PhD thesis
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Regulation of PDH, GS and insulin signalling in skeletal muscle; effect of physical activity level and inflammation

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Contents

Acknowledgements ..................................................................................................................5
Summary ....................................................................................................................................6
Resume (Danish summary).......................................................................................................8
List of manuscripts ..................................................................................................................10
INTRODUCTION ....................................................................................................................13
  Metabolic flexibility during rest and exercise .......................................................................13
  Insulin resistance ..................................................................................................................16
  Insulin signaling ....................................................................................................................17
    Protein kinase B/ Akt .............................................................................................................18
    Exercise-induced Akt regulation .......................................................................................18
  Glucose uptake and glucose metabolism .............................................................................19
    TBC1D4 ..............................................................................................................................19
    Insulin-induced TBC1D4 regulation .................................................................................19
    Exercise-induced TBC1D4 regulation .............................................................................20
    GLUT4 ................................................................................................................................20
    Exercise-induced GLUT4 regulation .................................................................................21
    HKII ...................................................................................................................................21
    Exercise-induced HKII regulation ...................................................................................21
    Fate of Glucose-6-phosphate ...........................................................................................21
  Glycogen synthase ...............................................................................................................22
    GS regulation ....................................................................................................................22
    Insulin-mediated GS regulation ........................................................................................22
    Exercise-induced GS regulation ........................................................................................23
  Pyruvate dehydrogenase ......................................................................................................24
    PDH structure ...................................................................................................................24
    PDH regulation ..................................................................................................................25
    Diabetes and PDK4 ............................................................................................................26
    Fasting and HFD-induced PDH regulation .....................................................................27
    Exercise-induced PDH regulation ....................................................................................28
    PDH and inflammation .......................................................................................................29
  Physical activity ...................................................................................................................30
    Physical activity level – physiological adjustments .........................................................30
    Physical activity level – and molecular adaptations .........................................................32
METHODS

AIMS

35

Statistics

Laboratory analyses

39

In vivo tests

38

Experimental protocols

36

Bed rest

36

LPS

36

Catheterization

37

Hyperinsulinemic euglycemic clamp

37

Interleukin-6 injection

37

In vivo tests

38

Oral glucose tolerance test

38

Exercise performance

38

VO_{2\text{max}} test

38

Laboratory analyses

39

Muscle biopsies

39

Freeze-drying

39

Muscle glycogen

39

Muscle creatine

40

Muscle G-6-P and lactate

40

mRNA isolation and PCR

41

SDS-page and immunoblotting

41

Gels

42

GS activity

43

PDHa activity

43

Enzyme activity

45

Statistics

45

INTEGRATED DISCUSSION

47
Physical activity level.........................................................................................................................47
Effects of physical activity level on key proteins in insulin signaling and glucose uptake in skeletal muscle........................................................................................................47
Effects of physical activity level on GS regulation in skeletal muscle ..............................................50
Effects of physical activity level on PDH regulation in skeletal muscle ...........................................53
Regulation of PDH during and after exercise ......................................................................................56
IL-6.....................................................................................................................................................56
Inflammation and PDH regulation during exercise ..........................................................................57
Conclusion..........................................................................................................................................60
Perspective...........................................................................................................................................62
Reference List .....................................................................................................................................63

Study I including co-authorship statement
Study II including co-authorship statement
Study III including co-authorship statement
Study IV including co-authorship statement
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Summary

The aims of the present thesis were to investigate 1) The impact of physical inactivity on insulin-stimulated Akt, TBC1D4 and GS regulation in human skeletal muscle, 2) The impact of exercise training on glucose-mediated regulation of PDH and GS in skeletal muscle in elderly men, 3) The impact of inflammation on resting and exercise-induced PDH regulation in human skeletal muscle and 4) The effect of IL-6 on PDH regulation in mouse skeletal muscle.

Study I demonstrated that bed rest–induced insulin resistance was associated with reduced insulin-stimulated GS activity and Akt signaling as well as decreased protein level of HKII and GLUT4 in skeletal muscle. In addition, the ability of acute exercise to increase insulin-stimulated glucose extraction was maintained after 7 days of bed rest. However, acute exercise after bed rest did not fully normalize the ability of skeletal muscle to extract glucose to the level seen when exercise was performed before bed rest.

Study II demonstrated that exercise training-improved glucose regulation in elderly healthy subjects was associated with increased HKII, GLUT4, Akt2, PDK2, GS and PDH-E1α protein content. Moreover, exercise training resulted in an enhanced response of TBC1D4 and GS and a reduced PDHa activity in response to glucose intake relative to before training.

Study III demonstrated that LPS-induced inflammation with elevated plasma TNFα concentration did not affect resting or exercise-induced AMPK and PDH regulation in human skeletal muscle.

Study IV demonstrated that an IL-6 injection reduced PDHa activity in skeletal muscle from fed mice and increased the PDHa activity in skeletal muscle from fasted mice without any change in phosphorylation level. An IL-6 injection increased AMPK and ACC phosphorylation in mouse skeletal muscle only in the fasted state. In addition, the effects of IL-6 on PDH were rather modest relative to the changes observed with fasting.

In conclusion, 1) decreased glucose transport/phosphorylation and decreased non-oxidative glucose metabolism seemed to contribute to the decreased skeletal muscle glucose extraction with physical inactivity in humans, and physical inactivity did not affect the ability of exercise to enhance insulin-mediated skeletal muscle glucose extraction. 2) Exercise training-improved glucose handling in aged human skeletal muscle was associated with increased content of key proteins in glucose metabolism and acute molecular changes towards improved glucose uptake and storage. 3) Short-term inflammation did not seem to influence resting or exercise-induced fat and carbohydrate utilization in human skeletal muscle. 4) IL-6 may regulate the PDHa activity in mouse skeletal muscle.
muscle, but the effect seems to depend on the energy state of the muscle, and AMPK may be involved in the fasted state.
Resume (Danish summary)

Formålet med denne PhD afhandling var at undersøge 1) effekt af fysisk inaktivitet på den insulin stimulerede regulering af Akt, TBC1D4 og GS i human skeletmuskulatur, 2) effekt af træning på glukose-induceret PDH og GS regulering i skeletmuskulaturen hos ældre mænd, 3) effekt af inflammation på hvile og arbejds-induceret PDH regulering i human skeletmuskulatur og 4) effekt af en enkelt IL-6 injektion på PDH regulering i skeletmuskulatur hos mus.

Studie 1 viste, at sengeleje-induceret insulinresistent var associeret med reduceret insulin-stimuleret GS-aktivitet og Akt-signalering, samt reduceret HKII og GLUT4 proteinindhold i skeletmuskulaturen. Desuden var evnen til at øge den insulin-stimulerede GS aktivitet efter akut arbejde bibeholdt efter 7 dages sengeleje. Efter 7 dages sengeleje var et enkelt akut arbejdet dog ikke nok til at normalisere muskulturens glukoseekstraktion til det samme niveau, som før sengeleje.

Studie II viste, at trænings-induceret forbedring af glukosereguleringen hos ældre, raske mænd var associeret med øget proteinindhold af GLUT4, HKII, Akt2, PDK2, GS og PDHH-E1α i skeletmuskulaturen. Derudover øgede træning det glukose-medierede respons på TBC1D4 i forhold til før træning, og glukoseindtag øgede GS-aktiviteten samt reducerede PDHa aktiviteten i skeletmuskulaturen kun i den trænede tilstand.

Studie III viste, at LPS-medieret inflammation, med forhøjet plasmakoncentration af TNFα ikke ændrede hvile eller arbejds-induceret AMPK og PDH regulering i human skeletmuskulatur.

Studie IV viste, at en IL-6 injektion reducerede PDHa aktiviteten i skeletmuskulaturen hos mus og øgede PDHa aktiviteten i fastede mus dog uden at ændre på fosforyleringsniveauet. En IL-6 injektion øgede kun AMPK og ACC fosforyleringen i fastede mus. Endvidere var de IL-6-medierede effekter på PDH kun moderate i forhold til de ændringer, der sås ved faste.

Samlet kan det konkluderes, at 1) reduceret glukosetransport/fosforylering og reduceret non-oxidativ glukosemetabolisme kan være årsagen til den reducerede glukoseekstraktion i skeletmuskulaturen, som forekommer ved fysisk inaktivitet. Fysisk inaktivitet synes ikke at ændre effekten af et forudgående arbejde på skeletmuskulaturens insulinfølsomhed. 2) den forbedrede glukosehåndtering efter træning hos ældre mænd var associeret med øget proteinindhold af centrale proteiner i glukosemetabolismen og akutte molekyldre ændringer mod forbedret optag og lagring af glukose. 3) Akut inflammation ændrede tilsyneladende ikke hvile eller arbejds-induceret
substratvalg i human skeletmuskulatur. 4) IL-6 synes at regulere PDH i skeletmuskulaturen, men energitilstanden synes at influere på denne effekt, og AMPK kan muligvis være involveret ved lav energitilstand i muskulaturen.
List of manuscripts

The present thesis is based on the following manuscripts referred to through this thesis as study I-IV

Study


Work was contributed to the following manuscript not included in the present thesis


Kenneth Allen Dyar, Stefano Ciciliot, Lauren Emily Wright, Rasmus Sjørup Biensø, Guidantonio Malagoli Tagliazucchi, Vishal Rajesh Patel, Mattia Forcato, Marcia Ivonne Peña Paz, Anders Gudiksen, Francesca Solagna, Mattia Albiero, Irene Moretti, Kristin Lynn Eckel-Mahan, Pierre Baldi, Paolo Sassone-Corsi, Rosario Rizzuto, Silvio Bicciato, Henriette Pilegaard, Bert Blaauw, Stefano Schiaffino. Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock Molecular Metabolism. 01/2013


INTRODUCTION

The development of lifestyle related metabolic diseases including insulin resistance and type 2 diabetes (T2D) are increasing all over the world. It was estimated that 200 million people were glucose intolerant (Zimmet et al. 2001) and 171 million people had T2D in 2000 and a prognosis estimates that this number will increase to 366 million people in the year 2030 (Wild et al. 2004). The development of glucose intolerance, insulin resistance and T2D is strongly correlated with aging and the number of elderly people with T2D is predicted to be doubled in the year 2030 (Glumer et al. 2003;Wild et al. 2004). Together this underlines the importance of understanding the etiology of lifestyle related metabolic diseases in order to be able to cope with the future challenges and hopefully prevent these predictions.

Metabolic flexibility during rest and exercise

Skeletal muscle is one of the largest organs in the body and skeletal muscle metabolism has therefore a major impact on whole body metabolism. Skeletal muscle has normally an incredible ability to adjust the substrate utilization according to the demands. This includes the changes in substrate utilization from rest to exercise, where the energy turn-over increases and there is a need for a marked increase in substrate utilization, as well as the ability to shift between fat and carbohydrates, when substrate availability changes after a meal or during prolonged exercise. This ability of skeletal muscle to adjust according to demands can be termed metabolic flexibility (Corpeleijn et al. 2009;Kelley et al. 1999;Kelley, Mandarino 2000;Storlien et al. 2004). Previous studies have most often used “metabolic flexibility” to describe the ability to switch between fat and carbohydrate oxidation (Corpeleijn et al. 2009;Kelley et al. 1999;Kelley, Mandarino 2000;Meex et al. 2010;Storlien et al. 2004), but also the regulation of fat oxidation (Corpeleijn et al. 2009;Kelley, Mandarino 2000) and the ability to increase glucose utilization in response to enhanced availability and increased workload (Crewe et al. 2013;Prior et al. 2013). Metabolic flexibility was first introduced by Kelley and Mandarino in 2000 (Kelley, Mandarino 2000), where they described the metabolic inflexibility of oxidative fuel selection in skeletal muscle of insulin resistant, obese subjects and in T2D subjects emphasizing that a key aspect of skeletal muscle metabolic fitness is the capacity to switch between fuels. They illustrated (Figure 1, left) that insulin resistant, obese subjects and T2D patients had a diminished or blunted ability to increase respiratory quotient (RQ)
during insulin stimulation due to a reduced insulin-mediated suppression of fat oxidation and they suggested this as part of the observed insulin resistance (Kelley, Mandarino 2000). This point has

![Fig 1. Illustration of metabolic flexibility in obese subjects and T2D patients as the ability to increase the respiratory quotient (RQ) during a hyperinsulinemic euglycemic clamp (left) (Kelley et al. 2000), and metabolic flexibility and inflexibility after a meal (right) (Corpeleijn et al. 2008).](image1)

also schematically been illustrated by the response of lipid and carbohydrate oxidation after a meal in a metabolic flexible and inflexible subject (Figure 1, right).

Exercise is the most potent physiological enhancer of whole body and muscle metabolism with an intensity dependent increase in energy expenditure of the working muscle (van Loon et al. 2001). While carbohydrate utilization dominates the initial phase of exercise (Figure 2, left), a switch towards fat oxidation takes place as the exercise is prolonged. This was presented already 75 years ago by Christensen and Hansen showing a decline in RQ during prolonged exercise reflecting increased fat oxidation (E. Christensen, O. Hansen 1939; E. Christensen, O. Hansen 1939) (Figure 2, right).

![Fig 2. The respiratory quotient (RQ) during 30 min (left) and up to 240 min (right) of exercise. The figure to the right shows the RQ during exercise when on a carbohydrate (K-h) diet, protein rich (N) diet and fat (F) diet (Christensen et al. 1939).](image2)
This shift in substrate choice takes place as carbohydrate availability decreases and enables muscle contractions to continue although potentially at a lower exercise intensity. The mechanisms behind this shift is not fully understood, but the interplay between fat and carbohydrate utilization is thought to be a contributing factor (Garland et al. 1962; Randle et al. 1963; Randle et al. 1978; Randle 1986; Randle et al. 1988) as originally described in the glucose fatty-acid cycle for resting conditions proposed by Randle et al. The original idea was that muscle derives energy through a tight regulation between glucose and fat metabolism (Randle et al. 1963), where fatty acids released from muscle or adipose tissue impose restrictions on muscle glucose metabolism, and where glucose uptake imposes restrictions on fatty acid release from triglycerides (Randle et al. 1963). This idea was later modified with the discovery that lipolysis is independent of glucose metabolism and regulated by hormones, and the observation that FFA oxidation can inhibit glucose oxidation (Randle et al. 1988).

Recently, a less marked increase in the respiratory exchange ratio (RER) during exercise was demonstrated in obese, older glucose intolerant subjects than in normal glucose tolerant subjects reflecting metabolic inflexibility during submaximal exercise (Prior et al. 2013) (Figure 3). This suggests a link between insulin-mediated and exercise-induced metabolic inflexibility in glucose intolerant subjects.

![Figure 3. Respiratory exchange ratio (RER) during exercise at 50% and 60% of VO2max in normal glucose tolerant subjects (NGT) and subjects with impaired glucose tolerance (IGT) (Prior et al. 2013).](image)

Taken together, disturbances in the interaction between fat and carbohydrate may lead to inflexible skeletal muscle fat and carbohydrate utilization, which may influence skeletal muscle and whole body metabolism both during exercise and at rest, where it may result in insulin resistance.
Insulin resistance

Insulin regulates a large number of cellular processes in many tissues including adipose tissue, liver and skeletal muscle. In adipose tissue insulin stimulates glucose uptake and triglyceride synthesis and inhibits the release of FFA (Kiens et al. 1989; NOVAK et al. 1965), while insulin inhibits the release of glucose from the liver by inhibiting hepatic gluconeogenesis and glycogenolysis and stimulating glycogen storage (DeFronzo et al. 1981; Donkin et al. 1997). In skeletal muscle, insulin stimulates glucose uptake, storage and oxidation and inhibits glycogenolysis (Bogardus et al. 1984; Cohen 1979; DANFORTH 1965; DeFronzo et al. 1979; DeFronzo et al. 1981), and prior exercise has been shown to enhance the insulin-mediated effects on glucose uptake and glycogen storage in skeletal muscle (Wojtaszewski et al. 2000; Bergstrom, Hultman 1966). Insulin resistance is defined as reduced insulin-mediated cellular responses (Hojlund, Beck-Nielsen 2006; Mandarino et al. 1987; Mandarino et al. 1996; Perseghin et al. 1996; Schinner et al. 2005) and T2D is most often characterized by the combined existence of peripheral insulin resistance and β-cell dysfunction (Donath et al. 2009; Kahn et al. 2006; Stumvoll et al. 2005). The general view is that insulin resistance is the first step in the development of T2D and that T2D only develops if the beta-cells are unable to compensate for the reduced insulin sensitivity (Kahn et al. 2006; Stumvoll et al. 2005). Hence, while a normal glucose tolerant individual will compensate for reduced insulin sensitivity with increased β-cell insulin release, a person with impaired glucose tolerance will respond with a reduced insulin secretion and T2D patients will be unable to compensate for reduced insulin sensitivity due to β-cell dysfunction. A schematic illustration (Figure 4) of this scenario originally presented by Stumvoll et al (Stumvoll et al. 2005) places insulin resistance as the first step in the development of T2D and underlines the utmost importance in understanding how to prevent and potentially reverse the insulin resistant state before β-cell dysfunction sets in.
Insulin resistance may occur in several insulin-sensitive tissues (Schinner et al. 2005; Tsatsoulis et al. 2013). Although the insulin regulated processes in adipose tissue and liver may be impaired and contribute to insulin resistance (DeFronzo et al. 1985; Smith et al. 1999; Tsatsoulis et al. 2013), skeletal muscle is at least quantitatively by far the most important insulin sensitive tissue (Baron et al. 1988). Hence, glucose uptake by skeletal muscle constitutes 75-95% of insulin-stimulated glucose uptake (Baron et al. 1988). Therefore even small changes in skeletal muscle glucose uptake and glucose metabolism can have a major impact on whole body metabolism. This includes skeletal muscle insulin resistance characterized by for example reduced insulin-stimulated glucose uptake and glycogen storage, compared with normal insulin sensitive muscles (DeFronzo et al. 1985; Himsworth 2011; Mandarino et al. 1987). An understanding of insulin signaling in skeletal muscle in both insulin sensitive and insulin resistant states is therefore important.

**Insulin signaling**

The plasma glucose concentration is regulated to be kept around 5 mM in the post absorptive phase and during fasting (Hommes et al. 1991). But after a meal, the blood glucose concentration will increase and glucose will activate secretion of insulin from the pancreas. The increased insulin level will stimulate signaling pathways in skeletal muscle leading to increased glucose uptake, storage
and oxidation. The first step in the insulin signaling pathway is the activation and phosphorylation of the insulin receptor (IR) at the plasma membrane. This is followed by the docking and phosphorylation of the Insulin receptor substrate (IRS)1 (Yenush, White 1997). Phosphatidylinositol 3-kinase p85 regulatory subunit binds to IRS and activates the p110 catalytic subunit, that becomes active and phosphorylates phosphatidylinositol (4,5)-biphosphate (PIP₂) to phosphatidylinositol (3,4,5)-triphosphate (PIP₃) (Summers, Birnbaum 1997). The increase in PIP₃ will phosphorylate and activate the phosphatidylinositol dependent kinase, which then phosphorylates protein kinase B (Akt).

Protein kinase B/ Akt
Akt exists as 2 isoforms, Akt1 and Akt2, and it has been suggested that Akt2 plays a larger role in insulin-stimulated glucose uptake than Akt1 (Vind et al. 2012; Whiteman et al. 2002). Akt is activated by phosphorylation on two phosphorylation sites termed Akt Thr³⁰⁸ and Akt Ser⁴⁷³ (Alessi et al. 1996; Summers et al. 1999) and concomitantly phosphorylate downstream targets including TBC1D4 and glycogen synthase kinase (GSK)-3. As TBC1D4 is a key factor in GLUT4 translocation and GSK3 involved in regulating glycogen synthase (GS), Akt is seen as a central factor in insulin-mediated intracellular signaling towards glucose uptake and glycogen storage (Figure 4 and 5). While there is no clear line regarding the effect of aging on Akt protein content in skeletal muscle (Consitt et al. 2013; Leger et al. 2008; Wilkes et al. 2009), lower insulin-stimulated Akt Ser⁴⁷³ phosphorylation and Akt activity has been observed with aging (Leger et al. 2008; Wilkes et al. 2009), although Akt phosphorylation has also been reported to be similar in young and elderly subjects (Consitt et al. 2013). The reduced Akt phosphorylation may be compared with the lower insulin-stimulated Akt Thr³⁰⁸ and Akt Ser⁴⁷³ phosphorylation observed in insulin resistant subjects (Hojlund, Beck-Nielsen 2006; Hojlund et al. 2009; Karlsson et al. 2005; Meyer et al. 2002; Vind et al. 2011; Vind et al. 2012) indicating that an impaired insulin-stimulated Akt response in aged skeletal muscle may have similarities with observations in insulin resistant subjects.

Exercise-induced Akt regulation
An increase in Akt Thr³⁰⁸ and Akt Ser⁴⁷³ phosphorylation has been observed in rodent skeletal muscle immediately after exercise relative to resting skeletal muscle (Howlett et al. 2006; Sakamoto et al. 2004). However, Akt phosphorylation is similar in a rested and a prior exercised muscle in both humans and rodents 3-4 h after exercise (Wojtaszewski et al. 1997; Wojtaszewski et al. 2000; Wojtaszewski et al. 2002b; Arias et al. 2007; Hansen et al. 1998).
**Glucose uptake and glucose metabolism**

**TBC1D4**

The TBC1D4 protein has been shown to be a direct target of Akt, hence the name Akt substrate of 160 kD (zeiger 04). TBC1D4 contains a Rab GTPase activating protein, which has a reduced activity when phosphorylated leading to an increase in Rab-GTP and the exocytosis of the GLUT4 vesicles to the plasma membrane (Bruss et al. 2005;Cartee, Wojtaszewski 2007;Karlsson et al. 2005;Zeigerer et al. 2004) (Figure 5).

![Fig 5 Insulin stimulated and contraction stimulated phosphorylation of TBC1D4 and the following exocytosis of GLUT4 vesicles (CArtee and wojtaszewski 2007).](image)

**Insulin-induced TBC1D4 regulation**

The insulin-stimulated GLUT4 translocation is dependent on the RabGAP on TBC1D4 observed in mice skeletal muscle (Zeigerer et al. 2004;Karlsson et al. 2005). TBC1D4 has been shown to be phosphorylated on 9 different phosphorylation sites (REF). Furthermore it has been observed in humans that insulin stimulation further increases the phosphorylation on several specific phosphorylation sites (Consitt et al. 2013;Treebak et al. 2009;Treebak et al. 2010;Treebak et al. 2014;Vind et al. 2011). The insulin-stimulated phosphorylation of specific sites on TBC1D4 in skeletal muscle is reduced in type 2 diabetic patients compared with healthy subjects (Consitt et al. 2013;Hojlund et al. 2008;Vind et al. 2011) and this effect has been suggested to contribute to the
reduced glucose uptake observed in insulin resistant skeletal muscle (Hojlund et al. 2008; Vind et al. 2011). In addition, insulin-stimulated TBC1D4 phosphorylation has been shown to be reduced in skeletal muscle of elderly subjects (Consitt et al. 2013)

**Exercise-induced TBC1D4 regulation**

TBC1D4 was first shown to be phosphorylated by insulin, and shortly after it was observed that insulin and exercise together increased the overall phosphorylation of TBC1D4 in mouse (Bruss et al. 2005) and human skeletal muscle (O’Gorman et al. 2006; Treebak et al. 2009).

A single 1 hour exercise bout or longer has been reported to increase TBC1D4 phosphorylation in a site-specific manner (Howlett et al. 2008; Treebak et al. 2009; Treebak et al. 2014) and with an elevated phosphorylation level 3 hours into recovery (Howlett et al. 2008; Treebak et al. 2009). The phosphorylation level in recovery from exercise was further increased with insulin stimulation (Treebak et al. 2009; Vind et al. 2011), but this seemed to be exercise intensity dependent. In one study the subjects exercised 60 min at 60% of VO$_{2\text{max}}$ (Howlett et al. 2008) and in another study 60 min at 80% of peak work-load and 5 min with 100% peak work load. The more intense workload in the Treebak et al study resulted in the detection of increased site-specific phosphorylation on TBC1D4 (Treebak et al. 2009). In addition, the enhanced phosphorylation of TBC1D4 by combining exercise and insulin could explain some of the increased glucose uptake in the prior exercised muscle (Treebak et al. 2009).

**GLUT4**

Because glucose transport across the sarcolemma in part is determined by both the protein content of GLUT4 and by the translocation of GLUT4 to the membrane (Goodyear, Kahn 1998; Mueckler 1990), improvements in either of these can enhance glucose uptake. In accordance, the insulin stimulated glucose uptake correlates with the GLUT4 protein content in the muscle (Christ-Roberts et al. 2004; Dela et al. 1993; Dela et al. 1994b; Frosig et al. 2007).

Aging has often been associated with decreased GLUT4 protein in human (Houmard et al. 1995) and mouse skeletal muscle (Kern et al. 1992), although no change in GLUT4 protein content has also been reported with age (Cox et al. 1999). As with aging there is some controversy about whether or not the GLUT4 protein content in skeletal muscle is changed with insulin resistance and T2D, because some studies have reported no change (Kahn et al. 1992; Pedersen et al. 1990; Dela et al. 1994b) and some a decrease in GLUT4 protein content in skeletal muscle with aging and T2D (Kampmann et al. 2011).
Exercise-induced GLUT4 regulation
Even a single exercise bout has been observed to increase the GLUT4 protein content in rat (Ren et al. 1994) and human skeletal muscle. The contraction-induced translocation of GLUT4 to the plasma membrane occurring already during exercise is in part mediated by AMPK as shown in AMPK KO mice (Kramer et al. 2006). Studies suggest that GLUT4 re-locates back to the cytoplasmic vesicles within 2h after the end of exercise (Goodyear et al. 1990). This indicates that the increased insulin-stimulated glucose uptake several hours after an exercise session is not due to an initially elevated number of GLUT4 in the plasma membrane.

HKII
When glucose has entered the muscle cell through GLUT4, it is phosphorylated by hexokinase (HK)II, into glucose-6-phosphate (G-6-P), which is unable to leave the muscle cell. The phosphorylation of glucose by HKII has been suggested as a limiting step in glucose uptake and studies in genetically manipulated mice support this possibility (Fueger et al. 2004a; Fueger et al. 2004b; Wasserman et al. 2011; Wasserman, Ayala 2005). In addition, insulin resistant subjects and T2D patients have reduced HKII activity (Vestergaard et al. 1995) and reduced insulin-stimulated muscle G-6-P compared with healthy subjects (Cline et al. 1999; Rothman et al. 1995). Furthermore as aging can be associated with reduced insulin sensitivity (Consitt et al. 2013; Cox et al. 1999; Dela et al. 1996) and HKII activity also is reduced with age, HKII may be suggested to contribute to the reduced glucose uptake with aging (Consitt et al. 2013; Cox et al. 1999; Dela et al. 1996).

Exercise-induced HKII regulation
Even a single exercise bout has been shown to increase HKII activity as well as HKII transcription and mRNA content in human skeletal muscle in recovery from exercise (O’Doherty et al. 1996; Pilegaard et al. 2000). In addition, the exercise-induced HKII mRNA response has been reported to be similar in T2D patients and control subjects, while the HKII activity has been reported to be lower in T2D patients (Cusi et al. 2001; Vestergaard et al. 1995) suggesting a less efficient phosphorylation of glucose in T2D patients than in healthy controls.

Fate of Glucose-6-phosphate
The G-6-P in the muscle cell will either be incorporated into muscle glycogen or through glycolysis be converted to pyruvate, which either will be converted to acetyl CoA in the mitochondria or lactate in the cytosol (Randle et al. 1978; Randle 1986; Randle et al. 1988; Randle et al. 1963). GS catalyzes the incorporation of G-6-P into glycogen in the cytosol (Leloir, CARDINI 1955; Leloir,
CARDINI 1957), while the pyruvate dehydrogenase (PDH) complex (PDC) catalyzes the oxidative
decarboxylation of pyruvate to acetyl CoA in the mitochondria (Randle et al. 1978; Randle 1986).
Hence GS and PDC are key enzymes in non-oxidative and oxidative skeletal muscle glucose
metabolism, respectively.

Glycogen synthase

GS regulation
Glycogen is a branched polymer and GS incorporates glucose from uridine diphosphate glucose
(UDP-gluc) into glycogen (Leloir, CARDINI 1957). GS is a member of the A-type
glycosyltransferase family that catalyzes the formation of the glycosidic linkages of glycogen using
UDP-glucose as the donor (Roach 2002; Roach et al. 2012). GS is regulated by phosphorylation on
at least 9 serine residues (Roach et al. 1977; Roach 1990; Roach 2002; Roach et al. 2012; Roach,
Larner 1977; Skurat et al. 1994; Skurat, Roach 1995; Skurat, Roach 1996) and allosterically by G-6-P
(Leloir, CARDINI 1957; Roach et al. 1976; Roach, Larner 1976; Roach et al. 1977) (Figure 6). When
GS is phosphorylated it becomes inactivated, however the allosteric regulation by G-6-P overrules
the covalent modification (Leloir, CARDINI 1957; Roach et al. 1976; Roach, Larner 1976; Roach et
al. 1977). The main regulatory sites on GS are 2+2a, 3a and 3b (Skurat et al. 1994; Skurat, Roach
1995).

Insulin-mediated GS regulation
Insulin is a very potent activator of GS activity both through the increase in cellular G-6-P and
through reduced GSK-3 activity by Akt-mediated phosphorylation, leading to dephosphorylation of
sites 3a, 3b, 3c and 4 (Roach et al. 1977; Skurat, Roach 1995; Skurat et al. 2000) (Figure 6). GS is
dephosphorylated by protein phosphorylase (PP1) (Cohen 1989), the activity of which is regulated
by its subunit G_M. When G_M is bound to PP1, the PP1-G_M complex is active and dephosphorylates
GS (Cohen 1989; Nielsen, Wojtaszewski 2004; Roach 2002). Insulin resistant subjects and T2D
patients have reduced GS activity and a reduced insulin-mediated effect on GS regulation (Christ-
et al. 1996; Vind et al. 2011; Cline et al. 1999). One of the molecular changes observed in skeletal
muscle of insulin resistant subjects is a defect in insulin-mediated dephosphorylation of GS site
2+2a (Hojlund et al. 2003; Hojlund et al. 2009; Glintborg et al. 2008; Vind et al. 2011) resulting in a
hyper-phosphorylation of GS site 2+2a (Hojlund et al. 2003; Vind et al. 2011), although not
observed in all studies (Hojlund et al. 2006).
Fig 6. The regulation of glycogen synthase (GS) by insulin (left) and exercise (right). Insulin stimulated the Akt/PKB-GSK-3 pathway (modified from Roach 2002 and Nielsen et al 2004)

In addition the insulin-stimulated GS activity in elderly subjects has been reported to be reduced despite a reduced phosphorylation level on GS site 2 and site 3a compared with young subjects (Poulsen et al. 2005). Furthermore, the GS protein content has also been shown to be reduced in aged subjects (Poulsen et al. 2005). Indicating a discrepancy between GS activity and phosphorylation level in elderly subjects

**Exercise-induced GS regulation**

During exercise glycogen is a key substrate for supplying the muscle with energy and restoring glycogen after use has high priority for the muscle (E Christensen, O Hansen 1939;Green 1991). GS activity has been shown to increase in human skeletal muscle after a single bout of exercise in part due to dephosphorylation of GS site 2a, 3a and 3a+3b (Jensen et al. 2012;Prats et al. 2009), while several studies have reported that GS activity is not increased during exercise, but immediately after exercise (Chasiotis et al. 1983;Katz, Raz 1995;Kida et al. 1989). As phosphorylation on site 2+2a is regulated by AMPK (Huang, Krebs 1977;Jorgensen et al. 2004;Wojtaszewski et al. 2002a), adrenaline (Hiraga, Cohen 1986) and the glycogen content in the muscle (DANFORTH 1965;Nielsen, Wojtaszewski 2004;Wojtaszewski et al. 2002a) (Figure 6,7), these factors are thought to mediate the observed changes at 2+2a in response to exercise. GS remains dephosphorylated and GS activity elevated for several hours after a single bout of exercise (Prats et al. 2005;Wojtaszewski et al. 1997;Wojtaszewski et al. 2000;Wojtaszewski et al. 2003). This increased GS activity after exercise is related to lower glycogen content in skeletal muscle as
both observed in rats (DANFORTH 1965) and humans (Nielsen et al. 2001; Wojtaszewski et al. 2001).

![Graph showing relationship between GS activity and glycogen content](image)

Figure 7. Relationship between GS activity and glycogen content in skeletal muscle in fed and 16h fasted mice as well as after treatment with epinephrine (DANFORTH 1965).

The elevated GS activity in recovery from exercise can be further increased with insulin stimulation (Christ-Roberts, Mandarino 2004; Frosig et al. 2007; Prats et al. 2009; Wojtaszewski et al. 1997; Wojtaszewski et al. 2000; Wojtaszewski et al. 2003), which is associated with a further dephosphorylation of GS site 2+2a and 3a (Prats et al. 2009). This elevated GS activity is thought to be a key factor in the effect of prior exercise on insulin-stimulated glucose uptake (Wojtaszewski et al. 1997; Wojtaszewski et al. 2000; Wojtaszewski et al. 2003).

**Pyruvate dehydrogenase**

The PDC is the only entry for carbohydrate-derived substrate into the mitochondria for oxidation and is therefore central in the switching between carbohydrates and fat oxidation. PDC ensures carbohydrate oxidation when carbohydrates are abundant and inhibition of carbohydrate oxidation when carbohydrates are scarce (Peters et al. 1998; Putman et al. 1993; Randle et al. 1978; Randle et al. 1988; St Amand et al. 2000).

**PDH structure**

PDC is a multi-enzyme complex composed of several copies of the heterotetramer (α2β2) pyruvate dehydrogenase (PDH) (E1), dihydrolipoamide acetyltransferase (E2), dihydrolipoamide
dehydrogenase (E3) and dihydrolipoamide dehydrogenase-binding protein (E3BP) (Holness, Sugden 2003; Patel, Korotchkina 2001; Sugden, Holness 1994).

**PDH regulation**

The activity of PDH in the active form (PDHa) is tightly regulated when energy demand and substrate availability changes during exercise and when availability of glucose changes during glucose intake, fasting and high fat diet (HFD). Although the activity of PDC to some extend is regulated by product inhibition and stimulated by the substrates (Garland et al. 1963; Kanzaki 1969; Kelley et al. 1993), the main regulation is thought to be by phosphorylation of at least 4 serine residues in PDH-E1α located at 232 (site 3), 293 (site 1), 300 (site 2) and 295 (site 4) (Gnad et al. 2011; Kiilerich et al. 2010a; Sugden et al. 1978; Linn et al. 1969). More recently, PDH has also been reported to be regulated by acetylation, but with the effects exerted on the phosphorylation level of PDH-E1α (Gnad et al. 2011; Jing et al. 2013).

The phosphorylation level of these sites is regulated by 4 isoforms of PDH kinase (PDK) (Jing et al. 2013; Korotchkina et al. 1995; Korotchkina, Patel 2001; Patel, Korotchkina 2001), which phosphorylate and inactivate the complex, and 2 isoforms of PDH phosphatase (PDP) (Patel, Korotchkina 2006; Roche et al. 2001) (Fig 7), which dephosphorylate and activate the complex (Figure 8). The distribution of the 4 PDK’s is tissue specific. While PDK1 is primarily expressed in heart and pancreas islets (Bowker-Kinley et al. 1998; Sugden, Holness 2003), PDK2 is expressed in most tissues (Bowker-Kinley et al. 1998), and PDK3 is expressed in testis, kidney and brain (Bowker-Kinley et al. 1998; Huang et al. 1998). PDK4 is primarily expressed in heart, skeletal muscle, liver, kidney and pancreatic islets (Bowker-Kinley et al. 1998; Holness et al. 2000). PDP1 is primarily expressed in heart, brain and skeletal muscle (Huang et al. 1998; Huang et al. 2003), while PDP2 is expressed in liver, adipose tissue and kidney (Huang et al. 1998; Huang et al. 2003). Furthermore both PDP isoforms have been detected at the mRNA level in human skeletal muscle by using real time PCR (Pilegaard et al. 2006). The phosphorylation level of PDH-E1α will therefore at any given time depend on the balance between the activities of the PDK’s and PDP’s, In addition, the different PDK isoforms have different affinities for the various phosphorylation sites on the PDH-E1α subunit (Korotchkina, Patel 2001), which can further play a role in the regulation of the phosphorylation state.

The activities of the PDK’s and PDP’s can be modified both by changes in expression, phosphorylation and/or allosteric regulation of the proteins (Patel, Korotchkina 2006). The activity of the PDK’s is regulated by the acetylation and reduction state of PDH-E2 with PDK activated by
an increase in mitochondrial acetyl CoA/CoA and NADH/NAD⁺ concentration ratios, which may occur during high rates of β-oxidation as well as PDH activity (Cate, Roche 1978; Randle et al. 1988; Roche et al. 1989). Furthermore, PDK activity is reduced by pyruvate (Cooper et al. 1974; Cooper et al. 1975; Priestman et al. 1996). PDP1 is stimulated by Ca²⁺ (Huang et al. 1998) and PDP2 activity has previously been reported to be regulated by insulin in skeletal muscle (Caruso et al. 2001) through phosphorylation-induced translocation to the mitochondria (Caruso et al. 2001) (Figure 8).

![Diagram of the regulation of the pyruvate dehydrogenase complex (PDC) by PDP and PDK, and the interaction between the different factors involved in PDK and PDP regulation](holness2003_diagram.png)

Fig 8. Illustration of the regulation of the pyruvate dehydrogenase complex (PDC) by PDP and PDK, and the interaction between the different factors involved in PDK and PDP regulation (Holness et al. 2003).

**Diabetes and PDK4**

An increased protein content of PDK4 has been observed in a rat model for diabetes and the authors suggest that the higher PDK4 protein content was induced by elevated FFA plasma levels (Bajotto et al. 2004). In a human study, PDH activity during a hyperinsulinemic euglycemic clamp was reduced in T2D patients relative to healthy subjects (Mandarino et al. 1990; Mandarino et al. 1996). The lower PDH activity indicates a reduced glucose oxidation and this could be a possible mechanism behind insulin resistance. This is in line with the observed reduced glucose oxidation and increased lipid oxidation in skeletal muscle during insulin stimulation in diabetic rats (Wu et al. 1998) and in T2D patients (Kelley, Mandarino 2000). In addition, it has been reported that T2D patients have elevated PDK4 mRNA content in skeletal muscle compared with glucose tolerant subjects (Kulkarni et al. 2012).
Fasting and HFD-induced PDH regulation

Fasting has been shown to increase PDK4 transcription and mRNA content in human skeletal muscle (Pilegaard et al. 2003a) and PDK4 mRNA and protein content in rat (Wu et al. 1998; Wu et al. 2000) and mouse (Kiilerich et al. 2010a) skeletal muscle with no change in skeletal muscle PDK2 protein content (Wu et al. 1998; Wu et al. 2000). Similarly skeletal muscle PDK activity has been reported to increase in human and rat skeletal muscle with fasting (Fuller, Randle 1984; Peters et al. 1998; Sale, Randle 1980). In accordance with the observed PDK regulation, the two PDP isoforms have been shown to be down regulated in rat skeletal muscle at both the mRNA and protein level by 48 hours of fasting (Huang et al. 2003). Hence, the hyper-phosphorylation of PDH-E1α during fasting shown in mouse skeletal muscle (Kiilerich et al. 2010a) seems to be due to a combination of increased PDK4 (Kiilerich et al. 2010a; Wu et al. 1998; Wu et al. 2000) and decreased PDP protein content. Furthermore, in agreement with an the increased PDH-E1α phosphorylation, skeletal muscle PDHa activity has been shown to be reduced in mice after 24 hours of fasting (Kiilerich et al. 2010a) suggesting that inhibition of PDHa activity contributes to reducing carbohydrate oxidation and increasing fat oxidation in skeletal muscle during fasting.

Another model used to investigate the regulation of PDH during changes in substrate availability and use is high fat diet (HFD). Intake of saturated fat has been shown to increase PDK activity in rat oxidative skeletal muscle (Peters et al. 2001a) and a human study has shown that decreased RER after 3 days on low carbohydrate diet/rich in saturated fat was associated with increased PDK activity and reduced PDHa activity in skeletal muscle (Peters et al. 2001b). This suggests that PDK-mediated inhibition of PDH contributes to an increased fat oxidation during HFD (Jansson, Kaijser 1982; Peters et al. 1998; Peters et al. 2001b; Putman et al. 1993). Furthermore, intake of a carbohydrate rich breakfast meal after an overnight HFD has been shown to reduce PDK4 protein content in human skeletal muscle relative to a high fat rich breakfast after an o/n HFD demonstrating very rapid regulation of PDK4 protein in humans by changes in carbohydrate availability (Kiilerich et al. 2010b). A tendency for an increase in PDK4 mRNA content in human skeletal muscle after only 4h of intralipid infusion (Pilegaard et al. 2006) supports that fatty acids play a role in this regulation of PDH during fasting and HFD.

Only limited information exists on PDK4 regulation with aging, but there are indications that PDK4 at least in rat muscle is increased with aged (Bajotto et al. 2004).
Exercise-induced PDH regulation

The first study to show an exercise-induced increase in PDHa activity in human skeletal muscle used cycling exercise with 1 min exercise and 3 min rest until exhaustion (Ward et al. 1982). Later it has been demonstrated that PDHa activity increases in human skeletal muscle already after just 1 min (Howlett et al. 1998) and that the PDHa activity is increased in an exercise intensity dependent manner (Howlett et al. 1998). A proposed contributing mechanism for the increase in PDHa activity during exercise is a combination of increased accumulation of Ca\(^+\) and pyruvate (Constantin-Teodosiu et al. 2004; Huang et al. 2003) with concomitant effects on the activity of PDP1 and PDK’s. In accordance, a single exercise bout has been shown to result in marked dephosphorylation of PDH-E1\(\alpha\) in human skeletal muscle (Kiilerich et al. 2008; Pilegaard et al. 2006; Kiilerich et al. 2010b; Kiilerich et al. 2011) supporting a key role of phosphorylation status in PDHa activity also during exercise. The impact of plasma FFA concentration on exercise-induced PDH regulation has been examined by modulating the FFA concentration through HFD, nicotinic acid and intralipid infusion (Kiilerich et al. 2010b; Pilegaard et al. 2006; Stellingwerff et al. 2003). Reduction in plasma free fatty acid (FFA) concentration by ingestion of the antilipolytic dryg nicotinic acid resulted in a higher level of PDHa activity and higher RER value in response to exercise (Stellingwerff et al. 2003). In accordance, enhanced plasma FFA concentration by o/n HFD has been shown to reduce the exercise-induced increase in PDHa activity and reduced the decrease in PDH phosphorylation at Ser\(^{300}\) (Kiilerich et al. 2010b).

Further studies have shown that the PDHa activity in human skeletal muscle remains elevated at a rather constant level up for to about 2 hours of low intensity exercise followed by a decrease towards the resting level as exercise proceeds above 2 hours (Mourtzakis et al. 2006; Pilegaard et al. 2006; Watt et al. 2004). Although a single exercise bout has been shown to increase PDK4 transcription and mRNA content in human skeletal muscle in response to exercise (Pilegaard et al. 2002; Pilegaard et al. 2003a), the increase is first pronounced some hours into recovery (Pilegaard et al. 2000; Pilegaard et al. 2002). In accordance, the decrease in PDHa activity during prolonged exercise, as the duration exceeds 2 hours of exercise, does not seem to be explained by an increase in PDK4 protein content, but may be due to an increase in PDK activity (Watt et al. 2004). Opposite of expected, intralipid infusion resulting in elevated plasma FFA level, maintained the level of PDHa activity and of PDH dephosphorylation after 3 hours of two-legged knee extensor exercise relative to a decrease observed at 3 hours of exercise in the control trial. (Pilegaard et al. 2006).
This suggests that elevated circulating FFA are not sufficient to obtain the down-regulation of PDHa activity late during prolonged exercise and that additional factors also play a role. Previous findings in gene modified mice showing that AMPK KO mice exhibited a more marked exercise-induced increase in PDHa activity and PDH dephosphorylation than wildtype mice indicated that AMPK may exert an inhibitory effect on exercise-induced PDH regulation. The concomitant observation that in vitro muscle incubation with AICAR had no effect on PDH phosphorylation did however not support such a link between AMKP and PDH (Klein et al. 2007). On the other hand, the observations in AMPK KO mice (Klein et al. 2007) combined with the observations that plasma interleukin (IL)-6 increased during prolonged exercise (Keller et al. 2001;Steensberg et al. 2002), IL-6 infusion increased fat oxidation in human skeletal muscle (van et al. 2003;Wolsk et al. 2010) and IL-6 injections increased AMPK phosphorylation in rat skeletal muscle (Kelly et al. 2009;Ruderman et al. 2006) may suggest that an IL-6-AMPK-PDH mediated regulation is involved in regulating the shift towards fat oxidation in skeletal muscle during prolonged exercise. However, the potential role of IL-6 in regulating skeletal muscle PDH regulation is unresolved.

PDH and inflammation
One of the factors suggested to play a role in lifestyle related metabolic diseases is low-grade inflammation characterized by 2-3 fold elevated level of circulating cytokines (Dandona et al. 2004). One of these cytokines is the pro-inflammatory cytokine, tumor necrosis factor (TNF)α, which has been observed to be elevated in the plasma of obese individuals and T2D patients as well as in elderly subjects (Bruunsgaard et al. 2001;Bruunsgaard, Pedersen 2003;Hotamisligil 1999;Plomgaard et al. 2005;Stephens et al. 1997). Furthermore TNFα has been reported to induce insulin resistance in cell culture as well as in mouse and human skeletal muscle (Hotamisligil 1999;Hotamisligil 1999;Plomgaard et al. 2005;Stephens et al. 1997). Hence, 2 hours of TNFα infusion has been shown to reduce glucose uptake during a hyperinsulinemic euglycemic clamp in humans (Plomgaard et al. 2005) suggesting that elevated circulating TNFα plays a role in the development of insulin resistance. Although very little information exists on this, results from a few studies do indicate an inhibitory effect of inflammation on PDHa activity. Hence, incubation of cardio myocytes with TNF-α for 24 hours has been shown to reduce the PDH activity (Zell et al. 1997). Furthermore, 24 hour of LPS infusion increased PDK4 mRNA and protein content, reduced PDP1 mRNA content and reduced the PDHa activity in rat skeletal muscle (Crossland et al. 2008). Similarly, sepsis in rats induced by E. coli has been shown to decrease PDH activity (vary 1999,
vary martin 1993) and increase PDK activity in isolated mitochondria from skeletal muscle (Vary, Martin 1993; Vary et al. 1998; Vary, Hazen 1999). However the impact of inflammation on PDH regulation in human skeletal muscle at rest and during exercise is unknown.

**Physical activity**

Several factors contribute to the development of metabolic inflexibility including insulin resistance in skeletal muscle including genetics, microbiota and lifestyle related factors like obesity and physical inactivity (Fletcher et al. 2002; Meng et al. 2013; Morine et al. 2010). The major impact of a physical active lifestyle in reducing the risk of developing lifestyle related diseases and in improving health in general underlines, however, the special importance of physical activity in this (Pedersen, Saltin 2006).

The effects of endurance exercise training in preventing lifestyle related metabolic diseases involve numerous types of adaptations in several tissues of the body including skeletal muscle. These adaptations are initiated in response to each single exercise bout and are thought to arise from cumulative effects of each exercise bout (Pilegaard et al. 2000). However, the metabolic challenges during exercise with enhanced fat and carbohydrate utilization in skeletal muscle may also provide beneficial effects through the increased removal of fatty acids and/or glucose from the blood. In addition, the molecular changes in skeletal muscle induced by endurance exercise training improve both metabolic capacity and flexibility (Meex et al. 2010), which together will improve metabolic regulation and exercise endurance performance. Hence, an understanding of the impact of physical activity in the prevention of lifestyle related metabolic diseases requires both an examination of metabolic regulation during exercise as well as the adaptations to exercise training.

**Physical activity level – physiological adjustments**

It is well established that the physical activity level has a major impact on both cardiovascular and metabolic parameters with effects on maximal oxygen uptake, exercise endurance and metabolic flexibility. Previous studies have used both decreased and increased level of physical activity to examine the impact of physical activity on skeletal muscle metabolism.

Several studies have reported that exercise training improved insulin sensitivity and/or insulin responsiveness in skeletal muscle (Dela et al. 1994a; Mikines et al. 1988; Consitt et al. 2013; Frosig et al. 2007), which is related to the improved capacity for glucose handling. Similarly, many studies have demonstrated increased exercise endurance using for example an ergometer bike or one-legged knee extensor exercise (Meex et al. 2010; Perry et al. 2008; Yeo et al. 2008; Pilegaard et al.
Lifelong exercise trained subjects have also been shown to have markedly higher exercise endurance during cycling than age-matched untrained subjects (Iversen et al. 2011). Furthermore, exercise training has been reported to improve glucose regulation both using hyperinsulinemic euglycemic clamp and an oral glucose tolerance test (REF; REF). This indicates that elderly subjects have maintained the ability to adapt to exercise training (Consitt et al. 2013; Cox et al. 1999; Iversen et al. 2011). However, the underlying mechanisms behind the improved glucose regulation with exercise training in elderly subjects are not fully resolved.

The National Space Agency (NASA) was among the first to investigate the effect of physical inactivity on the human body through the investigations of the effects of weightlessness during space travel (Carlson 1967; Lipman et al. 1970; Lipman et al. 1972; Lutwak L, Whedon GD 1959; Mikines et al. 1989; Mikines et al. 1991; Saltin et al. 1968). In addition, one of the first studies investigating the impact of physical inactivity on cardiovascular functions showed that only 20 days of bed rest elevated the heart rate during submaximal exercise due to reduced stroke volume and reduced maximal oxygen uptake due to reduced maximal stroke volume and cardiac output (Saltin et al. 1968).

Mikines et al (Mikines et al. 1989; Mikines et al. 1991) investigated the effect of 7 days of bed rest on both whole body and skeletal muscle insulin sensitivity using the hyper-insulinemic euglycemic clamp technique. They observed that insulin-stimulated glucose uptake in the legs was reduced following 7 days in bed, clearly demonstrating a decreased skeletal muscle insulin sensitivity. Furthermore, using a glucose clamp, the same study also showed that the glucose-stimulated insulin secretion was increased after bed rest (Mikines et al. 1989). Even as little as 3 days in bed have later been shown to increase heart rate during a submaximal exercise bout, decrease glucose tolerance and increase the plasma insulin level during an oral glucose tolerance test (Smorawinski et al. 2000).

Detraining can be used as another model of physical activity level to study insulin sensitivity. Dela et al. observed a decrease in insulin sensitivity after only 6 days of detraining, underlining the impact of the physical activity level on skeletal muscle insulin sensitivity (Dela et al. 1992).
Physical activity level – and molecular adaptations

Akt
Several studies have demonstrated that exercise training increases both Akt 1 and Akt 2 protein content in human skeletal muscle (Consitt et al. 2013; Vind et al. 2011; Yfanti et al. 2011). However, no studies have reported the impact of physical inactivity on insulin-mediated Akt regulation.

TBC1D4
Exercise training has been reported to increase TBC1D4 protein content in young subjects (Frosig et al. 2007), but not in aged subjects (Consitt et al. 2013; Vind et al. 2011). Furthermore, exercise training has been shown to reverse the impaired insulin-stimulated TBC1D4 phosphorylation observed in T2D patients (Vind et al. 2011) and elderly subjects (Consitt et al. 2013) underlining the impact of exercise training on key factors in insulin-mediated cellular responses. However, no studies have examined the effect of physical inactivity on insulin-mediated TBC1D4 regulation.

GLUT4
Skeletal muscle GLUT4 protein content has been reported to be reduced following 10 days of detraining (McCoy et al. 1994) and in accordance exercise training has in many studies been shown to increase the GLUT4 protein content in skeletal muscle of both young and aged subjects (Consitt et al. 2013; Cox et al. 1999; Dela et al. 1994c; Frosig et al. 2007). However, no studies have investigated the effect of physical inactivity on GLUT4 protein content in skeletal muscle.

HKII
Although hexokinase is considered a glycolytic enzyme, numerous studies have shown that HKII activity (Bernadr R, Peter J. 1969) and HKII protein content can increase in mouse skeletal muscle and skeletal muscle of young subjects with endurance exercise training (Frosig et al. 2007; Leick et al. 2008). However, no studies have investigated the effect of physical inactivity on HKII protein content in skeletal muscle or whether exercise training can also enhance HKII protein in elderly subjects.

GS
Exercise training increases total GS activity in human skeletal muscle both in healthy subjects (Frosig et al. 2007; Vind et al. 2011) and in T2D patients (Perseghin et al. 1996; Vind et al. 2011). In line, skeletal muscle GS protein content has been reported to increase following 10 weeks of
endurance exercise training (Vind et al. 2011), but not to change with 3 weeks of one-legged knee extensor exercise training (Frosig et al. 2007). The improved insulin-stimulated glucose uptake observed after an exercise training period may thus in part be due to an increased capacity for GS synthesis if the exercise training period is of sufficient duration (Christ-Roberts et al. 2004;Frosig et al. 2007;Vind et al. 2011). The GS activity has also been observed in T2D patients indicating that improved glucose handling in T2D patients with exercise training include increased GS activity (Perseghin et al. 1996;Vind et al. 2011). However the impact of physical inactivity on prior exercise enhancement of insulin-stimulated GS regulation and the impact of exercise training on skeletal muscle GS regulation in elderly subjects are unresolved.

**PDH**

The protein content of PDH-E1α in skeletal muscle is increased in human skeletal muscle with exercise training (Burgomaster et al. 2008;LeBlanc et al. 2004b;Sjogaard et al. 2013) and the oxidative capacity has been shown to correlate with the protein content of PDH-E1α (LeBlanc et al. 2004a;Love et al. 2011). In line with the increased PDH-E1α protein content, PDK2 protein has also been shown to increase with exercise training, while PDK4 protein has been reported to remain unchanged with exercise training (LeBlanc et al. 2004b). The exercise-induced PDHa activity has been shown to depend on the relative exercise intensity (Howlett et al. 1998). In accordance, PDHa activity during exercise at the same absolute workload was demonstrated to be lower after an exercise training period than before (LeBlanc et al. 2004a). However, 7 days in bed have been reported not to affect the exercise-induced PDH regulation in human skeletal muscle (Kiilerich et al. 2011) indicating that PDH regulation is affected differently by increased and increased physical activity.

**Oxidative proteins**

Numerous previous studies have demonstrated that endurance exercise training increases skeletal muscle oxidative capacity through enhanced activity and/or protein content of oxidative proteins and increased capillarization (Henriksson, Reitman 1977;Iversen et al. 2011;Klausen et al. 1981;LeBlanc et al. 2004b;Leick et al. 2008). In accordance, RER during submaximal exercise has repeatedly been shown to be lower after endurance exercise training than before (Henriksson, Reitman 1976;Kiens et al. 1993) reflecting a change in substrate utilization towards increased fat oxidation (Kiens 2006). Furthermore, 6 weeks of exercise training followed by detraining demonstrated that the activity of oxidative enzymes decreased abruptly when the exercise training
was terminated (Henriksson, Reitman 1977). In line several studies have later reported reduced citrate synthase (CS) activity after a period with physical inactivity (Coyle et al. 1985; Jansson et al. 1988; Richter et al. 1989), while 7 days of bed rest only resulted in a visual decrease in skeletal muscle CS and 3-hydroxyacyl-CoA-dehydrogenase (HAD) activity. Such reduced capacity for substrate oxidation may reduce the ability of skeletal muscle to remove both glucose and FFA from the blood (Mikines et al. 1991).
AIMS

The overall aim of this PhD thesis was to investigate 1) the impact of physical activity level on glucose/insulin mediated regulation of PDH, GS and insulin signaling in skeletal muscle and 2) the role of IL-6 and inflammation in exercise-induced PDH regulation in skeletal muscle.

The following questions will be addressed

1) The impact of physical inactivity on insulin-stimulated Akt, TBC1D4 and GS regulation in human skeletal muscle.

2) The impact of exercise training on glucose-mediated regulation of PDH and GS in skeletal muscle in elderly men.

3) The impact of inflammation on resting and exercise-induced regulation in human skeletal muscle.

4) The effect of IL-6 on PDH regulation in mouse skeletal muscle
METHODS
In the following section a description of the methods used in this thesis will be described.

Human subjects
Human subjects participated in study I, II, and III. In study I, 12 normally physically active young male subjects with an average age 26±3 years. In study II, 13 elderly male subjects with an average aged of 65±1 years and in study III 9 untrained subjects age with an average age of 20.8±1 years participated. Ethical approvals study I, H-C-2007-0085, study II H-2-2011-079 and study III, H-1-2012-108. All the subjects were informed of the risk and discomfort associated with the participation in all studies and the experimental protocols were explained in details. The subjects all provided there oral and written consent to participate. All studies were conducted in accordance with the guidelines of the Declaration of Helsinki.

Mice
In study IV, 8 weeks old female C57/B6 mice (Taconic, Lille Skensved, Denmark) were used. The mice had free access to a normal chow diet (Altromin 1324, Brogaarden, Lynge, Denmark) and water ad libitum. The mice had a 12:12 light-dark cycle. The mice were housed together until the day before the experimental day, where they were housed individually. The ethical approval in study IV, 2009, 561 1607.

Experimental protocols

Bed rest
The protocol used in study I, were a 7 days bed rest with performance tests and hyperinsulinemic euglycemic clamp before and after the. The subjects performed a one-legged knee extensor endurance exercise test to exhaustion and a VO2max test before and after the 7 days in bed. The subjects were allowed and encouraged to sit in a wheel chair for up to 5 hours a day, to avoid cardiovascular complication. The subjects were transported in wheelchairs to the bathroom. Rigshospitalets kitchen delivered all meals to the subjects during the bed rest period, where the subjects were allowed to eat ad libitum.

LPS
Lipopolysaccharide (LPS) endotoxin is a protein located in the cell wall of gram negative bacteria and is the compound that our immune system reacts to during a bacterial infection (Elin et al. 1981). The LPS can create acute inflammation in subjects when injected with a dose of LPS. The LPS will
indorse the macrophages to produce TNFα and IL-6 (Andreasen et al. 2008;Frost et al. 2002). In study III untrained and trained subjects were injected with 0.3 ng/kg LPS based on previous studies (Andreasen et al. 2008;Andreasen et al. 2011) and pilot trials on 3 subjects. The LPS injection was given 2 hours before a 10 min one-legged knee extensor exercise bout at 60% of Wattpeak. Biopsies were obtained before and after the exercise bout.

Catheterization
In study I and III, the subjects had catheters placed in the femoral vein (18 Ga. X 8”, Arrow, Athlone, Ireland) and femoral artery (20 Ga. X 5”, Arrow, Athlone, Ireland). In study I catheters were placed in the femoral vein of both legs and one femoral artery, in the rested and exercise leg respectively. The femoral vein and artery was found with a combination of palpation and ultrasound. The area where the catheter was inserted was shaved free of hair and disinfected with ethanol. When the area is clean it was covered with a sterile cloth. The vein was localized with ultrasound punctuated with a needle and the catheter was inserted via a guide wire. A similar procedure was performed with the artery. The catheters was flushed with sterile saline and taped to the skin to minimize irritation during the exercise bout.

Hyperinsulinemic euglycemic clamp
There are several ways to assess insulin sensitivity. One method is with the hyper insulinemic euglycemic clamp technic first described by deFronzo in 1979 (DeFronzo et al. 1979). Insulin (Actrapid, Novo nordisk, Denmark) was infused into an arm vein in the subject at a given infusion rate while the blood glucose concentration was kept at approximately 5mM by a continuous infusion of glucose. The amount of glucose infused to keep glucose 5mM was a reflection of whole body insulin sensitivity (DeFronzo et al. 1979). The clamp technique was used in study I with an insulin infusion rate of 50 mU min⁻¹·m⁻². This results in plasma concentration between 400-500 pmol/l that was within the normal physiological range. The blood glucose concentration was measured during the clamp to make sure that the blood glucose does not drop to a critical low level. The plasma glucose was measured on a Radiometer ABL 725 series every 5-10 min and the glucose infusion rate was adjusted accordingly. The muscle tissue will during a hyper insulinemic clamp be affected by elevated plasma insulin, but the plasma glucose concentration around 5mM.

Interleukin-6 injection
In study IV, mice were injected with 3 ng/g recombinant mouse (rm) IL-6 or phosphate buffer saline (PBS) in the fed or fasted state (16-18h). The quadriceps muscles and trunk blood were obtained 30 min or 60 min after the injection.
In vivo tests

Oral glucose tolerance test
Another and more simple way to examine potential changed in glucose metabolism was to use an oral glucose tolerance test (OGTT) that was first described by Boudouin 1908 with a 100 gr. glucose load. In study II the subjects consumed a glucose drink containing 1 g glucose per kg body weight. Blood samples were obtained at 15, 30, 45, 60, 90 and 120 min and plasma glucose, insulin and C-peptide concentrations were measured. This will give an indication of the glucose tolerance of the subjects. The muscle tissue during a OGTT potentially be affected by the transient elevation in both plasma glucose and insulin concentrations.

Exercise performance
Performance tests were used in study I, II and III to determine fitness and exercise endurance of the subjects. A one-legged knee extensor exercise was used in study 1, 3 and 4. The one-legged knee extensor exercise was first described by Andersen et al (Andersen et al. 1985), where they used a modified Krogh bicycle ergometer. For the studies described in this thesis the test was performed on a modified Monach Ergometer bicycle ergometer, but with almost the same setup as in 1985. The bike was mounted with a rod and attached to one side of the crankset, making it possible for the subject to kick either with the right or left leg. The subject was instructed to continue until exhaustion, that was when it was no longer possible to keep up a frequency of 60 per min. The subjects kicked with a frequency of 60 kicks/min. The test begins with a 5 min warm up followed by the test a graded incremental test with an increase in resistance (6W) every second minute. The endurance test was started with different load with either untrained (18W) or trained (24W).

VO\textsubscript{2max} test
The subjects maximal oxygen uptake in study I, II and III using a graded bicycle ergometer test. The test is performed on an electric Monach Ergometer bike (Monark 839E, Sweden), a modified version of the original first described by August Krogh in 1911-1913 (Krogh 1913). Oxygen uptake was determined using an online system (COSMED Quark b2). The test was started with a 5 min warm up, followed by an incremental increase with 30 W every minute. The subjects respiratory exchange ratio should be ∼1.10-1.15 at exhaustion and the test should optimally last between 5-10 min (Astrand, Rodahl 1977).
Laboratory analyses

Muscle biopsies
Muscle biopsies were obtained from the vastus lateralis muscle in study I, II and III. Before the muscle biopsy was obtained, the area on the thigh was wiped with chlorhexidine and the skin area was anesthetized (lidocain) and an insertion was made in the skin. Muscle biopsies were obtained using the percutaneous needle biopsy technique (Bergstrom 1962) modified with suction (Evans et al. 1982) from individual insertions in the skin. The biopsies were quickly dissected free from connective tissue and visual blood and frozen in liquid nitrogen within 25 sec and stored at -80°C.

Freeze-drying
In study I, II and III the muscle samples were freeze-dried to make it possible to separate blood and connective tissue from the muscle tissue. Muscle pieces to measure PDHa activity and mRNA were cut off while 80-100 mg of the remaining samples were freeze-dried. The samples were placed in -25°C and 0.1 bar pressure for at least 48 h that allows the frozen water in the muscle samples to evaporate. After 48 h, the samples were moved to room temperature for 1 h ad 0.1 bar to be ready for dissection. Before starting the dissection, a water calibration was made to make it possible to calculate the exact dry weight of the samples. One sample was weighted at 20 s, 30 s, 40 s, 50 s, 60 s, 90 s and 120 s after the release of the vacuum. The muscle samples were dissected free from blood and connective tissue and weighted out to western blotting analyses, glycogen content, enzyme activity and perchloric acid (PCA) extract. Due to the very low water content within the sample <10% the protein phosphorylation and enzyme activity is maintained in the freeze-dried muscle samples (Essen et al. 1975; Schantz, Henriksson 1987).

Muscle glycogen
The muscle glycogen content was determined in ~0.8 mg dry weight or 225 µg protein in homogenate samples in study I, III, and IV and in ~20 mg wet weight in study II as previously described (Passonneau, Lauderdale 1974) was boiled for 2 hours in 1 M HCl, which hydrolyses the glycogen into glycosyl units. The samples were quick spun and the solution free of tissue is transferred to a new tube. The samples were then loaded to a white mikrotiter plate (Whatman, Frisenette, Ebeltoft, Denmark) and a reaction mix is added. The mix contains Trisbuffer, H₂O, ATP, MgCl, DTT and NADP⁺. This assay is based on the auto fluorescence properties of NADPH. By adding the enzyme G-6-PDH, any G-6-P and NADP⁺ are converted into 6- phosphogluconic acid and NADPH that was measured spectrophotometrically.

Glycogen --->boiling for 2hours ----> Glucose
G-6-P + NADP⁺  ---G-6-PDH  --->  6PG + NADPH + H⁺
Glucose + ATP  ---> HK  ---> G-6-P + ADP

The glycogen concentration was based calculated on a standard curve made from standards loaded together with the samples on the plate.

**Muscle creatine**
The creatine content was determined in the homogenate used for determining PDHa activity. The creatine content was use to normalized the PDHa activity, to the total muscle content in each sample. The creatine was extracted with 0.6 M PCA for 1 hour at room temperature. The reaction was neutralized with KHCO₃ and the samples were centrifuges at 10.000 g for 3 min and supernatant was transferred to new tubes. The creatine content was determined by using the auto fluorescence of NADPH in the reaction described in fig xx. The samples and a standard with a known concentration of creatine is loaded to a white microtiter plate a mix containing: Hepes, MgCl₂, phosphoenol pyruvate, NADH, pentaphosphate pentasodium, ATP, pyruvate kinase (PK) and lactate dehydrogenase (LDH) is added to each well on the plate and incubated for 20 min. The plate was then analyzed in a Flouroscan (Thermo Sientific, Rockford, IL, USA) and creatine kinase (CK) was added to start the reaction described below.

Creatine + ATP  ----CK----> P-Creatine + ADP
ADP + P-Pyruvate  ----PK---->  ATP + Pyruvate
Pyruvate + NADH + H  ---LDH----> Lactate + ADP

**Muscle G-6-P and lactate**
For measuring lactate and G-6-P in muscle 0.8 mg of freeze dried muscle was hydrolyzed with 0.6 M PCA to extract the metabolites (Bergmeyer H.U. 1965). The samples were kept on ice and the PCA was added the samples were then placed 30 min at 5C°. The sample was transferred to new tubes and neutralized with KHCO₃.
The G-6-P content in the muscle samples was determined in the PCA extract. In a similar way described above in the glycogen and creatine assay. The samples was loaded on a white microtiter plate and a mix containing Trisbuffer, H₂O, ATP MgCl, DTT and NADP was added and analyses in a flouroscan G-6-PDH enzyme was added to start the reaction described below. The samples was incubated for 30 min and analyzed again.

G-6-P + NADP⁺  ---G-6-PDH  --->  6PG + NADPH + H⁺
Glucose + ATP $\rightarrow$ HK $\rightarrow$ G-6-P + ADP

The muscle content of lactate was determined in the PCA extract from the muscle samples. The samples were loaded on a white microtiter plate and a mix containing Glycylglycine, H₂O, NAD⁺ and Glutamic acid. The plate was analyzed in a floroscan and a enzyme mix containing LDH and glutamic-pyruvate transaminase was added to start the reaction described below and incubated for 60 min. The plate was then analyzed in a flouroscan and the lactate content was calculated.

L-Lactate + NAD⁺ $\rightarrow$ LDH $\rightarrow$ Pyruvate + NADH + H⁺
Pyruvate + Lglutamate $\rightarrow$ GPT $\rightarrow$ Lalanine + α-Oxoglutarate

**mRNA isolation and PCR**

Total RNA was isolated from ~25 mg muscle with the guanidinium thiocyanate (GT)-phenol-chloroform extraction method as previously described (Chomczynski, Sacchi 1987) and modified by Pilegaard et al (Pilegaard et al. 2000). The muscle samples were weighted in a sterile tube in a -20°C freezer. The samples were homogenized in an ice-cold GT and β-mercaptoethanol (BME) solution using a TissueLyserII (Quiagen, Germany) and placed on ice. The GT and BME denature proteins including RNases. The RNA was extracted by adding phenol, chloroform:isoamyl (49:1) and NaOAc and the samples were shaken vigorously and placed on ice for 15 min followed by centrifugation at 12,000 g for 20 min at 4°C. This results in separation into an upper aqueous phase and a lower organic phase. The organic phase contains proteins while the aqueous phase contains the RNA. The aqueous phase was carefully transferred to a new tube. Ice-cold isopropanol (-20°C) was added and the samples vortexed vigorously followed by incubation at -20°C for at least 15 min. The samples were centrifuged at 12,000 g for 10 min at 4°C resulting in an RNA pellet. The supernatant was poured off and 75% EtOH in DEPC H₂O was added followed by centrifugation at 12,000 g for 5 min at 4°C. The wash step was repeated followed by vacuum drying of the tubes and resuspension of the pellet in 1 µl/mg DEPC H₂O containing 0.1 mM EDTA. It is important to work with sterile tubes and tips and to keep the samples on ice at all times to keep RNase inactive.

**SDS-page and immunoblotting**

The method was described and presented in 1979 (Towbin et al. 1979) and later modified (Burnette 1981). The method used in our laboratory is further modified from the original description. The analyses were made with either homogenate or lysate made from the homogenized samples. The
muscle samples were homogenized in an ice cold buffer containing 50 mmol/l HEPES, 150 mmol/l NaCl, 20 mmol/l Na4P2O7, 20 mmol/l β-glycerophosphate, 10 mmol/l Na3VO4, 2 mmol/l EDTA, 1%, NP-40, 10%, glycerol, 2 mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin and 3 mmol/l benzamidine using a TissuLyserII (Quiagen, Germany) for 2 min. Freeze-dried samples from the human studies (study I, II and III) were diluted 1:80, while wet weight samples were diluted 1:20 in the mouse study (Study IV). Afterwards the samples were rotated end over end for 1 hour. A part of the homogenate is transferred to new tubes, while the remaining of the samples were centrifuged at 17500 g at 4°C for 20 min to obtain lysate (the supernatant), that were transferred to new tubes. The homogenates and lysates are stored at -80°C.

The protein concentration in the homogenates and lysates was determined in microtiter plates using the bicinchoninic acid method (Pierce Chemical). This method uses that the reaction between Cu²⁺ and protein under alkali conditions reduced Cu²⁺ to Cu⁺ that produces a color shift, detectable at a wavelength at 550 nm. This value is converted to a protein concentration based on albumin standards (Thermo Sientific, Rockford, IL, USA) loaded on the plate.

To prepare the samples for SDS-PAGE the homogenate and lysate samples were mixed with sample buffer, containing Tris Base, Dithiothreitol (DTT), sodium dodecyl sulfate (SDS), glycerol and bromphenol blue. The samples were heated to 96 °C for 3 min, that denatures the proteins and SDS coats the proteins with negative charges, according to the size of the protein approximately one SDS molecule for every two amino acid residues.

**Gels**

The hand casted or pre-made gels (24 well, Bio-Rad, Hercules, CA, USA) were placed into a gel electrophoresis cell and running buffer solution containing Tris-base, glycine, SDS amd H₂O is added. The samples were loaded onto the gel, together with a protein standard and a protein ladder in each side of the gel. The protein standards were used to normalize between gels and to evaluate both the run across each gel and the quality of the blotting process. The ladder helps to identify the relevant part of the gel for visualizing a specific protein of interest. An electric current was applied to the electrophoresis cell, 150 V and 40 mA per gel in the cell. The SDS coated proteins is negatively charged and will know be pulled through the gel according to charge and therefore the size of the protein. When the samples were running through the gel, a color front was created by the sample buffer, helping to estimate when the samples have proceeded sufficiently, through the gel.

The proteins in the gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P membrane, Millipore, Billerica, MA, USA) by using the semi dry blotting. The gel
piece of interest was cut out of the gel and placed in a sandwich consisting of 3x2 pieces of filter paper with the gel in between. The sandwich was stacked on a blotting apparatus (Transblot DF semi-dry Electrophoretic transfer cell, Bio-Rad, Hercules, CA, USA) and an electric field was applied at 20 V and 0.8 mA per cm² to horizontally draw out the proteins and captured them in the membrane.

After the protein transfer it is important to block the membrane in either bovine serum albumin (BSA), skin milk solution or fish gelatin to ensure that the PVDF membrane was blocked with protein. This improves specific binding of the primary antibody to the protein of interest. The membrane was then incubated overnight in primary antibodies diluted in BSA.

The following day the membrane was washed in tris buffered saline (TBS) with tween 20 (TBST) and species specific secondary antibodies (DAKO, Glostrup, Denmark) against the primary antibody was added. Horse radish peroxidase is attached to the secondary antibody and addition of ECL (Luminata, Millipore, Billerica, MA, USA) reagent containing luminol creates a photon which is detected by a digital photo analyzing system (Image Quant LAS4000, Munich, Germany and Carestream Health, Rochester, NY, USA). The proteins will in this way be visualized as bands on the PVDF membrane. The bands can then be analyzed and quantified using computer software (Image Quant TL, Munich, Germany and Carestream MI SE, Rochester, NY, USA). The intensity of the bands on each gel was divided with the average of the loaded standard on the same gel, making it possible to compare the intensity of samples loaded on different gels.

GS activity
The GS activity was determined in muscle homogenate as previously described (Thomas et al. 1968) but modified to microtiter plates. A total of 125 µg protein was used from each sample and diluted to a concentration of 1 µg/µl in a new tube. The GS activity was calculated as UDP-glucose incorporated into glycogen per minute per gram tissue in the presence of low concentration of G-6-P (0.02 mM), a physiological concentration (0.17 mM) of G-6-P and a high concentration (8 mM) of G-6-P to get the total activity. These values were then used to calculate the percentage of GS G-6-P independent GS activity (0.02mM / 8 mM) (GS I-form) and the GS fractional velocity (GS %FV) (0.17mM/ 8 mM).

PDHa activity
The activity of PDH in the active form (PDHa) is determined based on the rate of acetyl-CoA formation and the following determination of acetyl-CoA using a radioisotopic assay.
The protocol was modified from the methods described in two original manuscripts (Cederblad et al. 1990; Constantin-Teodosiu et al. 1991). Approx. ∼10 mg of frozen muscle sample was homogenized in 225 µl ice-cold homogenizing buffer containing Sucrose, KCl, MgCl₂, EGTA, Tris HCl, NaF, DCA and Triton X-100 in a glass tube on ice for 50 sec using a motor-driven homogenizer and a glass pluger (Kontest, Vineland, NJ, USA). This process will gently tear apart the tissue and the without disrupting the PDC in the mitochondria. The homogenate is transferred to a tube containing the rest of the homogenizing buffer up to a total volume given by 30 x weight of the sample and the sample is quickly frozen in liquid nitrogen.

The homogenate was transferred to an assay buffer solution containing Tris-base, EDTA and MgCl₂ and kept at 37°C. To start the reaction, pyruvate is added, and after 45 s, 90 s and 135 s sample was transferred to a new tube containing PCA. Duplicates are made and a reaction with water added instead of pyruvate is also performed to serve as “black”. The total of 9 samples was neutralized after 5 min incubation by addition of potassium bicarbonate. The samples were centrifuged at 10000 g for 3 min. The supernatant contains the acetyl-CoA of which the content was determined as previously described (Cederblad et al. 1990) the following 2 enzymatic reactions. A) $^{14}$C-aspartate + 2-oxogluterat $\rightleftharpoons$ $^{14}$C-Oxaloacetate + L-glutamate catalyzed by aspartate aminotransferase and B) $^{14}$C-Oxaloacetat + acetyl CoA $\rightleftharpoons$ $^{14}$C-citrate + CoASH catalyzed by citrate synthase. Reaction A was necessary because $^{14}$C- oxaloacetate is not commercially available. After incubation the unreacted $^{14}$C- oxaloacetate is transaminated back to $^{14}$C-aspartate in a reaction catalyzed by glutamic oxaloacetic transaminase (GOT) followed by separation of $^{14}$C-aspartate from the formed $^{14}$C-citrate using DOWEX, which is a cation exchange resin (Figure 9).

Standards with different concentration of acetyl-CoA were running along with the samples and were used to create a standard curve. $^{14}$C-citrate was measured in the scintillation counter. The count for each of the samples was related to the standard curve and an average of the duplicates was calculated. After taking dilutions during the assay into account, the PDHa activity was given in mmol·min⁻¹·kg⁻¹.

The total creatine content in each sample is extracted using the PCA method (Bergmeyer H.U. 1965). The method is described under creatine content.

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).
Fig 9 the reactions in the PDHa assay drawn by Prof. Henriette Pilegaard (Cederblad et al. 1990; Constantin-Teodosiou et al. 1991)

**Enzyme activity**

The enzyme activity of 3-hydroxyacyl-CoA and citrate synthase in study I were measured in muscle homogenate as previously described (Lowry, Passonneau 1972).

**Statistics**

In all manuscripts the values were presented as mean±SE. Two way ANOVA tests were used to evaluate the effects in study I. A Two-way ANOVA with repeated measures was applied to evaluate the effect of exercise and insulin before as well as after bed rest. Also, a two-way ANOVA with repeated measures was used to evaluate the effect of bed rest and insulin within the rested leg as well as within the exercised leg. The data were log transformed if normality or equal variance tests failed. If significant main effects were found, the Student-Newman-Keuls post hoc test was used to locate differences. Differences were considered significant at P < 0.05. A tendency is reported for 0.05 ≤ P ≤ 0.1. Statistical calculations were performed using SigmaStat version 3.1.

In study II a student paired t-test was used to test the effect of exercise training on fasting plasma insulin and glucose as well as protein content. Two-way ANOVA with repeated measures was used to test the effect of exercise training and glucose intake on plasma glucose, insulin and c-peptide as well as skeletal muscle protein and protein phosphorylation levels. When a main effect was observed, a Student-Newman-Keuls post hoc test was used to locate differences between groups if the data set was log transformed if the data did not pass equal variance test. The data were
considered significant at P<0.05 and a tendency is reported when 0.05≤P<0.1. Statistical calculations were performed in SigmaPlot 11.0.

In study III A Two Way ANOVA with repeated measures was used to evaluate the effect of LPS and exercise. The data were log transformed if normality or equal variance test failed. If a significant main effect was detected, the student Newman-Keuls test was used to locate differences. Differences are considered significant at p<0.05 and a tendency is reported for 0.05≤p<0.1. Statistical calculations were performed using SigmaPlot 11.0

In study IV a two-way ANOVA tests were used to evaluate the effect of fasting and IL-6 injection by testing within each time point and within each condition. The data were log transformed if equal variance test failed. If a main effect was observed, the Student–Newman–Keuls post hoc test was used to locate differences. In addition, a Student’s t test was used to evaluate the effect of IL-6 within a given time point and condition. Differences were considered significant when P <0.05 and a tendency is reported for 0.05≤P<0.1. The statistical tests were performed using SigmaPlot 11.0.
INTEGRATED DISCUSSION

In the following the data from the 4 manuscripts of the present thesis will be discussed. Additional data from the 4 studies not included in the manuscripts and unpublished data from a mouse HFD/exercise training study, a single LPS injection in mice, a single AICAR injection in mice and a study examining the impact of glucose intake in recovery from exercise in elite athletes will also be integrated.

Physical activity level

Effects of physical activity level on key proteins in insulin signaling and glucose uptake in skeletal muscle

The findings in Study I that 7 days of bed rest reduced whole body and skeletal muscle insulin sensitivity is in accordance with previous bed rest studies (Mikines et al. 1989; Mikines et al. 1991). Similarly, in Study I prior exercise enhanced the insulin-induced increase in the arterio-venous glucose difference as previously demonstrated (Wojtaszewski et al. 1997; Wojtaszewski et al. 2000). Together this provides the setting for studying the underlying mechanisms behind physical inactivity induced insulin resistance. Study I shows also for the first time that bed rest did not abolish the ability of exercise to increase insulin sensitivity of skeletal muscle 3h after exercise, although the glucose uptake was at a lower level than before bed rest. Furthermore, the observations that exercise training in elderly men resulted in lower plasma insulin concentration during an OGTT in the trained state than the untrained together with similar plasma glucose responses indicate that exercise training induced an increase in insulin sensitivity in aging skeletal muscle. This is in agreement with previous studies using either an OGTT (Cox et al. 1999) or a hyperinsulinemic euglycemic clamp (Dela et al. 1996) and provides the background for exploring the underlining mechanisms behind exercise training-induced improvements in glucose handling in elderly subjects.

The decrease in HKII and GLUT4 protein content in skeletal muscle of young subjects with bed rest in Study I is in accordance with many previous studies showing an increase with exercise training (Christ-Roberts et al. 2004; Cox et al. 1999; Dela et al. 1994c; Frosig et al. 2007). In addition, the increase in GLUT4 and the tendency for an increase in HKII protein with exercise training in the elderly subjects in Study II show that this ability was maintained in the elderly subjects as was also observed for oxidative proteins in the same study (Olesen et al., unpublished data). Together these findings support that the protein level of both HKII and GLUT4 depends on the physical activity level in both young and aged individuals. Because HKII and GLUT4 are
central in skeletal muscle glucose uptake, these changes have likely contributed to the observed changes in glucose regulation in Study I and II.

In study I, 7 days of bed rest resulted in lower protein content of Akt1 and Akt2 in skeletal muscle to about 50-70% of the level before bed rest and reduced the Akt Thr\(^{308}\) and Ser\(^{473}\) phosphorylation level even after normalization to Akt protein in the resting leg before insulin and in both legs after insulin infusion. However, the insulin-induced increase in Akt Thr\(^{308}\) and Ser\(^{473}\) phosphorylation during a hyperinsulinemic euglycemic clamp was unaffected by bed rest. The reduced Akt1 and Akt2 protein content in Study I is in contrast to the lack of change in Akt protein in a similar bed rest study in subjects with low birth weight (Mortensen et al. 2013), while the reduced Akt phosphorylation level with bed rest in Study I is in accordance with the reduced Akt Ser\(^{473}\) phosphorylation in the previous bed rest study (Mortensen et al. 2013). The reduced Akt1 and Akt2 protein levels in Study I are however in line with the observed increase in total Akt protein in previous exercise training studies in young subjects (Frosig et al. 2007; Vind et al. 2011) and does suggest that the physical activity level can influence Akt protein and phosphorylation level. Similarly, the observed almost 2 fold increase in Akt2 protein and the more marked increase in glucose-stimulated absolute Akt Ser\(^{473}\) phosphorylation in the elderly subjects after exercise training in Study II are in accordance with the findings in a previous study examining elderly aged subjects (Vind et al. 2011). These observations support that the Akt protein level is sensitive to the physical activity level and demonstrates that aging muscle maintains the ability to adapt in insulin signaling components like Akt to exercise training as for HKII and GLUT4. Of notice is that the glucose intake-induced increase in Akt phosphorylation in Study II was similar before and after exercise training, when Akt phosphorylation was normalized to the Akt2 protein level. This suggests that changes in Akt protein content underlines the changes observed in Akt phosphorylation with exercise training in the elderly subjects, while physical inactivity in young subjects influences not only protein content, but also additional mechanisms. Although this mechanism may be lost with aging it may suggest that the changes induced with physical inactivity and exercise training are not direct opposites, when it comes to Akt regulation.

Previous studies have reported that the protein content of Akt1 and Akt2 in skeletal muscle was unaffected in skeletal muscle of T2D patients (Vind et al. 2011), although total Akt protein content has been found to be reduced in another T2D human study (Christ-Roberts et al. 2004). In addition, as Akt is central in insulin signaling (Hojlund, Beck-Nielsen 2006; Hojlund et al. 2009; Karlsson et al. 2005; Meyer et al. 2002; Vind et al. 2012) the observed changes in Akt in T2D patients have been
suggested to contribute to insulin resistance (Hojlund, Beck-Nielsen 2006; Hojlund et al. 2009; Karlsson et al. 2005; Meyer et al. 2002; Vind et al. 2012). Therefore, the observed changes in Akt protein and Akt phosphorylation level in Study I and II may in part explain the reduced insulin sensitivity with bed rest observed in Study I and the seemingly increased insulin sensitivity with exercise training in Study II.

In addition, the observations that bed rest in Study I lowered both Akt1 and Akt2 protein content in skeletal muscle, while exercise training only increased Akt2 protein content in the elderly subjects in Study II may suggest that Akt1 is sensitive to physical inactivity but not exercise training or that the exercise training-induced regulation of Akt1 is age-dependent. While the previous exercise training studies in young subjects did not discriminate between the isoforms (Christ-Roberts et al. 2004; Frosig et al. 2007), a comparison of the Akt1 and Akt2 protein levels in skeletal muscle of the untrained subjects in Study III and well trained young subjects, shows that both Akt1 and Akt 2 protein are higher in exercise trained than untrained young subjects (Figure 10).

![Akt1 and Akt2 protein content in untrained and trained subjects. The untrained subjects are from Study III. #:significantly different from untrained, P<0.05](image)

Although it is possible that an exercise training-induced increase in Akt1 protein requires more than 8 weeks of regular physical activity, the present findings do bring up the possibility that the ability of exercise training to increase Akt1 protein in skeletal muscle is lost with age. As results from Akt1 overexpression in mice (Lai et al. 2004) have suggested that especially Akt1 plays a role in translational regulation and hence potentially hypertrophy, an age-related impairment in Akt1 regulation may be speculated to affect the ability to increase muscle mass rather than to improve glucose regulation with exercise training at increasing age.

The observations that 7 days of bed rest in Study I did not affect either TBC1D4 protein level or the insulin-induced TBC1D4 phosphorylation at Ser^{588}, Thr^{642} or Ser^{751} are surprising based on the
reduced Akt phosphorylation level. However, a similar lack of change in both total TBC1D4 protein and insulin-stimulated TBC1D4 phosphorylation has been observed in normal birth weight subjects in a previous 10 day bed rest study (Mortensen et al. 2013). Moreover, in accordance with the unchanged TBC1D4 protein with physical inactivity in Study I, exercise training in Study II did not affect TBC1D4 protein in skeletal muscle of the elderly subjects. On the other hand, previous studies have reported that TBC1D4 protein tended to increase with exercise training in young subjects (Frosig et al. 2007) and increased in aged subjects (Vind et al. 2012), while another study reported no change (Consitt et al. 2013). This may indicate that physical inactivity and exercise training do not necessarily impose opposite effects on TBC1D4 protein in young subjects and that the ability to increase TBC1D4 protein with exercise training depends on the exact exercise training protocol. Moreover, the previous finding that exercise training enhanced the insulin-stimulated TBC1D4 phosphorylation in skeletal muscle of young subjects (Consitt et al. 2013; Frosig et al. 2007) suggests that insulin-stimulated TBC1D4 phosphorylation is not inversely affected by physical inactivity and exercise training in young subjects as observed for Akt1, Akt2, GLUT4 and HKII. Furthermore, the observation that glucose intake-induced TBC1D4 phosphorylation was enhanced after the exercise training period in the elderly subjects in Study II is in accordance with a previous finding in elderly subjects (Consitt et al. 2013), and suggests that the ability to enhance insulin-stimulated GLUT4 translocation with exercise training is maintained in aged skeletal muscle.

The similar regulation of TBC1D4 before and after bed rest in Study I together with the similar insulin-stimulated increase in the arterio-venous glucose difference before and after bed rest supports that TBC1D4 is important in glucose uptake. It has been suggested that TBC1D4 in part can explain the ability of prior exercise to enhance insulin-stimulated skeletal muscle glucose uptake. However, the observations in Study I, that the insulin-stimulated regulation of TBC1D4 phosphorylation was similar in the rested and prior exercised leg, although the arterio-venous glucose difference was higher in the prior exercised leg than the rested leg, do not support an important role of TBC1D4 in this regulation.

**Effects of physical activity level on GS regulation in skeletal muscle**

In study I, bed rest did not affect GS protein content or total GS activity in skeletal muscle of young healthy subjects, although the latter seemed lower than before bed rest. These observations are in accordance with previous findings showing that exercise training in young subjects did not change GS protein content in skeletal muscle (Frosig et al. 2007). On the other hand, GS protein content
and total GS activity increased in skeletal muscle with exercise training in the elderly men in study II, which is in line with previous observations in aged subjects (Vind et al. 2011). The different effects of exercise training in young (Frosig et al. 2007) and elderly subjects (Study II) with an increase in both GS protein and GS activity only in the elderly subjects may suggest that an initial lower level in the elderly subjects than the young has resulted in a more marked effect of exercise training in the aged subjects. However, the previous findings that GS protein level and total GS activity in skeletal muscle were similar in T2D patients and healthy controls (Vind et al. 2011) may suggest that the different observations rather are due to the exercise training protocols. Hence, the high-intensity bicycling and cross fit performed by the elderly subjects in Study II may have provided a stronger stimulus for GS regulation than the one-legged knee extensor exercise used in the previous study (Frosig et al. 2007).

While bed rest in Study I did not influence the insulin-stimulated dephosphorylation of GS site 3a, and insulin did increase GS activity also after bed rest, the GS activity tended to be lower and the insulin-induced dephosphorylation of GS site 2+2a was blunted in the rested leg after 7 days in bed in Study I. This may in part resemble the impaired insulin-stimulated GS activation and GS site 2+2a dephosphorylation reported in skeletal muscle of insulin resistant subjects and T2D patients (Glintborg et al. 2008;Hojlund et al. 2003;Hojlund et al. 2006;Hojlund et al. 2008;Vind et al. 2011). Moreover, the elderly subjects in Study II had before exercise training no change in GS activity or GS site 2+2a phosphorylation after glucose intake, but GS activity increased and GS site 2+2a phosphorylation decreased in response to glucose intake after the exercise training period. Taken together, it may therefore be speculated that 7 days in bed resulted in an impaired insulin-induced regulation of GS activity due to dysregulation of GS site 2+2a and that this modification is similar to the changes observed in skeletal muscle of T2D patients (Vind et al. 2011) and potentially also elderly untrained subjects. In addition, as exercise training in young subjects has been shown not to change insulin-induced GS activity and/or GS site 2+2a phosphorylation in skeletal muscle, the changes observed in the elderly subjects in Study II may be explained by an initial impairment in GS regulation in the elderly subjects in Study II and in previous studies (DeFronzo 1981;Dela et al. 1996;Poulsen et al. 2005).

In study I, a single bout of exercise resulted in higher GS activity in the prior exercised leg before insulin and GS activity appeared higher in the prior exercised leg than the rested after insulin-stimulation. This may suggest that regulation of GS at least in part contributed to the enhanced insulin-stimulated arterio-venous glucose difference in the prior exercised leg relative to the rested
as previously suggested (Wojtaszewski et al. 1997; Wojtaszewski et al. 2000). Furthermore, the observations that insulin decreased GS site 3a phosphorylation only in the prior exercised leg and the 2+2a phosphorylation level was lower in the prior exercised leg than in the rested leg before and after insulin support that modifications of sites 3a and 2+2a likely have played a role in the higher GS activity in the prior exercised leg than the rested leg in Study I. This is however different from a previous study showing that prior exercise resulted in a further dephosphorylation of GS site 2+2a (Prats et al. 2009). In addition, Study I showed for the first time that 7 days in bed did not abolish the ability of prior exercise to enhance insulin-stimulated skeletal muscle glucose removal, although the arterio-venous glucose difference was at a lower level after bed rest than before. This ability may in part be explained by the maintained effect of prior exercise on basal GS activity, although GS activity only tended to be higher in the prior exercised leg than the rested leg after bed rest. Furthermore, the exercise bout seemed to prevent the bed rest-induced GS site 2+2 hyperphosphorylation, but the insulin-mediated regulation of GS site 2+2a was not restored. Together these findings support that GS site 2+2a regulation is important in the observed GS dysregulation in Study I and II.

As muscle glycogen is one of the factors thought to regulate GS site 2+2a phosphorylation (Nielsen, Wojtaszewski 2004), the observed changes in muscle glycogen may have played a role in the modifications in GS site 2+2a phosphorylation in Study I and II. Hence, the lower muscle glycogen in the prior exercise leg both before and after bed rest in Study I likely contributed to the higher GS activity in that muscle as previously suggested (Wojtaszewski et al. 1997; Wojtaszewski et al. 2000; Wojtaszewski et al. 2002b). Similarly, the elevated muscle glycogen levels in both legs after bed rest relative to before in Study I and the elevated muscle glycogen after exercise training relative to before in Study II may have contributed to the observed elevated GS site 2+2a phosphorylation, although other factors most likely also have been in play. The link between muscle glycogen levels and GS site 2+2a phosphorylation during recovery from exercise was further investigated in an additional study, where elite athletes exercised for 4h at 73% of HRmax and received either glucose or water for the initial 4h of recovery (Figure 10). During the 24h of recovery the energy intake was similar in the two groups. Muscle glycogen was markedly reduced after exercise in both groups, and while glucose intake resulted in an increase in muscle glycogen at 4h of recovery, no change was observed with H2O intake. However, muscle glycogen was similar at 24 h of recovery. These changes in muscle glycogen were associated with lower GS site 2+2a phosphorylation in the H2O group than the glucose group at 4h and seemingly less than half in the
H₂O group than in glucose group at 24h of recovery. As reduced GS site 2+2a phosphorylation reflects increased GS activity (Jensen et al. 2012; Prats et al. 2009), a higher GS activity may be assumed in the H₂O group than in the glucose group at 4h of recovery, which is in accordance with the expected effect of reduced muscle glycogen on GS activity (Nielsen et al. 2001; Wojtaszewski et al. 1997; Wojtaszewski et al. 2000). However, these findings may also suggest that suppression of muscle glycogen resynthesis by H₂O intake after exercise results in elevated GS activity even 24h after exercise although muscle glycogen has been restored (Figure 11 left, 11 right).

Fig 11 Muscle glycogen (Left) and GS site 2+2a phosphorylation (Right) in elite athletes before (Pre) and after 4h of exercise (Post) as well as at 4h of recovery with either H₂O (black) or glucose (grey) intake from Post to 4h (4h) and at 24h of recovery (24h). GS phosphorylation is normalized to GS protein content and given in arbitrary units (AU). Values are mean±SE. *: significantly different from Pre within given group, P<0.05; #: significantly different from H₂O group at given time point, P<0.05; §: significantly different from post exercise, P<0.05 and £: significantly different from 4h after exercise, P<0.05 and † significantly different from H₂O group located with t-test. Parentheses indicate tendency (Bogardus et al. 1984; Frosig et al. 2007; Perseghin et al. 1996).

d Effects of physical activity level on PDH regulation in skeletal muscle

Previous published results from the same experiment as Study I showed that 7 days of bed rest did not affect resting PDH-E1α protein, PDH phosphorylation levels or exercise-induced PDH regulation in human skeletal muscle (Kiilerich et al. 2011). This is in contrast to previous studies demonstrating that PDH-E1α protein content increases with exercise training in skeletal muscle of young subjects (LeBlanc et al. 2004a) and unpublished results showing that the PDH-E1α protein content and the PDH phosphorylation level are higher in well trained young subjects than in the untrained young subjects from Study III (FIGUR 12). The different sensitivity of PDH-E1α protein
to decreased and increased physical activity indicates that basal PDH-E1α maintenance does not require regular muscle contractions, while increased muscle activity will elicit an increased capacity for glucose oxidation in accordance with an increased demand from the working muscle.

The observation that PDH-E1α protein content and PDH phosphorylation increased with exercise training in the elderly subjects in Study II as observed in young subjects (LeBlanc et al. 2004a) shows that aged skeletal muscle maintains the ability to increase the capacity for glucose oxidation with exercise training. The similar increase in PDH-E1α and PDK2, but no change in PDK4 protein content, in study II suggests that PDK2 may have mediated the increased phosphorylation of PDH in the trained state in Study II. This is in accordance with unpublished results showing higher PDK2 protein in well trained young subjects than the untrained young subjects in Study III (Figure 11). The observations that PDK4 protein did not follow the increase in PDH-E1α protein with exercise training in Study II and the similar level of PDK4 protein in untrained and trained young subjects (unpublished results; Figure 11) may suggest that a trained muscle independent of age possesses less capacity for PDK4-mediated PDH inactivation per PDH-E1α molecule. Unpublished results from a mouse study demonstrated a similar parallel increase in PDK2 and PDH-E1α in skeletal muscle with exercise training of mice on HFD, but also a parallel increase in PDK4 protein matching the increase in PDH-E1α. On the other hand, while HFD did not affect PDK2 and PDH-E1α protein, PDK4 protein content increased providing a likely mechanism for a reduced PDHa activity as observed in untrained HFD mice (also relative to PDH-E1α protein) reflecting an enhanced fatty acid oxidation during the HFD. Although the increase in PDK4 protein with exercise
training in the mouse study (Figure 13) may be species specific, it seems likely that it is due to the exercise training being combined with HFD intake. Hence, taken together these findings support previous studies (LeBlanc et al. 2004b; LeBlanc et al. 2004a) and show that endurance exercise training increases PDH-E1α protein content in skeletal muscle and that physical activity level regulates PDK2 protein, while PDK4 protein mainly is regulated by substrate availability (Peters et al. 1998; Peters et al. 2001b).

In accordance with previous studies (Peters et al. 1998; Peters et al. 2001b; Peters et al. 2001a). The above mentioned HFD mouse study demonstrated that 4 month of HFD resulted in decreased resting PDHa activity in mouse skeletal muscle (unpublished data). In addition, 4 month of HFD combined with exercise training in mice increased resting PDHa activity relative to sedentary HDF mice. This may suggest that the exercise trained muscle maintained the ability to oxidize carbohydrates to the same extend as chow fed mice. Still, no difference was observed in the RER during submaximal treadmill exercise. However, it may be speculated that the effect of the enhanced resting PDHa activity, which was matched by a similar increase in PDH-E1α protein, first becomes evident during more intense exercise, where carbohydrate oxidation is dominant and required (unpublished data). This remains to be determined.

Previous unpublished results from our group has shown that PDH phosphorylation and PDHa activity were unaffected during the 3h hyperinsulinemic euglycemic clamp in the same experiment as used in Study I (Kiilerich, Biensø, Ringholm, Wojtaszewski, Calbet, Pilegaard) both before and
after bed rest. However, while insulin was elevated, the plasma glucose concentration was kept constant and relatively low (5 mM) in Study I, and the use of an OGTT in Study II aimed at providing a more physiological condition with a transient increase in plasma glucose and plasma insulin. The findings in Study II that glucose intake did not affect PDHa activity in skeletal muscle of the elderly subjects in the untrained state and even reduced PDHa activity after the exercise training period were therefore unexpected. Because a previous study has reported increased PDHa activity in human skeletal muscle with glucose intake (Pehleman et al. 2005), the lack of change in Study II may indicate that age or training status has played a role. However, the observation that glucose intake reduced the PDHa activity in the elderly subjects only after the exercise training period may suggest that exercise training prevented an otherwise impaired regulation in the elderly untrained subjects in Study II. In addition, the observed combined regulation of PDH and GS after the exercise training period may suggest that glucose oxidation is inhibited to prioritize glycogen storage after the glucose intake, although this suggestion remains to be examined.

**Regulation of PDH during and after exercise**

**IL-6**

The previous finding that PDHa activity after an initial increase decreases towards resting level as the exercise duration proceeds above 2h has led to the suggestion that PDH is involved in regulating the switch from carbohydrate to fat oxidation during prolonged exercise, although PDH does not seem to be the only factor (Mourtzakis et al. 2006; Pilegaard et al. 2006; Watt et al. 2004). Study IV aimed at elucidating the potential role of IL-6 in this regulation. A single injection of recombinant IL-6 was given to fed and fasted mice to mimic the transient IL-6 increase in response to exercise during different metabolic states. The injection increased the plasma IL-6 9.5 fold, which is rather similar to what has previously been reported in mice running on a treadmill (Nedachi et al. 2008). The observation that IL-6 reduced PDHa activity in the fed state was in line with the hypothesis that IL-6 reduced glucose oxidation in skeletal muscle through an inhibition of PDH. The finding, that IL-6 did not affect AMPK and ACC phosphorylation in the fed state, indicated however that this IL-6-mediated effect on PDH was not through AMPK, which is not in agreement with a previous study in rats reporting that IL-6 increased the AMPK phosphorylation in skeletal muscle (Kelly et al. 2009). However, the observation that AMPK and ACC phosphorylation was increased in the fasted mice 1h after the injection together with an IL-6-induced increase in PDHa activity may suggest that an IL-6-AMPK-PDH pathway does exist in fasting conditions although the effect is opposite of expected based on a previous study in AMPK KO mice (Klein et al. 2007). The idea
that PDH should be a direct target for AMPK was also investigated in a pilot study. Fed mice were injected with the AMPK activator AICAR. AMPK phosphorylation increased but no change was observed in PDHa activity or PDH phosphorylation (data not shown). Furthermore, IL-6 was also injected at a higher dose resulting in 10 fold higher IL-6 plasma concentration than in Study IV (data not shown), but this did not affect PDHa activity or PDH phosphorylation in skeletal muscle. Taken together, it seems that IL-6 can regulate PDH in mouse skeletal muscle, but that the level of IL-6 and the metabolic condition of the muscle influence this regulation. In addition, whether such an IL-6-mediated regulation of PDH takes place during prolonged exercise remains to be determined.

**Inflammation and PDH regulation during exercise**

Several studies have found that insulin resistant subjects have an impaired ability to switch to glucose oxidation during an hyperinsulinemic euglycemic clamp (Kelley, Mandarino 2000;Mandarino et al. 1996). The aim of Study III was to investigate whether inflammation is associated with a similar metabolic inflexibility in exercise-induced PDH and AMPK regulation in human skeletal muscle reflecting the ability to enhance substrate oxidation. The observation in Study III that PDH and AMPK regulation in human skeletal muscle was not changed at rest or during exercise after LPS injection resulting in a 17 fold increase in plasma TNFα indicates that low grade inflammation does not affect substrate oxidation at rest and in the initial phase of exercise. These findings are in line with the observation that local infusion of TNFα or LPS did not change PDH phosphorylation in human skeletal muscle (Bach et al. 2013;Buhl et al. 2013), but different from studies in rats and another study in humans reporting that PDH or AMPK regulation was changed during sepsis or LPS infusion (Andreasen et al. 2008;Andreasen et al. 2011;Crossland et al. 2008;Vary, Martin 1993;Vary et al. 1998;Vary, Hazen 1999). Additional observations in a pilot study that PDH Ser³⁰⁰ phosphorylation increased in mouse skeletal muscle 2h after a single LPS injection compared with saline (unpublished results; FIGURE 14) do support the latter findings in rats and humans. Although species differences may explain the different effects of LPS on PDH regulation in Study III and the previous studies (Crossland et al. 2008;Vary, Martin 1993;Vary et al. 1998;Vary, Hazen 1999), it is also possible that the level of cytokines like TNFα is important for the effects on PDH. Hence, the pilot study in mice resulted in 23 fold increase in plasma TNFα, while plasma TNFα increased 17 fold in Study III. But this remains to be clarified.
Fig 14 PDH Ser^{300} phosphorylation 2h after saline or LPS injection. Values are mean±SE. (*): tendency to be significantly different from saline, 0.05≤p<0.1.

As mentioned previous published results (Kiilerich et al. 2011) from the same experiment as Study I have shown that the exercise-induced regulation of PDHa activity and PDH phosphorylation was similar before and after bed rest, even though Study I shows that muscle insulin sensitivity was reduced. However, the exercise-induced AMPK phosphorylation was abolished after the bed rest (Ringholm et al. 2011). In accordance with the observations in Study III, this effect did not seem to be caused by inflammation, because systemic inflammation was not detected after bed rest (Olesen et al, unpublished data). Taken together, these observations suggest that the exercise-induced regulation of PDH is rather robust, although previous studies have observed effects of intralipid and high fat diet (Kiilerich et al. 2011;Pilegaard et al. 2006). In addition, the maintained exercise-induced PDH and AMPK regulation in Study III together with previous studies reporting that inflammation induces insulin resistance (Bach et al. 2013;Buhl et al. 2013;Plomgaard et al. 2005) suggests that insulin sensitivity and exercise-induced substrate regulation are affected differently during inflammation, although it cannot be ruled out that more prolonged inflammation also will affect exercise-induced metabolic flexibility.

PDH regulation during recovery from exercise
PDH has also been suggested to play a role in substrate regulation in recovery from exercise. To investigate this further, PDH regulation was examined in the elite athletes described above after prolonged exercise with either glucose or only water during the initial 4h of recovery (Figure 15). PDH phosphorylation was at 4h lower in the glucose group than the water group indicating a higher
glucose oxidation when glucose was available. However, the PDH phosphorylation level was still higher, reflecting reduced PDHa activity, 4h after glucose intake than before exercise, which suggests that the muscle prioritizes glycogen resynthesis rather than glucose oxidation in recovery from prolonged endurance exercise, when glucose is available during recovery, as also suggested above after glucose intake without acute exercise in Study II.

Fig 15. Phosphorylation of PDH Ser^{300} (site p2) in elite athletes before (Pre) and after 4h of exercise at 73% HR_{max} (Post), 4h after exercise with H_{2}O (black) or glucose intake (grey) from Post to 4h (4h) and at 24h of recovery (24h). Values are mean±SE. *: significantly different from Pre within given group, P<0.05; #: significantly different from H_{2}O group at given time point, P<0.05; §: significantly different from post exercise, P<0.05. Parentheses indicate a tendency.
Conclusion

• Study I demonstrated that bed rest–induced insulin resistance was associated with reduced insulin-stimulated GS activity and Akt signaling as well as decreased protein level of HKII and GLUT4 in skeletal muscle. This suggests that decreased glucose transport/phosphorylation and decreased non-oxidative glucose metabolism contributed to the decreased skeletal muscle glucose extraction with physical inactivity in humans. In addition, the ability of acute exercise to increase insulin-stimulated glucose extraction was maintained after 7 days of bed rest. However, acute exercise after bed rest did not fully normalize the ability of skeletal muscle to extract glucose to the level seen, when exercise was performed before bed rest.

• Study II demonstrated that exercise training-improved glucose regulation in elderly healthy subjects was associated with increased HKII, GLUT4, Akt2, PDK2, GS and PDH-E1α protein content. Moreover, exercise training resulted in an enhanced glucose-stimulated TBC1D4 response relative to before training and GS activity increased while PDHa activity decreased after glucose intake only in the trained state. This shows that aged human skeletal muscle can adapt to exercise training. In addition, these results suggest that increased content of key proteins in glucose metabolism and acute molecular changes towards improved glucose uptake and storage contributed to the observed exercise training-improved glucose handling in aged human skeletal muscle.

• Study III demonstrated that LPS-induced inflammation with elevated plasma TNFα concentration did not affect resting or exercise-induced AMPK and PDH regulation in human skeletal muscle. This suggests that short-term inflammation did not influence resting or exercise-induced fat and carbohydrate utilization in human skeletal muscle.

• Study IV demonstrated that IL-6 reduced PDHa activity in skeletal muscle from fed mice, and increased the PDHa activity in skeletal muscle from fasted mice without any change in phosphorylation level. IL-6 injection increased AMPK and ACC phosphorylation in mouse skeletal muscle only in the fasted state. This suggests that IL-6 regulates PDHa activity in mouse skeletal muscle, but that the effect depends on the energy state of the muscle. In addition, AMPK may be involved in the fasted state.

Taken together the present findings suggest that modifications in key factors in skeletal muscle glucose uptake and storage contribute to the altered insulin-stimulated glucose handling with changes in physical activity level. Furthermore, the maintained exercise-induced metabolic regulation in skeletal muscle during acute inflammation suggests that exercise-induced metabolic
flexibility is unaffected during low grade inflammation. In addition, IL-6 may regulate substrate utilization in skeletal muscle through effects on PDH.
**Perspective**

Several questions have already been answered with this thesis, but there were several interesting findings that could be further pursued.

In study II the unchanged PDHa activity with glucose stimulation before the training intervention in the elderly subjects was unexpected and the decreased PDHa activity after the 8 weeks of training was also unexpected. Because a previous study observed increased PDHa activity with glucose stimulation (Pehlemand), it may be speculated that aging caused this. Hence, it would be interesting to conduct the same experiment in untrained and trained young subjects. In line with this a similar experiment would be interesting to perform in T2D patients.

Most of the previous studies investigating the effect of insulin on PDH regulation have used a hyperinsulinemic euglycemic clamp and observed no effect of insulin. It may be speculated that the lack of an elevation in plasma glucose concentration is the reason for the absent PDH regulation. Therefore it would be interesting to perform a hyperglycemic clamp in both healthy subjects and insulin resistant subjects.

The current study did not observe any effects of acute inflammation on PDH and AMPK regulation. However, it may be due to the relatively short period of inflammation. Therefore, it would be interesting to investigate the effect of more prolonged inflammation on PDH regulation in skeletal muscle both in humans and rodents, with both healthy and diseased individuals.

The present findings indicated that IL-6 may regulate PDH in skeletal muscle. However this was based on injection of recombinant IL-6. Therefore it would be interesting to further examine the potential role of IL-6 in PDH regulation during prolonged exercise for example by using IL-6 knockout mice.

Three phosphorylation sites in PDH-E1α have been analyzed in the current PhD studies. However, a fourth site is known (Ser232). This site was previously thought not to play a role in skeletal muscle, but recent studies do indicate that this may not be a correct conclusion. Therefore, it would be interesting to supplement the present analyses with phosphorylation status of this site, Ser232.
Reference List


glucose transport is dependent on glucose phosphorylation capacity. *Endocrinology* **145**:4912-4916.


Study I

GLUT4 and Glycogen Synthase Are Key Players in Bed Rest–Induced Insulin Resistance

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To elucidate the molecular mechanisms behind physical inactivity–induced insulin resistance in skeletal muscle, 12 young, healthy male subjects completed 7 days of bed rest with vastus lateralis muscle biopsies obtained before and after. In six of the subjects, muscle biopsies were taken from both legs before and after a 3-h hyperinsulinemic euglycemic clamp performed 3 h after a 45-min, one-legged exercise. Blood samples were obtained from one femoral artery and both femoral veins before and during the clamp. Glucose infusion rate and leg glucose extraction during the clamp were lower after than before bed rest. This bed rest–induced insulin resistance occurred together with reduced muscle GLUT4, hexokinase II, protein kinase B/Akt1, and Akt2 protein level, and a tendency for reduced 3-hydroxyacyl-CoA dehydrogenase activity. The ability of insulin to phosphorylate Akt and activate glycogen synthase (GS) was reduced with normal GS genase activity. The ability of insulin to phosphorylate Akt and activate glycogen synthase (GS) was reduced with normal GS site 3 but abnormal GS site 2+2a phosphorylation after bed rest. Exercise enhanced insulin-stimulated leg glucose extraction both before and after bed rest, which was accompanied by higher GS activity in the prior-exercised leg than the rested leg. The present findings demonstrate that physical inactivity–induced insulin resistance in muscle is associated with lower content/activity of key proteins in glucose transport/phosphorylation and storage. Diabetes 61:1090–1099, 2012

Lifestyle-related diseases like type 2 diabetes are rapidly increasing worldwide, and there is strong evidence that physical inactivity contributes to this development (1). The prediabetic and diabetic states are characterized by peripheral insulin resistance (2), and due to the large mass of skeletal muscle, the insulin action in this tissue has major influence on whole-body insulin sensitivity, especially in lean adults (3). Insulin sensitivity in skeletal muscle is to a large extent a dynamic parameter that can be enhanced by exercise training and decreased by physical inactivity (4–7). Thus, research attempting to combat diseases related to insulin resistance has focused on mechanisms by which the sensitivity of the glucose uptake to insulin is regulated.

Type 2 diabetes patients with peripheral insulin resistance have impaired GLUT4 translocation in skeletal muscle (8). Moreover, muscle insulin–resistant individuals and type 2 diabetes patients have impaired insulin-stimulated Akt Thr308 and Ser473 phosphorylation (9–11), lower insulin-stimulated Akt substrate of 160 kDa (AS160/TBC1D4) phosphorylation on multiple sites (7,9–11), and impaired insulin-stimulated glycogen synthase (GS) activity (9). The impaired GS activity in type 2 diabetes patients is associated with hyperphosphorylation of GS site 2+2a during insulin stimulation, whereas GS site 3a phosphorylation appears to be unaffected (9,12), indicating that GS phosphorylation downstream of Akt is maintained despite lower Akt phosphorylation. Exercise training has been shown to enhance the capacity for glucose transport and glycogen storage in skeletal muscle by increasing GLUT4, hexokinase II (HKII), and TBC1D4 protein content as well as GS activity in both healthy and type 2 diabetic patients, and these adaptations may contribute to the training-induced improvements in insulin sensitivity (13–15). In addition, the improved insulin sensitivity observed after exercise training has been reported to be associated with improved, and in fact normalized, insulin-induced phosphorylation of TBC1D4 in type 2 diabetes (7). In line with the changes observed with training, physical inactivity has been demonstrated to induce insulin resistance (5,16). However, to the best of our knowledge, no study has reported the effects of physical inactivity on key proteins in glucose metabolism in human skeletal muscle.

Muscle insulin sensitivity has been shown to be increased in the period after a single bout of exercise (17). Thus, enhanced insulin-mediated glucose clearance is observed for up to 48 h after a single exercise bout (17–19). It has been shown in rat skeletal muscle that the increased insulin sensitivity to glucose uptake is associated with an increased GLUT4 translocation to the plasma membrane (20). Moreover, multiple studies have documented that insulin-mediated activation of the most proximal insulin signaling elements (e.g., insulin receptor, insulin receptor substrate, phosphatidylinositol-3 kinase, and Akt) is not enhanced after a single bout of exercise (21–23). Interestingly, recent studies have revealed that basal and insulin-induced TBC1D4 phosphorylation are increased in the period after a single bout of exercise (24,25), although unchanged TBC1D4 phosphorylation has also been reported, but in a study not detecting increased glucose uptake after exercise (26). Studies have also reported an increased GS activity 3–4 h after exercise (27), and thus a combination of increased phosphorylation of TBC1D4 (regulating glucose transport)
and increased GS activity (regulating glycogen synthesis) may explain the increased sensitivity of insulin to stimulate glucose uptake 3–4 h after a single bout of exercise.

Knowing the underlying molecular mechanisms behind physical inactivity–induced insulin resistance and the impact of physical inactivity on exercise-induced insulin sensitivity may provide the basis for developing pharmacological agents and interventions that can counteract the development of insulin resistance in skeletal muscle during bed rest after surgery/illness as well as induced by a physically inactivel lifestyle. The aim of the current study was to test the hypotheses that 1) physical inactivity–induced insulin resistance in human skeletal muscle is due to dysregulation of both GS and TBC1D4 and 2) exercise-induced enhancement of insulin sensitivity is lost with physical inactivity.

RESEARCH DESIGN AND METHODS

Subjects. Twelve young, healthy male subjects participated in the study (Table 1). The subjects were physically active, as they all walked or bicycled for >1 h/day. The subjects were given both oral and written information about the experimental protocol and procedures as well as the discomfort they might experience during the experiment. After having received this information, the subjects gave their written consent. The study was performed according to the Declaration of Helsinki and was approved by the Copenhagen and Frederiksberg Ethics Committee in Denmark (H-C-2007-0085).

Bed rest. The subjects were placed in bed for 7 days and were given information about the importance of not moving their legs. All transport of subjects took place in wheelchairs and subjects were told to sit down during showers. The subjects were encouraged to sit in the wheelchair for up to 5 h per day to avoid vascular complications. The daily energy intake during the bed rest came from meals containing 10–20% protein, 50–60% carbohydrates, and 25–35% fat. The meals were served ad libitum.

Physical performance and body composition. Twelve subjects were included in the bed rest part (Table 1). Six of these subjects were chosen randomly to follow a study protocol involving one-legged exercise and a hyperinsulinemic euglycemic clamp. The characteristics of these subjects are given in brackets in Table 1.

Approximately 2 weeks before the bed rest, maximal oxygen uptake (VO2max) was determined by an incremental test on a cycle ergometer (Monarch Ergometer). VO2max measures was applied to evaluate the effect of exercise and insulin before as described (25,34).

Experimental protocol. The day before the experimental day (both before and after the bed rest period), the subjects consumed a prepackaged, standardized meal and evening snack regulated for body weight (14.3 kcal/kg and 2.9 kcal/kg, respectively). On the experimental day, the subjects arrived to the laboratory in the morning. Catheters were placed in the femoral vein of each leg and in one femoral artery. Three and a half hours after consuming a standardized breakfast (2.9 kcal/kg), the subjects completed 45 min of one-legged knee extensor exercise at 60% of Wattmax. This relatively low intensity was chosen to ensure that the subjects would be able to complete the exercise bout after the bed rest. Three hours into recovery, a 3-h euglycemic hyperinsulinemic clamp with a constant rate of insulin infusion (50 mU/[min · m2]) (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was performed. Arterial blood samples were obtained every 5–10 min for immediate determination of plasma glucose concentration (Radiometer ABL 725 series Acid-Base Analyser). Euglycemia was maintained by subsequent adjustment of the glucose infusion rate (GIR). The duration of 3 h was chosen to approach clamp steady-state conditions at the sampling time points.

Muscle biopsies were obtained from the vastus lateralis of both the previously exercised (Ex leg) and the rested leg (Rest leg) both before (B) and after (A) the clamp using the needle biopsy technique (29) with suction. Incisions for biopsies were made under local anesthesia (lidocaine; AstraZeneca, Sodertalje, Sweden) and individual incisions were used for each biopsy. The biopsies were quickly frozen in liquid nitrogen (within 10–20 s) after removing visual blood and connective tissue.

Before as well as at 2, 2.5, and 3 h of the insulin clamp, blood samples were drawn from the three femoral catheters and placed in EDTA-treated tubes. Plasma was collected by centrifugation and stored at –80°C.

Plasma analyses. The femoral arterial and venous plasma glucose concentrations were measured using an acid-base analyzer (Radiometer ABL 725 series Acid-Base Analysers), and arterial insulin concentration was measured by ELISA (K6219; DAKO, Glostrup, Denmark). Glucose extraction across the leg was calculated as the difference between the glucose concentration in the arterial and venous plasma samples. The plasma palmitate concentration was determined by gas chromatography (30).

Muscle samples were freeze dried for >48 h. Visible blood, fat, and connective tissue were removed under a microscope.

Glycogen was measured as glycosyl units after acid hydrolysis using an automatic spectrophotometer as previously described (31,32).

Enzyme activities. The activity of 3-hydroxyacyl-CoA dehydrogenase (HAD) and citrate synthase (CS) was analyzed spectrophotometrically as previously described (32). The GS activity was determined in muscle homogenates as previously described (22,33).

SDS-PAGE and Western blotting. Muscle homogenate, muscle lysate, and protein concentration determination were prepared as previously described (25,34).

The protein or phosphorylation of β-actin, Hki, GLUT4, Akt, GS kinase β (GS-β), and TBC1D4 in muscle lysates and GS protein and protein phosphorylation in muscle homogenates were measured by SDS-PAGE and Western blotting (25,34).

Primary polyclonal antibodies against β-actin (4967, Cell Signaling Technology, Beverly, MA), Hki2 (2867; Cell Signaling Technology), GLUT4 (ABR-PAIL065; Thermo Scientific, Golden, CO), Akt1 and Akt2 (2867 and 3063, respectively; Cell Signaling Technology), GS-α and -β (9338 and 9315, respectively; Cell Signaling Technology), and TBC1D4 protein (ABCAM, Cambridge, UK) as well as against protein phosphorylation of Akt Thr308 (05-669; Upstate Biotechnology, Lake Placid, NY) and Ser473 (05-802; Millipore, Bedford, MA), GS-3α Ser21 and GS-3β Ser2 (9331 and 9323, respectively; Cell Signaling Technology), TBC1D4 Ser33 (3028-P2 and 3028-P1, respectively; Synnaxis, Auckland, N.Z.), and TBC1D4 Ser35 (31, provided by Graham Hardie, Dundee University, Dundee, U.K.) were used. Primary polyclonal antibodies against GS protein (provided by Olfur Pedersen, University of Copenhagen) and phosphospecific antibodies against 2-4a, 1b (provided by Graham Hardie), and 3a (3891; Cell Signaling Technology) were also used. Membranes probed with phosphospecific antibodies were stripped to allow for determination of the level of the corresponding protein as previously described (25).

Statistics. Values presented are means ± SE. Two-way ANOVA with repeated measures was applied to evaluate the effect of exercise and insulin before as well as after bed rest. Also, a two-way ANOVA with repeated measures was applied to evaluate the effect of exercise and insulin before as well as after bed rest. Values are means ± SE, n = 12. Values given in parentheses represent the six subjects randomly chosen for the invasive exercise and clamp study. *Significantly different from before bed rest, P < 0.05.

<table>
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<tr>
<th>TABLE 1 Subject characteristics before and after bed rest</th>
<th>Before bed rest</th>
<th>After bed rest</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>26 ± 3 (29 ± 5)</td>
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<tr>
<td>Height (cm)</td>
<td>181 ± 2 (183 ± 7)</td>
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<td>Weight (kg)</td>
<td>75.2 ± 3.2 (81.6 ± 4.9)</td>
<td>75.1 ± 3.3 (81.4 ± 5.0)</td>
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<td>VO2max (L/min)</td>
<td>3.9 ± 0.2 (4.1 ± 1.0)</td>
<td>3.7 ± 0.2 (3.9 ± 0.4)</td>
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<td>Leg muscle mass (kg)</td>
<td>20.6 ± 1.0 (21.7 ± 1.9)</td>
<td>20.0 ± 0.9 (21.0 ± 1.7)</td>
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<tr>
<td>Percentage of whole body fat (kg)</td>
<td>18.2 ± 2.1 (21.7 ± 3.3)</td>
<td>18.5 ± 2.1 (21.7 ± 3.1)</td>
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Age, height, weight, VO2max, leg muscle mass, and percentage of whole body fat before and after 7 days of bed rest. Values are means ± SE; n = 12. Values given in parentheses represent the six subjects randomly chosen for the invasive exercise and clamp study. *Significantly different from before bed rest, P < 0.05.
used to evaluate the effect of bed rest and insulin within the rested leg as well as within the exercised leg. The data were log transformed if normality or equal variance tests failed. If significant main effects were found, the Student-Newman-Keuls post hoc test was used to locate differences. Differences were considered significant at \( P < 0.05 \). A tendency is reported for \( 0.05 \leq P < 0.1 \). Statistical calculations were performed using SigmaStat version 3.1.

RESULTS

Physical characteristics and performance. Total body weight and BMI were unaffected by 7 days of bed rest (Table 1). Leg muscle mass was reduced 3 ± 0.7\% (\( P < 0.05 \)), whereas whole body fat did not change significantly with bed rest (Table 1). Seven days of bed rest reduced whole body \( V_{O_{2\max}} \) 5 ± 1.4\% (\( P < 0.05 \)) (Table 1).

Protein content and enzyme activity. Skeletal muscle HAD activity tended to decrease 8 ± 4.7\% (0.05 ≤ \( P < 0.1 \)) by bed rest, whereas CS activity did not change significantly (Table 2). The protein content of HKII (66 ± 15\%), GLUT4 (74 ± 14\%), Akt1 (56 ± 6\%), and Akt2 (68 ± 7\%) was reduced after bed rest relative to before (\( P < 0.05 \)), whereas the protein level of TBC1D4 and GS and GS total activity were unchanged with bed rest (Fig. 1 and Table 2).

Plasma insulin. The average plasma insulin concentration before exercise, immediately after, and 3 h into recovery was not significantly different before and after bed rest, with an average of 32 ± 15 pmol/L before and 74 ± 20 pmol/L after bed rest. However, during the last hour of the clamp, plasma insulin concentration tended to be higher after (452 ± 66 pmol/L) than before (341 ± 30 pmol/L) bed rest (0.05 ≤ \( P < 0.1 \)) (Table 3).

Plasma glycerol. The plasma glycerol concentration was unaffected by bed rest both before and after the insulin clamp. Insulin reduced the plasma glycerol concentration both before and after bed rest (\( P < 0.05 \)).

Plasma glucose and GIR. The arterial plasma glucose concentration during the clamp was similar, 4.9 ± 0.1 mmol/L before and after bed rest. The GIR did not reach a steady state even after 180 min (Fig. 2A). At all time points after 50 min, GIR was higher before than after bed rest (\( P < 0.05 \)). During the last hour of the clamp, GIR after bed rest was 75 ± 12\% of the level before bed rest (\( P < 0.05 \)) (Fig. 2A).

Glucose extraction. Glucose extraction was evaluated during the last hour as the arterio-venous concentration difference across the leg. It has previously been reported that glucose extraction increased only in the exercising leg during the one-legged exercise (35). Three hours after exercise, glucose extraction across the leg was similar in the exercised and rested leg, reflecting that the acute exercise effect on glucose extraction was fully diminished at 3 h of recovery from exercise (corresponding to pre-insulin) (Fig. 2B). This was seen both before and after bed rest. Insulin infusion increased glucose extraction both in the prior-exercised and the rested leg, both before and after bed rest (Fig. 2B). During the last hour of the clamp before bed rest, glucose extraction in the rested leg was 11-fold (\( P < 0.05 \)), and the prior-exercised leg sixfold (\( P < 0.05 \)), higher after insulin than at basal. After bed rest, glucose extraction was six- and sevenfold (\( P < 0.05 \)) higher after insulin than at basal in the rested and prior-exercised leg, respectively. The insulin-stimulated glucose extraction in the rested leg after bed rest was 78 ± 10\% (\( P < 0.05 \)) of the level before bed rest. Also, insulin-stimulated glucose extraction before bed rest was 1.6 ± 0.2-fold (\( P < 0.05 \)), and after bed rest 1.9 ± 0.2-fold (\( P < 0.05 \)), higher in the prior-exercised than in the rested leg (Fig. 2B).

Muscle glycogen. Three hours after exercise, muscle glycogen content was ~20\% (\( P < 0.05 \)) lower in the prior-exercised than the rested leg. This was observed both

![FIG. 1. Representative blots showing GLUT4, HKII, Akt1, Akt2, TBC1D4, and GS protein before and after 7 days of bed rest in rested (Rest) and prior-exercised (Ex) leg for the same subject. Although the average Akt2 protein content of all subjects is reduced with bed rest (Table 2), it may be noted that the abundance of Akt2 is not reduced with bed rest in the subject used for the representative blots. kD, kilodaltons.](image-url)
before and after bed rest. There was no change in the muscle glycogen level in response to insulin infusion. Bed rest per se tended to enhance the muscle glycogen content ~20% (0.05 ≤ P < 0.1) in the rested leg (Fig. 2C).

**Muscle signaling**

**Akt phosphorylation.** Akt Thr308 phosphorylation was similar in the rested and exercised leg at 3 h of recovery both before and after bed rest. Insulin infusion increased Akt Thr308 phosphorylation similarly four- to fivefold in both legs before and after bed rest. No effect of prior exercise was observed on insulin-induced Akt phosphorylation. However, bed rest decreased Akt Thr308 phosphorylation 20–30% (P < 0.05) after the clamp (Fig. 3A and C). This decrease was observed in both the prior-exercised and rested leg. A similar phosphorylation pattern was observed for Akt Ser473 (data not shown). Thus, the insulin resistance induced by bed rest was associated with decreased signaling through Akt, even after accounting for decreased Akt2 protein expression (Table 2).

**GSK-3 phosphorylation.** The phosphorylation pattern of Akt target sites (GSK-3α Ser21 and GSK-3β Ser9) did not reflect the 20–30% lower Akt phosphorylation after bed rest. Phosphorylation of GSK-3β Ser9 was however 10–20% (P < 0.05) lower in the prior-exercised than the rested leg at 3 h recovery both before and after bed rest, but the ability of insulin to induce phosphorylation was apparently not significantly changed by either bed rest or prior exercise (Fig. 3B and C). Effects of insulin on GSK-3α Ser21 phosphorylation were too small to be detected with the antibody applied (data not shown).

**TBC1D4 phosphorylation.** Being a direct target of Akt TBC1D4 phosphorylation was evaluated by the use of phosphospecific antibodies toward three phosphorylation sites (Ser308, Thr422, and Ser421). The general phosphorylation pattern revealed by these antibodies was similar (Fig. 4). Thus, both before and after bed rest, TBC1D4 phosphorylation 3 h after exercise was similar in the rested and prior-exercised leg. Insulin stimulation increased the TBC1D4 phosphorylation 1.5- to 3-fold (P < 0.05) in both legs before and after bed rest, and there was no change in the insulin-stimulated TBC1D4 phosphorylation with bed rest (Fig. 4).

**Glycogen synthase activity and phosphorylation**

**GS activity.** The GS activity was evaluated both as %I-form (not shown) and percent fractional velocity (%FV). The two measures of GS activity revealed the same pattern of regulation. GS %FV activity was enhanced (P < 0.05) in the prior-exercised leg relative to the rested leg 3 h after exercise both before (P < 0.05) and after bed rest (0.05 ≤ P < 0.1). Insulin increased GS activity in both legs before and after bed rest and with a similar magnitude (P < 0.05). However, in both legs, the level of GS activity tended to be lower after bed rest than before (0.05 ≤ P < 0.1) (Fig. 5A).

**GS phosphorylation.** GS phosphorylation at site 2+2α was, in general, elevated after bed rest relative to before (P < 0.05). Site 2+2α phosphorylation was 30% (0.05 ≤ P < 0.1) lower in the prior-exercised leg than in the rested leg 3 h after exercise only before bed rest (Fig. 5B and C). Insulin stimulation decreased site 2+2α phosphorylation by 70% (P < 0.05) before but not after bed rest.

GS site 3α phosphorylation was reduced 40% (P < 0.05) by insulin in the rested leg after bed rest and was reduced 45% in the prior-exercised leg (P < 0.05) before and tended to be reduced 35% (0.05 ≤ P < 0.1) after bed rest in response to insulin. Bed rest resulted in an apparent (P < 0.05) 1.2-fold higher GS site 3a phosphorylation in both legs 3 h after exercise (Fig. 5B and D). GS phosphorylation at site 1b was not different between legs and was not affected by insulin, exercise, or bed rest (data not shown).

**DISCUSSION**

The main findings of the current study are that bed rest–induced insulin resistance is associated with reduced insulin-stimulated muscular GS activity and Akt signaling as well as decreased protein level of HKII and GLUT4. Thus, this study may indicate that decreased glucose extraction with bed rest occurs as a consequence of both decreased glucose transport/phosphorylation as well as decreased nonoxidative glucose metabolism in skeletal muscle. In addition, the ability of acute exercise to increase insulin-stimulated glucose extraction is well maintained even after 7 days of bed rest. However, acute exercise after bed rest does not fully normalize the ability of skeletal muscle to extract glucose to the level seen when exercise is performed before bed rest.

The observation that 7 days of bed rest resulted in lower insulin-stimulated glucose extraction across the leg is in accordance with previous bed rest studies (5,16). Unfortunately, we are not able to evaluate whether this relates to changes in blood flow. However, based on previous observations, insulin-stimulated blood flow is expected to decrease with bed rest (5,36). Thus, it seems unlikely that the decreased glucose extraction observed after bed rest is a compensation for an increased blood flow. Thus, we are confident that the decreased leg glucose extraction observed reflects decreased leg glucose uptake after bed rest.

The current study provides some indications for the molecular mechanisms behind the observed bed rest–induced insulin resistance. Although it is assumed that the observed

### TABLE 3

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<td><strong>Glucose (mmol/L)</strong></td>
<td>5.3 ± 0.03</td>
<td>4.9 ± 0.1*</td>
<td>5.3 ± 0.08</td>
<td>5.0 ± 0.07*</td>
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<tr>
<td><strong>Insulin (pmol/L)</strong></td>
<td>32 ± 15</td>
<td>341 ± 30*</td>
<td>74 ± 20</td>
<td>452 ± 66* (†)</td>
</tr>
<tr>
<td><strong>Palmitate (µmol/L)</strong></td>
<td>183 ± 11.1</td>
<td>20 ± 1.3*</td>
<td>166 ± 9.8</td>
<td>20 ± 3.3*</td>
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Values are means ± SE, n = 6. *Significantly different from B, P < 0.05. †Tends to be significantly different from before bed rest, 0.05 ≤ P < 0.1.
molecular changes are due to the physical inactivity in itself, a contribution from a potential positive energy balance cannot be ruled out (37). But the finding that the body weight was maintained by the ad libitum food intake during the bed rest period indicates that the subjects were in energy balance. Central to the observed bed rest–induced insulin resistance is the finding that both basal and insulin-stimulated GS activity was lower after bed rest than before. This impairment is likely one explanatory factor for the decreased leg glucose extraction. The observed lower GLUT4 protein content after bed rest is similar to the previously reported decrease in GLUT4 protein after a period of physical inactivity in trained humans (38) and rats (39). This suggests a similar effect of bed rest and detraining on the regulation of GLUT4 expression. Although our data suggest that physical inactivity–induced insulin resistance is associated with reduced GLUT4 abundance, it is notable that other studies have found no relationship between GLUT4 protein content and insulin action (40). Furthermore, the lower HKII and GLUT4 protein levels after bed rest likely decrease glucose transport and phosphorylation in skeletal muscle. These modifications are likely additional explanatory factors. Although we can only speculate as to the mechanism for the latter observations, it is tempting to suggest that the absence of muscle use during bed rest leads to lack of stimulation of transcriptional/translational processes normally seen in contracting skeletal muscle (41,42).

The current study does not provide insight as to whether translocation of GLUT4 to the plasma membrane is also compromised in bed rest–induced insulin resistance as seen in, for example, insulin-resistant muscle (40). But the 20–30% decreased protein and phosphorylation level of Akt after bed rest, similar to what has recently been observed with reduced levels of physical activity (43), may suggest such a mechanism because impaired Akt phosphorylation is seen in skeletal muscle of type 2 diabetes subjects. A possible upstream regulator responsible for the observed decreased Akt phosphorylation could be phosphatidylinositol-3 kinase, but due to lack of sufficient tissue, this cannot be elucidated further in the current study. However, the impaired phosphorylation on both of the key regulatory sites for Akt activity did not translate to two endogenous substrates of Akt. Thus, in contrast to insulin resistance in type 2 diabetes subjects (7), basal and insulin-induced phosphorylation of TBC1D4 was fully normal on multiple proposed Akt target sites after bed rest. In addition, the present observations do not indicate that reduced TBC1D4 phosphorylation is responsible for muscle insulin resistance after bed rest. However, other mechanisms involved in the GLUT4 recruitment to the plasma membrane, dependent on Akt, may be impaired (e.g., cytoskeleton rearrangements) (44).

Interestingly, the impaired activity and activation of GS after bed rest might be linked to impaired covalent regulation of the enzyme. Thus, whereas GS site 3a dephosphorylation was not compromised after bed rest, the ability of insulin to induce dephosphorylation on site 2+2a was lost. Furthermore, the general level of site 2+2a phosphorylation was increased and potentially contributing to the suppressed GS activity after bed rest. Such a pattern of dysregulation of site 2+2a rather than site 3a is

![FIG. 2.](image-url)
FIG. 3. Akt Thr\textsuperscript{308} phosphorylation (A), GSK-3β Ser\textsuperscript{9} phosphorylation (B), and representative blots (C) in rested (Rest leg) and prior-exercised (Ex leg) vastus lateralis before (B) and after (I) 3-h hyperinsulinemic euglycemic clamp performed 3 h after one-legged knee extensor exercise before and after 7 days of bed rest. AU, arbitrary units; kD, kilodaltons. Values are means ± SE; n = 6. *Significantly different from B, P < 0.05. †Significantly different from before bed rest, P < 0.05. #Significantly different from Rest leg at given time point, P < 0.05. (#)Tendency, 0.05 ≤ P < 0.1.
in agreement with observations in muscle insulin resistance related to obesity and type 2 diabetes (9,12). Site 2+2a is apparently not a direct target of either GSK-3 or Akt. Yet it is uncertain whether the impaired Akt signaling indirectly relates to the lack of site 2+2a dephosphorylation seen after bed rest. Site 2+2a is directly targeted by multiple kinases, but so far we have not been able to find indications that these kinases are dysregulated with bed rest. Thus, the fully nonresponsive site 1b phosphorylation indicates that neither calcium calmodulin–dependent kinase nor cAMP-dependent protein kinase is regulated under these conditions (45). Also, AMP-activated protein kinase activity/phosphorylation is not regulated by either insulin or bed rest (data not shown). In addition, both GS site 3 and site 2+2a are dephosphorylated by the multisubstrate phosphatase (PP1) (46), and a dysregulation of PP1 activity would be expected to affect phosphorylation at both sites, and not only site 2+2a as seen in the current study.

Previous studies have suggested that GS site 2+2a phosphorylation is affected by muscle glycogen levels, and that GS site 2+2a phosphorylation is a possible link between the inverse relationship between muscle glycogen and GS activity (47,48). Thus, although mechanistically unresolved, the lower GS activity and higher site 2+2a phosphorylation after bed rest might be related to the higher muscle glycogen level after bed rest. Of notice is, however, that the differences in muscle glycogen concentration eliciting changes in GS activity in previous studies (47–50) are larger than the 20% difference seen in the current study. This makes it less likely that the enhanced glycogen level is the only reason for the differences observed in the current study. The findings that resting muscle glycogen was elevated despite impaired insulin-induced glucose extraction and reduced GS activity after bed rest may at first seem contradictory. However, the impact of the dysregulated GS activation on the glycogen level likely first becomes evident when a significant amount of glycogen has to be synthesized (e.g., in the period after glycogen-depleting exercise).

The finding that insulin-stimulated GS activity in the prior-exercised leg was higher than in the rested leg both before and after bed rest is in line with previous studies (14,22) and indicates that activation of GS may play a role in the exercise-induced enhanced glucose uptake several hours after exercise both before and after the inactivity period. The similar insulin-mediated regulation of GS site 3a and Akt phosphorylation in the rested and prior-exercised leg both before and after bed rest is also in accordance with earlier studies (22,48) and suggests that changes in
GS site 3a and Akt phosphorylation do not contribute to the observed exercise-induced effects on GS activity. Previous findings show that reduced muscle glycogen is associated with dephosphorylation of GS site 2+2a (34), and thus the more marked dephosphorylation of GS site 2+2a could be a link between the reduced muscle glycogen and the increased GS activity in the prior-exercised leg. Previous studies showing that prior exercise increased TBC1D4 phosphorylation both before and after insulin stimulation (25) suggest a role of TBC1D4 in the enhanced glucose clearance in a prior-exercised muscle. However, the current study does not confirm these observations, although it cannot be excluded that a more intense or more prolonged bout of exercise would elicit such changes. Thus, the analyses performed in this study do not bring further insights to the mechanisms underlying the enhanced glucose clearance in the prior-exercised leg. Previous studies showing that prior exercise increased TBC1D4 phosphorylation both before and after insulin stimulation (25) suggest a role of TBC1D4 in the enhanced glucose clearance in a prior-exercised muscle. However, the current study does not confirm these observations, although it cannot be excluded that a more intense or more prolonged bout of exercise would elicit such changes. Thus, the analyses performed in this study do not bring further insights to the mechanisms underlying the enhanced glucose clearance in the prior-exercised leg. Previous studies showing that prior exercise increased TBC1D4 phosphorylation both before and after insulin stimulation (25) suggest a role of TBC1D4 in the enhanced glucose clearance in a prior-exercised muscle. However, the current study does not confirm these observations, although it cannot be excluded that a more intense or more prolonged bout of exercise would elicit such changes. Thus, the analyses performed in this study do not bring further insights to the mechanisms underlying the enhanced glucose clearance in the prior-exercised leg.

ACKNOWLEDGMENTS
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No potential conflicts of interest relevant to this article were reported.
R.S.B. designed the study, took part in conducting the human experiments, performed the laboratory analyses, wrote the manuscript, and commented on the manuscript. S.R. designed the study, took part in conducting the human experiments, performed the laboratory analyses, and commented on the manuscript. K.K., C.L., and P.P. designed the study, took part in conducting the human experiments, and commented on the manuscript. J.T.T. provided valuable guidance for specific laboratory analyses and commented on the manuscript. B.S. designed the study and commented on the manuscript. H.P. and J.F.P.W. designed the study, took part in conducting the human experiments, assisted with writing the manuscript, and commented on the manuscript. J.F.P.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this study were presented in poster form at the 70th Scientific Sessions of the American Diabetes Association, Orlando, Florida, 25–29 June 2010.

The authors would like to thank the subjects for their extraordinary effort during the study. In addition, the authors have sincere appreciation for the whole Copenhagen 2008 Bed Rest Team for a fantastic collaboration. The authors are grateful to Oluf Pedersen (University of Copenhagen), Graham Hardie, and Carol Mackintosh (Dundee University) for donating antibodies essential for this study.

REFERENCES

2. Chen YD, Golay A, Swislocki AL, Reaven GM. Resistance to insulin suppression of plasma free fatty acid concentrations and insulin stimulation of glucose uptake in non-insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 1987;64:17–21
44. Thong FS, Dugani CB, Klip A. Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. Physiology (Bethesda) 2005;20:271–284
3A. Co-authorship statement

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

### 1. General information

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### 2. Title of PhD thesis

Regulation of PDH, GS and insulin signalling in skeletal muscle; effect of physical activity level and inflammation

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

GLUT4 and glycogen synthase are key players in bed rest-induced insulin resistance

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

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

Revised 29 January 2013
4. Declaration on the individual elements

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

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

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7. Signature

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

PhD student:  
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Date: 03/02/2014  (dd.mm/yyyy)
When completed, the form should be sent to the relevant department’s PhD secretary, who on your behalf will forward it to the PhD School (PhD@science.ku.dk).

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Study II

Effects of exercise training on regulation of skeletal muscle glucose metabolism in skeletal muscle of elderly men

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Short title: Skeletal muscle glucose regulation in elderly

Key word: Physical activity, aging, pyruvate dehydrogenase, glycogen synthase, insulin signaling

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Abstract

BACKGROUND: The aim was to investigate the molecular mechanisms behind exercise training-induced improvements in glucose regulation in aged subjects. METHODS: Twelve elderly males completed 8 weeks of exercise training. Before and after the training period, the subjects were given an oral glucose tolerance test (OGTT) and a muscle biopsy was obtained from the vastus lateralis before and 45 min into the OGTT. Blood samples were collected before and up to 120 min after glucose intake. RESULTS: exercise training increased Hexokinase II, GLUT4, Akt2, glycogen synthase (GS), pyruvate dehydrogenase (PDH)-E1α and PDK2 protein and glycogen content in skeletal muscle. Furthermore, in response to glucose GS activity was increased and the dephosphorylation on GS site 2+2a and 3a was enhanced after the training intervention. The glucose-mediated insulin stimulation of TBC1D4 Thr642 phosphorylation was increased. The PDHa activity was reduced following glucose intake and without changes in phosphorylation level on PDH-E1α. CONCLUSIONS: This suggests that exercise training improves glucose regulation in elderly subjects by enhancing the capacity for glucose uptake and improving glycogen storage rather than oxidation.
Introduction

Aging is associated with impaired glucose metabolism as demonstrated both by reduced glucose tolerance during an oral glucose tolerance test [1] and reduced glucose disposal during a hyperinsulinemic euglycemic clamp [2;3]. Such changes may pre-dispose to diseases like Type 2 diabetes [4] and prevention of age-related dysfunctional glucose metabolism through physical activity is therefore important. Regular physical activity has been shown to increase glucose tolerance [1;5] and insulin sensitivity in elderly subjects [3] to a similar extent as in young subjects [6;7]. Aging and exercise training associated changes in glucose tolerance and insulin sensitivity involve several tissues, but changes in skeletal muscle metabolism have a particularly large impact on whole body metabolism [8].

Insulin-stimulated glucose uptake in skeletal muscle involves glucose transporter (GLUT)4 translocation to the plasma membrane regulated through phosphorylation and activation of Akt and concomitant phosphorylation and inactivation of Akt substrate of 160 kD (AS160/TBC1D4) [9]. G-6-P will in a resting muscle cell either be incorporated into glycogen or be oxidized within the mitochondria. The rate limiting enzyme in glycogen formation is glycogen synthase (GS), while the pyruvate dehydrogenase (PDH) complex converts pyruvate to acetyl CoA in an irreversible step, which represents the only entry of carbohydrate-derived substrates into the mitochondria for oxidation [10]. GS is stimulated allosterically by G-6-P [11] and inactivated by phosphorylation [12;13]. Insulin is known to increase the activity of GS through insulin-stimulated activation of Akt and concomitant phosphorylation and inactivation of GSK3[12;13]. Activity of PDH in the active form (PDHa) is primarily determined by the phosphorylation level of the PDH-E1α subunit regulated by PDH kinases (PDK), which phosphorylate and inactivate PDH, and PDH phosphatases (PDP), which dephosphorylate and activate PDH [14;15]. PDHa activity has previously been shown
to increase in human skeletal muscle in response to oral glucose intake [16], and to either increase [17] or remain unchanged [18] during a hyperinsulinemic euglycemic clamp in humans. In accordance with an insulin-mediated regulation of PDHa activity, insulin has been reported to increase PDP activity [19] and to reduce PDK4 protein in rat skeletal muscle [20].

Previous studies have observed either reduced or unchanged GLUT4 [21;22] protein content in human skeletal muscle with increasing age. Moreover, the recent finding that aging was associated with impaired insulin-induced TBC1D4 phosphorylation in skeletal muscle [2] suggests that reduced insulin-mediated GLUT4 translocation may contribute to insulin resistance in aged subjects. Furthermore, skeletal muscle HKII activity has been shown to be lower in elderly than in young subjects [23] indicating that a reduced ability to maintain the transmembrane glucose gradient also may affect skeletal muscle glucose uptake in aged subjects. Knowledge about potential age-related effects on GS and PDH regulation in skeletal muscle is scarce. However total GS activity has been reported to be reduced with age [24]. Moreover insulin-stimulated GS activation has been demonstrated to be impaired in Type 2 diabetic (T2D) patients [22;25]. Similarly, an insulin-induced increase in PDHa activity has been reported to be abolished in Type 2 diabetes patients [17]. In line with this observation, T2D has been shown to be associated with impaired insulin-mediated down-regulation of PDK4 in skeletal muscle suggesting that PDK4-induced inhibition of PDH and hence inhibition of glucose oxidation may contribute to insulin resistance [26]. Together, this indicates that both oxidative and non-oxidative glucose removal are reduced in skeletal muscle of T2D patients. This may suggest that GS and PDH dysregulation also contributes to age-associated glucose intolerance and insulin resistance.

Exercise training has previously been shown to increase skeletal muscle protein content of GLUT4, HKII, Akt, TBC1D4, GS, PDK2 and PDH-E1α in young subjects [7;22;27] and similarly for GLUT4 and Akt2, but not TBC1D4 in elderly subjects [2;28]. Furthermore, exercise training has
been demonstrated to enhance insulin-mediated TBC1D4 and GS regulation in young subjects as well as to reverse the age-associated impairment of insulin-stimulated TBC1D4 phosphorylation [2;22].

Therefore, the purpose of the present study was to test the hypotheses that exercise training-induced improvements in whole body glucose metabolism in elderly men are associated with increased content of key factors involved in GS and PDH regulation in skeletal muscle, and enhanced acute regulation of GS and PDH in skeletal muscle upon glucose intake.
Methods

Results from the current experiment have previously been published [29].

Subjects. Twelve physically inactive but healthy male subjects 60-72 years of age with a average body mass index 26.0±0.5 participated in the study. Subject characteristics have previously been published [29] including fasting glucose (5.3±0.1 mmol/l) and fasting blood insulin concentration (49.3±12 pmol/l) [29].

Exercise training. The subjects exercise trained for 8 weeks, 4 days a week as previously described [29]. The training was a combination of high intensity cycling exercise (Spinning) 2 times per week, strength and mobility training (crossfit) once per week and a 5 km walk once a week.

Experimental setup. Before and after the intervention period, the subjects were challenged with an oral glucose tolerance test (OGTT; 1g·kg⁻¹ body mass). A muscle biopsy was obtained from vastus lateralis using the Bergström needle biopsy method with suction and blood samples were obtained before and up to 2h after (see supplementary for details).

Plasma and muscle analyses: see supplementary

Statistics: see supplementary
Results

Performance

Exercise training increased \((p<0.05)\) VO\(_{2\text{max}}\) as previously published [29].

Blood analyses

Fasting glucose and insulin

Fasting plasma insulin and plasma glucose concentrations were not different before and after the 8 weeks of exercise training as in part previously reported [29].

OGTT

Plasma glucose: The plasma glucose response during the OGTT was similar in the untrained and trained state increasing \((p<0.05)\) in both conditions to approximately 8 mM 30 min after glucose intake and returning to the basal level (~5 mM) at 120 min after intake. There was no difference in the plasma glucose concentration between the untrained and the trained state (Fig 1A).

Plasma Insulin: The plasma insulin concentration increased \((p<0.05)\) ~10 fold 60 min after the glucose intake and remained elevated \((p<0.05)\) 120 min after intake relative to before both in the untrained and trained state. Moreover, the plasma insulin concentration was at 15 min after glucose intake higher \((p<0.05)\) and at 90 and 120 min lower \((p<0.05)\) in the trained than in the untrained state (Fig 1B). The insulin AUC was ~15% lower \((p<0.05)\) after exercise training than before exercise training. There was no difference in the HOMA-IR index before and after exercise training.

Plasma C-peptide: The plasma C-peptide concentration increased \((p<0.05)\) similarly in the untrained and trained state to ~5 fold at 60 min after glucose intake and remained in both conditions elevated \((p<0.05)\) at 120 min relative to before intake. Moreover, as for the plasma insulin
concentration, the C-peptide concentration was at 90 and 120 min after glucose intake lower (p<0.05) in the trained than in the untrained state (Fig 1C). The C-peptide AUC was ~15% lower (p<0.05) after exercise training than before.

**Muscle analyses**

**Muscle glycogen**

The muscle glycogen concentration increased (p<0.05) from 496±29 mmol·kg⁻¹·dw before to 686±43 mmol·kg⁻¹·dw after exercise training.

**Protein content**

Skeletal muscle protein content of GLUT4, Akt2, GS, PDK2 and PDH-E1α (all p<0.05) and HKII (0.05≤p<0.1) increased 1.2 to 1.8 fold with exercise training. There was no difference in Akt1, TBC1D4, GSK-3β or PDK4 protein content before and after exercise training (Fig 2).

**Intracellular signaling**

**Akt phosphorylation:** Forty five min after glucose intake, the absolute Akt Thr³⁰⁸ phosphorylation increased (p<0.05) 2.8 and 3.7 fold in the untrained and trained skeletal muscle, respectively, with no difference between training status (Fig 3A). Insulin-stimulated Akt phosphorylation occurs primarily if not exclusive on Akt2 in human skeletal muscle [36], The absolute phosphorylation on Akt Ser⁴⁷³ increased (p<0.05) 2.1 and 3 fold in the untrained and trained state, respectively, reaching a 2 fold higher (p<0.05) level in the trained than untrained state (Fig 3B)
**TBC1D4 phosphorylation:** The absolute TBC1D4 Thr$^{642}$ phosphorylation in skeletal muscle increased (p<0.05) 1.8 fold in the untrained state and 2.5 fold in the trained state reaching a higher (p<0.05) level in the trained than the untrained condition (Fig 3C).

**GSK3β phosphorylation:** Absolute GSK3β Ser$^9$ phosphorylation in skeletal muscle increased (p<0.05) similarly 1.3-1.4 fold 45 min after glucose intake before and after the exercise training period with no difference between the two conditions (Fig 4A).

**Glycogen synthase regulation**

**GS site 2+2a:** Glucose intake had no effect on the absolute GS site 2+2a phosphorylation in skeletal muscle before exercise training, but after exercise training the absolute GS site 2+2a phosphorylation decreased (p<0.05) 20% in response to glucose intake. Furthermore, the absolute GS site 2+2a phosphorylation was in the trained state 1.4 fold higher (p<0.05) in the basal state and tended to be 1.2 fold higher (0.05≤p<0.1) 45 min after glucose intake than in the untrained state (Fig 4B).

**GS site 3a:** The absolute GS site 3a phosphorylation in skeletal muscle tended to decrease (0.05≤p<0.1) 25% in response to glucose intake before exercise training and decreased (p<0.05) 40% with glucose intake after exercise training. The absolute GS site 3a phosphorylation was 1.3-1.7 fold higher (p<0.05) in the trained state than in the untrained state (Fig 4C).

**GS activity:** The GS activity (I-form) in skeletal muscle did not change with glucose intake before exercise training, but increased (p<0.05) 1.3 fold 45 min after glucose intake in the exercise trained state. There was no difference in GS activity between the untrained and trained state (Fig 4D).

**PDH regulation**
**PDH phosphorylation:** There was no effect of glucose intake on the absolute or the normalized PDH site 1 and site 2 phosphorylation in skeletal muscle. Exercise training increased (p<0.05) the absolute phosphorylation level of both sites with 1.3-1.8 fold, but this effect was removed by normalization to PDH-E1α protein, except for site 2 phosphorylation in the basal state (Fig 5A, Fig 5B; Supplementary Fig 3A; Fig 3B).

**PDHa activity:** There was no effect of glucose intake on skeletal muscle PDHa activity before exercise training, but glucose intake decreased (p<0.05) the PDHa activity 30% after the exercise training period (Fig 5C).

**PDK4 protein:**

PDK4 protein content overall (untrained and trained state together) tended to be lower (0.05≤p<0.1) after glucose intake (Fig 6B).
Discussion

The main findings of the present study are that exercise training-improved glucose regulation in elderly healthy subjects was associated with increased HKII, GLUT4, Akt2, PDK2, GS and PDH-E1α protein content. Moreover, exercise training resulted in an enhanced response of TBC1D4 and GS and a reduced PDHa activity in response to glucose intake relative to before training.

The present observation that the plasma glucose response to an oral glucose intake was similar before and after the exercise training period shows that glucose tolerance was unaffected by the exercise training intervention. However, there was a strong indication of improved insulin sensitivity after training in the present study as the insulin and C-peptide plasma concentrations as well as AUC for insulin and C-peptide were lower during the OGTT after training than before. Such an exercise training effect is in accordance with the observations in previous studies using hyperinsulinemic euglycemic clamp in elderly subjects demonstrating enhanced whole body insulin action [1;2] as well as improved insulin sensitivity in skeletal muscle after exercise training [2;6].

The finding in the current study that glucose intake induced a more marked absolute TBC1D4 phosphorylation after exercise training than before is in accordance with a recent study demonstrating that aged subjects increased the insulin-stimulated TBC1D4 phosphorylation during a hyperinsulinemic euglycemic clamp [2]. This indicates that exercise training enhances skeletal muscle GLUT4 translocation upon glucose intake in elderly subjects as previously reported in young subjects [2]. Although the effect of exercise training on TBC1D4 phosphorylation normalized to TBC1D4 protein did not reach statistical significance in the present study, the lack of change in TBC1D4 protein content with exercise training both in the present and the previous study [2;22] suggests that the observed training effect on TBC1D4 phosphorylation upon glucose intake is due to acute regulation rather than a change in TBC1D4 protein content. Although Akt Thr308
phosphorylation after glucose intake was similar in the untrained and trained state, the present observation that Akt Ser\textsuperscript{473} phosphorylation level after glucose intake was higher in the trained muscle than the untrained is in line with the higher TBC1D4 phosphorylation after the training period. This indicates that Akt mediate the exercise training effect on TBC1D4 phosphorylation in the present study, which is different from previous findings [2] reporting that insulin-stimulated Akt Ser\textsuperscript{473} phosphorylation could not explain the observed exercise training-induced changes in TBC1D4 phosphorylation. This difference between the two studies may be related to the use of hyperinsulinemic euglycemic clamp [2] and an oral glucose intake. In addition, the observed higher plasma insulin concentration in the trained state than in the untrained 15 min after glucose intake may have contributed to the enhanced Akt Ser\textsuperscript{473} response in the present study. The observed increase in GLUT4 and HKII protein content with exercise training in the current study is in line with previous studies showing that exercise training increases skeletal muscle GLUT4 protein content in aged subjects [1;28]. Together these observations indicate that exercise training-induced increased capacity for glucose transport and phosphorylation also contributes to improved regulation of glucose uptake in elderly subjects.

The observation that glucose intake did not affect GS activity in the elderly subjects in the untrained state is in contrast with previous observations in young subjects [16] suggesting an age-associated impairment in GS regulation [24]. However, the finding that glucose intake induced an increase in GS activity in the elderly subjects after the exercise training period in the present study may indicate that exercise training restored the ability of the aged muscle to activate GS in response to glucose intake.

The observation that glucose intake in the untrained state did not change GS site 2+2a phosphorylation is in contrast to the previously observed GS site 2+2 dephosphorylation in skeletal muscle of middle aged [25] and young subjects [24;25;31], but is in line with the unchanged GS
activity in the present study. Furthermore, the observed dephosphorylation of GS site 2+2a in response to glucose intake after the exercise training period is in accordance with the observed increase in GS activity upon glucose intake in the exercise trained state. The present observation that glucose uptake-stimulated phosphorylation of GSK-3β was similar before and after the exercise training period suggests that a difference in GSK-3 is not a likely explanation for the changes in GS regulation with exercise training. However, previous studies have indicated that GS site 2+2a hyperphosphorylation may contribute to impairment of insulin-induced GS activation in T2D patients [25] and in young subjects after 1 week of bed rest [31]. This suggests that the exercise training-induced change in glucose intake-stimulated site 2+2a phosphorylation may explain the associated changes in GS activation in the present study. The increased muscle glycogen concentration with exercise training is a possible reason for the elevated level of GS 2+2a phosphorylation after exercise training, because GS site 2+2a is regulated by the glycogen level [37]. But the lack of change in muscle glycogen after glucose intake does not support that muscle glycogen influenced the acute changes in GS site 2+2a phosphorylation in the present study.

The finding that PDHa activity was unaffected by the glucose intake in the untrained state is not in accordance with the previously reported increase in PDHa activity in young subjects during an OGTT [16]. It is possible that the elderly subjects in the present study had an impaired PDH regulation in response to glucose intake in the untrained state, but this needs to be further investigated. The finding that glucose intake even reduced skeletal muscle PDHa activity in the trained state was unexpected and has not previously been reported. However, it may suggest that downregulation of PDHa activity combined with an increased GS activity after glucose intake in the trained state serves to reduce carbohydrate oxidation ensuring that carbohydrate enters glycogenesis rather than oxidation.
The observation that the exercise training-induced elevation in basal PDH-E1α phosphorylation was associated with a similar increase in PDH-E1α protein content indicates that the same fraction of PDH-E1α molecules was phosphorylated in the trained and the untrained state. Furthermore, the concomitant exercise training-induced increase in PDK2 protein suggests that the enhanced PDH-E1α phosphorylation was executed by PDK2. In addition the observed overall tendency for reduction in PDK4 protein in response to glucose intake may be expected to influence the phosphorylation status of PDH-E1α and thus affect PDHa activity. However the lack of change in PDH-E1α phosphorylation with glucose intake in the trained state shows that the regulation of PDHa activity was independent of the phosphorylation status of site 1 and 2 on PDH-E1α. As PDH-E1α has been shown to be phosphorylated on at least 2 further sites [38] as well as being regulated by acetylation [38], additional post-translational modifications may explain the observed regulation of PDHa activity in the trained state.

In conclusion, the present findings suggest that exercise training-improved glucose regulation in elderly subjects is associated with enhanced potential for GLUT4 translocation, glucose phosphorylation and intracellular glucose removal to glycogen synthesis rather than oxidation.
Funding

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Acknowledgement

We would like to thank Ninna Iversen, Marie Henriksen, Line Nielsen, Simon Granjean and Sebastian Peronard for technical assistance. The authors are grateful to Oluf Pedersen (University of Copenhagen), Graham Hardie and Carol Mackintosh (Dundee University) for donating antibodies essential for this study.


Fig. 1. Plasma glucose (A), plasma insulin (B) and plasma C-peptide (C) during a 120 min oral glucose tolerance test in untrained (UT) (●) and exercise trained (T) (○) aged male subjects. *: Significantly different from before glucose intake OGTT, p<0.05. #: Significantly different from UT, p<0.05. Symbols within parentheses indicate tendency for statistical significance, 0.05≤p<0.1.

Fig. 2. Skeletal muscle protein content of hexokinase (HK)II, glucose transporter (GLUT)4, protein kinase B(Akt)1, Akt2, TBC1D4, glycogen synthase (GS), pyruvate dehydrogenase (PDH)-E1α and glycogen synthase kinase (GSK)3β in untrained (UT) and exercise trained (T). #: Significantly different from UT, p<0.05. Symbols within parentheses indicate tendency for statistical significance, 0.05≤p<0.1.

Fig. 3. Skeletal muscle Akt Thr\textsuperscript{308} phosphorylation (A), Akt Ser\textsuperscript{473} (B) and TBC1D4 Thr\textsuperscript{642} phosphorylation (C) and glycogen synthase kinase (GSK)-3 Ser\textsuperscript{9} phosphorylation (D) before (Pre) and 45 min into and oral glucose tolerance test (Post) in skeletal muscle of untrained (Untrained) and exercise trained (Trained) aged male subjects. *: Significantly different from Pre p<0.05. #: Significantly different from UT, p<0.05.

Fig. 4. Skeletal muscle Glycogen synthase (GS) site 2+2a phosphorylation (A), GS site 3a phosphorylation (B) and GS activity (I-form) (C) before (Pre) and 45 min into and oral glucose tolerance test (Post) in untrained (Untrained) and exercise trained (Trained) aged male subjects. *: Significantly different from Pre p<0.05, #: Significantly different from UT, p<0.05. Symbols within parentheses indicate tendency for statistical significance, 0.05≤p<0.1.
Fig. 5. Skeletal muscle pyruvate dehydrogenase (PDH) site 1 phosphorylation (A), PDH site 2 phosphorylation (B) and PDH activity in the active form (C) before (Pre) and 45 min into and oral glucose tolerance test (Post) in skeletal muscle of untrained (Untrained) and exercise trained (Trained) aged male subjects. *: Significantly different from Pre p<0.05, #: Significantly different from UT, p<0.05. Symbols within parentheses indicate tendency for statistical significance, 0.05≤p<0.1.

Fig. 6. Skeletal muscle pyruvate dehydrogenase kinase (PDK)2 (A) and PDK4 (B) before (Pre) and 45 min into and oral glucose tolerance test (Post) in untrained (Untrained) and exercise trained (Trained) aged male subjects. *: Significantly different from Pre p<0.05, #: Significantly different from UT, p<0.05. Symbols within parentheses indicate tendency for statistical significance, 0.05≤p<0.1.

Fig. 7 Representative blots before (B) and 45 min into and oral glucose tolerance test (G) in skeletal muscle of untrained (UT) and exercise trained (T) subjects.
Fig. 1

A) Plasma glucose (mmol/l)

B) Plasma insulin (pmol/l)

C) Plasma C-peptide (pmol/l)
Fig. 2

Protein content (AU.)

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(Valid)
Fig. 3

A)Akt Ser473 phosphorylation (AU)

B)Akt Thr308 phosphorylation (AU)

C)TBC1D4 Thr642 phosphorylation (AU)

D)GSK3-β Ser9 phosphorylation (AU)
### PDH Site 1 Phosphorylation (AU)

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### PDH Site 2 Phosphorylation (AU)

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### PDHa Activity / Total Cr (mmol·kg⁻¹·min⁻¹)

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Fig. 5

A) PDH site 1 phosphorylation (AU)

- **Pre**
- **Post**

B) PDH site 2 phosphorylation (AU)

C) PDHa activity / total Cr (mmol·kg⁻¹·min⁻¹)
Fig. 6

A) Untrained vs. Trained PDK2 protein (AU) comparison with pre- and post-training conditions. Pre-training shows lower protein levels compared to post-training.

B) Untrained vs. Trained PDK4 protein (AU) comparison with (*) indicating a statistically significant difference. Trained samples show higher protein levels, with (*) indicating a significant increase post-training.
### Fig. 7

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**Activation Sites:**
- Akt Thr^308^
- Akt Ser^473^
- GSK-3β Ser^9^
- TBC1D4 Thr^642^
- GS site 2+2a
- GS site 3a
- PDH site 1
- PDH site 2
- PDK4
- PDK2

**Molecular Weights:**
- 50 kD
- 37 kD
- 75 kD
- 100 kD
- 150 kD
- 250 kD
- 250 kD
- 37 kD
- 50 kD
- 75 kD
- 100 kD
- 150 kD
- 250 kD
- 37 kD
- 50 kD
- 75 kD
- 100 kD
- 75 kD
- 50 kD
- 37 kD
Supplementary methods

**Ethics:** The subjects were informed of the risk and discomfort associated with the experimental protocol and provided oral and written consent to participate. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg communities (H-2-2011-079) and was conducted in accordance with the guidelines of the *Declaration of Helsinki*.

**Experimental setup.** The subject arrived to the laboratory after 10-12h of fasting and 24h after the last exercise session. A venflon was inserted into an antecubital vein in the forearm and a blood sample was taken. The glucose beverage was ingested within 4 min and additional blood samples were obtained 15 min, 30 min, 45 min, 60 min, 90 min and 120 min after the glucose intake and a second biopsy 45 min after the glucose intake. The incisions for the biopsy needles were made under local anesthesia (lidocain; AstraZeneca, Södertälje, Sweden) and new incisions were made for each biopsy. Superficial blood was quickly removed from the biopsies, which were frozen in liquid nitrogen within 20 seconds and stored at -80°C.

**Blood parameters:** The blood plasma was analyzed for glucose, insulin and C-peptide concentrations at Department of Clinical Biochemistry at Rigshospitalet, Copenhagen, Denmark.

**Muscle analyses**

**Muscle biopsies.** Each muscle biopsy was cut in smaller pieces for determination of PDHa activity and the rest was freeze-dried for at least 48h. The freeze-dried samples were dissected free of blood, fat and connective tissue under the microscope.

**Muscle glycogen.** The muscle glycogen concentration was determined as previously described [30]
**SDS-PAGE and western blotting.** The freeze-dried muscle tissue was homogenized using a Tissue LyserII (Qiagen, Hilden, Germany) and muscle lysate was prepared as previously described [31]. SDS-PAGE and western blotting were performed as previously described [31] using hand casted gels. Protein and phosphorylation levels were determined in muscle lysate using antibodies towards HKII, GSK-3β Ser9, GSK-3β, Akt Ser473, Akt1 and Akt2 (#2867, #9323, #9315, #9271, #2967, #3063 respectively; Cell Signaling Technology, Beverly, MA, USA), GLUT4 (#998-101; ABR, Golden, CO, USA), PDK2 (#ST1643, Millipore, Billerica, MA, USA), Akt Thr308 (#05-669, Upstate Biotechnology, Lake Placid, NY, USA), TBC1D4 (#AB24469; Abcam, Cambridge, MA, USA), TBC1D4 Thr642 (#3028 p1; Symansis, New Zealand) as well as PDH site1 (Ser293), PDH site 2 (Ser300) phosphorylation, PDH-E1α and PDK4 (provided by Graham Hardie, Dundee University, Dundee, U.K) or in muscle homogenate using antibodies towards GS 2+2a, GS 3a (provided by Graham Hardie) and GS (provided by Oluf Pedersen, University of Copenhagen, Denmark). The bands on the membranes were visualized with ECL reagent (Millipore, Billerica, MA, USA) in a digital image system (GE healthcare, München, DE).

**PDHa activity.** PDHa activity in each sample was determined in muscle homogenate as previously described [32-34].

**GS activity.** GS activity in each sample was determined in muscle homogenate as previously described [35]

**Statistics.** Values are presented as mean ± SE unless otherwise stated. A student paired t-test was used to test the effect of exercise training on fasting plasma insulin and glucose as well as protein content. Two-way ANOVA with repeated measures was used to test the effect of exercise training and glucose intake on plasma glucose, insulin and c-peptide as well as skeletal muscle protein and protein phosphorylation levels. When a main effect was observed, a Student-Newman-Keuls post
hoc test was used to locate differences between groups if the data set was log transformed if the data did not pass equal variance test. The data were considered significant at $P<0.05$ and a tendency is reported when $0.05\leq P<0.1$. Statistical calculations were performed in SigmaPlot 11.0.

**Supplementary Results**

*Plasma glucose*

There was no difference in plasma glucose when comparing the area under the curve (AUC) for glucose before and after the training intervention.

*Intracellular signaling*

**GSK3β phosphorylation:** Normalized GSK3β Ser$^9$ phosphorylation in skeletal muscle increased ($p<0.05$) similarly 1.3-1.4 fold 45 min after glucose intake before and after the exercise training period with no difference between the two conditions (Fig 1A).

**Akt phosphorylation:** Akt Thr$^{308}$ phosphorylation normalized to Akt2 protein content increased ($p<0.05$) ~2.5 fold after glucose intake both before and after exercise training with no difference between the conditions (supplementary Fig 1B).

Akt Ser$^{473}$ phosphorylation normalized to Akt2 protein increased ($p<0.05$) similarly 2.2 fold and 3.5 fold in the untrained and trained state, respectively, with no difference between training status (supplementary Fig 1C).

**TBC1D4 phosphorylation:** TBC1D4 Thr$^{642}$ phosphorylation normalized to TBC1D4 protein content increased ($p<0.05$) similarly ~2.5 fold in response to glucose intake in the untrained and trained
state with no difference in TBC1D4 phosphorylation level before and after exercise training (supplementary Fig 1D).

**Glycogen synthase regulation**

**GS site 2+2a:** GS site 2+2a phosphorylation normalized to GS protein increased (p<0.05) ~1.8 fold with glucose intake before exercise training, but was unchanged in response to glucose intake after the exercise training period (Supplementary Fig 2A).

**GS site 3a:** GS site 3a phosphorylation normalized to GS protein showed a similar pattern as the absolute phosphorylation level both in response to glucose intake and exercise training (Supplementary Fig 2B).

**PDH regulation**

**PDH phosphorylation:** There was no effect of glucose intake on the normalized PDH site 1 and site 2 phosphorylation in skeletal muscle. Exercise training had no effect on PDH phosphorylation when normalization to PDH-E1α protein, except for site 2 phosphorylation in the basal state (Supplementary Fig 3A, 3B).

**Supplementary figure legends**

**Supplementary figure 1.** Skeletal muscle protein kinase B (PKB/Akt) Thr^{308} normalized to Akt2 protein (A) Akt Thr^{473} normalized to Akt2 protein (B) glycogen synthase kinase (GSK)-3β Ser^{9} phosphorylation normalized to GSK3β protein (C) and TBC1D4 Thr^{642} phosphorylation normalized
to TBC1D4 protein (D) before (Pre) and 45 min into and oral glucose tolerance test (Post) in untrained (Untrained) and exercise trained (Trained) aged male subjects. *: Significantly different, from Pre p<0.05, #: Significantly different from UT, p<0.05. Symbols within parentheses indicate tendency for statistical significance, 0.05≤p<0.1.

**Supplementary figure. 2.** Skeletal muscle glycogen synthase (GS) site 2+2a phosphorylation normalized to GS protein (A) and GS site 3a phosphorylation normalized to GS protein (B) before (Pre) and 45 min into and oral glucose tolerance test (Post) in skeletal muscle of untrained (Untrained) and exercise trained (Trained) aged male subjects. *: Significantly different, from Pre p<0.05, #: Significantly different from UT, p<0.05. Symbols within parentheses indicate tendency for statistical significance, 0.05≤p<0.1.

**Supplementary figure. 3.** Skeletal muscle pyruvate dehydrogenase (PDH) site 1 phosphorylation normalized to PDH-1Eα protein (A) and PDH site 2 phosphorylation normalized to PDH-1Eα protein (B) before (Pre) and 45 min into and oral glucose tolerance test (Post) in untrained (Untrained) and exercise trained (Trained) aged male subjects. #: Significantly different from UT, p<0.05.
Supplementary fig. 1

A) GSK3β Ser9 phosphorylation / GSK3β protein (AU)

B) AKT Thr308 phosphorylation / AKT protein (AU)

C) Akt Ser473 phosphorylation / Akt2 protein (AU)

D) TBC1D4 Thr642 phosphorylation / TBC1D4 protein (AU)
Supplementary fig. 2

A) 

GS site 2-2a phosphorylation / GS protein (AU)

- Pre
- Post

Untrained Trained

B) 

GS site 3a phosphorylation / GS protein (AU)

- Pre
- Post

Untrained Trained

C)
Supplementary fig. 3

A) Untrained Trained
PDH site 1 phosphorylation/ PDH-E1α protein (AU)

B) Untrained Trained
PDH site 2 phosphorylation / PDH-E1α protein (AU)
3A. Co-authorship statement

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

1. General information

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2. Title of PhD thesis

Regulation of PDH, GS and insulin signalling in skeletal muscle; effect of physical activity level and inflammation

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

Effects of exercise training on regulation of skeletal muscle glucose metabolism in skeletal muscle of elderly men

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

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

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

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

3. **Involvement in the analysis or the concrete experiments/investigation.**

4. **Presentation, interpretation and discussion of the results obtained in article form.**

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*Articles/work published in connection with another degree/thesis must not form part of the PhD thesis. Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.*

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### 6. Signatures of co-authors:

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**7. Signature**

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

PhD student: [Signature]  
Date: 03.02.2014 (dd.mm.yyyy)

When completed, the form should be sent to the relevant department's PhD secretary, who on your behalf will forward it to the PhD School (PhD@science.ku.dk).

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Study III

Exercise-induced AMPK and pyruvate dehydrogenase regulation is maintained during LPS-induced inflammation

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Keywords: LPS, TNFα, skeletal muscle, substrate oxidation, human, AMPK, exercise

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ABSTRACT

The aim of the present study was to examine the effect of LPS-induced inflammation on AMPK and PDH regulation in human skeletal muscle at rest and during exercise. Nine young healthy physically inactive male subjects completed two trials. In an LPS trial, the subjects received a single lipopolysaccharide (LPS) injection (0.3ng/kg body weight) and blood samples and vastus lateralis muscle biopsies were obtained before and 2h after the LPS injection and immediately after a 10 min one-legged knee extensor exercise bout performed approximately 2½ hour after the LPS injection. The exercise bout with muscle samples obtained before and immediately after was repeated in a control trial without LPS injection. The plasma tumor necrosis factor (TNF)α concentration increased 17 fold 2h after LPS relative to before. Muscle lactate and muscle glycogen were unchanged from before to 2h after LPS and exercise increased muscle lactate and decreased muscle glycogen in the control (P<0.05) and the LPS (0.05≤P<0.1) trial with no differences between the trials. AMP-activated kinase (AMPK), acetyl-CoA carboxylase (ACC) and pyruvate dehydrogenase (PDH) phosphorylation as well as PDHa activity were unaffected 2h after LPS relative to before. Exercise decreased (P<0.05) PDH and increased (P<0.05) AMPK and ACC phosphorylation as well as increased (P<0.05) PDHa activity similarly in the LPS and control trial. In conclusion, LPS-induced inflammation does not affect resting or exercise-induced AMPK and PDH regulation in human skeletal muscle. This suggests that metabolic flexibility during exercise is maintained during short-term low grade inflammation in humans.
INTRODUCTION
Skeletal muscle has an extraordinary ability to regulate substrate choice and utilization according to availability [13;32]. The exercise-induced enhancement of glucose and fat oxidation in skeletal muscle ensures ATP production for muscle contractions and the interaction between fatty acids and glucose regulates the relative fatty acid and glucose oxidation contributes to efficient substrate utilization [32]. Regulation of substrate choice and substrate utilization may however be influenced by metabolic changes and contribute to metabolic dysfunction. For example chronically elevated plasma free fatty acid (FFA) levels will inhibit glucose oxidation and elevated plasma glucose may inhibit fat oxidation in skeletal muscle [19;23;27;32]. Similarly, metabolically related diseases are often associated with low grade inflammation characterized by chronically elevated levels of circulating cytokines [25] like the pro-inflammatory cytokine tumor necrosis factor (TNF)α. Previous studies have linked TNFα to insulin resistance in rat and human skeletal muscle [17;30], as well as indicated TNFα-mediated effects on substrate utilization [35;40;47].

The pyruvate dehydrogenase (PDH) complex has a key position in the regulation of substrate choice as it catalyzes the decarboxylation of pyruvate to acetyl CoA, which represents the entry of carbohydrate-derived substrate into the mitochondria for oxidation [33]. In accordance, exercise has been shown to induce a rapid increase in the activity of PDH in the active form (PDHa) in human skeletal muscle [18] concomitant with increased glucose oxidation [31]. Furthermore, elevated plasma FFA has been shown to reduce the exercise-induced increase in PDHa activity in human skeletal muscle [20] supporting that PDH contributes to regulating substrate utilization during exercise as part of the interaction between fatty acids and carbohydrates [32;33]. The PDHa activity is thought mainly to be regulated by phosphorylation of the PDH-E1α subunit [22;28] determined by the activity of PDH kinases (PDK), which phosphorylate and inactivate the enzyme and PDH phosphatases (PDP), which dephosphorylate and activate PDH. Previous studies have shown that
the PDK4 protein content is up-regulated in rat and/or human skeletal muscle by fasting and high-fat diet [26;46] and this regulation has been suggested to contribute to the associated changes in PDHa activity [16]. Although the PDK4 protein content has been shown to be unaffected during even prolonged exercise, the PDK4 protein content has been demonstrated to be rapidly regulated in human skeletal muscle by carbohydrate availability [20] indicating a potential role of acute changes in PDK4 protein content in PDH regulation.

Several previous studies suggest that inflammation influences PDH regulation. Repeated E-coli injections in rats have been shown to reduce the concentration of active PDH complex (PDC) and increase PDK activity [39;40]. Moreover the observations that treatment with TNFα reduced PDH activity in rat cardiomyocytes [47] and human immune cells [36] as well as the finding that treatment with TNFα binding protein alleviated the effects of inflammation on PDH activity [40] indicate that TNFα influences PDH regulation. These findings are further supported by a recent study showing that LPS infusion for 24 hours decreased PDHa activity and increased PDK4 protein content in rat skeletal muscle [12]. On the other hand, local infusion of LPS or TNFα in one leg did not change resting PDH-E1α phosphorylation in human skeletal muscle [6;9], which may suggest that the impact of inflammation on PDH regulation at rest depends on species, dose or duration of the treatment.

The intracellular energy sensor, AMP-activated protein kinase (AMPK), is also a key factor in the regulation of substrate utilization. AMPK activity is increased both by AMP mediated allosteric regulation and by phosphorylation [44] leading to stimulation of several downstream processes aiming at increasing ATP production. In accordance, exercise increases AMPK phosphorylation and activity in skeletal muscle leading to an enhancement of fat oxidation in skeletal muscle through AMPK-mediated phosphorylation and inactivation of acetyl-CoA carboxylase (ACC) [43]. As ACC catalyzes the production of malonyl CoA, an inactivation of ACC leads to reduced
production of malonyl-CoA with concomitantly less inhibition of carnitine palmitoyltransferase I and an increased fatty acid oxidation [15].

AMPK has been suggested to have anti-inflammatory effects [14], and AMPK phosphorylation has been reported to increase in human skeletal muscle after an LPS injection, although a concomitant increase in ACC phosphorylation was not observed [3]. This may suggest that inflammation modify AMPK-mediated intracellular signaling in resting skeletal muscle. Although previous studies indicate that inflammation affects PDH and AMPK regulation, it is yet unresolved how inflammation influences exercise-induced AMPK and PDH regulation in human skeletal muscle. Therefore the aim of the present study was to investigate the effects of LPS-induced inflammation on AMPK and PDH regulation in human skeletal muscle at rest and in response to an acute exercise bout.
METHODS

Subjects

Nine physically inactive young healthy male subjects in the range from 20-26 years of age with an average body mass index of 25.6±1.3 (mean±SE) participated in the study. The subjects were physically active physical less than 1h per week. Each subject underwent a health examination by a medical doctor. The subjects could participate in the study if the VO$_{2\text{max}}$ was less than 45 ml·min$^{-1}$·kg$^{-1}$ and was approved by the medical doctor. The subjects were given both written and oral information about the study and the subjects gave their written consent to participate in the study. The study performed according to the Declaration of Helsinki and approved by the Copenhagen and Frederiksberg Ethical committee in Denmark (H-1-2012-108).

Pre-testing

The VO$_{2\text{max}}$ was measured for each subject using an incremental ergometer bicycle test (Monarch Ergomedic 839E) for use as inclusion criteria. In addition, Watt$_{\text{max}}$ was determined during an incremental one-legged knee extensor exercise test on a modified ergometer bicycle as previously described [29]. The Watt$_{\text{max}}$ was used to determine the resistance during the experimental trials.

Experimental protocol

The subjects completed a LPS trial and a control trial separated by at least 7 days. For both trials, the subjects were instructed to eat a carbohydrate rich meal 1h before arriving to the laboratory.

**LPS trial.** After arriving to the laboratory, a catheter was placed in the femoral artery and in the femoral vein of one leg and a venflon was placed in an antecubital vein in the forearm. Blood samples were obtained from the femoral catheters and a muscle biopsy was obtained from the
vastus lateralis muscle using the needle biopsy method [8] with suction. Insertions for biopsies were made under local anesthesia (Lidocaine, AstraZeneca, Södertälje, Sweden). A LPS solution (100 ng/ml) was either freshly prepared from a LPS stock (The Clinical Center, Critical Care Medicine Department, Bethesda, MD, USA) or used within a week from the preparation with storage at -20°C. An intravenous injection of 0.3 ng/kg LPS was given through an arm venflon approximately 2.5 hours after the subjects had eaten breakfast. Additional blood samples were obtained 30 min, 60 min, 90 min and 120 min after LPS injection and additional muscle biopsies at 1 hour and 2 hour after LPS was given. The 1 hour biopsy, all venous blood samples and arterial blood samples obtained at 30, 60 and 90 min after the LPS injection are not used in the present study except the venous sample before LPS for comparison of the arterial and venous TNFα level. After the 2h blood and tissue sampling, the subjects were transferred to a chair connected to a one-legged knee extensor ergometer bicycle with the back of the chair lying down. The right foot of the subject was tied to a rod connected to the crank set of the modified ergometer bicycle. The subjects first performed 1 min passive knee extensions to warm up the leg followed by 5 min exercise at 50% of Watt_{max} and 5 min exercise at 60% of Watt_{max}. Additional blood samples were obtained 8 min into the exercise and an additional muscle biopsy was obtained from the vastus lateralis muscle of the working leg immediately as the exercise was terminated after 10 min of exercise. Individual insertions were made for each biopsy. Visual fat, connective tissue and blood were removed from the biopsies, which were quickly frozen in liquid nitrogen. The muscle biopsies were stored at -80°C. The blood was collected in EDTA containing tubes, which were centrifuged and plasma was collected and stored at -80°C.

Ear temperature, mean arterial blood pressure (MAP) and heart rate were recorded every 15 min until 3h after the LPS injection. MAP and heart rate monitoring continued until at least 4 hours after the LPS injection to insure that normal blood pressure regulation was re-established.
Control trial. After arriving to the laboratory, a venflon was inserted in a vein in the forearm and a blood sample was obtained through the venflon. In addition, a muscle biopsy was obtained from the vastus lateralis muscle as described above. The subjects were placed in the chair connected to the one-legged knee extensor ergometer bicycle with the back of the chair lying down. The right leg of the subject was tied to the modified ergometer bicycle and 1 min of passive knee extensions were performed followed by 5 min exercise at 50% Watt\textsubscript{max} and 5 min exercise at 60% Watt\textsubscript{max} as in the LPS trial. An additional blood sample was drawn after 8 min of exercise and an additional Vastus lateralis muscle biopsy was obtained from the working leg immediately after 10 min of exercise. Blood and muscle biopsies were handled as described for the LPS trial above.

Plasma analyzes

*Plasma glucose and insulin.* The blood was analyzed immediately for blood glucose (ABL, Radiometer 725 series Acid-Base Analyzer, Denmark) and insulin was measured at The Department of Clinical Biochemistry at Rigshospitalet, Copenhagen, Denmark.

*Plasma TNF\(\alpha\).* The plasma TNF\(\alpha\) was determined using a MSD multi-spot 96 wells plate with pre-coated antibodies (MesoScaleDiscovery, Gaithersburg, ML, USA). The plates were measured on MSD Sector Image 2400 plate reader. The data were analyzed using the Discovery Workbench 3.0 (MSD). The results were converted to a concentration by use of a standard curve constructed from a serial dilution of recombinant TNF\(\alpha\) run alongside on each plate.

Muscle analyses
**Freeze-drying.** Muscle biopsies were freeze-dried for at least 48 hours and the samples were dissected free from visual blood and connective tissue under the microscope. The muscle tissue was weighted out for the different analyses and stored at -80°C.

**Muscle glycogen.** The muscle glycogen concentration was determined on freeze-dried muscle tissue as glycosyl units after acid hydrolysis as previously described [24].

**Muscle lactate.** PCA extract was made on freeze-dried muscle tissue as previously described [7]. Muscle lactate was measured using the auto-fluorescence ability of NADH as previously described [7].

**SDS-PAGE and Western blotting.** The freeze-dried muscle was homogenized using a Tissue LyserII (Qiagen, Hilden, Germany) and the homogenized muscle samples were made into lysates as previously described [38]. The protein concentration was determined with the bicinechonic acid method (Pierce, ThermoScientific, Rockford, IL, USA) using BSA as a standard. The lysate samples were loaded on hand casted gels (7.5%-10%). After the gel electrophoresis, the proteins were blotted from the gel to a PVDF membrane (Millipore, Bedford, USA), blocked in 3% fish gelatin solution and incubated with antibodies. The protein content and phosphorylation level were determined using antibodies towards AMPK Thr\(^{172}\) phosphorylation (2535; Cell Signaling Technology, Beverly, MA, USA), ACC Ser\(^{79}\) phosphorylation (07-303; Millipore, Bedford, USA), PDH Ser\(^{293}\) (site1), PDH Ser\(^{300}\) (site 2), PDH Ser\(^{295}\) (site 4) phosphorylation, PDH-E1\(\alpha\) protein, AMPK\(\alpha\)2 protein and PDK4 protein (provided by Graham Hardie, Dundee University, Dundee, U.K). ACC protein content was detected using streptavidin-HRP (Dako, Glostrup, Denmark). The
bands on the membranes were visualized with ECL reagent (Millipore, Billerica, MA, USA) in a
digital image system (GE healthcare, München, DE).

**PDHa activity.** The PDHa activity was determined as previously described [10;11;31]). In brief, muscle homogenate was prepared on ice from wet weight muscle tissue using a glass homogenizer (Kontes, Vineland, NJ, USA). Pyruvate was converted to acetyl CoA followed by determination of the acetyl CoA content by use of a radioactive assay. The PDHa activity was determined as the rate of conversion of pyruvate to acetyl CoA and normalized to the total creatine content in each sample as previously described [34].

**Statistics**

Values presented are means±SE. A paired t-test was used to test the effect of LPS at rest. A Two Way ANOVA with repeated measures was used to evaluate the effect of LPS and exercise. The data were log transformed if normality or equal variance test failed. If a significant main effect was detected, the student Newman-Keuls test was used to locate differences. Differences are considered significant at p<0.05 and a tendency is reported for 0.05≤p<0.1. Statistical calculations were performed using SigmaPlot 11.0
RESULTS

Physical parameters

The mean arterial pressure was stable around 85-90 mmHg and the ear temperature was unchanged around 37-37.6°C. The heart rate increased (P<0.05) from 63±3 before LPS to 83±4 beats/min 3h after the LPS injection (Table 1).

Plasma parameters

Plasma glucose and insulin

The arterial plasma glucose concentration decreased (P<0.05) 2h after LPS relative to before the LPS injection. Exercise did not change the plasma glucose concentration in the LPS trial (Table 1). The arterial plasma insulin concentration decreased (P<0.05) 2h after LPS injection relative to before LPS. Exercise did not affect the plasma insulin level in the LPS trial (Table 1).

Plasma TNFα

Plasma TNFα was determined as a measure of the LPS induced inflammation. The plasma TNFα concentration before LPS injection was similar in the femoral artery and femoral vein suggesting that arterial and venous levels can be compared at least in a non-inflammatory state. The arterial plasma TNFα concentration increased (P<0.05) ~17 fold 2h after LPS injection relative to before LPS. Exercise did not change the plasma TNFα concentration in either trial. The arterial plasma TNFα concentration was 13-15 fold higher (P<0.05) in the LPS trial than the venous plasma TNFα in the control trial before and at the end of exercise (Table 2).
Muscle analyses

Muscle glycogen

The muscle glycogen concentration was unaffected from before LPS injection to 2h after LPS. Exercise reduced (p<0.05) muscle glycogen 27% in the control trial and tended to reduce (0.05<P<0.1) muscle glycogen 6% in the LPS trial. There was no significant difference in the muscle glycogen concentration between the two trials neither before nor after exercise (Table 3).

Muscle lactate

Muscle lactate did not change from before the LPS injection to 2h after LPS. Exercise increased (P<0.05) muscle lactate 6.8 fold in the control trial and tended to increase (0.05<P<0.1) muscle lactate 4.9 fold in the LPS trial with no difference between the two trials neither before exercise nor after exercise (Table 3).

AMPK and ACC phosphorylation

There was no difference in AMPK Thr$^{172}$ and ACC Ser$^{79}$ phosphorylation in skeletal muscle before and 2 hours after LPS injection (Figure 1A and 1C).

The exercise bout increased (P<0.05) skeletal muscle AMPK phosphorylation ~3 fold and ACC phosphorylation 6-7 fold in the control and LPS trial with no difference in the responses between the trials. The AMPK and ACC phosphorylation levels were similar in the two trials both before and after exercise (Figure 1B and 1D).

PDH-E1α phosphorylation
PDH-E1α phosphorylation at Ser^{293}, Ser^{300} and Ser^{295} was unchanged 2 hours after LPS injection relative to before (Figure 2A, 2C and 2E).

Exercise decreased (P<0.05) the PDH-E1α phosphorylation at site Ser^{293} 40-50%, site^{300} 60-70% and site Ser^{295} 30-40 % in the control and LPS trial with no difference in the responses between the trials. The PDH-E1α phosphorylation level was for each of the three sites similar in the control and LPS trial both before and after exercise (Figure 2B, 2D,2F).

PDHa activity

The PDHa activity was unchanged 2 hours after LPS injection relative to before LPS. Exercise increased (P<0.05) the PDHa activity ~1.8 fold in the control trial and ~2.2 fold in the LPS trial with no difference in the response between the trials. The PDHa activity was similar in the two trials both before and after exercise (Figure 3).
DISCUSSION

The main findings of the present study are that LPS-induced inflammation with elevated plasma TNFα concentration does not affect the exercise-induced AMPK and PDH regulation in human skeletal muscle. In addition, short-term inflammation does not affect AMPK and PDH regulation at rest.

The present human study used a single LPS injection to induce a controlled inflammation in young volunteers as a model for low grade inflammation as frequently used [4;5;36;37]. The present observation that the LPS injection increased the plasma TNFα concentration 17 fold to ~15 ng/l 2 hours after the LPS injection is in accordance with several previous studies [4;5;37] and shows that the anticipated inflammatory state was obtained.

The observation that skeletal muscle PDHa activity and PDH phosphorylation did not change 2h after LPS injection in the current study is different from previous observations in rats [12;39;40]. Hence 24h of LPS infusion in rats has been shown to reduce the PDHa activity in skeletal muscle [12] and sepsis induced by repeated treatments with E-coli was associated with reduced concentration of active PDC in skeletal muscle [41]. The additional finding that treatment with TNFα binding protein prevented the E-coli-induced reduction in PDH activity suggested that TNFα is important in the LPS-induced effects on PDH during sepsis in rats [40]. Although the different observations may be due to species or model differences, it seems possible that the dose and the duration of treatment could be important. While a single injection of 0.3 ng·kg⁻¹ was used in the present study, effects on PDH was observed in rats with 24h of LPS infusion resulting in an 8.9 fold increase in plasma TNFα [2;12]. In addition, while the present study did not observe any change in muscle lactate 2h after the LPS injection, a previous rat study using a high dose of LPS infusion
(150µg·kg⁻¹·h⁻¹) reported that muscle lactate increased after 2 hours of LPS treatment [2] indicating that the inflammation inhibited the PDH.

Previous studies have also indicated a link between TNFα and AMPK, although different effects have been reported. The unchanged AMPK phosphorylation 2h after the LPS injection in the present human study is hence not in accordance with a previous study showing that treatment of muscle cells with TNFα decreased the AMPK activity [35]. The current finding is in line with previous observations in humans, although AMPK phosphorylation increased in human skeletal muscle 4h after a single LPS injection [3]. This may suggest that the different observations are due to model differences and the present findings do not oppose the possibility that LPS-induced inflammation in humans increases AMPK phosphorylation in skeletal muscle.

The present study is (to our knowledge) the first to examine the impact of LPS-induced inflammation on exercise-induced metabolic regulation in humans. The exercise-induced decrease in PDH phosphorylation and increase in AMPK and ACC phosphorylation as well as in PDHa activity in the control trial are as expected based on many similar previous studies [20;21;28;45]. The present finding that the exercise-induced regulation of PDH phosphorylation and PDHa activity was similar in the LPS trial, where TNFα was elevated, as in the control trial is however not as hypothesized. As PDH activation increases carbohydrate oxidation [31;42] and AMPK-mediated ACC inactivation increases fat oxidation [15;43], these observations suggest that human skeletal muscle maintains the ability to increase carbohydrate and fat oxidation in response to exercise despite the presence of short-term systemic inflammation. This conclusion is supported by the similar increase in the muscle lactate concentration in the two trials in the present study.

The lack of effect of inflammation on exercise-induced PDH and AMPK regulation suggests that exercise-induced metabolic flexibility is maintained during inflammation, which is in contrast to
previous studies showing that elevated systemic TNFα levels [30] and a single LPS injection [1] induce whole body insulin resistance in humans. These findings may suggest that insulin-signaling and exercise-induced metabolic regulation are affected differently by inflammation. However of notice is that the lack of effect on the exercise-induced responses was observed 2½ hours after LPS injection, while the LPS-induced lowering of insulin sensitivity was demonstrated 420 min after LPS was injected. On the other hand, the previously reported TNFα-induced insulin resistance was observed within 2h of TNFα infusion with similar plasma levels of TNFα [30] as in the present study, while the previous LPS study resulted in more than 50 fold higher plasma TNFα concentration [1] than in the present and previous TNFα study [30]. Although the TNFα infusion did result in a constant elevation in plasma TNFα, while the present study induced a gradual increase in plasma TNFα, these considerations do support a different impact of TNFα on insulin-mediated glucose uptake than on exercise-induced AMPK and PDH regulation in human skeletal muscle.

As the purpose of the present study was to examine the potential effect of inflammation with elevated plasma TNFα, the exercise bout was placed at the time point where the plasma TNFα concentration was expected to peak. In accordance, the observation that plasma TNFα was at a similar level (15 fold elevated) and not further increased at the sampling time point during exercise (approximately 2½ h after LPS injection) relative to 2h after LPS is in line with previous studies showing that plasma TNFα peaks at approximately 2h after a single LPS injection [1;3;5;37]. However, it is certainly possible that more long-term inflammation with more sustained elevation in plasma TNFα will affect the exercise-induced AMPK and PDH regulation in skeletal muscle, but this remains to be determined.
As previous studies examining the impact of LPS-induced inflammation and TNFα on PDH regulation have observed that a down-regulation of the PDHα activity was associated with increased PDK activity [39,40] and PDK4 protein content [12], it may be that inflammation-induced effects on PDH regulation require changes in PDK4 expression. The present observation that the lack of effect of inflammation on PDH regulation was associated with unchanged PDK4 protein content in skeletal muscle does support this possibility that changes in PDK4 protein underlies part of the effect of inflammation on PDH.

In conclusion, a single LPS injection resulting in 17 fold increase in plasma TNFα concentration did not change AMPK and PDH phosphorylation and/or activity in human skeletal muscle at rest and did not affect exercise-induced AMPK and PDH regulation in human skeletal muscle. This suggests that the ability of skeletal muscle to increase glucose and fat oxidation during exercise is maintained during short-term inflammation with elevated plasma TNFα levels. Hence, short-term low grade inflammation does not seem to elicit metabolic inflexibility during exercise in humans as has been reported for insulin resistance.
Acknowledgement

We would very much like to thank to subjects for the participation in the study. In addition, we are very grateful to Professor AF. Suffredini, National Institutes of Health, Bethesta, USA, for providing the LPS for the present study. The study was supported by The Danish Council for Independent Research, Danish Medical Research Council and The Danish Ministry of Culture for Sports Research. The Centre of Inflammation and Metabolism (CIM) is supported by a grant from the Danish National Research Foundation (DNRF55). The Centre for Physical Activity Research (CFAS) is supported by a grant from Trygfonden. CIM is part of the UNIK Project: Food, Fitness & Pharma for Health and Disease, supported by the Danish Ministry of Science, Technology, and Innovation. CIM is a member of DD2 - the Danish Centre for Strategic Research in Type 2 Diabetes (the Danish Council for Strategic Research, grant no. 09-067009 and 09-075724). The Copenhagen Muscle Research Centre (CMRC) is supported by a grant from the Capital Region of Denmark.


Table 1: Physiological and plasma parameters in the LPS trial

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<tr>
<th></th>
<th>Pre</th>
<th>2h LPS/Pre exercise</th>
<th>3h LPS</th>
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<tr>
<td>Temperature (°C)</td>
<td>37.4±0.2</td>
<td>37.3±0.1</td>
<td>37.6±0.2</td>
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<tr>
<td>MAP (mmHg)</td>
<td>87.8±2.9</td>
<td>88.0±3.4</td>
<td>87.9±3.6</td>
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<td>Heart rate (BPM)</td>
<td>63±3</td>
<td>68±4</td>
<td>86±4*</td>
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<tr>
<td>Arterial plasma glucose (mmol/l)</td>
<td>6.4±0.6</td>
<td>5.0±0.1 *</td>
<td>4.9±0.2</td>
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<tr>
<td>Arterial plasma insulin (pmol/l)</td>
<td>135.8±18.2</td>
<td>65.3±10.8*</td>
<td>69.3±8.9</td>
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Table 2: Plasma TNFα concentrations

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<th>2h LPS/Pre Exercise</th>
<th>Exercise</th>
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<tr>
<td>Control</td>
<td>-</td>
<td>0.97±0.1</td>
<td>1.0±0.1</td>
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<tr>
<td>LPS</td>
<td>0.90±0.1</td>
<td>15.5±1.6 *#</td>
<td>13.6±1.3*#</td>
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Table 3: Muscle glycogen and lactate concentrations

<table>
<thead>
<tr>
<th>Glycogen (mmol·kg⁻¹ dw)</th>
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<th>2h LPS/Pre Exercise</th>
<th>Post Exercise</th>
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<tr>
<td>Control</td>
<td>-</td>
<td>365±38</td>
<td>267±44*</td>
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<tr>
<td>LPS</td>
<td>329±32</td>
<td>391±49</td>
<td>367±35(*)</td>
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<td>Lactate (mmol·kg⁻¹ dw)</td>
<td>Control</td>
<td>-</td>
<td>9.7±1.9</td>
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<tr>
<td></td>
<td>LPS</td>
<td>10.9±1.3</td>
<td>66.4±15.6*</td>
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<tr>
<td></td>
<td></td>
<td>10.8±1.4</td>
<td>53.2±13.3(*)</td>
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Table and figure legends

Table 1
Ear temperature (˚C), mean arterial pressure (MAP; mmHg) and heart rate (beats/min) before (Pre LPS) and 2h (2h LPS) and 3h (3h LPS) after a single LPS injection as well as arterial glucose (mmol/liter) and arterial insulin (pmol/liter) at Pre LPS, 2h LPS corresponding to before exercise (Pre Exercise) and at 8 min of exercise (Exercise). The values are mean±SE. *: significantly different from Pre LPS, P<0.05.

Table 2
Arterial (LPS trial) and venous (control trial) plasma tumor necrosis factor (TNF)α (ng/liter) concentration before a single LPS injection (Pre LPS) and 2h after the LPS injection corresponding to before exercise (2h LPS/Pre Exercise) and 8 min into an one-legged knee extensor exercise bout (Exercise). The values are mean±SE. *: significantly different from Pre LPS, P<0.05; #: significantly different from control at the given time point, P<0.05.

Table 3
Glycogen (mmol·kg⁻¹ dry weight) and lactate (mmol·kg⁻¹ dry weight) concentration in the vastus lateralis muscle before a single injection of LPS (Pre LPS), 2h after the LPS injection corresponding to before exercise (2h LPS/Pre Exercise) and immediately after 10 min of one-legged knee extensor exercise (Post exercise). The values are mean±SE. *: significantly different from Pre LPS, P<0.05; (*): tends to be significantly different from Pre LPS, 0.05≤P<0.1

Figure 1
AMPK Thr172 phosphorylation normalized to AMPKα2 protein content A) before (Pre LPS) and 2 h after LPS (2h LPS) and B) before (Pre Exercise) and immediately after 10 min of one-legged knee extensor exercise (Post Exercise). ACC Ser 79 phosphorylation normalized to ACC2 protein content C) Pre LPS and 2h LPS as well as D) Pre Exercise and Post Exercise. The results are presented as arbitrary Units (AU). Values are mean±SE. *: significantly different from Pre Exercise, P<0.05.

Figure 2

PDH Ser293 phosphorylation normalized to PDH-E1α protein content A) before (Pre LPS) and 2 h after LPS (2h LPS) and B) before (Pre Exercise) and immediately after 10 min of one-legged knee extensor exercise (Post Exercise). PDH Ser300 phosphorylation normalized to PDH-E1α protein content C) Pre LPS and 2h LPS as well as D) Pre Exercise and Post Exercise. PDH site Ser295 phosphorylation normalized to PDH-E1α protein content E) Pre LPS and 2h LPS as well as F) Pre Exercise and Post Exercise. The results are presented as arbitrary Units (AU). Values are mean±SE. *: significantly different from Pre Exercise, P<0.05.

Figure 3

PDHa activity (mmol ·min⁻¹·kg⁻¹) A) before (Pre LPS) and 2 h after LPS (2h LPS) and B) before (Pre Exercise) and immediately after 10 min of one-legged knee extensor exercise (Post Exercise). The PDHa activity is normalized to total creatine in the samples. Values are mean±SE. *: significantly different from Pre Exercise, P<0.05.
Figure 1

A) Rested

AMPK Thr^{172} phosphorylation / AMPK α2 protein (AU)

Pre LPS 2 h LPS

B) Exercise

AMPK Thr^{172} phosphorylation / AMPK α2 protein (AU)

Pre Exercise Post Exercise

*C  **

C)

ACC Ser^{79} phosphorylation / ACC protein (AU)

Pre LPS 2 h LPS

D)

ACC Ser^{79} phosphorylation / ACC protein (AU)

Pre Exercise Post Exercise

*  **
Figure 2

A) PDH Ser\textsuperscript{295} phosphorylation / PDH-E1\textsuperscript{α} protein (AU)

B) PDH Ser\textsuperscript{295} phosphorylation / PDH-E1\textsuperscript{α} protein (AU)

C) PDH Ser\textsuperscript{293} phosphorylation / PDH-E1\textsuperscript{α} protein (AU)

D) PDH Ser\textsuperscript{295} phosphorylation / PDH-E1\textsuperscript{α} protein (AU)

E) PDH Ser\textsuperscript{295} phosphorylation / PDH-E1\textsuperscript{α} protein (AU)

F) PDH Ser\textsuperscript{295} phosphorylation / PDH-E1\textsuperscript{α} protein (AU)

Pre LPS 2 h LPS

Pre Exercise Post Exercise

* *
Figure 3

A) 

B)
3A. Co-authorship statement

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

### 1. General information

<table>
<thead>
<tr>
<th>PhD student</th>
<th>Name</th>
<th>Rasmus Sjørup Bienso</th>
</tr>
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<td></td>
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<td>PhD Scheme</td>
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<tr>
<td>Principal supervisor</td>
<td>Name</td>
<td>Henriette Pilegaard</td>
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<td></td>
<td>E-mail</td>
<td><a href="mailto:HPilegaard@bio.ku.dk">HPilegaard@bio.ku.dk</a></td>
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</tbody>
</table>

### 2. Title of PhD thesis

Regulation of PDH, GS and insulin signalling in skeletal muscle; effect of physical activity level and inflammation.

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

Exercise-induced AMPK and pyruvate dehydrogenase regulation is maintained during LPS-induced inflammation.

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

A. has contributed to the work (0-33%)

B. has made a substantial contribution (34-66%)

C. did the majority of the work independently (67-100%).

Revised 29 January 2013
4. Declaration on the individual elements

<table>
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<tr>
<td>2. Planning of experiments/analyses and formulation of investigative methodology in such a way that the questions asked under (1) can reasonably be expected to be answered, including choice of method and independent methodological development.</td>
</tr>
<tr>
<td>3. Involvement in the analysis or the concrete experiments/investigation.</td>
</tr>
<tr>
<td>4. Presentation, interpretation and discussion of the results obtained in article form.</td>
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</table>

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| Articles/work published in connection with another degree/thesis must not form part of the PhD thesis. Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study. |

| Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your masters degree) | Yes: □ |
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| Percentage of the paper that is from the PhD degree work | % □ |
| Percentage of the paper that is from the other degree / thesis | % □ |

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

6. Signatures of co-authors:

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<th>Date</th>
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<td>Lasse Gliemann</td>
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Revised 29 January 2013
By signing the document, the PhD student hereby declares that the above information is correct.

**PhD student:** [Signature]

**Date:** 03/02/2014

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Study IV

Effects of IL-6 on pyruvate dehydrogenase regulation in mouse skeletal muscle

Rasmus S. Biensø · Jakob G. Knudsen · Nina Brandt · Per A. Pedersen · Henriette Pilegaard

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Abstract Skeletal muscle regulates substrate choice according to demand and availability and pyruvate dehydrogenase (PDH) is central in this regulation. Circulating interleukin (IL)-6 increases during exercise and IL-6 has been suggested to increase whole body fat oxidation. Furthermore, IL-6 has been reported to increase AMP-activated protein kinase (AMPK) phosphorylation and AMPK suggested to regulate PDHa activity. Together, this suggests that IL-6 may be involved in regulating PDH. The aim of this study was to investigate the effect of a single injection of IL-6 on PDH regulation in skeletal muscle in fed and fasted mice. Fed and 16–18 h fasted mice were injected with either 3 ng·g\(^{-1}\) recombinant mouse IL-6 or PBS as control. Fasting markedly reduced plasma glucose, muscle glycogen, muscle PDHa activity, as well as increased PDK4 mRNA and protein content in skeletal muscle. IL-6 injection did not affect plasma glucose or muscle glycogen, but increased AMPK and ACC phosphorylation and tended to decrease p38 protein content in skeletal muscle in fasted mice. In addition IL-6 injection reduced PDHa activity in fed mice and increased PDHa activity in fasted mice without significant changes in PDH-E1\(\alpha\) phosphorylation or PDP1 and PDK4 mRNA and protein content. The present findings suggest that IL-6 contributes to regulating the PDHa activity and hence carbohydrate oxidation, but the metabolic state of the muscle seems to determine the outcome of this regulation. In addition, AMPK and p38 may contribute to the IL-6-mediated PDH regulation in the fasted state.

Keywords IL-6 · Pyruvate dehydrogenase activity · AMPK · p38 · Skeletal muscle

Introduction

Skeletal muscle has an exceptional ability to adjust the substrate choice during fasting and exercise according to the substrate availability [9, 24]. During prolonged exercise, skeletal muscle first oxidizes blood glucose and stored glycogen, but an increasing fraction of the energy is derived from fat oxidation as the exercise proceeds [37, 38]. This ensures that exercise can continue, although at a lower exercise intensity. Similarly, fasting is associated with a switch in substrate oxidation from carbohydrates to fat markedly increasing survival without food intake.

The pyruvate dehydrogenase complex (PDC) is central in the regulation of substrate choice in skeletal muscle. PDC converts pyruvate into acetyl CoA thereby linking glycolysis with the citric acid cycle and represents the only entry for carbohydrate-derived substrate into the mitochondria for oxidation. PDC is composed of several copies of three catalytic enzymes, which includes the pyruvate dehydrogenase (PDH)-E1\(\alpha\). Fasting has been shown to downregulate the activity of PDH in the active form (PDHa) [43]. In addition, PDHa activity increases with exercise [37], but declines towards resting level as the exercise duration exceeds 2 h [25, 39].
Although PDHa activity can be allosterically regulated [2], phosphorylation/dephosphorylation of the α subunit of PDH-E1 is thought to be the main mechanism regulating the activity of PDH. Phosphorylation and concomitant inactivation of PDH is catalyzed by pyruvate dehydrogenase kinases (PKD), and dephosphorylation and concomitant activation of PDH is catalyzed by pyruvate dehydrogenase phosphatases (PDP). The decrease in PDHa activity during fasting seems to be due to increased PDK4 protein and decreased PDP1 protein content in the muscle [43], while the reduction during prolonged exercise does not appear to be caused by increased PDK4 protein content but may be due to increased PDK activity as previously reported [39]. The underlying mechanisms initiating the regulation of PDH during prolonged exercise remains unresolved.

Interleukin (IL)-6 is a myokine released from contracting muscle during exercise and suggested to exert autocrine, paracrine and endocrine effects [23]. The plasma IL-6 concentration has been reported to increase in both humans and mice during prolonged exercise [20, 21]. In humans, the increase in plasma IL-6 concentration during exercise is detectable after about 2 hour with moderate exercise intensity and increases further with exercise duration [12, 34], while an increase in plasma IL-6 has been detected already after 30 min of intense exercise in mice [20]. Recombinant IL-6 has been shown to increase free fatty acid release from myotubes [1] and increase fat oxidation in human skeletal muscle in vivo [35, 42]. In addition, IL-6 has been reported to increase AMP-activated protein kinase (AMPK) phosphorylation in rat skeletal muscle [13], and rodent studies suggest that AMPK may regulate PDH [17, 32]. Furthermore, IL-6 has been shown to increase phosphorylation of the stress mitogen activated protein (MAP) kinase p38 in incubated human muscle [6] and MAPK p38 has been demonstrated to be involved in the regulation of IL-6 transcription [36]. Furthermore, previous findings suggest an association between increased p38 phosphorylation and decreased PDHa activity in cardiomyocytes [29]. Together this suggests that IL-6 could be involved in regulating the substrate choice of muscle either by directly regulating PDH or potentially via AMPK and p38. Therefore, the aim of the present study was to examine the isolated effects of a single injection of recombinant mouse (rm) IL-6 on PDH regulation in mouse skeletal muscle. As IL-6 normally is elevated during exercise in the presence of severe metabolic challenge, the effect of IL-6 injection was determined in both the fed and fasted state.

**Methods**

**Mice** Female C57BL/6 mice (Taconic, Lille Skensved, Denmark) 8 weeks of age were used in the study. Female mice were used because a previous study showed no indications of gender-specific regulation of PDH [14] and because regular oestrous cycle has been reported to be absent in the majority of female mice housed in large groups [41]. The mice had ad libitum access to water and normal chow diet (Altromin 1324, Brogaarden, Lyngby, Denmark) and had a 12:12 light–dark cycle. The experiments were approved by the Danish Animal Experimental expektorator (license no. 2009, 561 1607) and complied by the European Convention for the protection of vertebrate animals used for experiments and other scientific purpose (Council of Europe no. 123. Strasbourg, France 1985).

**Experimental setup** The mice were housed individually 24 h before initiation of the experiment and divided into two groups either fed or fasted for 16–18 h. The mice were given an intraperitoneal injection of either phosphate-buffered saline (PBS) or rm IL-6 (3 ng·g−1) dissolved in PBS. The mice were euthanized by cervical dislocation 30 or 60 min after the injection. Quadriceps muscles were removed and frozen in liquid nitrogen. Trunk blood was collected in EDTA containing tubes and plasma was collected after centrifugation at 2,600×g, 15 min, 4 °C. Muscle and plasma samples were stored at −80 °C.

**Plasma glucose and plasma IL-6** Plasma glucose concentration was analyzed fluorometrically [18]. The plasma IL-6 concentration was determined using Meso Scale Discovery (Rockville, MD, USA) as described by the manufacturer.

**Muscle glycogen** Muscle samples were hydrolyzed in 1 M HCl and the muscle glycogen concentration was determined fluorometrically as glycosyl units as previously described [22].

**RNA isolation and reverse transcription** Quadriceps muscles from the mice were crushed in liquid nitrogen to ensure homogeneity of each sample. RNA was isolated using a guanidinium thiocyanate phenol-chloroform method as previously described [4, 26] except that the samples were homogenized using a Tissue LyserII (Qiagen, Hilden, Germany). Reverse transcription was performed using the Superscript II RNase H− system (Invitrogen, Carlsbad, CA, USA) as previously described [26].

**Real time PCR** mRNA content was determined by use of the 5′ fluorogenic nuclease assay with TaqMan probes (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Foster City, CA, USA) as previously described [19]. Primer and probe sequences are given in Table 1. Cycle threshold values reflecting the content of a specific mRNA were converted to an arbitrary amount by use of a standard curve, constructed from a serial dilution of a representative sample run together with the unknown samples. For each sample the amount of target cDNA was normalized to the total single stranded (ss)DNA content in the sample determined by OliGreen as previously described [19].
SDS-PAGE and western blotting: Crushed quadriceps muscles were homogenized using a Tissue LyserII (Quagen, Hilden, Germany) and muscle lysate was prepared as previously described [25]. The protein content in each sample was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA).

Protein content and protein phosphorylation were determined in muscle lysates using pre casted gels (Biorad, Hercules, CA, USA) or hand-casted gels and SDS-PAGE and Western blotting as previously described [16, 25] with primary polyclonal antibodies against Signal Transducer and Activator of Transcription (STAT)3 (9139; Cell Signaling Technology, Beverly, MA, USA), p38 (9212; Cell Signaling Technology), acetyl CoA carboxylase (ACC; P0397; DAKO, Glostrup, Denmark), AMPKα2, PDP1, PDK4, and PDH-E1α (provided by Professor Graham Hardie, Dundee University, Dundee, UK) and primary phospho-specific antibodies against STAT3 Tyr705 and AMPK Thr172 (9138 and 2535, respectively, Cell Signaling Technology), ACC Ser79 (07-303; Millipore, Bedford, USA), PDH-E1α site 1 (Ser293), site 2 (Ser300), and site 4 (Ser295; provided by Professor Graham Hardie). Secondary antibodies used were all species-specific horseradish peroxidase conjugated immunoglobulin (DakoCytomation, Glostrup, Denmark). The bands were visualized with ECL reagent (Millipore, Billerica, MA, USA) in a photo image system and quantified (Carestream Health, Rochester, NY, USA). The PDP1 protein band was verified with recombinant protein. GAPDH protein was determined (2118, Cell Signaling Technology) and showed no changes between groups.

PDHa activity: PDHa activity was determined in muscle homogenates as previously described [3, 5, 25, 31]. PDHa activity in each sample was adjusted to the total creatine content in the homogenate as previously described [33].

Statistics: Values are presented as means±SE. Two-way ANOVA tests were used to evaluate the effect of fasting and IL-6 injection by testing within each time point and within each condition. The data were log transformed if equal variance test failed. If a main effect was observed, the Student–Newman–Keuls post hoc test was used to locate differences. In addition, a Student’s t test was used to evaluate the effect of IL-6 within a given time point and condition. Differences were considered significant when P<0.05 and a tendency is reported for 0.05≤P<0.1. The statistical tests were performed using SigmaPlot 11.0.

Results

Plasma IL-6

The plasma IL-6 concentration was measured to examine the time course of plasma IL-6 after an IL-6 injection. The IL-6
injection increased \( (P<0.05) \) the plasma IL-6 concentration 9.5-fold in fed mice and 40-fold in fasted mice 30 min after the injection relative to the PBS injected mice. After 60 min, the plasma IL-6 concentration was still elevated \( (P<0.05) \) 5-fold in fed mice and 12-fold in fasted mice relative to the PBS-injected mice (Fig. 1a).

In the IL-6-injected mice, the plasma IL-6 level was 2.4-fold higher \( (P<0.05) \) in fasted than fed mice at 30 min, which may be due to differences in blood volume or differences in uptake or removal of IL-6 from the blood. In the PBS-injected mice, fasting reduced \( (P<0.05) \) the plasma IL-6 concentration to 60 % compared with the fed mice (Fig. 1a). However, of notice is that such effect was not present at 30 min suggesting that additional factors have contributed to the effect observed at 60 min (Fig. 1a).

**STAT3 phosphorylation**

STAT3 phosphorylation was determined in skeletal muscle to confirm that IL-6 injections induced IL-6-mediated intracellular responses.

IL-6 injection increased \( (P<0.05) \) STAT3 Tyr\(^{705}\) phosphorylation in skeletal muscle 2-fold in fed mice and 2.3-fold in fasted mice relative to PBS at 30 min after injection. At 60 min, the injection of IL-6 increased \( (P<0.05) \) the STAT Tyr\(^{705}\) phosphorylation in skeletal muscle 2.2-fold in fed and 2-fold in fasted mice relative to PBS-injected mice (Fig. 1b).

In the IL-6-injected mice, STAT3 Tyr\(^{705}\) phosphorylation was at 30 min 1.8-fold higher \( (P<0.05) \) in the fasted than the fed mice (Fig. 1b).

**Plasma glucose**

The blood glucose concentration was measured to describe the metabolic status of the mice during fasting and after IL-6 injection. The IL-6 injection did not change the plasma glucose concentration relative to PBS, neither in the fed nor the fasted mice (Fig. 2a).

Plasma glucose concentration was at approximately 8 mM in fed mice independent of injection and decreased \( (P<0.05) \) to approximately 5 mM in the fasted mice both in the PBS and the IL-6-injected mice (Fig. 2a).

**Muscle glycogen**

Muscle glycogen concentration was measured to describe the metabolic status of the mice during fasting and after IL-6 injection. There was a main effect \( (P<0.05) \) of IL-6 injection on muscle glycogen content at 30 min, but no changes were observed at 60 min (Fig. 2b).

Fasting reduced \( (P<0.05) \) the muscle glycogen content with 50–60 % compared with the fed mice independent of injection (Fig. 2b).

**Signaling**

AMPK, ACC and p38 phosphorylation were determined to examine the effects of IL-6 on intracellular signaling in skeletal muscle.

**AMPK**

There were no effects of IL-6 on AMPK Thr\(^{172}\) phosphorylation in skeletal muscle of the fed mice. In the fasted mice, the IL-6 injection increased \( (P<0.05) \) AMPK Thr\(^{172}\) phosphorylation 1.3-fold relative to PBS at 60 min (Fig. 3a).

In addition, at 30 min fasting tended to increase \( (0.05 \leq P < 0.1) \) AMPK Thr\(^{172}\) phosphorylation 1.2-fold in the PBS-injected mice and in the IL-6-injected mice fasting increased \( (P<0.05) \) AMPK phosphorylation 1.2-fold relative to fed (Fig. 3a).
ACC

The IL-6 injection did not change ACC Ser$^{79}$ phosphorylation in skeletal muscle of the fed mice. In the fasted mice, the IL-6 injection increased ($P<0.05$) the ACC phosphorylation 1.4-fold in the fasted mice relative to the PBS mice at 60 min when using a $t$ test (Fig. 3b).

In line with the AMPK results, 30 min after injection, ACC Ser$^{79}$ phosphorylation was 1.5-fold higher ($P<0.05$) in fasted than in fed mice injected with PBS and tended to be 1.4-fold higher ($0.05 \leq P < 0.1$) in fasted than in fed mice injected with IL-6 (Fig. 3b).

p38

The IL-6 injection did not change the phosphorylation level of p38 when normalized to p38 protein content in skeletal muscle of fed or fasted mice (Fig. 3c). However, the IL-6 injection tended to decrease ($0.05 \leq P < 0.1$) the p38 protein content with 70–85% compared with PBS injection both at 30 and 60 min (Fig. 3c, d).

In addition, p38 protein content was 1.5- to 1.8-fold higher ($P<0.05$) in the fasted than in the fed mice both at 30 and 60 min (Fig. 3c, d).

IL-6 mRNA

IL-6 injection did not change the mRNA content of IL-6 in skeletal muscle of the fed and fasted mice (Fig. 4).

Furthermore, fasting increased ($P<0.05$) the IL-6 mRNA level 1.8- to 2.0-fold at 30 and 60 min but only in the IL-6-injected mice (Fig. 4).

PDH regulation

The mRNA and protein levels of PDK4 and PDP1 were determined together with the PDHa activity and PDH phosphorylation to examine the effects of fasting and IL-6 injection on PDH regulation.

PDK4 and PDP1 mRNA

IL-6 injection did not change the PDK4 mRNA content in skeletal muscle of the fed and fasted mice (Fig. 5a), but the PDP1 mRNA content tended to be higher ($0.05 \leq P < 0.1$) 60 min after IL-6 injection than PBS (Fig. 5c).

Fasting increased ($P<0.05$) the PDK4 mRNA content 5- to 6-fold (Fig. 5a) and decreased ($P<0.05$) the PDP1 mRNA content with 60% (Fig. 5c).

PDK4 and PDP1 protein

The PDK4 protein content in skeletal muscle increased ($P<0.05$) 1.6-fold in the fasted mice relative to the fed with no effect of IL-6 injection (Fig. 5b). The PDP1 protein content did not change with IL-6 injection or with fasting (Fig. 5d).

PDHa activity

The PDHa activity in skeletal muscle tended overall to be lower ($0.05 \leq P < 0.1$) in the IL-6-injected fed mice than the PBS injected fed mice. In addition, the PDHa activity was in the fed mice at 30 min 30% lower ($P<0.05$) after IL-6 injection than after PBS when using a $t$ test. IL-6 injection in the fasted mice increased ($P<0.05$) PDHa activity 2-fold at 30 min and 1.8-fold at 60 min.

Furthermore, fasting overall reduced ($P<0.05$) the PDHa activity in skeletal muscle to 15–30% of the level in fed mice (Fig. 6a).

PDH-E1$\alpha$ phosphorylation

Overall, the effects on PDH-E1$\alpha$ phosphorylation were similar for the investigated PDH-E1$\alpha$ phosphorylation sites. IL-6

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Fig. 2 Plasma glucose concentration and muscle glycogen. Plasma glucose concentration (millimolar) (a) and muscle glycogen (millimoles per kilogram) (b), in skeletal muscle of fed (FED) and fasted (FASTED) mice 30 and 60 min after a single injection of either PBS or rmIL-6. Values are means±SE; $n=8$. *$P<0.05$, significantly different from PBS within given condition and time point; #$P<0.05$, significantly different from FED within given treatment and time point. Line indicates an overall effect.
injection did not change PDH-E1α phosphorylation in the fed or fasted mice (Fig. 6b–d).

Fasted mice had 1.7- to 2.1-fold higher ($P < 0.05$) PDH-E1α phosphorylation than fed mice independent of injection (Fig. 6b–d).

**Discussion**

The main finding of the present study is that IL-6 injections regulated PDHa activity in mouse skeletal muscle but differently in fed and fasted conditions. In addition, an IL-6 injection increased AMPK and ACC phosphorylation in mouse skeletal muscle only in the fasted state. This indicates that

AMPK did not mediate the IL-6-induced reduction in PDHa activity in the fed state but could potentially be involved in the increase in the fasted state. Furthermore, IL-6 reduced p38 protein content in skeletal muscle in the fasted state, making it possible that p38 could play a role in the observed IL-6-induced change in PDHa activity in the fasted state.

Skeletal muscle regulates substrate choice according to availability and is therefore capable of coping with major metabolic changes during exercise and fasting [2, 38]. The previous findings that IL-6 infusion in humans enhances fat oxidation in skeletal muscle [35] and that plasma IL-6 is increased during prolonged exercise [21] suggest that IL-6 could be involved in regulating substrate choice during exercise. The observation that the increase in plasma IL-6 after the

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**Fig. 3** Phosphorylation of AMPK, ACC, and p38. AMPK Thr<sup>172</sup> phosphorylation (a), ACC Ser<sup>79</sup> phosphorylation (b), p38 Ser<sup>15</sup> phosphorylation (c), and p38 protein content (d) in skeletal muscle of fed (FED) and fasted (FASTED) mice 30 and 60 min after a single injection of either PBS or rmIL-6. Values are means±SE; $n=8$. *$P<0.05$, significantly different from PBS within given condition and time point; #$P<0.05$, significantly different from FED within given treatment and time point. Symbols within parentheses indicate a statistical tendency, 0.05 ≤ $P$ < 0.1. Line indicates an overall effect.
IL-6 injection in the present study was similar to the increase reported after a single exercise bout in mice [20], shows that the obtained IL-6 levels are at a physiologically relevant level. Furthermore, the increase in phosphorylation of the IL-6 signaling marker STAT3 in skeletal muscle in the current study demonstrates that IL-6 elicited an intracellular response in skeletal muscle at the investigated time points. Together this suggests that the present experimental setting can be used as a model to investigate the isolated effects of IL-6 on PDH regulation in skeletal muscle during exercise. In addition, the pronounced fasting-induced decrease in muscle glycogen and plasma glucose concentration shows that the fasted mice were severely metabolically challenged. This provides a model to investigate the potential impact of the metabolic state for IL-6-mediated effects on PDH regulation in skeletal muscle.

The observation that IL-6 downregulated PDHa activity in skeletal muscle in the fed state is in accordance with the

IL-6 mRNA content. IL-6 mRNA in skeletal muscle of fed (FED) and fasted (FASTED) mice 30 and 60 min after a single injection of either PBS or rmIL-6. Values are means±SE; n=8. #P<0.05, significantly different from FED within given condition and time point.

Methylation

## Fig. 4

IL-6 mRNA content. IL-6 mRNA in skeletal muscle of fed (FED) and fasted (FASTED) mice 30 and 60 min after a single injection of either PBS or rmIL-6. Values are means±SE; n=8. #P<0.05, significantly different from FED within given condition and time point.

PDK4 mRNA / ssDNA

PDK4 protein (AU)

#P<0.05, significantly different from PBS within given condition and time point; #P<0.05, significantly different from FED within given treatment and time point. Symbols within parentheses indicate a statistical tendency, 0.05 ≤ P<0.1. Line indicates an overall effect.
previously shown IL-6-induced fat oxidation [35, 42] and suggests that IL-6 may contribute to regulating substrate choice during exercise towards increased skeletal muscle fat oxidation in part by regulating PDH. The previous observation that an IL-6 injection increased AMPK phosphorylation in rat skeletal muscle suggests that AMPK may be mediating IL-6-induced regulation [13]. Furthermore, AMPKα2 knockout (KO) mice have been reported to exhibit an enhanced exercised-induced increase in PDHα activity [17] and the AMPK activator AICAR has been shown to increase PDK4 mRNA in skeletal muscle [11]. Together this suggests that IL-6 mediates effects on PDH via AMPK. However, the present observation that AMPK and ACC phosphorylations were unaffected by IL-6 injections in the fed state does not support that AMPK was directly involved in the observed IL-6-induced reduction in PDHα activity in the fed state.

The present findings that IL-6 elicited an increase in skeletal muscle PDHα activity in the fasted state, despite the IL-6-induced downregulation in the fed state, indicate that the metabolic status influences the impact of IL-6 on PDH regulation. As IL-6 injections in the fasted state were associated with increased AMPK and ACC phosphorylation at 60 min, it is possible that AMPK was involved in the IL-6-mediated PDH regulation at this time point. However, this potential effect of AMPK on PDH is opposite of the apparent AMPK-mediated suppression of the exercise-induced PDHα activation, as suggested from the more marked exercise-induced increase in PDHα activity in AMPKα2 KO than wild-type (WT) mice [17]. However, the AMPKα2 KO mice in the previous study had lower muscle glycogen and plasma glucose after exercise than WT [17]. Therefore, the more marked PDH activation in AMPKα2 KO mice may not be due to the
lack of AMPK, but rather that the AMPKα2 KO mice were more metabolically challenged than WT as also suggested in the previous study [17, 25]. Furthermore, as low muscle glycogen has been suggested to enhance exercise-induced IL-6 expression and release from skeletal muscle [12] the more marked PDHa activation in the AMPKα2 KO mice in the previous study [17] may have been due to increased IL-6 levels rather than lack of AMPK. This possibility is supported by the previous finding that AICAR incubation elicited a higher IL-6 release from AMPK kinase dead mouse muscle than WT muscle [7] and is also in line with the present IL-6-mediated increase in PDHa activity in the fasted state. In addition, the present findings that IL-6 injection tended to reduce p38 protein content in the fasted state may suggest that p38 potentially have contributed to the observed IL-6-induced increase in PDHa activity, because increased p38 phosphorylation has been associated with decreased PDHa activity in cardiomyocytes [29]. Of notice, the IL-6-induced downregulation of p38 protein only 30 and 60 min after injection is surprisingly fast and indicates that IL-6 either inhibits p38 synthesis or increases degradation. While a previous study has shown that IL-6 regulates p38 phosphorylation in skeletal muscle [40], no previous studies have to our knowledge reported IL-6-mediated regulation of p38 protein. However, it may be speculated that IL-6 mediates the regulation of p38 mRNA targeting miRNA’s leading to reduced translation of p38, but this remains to be determined.

As PDHa activity is known to be regulated by changes in NADH/NAD+, ATP/ADP, and acetyl CoA/acetyl [9, 24, 30], it may be speculated that IL-6 has mediated effects via changes in one of these ratios. Indeed a previous study has shown that IL-6 incubation of rat EDL decreased the ATP concentration and elevated the AMP concentration suggesting that IL-6-induced changes in nucleotides could play a role in the observed IL-6-mediated regulation of PDHa activity in the present study. An IL-6-induced decrease in ATP and increase in AMP concentrations would be expected to be associated with an enhanced PDHa activity, which is in accordance with the changes observed in the fasted state in the present study. However, changes in ATP, ADP and AMP as well as NADH/NAD+ and acetyl CoA/acetyl are thought to exert effects on PDHa activity through changes in PDH-E1α phosphorylation [10, 24]. Because IL-6 injections did not affect PDH-E1α phosphorylation significantly neither in the fed nor the fasted state in the present study, changes in these parameters do not appear to be a likely mechanism for the observed IL-6-induced effects on PDH regulation.

It should be noted that IL-6 induced an increase in PDHa activity when PDHa activity was at a very low level due to the fasting conditions. This means that the PDHa activity after IL-6 injections in the fasted state still was much lower than the level of PDHa activity after IL-6 induced downregulation of PDH in the fed state. The mechanism behind such a switch is unknown and additional studies are needed to answer this. The observations that PDHa activity, PDH-E1α phosphorylation, PDK4 and PDP1 mRNA, as well as PDK4 protein were markedly changed by fasting in the present study are in accordance with previous studies [27, 28]. This shows that differences could be detected at the mRNA, protein phosphorylation, and activity level. Furthermore, the finding that fasting elicited 90 % reduction in PDHa activity in PBS, while IL-6 injection only resulted in a 30 % reduction demonstrates that the effect of 18 h of fasting is much more marked than a single IL-6 injection. This may indicate that IL-6 only contributes to the regulation of PDH for example by sensitizing PDHα to the metabolic needs of the cell. However, it may also be worth noting that the magnitude of reduction in PDHa activity in response to IL-6 injection is quite similar to the reduction previously reported in the exercise-induced increase in PDHa activity in human skeletal muscle, when muscle glycogen had been lowered prior to exercise [15]. This makes it possible that an enhanced IL-6 release contributed to the smaller exercise-induced increase in PDHa activity when muscle glycogen was reduced in the previous study [15]. Moreover, the similar magnitude of change in the present study and the previous human study indicates that the observed change in PDHa activity with IL-6 injections is similar to changes observed in a physiological setting in humans [15].

PDH-E1α phosphorylation is known to be the dominant regulatory mechanism determining PDHa activity. The unchanged PDH-E1α phosphorylation with IL-6 injection in the present study indicates that the observed changes in PDHa activity was not due to changes in phosphorylation level. Such discrepancy between PDHa activity and PDH-E1α phosphorylation has previously been reported in resting human and mouse skeletal muscle [14, 25], although the previous studies observed changes in PDH-E1α phosphorylation without changes in PDHa activity. As effects of PDK and PDP expression and/or activity would be expected to influence the phosphorylation level of PDH-E1α, these PDH regulatory proteins do not seem to be in play in the IL-6-mediated effects. This suggestion is in line with the unaffected PDK4 and PDP1 protein level with a single IL-6 injection. Furthermore, the unchanged plasma glucose concentration and unchanged muscle glycogen in the fed state upon IL-6 injection (although an overall difference was observed for muscle glycogen) indicate that IL-6 did not change the carbohydrate availability in the fed state. Thus, it seems unlikely that changes in the metabolic state have contributed to the observed downregulation of PDHa activity. It may therefore be speculated that IL-6 has elicited alternative mechanisms like changes in acetylation state, because several acetylation sites have been identified on PDH [8].

Based on the previous findings that reduced muscle glycogen has been shown to enhance IL-6 transcription in human skeletal muscle [12] it may be expected that fasting would
elevate muscle IL-6 mRNA levels. However, the finding that a fasting-induced elevation in IL-6 mRNA only was observed in IL-6 injected mice suggests that low glycogen is not sufficient to increase IL-6 mRNA, and that IL-6 exerts a positive feedback on the expression of IL-6 when muscle glycogen levels are low. In addition, the IL-6 induced intracellular signaling observed both in the fed and fasted state while IL-6 mRNA only increased significantly in the fasted state in the present study, may suggest that an IL-6-mediated effect on IL-6 expression requires a factor which is only available when muscle glycogen is low. As p38 has been implicated in regulating IL-6 expression [36] the observed fasting-induced increase in p38 protein content may have contributed to the elevated IL-6 mRNA. However, the observed reduction in p38 protein content with IL-6 injection does not support this possibility. Hence, the mechanism behind the fasting-induced increase in skeletal muscle IL-6 mRNA only when IL-6 was injected remains to be determined.

In conclusion, a single IL-6 injection reduces PDHa activity in mouse skeletal muscle in the fed state and increases PDHa activity in the fasted state. This suggests that IL-6-mediated PDH regulation contributes to regulating substrate choice, but that the metabolic state determines the outcome. IL-6 appears to regulate PDHα activity without clear changes in PDH-E1 phosphorylation and AMPK and p38 may be involved in the IL-6-mediated PDH regulation in the fasted state.

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References