle AMPK$^{\text{Thr172}}$ phosphorylation was in untrained subjects higher (P<0.05) at all time points during exercise than Pre and in trained subjects higher (P<0.05) at Exh than Pre. In addition, AMPK$^{\text{Thr172}}$ phosphorylation was lower (P<0.05) in trained than untrained subjects at all time points during exercise (Figure 7a). Furthermore, AMPKα2 protein was not affected by exercise and was similar in untrained and trained subjects.
ACC$^{\text{Ser221}}$ phosphorylation in muscle was in both untrained and trained subjects higher (P<0.05) at all time points during exercise than Pre with no difference between untrained and trained subjects (Figure 7b). In addition, muscle ACC2 protein was unaffected by exercise and similar in untrained and trained subjects.

**Discussion**

The main findings of the present study are that exercising at the same relative intensity at steady state and with a short-term increase in intensity, elicited similar PDH activation in skeletal muscle from untrained and trained subjects. Skeletal muscle PDHa activity was higher in trained than untrained at exhaustion providing a contributing mechanism for the augmented capacity for carbohydrate oxidation in the trained state. This reflects a well-controlled tight regulation of PDH together with the ability to rely on fat oxidation at a higher exercise intensity in the trained than untrained state. This response was associated with higher PDH-E1α content, PDH phosphorylation and PDH acetylation as well as protein content of PDH regulators. PDH acetylation increased in response to exercise only in trained subjects, and although the time course and relative change in PDH dephosphorylation with exercise in general was similar in untrained and trained subjects, effects of site-specific differences in phosphorylation cannot be excluded. However, the difference in PDHa activity at exhaustion between trained and untrained seems to be related to differences in glycogen utilization rather than clear differences in PDH phosphorylation and acetylation state.

The finding that skeletal muscle PDHa activity was similar in untrained and trained subjects when exercising at 50% IPPO indicates that PDHa activity depends on the relative rather than the absolute exercise intensity. This is in accordance with another human study demonstrating that PDHa activity was lower when exercising at the same absolute intensity after completing a 7 week endurance training program than before (LeBlanc et al., 2004a). Furthermore, the observation that the muscle acetyl CoA concentration also was similar in trained and untrained subjects at 50% IPPO may suggest a similar flux through PDH and
hence equal carbohydrate oxidation although the trained subjects exercised at a higher absolute intensity. The observed lower RER in trained than untrained subjects during the submaximal steady-state exercise, indicates higher fat oxidation in the trained, which is in accordance with numerous studies (Henriksson, 1977; Holloszy & Booth, 1976; Kiens et al., 1993; Klein et al., 1994). Such an increased fat utilization will also provide the additional ATP required by the trained subjects exercising at a higher absolute work load. In addition, although the untrained subjects used more muscle glycogen during the initial 30 min of exercise and muscle G-6-P was higher in the untrained than the trained subjects at 50% IPPO, the higher plasma and muscle lactate in the untrained than the trained still supports the possibility of equal PDH flux and carbohydrate oxidation in the trained and untrained. Studies have reported that PDHa activity increased in human skeletal muscle after only 6 seconds of exercise (Parolin et al., 1999) indicating a highly responsive system. However, the present finding that the level of PDHa activity did not increase any further in response to 1 min of exercise at the increased intensity may suggest that muscle PDH regulation requires longer time to adjust.

The observation that muscle PDHa activity and acetyl CoA were higher in the trained than untrained at exhaustion is in accordance with the augmented PDH-E1α protein content and hence capacity for PDH activation and indicates elevated carbohydrate oxidation in the trained subjects at exhaustion. This finding is not in accordance with findings demonstrating that an increased carbohydrate oxidation at maximal exercise after high intensity exercise training was not associated with a corresponding change in PDHa activity (Perry et al., 2008). A higher flux through PDH in the trained than the untrained subjects at exhaustion in the present study is in accordance with the higher glycogen use in the trained than the untrained between the last two biopsies potentially providing additional substrate for PDH in the trained subjects. This is further supported by the similar plasma and lower muscle lactate concentration in trained than untrained subjects at exhaustion. Furthermore, the elevated muscle glucose in the trained than the untrained at exhaustion may suggest that glucose derived from the blood has contributed more to carbohydrate oxidation in the trained than the untrained subjects, although interstitial glucose likely
contributes to this measure. In addition, the higher plasma glucose level in trained than untrained subjects throughout the exercise from 30 min of exercise, may suggest an increased hepatic gluconeogenesis in trained than untrained. However, the observations that plasma adrenaline was similar in trained and untrained subjects throughout the exercise and plasma IL-6 higher in untrained than trained at 41 min and exhaustion do not support that adrenaline or IL-6 mediated this effect.

The finding that exercise induced a dephosphorylation of PDH293 and PDHSer300 in association with the increase in PDHa activity is in line with previous findings in humans (Kiilerich et al., 2008; Kiilerich et al., 2010c; Pilegaard et al., 2006), while the unaffected PDHSer295 phosphorylation differs from a previously shown down-regulation (Bienso et al., 2015b). Furthermore, the finding that exercise dephosphorylated PDHSer232 similarly in human skeletal muscle apparently independent of exercise intensity is novel. Because PDHSer232 is thought to be regulated mainly by PDK1 (Korotchkina & Patel, 2001), the present observations support the possibility that PDK1 contributes to PDH regulation in human skeletal muscle. Together, these findings demonstrate site-specific regulation in response to exercise.

The overall similar time course of exercise-induced PDH dephosphorylation in trained and untrained subjects in the present study is in accordance with the similar PDHa activity in trained and untrained at 50% and 65% IPPO demonstrating a well-controlled regulation of PDH dephosphorylation adjusted to the relative exercise intensity. Furthermore, the observation that the phosphorylation level of PDHSer293 and Ser295 was similar at exhaustion, while the normalized PDHSer 232 was lower in trained than untrained and Ser300 higher in trained both as absolute and normalized may suggest that site-specific PDH regulation contributed to the difference in PDHa activity at exhaustion. Of notice is however, that only the lower phosphorylation at PDHSer232 would be expected to be associated with higher PDHa activity and this site has been reported only to be regulated by PDK1, which is low expressed in skeletal muscle (Bowker-Kinley et al., 1998). Taken together this seems to indicate that differences in PDH phosphorylation likely only have had minor contributions to the observed training state difference in PDHa activity at exhaustion. However, the observation that PDH-E1α protein content was higher in trained than untrained individuals in the
The present study is in accordance with previous studies (Consitt et al., 2016; Kiilerich et al., 2010b; LeBlanc et al., 2004b) and indicates an enhanced capacity for carbohydrate flux into the mitochondria in trained. Furthermore, the higher level of phosphorylation at all four sites and the higher protein content of PDK1, PDK2, PDK4 and PDP1 in skeletal muscle from the trained subjects as previously reported for PDK2, PDK4 and PDP1 with exercise training (Consitt et al., 2016; LeBlanc et al., 2004b) provide evidence that the capacity to regulate PDHa activity follows the overall capacity of the enzyme. This ensures that the flexibility of PDH regulation through modifications of the phosphorylation state is maintained despite a higher PDH-E1α content in a trained state. In addition, the finding that PDP1 protein abundance increased during exercise only in trained subjects has not previously been reported and may suggest an additional regulatory mechanism to increase PDHa activity in the trained state.

The present observations that muscle PDH-E1α lysine acetylation was higher in the trained than untrained subjects and increased in response to exercise in trained are novel findings. Acetylation has been reported to both activate and inactivate enzymes in the TCA cycle (Guan & Xiong, 2011) and the impact of acetylation on PDHa activity in human skeletal muscle remains uncertain. Moreover, the present study demonstrating that SIRT3 protein was higher in skeletal muscle of trained than untrained subjects is in accordance with a study showing that SIRT3 protein increased with exercise training (Vargas-Ortiz et al., 2015), while others have reported that 3 weeks of knee extensor endurance training had no effect on SIRT3 protein (Brandauer et al., 2015). Considering the inherent function of SIRT3 as a powerful deacetylase, increased SIRT3 abundance does not readily explain that lysine acetylation in trained subjects was higher at all time points. However, SIRT3 requires NAD⁺ as an obligatory co-factor for deacetylase activity (Schwer et al., 2002), and it may therefore be speculated that trained subjects maintain a higher mitochondrial NADH/NAD⁺ ratio and thus a lower intrinsic SIRT3 activity during exercise than untrained due to mitochondrial adaptations (Phillips et al., 1996). In vivo shifts in [NAD⁺] are difficult to measure (White & Schenk, 2012) and the available literature provides conflicting evidence regarding changes in the NADH/NAD⁺ ratio in human skeletal muscle during submaximal short-term exercise (Graham & Saltin,
Nevertheless, the higher SIRT3 protein content in trained subjects provides evidence for an increased capacity for mitochondrial acetylome-regulation with exercise training. The observed attenuation in AMPK phosphorylation in trained compared to untrained subjects during exercise is in line with findings that short-term exercise training reduces AMPK activity during exercise (McConell et al., 2005). This may be due to diminished levels of free AMP (Green et al., 1991) and free ADP (LeBlanc et al., 2004a) in trained skeletal muscle reflecting an improved mitochondrial capacity to sustain ATP supply and energy homeostasis. However, the finding that the downstream target of AMPK, ACC, was equally phosphorylated in trained and untrained subjects indicates a dissociation between AMPK activation and ACC deactivation in trained subjects. Although such dissociation has been reported previously in human skeletal muscle during prolonged exercise (Wojtaszewski et al., 2002), it may be speculated that residual ACC deactivation is mediated in a β-adrenergic dependent manner through PKA (Winder et al., 1997) although plasma adrenaline levels were similar in untrained and trained subjects.

The present findings that plasma adrenaline and noradrenaline increased similarly in the trained and untrained indicate that these catecholamines cannot explain the elevated glycogenolysis in the trained during the last part of the exercise and does not seem to have contributed to the difference in PDHa activity at exhaustion. Furthermore, the similar increase in plasma IL-6 in trained and untrained subjects does not support a role of circulating IL-6 in the observed PDHa activity at exhaustion. However, the observation that STAT3 phosphorylation, a marker of IL-signaling, was distinctly higher in untrained than trained subjects at exhaustion, may suggest a higher autocrine IL-6 signaling within untrained than trained muscle tissue. Because previous studies in mice have indicated that IL-6 reduces PDHa activity in skeletal muscle (Bienso et al., 2014;Gudiksen et al., 2016), it is possible that IL-6 has dampened the PDHa activity at exhaustion in the untrained subjects, but this remains to be confirmed.

In conclusion, the results from the present study demonstrates that that exercise-induced skeletal muscle PDH activation is closely matched to the relative exercise intensity at submaximal exercise, while reaching a
higher level at maximal exercise in trained individuals. These responses are associated with increased PDH phosphorylation, acetylation and content of covalent regulators.
Reference List


Table and Figure legends

Table 1. Markers of training status.

Maximal oxygen uptake (VO2max) given in liter/min and ml·min\(^{-1}\)·kg\(^{-1}\), as well as basal CS activity and OXPHOS protein content in vastus lateralis from untrained (UT) and trained (T) subjects. #: Significantly different from untrained, P<0.05.

Table 2. Whole-body calorimetry during exercise.

Respiratory exchange ratio (RER) in untrained (UT) and trained (T) subjects at steady state submaximal exercise. #: Significantly different from untrained within given time interval, P<0.05.

Table 3. Venous blood metabolites.

Venous plasma glucose, lactate, and plasma non-esterified fatty acids (NEFA) in untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Values are given as mean ± SE; (n=8-9). *: Significantly different from Pre within given group, P<0.05. #: Significantly different from untrained within time point, P<0.05. (*): Tendency to be significantly different from Pre within given group, 0.05<P<0.01. (#): Tendency to be significantly different from untrained within given time point, 0.05<P<0.01.

Table 4. Muscle metabolites.
Glucose and glucose-6-phosphate (G-6-P) concentration in vastus lateralis muscle from untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Values are given as mean ± SE; (n=8-9). *: Significantly different from Pre within given group, P<0.05. #: Significantly different from untrained within given time point, P<0.05. (*): Tendency to be significantly different from Pre within given group, 0.05<P<0.01. (#): Tendency to be significantly different from untrained within given time point, 0.05<P<0.01.

Figure 1. Study design.

Schematic illustration of the incremental exercise protocol. The individual workloads to obtain 50%, 65%, and 80% incremental peak power output (IPPO) were calculated based VO2max.

Figure 2

a) Muscle glycogen b) Muscle lactate and c) Acetyl CoA in vastus lateralis muscle from untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Values are given as mean ± SE; (n=8-9). *: Significantly different from Pre within given group, P<0.05. #: Significantly different from untrained within given time point, P<0.05.

Figure 3

a) PDHa activity b) PDH-E1α protein in vastus lateralis muscle from untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Values are given as mean ± SE; (n=8-9). Protein levels are given in arbitrary units (AU). *: Significantly different from Pre within
given group, P<0.05. #: Significantly different from untrained within given time point, P<0.05. (#): Tendency to be significantly different from untrained within given time point, 0.05<P<0.01.

Figure 4

a) PDH Ser293 phosphorylation  b) PDH Ser293 phosphorylation normalized to PDH-E1α protein content  c) PDH Ser300 phosphorylation  d) PDH Ser300 phosphorylation normalized to PDH-E1α protein content  e) PDH Ser232 phosphorylation  f) PDH Ser232 phosphorylation normalized to PDH-E1α protein content  g) PDH Ser295 phosphorylation  h) PDH Ser295 phosphorylation normalized to PDH-E1α protein content in vastus lateralis muscle from untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Values are given as mean ± SE; (n=8-9). Protein and phosphorylation (phos) levels are given in arbitrary units (AU). *: Significantly different from Pre within given group, P<0.05. #: Significantly different from untrained within given time point, P<0.05. (*): Tendency to be significantly different from Pre within given group, 0.05<P<0.01. (#): Tendency to be significantly different from untrained within given time point, 0.05<P<0.01.

Figure 5

a) PDK1 protein content  b) PDK2 protein content  c) PDK4 protein content and  d) PDP1 protein content in vastus lateralis muscle from untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Values are given as mean ± SE; (n=8-9). Protein levels are given in arbitrary units (AU). *: Significantly different from Pre within given group, P<0.05. #: Significantly different from untrained within given time point, P<0.05. (*): Tendency to be significantly different from untrained within given time point, 0.05<P<0.01. (#): Tendency to be significantly different from untrained within given time point, 0.05<P<0.01.
**Figure 6**

**a)** SIRT3 protein content  **b)** total lysine acetylation of immuno-precipitated PDH-E1α protein in vastus lateralis muscle from untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Values are given as mean ± SE; (n=8-9). Protein levels are given in arbitrary units (AU). *: Significantly different from Pre within given group, P<0.05. #: Significantly different from untrained within given time point, P<0.05. (*): Tendency to be significantly different from untrained within given time point, 0.05<P<0.01.

**Figure 7**

**a)** AMP-activated protein kinase (AMPK) Thr172 phosphorylation  **b)** Acetyl-CoA carboxylase 2 (ACC2) Ser212 phosphorylation  **c)** p38 MAPK Thr180/Tyr182 phosphorylation and  **d)** STAT3 Tyr705 phosphorylation in vastus lateralis muscle from untrained (UT) and trained (T) subjects before exercise (Pre) and after 30 min and 41 min of exercise and at exhaustion (Exh). Phosphorylation (phos) levels are given in arbitrary units (AU). Values are given as mean ± SE; (n=8-9, except for ACC, where n=7-8). *: Significantly different from Pre within given group, P<0.05. #: Significantly different from untrained within given time points, P<0.05. (*): Tendency to be significantly different from Pre within given group, 0.05<P<0.01.

**Figure 8** representative western blots of PDH-E1α, PDHSer293, PDHSer300, PDHSer232, PDHSer295, PDK1, PDK2, PDK4, PDP1, SIRT3, acetylated (ac) PDH-E1α, AMPKThr172, AMPKα2, ACC2, P38Thr180Tyr182, p38,
STAT3, STAT3Tyr705. For each protein and phosphorylation site blots for untrained (UT) and trained (T) at Pre, 50% IPPO, 65% IPPO and EXH, were taken from parts of the same respective membrane.
Figure 1

- 50% IPPO
- 50% IPPO
- 65% IPPO
- 80% IPPO

Muscle biopsy
Blood sample

Pre 0’
30’ 33’
40’ 41’ 43’
50’
Exh

Muscle biopsy
Blood sample
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<td>G-6-P (mmol \cdot kg\textsuperscript{-1} \cdot dry weight)</td>
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Figure 2

(a) Untrained vs. Trained Acetyl CoA (mol kg\(^{-1}\))

(b) Pre vs. trained Muscle lactate (mmol kg\(^{-1}\) dry weight)

(c) Glycogen (mmol kg\(^{-1}\) dry weight)

Legend:
- Untrained
- Trained

Significance:
- * p < 0.05
- # p < 0.01
Figure 3

(a) Pre-PDHα activity (mmol \cdot min^{-1} \cdot kg^{-1})

(b) Pre-PDH-E1 protein (AU)
Figure 4
Figure 5
Figure 6

(a) SIRT3 protein (AU)

(b) Total lysine acetylation/PDH-E1 (AU)
Figure 7
<table>
<thead>
<tr>
<th>Protein</th>
<th>UT Pre 50% 41% Exh</th>
<th>UT 50% 41% Exh</th>
<th>T Pre 50% 41% Exh</th>
<th>T 50% 41% Exh</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDH E1α</td>
<td></td>
<td></td>
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<tr>
<td>PDH Ser293</td>
<td></td>
<td></td>
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<tr>
<td>PDH Ser100</td>
<td></td>
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<tr>
<td>PDH Ser232</td>
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<td></td>
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<tr>
<td>PDH Ser295</td>
<td></td>
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<tr>
<td>PDK1</td>
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<tr>
<td>PDK2</td>
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<tr>
<td>PDK4</td>
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<tr>
<td>PCK1</td>
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<td></td>
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<tr>
<td>PCK2</td>
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<tr>
<td>PDK1</td>
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<tr>
<td>PDK4</td>
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</tr>
<tr>
<td>PDP1</td>
<td></td>
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</tr>
<tr>
<td>SIRT3</td>
<td></td>
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<tr>
<td>acPDH E1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 8
Figure 1

Muscle biopsy
Blood sample

- 50% IPPO
- 50% IPPO
- 65% IPPO
- 80% IPPO

Pre 0' 30' 40' 50' Exh
<table>
<thead>
<tr>
<th>Markers of training status</th>
<th>UT</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>VO$_2$max (ml · min$^{-1}$)</td>
<td>3573±382</td>
<td>4653±282#</td>
</tr>
<tr>
<td>VO$_2$max (ml · kg$^{-1}$ · min$^{-1}$)</td>
<td>39.4±6.1</td>
<td>59.2±3.3#</td>
</tr>
<tr>
<td>CS activity (mmol · min$^{-1}$ · mg protein$^{-1}$)</td>
<td>138.9±8.7</td>
<td>249.4±13.5#</td>
</tr>
<tr>
<td>OXPHOS (AU)</td>
<td>3.6±0.2</td>
<td>4.9±0.2#</td>
</tr>
</tbody>
</table>
Table 2. Respiratory exchange ratio

<table>
<thead>
<tr>
<th></th>
<th>20-25 min</th>
<th></th>
<th>45-50 min</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>0.97±0.02</td>
<td>T</td>
<td>0.94±0.02#</td>
<td>UT</td>
</tr>
<tr>
<td>T</td>
<td>1.09±0.02</td>
<td></td>
<td>0.99±0.02#</td>
<td></td>
</tr>
<tr>
<td>RER (VCO₂/VO₂)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


### Table 3. Venous blood metabolites

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>50% IPPO</th>
<th>65% IPPO</th>
<th>Exh</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UT</td>
<td>T</td>
<td>UT</td>
<td>T</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.5±0.2</td>
<td>5.6±0.2</td>
<td>4.9±0.2</td>
<td>6.0±0.3#</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>1.2±0.1</td>
<td>1.5±0.2</td>
<td>3.7±0.4*</td>
<td>1.8±0.2#</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.07±0.01</td>
<td>0.07±0.03</td>
<td>0.11±0.02</td>
<td>0.22±0.04#</td>
</tr>
<tr>
<td>Noradrenaline (pg/ml)</td>
<td>359±68</td>
<td>548±80</td>
<td>1729±178</td>
<td>1468±113</td>
</tr>
<tr>
<td>Adrenaline (pg/ml)</td>
<td>40±9</td>
<td>72±18</td>
<td>162±13</td>
<td>224±32(*)</td>
</tr>
<tr>
<td>Plasma IL-6 (pg/ml)</td>
<td>0.23±0.04</td>
<td>0.11±0.01#</td>
<td>0.35±0.07(*)</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>Table 4. Muscle metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pre</strong></td>
<td><strong>50% IPPO</strong></td>
<td><strong>65% IPPO</strong></td>
<td><strong>Exh</strong></td>
<td></td>
</tr>
<tr>
<td>UT</td>
<td>T</td>
<td>UT</td>
<td>T</td>
<td>UT</td>
</tr>
<tr>
<td>Glucose (mmol·kg(^{-1}) dry weight)</td>
<td>9.0±1.7</td>
<td>11.9±2.0</td>
<td>5.7±0.9</td>
<td>9.0±1.7</td>
</tr>
<tr>
<td>G-6-P (mmol·kg(^{-1}) dry weight)</td>
<td>10.2±1.3</td>
<td>5.3±0.6</td>
<td>15.4±1.7(*)</td>
<td>9.7±1.3(#)</td>
</tr>
</tbody>
</table>
Figure 2

(a) Acetyl CoA (µmol·kg⁻¹)

(b) Muscle lactate (mmol·kg⁻¹ dry weight)

(c) Glycogen (mmol·kg⁻¹ dry weight)

Untrained vs. Trained comparison at different time points:
- * indicates significant difference
- # indicates another significant difference

Time points:
- Pre
- 50%
- 65%
- Exh

50% and 65% Exh show significant changes compared to Pre.
Figure 3

(a) PDHa activity (mmol·min⁻¹·kg⁻¹) from Pre to Exh.

(b) PDH-E1 protein (AU) from Pre to Exh.

Comparison between Untrained and Trained groups.

Key:
- *: Significant difference between groups
- #: Significant change within group
Figure 4
**Figure 5**

(a) Pre-PDK1 protein (AU)

(b) Pre-PDK2 protein (AU)

(c) Pre-PDP1 protein (AU)

(d) Pre-PDK4 protein (AU)
Figure 6
Figure 7

(A) AMPK Thr172 phosphorylation

(B) ACC Ser221 phosphorylation

(C) P38 Thr180/Tyr182 MAPK phosphorylation

(D) STAT3 Tyr705 phosphorylation
Figure 8

UT

Pre  50%  41%  Exh

PDH-E1a

PDHSer293

PDHSer300

PDHSer232

PDHSer295

PDK1

PDK2

PDK4

PDP1

SIRT3

acPDH-E1a

T

Pre  50%  41%  Exh

AMPKThr172

AMPKα2

ACC2

ACC2

P38Thr180Tyr182

p38

STAT3

STAT3Tyr705

UT

Pre  50%  41%  Exh

AMPKThr172

AMPKα2

ACC2

ACC2

P38Thr180Tyr182

p38

STAT3

STAT3Tyr705

T

Pre  50%  41%  Exh

AMPKThr172

AMPKα2

ACC2

ACC2

P38Thr180Tyr182

p38

STAT3

STAT3Tyr705