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Rita Rinnankoski-Tuikka

The Effects of Physical Activity and Diet Change on Intracellular Lipids and Metabolism in Mice with Diet-Induced Obesity

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To my father

Life is what happens,
while you're busy making other plans.
-John Lennon

ABSTRACT

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High energy intake, sedentary lifestyle, overweight and obesity are all considered to predispose to metabolic syndrome and the development of type 2 diabetes. Under these physiological conditions, excess lipids are stored ectopically in insulin-sensitive tissues, such as skeletal muscle and the liver. Several studies have demonstrated an association between elevated amounts of intramyocellular lipids (IMCL) and insulin resistance, which is one of the hallmarks of the metabolic syndrome and type 2 diabetes. However, the cellular pathogenesis leading to insulin resistance remains a matter of debate. Physical activity is shown to be one of the most potent remodelers of the lipid metabolism in skeletal muscle. The aim of this thesis was to investigate the effects of combined diet-induced obesity, voluntary wheel-running and diet change intervention on intracellular lipid accumulation and its role in the development of insulin resistance in the skeletal muscle and livers of C57BL/6J mice. In order to induce a condition mimicking the metabolic syndrome, male mice (n=90) were fed with a high-fat diet (60% energy from fat). These animals manifested typical characteristics of the metabolic syndrome after the 19-week experiment. Long-term high-fat feeding increased intracellular lipid accumulation and the amount of the lipid droplet-associated protein perilipin 5 in skeletal muscle, as well as in the liver. Surprisingly, an increased effect of oxidative capacity in skeletal muscle was observed, whereas in the liver oxidative capacity decreased with high-fat feeding. In addition, muscle capillarization seems to have even increased with the high-fat diet. In conclusion, it was discovered that many of the investigated variables benefit from voluntary physical activity, with or without a high-fat diet, and surprisingly the results showed similar positive effects even with high-fat diet alone (i.e. increased energy supply). However, the strongest beneficial effects in many of the variables were seen after diet change intervention, with no additional effect of physical activity. Thus, diet change intervention alone seems to play a more critical role in the prevention of insulin resistance than physical activity alone. A detailed understanding of these cellular mechanisms and pathways involved in obesity and complex metabolic diseases contributes to finding an efficient means of attenuating their global prevalence.

Keywords: physical activity, insulin resistance, metabolism, obesity, mice, diet change

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Jyväskylä 19.8.2014
Rita Rinnankoski-Tuikka

LIST OF ORIGINAL ARTICLES

This thesis is based on the following original research articles, which will be referred to in the text by their Roman numerals. Additionally, some unpublished data are included in the thesis.

I **Rinnankoski-Tuikka R**, Silvennoinen M, Torvinen S, Hulmi JJ, Lehti M, Kivelä R, Reunanen H, Kainulainen H. 2012. Effects of high-fat diet and physical activity on pyruvate dehydrogenase kinase-4 in mouse skeletal muscle. Nutrition and Metabolism (Lond) 9;9(1):53. doi: 10.1186/1743-7075-9-53.

II Silvennoinen M*, **Rinnankoski-Tuikka R***, Vuento M, Hulmi JJ, Torvinen S, Lehti M, Kivelä R, Kainulainen H. 2013. High-fat feeding induces angiogenesis in skeletal muscle and activates angiogenic pathways in capillaries. Angiogenesis 16(2):297–307. doi: 10.1007/s10456-012-9315-8. (*equal contribution)

III **Rinnankoski-Tuikka R**, Hulmi JJ, Torvinen S, Silvennoinen M, Lehti M, Kivelä R, Reunanen H, Kujala UM, Kainulainen H. 2014. Lipid droplet-associated proteins in high-fat fed mice with the effects of voluntary running and diet change. Metabolism 63(8):1031-40. doi: 10.1016/j.metabol.2014.05.010.

IV **Rinnankoski-Tuikka R**, Hulmi JJ, Reunanen H, Kainulainen H. 2014. Increased hepatic lipid accumulation and perilipin 5 in high-fat fed mice with the effects of exercise and diet change. Submitted for publication.

ABBREVIATIONS

BSA	Bovine serum albumin
CSA	Cross sectional area
CitSy	Citrate synthase
CytC	Cytochrome c
DC	Diet change
DCA	Diet change active
DCS	Diet change sedentary
EM	Electron microscopy
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HF	High-fat
HFA	High-fat active
HFD	High-fat diet
HFS	High-fat sedentary
HOMA-IR	Homeostasis model assessment of insulin resistance
IMCL	Intramyocellular lipid
IMTG	Intramyocellular triglyceride
LCM	Laser capture microdissection
LD	Lipid droplet
LF	Low-fat
LFA	Low-fat active
LFS	Low-fat sedentary
mRNA	Messenger RNA
NEFA	Non-esterified fatty acid
PDK4	Pyruvate dehydrogenase kinase 4
PLIN	Perilipin
RT-PCR	Reverse transcriptase-polymerase chain reaction
TG	Triglyceride

CONTENTS

1 INTRODUCTION

The pathogenesis of the metabolic syndrome and its possible progression to type 2 diabetes in some individuals is still largely unknown. Excessive energy intake, sedentary lifestyle, overweight and obesity are considered to be the major contributors and underlying causes. Together with possible genetic components, these factors interact and predispose to metabolic syndrome (Lakka & Laaksonen 2007). People with metabolic syndrome have four to six times the risk of developing type 2 diabetes. It was recently estimated that there are 382 million people with type 2 diabetes worldwide, and this is predicted to rise to almost 600 million by 2035. (Alberti & Zimmet 2014.) Globally, the exponential increase of metabolic disorders, especially type 2 diabetes and the metabolic syndrome preceding the onset of diabetes, comprise a major threat to public health. Since many type 2 diabetics may remain undiagnosed for years, the corollary conditions are expensive. Many people can manage their condition through a healthy diet and by increasing physical activity.

Fat accumulates subcutaneously and then in visceral fat deposits. In obesity, excessive fat is stored also ectopically in insulin-sensitive tissues. It is postulated that intramyocellular lipid (IMCL) accumulations or a disordered lipid metabolism disturb insulin signalling, leading finally to insulin resistance (Manco et al. 2004; Corcoran et al. 2007; Savage et al. 2007), although there have been conflicting results on the effects of a high-fat diet (Lowell & Shulman 2005; Schrauwen & Hesselink 2004). The diverse nature of lipids stored as lipid droplets (LDs) suggests that they may be involved in the pathogenesis of various disorders, including obesity and type 2 diabetes. There are indications that lipid droplets are large in size in insulin-resistant subjects and that the location and number of lipid droplet coat proteins and lipases differ significantly from normal homeostasis (Meex et al. 2009). However, the role of a single type of lipid droplet coat protein in the regulation of the fat content and insulin sensitivity of the body is still unclear. A growing body of evidence indicates that there is a similar mechanism for fat-induced insulin resistance in the liver as that found in muscle (Flamment et al. 2008).

Weight loss, with or without a concurrent exercise regime, is widely recommended in the management of obesity, metabolic syndrome and type 2 diabetes (Lakka & Laaksonen 2007). One of the beneficial effects that physical activity has been shown to offer is its ability to increase capillarization in order to augment efficient oxygen and nutrient supplies to skeletal muscle (Brouns & Vusse 1998). It has been proposed that the mitochondrial content and function in muscle are reduced as a consequence of physical inactivity and a sedentary lifestyle, whereas exercise has been shown to efficiently stimulate muscle oxidative capacity and thus correct the imbalance between fatty acid uptake and oxidation (Holloway et al. 2009).

Because of discrepancies and ambiguities in previous research results concerning the processes leading to an insulin-resistant state, it is important to understand the underlying molecular basis in the development of insulin resistance and its response and adaptations to exercise training and diet change interventions. After the scientific community has reached more consensus on the fundamental biological pathways involved, research towards even more efficient management, therapy and prevention can be commenced. Therefore, a complete understanding of the mechanism of lipid accumulations and their metabolism on a cellular level is fundamental for understanding the aetiology of insulin resistance.

The present study investigated the effects of voluntary wheel running exercise and diet change intervention on intracellular lipid accumulation and its role in the development of insulin resistance in skeletal muscle and the livers of diet-induced obese mice. In the studies, there were six groups. There were two low-fat diet groups, which served as control groups, and two high-fat fed mice groups. These were either physically active, having access to running wheels, or more sedentary in normal cages throughout the experiment. In addition, two diet change intervention groups were formed. The two groups were first fed the high-fat diet and were sedentary for nine weeks, after which their diet was changed to the low-fat diet. One of these two groups remained sedentary, while the other group had access to running wheels. (See Figure 6 for further details on study design.)

This thesis is a combination of a literature review and a critical summary of four original articles. The study was focused on the effects of high-fat diet, physical activity and diet change on accumulated lipids and the expression of the lipid droplet coat protein perilipin 5 (studies III and IV), the oxidative capacity of insulin-sensitive tissues (studies I, II and IV) and metabolic flexibility (studies I and III) during an insulin-resistant state.

2 LITERATURE REVIEW

2.1 Obesity and the metabolic syndrome

2.1.1 Prevalence and public health significance

Obesity and being overweight are continually increasing in Western countries, due to high intake of calories and sedentary lifestyle. According to the World Health Organization, worldwide in 2008 an estimated 1.4 billion adults or more were overweight and ~500 million were obese (WHO 2014). It is estimated that by 2015 these numbers will reach 2.3 billion and 700 million, respectively (Eckardt et al. 2011). Obesity is closely associated with metabolic disturbances, like dyslipidemia. Central obesity is indicated to be an important risk factor for type 2 diabetes and cardiovascular diseases. (Eckardt et al. 2011.) Early defects in type 2 diabetes pathogenesis include decreased glucose sensitivity, followed by insulin resistance in skeletal muscles, the liver and adipose tissue. As a consequence, there are disturbances in insulin action, leading to hyperglycaemia as well as compensatory hyperinsulinemia. (Zimmet et al. 2001; Eckardt et al. 2011.)

2.1.2 Excess calories from nutrition and sedentary lifestyle

The fundamental cause of obesity and overweight is an imbalance between nutritional intake and energy expenditure. Not only is there an increased intake of energy-dense food that is high in fat content, but physical inactivity and a sedentary way of life are also globally on the increase (WHO 2014). Under normal conditions, free fatty acid levels rise during fasting, while in a fed state the oversupply of energy is stored in the adipose tissue via insulin action. In obese people, however, this insulin action is impaired. In states of lipid oversupply, fat is stored in non-adipose tissues, such as skeletal muscle and the liver. (Unger & Scherer 2010.) Thus, increased amounts of lipids are found to be stored ectopically in obesity. Only a small amount of intracellularly stored triglycerides represent an important energy source during periods of low glucose supply. In

contrast, several studies demonstrate an association between elevated amounts of intramyocellular lipids (IMCL) and insulin resistance (Krssak et al. 1999; Moro et al. 2009; Bell et al. 2008). Insulin resistance develops gradually into type 2 diabetes, resulting in complications that are often irreversible. Therefore, it would be judicious to attempt to improve or prevent insulin resistance by all means possible before the onset of diabetes and its complications.

2.2 Rodent model for studying diet-induced obesity

Obesity is caused by several factors, such as dietary fat, energy intake, and energy expenditure. Many other things, such as gender, genetic predisposition, and social and cultural factors, also affect it. Due to these various reasons, it is very difficult to design a strictly controlled study in humans. (West & York 1998.) In contrast, using mice as a model has many advantages; in particular, genetic background and environmental circumstances are relatively easy to standardize. In order to study the development of insulin resistance in obesity, a model comparable to that of humans is needed. In studies investigating obesity and obesity-related diseases, placing rodents on a high-fat diet usually induces weight gain. (Oscai 1982; Collins & Surwit 1996; Kim et al. 2004.) Obesity results from an imbalance between caloric intake and energy expenditure, as excess energy is stored in tissues as glycogen and triglycerides. As a model affected by a high-fat diet, C57Bl/6J mice are widely used to investigate obesity-related diseases, such as type 2 diabetes and metabolic syndrome. These mice have been shown to have a predisposition to develop severe obesity, insulin resistance and disturbances in insulin secretion when fed a high-fat diet (HFD). (Surwit et al. 1995; Murase et al. 2001; Toye et al. 2005; Brownlow et al. 1996.) This mouse strain is inbred and 99.9% genetically identical. Therefore, any phenotypic variation is likely due to environmental influences. (Garcia-Valles et al. 2013.) Efforts to determine the effects of HFD on skeletal muscle have been considerable during recent years. A major focus has been on the development of insulin resistance and the accumulation of intramyocellular lipids that HFD often induces, especially in rodents. (Burcelin et al. 2002; Hancock et al. 2008.) The C57Bl/6J strain of mice has been observed to store excess triglycerides not only in adipose tissue, but additionally as ectopic deposits in muscle and the liver (Rossmeisl et al. 2003; Carr et al. 2012).

2.2.1 Physical activity

There is conclusive evidence that a lack of physical activity is one important cause of most chronic diseases (Booth et al. 2012). It is commonly believed that there is a causal relationship between obesity and decreased physical activity. It seems that obesity is not necessarily attributed to a behavioural difference, but influenced by multiple variables. Thus, it is plausible that in some obese subgroups with different aetiology and phenotype, decreased physical activity may

play a causal role, while in other groups some other factors predominate. (Brownlow et al. 1996.) However, as several recent studies have demonstrated, many pathological metabolic events associated with chronic diseases may be prevented or delayed when physical activity is commenced (Linden et al. 2013; Rector et al. 2010; Laye 2009; Lakka & Laaksonen 2007). Physical activity is defined by the Centers for Disease Control and Prevention (CDC) as "any bodily movement produced by the contraction of skeletal muscle that increases energy expenditure above a basal level. Physical activity generally refers to the subset of physical activity that enhances health." (CDC 2011.)

When given daily access to voluntary running wheels, C57Bl/6J mice spend about three hours per day running. Thus, voluntary wheel running can be categorized as one form of regular locomotor activity. (Harri et al. 1999.) Because the strain of mice used in this experiment is active and interested in wheel running, it is suitable for studying physical activity in a high-fat feeding setup together with obesity and insulin resistance (Brownlow et al. 1996; Garcia-Valles et al. 2013). Physical activity provides a plethora of physiological changes, in addition to overall health benefits. Due to increased energy expenditure, glycolysis in skeletal muscle and the liver are enhanced and lipolysis in adipose tissue is stimulated (Ahlborg et al. 1974). It is also reported that physical activity or exercise training increases the capacity for aerobic metabolism. This is observed as increased oxidative capacity and an augmented amount of mitochondria and mitochondrial oxidative enzyme levels in skeletal muscle, as well as increased capillarization. (Holloszy 1967; Molé et al. 1971.) In particular, endurance exercise has been shown to increase mitochondrial biogenesis and oxidative capacity (Molé et al. 1971). There is also plenty of evidence to show that by producing many metabolic adaptations, exercise training leads to improvements in insulin sensitivity in muscle and the whole body (Hawley & Lessard 2008; Hawley 2004; Hood et al. 2006; Zierath 2002). A single bout of exercise has been shown to increase skeletal muscle glucose uptake via an insulin-independent mechanism. However, this contraction-mediated insulin-sensitizing effect is short-lived. To improve the insulin sensitivity of tissues, a repeated physical activity (e.g. exercise training) regimen needs to be followed. (Hawley & Lessard 2008; Wojtaszewski et al. 2002.) There is irrefutable evidence that exercise training has a direct role in improving glucose tolerance and insulin action, as well as preventing insulin resistance (Hawley & Lessard 2007; Hawley & Houmard 2004; Albright et al. 2000). Identification of the underlying molecular mechanisms resulting from exercise training is essential in order to understand which metabolic pathways are involved in substrate turnover, especially in regard to the potential of improving insulin action.

2.2.2 Diet change intervention

Dietary fat intake and a sedentary lifestyle have been shown to play an important role in the development of obesity and insulin resistance, as well as type 2 diabetes. It has been demonstrated in numerous studies that high dietary fat intake correlates positively with body fat content in humans (Miller et al. 1990; Romieu et al. 1988; Tucker & Kano 1992), as well as in animals (Storlien et al. 1991, 1986; Rocchini et al. 1997). Furthermore, weight loss is caused in overweight subjects when dietary fat is reduced without restricting total energy intake (Astrup et al. 2000). Thus, it is of importance to reduce dietary fat in obese and overweight subjects as a treatment to manage the present worldwide epidemic of obesity. In addition to this, there must also be a reduction of energy intake, an increase in energy expenditure, or both. Reduction of energy intake can be achieved by restricting calorie intake or by shifting from a high-fat diet to lower dietary fat content. (Bray & Popkin 1998.) There are indications that a reduction in dietary fat content may not entirely reverse the development of obesity. However, once obesity has already developed, dietary fat content does seem to play an important role after all. (Bray & Popkin 1998.)

It has been shown in human subjects that if dietary fat content is reduced, insulin sensitivity improves (Bisschop et al. 2001; Lovejoy et al. 1998). Comparable to human studies, several studies performed in rodents show beneficial effects on body weight and glycemic control induced by either energy restriction or reduction of dietary fat (Cha et al. 2001; Harris & Kor 1992; Takahashi et al. 1999). However, switching from a high-fat diet to a low-fat diet has been shown to have more potential to improve high-fat diet-induced insulin resistance and plasma lipid levels than energy restriction (Muurling et al. 2002).

2.2.3 Translatability of mouse models to human disease

Similarly as in humans, a relationship between high-fat diet and obesity and type 2 diabetes has been found in rodents. The C57Bl/6J mouse strain is often used as a model because it is particularly susceptible to high-fat diet effects. This mouse strain is found to develop type 2 diabetes that closely resembles the common features of the human disease. (Surwit et al. 1995; Surwit et al. 1988.) Many similarities exist between the human metabolic syndrome and the rodent equivalent. The same impaired organs in rodents are also affected in human metabolic syndrome. For example, generalized lipodystrophy is accompanied by hepatic steatosis in both rodents and humans. Overall, ectopic lipid accumulation is observed, especially in insulin-sensitive tissues, such as skeletal muscle and the liver. (Boden & Shulman 2002.) Consequently, this mouse strain may be considered to be a relevant tool for translational studies investigating human chronic metabolic diseases.

2.3 Energy metabolism

Maintaining the balance between energy demand and energy supply is critical for health (Randle 1998). Primarily, it is muscle tissue, adipose tissue and the liver, together with the neuroendocrine system, that maintain the energy homeostasis of the whole body. The metabolic homeostasis of these tissues is in turn maintained and regulated by many hormones, of which insulin and glucagon are the most important. Insulin is excreted from the pancreatic β-cells in response to elevated plasma glucose levels. As the primary regulator of blood glucose concentration, insulin inhibits glucose production by the liver. As the most potent anabolic hormone known, from among its many other roles insulin also stimulates glucose uptake, utilization and storage. (Saltiel & Kahn 2001.) One of the most important functional roles of insulin is to control the switching between carbohydrate and lipid metabolism. As sources for cellular energy, glucose and lipids can compete and interact with each other. (Randle 1998.) Glucagon, excreted from pancreatic α-cells when blood glucose concentration falls, has several antagonist functions that are opposite to insulin action (Guyton & Hall 2006, 891-892).

2.3.1 Lipid metabolism

Intracellular lipid stores are considered to serve as a significant source of energy. The energy reserves are mainly deposited in subcutaneous and deep visceral adipose tissue. Smaller quantities of lipids circulate as lipoprotein particles in the blood stream and some may be deposited elsewhere, such as inside muscle fibres as intramyocellular triglyceride (IMTG). These intramyocellular lipid droplets can be used as a substrate for energy during rest and in more intense energy expenditure conditions, such as exercise. (Kiens 2006; van Loon 2004.) In lipid homeostasis, there needs to be a dynamic balance between IMTG breakdown (lipolysis) and lipid synthesis, both of which depend on the rate of mitochondrial fat oxidation and the availability of plasma free fatty acids (Figure 1) (Moro et al. 2008). The release of fatty acids from storage sites requires the enzymatic activity of lipases. There are observations that lipolysis is regulated differently in adipose tissue and in skeletal muscle (Watt & Steinberg 2008).

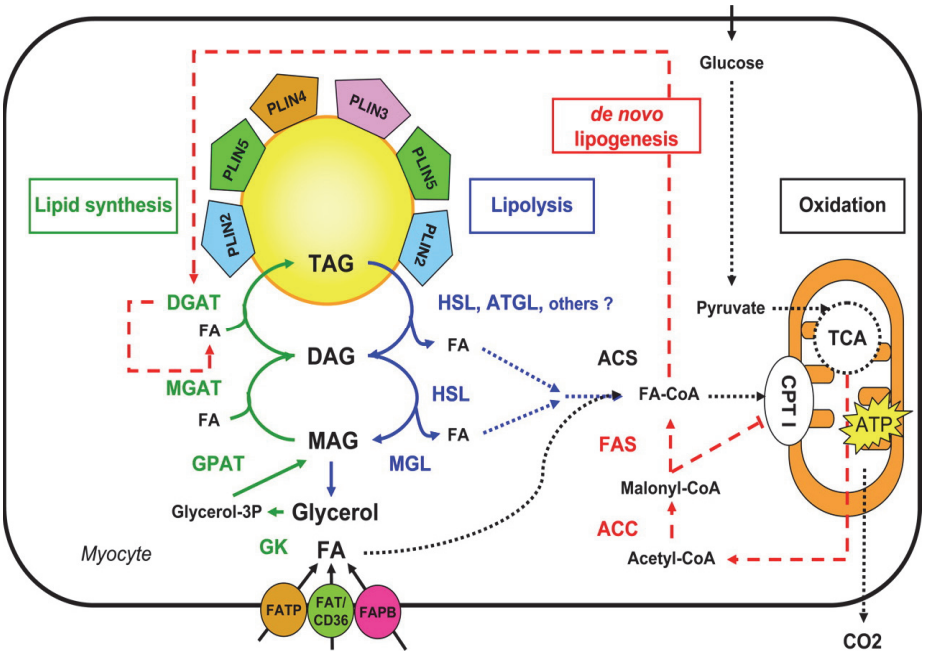

FIGURE 1 Biochemical pathways of lipid metabolism represented in a human myocyte. Fatty acids (FA) are transported into the myocyte through fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP) and fatty acid binding protein (FAPB). Free fatty acids are delivered to mitochondria via carnitine palmitoyl transferase (CPT-1) and go through mitochondrial β-oxidation. Lipids are stored in lipid droplets as triacylglycerides (TAG). The lipid synthesis pathway is represented in green. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are key enzymes involved in degradation of TAGs. Lipolysis pathway in blue, de novo lipogenesis in red, and mitochondrial oxidation in black. (GK=glycerol kinase, GPAT=glycerol-3-phosphate acyltransferase, MGL=monoglyceride lipase, MGAT=acyl-CoA:monoacylglycerol acyltransferase, ACC=acetyl-CoA carboxylase, ACS=acyl-coenzyme A synthase, FAS=fatty acid synthase, TCA=tricarboxylic acid cycle). Modified from Moro et al. 2008.

During rest and after fasting, plasma free fatty acids from adipose tissue lipolysis in skeletal muscle is the major fuel source in lipid metabolism, and only a small fraction seems to be provided from IMTG. Fat oxidation increases during moderate-intensity exercise and the contribution of plasma fatty acids decreases slightly, whereas the reliance on fatty acids from IMTG as an energy substrate is increased. With high-intensity exercise, the reliance shifts to plasma glucose and especially to muscle glycogen. (Moro et al. 2014; Jeukendrup 2002; Romijn et al. 1993.) Thus, physical activity is shown to be one of the most potential remodelers of the lipid metabolism in skeletal muscle. Due to these beneficial effects, it is often recommended as a treatment for metabolic diseases. (Meex et al. 2010.)

It has been shown that exercise training shifts fuel selection preference to fatty acid oxidation in muscle, thus possibly enhancing the capacity for fatty acid transport (Coen & Goodpaster 2012). During moderate-intensity endurance exercise, fat oxidation is shown to be highest (van Loon et al. 2001). During exercise, lipolysis in adipose tissue is stimulated and plasma fatty acid concentrations are elevated (Moro et al. 2014). Skeletal muscle fatty acid uptake is accordingly increased through sarcolemmal fatty acid transport and binding proteins (Bonen et al. 2007). Fatty acids are coupled to a co-enzyme A (CoA) group upon entry into muscle cells (Figure 1). After this they are transported towards either anabolic processes, such as incorporation into neutral lipids and stored as lipid droplets for later use, or catabolic processes as a main destination to be broken down into acetyl-CoA in β-oxidation. (Kanaley et al. 2009.) The first step in the use of fatty acids is their transport into the mitochondria. This is a carrier-mediated process that uses carnitine as the carrier substance (Figure 1). Once inside the mitochondria, the fatty acids split away from the carnitine and are degraded in β-oxidation and oxidized in the citric acid cycle. (Guyton & Hall 2006, 783-787; Nelson & Cox 2000, 603-607.)

In the literature, a frequently reported phenomenon in which individuals engaging in regular exercise training display normal insulin sensitivity and high oxidative capacity despite having high levels of intramyocellular triglycerides is referred to as the athlete's paradox (Goodpaster et al. 2001; van Loon & Goodpaster 2006). This observation may lead to a conclusion that lipid droplets *per se* are not detrimental. A current hypothesis suggests that high rates of IMCL oxidation allow for regular turnover of the intramuscular lipid pool. In other words, non-utilization of the fatty acid reservoir in parallel with an increased amount of IMCL is linked with the deleterious effects of accumulating metabolically active lipid intermediates, such as long-chain acyl-CoA, diacylglycerol, ceramides and acylcarnitines, which are believed to blunt insulin sensitivity in skeletal muscle. (Amati 2012; van Loon & Goodpaster 2006; Moro et al. 2008.) Moreover, physically inactive persons may have disturbances in their skeletal muscle lipid metabolism and turnover, so that lipids end up being accumulated (Kelley & Goodpaster 2001).

2.3.2 The metabolic flexibility and fuel selection

Skeletal muscle contributes to 80% of the insulin-stimulated glucose uptake in the body. Thus, it is the major site for glucose oxidation, as well as fatty acid metabolism. (de Lange et al. 2007.) Under normal conditions of alternating fed and fasting states in lean healthy individuals, skeletal muscle displays remarkable metabolic flexibility, having the capacity to switch from fat oxidation during the fasting state to glucose oxidation during the postprandial state (Kelley & Mandarino 2000). Impairment in the regulation of fuel oxidation is often referred to as metabolic inflexibility, which is considered to be one of the core components of metabolic syndrome (Figure 2). Even though the underlying mechanisms are still elusive, this phenomenon is used to describe one of the possible pathways causing the accumulation of intracellular lipids. In the skele-

tal muscle of obese insulin-resistant individuals, a failure was observed in substrate switching during the transition from fed to fasting states. Moreover, greater lipid oxidation rates were manifested, resulting in metabolic inflexibility. (Kelley & Mandarino 2000; Muoio 2012.)

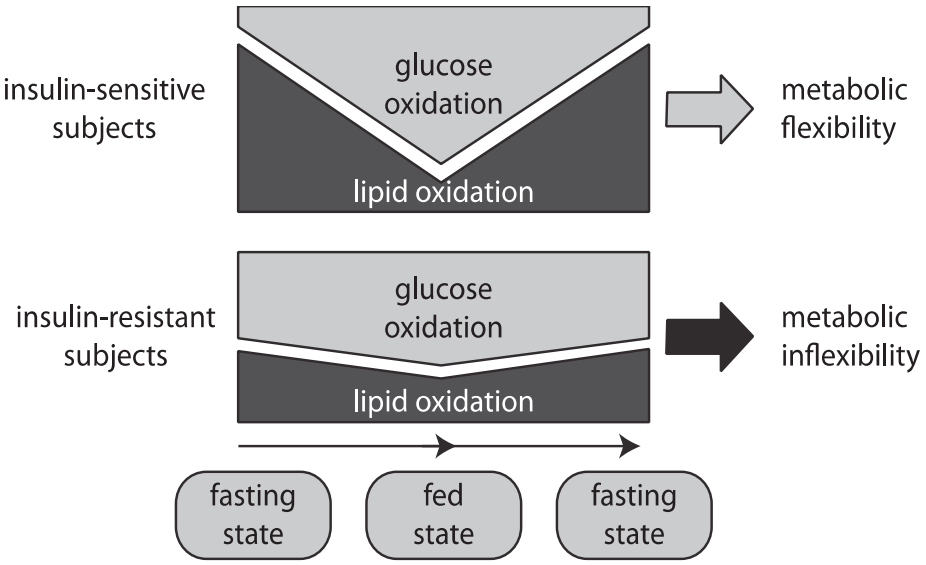

FIGURE 2 Illustration of metabolic inflexibility. For obese individuals, no modulation between reliance on lipid and glucose oxidation can be observed in fasting and insulin-stimulated conditions. During fasting, fat oxidation is impaired. Furthermore, the switch from fat to glucose oxidation is impaired after a meal or after insulin stimulation. From Eckardt et al. 2011, reproduced with permission from Springer.

Indeed, a failure to appropriately adjust mitochondrial fuel selection in response to nutritional cues causes insulin-resistant subjects to have an impaired capability to modulate between glucose and lipid oxidation. A state of metabolic inflexibility is produced when fat oxidation capacity is impaired upon increased fatty acid availability and glucose uptake, oxidation and storage are decreased under insulin-stimulated circumstances. (Kelley & Mandarino 2000; Corpeleijn et al. 2009.) Thus, fatty acids and glucose compete with each other for oxidation. One of the major enzymes responsible for metabolic flexibility is the pyruvate dehydrogenase complex (PDC), a mitochondrial multi-enzyme complex that catalyses the oxidative carboxylation of pyruvate and functions as a link in glucose metabolism, fatty acid metabolism and the tricarboxylic acid (TCA) cycle. Specific pyruvate dehydrogenase kinases (PDK) regulate PDC activity by means of phosphorylation. Thus, PDKs play a pivotal role in controlling metabolic flexibility. (Zhang et al. 2014.)

It has been suggested that during times of high fatty acid availability, cellular and mitochondrial fatty acid uptake may be limited and thus limit fat oxidation (Figure 3). This impairment to switch fuel sources may promote lipid accumulation in skeletal muscle. Increases in accumulated intramyocellular lipid deposits may subsequently contribute to the development of insulin resistance and possibly coincide with impaired mitochondrial content or function. (Kelley & Mandarino 2000; Corpeleijn et al. 2009.) The increase in muscle lipid content is observed especially in animal models, where high-fat diet-induced insulin resistance appears to relate to both the temporal development of insulin resistance and its severity (Pagliassotti et al. 1995; Storlien et al. 1991).

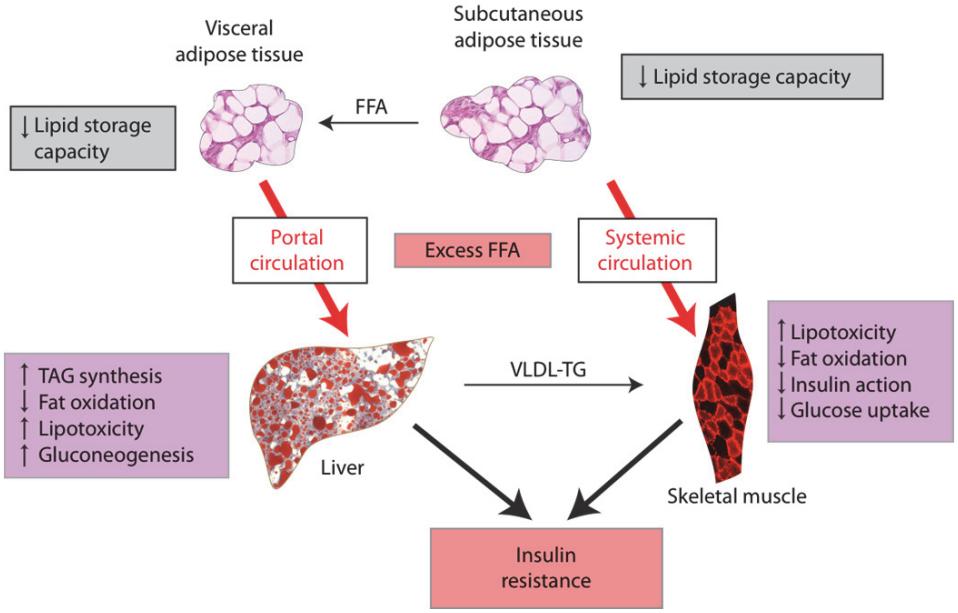

FIGURE 3 Model describing lipid-induced insulin resistance. Failure in the proper storage for lipids in subcutaneous adipose tissue will lead to deposition of lipids ectopically into visceral tissue and insulin-sensitive tissues, such as the liver and skeletal muscle. A state of lipotoxicity progressively develops and contributes to whole-body insulin resistance. (FFA=free fatty acid, VLDL-TG=very-low-density lipoprotein triglyceride, TAG=triacylglyceride) Modified from Galgani et al. 2008. Liver: Oil Red O stained murine liver specimen from Gorden et al. 2011. Adipose tissue: Hematoxylin & eosin stained adipose tissue specimen from AnatomyBox 2010. Skeletal muscle: Gastrocnemius muscle sample stained with HCS LipidTOX Neutral Lipid Stain (Invitrogen) after the 19-week high-fat feeding from Rinnankoski-Tuikka et al. (unpublished data).

2.3.3 The interplay between skeletal muscle and the liver

The role of skeletal muscle

Normal resting muscle membrane is only slightly permeable to glucose. Glucose enters muscle cells via insulin- and contraction-regulated glucose transporter isoform (GLUT4). Insulin increases glucose transport by stimulating translocation of GLUT4. Skeletal muscle is highly sensitive to insulin, and it is estimated that up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle. Only a small fraction of such disposal occurs in adipose tissue. (Klip & Pâquet 1990; Saltiel & Kahn 2001.) During exercise, muscles utilize glucose in large amounts, even in the absence of insulin. The current consensus is that muscle sarcolemmal and T-tubular glucose transport during exercise is primarily due to GLUT4 translocation. (Richter & Hargreaves 2013.)

The role of the liver

The role of the liver in the maintenance of whole-body energy homeostasis is essential. Because fat contains almost 2.5 times as many calories as glycogen, it is very useful to store excess carbohydrates in the form of fat by converting carbohydrates into triglycerides. Later, the fatty acids released from the stored triglycerides can be used for energy. Degradation and oxidation of fatty acids occurs in the mitochondria similarly as in the muscle. (Guyton & Hall 2006, 783-787; Nagle et al. 2009.) In addition to increased dietary lipids and hepatic triacylglycerol synthesis, lipids begin accumulating in the liver because of increased fatty acid delivery from lipolyzed adipose triacylglycerol or because of decreased hepatic fatty acid oxidation (Nagle et al. 2009). Insulin does not stimulate glucose uptake in the liver. By blocking glycogenolysis and gluconeogenesis, however, it does regulate fasting plasma glucose levels. (Saltiel & Kahn 2001.)

In addition to adipose tissue, skeletal muscle and liver are involved greatly in fatty acid metabolism. Stored triacylglycerides can be hydrolysed and these fatty acids are released into the circulation. In muscle, fatty acids are a substrate for oxidation. In the liver fatty acids are a substrate for a re-esterification to make triacylglyceride, which will be secreted as a very-low-density lipoprotein particles (Figure 3). There is a clear cooperation amongst these tissues. When the proper storage capacity exceeds in adipose tissue, lipids are deposited ectopically into insulin-sensitive tissues, such as skeletal muscle and liver, contributing to the development of insulin resistance. (Frayn et al. 2006.)

2.4 Insulin resistance

Insulin resistance can be defined as a state in which responsiveness to normal circulating levels of insulin is reduced (Savage et al. 2007). Insulin resistance is one of the hallmarks of metabolic syndrome and type 2 diabetes. This condition is typically associated with obesity, prolonged physical inactivity, and/or ectopic lipid accumulations. In fact, insulin resistance observed in obesity is hypothesized to be secondary to ectopic lipid accumulation. The suggestion that excess lipids might drive the molecular underpinnings of insulin resistance, because of the hypercaloric environment together with physical inactivity, has been repeatedly confirmed. (Koves et al. 2008; Unger & Scherer 2010; Booth et al. 2012; Thyfault & Cree 2007.) Several mechanisms have been postulated to be accountable in the development of insulin resistance. Because the principal site of insulin-stimulated glucose clearance is skeletal muscle, it is important to understand the mechanisms of lipid-induced metabolic derangement of skeletal muscle.

2.4.1 Randle cycle

Showing that fatty acids compete with glucose for substrate oxidation, Randle et al. have proposed the first mechanistic theory for fatty acid-induced muscle insulin resistance. It speculates that increased fatty acid oxidation may be responsible for insulin resistance. The pathway to increased intracellular glucose accumulation and decreased glucose uptake has been postulated to occur through the inactivation of pyruvate dehydrogenase and a subsequent increase in intracellular glucose-6-phosphate levels that inhibit hexokinase activity. (Randle et al. 1963.) However, this glucose-fatty acid cycle, the so-called Randle cycle, has faced criticism for not completely explaining the effects of free fatty acids on glucose metabolism. Recent studies have challenged the theory, while reduced levels of intracellular glucose-6-phosphate levels have been observed in patients with type 2 diabetes. (Roden & Price 1996; Rothman et al. 1995; Savage et al. 2007.) Furthermore, some studies have reported that glucose, rather than free fatty acids, can inhibit fat oxidation in skeletal muscle. This process has been designated as the 'reverse' Randle cycle (Sidossis & Wolfe 1996).

2.4.2 Accumulation of intracellular lipid derivatives

In a recent study, skeletal muscle oxidative capacity has been observed to be a better predictor of insulin sensitivity than intramuscular triglyceride concentration or long-chain fatty acyl-CoA content (Bruce et al. 2003). In addition, there are other studies that have observed a negative correlation or no association between intramyocellular triglyceride content and insulin sensitivity (Krssak et al. 1999; Goodpaster et al. 2001).

Later studies on the molecular level suggest that the mechanistic explanation for the suppressive effect of fatty acids on glucose metabolism is different

than Randle et al. first proposed. It seems that there might be even a direct effect of accumulated lipids or lipid derivatives involved, which interfere with insulin signalling and action (Itani et al. 2002). Abnormal accumulation of lipids (lipotoxicity) in ectopic tissues, such as skeletal muscle and the liver, is shown to be strongly associated with insulin resistance (Borén et al. 2013). Fatty acids accumulate intracellularly as long-chain fatty acyl-CoA, mono-, di- and triacylglycerol, phosphatidic acid and ceramides (Cooney et al. 2002; Itani et al. 2002; Summers 2006; Nagle et al. 2009). It has been suggested that these lipid intermediates cause perturbations in insulin signalling, rather than triglycerides, which are considered to be metabolically inert (Kraegen & Cooney 2008; Timmers et al. 2008). One hypothesis is that circulating lipids or accumulated lipids are diverted away from the mitochondrial oxidation pathway and instead directed toward the synthesis of signalling intermediates. These bioactive lipid molecules, such as diacylglycerol (DAG) and ceramides, are considered to be toxic when freely available in a cellular environment. Although there are some discrepancies in studies evaluating the relevance of the findings, it has been hypothesized that these lipotoxic molecules interfere with insulin signal transduction. (Holland et al. 2007; Yu et al. 2002; Itani et al. 2002.) The accumulation of DAG is associated with the activation of members of the protein kinase C (PKC) family. PKCs, as well as other kinases, have been postulated to catalyse serine residue phosphorylation in the insulin receptor (IRS-1), inhibiting its activity and blocking its downstream signal transduction. (Martins et al. 2012; Paz et al. 1997; Zhande et al. 2002; Evans 2005.) Impairment of the downstream signalling pathway leads to decreased glucose uptake and metabolism in response to insulin (Martins et al. 2012). However, one must take into consideration that impaired insulin-stimulated glucose uptake can occur without alterations in insulin signalling pathways (Goodpaster 2013).

2.4.3 Mitochondrial function

Many mechanisms are hypothesized to be accountable for the development of skeletal muscle insulin resistance induced by fatty acids. These include inflammation, modulation of gene expression and an increase in reactive oxygen species. Accruing evidence suggests that none of the mechanisms are exclusive, but more than one mechanism might be involved in the establishment of fatty acid-induced insulin resistance. A common factor in all of these has been proposed to be the importance of mitochondria and their function. (Martins et al. 2012; Aon et al. 2014.) Indeed, mitochondrial dysfunction has gained much research attention, and impaired mitochondrial uptake and oxidation of fatty acids are considered to be some of the most prominent theories on pathways leading to muscle insulin resistance (Koves et al. 2008; Morino et al. 2006; Ruderman et al. 1999).

There are several studies that show decreased mitochondrial content, mitochondrial function and oxidative capacity in insulin-resistant states, suggesting that mitochondrial dysfunction plays an important role in the pathophysiology of insulin resistance (Lowell & Shulman 2005; Holloway et al. 2009;

Morino et al. 2006). However, there are almost as many reports from human, animal and cell culture studies with conflicting results. Controversy on this issue will remain as long as there is a lack of knowledge about basic biological interactions in the context of fatty acid-induced insulin resistance. In addition, whether or not the defect in mitochondrial oxidative capacity precedes insulin resistance is under debate, and as yet no definitive answers have been provided. (Morino et al. 2006; Petersen & Dufour 2004; Baar et al. 2002; Toledo et al. 2008.) Alternatively, accumulated cellular fat may cause deleterious effects on mitochondrial function; this is often referred to as lipotoxicity. However, it cannot be excluded that mitochondrial dysfunction may be a consequence of the diabetic state, not a cause in the development of insulin resistance. (Eckardt et al. 2011; Wende et al. 2012.)

It has been suggested that the accumulation of intracellular lipids induced by high-fat feeding, especially in rodents (Burcelin et al. 2002; Iossa et al. 2007; de Wilde et al. 2008), is related to mitochondrial deficiency, leading to insufficient fatty acid oxidation capacity and subsequently causing insulin resistance via the accumulation of intramyocellular lipids (Kelley et al. 2002; Boyle et al. 2011). However, results on the effects of HFD on mitochondria have been conflicting, and there exist studies on rodents that refute the causal role of mitochondria on insulin resistance. It has been shown that high-fat feeding causes insulin resistance while increasing mitochondria in muscle. (Turner & Heilbronn 2008; Turner et al. 2007.) Furthermore, a number of studies have shown that high-fat feeding increases markers of mitochondrial enzymes (Hancock et al. 2008; Miller et al. 1984; Nemeth et al. 1992; Simi et al. 1991), mitochondrial biogenesis and fatty acid oxidative capacity in rodent skeletal muscle (Hancock et al. 2008; Garcia-Roves et al. 2007; Turner et al. 2007; Hoeks et al. 2011). In contrast, a study of obese human subjects has shown a correlation between markers of decreased mitochondrial capacity and insulin resistance (Simoneau & Veerkamp 1999) and a reduction in electron transport chain activity in the subsarcolemmal mitochondrial fraction (Ritov et al. 2005). Conclusions from various human studies suggest that impairments in mitochondrial function are probably due to environmental factors (Huang et al. 1999; Mustelin et al. 2008). Physical inactivity seems to be the major contributor, causing mitochondrial defects in the skeletal muscle of type 2 diabetics (Rimbert et al. 2004; van Tienen et al. 2012). All in all, it is difficult to determine whether a true functional deficit exists, as there seem to be pronounced differences in the mitochondrial phenotypes of obese rodents (Turner et al. 2007) and humans (Simoneau & Veerkamp 1999).

2.4.4 Alleviating insulin resistance

Lifestyle interventions (including physical activity, changes in diet, and weight loss) have the potential to improve metabolic flexibility and lipid handling in skeletal muscle (Corpeleijn et al. 2009). Several recent studies have made mechanistic advances into the cause of insulin resistance as it relates to altered neutral lipid storage (Amati 2012). There are contradictory results regarding chang-

es in IMCL content after diet change or exercise intervention. Both diet change and exercise training have been shown to improve insulin sensitivity (McAuley et al. 2002; Tuomilehto & Lindström 2001; Pan et al. 1997). Weight loss has also been shown to reduce IMCL content (Gray et al. 2003; Goodpaster et al. 2000; Toledo et al. 2008), although not significantly in all studies (Malenfant & Tremblay 2001). In addition to improved insulin sensitivity and reduced IMCL accumulation, weight loss combined with exercise training has been shown to also improve fat oxidation and mitochondrial function (Toledo et al. 2008).

In contrast, in some cases IMCL content has been reported to even increase after exercise (Meex et al. 2010; Goodpaster et al. 2001; Tarnopolsky et al. 2007). Thus, it seems that IMCL content and insulin resistance are correlated only in untrained individuals. However, the phenomenon of the "athlete's paradox" must be mentioned, because individuals who regularly engage in exercise training display normal insulin sensitivity and high oxidative capacity, despite having high levels of intramyocellular triglycerides (Goodpaster et al. 2001). Exercise training undeniably has several beneficial effects. It is well documented that in rats – as in humans – exercise training enhances the oxidative metabolism and glucose uptake of skeletal muscle (e.g. Holloszy & Booth, 1976). Although the mechanisms are still not fully understood, exercise training has been proven to mediate therapeutic effects on insulin sensitivity (Henriksen 2002; Holloszy 2005). While weight loss reduces the quantity of IMCL, exercise training without weight loss alters the physical properties of accumulated lipids and their metabolism (Bruce et al. 2006; Gan et al. 2003). The insulin-sensitizing effects of exercise training has been linked to enhanced mitochondrial activity (Bruce et al. 2003; Koves et al. 2005; Goodpaster et al. 2003). Furthermore, diabetic muscle might be able to circumvent upstream impairments in insulin signalling and insulin action by changing mitochondrial energy flux by means of acute exercise (Thyfault & Cree 2007). Although a considerable amount of information has been gathered on the post-exercise increase of insulin sensitivity in muscle, the role of the contraction/hypoxia-stimulated pathway of glucose transport – not only insulin-stimulated increases in sensitivity – remains to be elucidated in detail (Holloszy 2005). Taken together, these studies clearly indicate that lifestyle interventions may improve metabolic flexibility and thereby improve insulin sensitivity.

2.5 Oxidative capacity

2.5.1 Oxidative capacity in insulin resistance

The suggested reduction in oxidative capacity in insulin resistance may be either a defect or an adaptation, but its pathophysiological significance remains unclear (Toledo & Goodpaster 2013). The lower mitochondrial oxidative capacity may be a consequence of the insulin-resistant state or stress induced by nutrient overload, as suggested earlier (Anderson et al. 2009; Stump et al. 2003).

Factors affecting the oxidative capacity of muscles include the activity of mitochondrial oxidative enzymes (e.g. cytochrome c or succinate dehydrogenase), muscle fibre-type composition and availability of oxygen. Since the effect of mitochondrial function on the oxidative capacity of muscle was elucidated in the previous paragraph on mitochondrial function, the following paragraphs have an emphasis on oxygen availability.

2.5.2 Capillarization to increase oxidative capacity

To modulate insulin sensitivity, the oxidative capacity of skeletal muscle is suggested to be of great importance, boosting lipid oxidation to compensate for excess lipid supply (Galgani et al. 2008). If skeletal muscle cannot increase oxygen supply to the level of oxygen demand required in fat oxidation, deprivation of oxygen supply (i.e. hypoxia) will ensue. Hypoxia may thus be considered as the ultimate degree of mitochondrial dysfunction or nonfunction. (Holloszy 2009.) Therefore, adequate microcirculation to skeletal muscles is essential. Capillaries play a crucial role in the metabolism of muscles. Increased capillarization has been shown to be linked with an increase in the oxidative capacity of skeletal muscle (Hudlicka et al. 1992). The intrinsic signals within tissues that coordinate sprouting of new capillaries (i.e. angiogenesis) with tissues' metabolic needs are still poorly understood. Hypoxia, lowered oxygen tension and/or elevated metabolic activity are known to be inducers of angiogenesis. (Egginton 2009.) Endurance types of exercise have been shown to trigger mitochondrial biogenesis, changes in fibre composition and the growth of new blood vessels in skeletal muscle (Hood 2001; Wagner 2011). Angiogenic factors like the vascular endothelial growth factor (VEGF) are needed to trigger and regulate angiogenesis in a complex set of physiological processes. It has been shown that circulating levels of VEGF after acute exercise training increased with no difference between sedentary and trained subjects. (Kraus et al. 2004.) However, there seems to be a disparity in the localization of capillary growth between different metabolic regions of the muscle (Badr et al. 2003).

Many of the problems associated with type 2 diabetes are attributable to vascular complications, although the pathophysiological connections between obesity and microvascular dysfunction are unclear. Regular exercise has been shown to have positive effects on cardiovascular health, including positive influence on serum lipids and body fat content. (Blair 1984; Egginton 2009.) There are numerous observations that obesity and insulin resistance predispose to either reduced tissue capillary density or an impaired response to angiogenic stimulus, but the mechanisms underlying the effects of chronic exercise on skeletal muscle vascularity is less thoroughly investigated in subjects who are obese and insulin-resistant (reviewed in Frisbee, 2007).

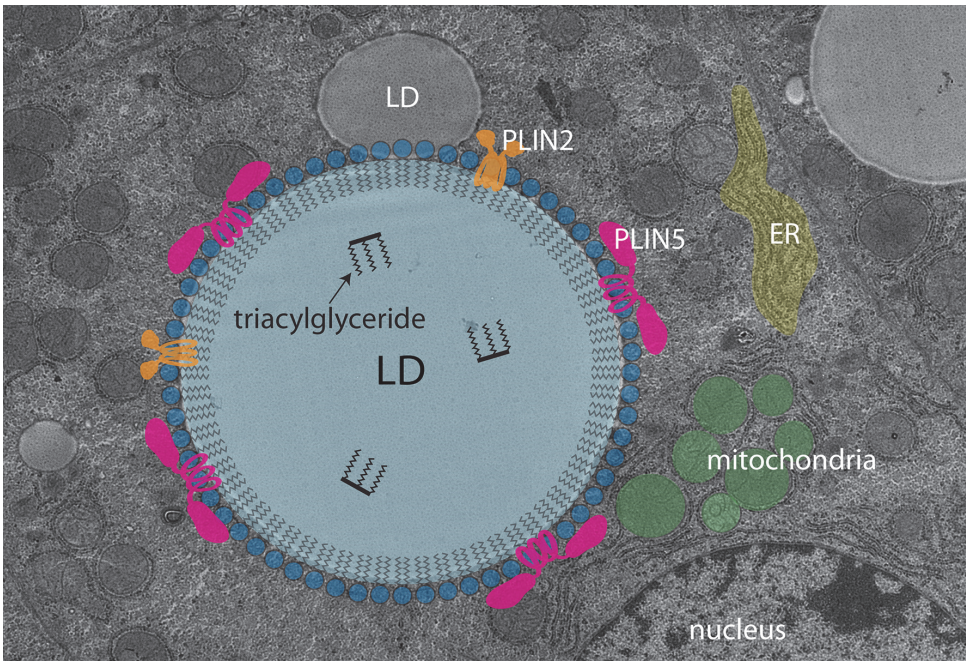

FIGURE 4 Organization of the lipid droplet. An electron micrograph showing a large lipid droplet (LD) in the liver specimen from the Study IV. The nonpolar core of LDs is surrounded by a phospholipid monolayer of polar surface lipids. A variety of coat proteins are embedded on the surface lipids, from which PLIN2 and PLIN5 are illustrated here. LDs are often with a close association with mitochondria and endoplasmic reticulum (ER) membranes.

2.6 Intracellular lipid accumulation

2.6.1 Lipid droplets

Neutral lipids, such as triglycerides, are insoluble in water. To facilitate their storage, the hydrophobic core is surrounded by a monolayer of phospholipids embedded with a variable set of proteins (Figure 4). These structures, called lipid droplets, are present in most eukaryotic cells. Lipid droplets (LD) are studied mostly in adipocytes and are only recently recognized as important and highly dynamic organelles. (Walther & Farese 2012; Farese & Walther 2009.) LD size and number differ considerably by cell type, and depending on metabolic status LDs can change their size rather rapidly. LDs serve as lipid reservoirs, while stored lipids are a source for membrane synthesis, protein modification, and generation of signalling molecules and other lipid products.

There are several models of LD formation and maturation, but consensus locates the formation of LDs on the luminal side of the endoplasmic reticulum (ER). The models for LD biogenesis include budding off from the ER, bicelle formation, budding as a fillable bilayer vesicle or as a concave depression of the

ER, which envelops LDs like a so-called "eggcup". (Walther & Farese 2012; Robenek et al. 2009.)

In addition to adipose tissue, the liver also has a great capacity to store lipids in LDs. In hepatocytes, LDs can store very large amounts of TGs, occupying almost the entire cell. The accumulation of LDs in hepatocytes causes some individuals to have pathologic changes in their hepatocyte architecture, which is referred to as hepatic steatosis, or fatty liver. (Hall et al. 2010; Walther & Farese 2012.) Depending on the size of accumulated lipid vacuoles, macrovesicular steatosis is seen in obesity, metabolic syndrome and type 2 diabetes (Marceau et al. 1999; Van Steenbergen & Lanckmans 1995). Hepatic steatosis has been closely associated with hepatic insulin resistance (Marceau et al. 1999), but additionally predisposes to the development of fibrosis and liver failure (Walther & Farese 2012; Hall et al. 2010).

The majority of studies focusing on LDs have been conducted in adipocytes and hepatocytes, and only very recently in skeletal muscle as well (Shaw et al. 2009). Slow-twitch muscle fibres (type I) are oxidative, having high rates of oxidative metabolism due to large amounts of mitochondria. These oxidative muscle fibres have been shown to also contain more LDs than glycolytic (type II), slow-twitch muscle fibres. (Walther & Farese 2012.) More accumulated IMTG in obese individuals has been observed in type I muscle fibres, and this is also associated with insulin resistance (Walther & Farese 2012; Malenfant & Tremblay 2001).

Frequently LDs are stored as small (<1 μm^2) droplets strategically located inside cells in near proximity to mitochondria or subsarcolemmal mitochondria in muscle, enabling tight coupling of stored lipids with efficient consumption of lipids for fuel (Hoppeler et al. 1973; Tarnopolsky et al. 2007; Shaw et al. 2008). In order to generate energy in β-oxidation or to synthesize membrane lipids, lipolysis is activated to mobilize fatty acids. LDs shrink as their core lipids are catabolized. (Walther & Farese 2012.)

Research on LD-coating proteins is still rather sparse, but there is accruing evidence that these proteins are involved in LD dynamics, including LD synthesis, growth and fusion, triglyceride metabolism, and organelle interactions, as well as intracellular trafficking (Bosma et al. 2012a; Coen & Goodpaster 2012).

2.6.2 Lipid droplet coat proteins

Excess fat is stored as lipid droplets in the cytoplasm. Embedded on the surface of LDs, diverse family of proteins guide and regulate the formation of lipid droplets. Perilipins (derived from Greek, meaning 'surrounding lipid') are the best-characterized and quantitatively most important family of proteins that coat lipid droplets. Perilipins (PLIN) are necessary for the biogenesis of lipid droplets, as well as for structure and function of lipid droplets. (Wolins et al. 2006a; Miura et al. 2002; Wolins et al. 2003.)

The PLIN protein family is the best characterized group of LD coat proteins (Bosma et al. 2012a). The protein family's five members show considerable variation in tissue distribution, but all are involved in lipid accumulation and

metabolism in LDs (Bickel et al. 2009; Brasaemle 2007). PLIN1 was the first member of the perilipin family found in white adipose tissue, and it is mainly expressed in adipocytes. PLIN2 (also known as ADRP, ADFP and adipophilin) is ubiquitously expressed and found to be constitutively bound to LDs. PLIN3 (also known as Tip47, M6PRBP1 and PP17) was originally reported to be involved in the intracellular transport of mannose-6-phosphate receptors. It is expressed diffusely throughout the cytosol and becomes localized to LDs in the presence of increased levels of fatty acids. (Bickel et al. 2009; Bartholomew et al. 2012; Brasaemle et al. 1997; Wolins et al. 2001.) PLIN4 (also known as S3-12) is mainly expressed in white adipose tissue and has some sequence similarity to other perilipins (Wolins et al. 2003). Perilipin 5 (PLIN5, also known as lsdp-5, MLDP, PAT-1 and OXPAT) is the most recently discovered member of the perilipin family. PLIN5 content seems to be proportional to LD content, with the most abundant expression in tissues with high oxidative capacity, such as type I skeletal muscle and the liver. (Kimmel et al. 2010; Minnaard et al. 2009; Wolins et al. 2006b.) PLIN5 is a so-called exchangeable protein, and it can be found on both lipid droplets and the cytosol (Bickel et al. 2009; Bartholomew et al. 2012).

PLIN5 overexpression is known to increase cellular triglyceride (TG) storage in parallel with increased fatty acid (FA) oxidation and induction of the expression of mitochondrial enzymes involved in oxidative metabolism (Wolins et al. 2006a). It has been suggested that PLIN5 protects the TG core of lipid droplets from both basal and stimulated lipolysis, consequently increasing TG storage (Dalen et al. 2007). Thus, it is probable that PLIN5 is not involved only in TG storage, but also in the oxidative degradation of LD-originated fatty acids, thus suggesting a dual role for PLIN5 in lipid turnover (Figure 5). Indeed, this finding further supports the theory that PLIN5 has the potential to modulate LD lipolysis by promoting interaction of LDs with a major triacylglycerol lipase ATGL. (Lass et al. 2006; Granneman et al. 2009; Wang et al. 2011a.)

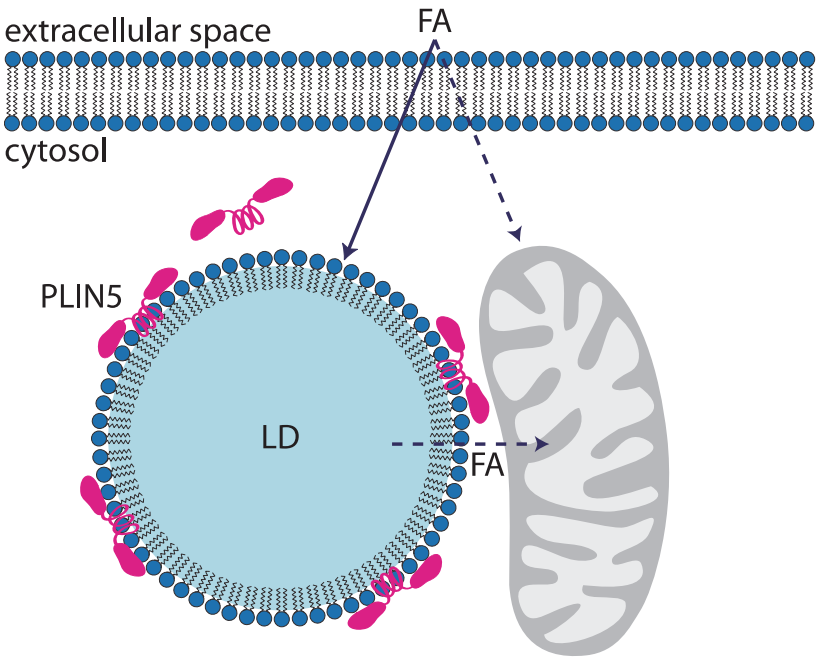

FIGURE 5 Model of the role of perilipin 5 (PLIN5) under physiological adaptation in oxidative tissues in response to excess fatty acid (FA) flux. Excess fatty acid supply leads to an increase in lipid droplet (LD) formation in oxidative cells, such as in skeletal muscle or the liver. PLIN5 is then recruited from the cytosol to the surface of newly formed LDs. The role of PLIN5 is then to regulate triacylglycerol hydrolysis to thus protect mitochondria against lipotoxicity. Modified from Wang & Sztalryd 2011.

Whether PLIN proteins correlate with energy balance or insulin sensitivity is still a matter of debate. Modifications in dietary fat and exercise training can promote changes in IMTG accumulation (Dubé et al. 2008; Schrauwen-Hinderling et al. 2005), but the effects of such interventions on the expression of PLIN proteins have only been sporadically investigated. The diverse nature of lipid droplets suggests that they are most probably involved in the pathogenesis of various disorders or diseases, such as obesity and type 2 diabetes. There are indications that lipid droplets are large in size in insulin-resistant subjects, where the location and number of PLIN proteins and lipases differ significantly from a normal situation. (Meex et al. 2009.) However, the role of a single PLIN protein in the regulation of fat content and the insulin sensitivity of the body is still unclear.

3 AIMS OF THE STUDY

The aim of this thesis was to investigate the role and significance of intracellular lipids in metabolic syndrome and obesity in a mouse model, with an emphasis on the effects of physical activity and diet change. Furthermore, the aim was to elucidate the effect of a high-fat diet on the oxidative capacity of skeletal muscle and the liver. Since skeletal muscle and the liver are both highly insulin-sensitive, their role in the development of insulin resistance was the leading feature of all research questions.

The specific aims were to study:

1. The effects of a high-fat diet and exercise-induced changes on the accumulation of both intramyocellular and intrahepatic lipids in mice after a 19-week experiment. (III, IV)

   Hypothesis: Long-term high-fat feeding increases, whereas physical activity in the form of voluntary wheel running exercise reduces, the amount of accumulated lipids in both skeletal muscle fibres and hepatic cells. A high-fat diet combined with voluntary exercise has similar effects as exercise alone. A diet change from high-fat to low-fat has similar effects as voluntary wheel running exercise.

2. The effect of a high-fat diet and voluntary wheel running on the PLIN proteins associated with lipid droplets and their metabolism in skeletal muscle and the liver. (III, IV)

   Hypothesis: A high-fat diet increases, whereas physical activity and diet change reduces, the amount of PLIN proteins concurrent with the amount of lipid droplets observed in skeletal muscle fibres and hepatocytes.

3. The oxidative capacity and metabolic flexibility of skeletal muscle and the liver, and track the effects of a high-fat diet and physical activity. (I, IV)

   Hypothesis: Markers of oxidative capacity in muscle and in the liver indicate decreased oxidative capacity after high-fat feeding. The quantity of mitochondria in muscle, as well as metabolic flexibility, is reduced with high-fat feeding. Diet change, as well as physical activity, has beneficial effects on the quantity of mitochondria, fatty acid oxidation and metabolic flexibility.

4. The capillarization of skeletal muscle and the effects of a high-fat diet and physical activity on angiogenesis. (II)

   Hypothesis: A high-fat diet decreases the capillarization of skeletal muscle, whereas physical activity increases skeletal muscle capillarization.

4 MATERIALS AND METHODS

4.1 Experiment design

The Animal Care and Use Committee of the University of Jyväskylä, Finland, approved all of the experiment procedures for the present study. All efforts were made that the use of animals caused the least possible amount of suffering. Male C57BL/6J mice (n = 88) were obtained from Taconic (Taconic Europe, Ejby, Denmark) at the age of six weeks and individually housed in standard conditions (temperature 22 °C, humidity 50 ± 10%, light from 8:00 am to 8:00 pm). After one week of adaptation to the new environment, the mice were matched by body-weight (20.8 ± 1.4 g) and sorted into six intervention groups. Groups of low-fat fed (LF) and of high-fat fed (HF) mice were either sedentary (LFS, n = 14 or HFS, n = 14) or physically active (LFA, n = 15 or HFA, n = 15) throughout the experiment (these four groups were examined in studies I and II). In addition, two further groups were first fed the HF diet and kept sedentary for 9 weeks, after which their diet was changed to the LF diet (DC). One of these two groups remained sedentary (DCS, n = 15), while the other group (DCA, n = 15) had access to running wheels (all six groups were examined in studies III and IV). The mice received *ad libitum* for 19 weeks either a lard-based purified high-fat diet (61% of energy from fat, 19% protein, 20% carbohydrates; 5.16 kcal/g; D12492-Euro) to induce obesity and insulin resistance or a low-fat diet as a control diet (10% of energy from fat, 19% protein, 71% carbohydrates; 3.78 kcal/g; D12450-Euro, Purina Mills TestDiet®, PMI® Nutrition International, Richmond, IN, USA). The nutritional profile of the fat content of the two diets was as follows (high-fat diet/low-fat diet): cholesterol 229/18 ppm, linoleic acid 3.97/1.39%, linolenic acid 0.36/0.19%, arachidonic acid 0.05/0.00%, omega-3 fatty acids 0.36/0.19%, total saturated fatty acids 10.54/1.14%, total monounsaturated fatty acids 10.84/1.30%.

The active mice groups of LFA, HFA and DCA were housed in cages where they had free access to custom-made vertical running wheels (diameter 24 cm, width 8 cm) 24 h/day. Total wheel revolutions were recorded daily and

the total running distance per day was determined by multiplying the number of wheel rotations by the circumference of the wheel. Sedentary animals were housed in similar cages without a running wheel.

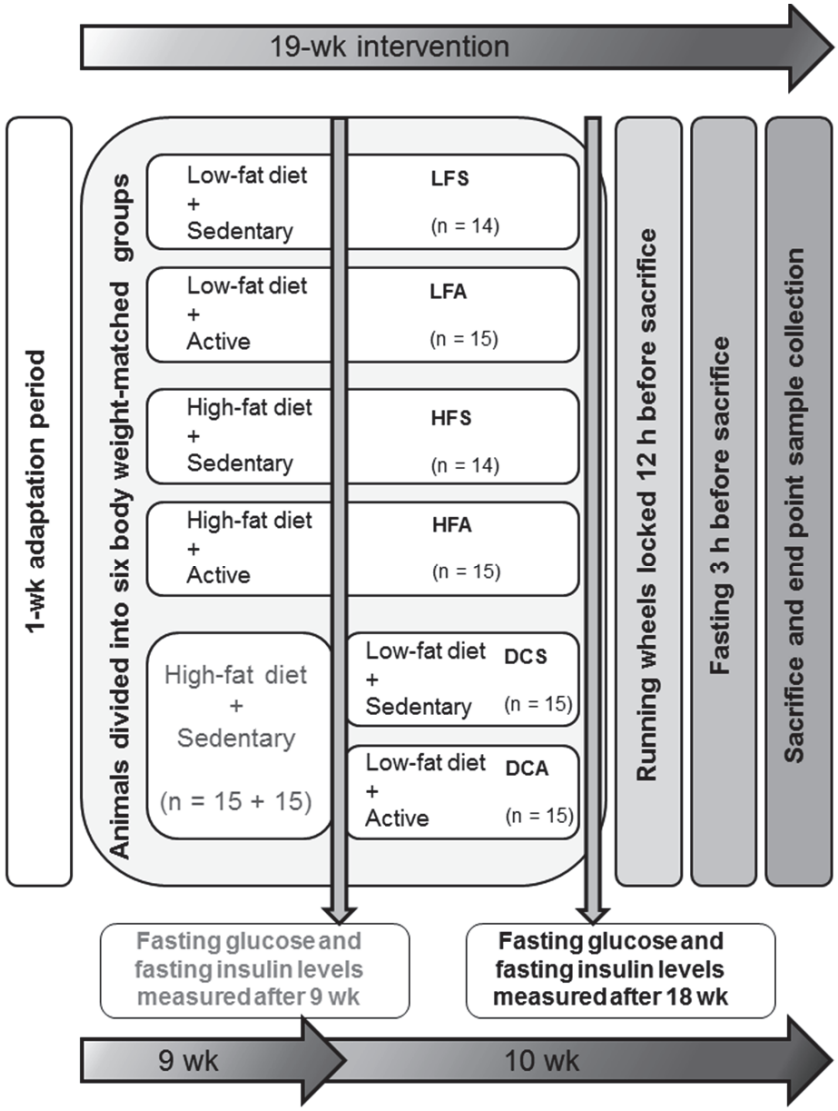

FIGURE 6 Summary of study design. This graph summarizes the experiment set-up and data collection points during the 19-week experiment.

4.1.1 Data and tissue collection

The experiment setup and data collection points are summarized in Figure 6. Body mass and food consumption were measured at three-week intervals throughout the study. The glucose balance of the mice was determined at week 18 of the intervention by analysing fasting glucose and fasting insulin concentrations. After 19-week intervention, the mice were sacrificed, after 3 h fasting and 12 h running restraint, by cervical dislocation and decapitation. Sacrification was performed during the day when the lights were on (light phase). Blood and serum samples were collected for the triglyceride, cholesterol and free fatty acid measurements and stored at -80 °C. The muscles extensor digitorum longus (EDL), soleus, gastrocnemius and quadriceps femoris (QF), epididymal fat pads and the liver were excised from the animals, weighed and prepared for further analysis.

4.1.2 Tissue preparation

For the immunohistochemical analyses, the middle section of the left QF muscle (study II) was used. After sacrifice, the muscle samples were dissected free from visible fat and connective tissue. Muscle samples were mounted in Tissue Tek O.C.T. embedding medium (Tissue Tek, Sakura Finetek Europe) under a microscope to orientate the muscle fibres vertically. They were snap frozen in isopentane (-160 °C) cooled with liquid nitrogen. For the biochemical analyses, the calf muscles (gastrocnemius and quadriceps femoris) and the liver were snap frozen. All samples were stored at -80 °C for further analysis. The soleus muscle (studies I and III) and a liver sample (study IV) were immediately cut into pieces and fixed for electron microscopy.

4.2 Blood analyses

Determined (HemoCue, Sweden) after overnight fasting, blood glucose concentrations were collected at intervention weeks 9 and 18. Serum insulin was analysed with an Ultra Sensitive Rat Insulin ELISA Kit, according to the manufacturer's protocol (Crystal Chem Inc., USA). The HOMA-IR index (homeostasis model assessment of insulin resistance) was calculated as follows: HOMA-IR = fasting blood glucose (mmol/l) x fasting serum insulin (mU/l)/22.5 (Matthews et al. 1985).

Triglycerides, total cholesterol and free fatty acids were measured from the end-point serum samples. Triglycerides and cholesterol were measured using the VITROS DT60 II Chemistry System (Ortho-Clinical Diagnostics, USA). The Wako NEFA C test kit (Wako Chemicals GmbH, Germany), scaled down to a microplate format, was used to determine free fatty acids (FFA).

4.3 Electron microscopy (I, III, IV)

Samples (n = 5 / group) for electron microscopy analysis were prepared by fixing pieces of soleus (study I and III) or pieces of liver (study IV) with 3% glutaraldehyde/0.1 M phosphate buffer for 2-2.5 h at +4 °C, and post-fixed with 1% osmium tetroxide/0.1 M phosphate buffer at +4 °C for 1 h. The specimens were stained in uranyl acetate, dehydrated in ethanol and embedded in LX-112 (Ladd). Semi-thin sections were cut, stained with toluidine blue and examined with light microscopy to optimize the transverse orientation of the muscle samples. After that, ultrathin sections were cut, mounted on grids and stained with uranyl acetate and lead citrate. Micrographs were taken from the best section of each block with a Jeol JEM-1200 electron microscope at 2500x primary magnification for muscle samples and at 4000x primary magnification for liver samples. Approximately 10–13 micrographs/section were analysed using AnalySIS software (Olympus, Tokyo, Japan). In total, 343 micrographs were analysed from the muscle samples. It was checked that micrographs were taken from different cells and that sarcolemmal areas were included. The quantity of subsarcolemmal mitochondria (study I) was expressed as mitochondrial area (μm^2) and related to the length of sarcolemma (μm). For each set of images, the average droplet size (i.e. an average LD surface area [μm^2]), total droplet amount (i.e. total amount of LDs per 1000 micrometre of cytoplasm [number × $(1000\ \mu m^2)^{-1}$]) and percentage of lipid droplet area density (i.e. the fraction of the cell area occupied by lipid droplets [%]) was also assessed (study III). From the liver samples (study IV), the nuclei, Kuppfer cells, bile canaliculi and central veins were cropped from the micrographs in the analysis of proportional volume of cytoplasmic fat (%). The total droplet amount (i.e. total amount of LDs per 100 micrometre of cytoplasm [number × $(100\ \mu m^2)^{-1}$]) and the average droplet size (i.e. average LD surface area [μm^2]) were also assessed from the total of 358 micrographs.

4.4 Immunohistochemistry (II)

For the visualization of capillaries (in study II), cross-sections (8 μm) of QF muscles were cut with cryomicrotome and then stained with Isolectin-GS-IB_4 containing fluorescent Alexa Fluor 488 dye (Molecular Probes, USA). To visualize muscle fibres, antidystrophin (Novocastra, UK) staining was combined with Alexa Fluor 555 secondary antibody (Molecular Probes). Stained sections were studied with a fluorescent microscope (Olympus BX-50F, Olympus Optical, Japan). TEMA image analysing software (Scan Beam, Denmark) was used to analyse the cross-sectional area of muscle fibres (CSA), capillary density (number of capillaries per mm^2) and capillary-to-fibre (C:F) ratio.

4.5 Laser Capture Microdissection (LCM) (II)

In study II, LCM was used to collect capillaries and pure muscle fibres from the QF muscles, as adopted from Milkiewicz & Haas (Milkiewicz & Haas 2005). Cryosections (8 μm) were cut on uncoated glass slides and fixed immediately with cold acetone. Capillaries were stained with Isolectin-GS-IB_4 Alexa Fluor 488 dye (Molecular Probes, USA), which was diluted in sterile PBS with RNase inhibitor (SUPERaseIn, Ambion, USA). Laser-capture microdissection for capillaries and pure muscle was performed with a Veritas microdissection system (Arcturus Engineering, USA). Samples were randomly collected from all parts of the muscle, including the deep oxidative and the superficial glycolytic portions. Capillaries were distinguished by size (<10 μm) from larger blood vessels. RNA was extracted with a PicoPure RNA isolation kit (Arcturus Engineering) according to the protocol. RNA was transcribed to cDNA with a Sensiscript reverse transcription kit (Qiagen), using both random and oligo(dT) primers (Ambion).

4.6 Analysis of messenger RNA (I, II, III)

Total RNA isolation, reverse transcription and cDNA synthesis of gastrocnemius muscle samples

Homogenization of the gastrocnemius muscle samples was done with a FastPrep (Bio101 Systems, USA) tissue homogenizer by using Lysing Matrix D (Q-Biogene, USA), and total RNA was isolated using the Trizol reagent (Invitrogen, USA). The quality of RNA was confirmed by spectrophotometry and agarose gel electrophoresis. Reverse transcription of mRNA was performed from total RNA (5 μg) by using anchored oligo$(dT)_{20}$ primers (Oligomer, Finland) and a SuperScript III Reverse Transcriptase kit (Invitrogen), according to the manufacturer's instructions.

Real-time RT-PCR

The mRNA expression was quantified with a real-time reverse transcriptase-PCR (RT-PCR) assay, using an ABI 7300 Real-Time PCR System (Applied Biosystems, USA). The probes and primers used were pre-designed transcripts validated by Applied Biosystems bioinformatics design pipelines. The used TaqMan primers and probes with Applied Biosystems assay ID are PDK4 (study I) Mm00443326_m1, VEGF-A (study II) Mm00437304_m1, PLIN5 (study III) (Mm00508852_m1) and GAPDH (Mm99999915_g1).

The gene expressions of muscle homogenates were quantified according to the corresponding gene-specific standard curve. For the LCM samples, the comparative CT method was utilized as outlines in Applied Biosystems User Bulletin 2 (Applied Biosystems). All samples were analysed in triplicate. The gene expressions were normalized to the expression of GAPDH to correct for

potential variation in RNA loading or amplification efficiency. GAPDH is considered the most stable internal control in endurance exercise studies (Mahoney et al. 2004; Jemiolo & Trappe 2004). Stability in these experiments was confirmed by the fact that there were no differences between groups in the GAPDH expression of the muscle homogenate samples when normalized to cDNA-RNA hybrid concentrations, as previously validated (Lundby et al. 2005). cDNA-RNA hybrid concentrations were measured using a Quant-iT™ PicoGreen® assay (Invitrogen), according to the manufacturer's instructions. The amplification efficiencies of all the target genes and GAPDH were checked to be equal over a range of serial dilutions.

4.7 Analysis of proteins with Western blotting (I, II, III, IV)

4.7.1 Homogenization of the samples

The QF muscle samples (study I and II) were hand-homogenized in a 4% homogenization buffer [10% SDS (w/v), 40 mM DTT, 5 mM EDTA, 0.1 M Tris-HCl pH 8 and protease inhibitors 40 µg/ml aprotinin, 80 µg/ml PMSF and 40 µg/ml leupeptin (Sigma, Saint Louis, USA)]. The gastrocnemius muscle samples (study III) were hand-homogenized with a dounce homogenizer in an ice-cold 4% homogenization buffer (20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% Triton X-100, 0.2% sodium deoxycholate, 30 µg/ml leupeptin, 30 µg/ml aprotinin, 60 µg/ml PMSF, and 1% phosphatase inhibitor cocktail (P 2850; Sigma, St. Louis, USA). The liver samples (IV) were pulverized in liquid nitrogen and then homogenized in an ice-cold 7% homogenization buffer (20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% NP-40, 0.2% sodium deoxycholate, 3% phosphatase inhibitor cocktail (P 2850; Sigma, St. Louis, USA).

4.7.2 Analysis of proteins with Western blotting

Homogenates were rotated for 30 min at 4 °C and then centrifuged at 10,000 *g* for 10 min at 4 °C to remove cell debris. Total protein content was determined using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, USA) in triplicates with a KoneLab device (Thermo Scientific Inc., Vantaa, Finland). Western immunoblot analyses from the sample lysates were solubilized in a Laemmli sample buffer. Samples contained 20 µg (study I and II), 30 µg (study III) or 60 µg (study IV) of total protein. The PVDF membranes were incubated overnight at 4 °C with a primary antibody against cytochrome C (CytC, 1:2000), VEGF-A (1:500; both Santa Cruz Biotechnology Inc., USA) and PLIN5 (1:6000 in muscle and 1:2000 in liver; PROGEN Biotechnik GmbH, Germany) in 2.5% milk. Membranes were incubated with horseradish peroxidase-conjugated secondary IgG antibodies (Jackson ImmunoResearch Laboratories, USA), which were di-

luted 1:50,000–1:70,000 depending on the protein. Uniformity of protein loading was confirmed by consistently staining the membrane with Ponceau S and by probing the membrane with an antibody against GAPDH (Abcam, Cambridge, UK). The results are presented as relative to Ponceau S, while earlier studies have confirmed a proportional linear relationship between amount of protein loaded and the strongest band in Ponceau S at ~42 kDa in quantification when 5–60 µg of protein was loaded (Hulmi et al. 2012). Proteins were visualized by ECL, according to the manufacturer's protocol (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology) and quantified using Chemi-Doc XRS Quantity One software (version 4.6.3. Bio-Rad, UK).

4.8 Citrate synthase activity (IV)

Liver samples were homogenized as described above. The activity of citrate synthase (CS) in the liver homogenate was measured using a kit (Sigma-Aldrich, CS0720) with an automated KoneLab device (Thermo Scientific). Enzyme activities were expressed as units per µg of dissolved protein.

4.9 Muscle and liver lipid extraction (III, IV)

Intracellular triglyceride (TG) and non-esterified fatty acid (NEFA) content from the gastrocnemius muscle (study III) samples and intrahepatic TG content from the liver (study IV) samples were determined by means of an enzymatic colorimetric technique (KoneLab 20XTi, Thermo Scientific). Total lipids were extracted from the pulverized muscle samples (30 mg) or from the liver (30 mg wet weight) in 4 ml of chloroform-methanol (2:1), using the method of Folch et al. (Folch et al. 1957). Homogenates were gently agitated in an orbital shaker for 2 h (muscle samples) or 20 min (liver samples). After that, the liver samples were additionally filtrated. The solvent was washed by adding 2 ml of 0.9% NaCl, and after separating the phases by centrifuging (2000 rpm, 10 min), the organic phase was evaporated in a vacuum (Speedvac Savant, Thermo Scientific). The dried lipid extract was dissolved in 250 µl ethanol and used for determination of TG and NEFA content.

4.10 Statistical methods

All data are presented as mean ± SD. The within-group normality and equality of the variance assumptions were not met in the parameters of interest and hence the nonparametric Kruskal-Wallis one-way analysis of variance was used for multiple comparisons and the Mann-Whitney *U* test for post hoc analysis.

All statistical analyses were carried out using PASW statistics software (versions 18.0–20.0, IBM Corporation, USA). Differences of $P < 0.05$ were considered significant.

5 RESULTS

The main findings of the present series of studies are presented below. For more details, the original articles (I–IV) should be consulted.

5.1 Effects of diet and exercise interventions

5.1.1 Obesity and the insulin-resistance state (I–IV)

The results indicate that HF animals were significantly more obese and insulin-resistant than LF and DC animals. After only one week of intervention, significantly higher body mass and epididymal fat mass were observed in the HF-fed mice, compared to both LF mice and DC mice (Table 1). Thereafter, the body weight of the HF mice increased continuously during the experiment. After seven weeks of intervention and throughout the rest of the intervention, a significant difference in body weight between the sedentary and their respective running groups was seen. By the end of the experiment, the body weight of the HF mice was significantly higher (39% and 52%, $P < 0.001$ in sedentary and active) than that of the respective LF ($P < 0.001$) mice. The body weight of DC mice was even lower when compared to LFS ($P < 0.001$) mice. There was a significant decreasing effect of physical activity on body weight in the LF and DC groups. The HOMA-index values indicated that HF mice were significantly more insulin-resistant than LF and DC mice. In addition, the HF-fed mice had lower levels of free fatty acids and higher levels of cholesterol in serum after 3 h fasting, compared to LF mice. Serum triglyceride concentrations were quite similar in all groups, except for HFA mice, which had a lower concentration than the other active groups.

TABLE 1 Body weight, liver mass, epididymal fat mass (Fat) and serum parameters (Chol, FFA and TG) were measured after 19 week experiment. Fasting glucose and fasting insulin values are those measured after 18 weeks. (HOMA= homeostasis model assesment of insulin resistance, Chol=total cholesterol, FFA=free fatty acid, TG=triglyceride, LF=low fat diet, HF=high fat diet, DC=diet change)
a= *P*<0.05 vs. LFS, **a**= *P*<0.01 vs. LFS, **A**= *P*<0.001 vs. LFS,
b= *P*<0.05 vs. LFS, **b**= *P*<0.01 vs. LFS, **B**= *P*<0.001 vs. LFS,
c= *P*<0.05 vs. LFS, **c**= *P*<0.01 vs. LFS, **C**= *P*<0.001 vs. LFS,
d= *P*<0.05 vs. DCA, **d**= *P*<0.01 vs. DCA, **D**= *P*<0.001 vs. DCA

	Sedentary			**Active**		
	LF	HF	DC	LF	HF	DC
Weight (g)	33 ± 3	45 ± 5^{A}	30 ± 1^{A,B,d}	29 ± 2^{A,B}	44 ± 3^{A,C,D}	28 ± 2^{A,B}
Liver (g)	1.4 ± 0	2.1 ± 0.2^{a}	1.2 ± 0^{a,B,d}	1.2 ± 0.1^{B}	1.8 ± 0.1^{A,C,D}	1.2 ± 0^{a,B}
Fat (g)	0.8 ± 0.3	1.8 ± 0.4^{A}	0.6 ± 0.1^{B,C,d}	0.4 ± 0.7^{A,B}	1.9 ± 0.5^{A,b,C,D}	0.5 ± 0.1^{a,B,c}
Glucose (mmol/l)	8.9 ± 1.1	9.4 ± 1.1	8.1 ± 1.4^{b}	8.4 ± 0.9^{b}	10.5 ± 0.7^{A,b,C,D}	8.3 ± 1.1^{b}
Insulin (mIU/L)	9.4 ± 5.3	49.5 ± 24.3^{A}	5.6 ± 3.5^{B}	5.9 ± 3.5^{B}	47.1 ± 18.0^{A,C,D}	7.9 ± 4.2^{B}
HOMA	3.9 ± 2.4	20.9 ± 10.2^{A}	2.1 ± 1.4^{B}	2.3 ± 1.5^{B}	22.1 ± 8.6^{A,C,D}	2.9 ± 1.4^{B}
Chol (mmol/l)	3.0 ± 0.9	4.9 ± 0.5^{A}	2.7 ± 0.5^{B}	2.7 ± 0.4^{B}	4.5 ± 0.5^{A,b,C,D}	2.7 ± 0.4^{B}
TG (mmol/l)	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.2	0.9 ± 0.1^{c,d}	1.1 ± 0.2
FFA (mmol/l)	0.8 ± 0.2	0.5 ± 0.1^{A}	0.9 ± 0.2	0.9 ± 0.2	0.4 ± 0.1^{A,D}	0.9 ± 0.1

5.1.2 Weekly running distance of active mice (I–IV)

The mice were caged separately. Active mice had access to running wheels. The weekly running distance gradually increased during the first weeks, and the maximum running distance was reached at the fourth week for the LF and HF groups and at the sixth week for the DC group (see original Article I, Figure 2, and Article III, Figure 2). After this, the running distance in all groups gradually declined until the end of the study. On the ninth week and from the twelfth week onwards, the LF mice ran significantly longer distances per week than the HF mice. The DCA mice did not show as dramatic an increase in their running distance at the beginning as the LFA and HFA mice, probably because the DCA mice were already heavier and 10 weeks older at that point. The LFA mice voluntarily ran a significantly longer distance during the study than the HFA mice (428 ± 110 km vs. 346 ± 138 km).

5.2 Lipids and lipid droplet-associated protein PLIN5

5.2.1 Accumulated intramyocellular lipids (III)

The proportional volume of lipid droplets (LD), their average size and their total number were examined by electron microscopy in the soleus muscle (see original Article III, Figure 3). HFS mice had significantly more LDs and larger droplets than LFS, and LDs seemed to also be in close proximity to subsarcolemmal mitochondria. It is worth noting that in the HFA group, both LD size and number did not increase significantly, when compared to either LFS or LFA mice. Myocellular triglycerides and non-esterified fatty acids (NEFA) were determined from the extracted gastrocnemius muscle samples by biochemical measurements. The HF diet increased the amount of muscular TG. HFA mice had significantly more muscular TG when compared to either LFA (P<0.01) or DCA (P<0.01) mice. There was also a statistical significance between LFS and LFA (P<0.05) mice. There were no statistical differences between any of the groups in muscular NEFA results.

TABLE 2 Soleus muscle tissue morphology was examined by electron microscopy. The size and amount of lipid droplets (LD) were analyzed from the micrographs (n=5/group). Triglyceride content (n=10/group) was determined from the gastrocnemius muscle homogenates. (LD vol.=LD proportional volume, LD count=amount of LDs, TG=triglyceride, LF=low-fat diet, HF=high-fat diet, DC=diet change)
a= P<0.05 vs. LFS, **a**= P<0.01 vs. LFS, **A**= P<0.001 vs. LFS,
b= P<0.05 vs. LFS, **b**= P<0.01 vs. LFS, **B**= P<0.001 vs. LFS,
c= P<0.05 vs. LFS, **c**= P<0.01 vs. LFS, **C**= P<0.001 vs. LFS,
d= P<0.05 vs. DCA, **d**= P<0.01 vs. DCA, **D**= P<0.001 vs. DCA

	Sedentary			**Active**		
	LF	HF	DC	LF	HF	DC
LD vol. [‰]	0.79 ± 0.4	3.45 ± 2.4^{a}	1.76 ± 1.3	0.83 ± 0.5	1.91 ± 2.2	1.60 ± 1.0
LD size [μm^2]	0.077 ± 0.01	0.135 ± 0.05^{A}	0.089 ± 0.02	0.085 ± 0.02	0.090 ± 0.03	0.088 ± 0.04
LD count [(100 μm^2)$^{-1}$]	9.8 ± 3.8	24.0 ± 8.9^{a}	18.2 ± 10.1	9.4 ± 5.7	17.7 ± 14.4	15.7 ± 9.1
Muscle TG [μmol/g]	36.3 ± 19.6	56.6 ± 33.3	28.4 ± 13.3^{b}	26.4 ± 22.1 ^{a,b}	55.0 ± 27.6 ^{c,d}	23.1 ± 9.2 ^{B}
NEFA [μmol/g]	16.2 ± 9.0	15.2 ± 8.5	17.6 ± 7.2	14.9 ± 8.6	14.3 ± 7.5	16.9 ± 4.6

5.2.2 Accumulated hepatic lipids (IV)

The proportional volume of lipid droplets (LD), their average size and their total amount were examined by electron microscopy in the liver (Table 3). The proportional volume of LDs was significantly highest in HFA mice (P<0.01), when compared to LFS and other active groups (i.e. LFA and DCA). HFS mice also had a significantly higher volume of LDs, compared to the DC group. There was a high within-group variation in many of the groups, especially in LFS and DCA mice. The amount of LDs per 100 micrometres was highest in HFA mice, compared to the LF groups. The largest LDs were in HF groups, although within-group variation was high in the LFS and HF groups.

From the liver extracts, the liver triglyceride content was measured. The highest liver triglyceride content was in the HF group, when compared to other diet groups. The triglyceride content was lowest in the DCS and LFA groups, which had significantly lower triglyceride content than LFS mice.

TABLE 3 Liver tissue morphological analysis of lipid droplets and triglyceride content in liver homogenate. Liver tissue morphology was examined by electron microscopy and lipid droplets calculated. The size and amount of lipid droplets (LD) were analyzed from the micrographs (n=5/group). Liver triglyceride content (n=10/group) was determined from the liver homogenates. (LD vol.=LD proportional volume, LD count=amount of LDs, TG=triglyceride, LF=low-fat diet, HF=high-fat diet, DC=diet change)
a= P<0.05 vs. LFS, **a**= P<0.01 vs. LFS, **A**= P<0.001 vs. LFS,
b= P<0.05 vs. LFS, **b**= P<0.01 vs. LFS, **B**= P<0.001 vs. LFS,
c= P<0.05 vs. LFS, **c**= P<0.01 vs. LFS, **C**= P<0.001 vs. LFS,
d= P<0.05 vs. DCA, **d**= P<0.01 vs. DCA, **D**= P<0.001 vs. DCA

	Sedentary			**Active**		
	LF	HF	DC	LF	HF	DC
LD vol.[%]	8.6 ± 8.9	22.5 ± 6.4	3.3 ± 0.9^{b}	2.8 ± 0.9^{b}	30.7 ± 6.3^{a,c,d}	2.9 ± 1.3^{b}
LD size [µm^2]	1.9 ± 2.0	4.0 ± 2.0	0.8 ± 0.2^{b}	0.7 ± 0.2^{b}	4.0 ± 2.0^{c,d}	0.6 ± 0.2^{b}
LD count [(100 µm^2)$^{-1}$]	4.2 ± 0.8	6.5 ± 2.7	4.1 ± 0.9	4.2 ± 1.8	8.7 ± 2.6^{a,c}	5.4 ± 2.9
Liver TG [µmol/g]	20.3 ± 15.6	39.8 ± 19.3^{a}	8.5 ± 4.8^{a,B}	9.6 ± 4.6^{a,B}	38.7 ± 18.8^{a,C,D}	11.5 ± 11.3^{B}

5.2.3 Skeletal muscle PLIN5 expression (III)

The protein expression level of PLIN5 (Figure 7A) in HF mice was significantly higher than in LF in both the sedentary and active mice. The sedentary HF mice had 322% higher and the active HF mice 368% higher PLIN5 protein expression levels than their LF counterparts. The expression was reversible, as shown by the markedly higher expression in HF mice than in DC mice, whereas no significant differences in the protein expression levels of PLIN5 were observed between the DC and LF mice.

The PLIN5 mRNA expression (Figure 7B) patterns of the different groups were very similar to the expression patterns observed for protein. The mRNA expression level was higher in other groups, compared to LFS mice, with the exception of DCS mice. The mRNA expression levels in the HF groups were markedly higher than their respective LF and DC groups.

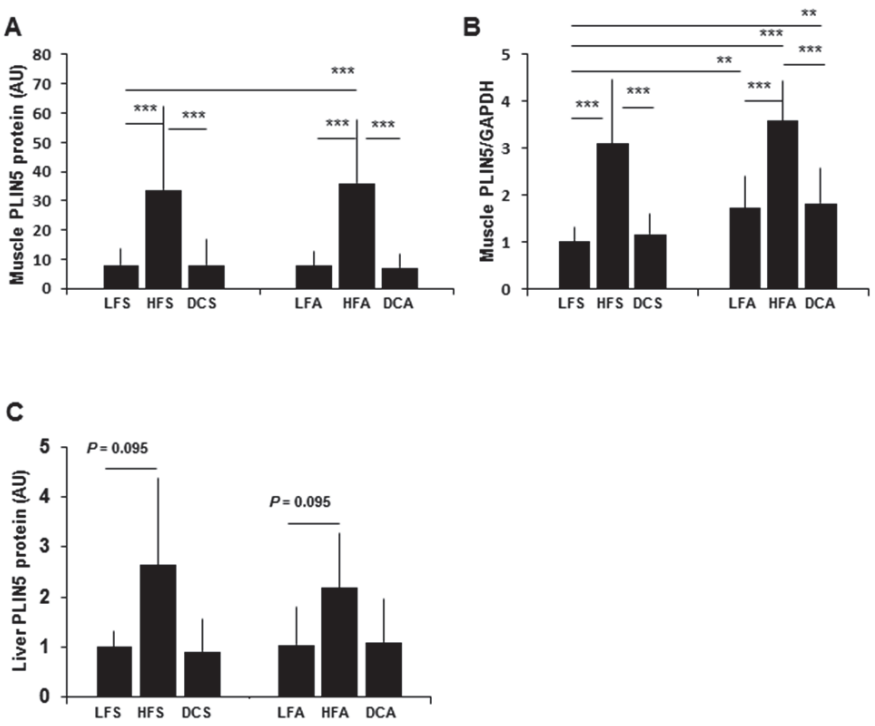

FIGURE 7 The protein expression level (A) and mRNA expression (B) levels of lipid droplet coat protein perilipin 5 (PLIN5) in muscle and PLIN5 protein expression level (C) in the liver were measured (AU = arbitrary units). PLIN5 expression was significantly higher in HF groups in muscle samples. In liver samples, the effect can be observed, although not statistically, due to small sample size (LFS = low fat sedentary, HFS = high fat sedentary, DCS = diet change sedentary, LFA = low fat active, HFA = high fat active, DCA = diet change active). ** $p<0.01$, *** $p<0.001$.

5.2.4 Liver PLIN5 protein expression (IV)

In the liver, the protein expression level of PLIN5 (Figure 7C) in HF mice was higher than in LF mice for both the sedentary and active groups, although the difference was not statistically significant (P = 0.095). The sedentary HF mice had 163% higher and the active HF mice 108% higher PLIN5 protein expression levels than their LF counterparts. The expression was reversible, as shown by

the higher expression in HF than in DC mice (189% and 98% higher in sedentary and active). None of the differences reached statistical significance, probably due to small group sizes (n = 5/group) in protein expression analysis in the liver.

5.3 Oxidative capacity and energy metabolism

5.3.1 Oxidative capacity in skeletal muscle (I)

The evaluation of the effect of a high-fat diet and physical activity on the oxidative capacity of skeletal muscle was done through analysis of the QF or soleus muscle. Results were reanalysed from the original publications I and II. The protein expression level of cytochrome C (CytC) as a mitochondrial quantity marker was measured. CytC protein expression (Figure 8A) was observed to be higher in the quadriceps femoris muscles of the LFA, HFS and HFA mice, compared to LFS mice, but no difference was observed between HFA and HFS mice. CytC protein expression in DCS was significantly lower than in HFS mice ($P < 0.05$) and at a similar level with LFS (previously unpublished data). There were no significant difference between the CytC protein expression levels of DCA and other active groups (LFA and HFA).

The quantity of mitochondria was analysed from subcellular mitochondria in the soleus muscle by means of electron microscopy. Clusters of mitochondria beneath the sarcolemma, often located near the capillaries and lipid droplets, were observed with the electron micrographs and quantified. The area occupied by mitochondria (Figure 8C) seemed to be ~20% larger in the HFA mice than in the HFS mice and ~25% larger than in the LF mice, although the differences were not statistically significant. The amount of mitochondria in the DC groups was also very similar to the respective LF groups (previously unpublished data). There was no increase in the amount of mitochondria in the active mice, compared to the respective sedentary groups. The ultrastructure of mitochondria seemed to be normal in all groups.

5.3.2 Oxidative capacity in liver (IV)

In order to evaluate oxidative capacity in the liver, the protein expression level of cytochrome c was investigated by means of Western blotting (Figure 8B). The lowest CytC protein expression level was in HFS mice, but the difference was significant only when compared to DCS mice ($P<0.05$). Furthermore, HFA mice had a lower expression level than the other active groups (approaching significance $P = 0.056$).

In addition, to increase the evidence on hepatic oxidative capacity, the citrate synthase activity level was measured (Figure 8D). The activity of citrate synthase was lowest in HFS mice. Physical activity seems to have an elevating effect on the activity level, at least in DC mice, although the difference was not

significant (P = 0.095). The highest activity was in DCA mice, which was significantly higher than the other active groups (P<0.01 in DCA vs. HFA and P = 0.056 in DCA vs. LFA).

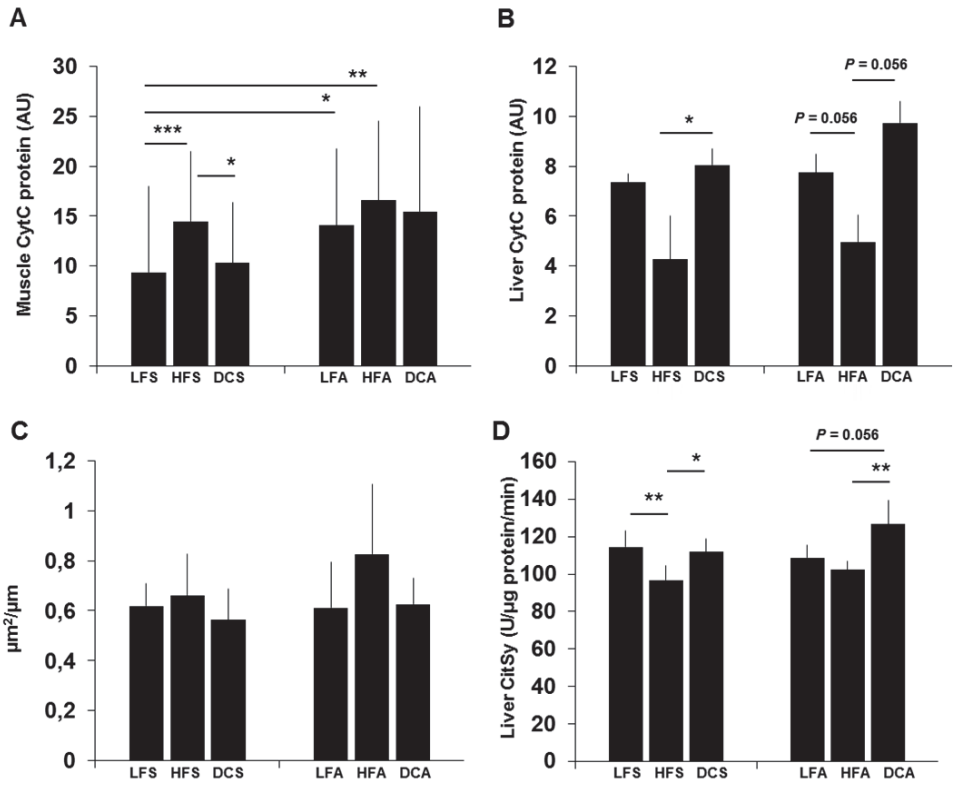

FIGURE 8 The oxidative capacity of muscle and the liver was evaluated. The protein expression level (AU = arbitrary units) of cytochrome c (CytC) in muscle (A) and in the liver (B). In skeletal muscle, the CytC expression indicates a higher oxidative capacity in HF groups, compared to LFS mice. In the liver, the CytC protein expression was lowest in HF groups, although not statistically significant when compared to LF mice. There were no statistical differences between any of the six groups in the area of subsarcolemmal mitochondria relative to the length of the sarcolemma analysed (µm²/µm) from electron micrographs in soleus muscle (C). Liver citrate synthase (CitSy) activity (U/µg protein/min) was determined from the liver homogenate (D). The CitSy activity was lowest in HF groups and highest in DCA mice (LFS = low fat sedentary, HFS = high fat sedentary, DCS = diet change sedentary, LFA = low fat active, HFA = high fat active, DCA = diet change active). * p<0.05, ** p<0.01, *** p<0.001.

5.3.3 Muscle capillarization and VEGF-A (II)

Analysis of skeletal muscle capillarization, muscle fibre cross-sectional area (CSA) and the expression level of the angiogenesis regulating factor VEGF-A in muscle homogenate as well as in capillaries were performed in low-fat and high-fat diet-fed mouse groups (Table 4). The effect of a high-fat diet and physical activity were measured for the variables mentioned above. The capillary density (CD) of sedentary DC mice was slightly higher than for LFS mice (P = 0.088), but lower (not statistically significant) when compared to HFS mice (previously unpublished data). In active DC mice, the capillary density was lower than in the respective LF and HF mice, although none of these differences reached statistical significance. The sedentary HF mice had higher capillary density (21%) and a higher capillary-to-fibre ratio (18%) in their quadriceps femoris muscles than the sedentary LF mice. The LFA mice had higher capillary density (32%) and capillary-to-fibre ratio (32%) than LFS mice. There were no significant differences between HFA and HFS mice in capillary density or capillary-to-fibre ratio. However, the capillary-to-fibre ratio was slightly higher in the HFA mice, with the difference approaching significance (P = 0.08). The muscle fibre cross-sectional area was similar between the LFS, LFA and HFS groups, but higher in HFA mice than HFS mice.

TABLE 4 Skeletal muscle capillaries and muscle fibre cross-sectional areas analysed with fluorescent microscopy. Capillary density (CD) calculated as capillaries per mm² (cap./mm²) was studied in all six groups. Capillary density was significantly higher in HFS, LFA and HFA mice, when compared to LFS mice. Fibre cross-sectional area (CSA) and capillary-to-fibre ratio (C:F) was not calculated in the DC mice. The highest CSA was in HFA mice. The C:F ratio was also highest in HF mice and in LFA mice, when compared to LFS mice.
(LF = low fat diet, HF = high fat diet, DC = diet change).
a= P<0.05 vs. LFS, **a**= P<0.01 vs. LFS, **A**= P<0.001 vs. LFS,
b= P<0.05 vs. LFS, **b**= P<0.01 vs. LFS, **B**= P<0.001 vs. LFS,
c= P<0.05 vs. LFS, **c**= P<0.01 vs. LFS, **C**= P<0.001 vs. LFS

	Sedentary			**Active**		
	LF	HF	DC	LF	HF	DC
CD (cap./mm²)	480 ± 64	582 ± 90 [a]	544 ± 91	632 ± 182 [a]	612 ± 145 [a]	550 ± 137
CSA (µm²)	3211 ± 514	3114 ± 246		3325 ± 537	3623 ± 404 [a,B,c]	
C:F	1.5 ± 0.2	1.8 ± 0.2 [a]		2.1 ± 0.5 [A]	2.2 ± 0.5 [A]	

Both physical activity in the LF group and a high-fat diet increased levels of VEGF-A protein expression (Figure 9A). However, no significant difference was observed between HFA and HFS mice. Although not a statistically significant difference, the VEGF-A protein expression of DC mice was at a similar level as LF mice (previously unpublished data). The difference of VEGF-A protein expression levels between DCS and HFS mice was nearly significant ($P = 0.094$).

The expression level of VEGF-A mRNA was also found to be higher in the muscle homogenates of HFA mice, compared to LFS mice (Figure 9B). The VEGF-A mRNA expression level of DC mice was at a similar level as LF mice in the muscle homogenate (Figure 9B), although the finding was not statistically significant (previously unpublished data). There were no significant differences in the mRNA expression levels of VEGF-A between the active and sedentary groups (LFAvs. LFS or HFA vs. HFS) in any sample type (Figure 9B, 9C and 9D), although the difference between HFS and HFA mice in the muscle homogenate was nearly significant ($P = 0.093$). In capillary samples, however, VEGF-A mRNA expression was slightly higher in LFA mice than LFS mice, with the difference ($P = 0.053$) being very near to the limit of statistical significance (Figure 9D). The mRNA levels of VEGF-A were higher in the muscle capillary samples of the HFS and HFA mice compared to LFS mice (89% and 108%, respectively). However, there were no differences between these groups in muscle fibres (Figure 9B). The mRNA levels of isolated muscle fibre and capillary samples were not determined in DC mice.

5.3.4 The effect of PDK4 on energy metabolism (I)

To evaluate the effects of high-fat feeding and physical activity on the energy homeostasis status in skeletal muscle, the pyruvate dehydrogenase kinase 4 (PDK4) expression levels were measured (Figure 10). Both physical activity and a high-fat diet significantly increased the protein expression of PDK4 ($P < 0.05$) in the LF group, compared to LFS mice (Figure 10A). There was a notable decrease in the PDK4 protein expression in DC mice, when compared to LF and HF mice (previously unpublished data). The mRNA expression level of PDK4 in the HF-fed animals, especially when combined with running, was significantly higher than in LFS mice (Figure 10B). No additional effects were observed with concurrent physical activity, in contrast to sedentary groups. Unlike the protein expression, no significant decrease in the mRNA expression level of DC mice was observed; the expression was similar to that of the LF mice (previously unpublished data).

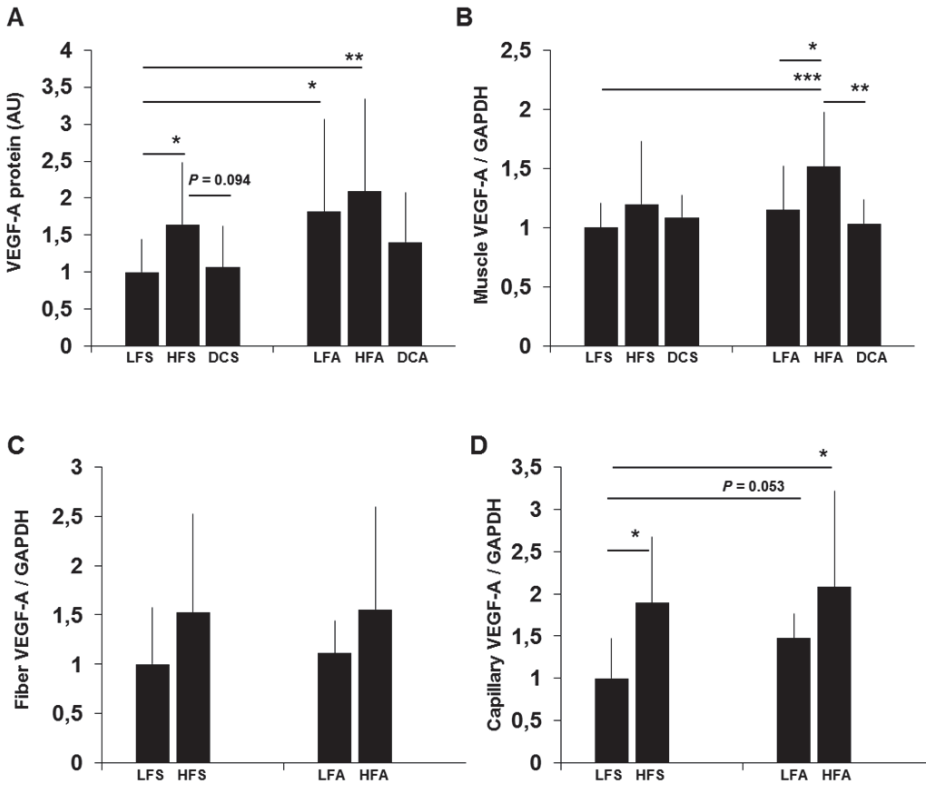

FIGURE 9 VEGF-A protein expression levels were measured from the whole muscle homogenate (AU= arbitrary units) (A). VEGF-A protein expression was highest in HF and LFA mice, compared to LFS mice. In addition, VEGF-A mRNA expression levels were measured from the whole muscle homogenate (B), from isolated muscle fibres (C) and capillaries (D). The highest VEGF-A mRNA expression levels in the muscle homogenate and in isolated capillaries were detected in HFA mice, compared to LFS mice. The isolated mRNA expression levels of VEGF-A were not determined in DC mice (LFS = low fat sedentary, HFS = high fat sedentary, DCS = diet change sedentary, LFA = low fat active, HFA = high fat active, DCA = diet change active). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

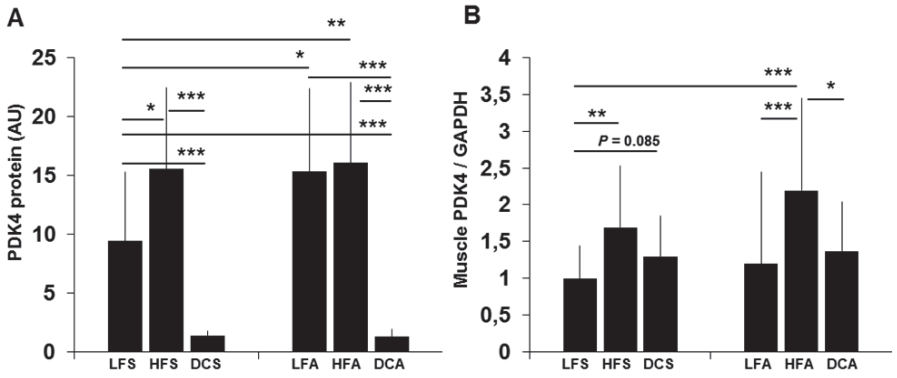

FIGURE 10 The protein (A) and the mRNA (B) expression levels of PDK4 were investigated. PDK4 protein expression (AU = arbitrary units) was highest in HF and LFA mice. Very low PDK4 protein expression was detected in DC mice. However, a similar effect was not seen at the mRNA level. The highest mRNA expression level was in HF mice, compared to LFS mice (LFS = low fat sedentary, HFS = high fat sedentary, DCS = diet change sedentary, LFA = low fat active, HFA = high fat active, DCA = diet change active). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6 DISCUSSION

This study investigated the role and significance of intracellular lipids in the development of insulin resistance in diet-induced obesity. The aim was to investigate the effects of long-term high-fat feeding or diet change intervention with or without voluntary wheel running exercise. The main findings of this study are that insulin resistance resulting from long-term high-fat feeding can be effectively reversed with diet change intervention, but not with concurrent physical activity. In addition, a similar effect was seen in cellular triglyceride concentrations that paralleled with perilipin 5 expression in both muscle and the liver. Under these circumstances, there was an increase in fat oxidation capacity in muscle after HF feeding, with the necessity to also increase the density of the muscular capillary bed.

6.1 Lipid accumulation and insulin resistance

To unravel the mechanisms behind high-fat diet-induced insulin resistance, research has focused on many different types of pathways. An association between excess lipid content and functional insulin resistance does exist, as do the positive effects of physical activity on insulin sensitivity (Goodpaster et al. 2001). In the present study, the diet-induced accumulation of ectopic lipids was investigated by determining muscular and hepatic triglyceride (TG) concentrations. HF mice showed higher TG concentrations in muscle, and especially in the liver, where the difference of the LF and DC groups was very significant. Moreover, HF-fed mice were more insulin resistant than their LF-fed counterparts, and insulin sensitivity after the diet change intervention was improved. Although the accumulation of IMTG *per se* may not induce insulin resistance (for a review, see Bosma et al. 2012a), it has been shown that effective shuttling of fatty acids is required to maintain low cellular concentrations of cytotoxic fatty acids (Bosma et al. 2012b). However, in order to increase fatty acid oxidation, the rate of fatty acid uptake also needs to be enhanced. This increase in fatty acid uptake

leads to triglyceride deposition in muscle and the detrimental effects associated with the development of insulin resistance (Bonen et al. 2004).

Intramyocellular TGs in large LDs are suggested to be more detrimental to insulin sensitivity (He et al. 2004). Smaller LDs have a higher surface-area-to-volume ratio, thus allowing lipolytic enzymes to have a greater disposable surface area for the interaction with regulatory proteins (Bosma et al. 2013). It has been suggested that most of the intrahepatic TGs (~75%) are stored as microvesicles (<1 μm^2) and that lipid droplets stored as macrovesicles (>1 μm^2) are more likely to cause HF diet-induced hepatic steatosis (Gauthier et al. 2003). Electron microscopy studies revealed that in sedentary mice after HF feeding, the intramuscular LDs were larger and higher in number than those of LFS mice, which lends further credence to the observation that HF mice were also severely insulin resistant. Similarly, the likelihood of hepatic steatosis is increased in the liver, while the LDs were larger and higher in number after HF feeding, compared to the respective LF or DC groups.

A recent publication has suggested that physical activity would be more efficient in reducing intrahepatic TG content when animals are fed high-fat diets (Magkos 2012). However, such an effect was not observed in the present study. In fact, the effect of exercise-reducing hepatic TG levels was significantly observed only in LF mice in a biochemically extracted measurement of TG levels. Previously, physical activity with a concurrent loss of body weight (e.g. by means of diet change intervention) has not been observed to mediate exercise-induced changes in intrahepatic TG content (Magkos 2012). In light of the present study results, this would seem to be the contrary; diet change intervention seemed to be more effective in reducing TG concentration and the amount and size of LDs than exercise alone. Thus, there remains controversy about the evidence that exercise reduces liver lipid accumulation induced by a HF diet. Possible reasons for conflicting results could include selection of species, fat content of the diets, duration of the experiment, the exercise regimen used and whether it was conducted concurrently or introduced during the course of a HF diet.

Perilipin 5 in skeletal muscle

It is still essentially unknown whether a change in PLIN density relative to IMTG content is important in regulating lipid storage and its possible subsequent effects on insulin sensitivity (Bosma et al. 2013). Increased muscular TG concentration and small LD size was observed in parallel with up-regulated PLIN5 expression in HFA mice. Previously it was shown that small LD size and up-regulated PLIN5 expression might be important in facilitating IMTG breakdown and mobilizing fatty acids for oxidation and preventing lipotoxic effects, especially during fluctuating energy demands, such as exercise (Shepherd et al. 2013).

Future studies are needed to clarify whether the increase in PLIN5 expression drives the accumulation of IMTGs or whether it is a consequence of the increase in IMTG content. While increased PLIN5 expression augments free fatty acid uptake in parallel with an increase in cellular triglyceride storage, PLIN5 may also improve oxidative capacity and increase the expression of genes involved in fatty acid oxidation (Wolins et al. 2006b). Increased PLIN5 may thus be an adaptive response in skeletal muscle to increase the availability of fatty acid to be used as fuel. For example, in the skeletal muscle of active mice on a HF diet with plenty of stored TGs to be converted back to fatty acids as fuel, the LD turnover promoted by triacylglycerol lipase would be efficient. Indeed, there is evidence that PLIN5 competes with lipases on the LD surface to lower basal lipolytic rates. Lipid turnover is determined not only by triglyceride synthesis, but also by triglyceride lipolysis. It has been suggested that PLIN5 could be an important regulator in lipogenesis as well as in lipolysis. (Bickel et al. 2009.) In highly oxidative tissues, PLIN5 may protect lipid droplet triglyceride from both basal and stimulated lipolysis, consequently increasing triglyceride storage (Dalen et al. 2007).

PLIN5 may be involved not only in triglyceride storage, but also in the oxidative degradation of LD-derived fatty acids, thus suggesting a dual role for PLIN5 in lipid turnover. Indeed, PLIN5 has been suggested to play a role in shuttling fatty acids from lipid droplets to mitochondrial oxidation and maintaining lower cellular concentrations of cytotoxic fatty acids. (Bosma et al. 2012b.) In order to be able to do this, LDs need to be spatially connected to triglyceride mobilization and fatty acid release to mitochondrial uptake and oxidation (Wang et al. 2011b; Bosma et al. 2012b). In the present study, EM observations revealed that not only were the LDs of HF mice larger and greater in number, but they were also in close proximity to the subsarcolemmal mitochondria. This observation, together with the increase in PLIN5 expression, is further corroborated by earlier studies, where PLIN5 recruits mitochondria to the proximity of LDs, facilitating the effective shuttling of fatty acids to mitochondria for oxidation (Bosma et al. 2012b).

Perilipin 5 in the liver

It has been shown that PLIN5 expression is induced in increased fatty acid oxidation states (e.g. fasting and insulin deficiency) in oxidative tissues, including skeletal muscle and the liver (Wolins et al. 2006b; Dalen et al. 2007; Wolins et al. 2006a; Yamaguchi et al. 2006). During lipid loading, PLIN5 has been shown to move to the LD surface in cultured cells (Bickel et al. 2009; Granneman et al. 2009), and corresponding to lipid overload PLIN5 is up-regulated in rat hepatocytes and might represent an early response to excess lipids (Grasselli & Voci 2010). However, whether PLIN5 expression is increased by a high-fat diet in the liver *in vivo* is not yet reported. The current study demonstrates that, similar to what has been found in skeletal muscle, a high-fat diet also induced PLIN5 protein expression in the liver.

PLIN5 seems to promote free fatty acid consumption and is postulated to regulate lipid storage for short-term utilization through oxidative pathways (Wolins et al. 2006b). A recent hypothesis suggests that PLIN5 reduces lipolysis in order to sequester lipotoxic fatty acids and acts as a component in controlling lipid homeostasis in the liver (Brasaemle 2013). When observations were made of cardiac myocytes of PLIN5 null mice (Kuramoto et al. 2012), and similarly when the roles of PLIN5 were studied *in vitro* in hepatocytes, the results indicated an important role for PLIN5 promoting TG accumulation by acting as a negative regulator of lipolysis and fatty acid β-oxidation (Li et al. 2012). The prevention of uncontrolled TG mobilization by PLIN5-coated LDs and by decreasing mitochondrial function as earlier suggested (Pollak et al. 2013; Langhi et al. 2014), as well as the effect of concurrent physical activity with long-term high-fat feeding or diet change, needs to be investigated *in vivo* in the liver. High-fat feeding increased and diet change intervention decreased PLIN5 expression, while physical activity was not observed to have a decreasing effect. These results support the importance of PLIN5 as a key player in overall intracellular lipid accumulation.

6.2 Metabolic flexibility and fuel selection in obesity

Reduced metabolic flexibility is associated with the accumulation of triacylglycerides in skeletal muscle and in the liver, and thus it is one of the early components in the development of insulin resistance (Sparks et al. 2009; van Herpen et al. 2011). In the present study, the HF mice with or without exercise were severely insulin-resistant, as indicated by their increased levels of fasting insulin and glucose, suggesting that they had developed a metabolic condition resembling metabolic syndrome (Han et al. 1997). The plasma free fatty acid concentration was found to be significantly lower in the HF mice at the end of the experiment. The preference for fatty acids as an energy source is reflected in elevated blood glucose levels. Higher fat oxidation and higher glucose oxidation/storage after insulin stimulation during fasting have been associated with the metabolically flexible phenotype (Kelley & Mandarino 2000). Thus, it seems that the skeletal muscle of HF mice was still metabolically flexible. However, this can also be interpreted as an adaptation to a chronic high-fat diet, in order to be able to better extract and oxidize circulating lipids.

While HF-fed mice were insulin-resistant and had more accumulated TGs, the underlying mechanism of the metabolic regulation of lipid storage and the downstream effects of insulin sensitivity remain unknown. One explanation might be metabolic switching and simple substrate competition to increase fatty acid usage, instead of carbohydrates. As the Randle effect suggests, elevated rates of fatty acid oxidation may lead to suppressed glucose utilization in obesity and diabetes (Garland & Randle 1964; Garland et al. 1964).

PDK4 is known to negatively regulate the pyruvate dehydrogenase complex and contributes to lipid-induced changes of glucose metabolism in rodent

and human studies (Sugden 2001; Chokkalingam et al. 2007; Holness et al. 2000). The expression of PDK4 is known to increase in fasting, diabetes and other conditions associated with switching from glucose oxidation to fatty acid oxidation (Wu et al. 2000). In the present study, there was an elevated expression of PDK4 in LF-fed mice with concurrent physical activity. Although a marked increase in PDK4 expression could also be seen after a long-term HF diet, no additional effects with concurrent physical activity in HF-fed mice were observed. It has been shown earlier that fatty acid oxidation in slow-twitch muscle of rats increased after a four-week HF diet, which was attributed to the up-regulation of PDK4 (Holness et al. 2000). An elevation of PDK4 expression was seen in humans, leading to inhibited PDC-controlled carbohydrate oxidation when the consumption of an isocaloric HF diet was maintained for three days and then followed by an exercise session (Constantin-Teodosiu et al. 2012). It is known that during long-term exercise or short-term fasting, the activity of pyruvate dehydrogenase complex is attenuated in conjunction with increased fatty acid usage (Roche & Hiromasa 2007). The present study showed also that the expression of PDK4 was significantly lower after diet change intervention, compared to HF mice. Thus, the effect of up-regulated PDK4 increasing fat oxidation and blunting glucose oxidation in a condition of high fatty acid availability is temporary. The switch back to using glucose when fatty acids are no longer abundantly available suggests that diet change intervention would be more beneficial in conditions of metabolic inflexibility than only adding physical activity. Taken together, these data suggest that both in chronic high-fat diet and in long-term exercise training, the switch of fuel usage from glucose to fatty acids is mediated by the elevated expression of PDK4. Thus, the role of PDK4 as a possible contributor to insulin resistance should be carefully considered as well, since it seems to control the metabolic flexibility in skeletal muscle under some extreme nutrient conditions.

6.3 High-fat diet-induced oxidative capacity

Previous studies have shown that a high-fat diet increases the amount of mitochondrial enzymes (Hancock et al. 2008; Miller et al. 1984; Nemeth et al. 1992; Simi et al. 1991; Garcia-Roves et al. 2007), the biogenesis of mitochondria, and fatty acid oxidative capacity in the skeletal muscle of mice and rats (Hancock et al. 2008; Turner et al. 2007; Garcia-Roves et al. 2007). These results were supported by the present finding that a HF diet increased the levels of the mitochondrial quantity marker protein cytochrome c. It can be suggested that not only in a state of increased energy demand, such as exercise, but also in the case of constantly increased energy supply with high fatty acid availability, the oxidation of fatty acids can be intensified. This paradigm is supported by the present data that shows slightly improved mitochondrial oxidative capacity in response to a long-term HF diet, when compared to LF or DC mice. Oxidative capacity seemed to be similar between the diet change intervention groups and

LF-fed groups. This further demonstrates the temporary effect of HF feeding on increased mitochondrial fat oxidation, which is also seen with the up-regulation of PDK4.

It has been suggested that impaired mitochondrial function in skeletal muscle may play an indirect role in the development of hepatic steatosis (Pessayre & Fromenty 2005). Increased rates of fatty acid oxidation in muscle have been associated with skeletal muscle insulin resistance (Koves et al. 2008). In contrast to muscle, the markers of hepatic mitochondrial content were reduced, indicating that oxidative capacity in the liver decreases after HF feeding, compared to LF and DC mice in the present study. Indeed, it has been suggested that impaired hepatic fatty acid oxidation might be either a primary cause of fatty liver or at least strongly associated with it (Thyfault et al. 2009; Rector et al. 2008). Physical activity did not seem to have an additional effect on the hepatic mitochondrial content of HF-fed mice, since their CytC expression level and citrate synthase activity were as low as those of HF sedentary animals. Interestingly, concurrent physical activity with diet change intervention showed the highest oxidative capacity in the liver. In order to increase mitochondrial content and the fatty acid oxidation potential of mitochondria, concurrent weight loss by diet change intervention after high-fat feeding combined with physical activity would be the most beneficial. Moreover, these results indicate that cellular reactions to ectopic lipid accumulations and molecular pathways differ between the liver and skeletal muscle. Thus, the mechanism for fat-induced insulin resistance in the liver is dissimilar to that of muscle.

It has been previously shown that obesity-induced insulin resistance could be protected by increasing fatty acid flux exclusively in mitochondria for β-oxidation (Orellana-Gavaldà et al. 2011). Because mitochondrial biogenesis normally occurs in response to increased cellular ATP demand, HF-induced mitochondrial biogenesis could be a consequence of insufficient capacity to oxidize fatty acids. In order to match oxygen supply with increased oxygen demand, long-term HF feeding led to an impressive increase in capillary density and the capillary-to-fibre ratio in the muscle, compared to LFS mice in the current study. This increased capillarization was accompanied by an increased level of the angiogenic factor VEGF-A protein in muscle tissue. The lack of an additive effect of voluntary running, combined with a HF diet, on capillary density may be partly explained by the detected muscle hypertrophy in HFA mice. Diet change intervention showed a slight, yet not statistically significant decrease in capillary density, compared to HF mice. Earlier reports have shown that short-term HF feeding combined with endurance training results in higher capillary density and capillary-to-fibre ratio in the muscle of rats. In sedentary rats with a HF diet, no significant difference was found in capillarization. (Roudier et al. 2009.) While it has been reported that high-fat diet-induced insulin resistance may impair oxygen delivery or release (Ellis et al. 2010; Costa et al. 2011), it may be that the increased capillarization after HF feeding is an adaptation to insufficient oxygen transport.

6.4 The effects of physical activity and diet intervention

Physical activity accompanied by a low-fat diet has long been prescribed for treatment in the management of obesity and type 2 diabetes. However, there is no firm conclusion about whether physical activity introduced concurrently with a long-term high-fat diet would be sufficiently beneficial, counteracting and preventing the detrimental metabolic effects of a high-fat diet (e.g. on insulin sensitivity and blood lipid profile). In human studies, a low degree of physical activity *per se* without restriction of energy intake is known to have an effect on body mass (Thomas et al. 2012). In the present study, a lower running distance, as well as a faster decrease of running distances compared to LF-fed mice, may partly explain the less pronounced effect of voluntary running on many of the measured variables of HF-fed mice. The favourable running effects on body mass and glucose balance variables were stronger only at the mid-point measurement after nine weeks, when running distances were still higher than at the end of the study. Although many favourable effects on the metabolic phenotype were observed, similar studies using a long-term experiment have also observed a weak effect of voluntary running to prevent high-fat diet-induced weight gain and insulin resistance (Bradley et al. 2008).

6.5 Strengths and limitations

The total length of the study was 19 weeks. The C57Bl/6J mouse strain is reported to have a longevity of approximately 30 months (Yuan et al. 2014). If the onset of obesity and insulin resistance is considered to come in later adulthood, this study design with this mouse model might not be as optimal as possible in mimicking the clinical situation in humans. However, these results indicate clearly that the mice were insulin-resistant after 19 weeks of high-fat feeding. This is in line with previous studies showing that C57Bl/6J mice given a high-fat diet develop obesity and type 2 diabetes, and these closely resemble features of the human disease (Surwit et al. 1988). This demonstrates that this time period was long enough to induce sufficient metabolic changes to study obesity and the insulin-resistant state. Although it has been reported that even short-term high-fat feeding (e.g. as short as 3 or 28 days) can successfully result in changes in energy metabolism and lipogenesis (de Wilde et al. 2008), the long-term effects of high-fat feeding are less studied, partly because of lengthy and costly design.

To our knowledge, this study is the first to report the combined effects of diet and physical activity on PLIN proteins in skeletal muscle and in the liver, as well as high-fat diet-induced capillarization. According to our results, it seems that diet alone plays a more prominent role on the investigated parameters. On the other hand, it may be that the effects of physical activity have been obscured due to the very high fat content (60%) of the diet used in this study.

One of the limitations of the study is that the quantified PLIN protein results were obtained from a mixed muscle (gastrocnemius) homogenate, whereas the characterization of lipid droplets was from oxidative muscle (soleus). Additionally, a fibre type-specific investigation would have been beneficial, since PLIN proteins are shown to be expressed more or less fibre-type-specifically in skeletal muscle. For example, PLIN5 has been shown to localize more oxidative type I muscle fibres (Minnaard et al. 2009). Naturally, high-fat diet-induced capillarization, as well as the investigations of oxidative capacity and metabolic flexibility, should also be studied separately in different types of muscle fibres, since fast fibres produce energy primarily by using glucose as a substrate.

It is probable that this experimental setup did not allow us to detect some important high-fat diet-induced changes in protein and mRNA levels. The measurements were performed in an adapted state after the intervention had lasted for 19 weeks, at a time when the mice had also fasted and were passive. Because mice are active and eat appreciably more at night, this might have been a better moment to measure many of the variables.

6.6 Future directions

Further studies are needed to confirm exact mechanisms, especially at the acute phase in high-fat exposure. It is also important to determine whether diet-induced adaptation in skeletal muscle occurs also in humans and, if so, whether this adaptation is relevant in the development of insulin resistance. In addition, further research is required to fully understand the causal relations of lipid droplets, lipid droplet coat proteins and muscle metabolism. The effect of physical activity on the amount of PLIN5, when it is pursued at the same time as a long-term high-fat diet or diet change intervention, needs to be further verified, especially regarding the effect of PLIN5 as a negative regulator of lipolysis in the liver. Once the regulation of lipid droplet dynamics in the skeletal muscle of experimental rodent models is understood in more detail, an integral part of overall lipid metabolism will be revealed. This will bring new insight into human lipid metabolism and the management of skeletal muscle insulin resistance as well.

7 MAIN FINDINGS AND CONCLUSIONS

The main results and conclusions of this thesis can be summarized as follows:

1. Long-term high-fat feeding engendered a condition mimicking the metabolic syndrome, since the mice were significantly more insulin-resistant. Physical activity (i.e. voluntary wheel running) seemed to be unable to ameliorate the condition, whereas change to a low-fat diet led to a significant improvement on the markers of insulin resistance. The positive effect of diet change intervention suggests that diet change alone plays a more important role in the prevention of insulin resistance than physical activity.

2. High-fat feeding led to an impressive increase in cellular triglyceride concentration, which was paralleled with increased PLIN5 expression in skeletal muscle and in the liver. Concurrent physical activity did not have any major decreasing effect; however, change to a low-fat diet showed significantly decreased triglyceride concentration and PLIN5 expression. Since the relation between accumulated lipids and the influence of physical activity on insulin sensitivity is not yet fully known, future research should place more emphasis on the proper balance between lipid droplet synthesis, lipolysis and their oxidative metabolism.

3. In muscle, oxidative capacity and capillarity were observed to have increased after high-fat feeding. In light of these results, it seems plausible that insulin resistance in skeletal muscle might be mediated by some other mechanism than decreased qualitative or quantitative properties of mitochondria. The role of PDK4 as a possible contributor to insulin resistance should be carefully considered, since it seems to control metabolic flexibility in skeletal muscle.

4. In contrast to muscle, the markers of oxidative capacity in the liver indicate decreased capacity after high-fat feeding, compared to low-fat feeding. Concurrent physical activity with diet change intervention showed the highest oxidative capacity in the liver. These results indicate that the cellular reactions to ectopic lipid accumulations and molecular pathways differ between these tissues. Thus, the mechanism for fat-induced insulin resistance may differ from that of muscle.

YHTEENVETO (FINNISH SUMMARY)

Tyypin 2 diabetes ja metabolinen oireyhtymä yleistyvät nopeasti Suomessa ja muualla maailmassa. Tämä on kohdistanut tiedemaailman kiinnostuksen tautiin johtavien perimmäisten solutason syiden selvittämiseen. Ylipainoisuuden ja lihavuuden tiedetään edesauttavan metabolisen oireyhtymän ja tyypin 2 diabeteksen kehittymisessä. Rasvaisen ruokavalion lisäksi ylipainoa ja liikalihavuutta on nopeasti lisännyt liian vähäinen fyysinen aktiivisuus maailmanlaajuisesti. Runsas rasvan saanti sekä vähentynyt energian kulutus edistävät ylimääräisen rasvan varastoitumista rasvakudokseen. Ensimmäisenä ylimääräinen rasva alkaa varastoitua viskeraalisesti, sisäelinten ympärille. Ylipainoisilla ja lihavilla tämän varastokapasiteetin loputtua rasvaa varastoidaan myös muihin kudoksiin, kuten insuliinille herkkiin luurankolihaksiin sekä maksaan. Useissa aikaisemmissa tutkimuksissa on todettu selvä yhteys lisääntyneen lihasten solunsisäisen rasvan kertymisen sekä insuliiniresistenssin välillä. Insuliiniresistenssi eli kudosten alentunut insuliiniherkkyys on eräs tärkeimmistä metabolisen oireyhtymän ja tyypin 2 diabeteksen tunnusmerkeistä. Solunsisäisten rasvojen kertymisen tarkkoja vaikutusmekanismeja insuliiniresistentin tilan kehittymiselle ei kuitenkaan vielä tiedetä. Insuliiniresistenssin kehittymisestä onkin tehty monia erilaisia hypoteeseja. Useimmille niistä on kuitenkin yhteistä se, että solunsisäiset rasvakertymät vaikuttavat suoraan tai välillisesti solujen normaalia toimintaa häiritsevästi, jolloin seurauksena on kudoksen heikentynyt vaste insuliinille ja insuliiniresistentti tila.

Fyysisen aktiivisuuden on osoitettu olevan hyvin hyödyllistä elimistölle. Erityisesti sen lihasten rasva-aineenvaihdunnalle suotuisien vaikutuksien ansiosta fyysistä aktiivisuutta ja liikuntaa pidetään yhtenä tärkeimmistä tekijöistä metabolisen oireyhtymän ja tyypin 2 diabeteksen hoidossa. Eräs fyysisen aktiivisuuden ja liikunnan suotuisista seurauksista on lihasten uusien hiusverisuonien eli kapillaarien muodostuminen. Kapillaareilla ja erityisesti kapillaaritiheydellä on tärkeä tehtävä ravinteiden ja hapen kuljettamisessa tehokkaasti paikkoihin, joissa sitä aineenvaihdunnallisesti tarvitaan. Vähentyneen fyysisen aktiivisuuden on arveltu johtavan lihasten mitokondrioiden määrän vähenemiseen ja niiden heikentyneeseen tai virheelliseen toimintaan. Tämän arvellaan johtavan epätasapainoon solujen vapaiden rasvahappojen sisäänotossa ja hapettamisessa, jolloin solujen ja kudosten oksidatiivisen kapasiteetin sanotaan vähentyneen. Tämän taas on arveltu johtavan kudosten solunsisäisen rasvan (edelleen) kertymiseen.

Tämän väitöskirjatyön tavoitteena oli tutkia hiirillä fyysisen aktiivisuuden ja korkearasvaisen ruokavalion sekä ruokavalion vaihtamisen vaikutuksia rasvan solunsisäiseen kertymiseen lihaksissa ja maksassa. Lisäksi tutkittiin pitkäaikaisen rasvasyötön vaikutuksia insuliiniresistenssiin johtavissa tapahtumissa.

Väitöskirjatutkimuksessa käytettiin hiirimallia, jota yleisesti käytetään rasvaisen ruokavalion aiheuttaman insuliiniresistenssin ja metabolisen oireyhtymän mallintamiseen. Tutkimuksessa C57Bl/6J-kannan uroshiiriä (n=90) syötettiin 19 viikon ajan korkearasvaisella ruokavaliolla (60 % energiasta rasvaa). Tutkimusjakson jälkeen havaittiin, että hiirillä oli tyypillisiä metabolisen oireyhtymän tunnusmerkkejä, kuten merkittävä insuliiniresistenssi. Lisäksi tutkimuksessa määritettiin niin kutsuttujen lipididroplettien eli rasvapisaroiden solunsisäistä kertymistä lihaksissa ja maksassa, sekä näiden lipididroplettien ympärillä olevan säätelyproteiinin, perilipiini 5:n määrää. Lihakseen ja maksaan kertyi selvästi enemmän rasvaa korkearasvaisella ruokavaliolla. Lisäksi lipidien aineenvaihdunnalle tärkeän perilipiini 5:n määrä lisääntyi.

Toisin kuin monessa muussa aikaisemmassa tutkimuksessa, lihasten oksidatiivinen kapasiteetti näytti yllättäen olevan parempi korkearasvaisella ruokavaliolla, minkä lisäksi myös kapillaareja havaittiin olevan enemmän. Rasvaiseen ruokavalioon yhdistetyllä fyysisellä aktiivisuudella ei juurikaan ollut merkittävää suotuisaa vaikutusta muuttujiin. Maksan oksidatiivinen kapasiteetti sen sijaan oli rasvaisella ruokavaliolla olleilla laskenut verrattuna ruokavalion vaihtajien ryhmään.

Pyruvaattidehydrogenaasikinaasi-4 (PDK4) on tärkeässä roolissa lihaksen sokeri- ja rasva-aineenvaihdunnan säätelyssä. PDK4-proteiinin määrän havaittiin nousseen rasvaisella ruokavaliolla, mutta laskeneen merkittävästi ruokavaliota vaihtaneiden ryhmässä hyvin alhaiselle tasolle. Tämä havainto osaltaan puhuu ruokavalion vähärasvaiseksi muuttamisen puolesta, ja korostaa sen tärkeyttä jopa enemmän kuin pelkän liikunnan hyötyvaikutuksia. Osaltaan se antaa viitteitä PDK4:n roolista solutason aineenvaihdunnan säätelijänä, ja näin ollen sen merkitystä insuliiniresistenssiin johtavien syiden listalla tulisi korostaa.

Tämä väitöskirja antaa viitteitä siitä, että insuliiniresistenssin kehittymiseen aiemmin sovelletut teoriat eivät välttämättä kaikilta osin pidä paikkaansa ainakaan käyttämämme koeasetelman kaltaisissa tilanteissa. Eläinten vapaaehtoinen juoksupyöräharjoittelu vaikutti useisiin muuttujiin edullisesti käytetyn ruokavalion rasvapitoisuudesta riippumatta. Yllättäen vastaavanlaisia positiivisia vaikutuksia havaittiin jopa pelkällä rasvaisella ruokavaliolla. Suotuisimmat vaikutukset havaittiin kuitenkin ruokavaliota rasvaisesta vähärasvaiseen vaihtaneilla hiirillä. Tehokkaiden hoito- ja ehkäisykeinojen löytäminen edellyttää vielä useiden insuliiniresistenssiin johtavien tapahtumaketjujen selvittämistä. Esimerkiksi solunsisäisen rasvan kertymisen merkitystä insuliiniresistenssin kehittymisessä ei vielä tunneta tarpeeksi hyvin.

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ORIGINAL PAPERS

I

EFFECTS OF HIGH-FAT DIET AND PHYSICAL ACTIVITY ON PYRUVATE DEHYDROGENASE KINASE-4 IN MOUSE SKELETAL MUSCLE

by

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Effects of high-fat diet and physical activity on pyruvate dehydrogenase kinase-4 in mouse skeletal muscle

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Abstract

Background: The expression of PDK4 is elevated by diabetes, fasting and other conditions associated with the switch from the utilization of glucose to fatty acids as an energy source. It is previously shown that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a master regulator of energy metabolism, coactivates in cell lines pyruvate dehydrogenase kinase-4 (PDK4) gene expression via the estrogen-related receptor α (ERRα). We investigated the effects of long-term high-fat diet and physical activity on the expression of PDK4, PGC-1α and ERRα and the amount and function of mitochondria in skeletal muscle.

Methods: Insulin resistance was induced by a high-fat (HF) diet for 19 weeks in C57BL/6 J mice, which were either sedentary or with access to running wheels. The skeletal muscle expression levels of PDK4, PGC-1α and ERRα were measured and the quality and quantity of mitochondrial function was assessed.

Results: The HF mice were more insulin-resistant than the low-fat (LF) -fed mice. Upregulation of PDK4 and ERRα mRNA and protein levels were seen after the HF diet, and when combined with running even more profound effects on the mRNA expression levels were observed. Chronic HF feeding and voluntary running did not have significant effects on PGC-1α mRNA or protein levels. No remarkable difference was found in the amount or function of mitochondria.

Conclusions: Our results support the view that insulin resistance is not mediated by the decreased qualitative or quantitative properties of mitochondria. Instead, the role of PDK4 should be contemplated as a possible contributor to high-fat diet-induced insulin resistance.

Keywords: Skeletal muscle, Mitochondria, Lipids, Glucose, Fuel switching

Background

A multitude of studies have postulated that obesity and the metabolic syndrome caused by sedentary lifestyle and western diet decrease the capacity of skeletal muscles to oxidize the accumulated lipids [1,2]. Previously this has been proposed to occur by decreased mitochondrial content as well as mitochondrial biogenesis and function [3-8] suggesting an association between mitochondrial dysfunction and insulin resistance, the qualitative and quantitative changes in mitochondria being potentially the ultimate cause [9,10]. However, recent studies have convincingly shown that high-fat diet actually increases mitochondrial biogenesis and fatty acid oxidative capacity in skeletal muscle [11-13] and that lipid-induced insulin resistance in the absence of physical activity is strongly associated to incomplete β-oxidation and mitochondrial overload or "mitochondrial stress" [14]. Mitochondrial defects per se, e.g. deficient electron transport chain, do not seem to be the cause of insulin resistance [15].

Although reduced muscle mitochondrial content and function have been proposed to be a consequence of physical inactivity and sedentary lifestyle, exercise efficiently stimulates muscle oxidative capacity and thus corrects the imbalance between fatty acid uptake and oxidation [16-18]. Furthermore, physical activity reduces the reliance on

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carbohydrates, thus increasing the proportion of fatty acids used as an energy source and enhancing muscle fatty acid oxidation, especially during submaximal exercise [19,20].Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a potential main regulator of the metabolic program that has been shown to be acutely activated by exercise training and down-regulated by high-fat feeding and sedentary lifestyle [21]. PGC-1α has known roles in mitochondrial biogenesis and fatty acid oxidation. The ability of PGC-1α to co-activate the orphan nuclear receptor ERRα (estrogen- related receptor) results in the activation of a broad mitochondrial program, including the induction of oxidative phosphorylation and mitochondrial biogenesis [22-25]. It has been demonstrated both in humans [26] and in rodents [27,28] that the expression of PGC-1α is induced by exercise [29,30] after the activation of PGC-1α promoter [28]. Despite the many functions of PGC-1α in overall energy homeostasis, its function as a potential regulator in glucose utilization pathways is not well characterized [31].

The pyruvate dehydrogenase kinases (PDKs) regulate the activity of pyruvate dehydrogenase complex (PDC), which catalyzes the oxidative decarboxylation of pyruvate in the glucose oxidation process. The isoform PDK4 is highly expressed in liver, heart and skeletal muscle and is regulated by exercise. Its expression is elevated with diabetes, fasting and other conditions associated with the switch from the utilization of glucose to fatty acids as an energy source [32,33]. It has been suggested that insulin resistance is associated with dysregulation of the PDC in skeletal muscle and that excess insulin would on the other hand down-regulate the expression of PDK4 [25,34,35]. Interestingly, transcription factor ERRα and transcriptional co- activator PGC-1α both induce PDK4 gene expression independently [31,36]. In addition, it has been shown that PGC-1α is recruited to the PDK4 promoter by ERRα, which stimulates further the expression of PDK4 [31,37,38]. Our primary aim was to study the effects of high-fat diet and physical activity on the expression of PDK4 and aspects of its regulation. We hypothesized that when dietary carbohydrates are replaced by fatty acids as a fuel for oxidation in muscle, the expression of PDK4 is increased, and this elevation is regulated by the PGC-1α/ERRα-pathway. Secondly, we studied the effect of high-fat diet and physical activity on the amount and function of mitochondria in skeletal muscle.

Methods

Animals and diets

Male C57BL/6 J mice (n = 58) were obtained from Taconic (Ejby, Denmark) at the age of 6 weeks and were individually housed in standard conditions (temperature 22°C, humidity 50 ± 10%, light from 8:00 am to 8:00 pm). After one week of adaptation to new environment, the mice were matched for body-weight (20.8 ± 1.4 g) and divided into four groups. The mice received for 19 weeks *ad libitum* either a lard- based purified high-fat diet (61% of energy from fat, 19% protein, 20% carbohydrates 5.16 kcal/g; D12492-Euro) to induce obesity and insulin resistance, or a low-fat diet as a control diet (10% of energy from fat, 19% protein, 71% carbohydrates, 3.78kcal/g; D12450-Euro, Purina Mills TestDiet®, PMI® Nutrition International, Richmond, IN, USA). The nutritional profile of the fat content of the two diets was as follows (high-fat diet/low-fat diet): cholesterol 229/18 ppm, linoleic acid 3.97/1.39%, linolenic acid 0.36/0.19%, arachidonic acid 0.05/0.00%, omega-3 fatty acid 0.36/0.19%, total saturated fatty acids 10.54/1.14%, total monounsaturated fatty acids 10.84/1.30%. The groups of low-fat fed (LF) or high-fat fed (HF) mice were either sedentary (LFsed or HFsed) or physically active (LFexe or HFexe) throughout the experiment. Mice were housed individually in cages and the physically active mice had access to a running wheel, as previously described [39]. The amount of running was monitored via a computerized system across the study period. All mice were weighed and food consumption was monitored at three-week intervals. Feeding efficiency was calculated (weight gained in mg per kilocalories consumed), but no numerical results are presented and only significant differences are mentioned in the results. The protocols were approved by the Animal Care and Use Committee of the University of Jyväskylä.

The running wheels were locked for 12 hours before sacrifice. After 3-hours' fasting the animals were weighed and then sacrificed by cervical dislocation. Blood and serum samples were collected for the triglyceride, cholesterol and free fatty acid measurements. The muscles extensor digitorum longus (EDL), soleus, gastrocnemius and quadriceps femoris (QF) and epididymal fat pads were excised from the animals, weighed and prepared for further analysis. Total RNA isolation was done from the left gastrocnemius. The muscle oxygen consumption measurements were done from the right QF and homogenates for the Western blotting and histological samples were prepared from the left QF. Histological samples were transversally oriented and mounted on OCT compound (Tissue Tek, Sakura Finetek Europe) and snap frozen in isopentane cooled with liquid nitrogen (−160°C). Electron microscopic analyses were done from the soleus muscle. The experiment set up and data collection points are summarized in Figure 1.

Serum analyses

After overnight fasting, a blood sample was collected at intervention weeks 9 and 18 and the blood glucose level was determined (HemoCue, Ängelholm, Sweden). Insulin was analyzed with an Ultra Sensitive Rat Insulin ELISA Kit according to manufacturer's protocol (Crystal

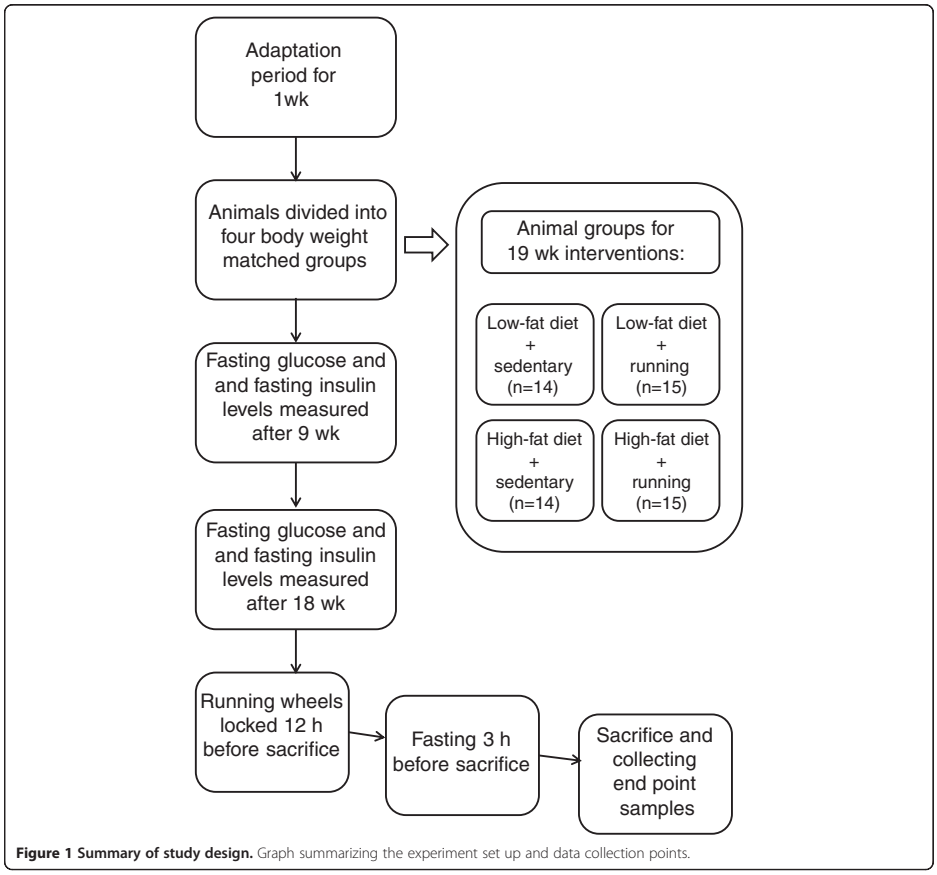

Figure 1 Summary of study design. Graph summarizing the experiment set up and data collection points.

Chem Inc., Downers Grove, IL, USA). Insulin resistance was estimated by multiplying the fasting values of glucose and insulin. Triglycerides, total cholesterol and free fatty acids were measured from the end-point serum samples, of which triglycerides and cholesterol were measured using the VITROS DT60 II Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY, USA). The Wako NEFA C test kit (Wako Chemicals GmbH, Neuss, Germany) scaled down to a microplate format was used to determine free fatty acids (FFA).

RNA extraction and cDNA synthesis

Total RNA was isolated from (approximately 50 mg of) the gastrocnemius with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Muscle samples were homogenized with a FastPrep (Bio101 Systems, USA) tissue homogenizer by using Lysing Matrix D (Q-Biogene, USA). The concentration and purity of RNA were determined photometrically at wavelengths of 260 nm and 280 nm. The integrity of RNA was checked with agarose gel electrophoresis. Five micrograms of total RNA was reverse transcribed to synthesize cDNA (SuperScript III Reverse Transcriptase kit, Invitrogen). For efficient mRNA transcription, a mixture of oligo primers (Oligomer, Helsinki, Finland), consisting of 20 dT residues followed by two additional nucleotides, which anneal only at the 5' end of the poly(A) tail of mRNA, was used.

Real-time quantitative PCR

The mRNA expression levels of ERRα, PCG-1α and PDK4 were determined with the ABI 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The TaqMan primer and probe sets were designed and synthesized by Applied Biosystems. The gene bank accession numbers and Applied Biosystems assay IDs, respectively were: NM_007953.2 and Mm00433142_m1 (ERRα), NM_008904.1 and Mm01208833_m1 (PGC-1α), NM_013743.2 and Mm00443326_m1 (PDK4). The PCR cycle parameters used were: +50°C for 2 min, +95°C for 10 min, 40 cycles at +95°C for 15 s, and +60°C for 1 min. All samples were analyzed in triplicate. The gene expressions were normalized using a Quant-iT™ PicoGreen® assay (Invitrogen) according to manufacturer's instructions. The PicoGreen method was used to quantitate the total amount of RNA-cDNA-hybrids from the solution of reverse- transcribed mRNA products [40].

Western blotting

The QF muscle samples were hand-homogenized in 4% homogenization buffer [10% SDS (w/v), 40 mM DTT, 5 mM EDTA, 0.1 M Tris–HCl pH 8 and protease inhibitors 40 μg/ml aprotinin, 80 μg/ml PMSF and 40 μg/ml leupeptin (Sigma, Saint Louis, USA)]. Western immunoblot analyses from the muscle lysates (samples containing 20 μg of total protein) were done as previously described [41,42]. Briefly, PVDF membranes were incubated overnight at 4°C with rabbit primary antibodies against PGC-1α (1:1000, Calbiochem, Merck KGaA, Darmstadt, Germany), PDK4 and ERRα (1:1200 and 1:3000 respectively, Novus Biologicals, Littleton, CO, USA), and with goat antibody against cytochrome c (CytC, 1:2000 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Membranes were incubated with horseradish peroxidase- conjugated secondary anti-rabbit or anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA) diluted 1:50 000 or 1:70 000, respectively, in TBS-Tween (0.1%) with 2.5% milk for 1 h followed by washing in TBS-T. Preliminary experiments confirmed a proportional linear relationship between protein loaded and, especially, Ponceau S but also α-actin (1:20 000, Sigma) in quantification between 5 and 60 μg, demonstrating the suitability of Ponceau S to be used as a method to control for loading [42]. Proteins were visualized by ECL according to manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, IL, USA) and quantified using ChemiDoc XRS Quantity One software (version 4.6.3. Bio-Rad, UK).

Image analysis of SDH activity

Serial cross-sections (8 μm) from the QF muscle were cut in a cryomicrotome (−25°C). The activity of succinate dehydrogenase (SDH) was used as a marker for muscle fiber oxidative capacity as described by Pette and Tyler [43].

The SDH-stained cross-sections (n = 4-12 animals/group) were captured in full color using light microscopy (Olympus BX-50, Olympus Optical, Tokyo, Japan). Digitally captured images (magnification 20 x) with a minimum of three fields-of-view per muscle cross-section were processed and analyzed using ImageJ software (NIH, Bethesda, MD, USA). The images were converted to 8-bit gray-scale (range of grey levels 0–255) images. An intensity threshold representing minimal intensity values corresponding to SDH activity was set manually and uniformly used for all images (least oxidative gray levels 46–90; most oxidative 140–255). The three intensity scaled fractions representing different oxidative capacities of fibers were expressed as the percentage of the measured area.

Electron microscopic analysis of mitochondrial content

Pieces of soleus (n = 5 animals/group) were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer for 2–2.5 h at +4°C, and post-fixed with 1% osmium tetroxide in the same buffer at +4°C for 1 h. The specimens were stained in uranyl acetate, dehydrated in ethanol and embedded in LX-112 (Ladd). Semithin sections were cut, stained with toluidine blue and examined with light microscope to optimize the transverse orientation. Thereafter, ultrathin sections were cut, mounted on grids and stained with uranyl acetate and lead citrate. Micrographs were taken from the best section of each block with a Jeol JEM-1200 electron microscope at 2500 x primary magnification. It was checked that micrographs were taken from different cells (10–13 cells/section) and that sarcolemmal areas were included. In total 343 micrographs were analyzed using AnalySIS software (Olympus). The amount of subsarcolemmal mitochondria was expressed as mitochondrial area (μm^2) and related to the length of sarcolemma (μm).

Measurements of mitochondrial respiration

The homogenization of QF muscle samples and isolation for the mitochondrial respiration measurements was done mainly according to Wardlaw *et al.* [44] with minor modifications. Briefly, mitochondrial respiration rates (30 μl of freshly prepared mitochondria) were measured at 25°C with a Clark-type oxygen electrode (Hansatech Instruments Ltd, England) in a reaction medium. Respiration rates were recorded in the presence of complex I substrates pyruvate (5 mM) and malate (2.5 mM). State 3 respiration was initiated by adding 150 mM ADP (1.5 mM in buffer). Oxygen consumption was related to the protein content of the suspension determined in triplicates according to manufacturer's instructions (BCA

assay kit, Pierce). Mitochondrial respiration rates in the QF muscle homogenates were measured using the same procedure as the respiration of isolated mitochondria.

Statistical analysis

All data are presented as mean ± SD. A repeated general linear model was used with weight gain, feeding efficiency and weekly running distance as parameters. Two-way ANOVA was used to determine the effect of diet (with 2 levels: low-fat diet and high- fat diet), exercise (with 2 levels: with and without voluntary running), and their interaction with the measured variables, as previously described [39]. Differences between the means of the intervention groups were evaluated, and the significance of differences was determined by Bonferroni post hoc testing. All statistical analyses were carried out using PASW statistics software release 18.0 (IBM Corporation, Armonk, NY, USA). Differences of $P < 0.05$ were considered significant.

Results

Food consumption, body mass and tissue weight

The development of body weight is shown in Figure 2A. After only 1 week of intervention, significantly higher

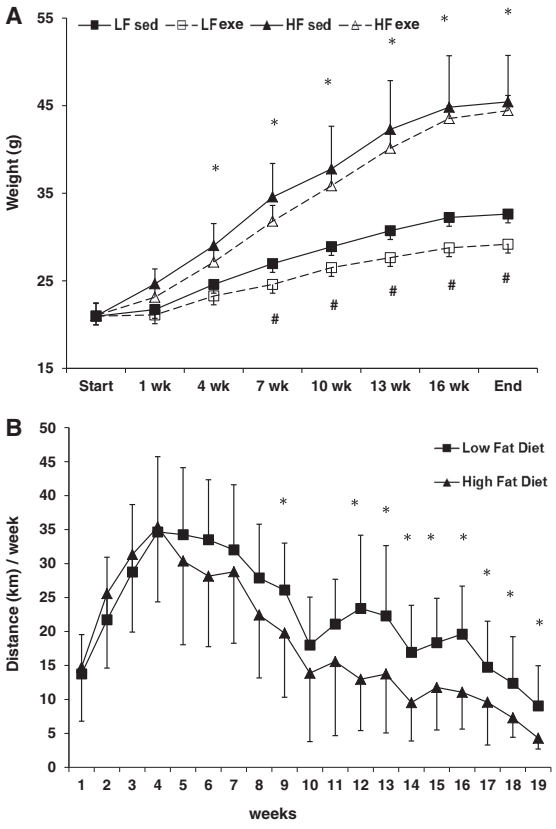

Figure 2 Body weight gain and voluntary running of the mice during the 19-week experiment. (A) After only 1 week, the body weight of low fat (LF) and high fat (HF) mice differed significantly (asterisk). After 7 weeks, the LFsed mice were significantly heavier than the LFexe mice (#). n = 14-15 animals/group. * $P < 0.001$, # $P < 0.05$. **(B)** Voluntary running distances per week on the running wheel during the 19-week intervention. After 9 weeks and after 12 weeks until the end of the experiment, the running distances differed significantly between the two diet groups. n = 14-15 animals/group. * $P < 0.05$ was considered significant. Results are means (±SD).

Table 1 Physiological characteristics

Basic data	Low-fat diet		High-fat diet		ANOVA *P*-value		
	Sedentary (n=14)	Running (n=15)	Sedentary (n=14)	Running (n=15)	Diet	Running	Diet*Running
Weight (g)[#]	32.6 ± 2.86	29.2 ± 1.72**	45.4 ± 5.29***	44.4 ± 3.12***,¤¤¤	<0.001	0.008	0.051
Fat (mg)	799.13 ± 345.1	424.04 ± 65.4*	1767.38 ± 383.2***	1925.06 ± 541.0***,¤¤¤	<0.001	0.275	0.009
Gastrocnemius (mg)	144.98 ± 12.6	141.12 ± 7.7	151.34 ± 8.6	154.81 ± 7.4**,¤¤¤	<0.001	0.936	0.137
Quadriceps femoris (mg)	206.53 ± 10.1	211.35 ± 11.8	220.61 ± 13.0**	228.23 ± 11.0***,¤¤¤	<0.001	0.045	0.646
EDL (mg)	12.96 ± 1.4	12.47 ± 1.1	13.19 ± 1.7	12.83 ± 1.1	0.721	0.500	0.404
Soleus (mg)	10.85 ± 1.6	11.81 ± 1.3	11.14 ± 1.2	13.80 ± 1.6***,§§§,¤¤¤	0.004	<0.001	0.028

[#]Logarithmic transformation for normality and comparison.
Body weight, epididymal fat mass, and the masses of gastrocnemius, quadriceps femoris, EDL and soleus muscles were measured at the end of the 19-week experiment. The muscle masses are average of both limbs. * = vs. LFsed ($P < 0.05$), ** = vs. LFsed ($P < 0.01$), *** = vs. LFsed ($P < 0.001$), §§§ = vs. HFsed ($P<0.001$), ¤¤¤ = vs. LFexe ($P < 0.001$). Results are means (±SD).

body weight was observed in the HF-fed mice compared to LF-fed mice. Thereafter, the body weight of the HF mice increased continuously during the experiment. After seven weeks of intervention a significant difference in body weight between the sedentary and their respective running groups was seen throughout the rest of the intervention. Consistent with their body weight, the HF mice had heavier epididymal fat pads and quadriceps femoris muscles (QF) than the LF mice (Table 1).

Feeding efficiency varied in the different groups throughout the experiment. The feeding efficiency values of the HF mice ranged from 16.75 ± 4.55 mg/kcal to 7.55 ± 4.15 mg/kcal during the three-week monitoring intervals, and were significantly higher than those of the LF mice (9.78 ± 2.25 mg/kcal and 3.85 ± 3.18 mg/kcal, respectively). Running induced a slight decrease in feeding efficiency in the LF mice.

Voluntary running

After four weeks of running, both the LF and HF groups reached their maximum weekly running distance, which then decreased gradually (Figure 2B). Consistent differences in the weekly running distance were observed after 12 weeks, the running distance of LF mice being significantly higher than that of HF mice. However, no statistically significant difference between the groups in total cumulative running distance (LF 422 ± 108 km, HF 339 ± 136 km) was observed.

Blood glucose, insulin and lipid profile

The fasting glucose levels were significantly higher in the HF compared to LF mice. There was also a difference within the group of HF mice, with the runners having higher fasting glucose (Table 2). The HF animals had significantly higher fasting insulin levels compared to LF animals. Estimated insulin resistance indicated that already after 9 weeks on the HF diet the HF mice were more insulin resistant than the LF mice and that a significant positive effect of running was seen in both diet groups (Figure 3). After 18 weeks on the HF diet the HF mice were significantly more insulin resistant than the LF mice. However, no statistical difference between the sedentary and running animals in the HF diet group was observed thereafter, which is concomitant with the decreased running activity seen in Figure 2B.

The high-fat diet had an effect on total cholesterol and on free fatty acids (FFA), the cholesterol levels being higher and, somewhat unexpectedly, the FFA levels lower in the HF groups (Table 2). The HFexe and LFexe groups also differed in total cholesterol, FFA and triglyceride levels.

mRNA expression

The expression level of PDK4 (Figure 4A) in the HF-fed animals, especially when combined with running, was significantly higher than in the LFsed animals. No

Table 2 Blood profiles of the mice after the 19-week experiment

Basic data	Low-fat diet		High-fat diet		ANOVA *P*-value		
	Sedentary (n=14)	Running (n=15)	Sedentary (n=14)	Running (n=15)	Diet	Running	Diet*Running
Total cholesterol (mmol/l)[#]	2.99 ± 0.88	2.70 ± 0.36	4.90 ± 0.54***	4.52 ± 0.49***,¤¤¤	<0.001	0.033	0.795
Triglycerides (mmol/l)	0.97 ± 0.23	1.05 ± 0.21	1.00 ± 0.24	0.90 ± 0.13¤	0.274	0.807	0.089
Free fatty acids (mmol/l)	0.82 ± 0.15	0.92 ± 0.16	0.49 ± 0.13***	0.44 ± 0.12***,¤¤¤	<0.001	0.572	0.108
Fasting glucose (mmol/l)	8.92 ± 1.17	8.45 ± 0.90	9.39 ± 1.12	10.53 ± 0.72***,§§,¤¤¤	<0.001	0.201	0.003
Fasting insulin (ng/ml)[#]	0.43 ± 0.24	0.27 ± 0.16*	2.25 ± 1.11***	2.14 ± 0.82***,¤¤¤	<0.001	0.125	0.112

[#]Logarithmic transformation for normality and comparison.
Fasting blood glucose and insulin were measured after 18 weeks. * = vs. LFsed($P < 0.05$), *** = vs. LFsed ($P < 0.001$), §§ = vs. HFsed ($P < 0.01$), ¤ = vs. LFexe ($P < 0.05$), ¤¤¤ = LFexe ($P < 0.001$). Results are means (±SD).

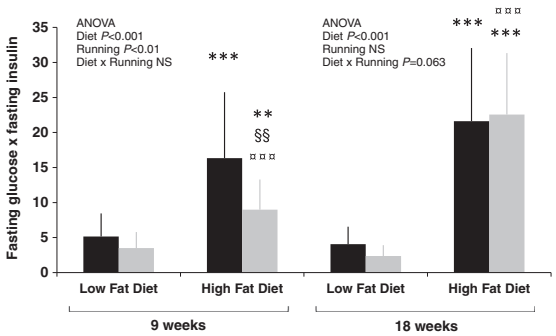

Figure 3 Estimated insulin resistance after 9 and 18 weeks of intervention. The insulin resistance of high fat diet groups differed significantly from the low fat diet groups. Additionally, after 18 weeks on HF diet, running no longer had an ameliorating effect. n = 14–15 animals/group. ** = vs. LFsed ($P < 0.01$), *** = vs. LFsed ($P < 0.001$), ¤¤¤ = vs. LFexe ($P < 0.001$), §§ = vs. HFsed ($P < 0.01$), NS =non-significant ($P > 0.1$). *Black bars* = sedentary, *grey bars* = running.

change in the expression of PGC-1α mRNA levels after HF diet or chronic exercise was observed (Figure 4B). The expression of ERRα (Figure 4C) was significantly up-regulated after HF feeding combined with running than it was in the three other groups (P <0.05-0.01).

Protein expression

Exercise and diet both significantly increased the expression of PDK4 ($P < 0.05$) compared to LFsed mice, but exercise had no additional effect on the HF mice (Figure 5A).

Although the change was most pronounced in PDK4, also PGC-1α (Figure 5B) and ERRα (Figure 5C) proteins showed a similar trend: both running and high-fat feeding increased the expression of each protein, but high-fat feeding combined with running had no additive effect on the protein expressions (no difference between the HF groups). PGC-1α expression showed a slight, although not statistically significant, effect for diet and for running. Exercise increased the expression of ERRα in the LF mice ($P < 0.05$).

Skeletal muscle oxidative capacity

Cytochrome c content measured by Western blotting showed no statistically significant differences between the groups (Figure 6A and 6B), although high-fat diet had nearly significant (P = 0.072) main effect and combination of HF and exercise showed significance compared to LF sedentary group. Oxygen consumption of the isolated mitochondria (Figure 6C) did not differ between the study groups. However, mitochondrial oxygen consumption in muscle homogenate (Figure 6D) was significantly increased in running groups ($P < 0.02$).

The soleus muscle was analyzed by electron microscopy, which showed clusters of mitochondria beneath the sarcolemma, often located near the capillaries and lipid droplets (Figure 7A and B). The area occupied by mitochondria was ~20% larger in the HFexe mice than HFsed mice and ~25% larger than in the LF mice (Figure 7A), although the differences were not statistically significant. The ultrastructure of mitochondria was normal in all groups.

Results from the SDH-staining of QF muscles (Table 3) showed that the HFexe mice had a larger proportion of the most oxidative fiber type area than the HFsed mice (P <0.05).

Discussion

In the present study we observed concomitantly with explicit insulin resistance an up- regulated PDK4 expression along with less prominent ERRα expression in response to the high-fat diet and/or to voluntary exercise. We also found that high-fat diet did not alter the oxidative capacity of isolated mitochondria or oxygen consumption in the muscle homogenate. Voluntary running exercise improved insulin sensitivity during the first 9 weeks of the high-fat diet, but no longer after 18 weeks, concomitantly with decreased running activity. The effects of exercise on the mitochondrial parameters were comparable or greater to those of the high-fat diet, but in most cases exercise and high-fat diet did not have additional/synergistic effects.

In addition to its ability to exert effects on oxidative metabolism in muscle [45], it has been suggested that PGC-1α controls skeletal muscle glucose metabolism by increasing the amount of PDK4 via a PGC-1α/

ERRα-dependent mechanism [31]. This is further supported by the finding that ERRα recruits PGC-1α to the PDK4 promoter [37,38]. Our results show distinct effects of high-fat diet and voluntary running on PDK4 protein expression and, more elaborately, an additive effect of both HF diet and voluntary running on mRNA expression but not on protein expression. Although we did not measure the activity of PDC in our experiment, it is known that PDK4 negatively regulates the PDC, thus inhibiting the entry of pyruvate to the Krebs cycle [46]. In addition, PDK4 has been found to be a contributor to lipid-induced changes of glucose metabolism in rodent and human studies [47-49]. We speculate that increased PDK4 expression after high-fat feeding and exercise is due to previous increase in PGC-1α and ERRα expression, which subsequently blunts cellular glucose oxidation. Our results suggest that in addition to molecular and cellular level *in vitro* [31], PGC- 1α/ERRα-dependent regulation of PDK4 expression also may operate *in vivo* in skeletal muscle.

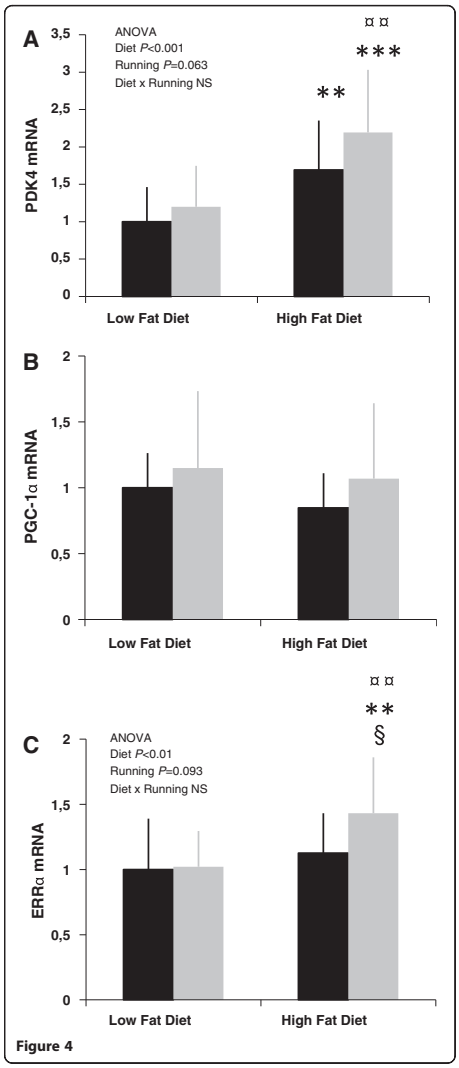

Figure 4

Figure 4 The mRNA expression levels measured with quantitative RT-PCR in gastrocnemius muscle. (A) In the mRNA expression levels of PDK4 there was a statistical effect of diet. The expression levels were significantly higher in HF mice groups compared to LFsed animals and additionally in HFexe group compared to LFexe. PGC-1α **(B)** expression level differences did not reach statistical significance between any of the groups. Also the mRNA expression levels of ERRα **(C)** showed a statistical effect of diet. HFexe mice had significantly higher expression in ERRα compared to other groups. The results are expressed in relation to the LFsed mean value. n = 14–15 animals/group. ** = vs. LFsed ($P < 0.01$), *** = vs. LFsed ($P < 0.001$), § = vs. HFsed ($P < 0.05$), ¤¤ = vs. LFexe ($P < 0.01$), NS = non-significant ($P > 0.1$). *Black bars* = sedentary, *grey bars* = running.

Previous studies have demonstrated that a high-fat diet can increase the biogenesis of mitochondria and fatty acid oxidative capacity in skeletal muscle [12,13]. It can be suggested that not only in the state of increased energy demand, such as exercise, but also in the case of constantly increased energy supply with high fatty acid availability, the oxidation of fatty acids can be intensified. This paradigm is supported by the present data, which shows improved total mitochondrial capacity in response to a high-fat diet for 19 weeks. These results contradict previous findings according to which a high-fat diet decreases the capacity of muscles to oxidize the accumulated lipids [1,2], which would occur owing to the decreased number of mitochondria, as reported in the offspring of type 2 diabetic parents [6].

PGC-1α is considered the master regulator that coordinates the gene expression of oxidative metabolism as well as mitochondrial biogenesis in skeletal muscle [50,51]. In our study the effect of chronic high-fat feeding for 19 weeks had no effect on the expression of PGC-1α. This is in contrast to a previous study that showed decreased PGC-1α expression in muscle after 1 week on a HF diet that persisted down-regulated over 11 weeks [52]. In other studies a high-fat diet for 4–5 weeks has even increased muscle PGC-1α protein expression and the number of

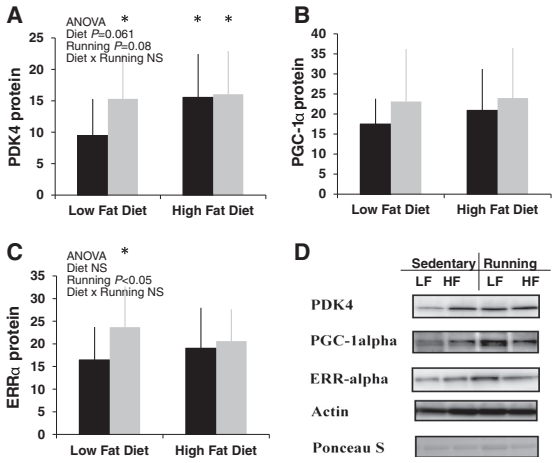

Figure 5 The protein expression levels in QF muscle. PDK4 **(A)** expression showed higher expression levels in all other groups, when compared to LFsed mice. PGC-1α **(B)** showed no statistical differences between the groups. ERRα **(C)** expression was considered to show a statistical effect of running. The protein expression results are normalized to the mean value of LFsed. **(D)** Representative Western blot images. n = 14-15 animals/group. * = vs. LFsed ($P < 0.05$), NS = non-significant ($P > 0.1$). *Black bars* = sedentary, *grey bars* = running.

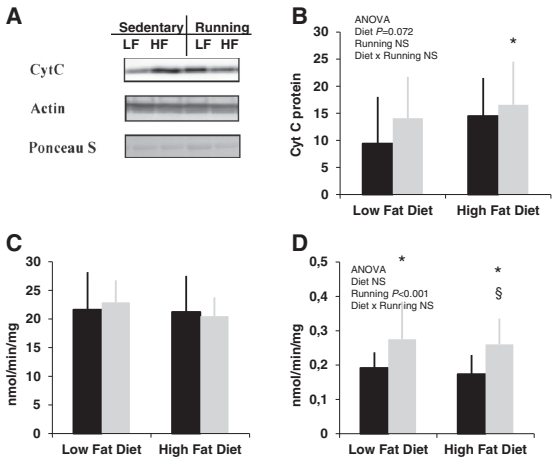

Figure 6 Measures of estimated oxidative capacity in skeletal muscle. Cytochrome *c* (CytC) content in Western blotting **(A)** when normalized to Ponceau S **(B)** showed a trend of HF diet main effect ($P = 0.072$). n = 14–15 animals/group. The oxygen consumption of mitochondria **(C)** in QF muscle did not show any statistical difference between the groups. n = 10–12 animals/group. **(D)** Oxygen consumption in muscle homogenates was higher in the running groups than in the respective sedentary groups. n = 10–12 animals/group. * = vs. LFsed ($P < 0.05$), § = vs. HFsed ($P < 0.05$), NS = non-significant ($P > 0.1$). *Black bars* = sedentary, *grey bars* = running.

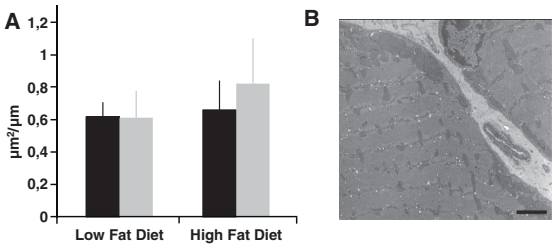

Figure 7 Oxidative capacity estimated by electron microscopy. (A) There were no statistical differences between any of the groups in the area of subsarcolemmal mitochondria relative to the length of the sarcolemma when electron microscopic micrographs were analysed. n = 5 animals/group. *Black bars* = sedentary, *grey bars* = running. **(B)** Electron microscopic image from the subsarcolemmal mitochondria. Scale bar 2 µm.

mitochondria [11,12]. These discrepancies may be partly due to differences in the fatty acid compositions of the diets, since it has been shown that, depending on their chain length and saturation level, fatty acids have greatly varying effects on PGC-1α expression [52]. PGC-1α mRNA and protein expression peak rapidly after a stimulus, such as an exercise bout [14,26,29] or an increase in the concentration of serum fatty acids [53]. After a period of adaptation, no change or only slight changes in PGC-1α mRNA and protein levels have been observed, after 4 weeks of high-fat diet [11] or, as in the present study, after a high-fat diet and/or exercise for 19 weeks. In addition to PGC-1α, ERRα, acting downstream of PGC-1α, is also a critical transcriptional regulator of mitochondrial biogenesis and cellular energy metabolism [24,28,31]. Moreover, ERRα is expressed in tissues demonstrating a high capacity for fatty acid β-oxidation [54,55]. In this study, we found a significant increase in ERRα mRNA expression after a high-fat diet combined with voluntary running, and in protein expression after a low-fat diet combined with running. We believe that the modest changes observed in PGC-1α and ERRα expression are remnants of previous high increases caused by every single exercise bout and/or dietary fatty acids. A limitation to our study is that we measured only the transcript levels of PGC-1α, but not alternative regulatory mechanisms. PGC-1α activity is also regulated by protein modifications, including phosphorylation, acetylation and ubiqitination [56].

High-fat feeding declines general physical activity in rodents [57,58]. Similarly, in this study high-fat feeding induced consistent reduction of wheel-running after 12 weeks of diet, although at the end of the experiment cumulative running distances did not statistically differ between LF and HF mice. Access to running wheels increases general cage activity and affect several components of energy balance (reviewed in Novak et al. [59]) that may have effects to the regulation of muscle metabolism. However, it is not possible to dissect the effects of these factors in this study. In this study the HF mice with or without exercise were severely insulin resistant, as indicated by their increased levels of fasting insulin and glucose, suggesting that they had developed a metabolic condition resembling metabolic syndrome or type 2 diabetes [60]. In our experiment, we found the plasma free fatty acid concentration to be significantly lower in the HF animals compared to LF animals. Conceivably, skeletal muscle had adapted to the chronic high-fat diet to be able to better extract and oxidize circulating lipids. The preference for fatty acids as an energy source is reflected in elevated blood glucose. Our data may suggest that both in chronic high-fat diet and in long-term exercise training, the switch of fuel usage from glucose to fatty acids is mediated by the elevated expression of PDK4. It is known that during long-term exercise or

Table 3 Oxidative capacity estimated by SDH staining

SDH stain intensity	Low-fat diet		High-fat diet	
	Sedentary (n=4)	Running (n=12)	Sedentary (n=8)	Running (n=7)
Least oxidative (%)	32.5 ± 18.0	39.4 ± 15.2	49.8 ± 18.4	30.3 ± 26.2
Intermediate (%)	42.8 ± 12.3	35.8 ± 7.3	33.4 ± 11.8	39.0 ± 15.6
Most oxidative (%)	24.7 ± 8.7	24.8 ± 11.1	16.8 ± 7.8	30.7 ± 16.8§

According to the SDH staining of the QF muscle, HFexe mice yielded the highest measure of SDH activity,i.e. the largest area of the most oxidative fiber types (vs. HFsed $P < 0.05$). § = vs. HFsed ($P < 0.05$).

short-term fasting, the activity of PDC is attenuated in conjunction with increased fatty acid usage [61]. Accordingly, the expression of PDK4 is increased in fasting, diabetes and other conditions associated with switching from glucose oxidation to fatty acid oxidation [62].

What is the mechanism behind high-fat diet-induced insulin resistance? It has been shown that chronic high-fat diet-induced insulin resistance, unlike insulin resistance induced by acute increase in plasma free fatty acids (i.e. Randle glucose fatty acid cycle), is not rapidly reversible [63]. On the basis of our studies, we agree that most probably it is not the decrease in the amount or intrinsic function of mitochondria that leads to increased intramyocellular lipids [12,13]. Our data on insulin resistance and normal mitochondrial function support the idea that lipids themselves or metabolites of lipid metabolism attribute to impaired response to insulin, e.g. via altered cell membrane properties [64] or by affecting IRS phosphorylation and GLUT4 translocation [65]. Our data further suggest that the inhibition of pyruvate dehydrogenase by PDK4 is a possible contributor to insulin resistance. In this scenario high-fat diet-induced insulin resistance may be a consequence of the continuing regulatory process of PGC-1α/ERRα activated by chronic high fatty acid availability. Our data also show that voluntary running exercise improved insulin resistance only transiently during the 19-week high-fat diet, implying that the regulatory power of fatty acids is superior to exercise. On the other hand, the inability of exercise to improve insulin sensitivity after the 19 weeks of wheel running in the experiment might be due to the reduced amount of running during the latter half of the experiment. The role of fatty acids in insulin resistance is a complex process, with some fatty acids inducing and others reversing skeletal muscle insulin resistance [66], suggesting that a balanced fatty acid composition in the diet would be beneficial for optimal muscle cell metabolism and function.

Conclusions

We conclude that a chronic high-fat diet does not have a negative effect on muscle mitochondrial function in spite of severe insulin resistance. This finding suggests that, contrary to frequent allegation, insulin resistance is not mediated by the decreased qualitative or quantitative properties of mitochondria. Instead, our data suggest that the role of PDK4 should be contemplated as a possible contributor to high-fat diet- induced insulin resistance.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RR-T participated in the design and execution of the study and drafted the manuscript, MS participated in the design and execution of the study, ST and HR participated in electron microscopic analyses, JJH participated in protein analysis, ML and RK participated in the execution of the study, HK participated in the design and coordination of the study and helped to draft the manuscript. All authors read, revised and approved the manuscript.

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II

HIGH-FAT FEEDING INDUCES ANGIOGENESIS IN SKELETAL MUSCLE AND ACTIVATES ANGIOGENIC PATHWAYS IN CAPILLARIES

by

Silvennoinen M*, Rinnankoski-Tuikka R*, Vuento M, Hulmi JJ, Torvinen S, Lehti M, Kivelä R, Kainulainen H. 2013

Angiogenesis 16(2):297-307

* These authors contributed equally to the paper.

III

LIPID DROPLET-ASSOCIATED PROTEINS IN HIGH-FAT FED MICE WITH THE EFFECTS OF VOLUNTARY RUNNING AND DIET CHANGE

by

Rinnankoski-Tuikka R, Hulmi JJ, Torvinen S, Silvennoinen M,Lehti M, Kivelä R, Reunanen H, Kujala UM, Kainulainen H. 2014

Metabolism: Clinical and Experimental 63(8):1031-40.

IV

INCREASED HEPATIC LIPID ACCUMULATION AND PERILIPIN 5 IN HIGH-FAT FED MICE WITH THE EFFECTS OF EXERCISE AND DIET CHANGE

by

Rinnankoski-Tuikka R, Hulmi JJ, Reunanen H, Kainulainen H. 2014

(submitted)