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MECHANISMS UNDERLYING MITOCHONDRIAL FUNCTION AND BIOGENESIS;
IMPLICATIONS FOR TYPE 2 DIABETES MELLITUS AND OBESITY

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Joy and woe are woven fine,
A clothing for the soul divine.
Under every grief and pine
Runs a joy with silken twine.

William Blake, *Auguries of Innocence*

To Kotten

ABSTRACT

The ample supply of food, in conjunction with a sedentary lifestyle and certain genetic risk factors contribute to the rise in obesity, insulin resistance and type 2 diabetes mellitus. Reduced mitochondrial capacity for oxidative metabolism has been implicated as one possible cause of insulin resistance in several tissues; such as liver and skeletal muscle. The adipose-derived hormone leptin and the metabolic sensor 5'-AMP-activated protein kinase, are two key regulators that modulate intracellular fuel handling. The aim of this thesis is to investigate the effects of these metabolic signals on tissue-specific mitochondrial respiration and biogenesis.

The aim of study I was to investigate the role of the AMPK γ3 subunit in determining mitochondrial function in glycolytic skeletal muscle. The AMPK signaling axis is a metabolic switch regulated by the intracellular energy charge. A single-nucleotide mutation (R225Q) in the AMPKγ3 subunit causes elevated basal enzyme activity. Transgenic expression in mice (Tg-AMPKγ3^{R225Q}) increased expression of regulators and mediators of substrate oxidation, as well as components of mitochondrial dynamics and electron transport. In summary, this single nucleotide mutation is associated with mitochondrial biogenesis, concomitant with increased expression of transcription factors that regulate mitochondrial proteins.

The focus of study II was to characterize tissue-specific mitochondrial function in permeabilized tissue from lean and leptin receptor-deficient obese *db/db* mice. Respiratory capacity in oxidative soleus muscle was similar between genotypes, except for decreased complex II function in *db/db* mice. Oxidative function in glycolytic EDL muscle was higher in *db/db* mice than in lean littermates; likely as a result of increased mitochondrial biogenesis. Maximal respiratory capacity in liver from *db/db* mice was blunted, concomitant with increased mitochondrial fission. In summary, mitochondrial respiratory performance is controlled by tissue-specific mechanisms and is not uniformly altered in obesity.

The aim of study III was to determine tissue-specific mitochondrial respiration in obese leptin-deficient *ob/ob* mice, and lean littermates, following treatment with leptin or saline. Oxidative capacity in soleus muscle was unaffected in saline- and leptin-treated *ob/ob* mice, whereas maximal electron transport capacity was increased with obesity in EDL muscle. Regulation of transcription and mitochondrial fission in EDL was altered in saline-treated *ob/ob* mice, and only partially normalized with leptin repletion. In liver, maximal respiratory capacity and mediators of lipid oxidation were reduced with in saline- and leptin-treated *ob/ob* mice; while leptin treatment normalized indicators of mitochondrial stress.

In conclusion, mitochondrial respiratory function is a dynamic process that is tightly regulated to meet the energy needs of the cell. Despite profound alterations in whole-body or intracellular energy sensing, mitochondrial adaptation can occur and respiratory adaptations are comparatively modest. This highlights the need to target several pathways of metabolic regulation to modulate mitochondrial function to improve systemic homeostasis.

Key words: mitochondria, mitochondrial biogenesis, mitochondrial dysfunction, respirometry, insulin resistance, type 2 diabetes mellitus, AMPK, leptin.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals.

I. Gain-of-function R225Q mutation in AMP-activated protein kinase gamma3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle. Pablo M. Garcia-Roves, Megan Osler, **Maria H. Holmström**, and Juleen R. Zierath. The Journal of Biological Chemistry. 283(51): 35724-35734, 2008

II. Tissue-specific control of mitochondrial respiration in obesity-related insulin resistance and diabetes. Maria H. Holmström, Eduardo Iglesias-Gutierrez, Juleen R. Zierath, and Pablo M. Garcia-Roves. American Journal of Physiologoy - Endocrinology Metabolism. 302(6): E731-739, 2012

III. Tissue-specific effects of leptin treatment on mitochondrial function in obese leptin-deficient *ob/ob* mice. Maria H. Holmström, Robby Z. Tom, Marie Björnholm, Pablo M. Garcia-Roves, and Juleen R. Zierath. [Manuscript submitted]

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ADP/AMP/ATP	Adenosine 5'-di-, mono- and triphosphate
AICAR	5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide
ALAS	δ-aminolevulinate synthase
AMI	According to the manufacturer's instruction
AMPK	5'-AMP-activated protein kinase
ATP5A1/ATP5B	Mitochondrial F_1-ATP synthase subunit α and β
C I/ C I+II	Mitochondrial respiration supported by complex I and I+II, respectively
CoA	Coenzyme A
COX4	Mitochondrial cytochrome c oxidase subunit 4 isoform 1
CPT1	Carnitine palmitoyltransferase 1
CS	Citrate synthase
DNM1L	Dynamin-1-like protein; dynamin-related protein 1 (DRP1) in study I
EDL	*Extensor digitorum longus* skeletal muscle
ERRα	Estrogen-related receptor α
ETC	Electron transport chain, complex I-IV
ETS	Electron transfer system, complex I-IV and ATP synthase
ETS II	Maximal mitochondrial respiratory electron flux mediated by II
ETS I+II	Maximal mitochondrial respiratory flux mediated by complex I+II
$FAD/FADH_2$	Flavin adenine dinucleotide oxidized/reduced
FCCP/FCCPH	Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone reduced/oxidized
FCR	Flux control ratio, mitochondrial respiration normalized by *ETS I+II*
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLP-1	Glucagon-like peptide 1
GLUT4	Glucose transporter 4
GTP	Guanosine triphosphate
Ig	Immunoglubulin
IRS-1	Insulin receptor substrate-1
LEAK	Mitochondrial respiration by endogenous uncoupling
LonP	Lon protease
LPL	Lipoprotein lipase
MCAD	Medium-chain acyl-CoA dehydrogenase
MFN-1/2	Mitofusin-1 and 2
MT-CO1	Cytochrome *c* oxidase subunit 1; COX1 in study I and II
mtDNA	Mitochondrial DNA
MyHC	Myosin heavy chain
NAD^+/NADH	Nicotinamide adenine dinucleotide oxidized/reduced
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate oxidized/reduced
NDUFA9	NADH dehydrogenase (ubiquinone) 1α subcomplex 9, 39 kDa
NEFA	Non-esterified fatty acids
NRF-1/2	Nuclear respiratory factor 1 and 2
OPA1	Mitochondrial dynamin-like 120 kDa protein
OXPHOS/Coupled	Coupled, ADP-stimulated mitochondrial respiration
PCR	Polymerase chain reaction

PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α/β	Peroxisomal proliferator-activated receptor gamma co-activator 1α and β
PPARα/δ/γ	Peroxisome proliferator-activated receptor α, δ (a.k.a. β in study I) and γ
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase, complex II
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.	Standard error of the mean
SOD2	Manganese superoxide dismutase 2
T2DM	Type 2 diabetes mellitus
TFAM	Mitochondrial transcription factor A
TNF-α	Tumor necrosis factor α
UCP3	Uncoupling protein 3
UQ/UQH	Ubiquinone reduced/oxidized
UQCRC1/2	Ubiquinol cytochrome *c* reductase subunit 1 and 2
ZERO	Residual respiration

1. RATIONALE

The incidence of obesity, insulin resistance and type 2 diabetes mellitus (T2DM) is increasing worldwide, as are the secondary complications associated with these states (1; 2). Lifestyle intervention is important (3), but the development of insulin resistance and β-cell defects often go undetected until the severity is of such magnitude that T2DM develops and pharmacological treatment is unavoidable (4-7). All tissues involved in the regulation of whole-body glucose homeostasis – including hypothalamus (8), liver (9; 10), β-cells (11; 12), skeletal muscle (13; 14) and white adipose tissue (15) – are affected in a progressively deteriorating manner during the pathogenesis of insulin resistance (16). Mitochondrial function may play a causal or supportive role in these pathological processes (17-22).

The aim of this thesis is to study how genetic alterations in energy metabolism influence mitochondrial function. Tissue-specific metabolic signaling and mitochondrial adaptations were studied using different mouse models, in which 5'-adenosine monophosphate-activated protein kinase (AMPK) or leptin signaling was altered.

2. INTRODUCTION

The link between obesity and T2DM was postulated in the late 19th century, along with the differentiation between adult T2DM and childhood type 1 diabetes mellitus (23). The clustering of hypertension, glucose intolerance and aging was suggested in a series of publications around the early 20th century (24). The association between traits of the metabolic syndrome (25) and insulin resistance is now widely accepted (1; 26; 27).

2.1 METABOLIC DEREGULATION

The core of insulin resistance and T2DM pathology is deregulation of whole-body glucose homeostasis. Decreased carbohydrate utilization in skeletal muscle and increased glucose production by liver are two key defects, which are propagated by the progressive inability to maintain appropriate levels of circulating glucose. Insulin resistance and T2DM are linked to obesity through increased lipid availability, in part through lifestyle and in part via insulin resistance in white adipose tissue; resulting in further metabolic derangements.

A central theme implicating mitochondrial function in the etiology of insulin resistance is the concept of metabolic flexibility (28). Metabolic flexibility refers to the ability to switch between oxidation/storage of carbohydrates or lipids in the fed and fasted state. Over the course of disease progression this ability is lost (20; 29) due to the convergence of insulin resistance and regulation of intracellular metabolism. Insulin resistant subjects who are obese and hyperinsulinemic, have normal to elevated glucose uptake, while insulin-stimulated glucose handling is reduced (20). Conversely, fasting lipid oxidation is blunted compared to lean subjects and insulin-stimulation fails to suppress lipid oxidation in favor of glucose metabolism (19; 20). The intracellular link between the aberrations in carbohydrate and lipid metabolism may be the formation of malonyl coenzyme A (CoA), catalyzed by acetyl-CoA carboxylase (ACC). Malonyl CoA, a precursor or fatty acid synthesis that is produced when citric acid cycle intermediates are abundant (30), is a dose-dependent inhibitor of the mitochondrial lipid transporter carnitine palmitoyltransferase 1 (31; 32). Hence, a relative increase in malonyl CoA suppresses fatty acid oxidation despite

increased lipid availability, thus exacerbating insulin resistance in a fashion dependent on mitochondrial oxidation efficiency. This thesis work is focused on mitochondrial oxidative function in liver and skeletal muscle because these organs hold a central place in whole-body metabolic homeostasis in both humans and mice Specific mitochondrial alterations in these tissues have also been implicated as a cause of metabolic dysfunction.

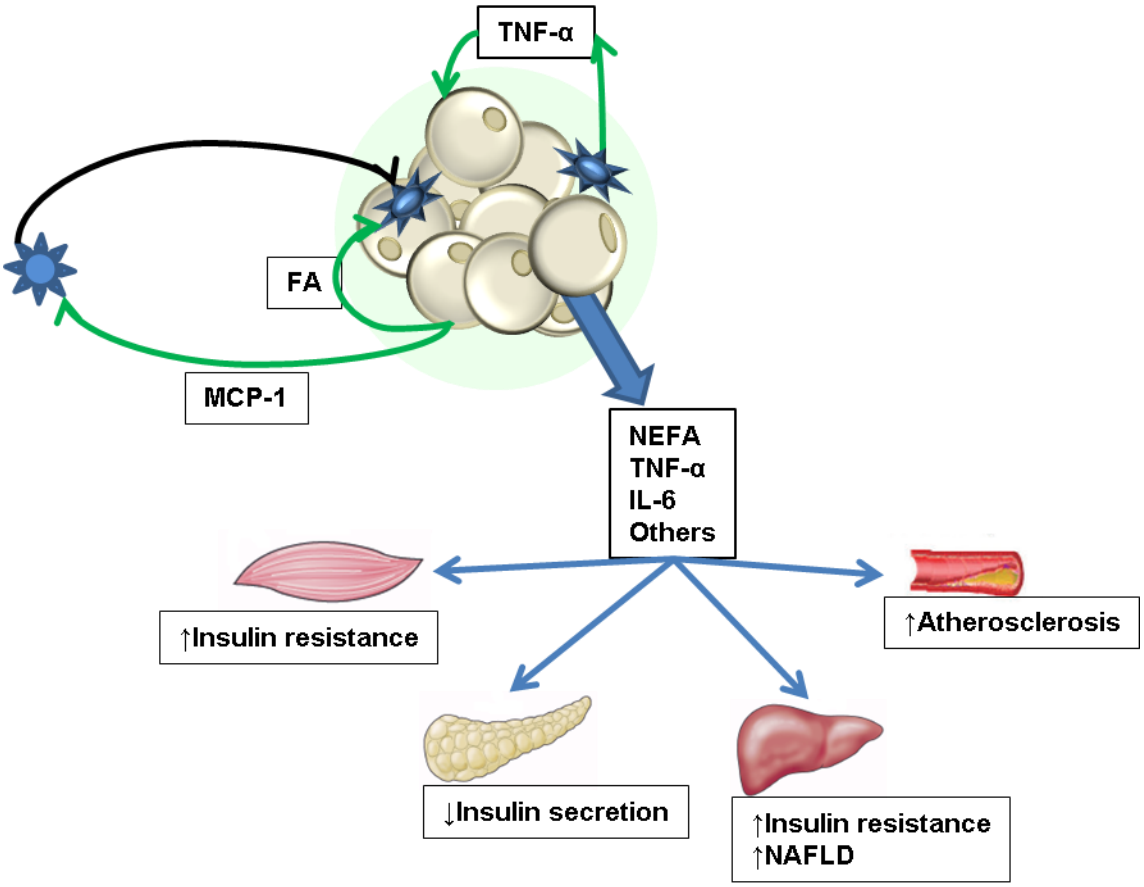

Figure 1. The proposed influence of white adipose tissue on whole-body metabolism in obesity associated with adipocyte hypertrophy. Adipocytes secrete inflammatory mediators that attract and activate stromal immune cells, such as macrophages. The immune cells can then propagate the inflammatory signal. The inflammatory mediators can also have systemic effects, together with enhanced release of non-esterified fatty acids. Adipokine signaling and ectopic lipid accumulation in non-lipid storage organs may cause disruption in insulin signaling, as well as other defects in intracellular signaling. FA – fatty acids, IL-6 – interleukin 6, MCP-1 – monocyte chemotactic protein-1, NAFLD – non-alcoholic fatty liver disease, NEFA – non-esterified fatty acids, TNF-α – tumor necrosis factor α.

2.1.1 Obesity

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. Obesity is associated with severe accretion of adipose tissue mass caused by caloric intake in excess of energy expenditure. Obesity is closely associated with insulin resistance and traits of the so-called metabolic syndrome; including hypertension, dyslipidemia, low-grade systemic inflammation, atherosclerosis and increased risk of cardiovascular disease (1; 33). The correlation is stronger for visceral adiposity, compared to subcutaneous fat depots (34-36), which

may be due to increased secretion of inflammatory markers (37; 38) and leptin, and enhanced lipolysis rate (39). However, the causality of the association between visceral adiposity and increased risk of e.g. cardiovascular disease and insulin resistance is subject of debate (40-43).

White adipose tissue has a nearly unlimited ability to expand in mass and is an important endocrine organ, secreting metabolic and immune cell regulators (44-47). Obesity and adipose cell hypertrophy (Fig. 1) are closely associated with increased plasma levels of tumor necrosis factor α (TNF-α) (48; 49), interleukin 6 (50; 51) and C-reactive protein (52). These inflammatory mediators act as chemoattractants for tissue macrophages, which may propagate the signal (53; 54). Expression of TNF-α and its receptor is upregulated in adipose tissue from obese subjects (55; 56) and circulating TNF-α has a negative systemic effect on insulin sensitivity by indirect inhibition of insulin receptor substrate 1 (IRS-1) (57-59). Localized TNF-α signaling in white adipose tissue reduces lipogenesis in favor of lipolysis (60) and increases the release of nonesterified fatty acids (NEFA), in part mediated by AMPK (61-63). Enhanced lipid mobilization is also associated with insulin resistance in white adipose tissue (10; 64), resulting in increased hepatic production of very low-density lipoproteins (65). In this manner obesity in itself contributes to, and is worsened by, the emergence of insulin resistance.

2.1.2 Insulin Resistance and Type 2 Diabetes Mellitus

Insulin resistance is a state in which insulin has less than the expected effect (66). Several intracellular defects causing reduced insulin response or sensitivity have been described in skeletal muscle; such as reduced IRS-1 phosphorylation and decreased activation of phosphoinositide 3-kinase and Akt (67-69). These impairments are also evident in otherwise healthy first-degree relatives of patients with T2DM (14; 70). Insulin resistance is often accompanied by compensatory hyperinsulinemia associated with increased β-cell mass (71) and function (72). Although the complete mechanism remains unresolved, it is likely stimulated by insulin itself (73), increased nutrient supply (74) and signaling by glucagon-like peptide 1 (75). With time, however, progressive plasma glucose derangement (25; 76-78) is followed by β-cell failure (79) due to functional impairments (11; 80-82) and progressive β-cell apoptosis (79) (Fig. 2).

T2DM is the point at which plasma glucose levels can no longer be controlled by compensatory mechanisms and hyperglycemia ensues (66). Diagnosis is made by repeated fasting plasma glucose measurements or measurement of the response to an oral glucose tolerance test (66; 83). The hyperglycemic state is characterized by pancreatic β-cell failure and a switch from hyperinsulinemia to relative hypoinsulinemia (11; 79-82) (Fig. 2). Altered regulation of lipid metabolism is also an early feature of T2DM pathology (84) and elevates the risk of cardiovascular disease (85), which may partly be explained by diabetes-associated atherosclerosis. In addition to elevated triglycerides and decreased high-density lipoprotein cholesterol in plasma, low-density lipoprotein particles are altered in T2DM (65). They are smaller, denser and more glycosylated in T2DM, which enhances their oxidation level and atherogenic properties (65). Thus, T2DM, as well as insulin resistance, constitutes profound deregulations in both lipid and carbohydrate homeostasis so that the integration of fuel metabolism and organ function is progressively disrupted.

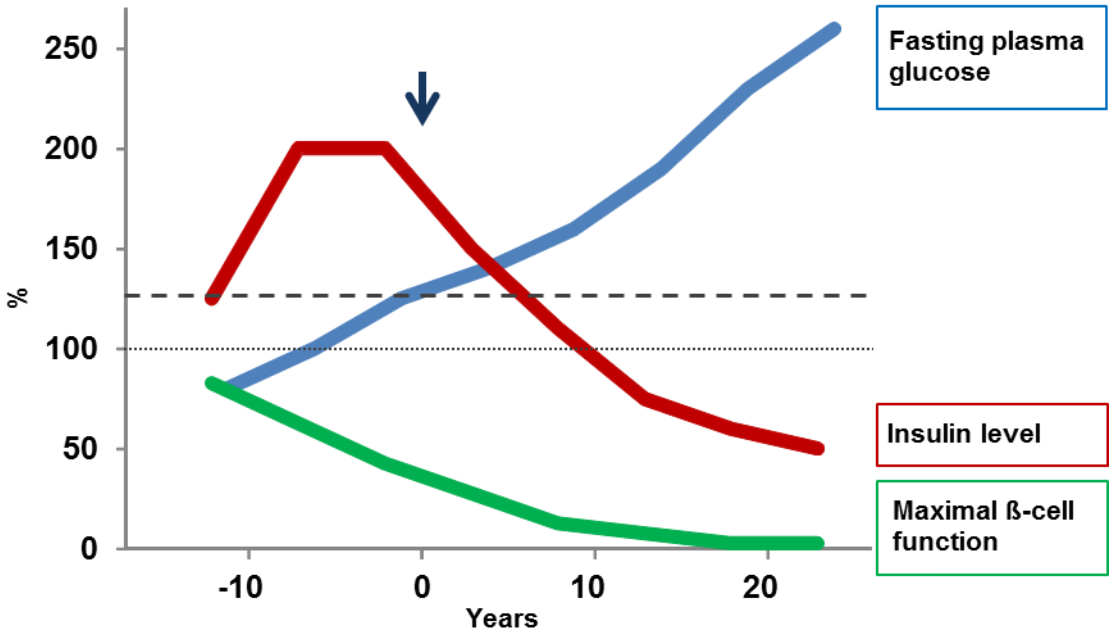

Figure 2. A generalized view of the natural history of the development of insulin resistance and type 2 diabetes. If left untreated, peripheral and central insulin resistance is continuously worsened so that higher concentrations insulin are required to maintain glucose homeostasis. As pancreatic β-cell function and number decline, insulin levels will eventually start to fall, with a concomitant rise in plasma glucose. Blue line – fasting plasma glucose, red line - relative postprandial insulin level (%) compared to normal, green line – relative maximal β-cell function (insulin secretion capacity per cell, %). Bold dashed line – the diagnostic plasma glucose level for diabetes (6.9 mM), thin dotted line – 100% referring to relative insulin level and β-cell function. The arrow marks time of diagnosis of type 2 diabetes mellitus.

2.1.2.1 Liver

Insulin signaling in liver inhibits hepatic glucose output (i.e. gluconeogenesis, ketogenesis and glygenolysis), increases fuel storage (glycogen and triglycerides), and biosynthesis of protein and very low-density lipoproteins (86-89). Hepatic insulin resistance results in increased gluconeogenesis and glucose output (9; 90). Hepatic glucose production is further increased by elevated glucagon (91) and accumulation of lipid and glucose. Indeed, the incidence of non-alcoholic fatty liver disease parallels that of obesity and is now the most common chronic liver disease (92; 93). Ectopic lipid accumulation results in increased expression of phosphoenolpyruvate carboxykinase and pyruvate carboxylase (94), which are rate-limiting enzymes for gluconeogenesis, and glucose-6-phosphatase, the rate-limiting enzyme for glucose release.

Many experimental animal models of obesity are characterized by ectopic hepatic lipid accumulation and are phenotypically similar to human non-alcoholic fatty liver disease. Severe non-alcoholic liver disease in humans is associated with reduced expression of genes regulating and encoding mitochondrial proteins (95; 96), reduced electron transport chain (ETC) enzyme activity (97), decreased resynthesis of adenosine 5'-triphosphate (ATP) (98) and altered mitochondrial structure (99). These aberrations are correlated with increased β-oxidation and elevated production of reactive oxygen species (ROS), as well as hepatocyte apoptosis due to

localized hypoxia (99; 100). In rodent models of obesity, β-oxidation is also enhanced with increased oxidative phosphorylation capacity in some studies (101-105), but not in others (106). The variation is likely due to methodological and strain/species differences, as well as adaptive responses that vary over time (107; 108). Animal models often develop severe obesity and pathology in a relatively short time-span, while human obesity develops in a more variable lifestyle setting against a complex background of genetic predisposition. The progressive tissue damage associated with hepatic lipid accumulation is however undisputed, regardless of study subject, although the specific molecular mechanisms may differ.

2.1.2.2 Skeletal Muscle

Skeletal muscle is central to glucose homeostasis in humans and is key to plasma glucose clearance (109). Obesity is correlated with muscle insulin resistance (110-112). Reduced glucose uptake in skeletal muscle can be caused by several intracellular deficiencies; insulin signaling (14; 67), and glucose transport (113; 114), phosphorylation (115) and oxidation (17; 116) are reduced to varying degrees in association with obesity, insulin resistance and T2DM (117); as well as glycogen synthesis (10; 118) and lipid oxidation (116). Insulin resistance and T2DM are further associated with excessive NEFA uptake, (119-121), dysregulated intramyocellular lipid handling in rodents (122-124), increased levels of intramyocellular lipids in humans (125) and inhibition of insulin signaling mediated by novel Ser/Thr protein kinase C (122; 126; 127). Enhanced secretion of inflammatory mediators from white adipose tissue and macrophages may also reduce muscle insulin sensitivity through the signaling pathway of Janus kinase 2/signal transducer and activator of transcription 3 (128; 129).

The metabolic properties of skeletal muscle and its impact on whole-body metabolism vary with fiber type. Oxidative fibers are more insulin-sensitive (130) and contain more mitochondria than glycolytic, fast-twitch type IIb fibers (131). Oxidative type I fiber percentage is inversely correlated to adiposity (132-134) and waist/thigh circumference ratio (135); and positively correlated to insulin action (135) and high-density lipoprotein cholesterol (136). However, indirect calorimetry during exercise shows either no association between slow-twitch fiber percentage and substrate oxidation (134) or a positive correlation with fat oxidation, depending on the specific protocol (132). Type IIb fiber percentage is increased in first-degree relatives of patients with T2DM (137) and with aging (138). Furthermore, type IIb fiber percentage is positively correlated to insulin resistance (135; 139; 140) and obesity (138) in *vastus lateralis* skeletal muscle, but not in gastrocnemius muscle (139). The additional influence of muscle group on metabolic properties was confirmed in middle-aged T2DM patients (141); comparing mitochondrial respiration and fiber type composition in locomotor *vastus lateralis* and supportive deltoideus muscle, mitochondrial function was negatively correlated with T2DM only in the *vastus lateralis* (141). Hence, the contribution of skeletal muscle and mitochondrial function to insulin resistance may be secondary to disuse, in addition to genetic risk factors.

2.2 LEPTIN AND AMPK SIGNALING

2.2.1 Leptin Regulation of Metabolism

Leptin is the product of the *ob* (obese) gene and is secreted from white adipose tissue (142-145). The leptin hormone regulates several aspects of whole-body metabolism; such as behavior (146),

fertility (147), food intake, metabolism and thermogenesis (Fig. 3) (145; 146; 148-150). Expression and secretion is in part regulated by hexosamine biosynthesis (151), a pathway sensing nutrient availability (152; 153), which is stimulated by both hyperglycemia and hyperlipidemia (151; 154). Plasma leptin levels are proportional to fat depot mass in healthy (155), obese (156) and insulin resistant humans (157). Key insulin-responsive tissues, such as liver (158), skeletal muscle (159) and white adipose tissue (160), as well as insulin-secreting β-cells (161), express the leptin receptor, suggesting a regulatory cross-talk (162; 163).

Mouse models of disrupted leptin signaling, such as the *db/db* (diabetes) (164; 165) and the *ob/ob* mouse (166), as well as humans with similar mutations (167), present severe symptoms of metabolic dysregulation; such as hyperphagia, obesity and reduced fertility. Leptin-mediated regulation of feeding occurs mainly at the level of the hypothalamus (148; 168-172), while thermogenic and metabolic control is exerted through both central and peripheral signaling (149; 170; 173-180). Leptin signaling in skeletal muscle occurs in a biphasic manner with an early, direct activation of the AMPK/ACC pathway; followed by indirect signaling through the hypothalamic/sympathetic signaling axis (174). While studies on the effects of leptin on glucose uptake are contradictory, leptin increases both glucose (173) and lipid oxidation (181), while insulin-stimulated lipogenesis and glycogenesis are inhibited (182) (Fig. 3). However, several aspects of leptin signaling through peripheral pathways, e.g. the direct pathway between leptin signaling and AMPK activation, remain unclear.

2.2.2 5'-AMP-Activated Protein Kinase

AMPK is a key metabolic switch between energy sparing and energy consuming processes (183-186). AMPK was first described as an enzyme regulating 3-hydroxy-3-methylglutaryl-CoA reductase (187), a key enzyme in hepatic cholesterol synthesis, and ACC (188). In addition to inhibiting biosynthesis of cholesterol, fatty acids (186), and glycogen (189); AMPK also enhances glucose (190-192) and lipid uptake (193) in response to contraction (194), glycolysis (195; 196), and fatty acid oxidation (32; 197).

AMPK is a heterotrimeric Ser/Thr kinase that consists of one catalytic subunit (three isoforms; denoted α1-3) and two regulatory subunits (β1-2; and γ1-3, respectively) (198). Diversity in tissue function and response to stimuli is conferred by specific subunit combinations, as well as upstream regulators (199). When the ATP/AMP ratio falls, e.g. as a result of fasting or cellular stress, AMP replaces ATP in the allosteric site on the γ subunit. A conformational change exposes Thr172 on the α subunit to phosphorylation by upstream kinases (200). Acute stimulation with 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR), an AMP analog, enhances the activity of mitochondrial enzymes in glycolytic skeletal muscle and inhibits ACC activity (201). The response in skeletal muscle to chronic changes in energy charge by AICAR treatment appears to be limited to glycolytic muscle and occurs in an AMPKα2-dependent manner (202); overlapping with expression of the γ3 subunit (203).

AMPK mediates changes in metabolism and mitochondrial function both directly, by phosphorylation of metabolic enzymes, and indirectly via gene expression. AMPK mediates contraction-induced translocation of glucose transporter 4 (GLUT4) to the plasma membrane independently of insulin action (204), as well as activation of hexokinase and inactivation of ACC (190). The stimulation of GLUT4 translocation increases glucose uptake, while ACC

phosphorylation inhibits fatty acid synthesis in favor of lipid oxidation (205). AMPK also phosphorylates peroxisomal proliferator-activated receptor γ co-activator 1α (PGC-1α) (206), which activates transcription of several mitochondrial genes in collaboration with nuclear respiratory factor 1 and 2 (NRF-1/2) (207). Thus, specific activation of AMPK signaling is a promising therapeutic target to improve fuel disposal by means of enhancing mitochondrial function, especially in skeletal muscle.

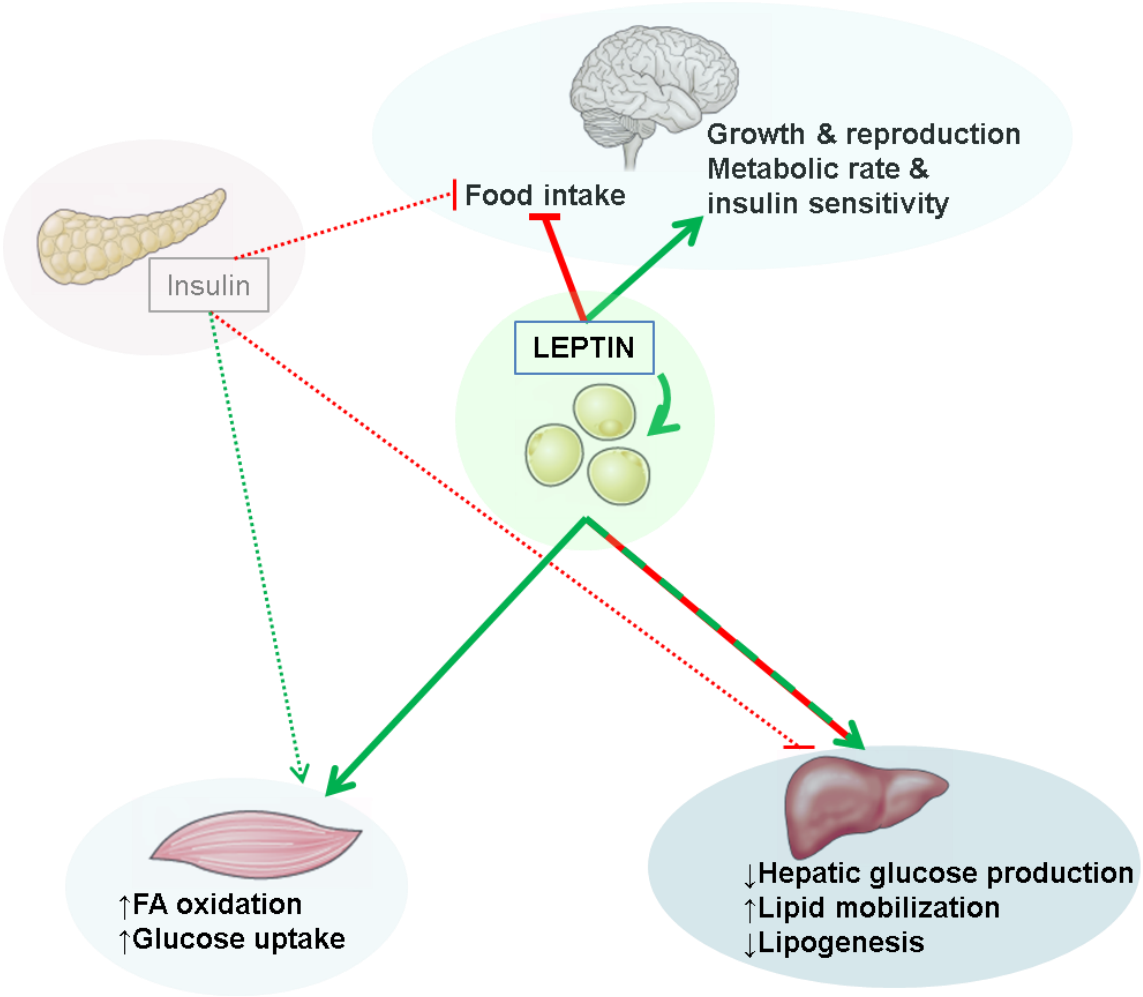

Figure 3. Integration of metabolism by leptin and AMPK, in relation to insulin. Leptin secretion from white adipose tissue provides an important positive signal of energy storage status. Acting on a variety of tissues, leptin participates in whole-body metabolic integration and modulates insulin signaling. The intracellular response to leptin is mediated by AMPK, among others. Hormonal stimulation by leptin enhances peripheral glucose disposal and lipid oxidation (most notably in skeletal muscle), and increases hepatic lipid mobilization. Hypothalamic leptin signaling reduces food intake and is important for reproduction, growth and temperature regulation.

2.2.2.1 The Gamma Subunit Confers Sensitivity to Energy Charge

The AMPK γ3 subunit is the predominant γ isoform in type II, fast-twitch glycolytic skeletal muscle fibers, as part of the α2β2γ3 heterotrimer (208). The γ3 subunit was implicated in skeletal muscle metabolism with the discovery of a spontaneous mutation in pigs. The phenotype, known as Rendement Napole (*RN*$^{-}$), results in increased growth rate and meat content (209), but reduced meat quality and yield (210) because of increased skeletal muscle glycogen content. The mutation was identified as *PRKAG3*^{R200Q} (211), a point mutation in the AMPKγ3 gene, causing an Arg→Gln substitution in the AMP/ATP binding site (212). This mutation results in a conformational change, which renders the heterotrimer insensitive to AMP/ATP regulation (213). A homologous mutation, AMPKγ3^{R225W} with similar effects in skeletal muscle has been identified in humans (214). In contrast, an Ile→Val substitution at a neighboring site in the γ3 subunit (215) causes reduced intramuscular glycogen content. Mutations in the γ2 subunit are linked to human cardiac disease (216); some of which are equivalent to the *RN*$^{-}$ (217) in terms or aberrant glycogen storage. In contrast, a similar mutation in the γ1 isoform, AMPKγ1^{R70Q}, confers increased PGC-1α expression, along with increased in slow-twitch glycolytic fiber percentage (218). Thus, exploring the role of the skeletal muscle-specific γ3 isoform could provide promising targets for pharmacological treatments of insulin resistance without interfering with AMPK activity in other tissues, such as the hypothalamus.

2.3 MITOCHONDRIA AND METABOLISM

Mitochondrial function was implicated in the development of insulin resistance and T2DM after observations of aberrant glucose and lipid oxidation in skeletal muscle (17; 219; 220). Reduced abundance of mitochondrial proteins (18; 221) and decreased mitochondrial respiratory capacity (19; 222) have been reported in skeletal muscle from obese insulin resistant humans or T2DM patients. Similar observations were made in first-degree relatives of T2DM patients, (20; 21), as well as in animal models of disease (101-104; 223). However, unaltered or even improved mitochondrial performance has been reported in skeletal muscle from T2DM patients (224; 225) and rodent models of obesity (108; 226-231). Furthermore, the capacity for exercise-mediated improvements in mitochondrial respiration, protein abundance (232-234), and metabolic adaptation (235; 236) are retained in human skeletal muscle. The disparity between these observations of reduced mitochondrial protein abundance and activity, and preserved mitochondrial function indicate alterations in mitochondrial quantity, as well as quality. Indeed, ATP synthesis (21) and citric acid cycle flux (237) are reduced by 30% in T2DM patients, compared to control subjects, along with a 38% decrease in mitochondrial density (70). Conversely, experimental models of respiratory chain dysfunction in skeletal muscle (caused by progressive reduction of mitochondrial transcription factor A, TFAM, expression), does not affect glucose homeostasis or peripheral insulin sensitivity in mouse (238). Hence, the pathogenic influence of mitochondrial adaptation in these disease states is far from clear and there are multiple factors (genetic or epigenetic predisposition, tissue-specificity, environment and behavior) that must be taken into account.

Increasing mitochondrial respiratory function in skeletal muscle counteracts insulin resistance in both T2DM patients and animal models (239; 240). Indeed, several of the commonly used pharmacological treatments have an indirect effect on mitochondria by stimulating glucose or lipid disposal and oxidation. Fibrates normalize plasma lipid levels by targeting peroxisome

proliferator-activated receptor alpha (PPARα) (241; 242). PPARα, a lipid-induced transcription factor, regulates expression of genes involved in mitochondrial fatty acid oxidation (243) and interacts with the transcriptional co-activator PGC-1α (244). In contrast, the biguanides (e.g. Metformin), lower plasma glucose by reducing hepatic glucose production and improving insulin sensitivity (245; 246). Hence, the further development of treatment strategies that target mitochondrial function is promising.

2.3.1 Mitochondrial DNA and Integration with the Nuclear Genome

Mitochondrial DNA (mtDNA) encodes less than 3% of all mitochondrial proteins (247), and so expression is highly integrated with transcriptional regulation in the nucleus. One mitochondrion normally contains several mtDNA copies packaged into a nucleoid by regulatory proteins, such as TFAM and Lon protease (248-250). However, mtDNA copy number is highly variable (251) and not directly associated with cell proliferation (252-258), mitochondrial oxidative capacity or density (259). For example, heart mitochondria are twice as large and possess higher oxidative capacity than liver mitochondria, but have lower amounts of mtDNA; while brain mitochondria are smaller and contain fewer mtDNA copies compared to both liver and heart (251).

The vast majority of all mitochondrial proteins are nuclear-encoded, including complex II and most of the constituents of complex I, III, IV and ATP synthase (247; 252; 260-262). Integration of cellular demand for mitochondrial activity and nuclear expression is primarily regulated on the transcriptional level (263) through gene-specific transcription factors and co-activators. Activation of PGC-1α (264) or PGC-1β (265) by reduced energy availability (266-269), adrenergic stimulation (264) or stimulation of gluconeogenesis (270), results in coordinated expression of specific gene sets, depending on the transcription factor partners. PGC-1α also participates in a positive transcriptional autoregulatory loop stimulated by sirtuin 1 (a sensor of nicotinamide adenine dinucleotide, NAD^+/NADH, energy charge), and under the control of the transcription factors myocyte-specific enhancer factor 2 and myoblast determination protein 1 (271). The PPAR family of transcription factors regulate lipid oxidation and membrane uncoupling (243; 244), while NRF-1 and NRF-2 induce genes mediating mitochondrial protein import and oxidative phosphorylation (207; 272). NRF-1/2 target sequences have been found in promoter regions of several mitochondrial enzymes and regulatory proteins; such as TFAM (273), cytochrome *c* (274; 275), δ-aminolevulinate synthase (ALAS) (276), and subunits of all of the electron transport system (ETS) complexes (277-282).

2.3.2 Mitochondrial Structure and Dynamics

Mitochondrial dynamics refers to intracellular distribution and fusion/fission of organelles. The importance of appropriate regulation is underscored by observations that many pathologies of mitochondrial dysfunction are also associated with abnormal mitochondrial shape and distribution, such as non-alcoholic fatty liver disease (283) and neurodegenerative conditions (284; 285).

The balance of membrane fusion and fission events is regulated by guanosine triphosphate hydrolases (GTPases) that are located in the mitochondrial membranes. There they ensure appropriate membrane function, distribution of matrix and membrane protein organization (286). Correct expression of mediators of mitochondrial dynamics is crucial to cell survival. Mitofusin

1 and 2 (MFN-1/2) are required for outer membrane fusion (223), and mitochondrial dynamin-like 120 kDa protein (OPA1) mediates fusion of the inner membranes (287; 288). MFN-2 deficiency causes human neurodegenerative disease (284) characterized by reduced respiratory function and mitochondrial membrane potential (289), and failure to organize nucleoids between organelles (290). Specific OPA1 mutations cause the most common form of hereditary optic nerve atrophy (285; 291), and deletion causes mitochondrial fragmentation and apoptosis (292). Mitochondrial fission is mainly dependent on the activity of dynamin-1-like protein (DNM1L) (293). DNM1L is required for both cell survival and regulated apoptosis in development (294). $DNM1L^{-/-}$ is embryonic lethal (295); not due to reduced energy production, but failed distribution of mitochondria into daughter cells (294). Interfering with fission events can delay, or even halt, apoptosis; e.g. by inhibiting DNM1L activity (296; 297) or overexpressing MFN-1 or MFN-2 (298). Downregulation of OPA1 protein abundance, or increased OPA1 proteolysis, however, results in enhanced apoptosis (292).

Appropriate mitochondrial structure and function are disrupted in hepatocytes (299), β-cells (81; 300; 301) and skeletal muscle (19; 302; 303) from humans and rodents with obesity, insulin resistance or T2DM. Thus, delineating mitochondrial structural regulation may be crucial to understanding how mitochondrial function influences tissue-specific adaptations to insulin resistance.

2.3.3 Reactive Oxygen Species and Antioxidant Defense

Mitochondrial ROS is generated when oxygen interacts with transient free radicals formed during electron transfer through complex I and III (304; 305). Excessive ROS production induces deleterious vascular effects associated with hyperglycemia (306). Intracellular ROS formation can cause damage in a number of ways: DNA damage, modifications of amino acids, chemical transformation of membrane lipids, or oxidation of lipids and proteins. However, a number of signaling processes are also associated with transient increases in ROS production; such as growth factor signaling (307) and exercise (308). Insulin signaling generates small ROS bursts (309), which serve as second messengers (310; 311) by oxidizing critical residues on target enzymes (312-314).

Antioxidant defense is performed by enzymes and compounds that are themselves oxidants, but less reactive (315; 316). Mitochondrial manganese superoxide dismutase 2 (SOD2) (317), glutathione peroxidase (318) and catalase (319) are key, antioxidant enzymes, which sequentially disarm the oxygen anion radical by the formation of water and molecular oxygen, via hydrogen peroxide. Mitochondrial DNA oxidative damage is implicated in several pathological conditions (320-323) and ageing (324) and mtDNA is particularly susceptible to oxidative damage by ROS (325). Consequently, mutations caused by oxidative damage are proposed to influence development of complications of insulin resistance and T2DM (326-329).

2.3.4 Oxidative Phosphorylation and Mitochondrial Respirometry

Mitochondrial respirometry is a method of directly measuring mitochondrial oxygen consumption, by supplying mitochondria with citric acid cycle substrates. Using permeabilized tissue, where the cell membrane is disrupted and soluble cytosolic components are lost, electron transport and oxidative phosphorylation can be studied independently of cytosolic metabolism.

Oxygen Flux Mediator	Symbol	State*	Substrate/ Inhibitor	Comment
Proton leak	*LEAK*	2/4	M & P (+)	Very low, NADH-dependent respiration due to absence of ADP.
Complex I, coupled	*Coupled, OXPHOS*	3	ADP (+)	Low respiration due to partial feedback inhibition of the citric acid cycle by high malate concentration. Can be used to calculate P/O ratio
Complex I, coupled	*C I*	3	G (+)	High respiration due to saturating amount of complex I substrate and ADP.
Complex I+II, coupled	*C I+II*	3	S (+)	Continued high respiration due to saturating amounts of ATP synthase, complex I and complex II substrates.
Electron transport system, complex I+II, uncoupled	*ETS I+II*	3u	FCCP (u)	High, uncoupled respiration (complex I-IV) independent of ATP synthase activity.
Electron transport system, complex II, uncoupled	*ETS II*	3u	Rot (-)	High uncoupled respiration (complex II-IV) independent of ATP synthase activity.
Residual respiration	*ZERO***	-	AnA (-)	Oxygen consumption in the absence of mitochondrial electron transfer.

Table 1. Comparison of oxygen flux and respiratory state. (+) = substrate, (-) = inhibitor, (u) = membrane uncoupler. ADP = ADP+ Mg^{2+}, AnA = Antimycin A, G = glutamate, M = malate, P = pyruvate, Rot = rotenone, S = succinate, *The conventional protocol (330) of respiratory states: State 1 – mitochondria alone, 2 – substrate, no ADP, 3 – limited amount of ADP, 4 – ADP depletion, 5 – anoxia. **Residual respiration is not presented in figures.

2.3.4.1 Oxidative Phosphorylation

The first step in respiration supported by NADH is the transfer of 2 electrons (e^-) from one NADH to ubiquinone (UQ) by ETC complex I, NADH-ubiquinone oxidoreductase. Simultaneously, 4 protons (H^+) are relocated to the intermembrane space (331-334). Electron transport supported by mitochondrial matrix-derived flavin adenine dinucleotide ($FADH_2$) starts at complex II, succinate dehydrogenase. Succinate dehydrogenase is part of the citric acid cycle and catalyzes the conversion of succinate to fumarate. In so doing, it transfers $2e^-$ to UQ without any charge movement across the membrane (335-338). Complex III, ubiquinol cytochrome *c* reductase, is a funnel through which complex I, II and other mitochondrial electron transferring complexes feed electrons to complex IV. It catalyzes the UQ/UQH_2 cycle, which returns one UQ and one UQH_2 to the quinone pool. During this process $2e^-$ are transferred to cytochrome *c*, and $2H^+$ per UQH_2 are moved to the intermembrane space (339). The final step of the ETC, mediated by cytochrome *c* and complex IV, cytochrome *c* oxidase, results in the transfer of $2e^-$ from cytochrome *c* by complex IV, reducing one half O_2 to H_2O and moving $2H^+$ into the intermembrane space. Complex IV function is crucial to the functionality of the entire electron transport chain as it catalyzes the reduction of the final electron acceptor (340-344). The proton-motive force generated by the ETC is then used to drive ATP synthesis by ATP synthase (345-351).

2.3.4.2 Mitochondrial Respirometry

The respirometry experiments included in this thesis employ the substrate-uncoupler-inhibitor protocol as outlined in Table 1. This protocol is designed to investigate endogenous uncoupling,

oxidative phosphorylation capacity, and maximal coupled and uncoupled respiratory capacity, with particular focus on complex I. The first substrate, malate, cannot support respiration since there is no acetyl CoA present for the synthesis of citrate (352). Subsequent addition of pyruvate results in three important reactions to consider: the conversion of pyruvate to acetyl CoA in the mitochondrial matrix, allowing citrate production; and the fates of malate and the α-ketoglutarate formed by the citric acid cycle. Malate reaches equilibrium with fumarate, which blocks oxidation of succinate to fumarate through feedback inhibition of succinate dehydrogenase (353). Citrate and α-ketoglutarate are exchanged for extra-mitochondrial malate by the tricarboxylate carrier (354); reducing the amount of substrates which would otherwise feed complex I. Addition of excess adenosine 5'-diphosphate (ADP) enables oxidative phosphorylation and oxygen consumption to increase. NADH production is still suboptimal because of the loss of citrate and α-ketoglutarate. ADP is followed by addition of cytochrome *c* as a test of inner mitochondrial membrane integrity (355; 356). Next, glutamate is added and readily converted to α-ketoglutarate and ammonia (357). However, fumarate-mediated feedback inhibition of succinate dehydrogenase persists; along with active exchange of glutamate (in) for aspartate (out) and malate (in) for inorganic phosphate (P_i) and α-ketoglutarate (out) by the malate-aspartate shuttle (358). Respiration rises as the conversion of α-ketoglutarate allows for another NADH-producing step (359). Subsequent addition of high concentrations of succinate will meet the levels of the other citric acid cycle intermediates and succinate dehydrogenase inhibition by fumarate is relieved (360). This protocol, combined with the appropriate tissue preparation and permeabilization, enables analysis of tissue-specific mitochondrial respiratory capacity in a variety of tissues.

3. AIMS

3.1 GENERAL AIM

The overall aim of this thesis was to investigate pathways that regulate mitochondrial biogenesis and to identify points of regulation of mitochondrial function, with an emphasis on ameliorating insulin resistance.

3.2 SPECIFIC AIMS

Study I: To determine if the γ3 subunit of the AMPK enzyme has a role in signaling pathways mediating mitochondrial biogenesis in skeletal muscle and if it is necessary for mitochondrial function.

Study II: To characterize tissue-specific mitochondrial function in leptin-receptor deficient *db/db* mice and lean littermates.

Study III: To characterize tissue-specific mitochondrial function in leptin-deficient *ob/ob* mice and lean littermates, and to determine if mitochondrial function can be modulated by exogenous leptin-repletion.

4. MATERIAL AND METHODS

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

4.1 GENETICALLY MODIFIED MICE

All animal experiments were approved by the regional animal ethical committee, Stockholm North, and mice were treated in accordance with regulations for protection of laboratory animals. Mice were housed in a temperature-, humidity- and light-controlled environment. They were maintained under a 12:12 h light:dark cycle and had free access to nesting material, water and standard rodent chow (4% fat, 16.5% protein, 58% carbohydrates, 3.0 kcal/g; Lantmännen, Stockholm, Sweden). Prior to tissue dissection, mice were anesthetized with an intraperitoneal injection of 2.5% (v/v) Avertin (0.02 ml/g body weight; 3.5 M 2,2,2-tribromoethanol in tertiary amyl alcohol). In study I, transgenic (Tg-AMPKγ3^{WT} and Tg-AMPKγ3^{R225Q}) or knock-out (AMPKγ3$^{-/-}$) homozygous mice were compared with homozygous wild-type littermates. In study II, homozygous *db/db* mice were compared with non-homozygous (lean, +/?) littermates. In study III, saline-treated *ob/ob* mice were compared with saline-treated non-homozygous (lean, +/?) littermates; and leptin-treated mice were compared with saline-treated mice of the same genotype.

All mouse models used in the described studies were bred into a C57Bl/6J background, making comparisons easier, albeit not without problems. The importance of genetic background is especially clear in the *ob/ob* and *db/db* mouse strains, which are commonly maintained on a 6J or Ks/J background. These "background genomes" have a profound effect on the homozygous mutant phenotype in both strains, which complicates data comparison between studies using strains from different genetic backgrounds. Both *db/db* and *ob/ob* mice bred into the Ks/J background have a more severe diabetic phenotype resulting in significantly shortened life span. Compared to 6J-bred mice, pancreatic islet function and atrophy, hyperglycemia and hyperinsulinemia are worse, and body weight and tissue accumulation of lipids is increased in C57BK/KsJ-*ob* mice (168; 361). When maintained on a 6J background, however, these characteristics are similar in the two genotypes (361). The phenotype of lean mice (heterozygous and wild-type, +/?) remains similar irrespective of breeding background (362). The precise source(s) of the differences due to genetic background is unknown, but the KsJ genome diverges from its 6J origin by almost 30% (363).

Circulating leptin levels in the lean, wildtype mouse is 3-12.5 ng/ml (364-366) and is increased up to tenfold in mouse and rat models of obesity (364). Leptin levels in lean humans lie in the range of 1-12 ng/ml and is lower in men, due to lower degree of adiposity (367). Obese humans typically have circulating leptin levels of 20-35 ng/ml (367; 368). A dose-response study performed in lean and *ob/ob* mice using peritoneal minipumps showed that doses of 1-42 µg/day correspond to 1.6-8.0 ng/ml in plasma (369). In the present study the dose was 1 mg/kg, which equals 30 µg/individual/day, but the leptin was administered as a bolus dose once per day. After a subcutaneous injection in humans, the half-life of the protein was estimated to 3 hours (370), while an intravenous injection in rat resulted in a plasma half-life of 21 minutes (369). However, the effects of hormonal signaling is not only dependent on the physical presence of the hormone, but are mediated by downstream effects on post-translational modification and gene expression.

4.1.1 AMPKγ3 Transgenic and Knock-Out Mice

Study I aimed to investigate the effect of the AMPKγ3 subunit on mitochondrial function and biogenesis in glycolytic skeletal muscle. For this purpose three different strains of mice were

used; AMPKγ3 wild-type transgenic (Tg-AMPKγ3^{WT}), AMPKγ3^{R225Q} transgenic (Tg-AMPKγ3^{R225Q}) and AMPKγ3 knock-out (AMPKγ3$^{-/-}$). The two transgenic strains express the transgene specifically in glycolytic skeletal muscle, under the control of the myosin light chain promoter. The AMPKγ3$^{-/-}$ strain is a whole-body knock-out model, but the AMPKγ3 isoform is specific to skeletal muscle and the predominant isoform in glycolytic fibers (213). All experiments were carried out on the white, glycolytic portion of the gastrocnemius skeletal muscle, unless state otherwise. All three strains were generated by using conventional gene targeting techniques (213).

The effects of these genetic modifications on whole-body metabolism and skeletal muscle fuel handling are described elsewhere (213). Briefly, AMPKγ3 is absent in the AMPKγ3$^{-/-}$ mice, while the transgene is expressed to varying degrees in skeletal muscle from the Tg-AMPKγ3^{WT} and Tg-AMPKγ3^{225Q} mice. Transgenic expression of AMPKγ3^{R225Q} is dominant over the endogenous AMPKγ3 and does not alter the expression of the other subunits (213; 371). AMPK basal and AICAR-stimulated activity was elevated in glycolytic skeletal muscle from Tg-AMPKγ3^{225Q} mice, and glycogen content increased. Following acute exercise, glycogen was depleted to a similar degree in all strains, but glycogen repletion was higher in Tg-AMPKγ3^{225Q} and lower in AMPKγ3$^{-/-}$, compared to controls. After high-fat diet, Tg-AMPKγ3^{225Q} mice had enhanced skeletal muscle fatty acid oxidation, reduced intramuscular lipid accumulation and were protected from insulin resistance (213).

4.1.2 Mouse Models of Obesity - The *db/db* and *ob/ob* Mouse

In study II, obese diabetic *db/db* mice and their lean littermates (+/?) were studied at 16-19 weeks of age. The *db/db* mouse is a model of obesity and T2DM and it expresses a nonfunctional splice variant of the long isoform of the leptin receptor; *Lepr^{db}* (372). This renders cells insensitive to leptin signaling. Mice homozygous for the mutation are hyperphagic and markedly heavier than control mice at a very young age. Furthermore, *db/db* mice present mild hyperglycemia, hyperinsulinemia, infertility and cold sensitivity (373; 374) due to inefficient brown adipose tissue response to noradrenaline (375).

In study III, obese *ob/ob* mice and lean littermates (+/?) were studied at 14-20 weeks of age. The *ob/ob* mouse has a point mutation in the *ob* gene, *Lep^{ob}*, which results in a premature stop codon and complete lack of the leptin hormone (142). Similar to the *db/db* mouse, the homozygous *ob/ob* mice are hyperphagic, obese, subfertile and incapable of cold adaptation (361; 374; 376). This is due to abnormal brown adipose tissue mitochondria. The *ob/ob* mouse also has reduced brown adipose tissue response to adrenergic signals (377), hypothyroidism (378) and reduced bone mass (379).

4.2 LEPTIN TREATMENT

In study III, *ob/ob* mice and lean (+/?) littermates were used to elucidate the effect of leptin repletion on mitochondrial function. The mice were matched for age and weight (within genotypes), and randomized to receive once-daily intraperitoneal injections with either sterile 0.9% saline or 1 mg/kg body weight of sterile leptin (Preprotech, Rocky Hill, NJ). Recombinant leptin was reconstituted according to the manufacturer's instructions (AMI) in sterile PBS, sterilized by filtration (0.2 µm acetate membrane; VWR) and stored at -20°C until use. Great

care was taken to avoid repeated freeze-thawing. Following a two-day lead-in with sterile 0.9% saline, the mice were treated with saline or leptin for five days at 15:00-17:00 for maximum effect during the wake period. Body weight, food intake and water consumption was monitored daily. Body composition (fat, lean, fluid and free water mass) was measured by magnetic resonance imaging (EchoMRI™, Houston, TX) before and after treatment. Tissues were collected the morning after the last treatment.

4.3 HISTOCHEMISTRY

Gastrocnemius, plantaris, soleus and *extensor digitorum longus (*EDL) skeletal muscles were dissected and frozen together in Tissue-Tek O.C.T. compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) in isopentane cooled in N_2 (l). 10 µm serial cross-sections were generated using a Microstat HM 500M cryostat (MICROM Laborgeräte GmbH, Walldorf, Germany). Sections were mounted on SuperFrost glass slides (Menzel GmbH & Co., Braunschweig, Germany) and stored at -20°C. Unless stated otherwise, all reagents were from Sigma-Aldrich.

4.3.1 Myosin Heavy Chain Isoform Immunohistochemistry

Immunohistochemical staining for myosin heavy chain (MyHC) isoforms was performed using rabbit anti-mouse monoclonal MyHC type IIa (SC-71) and MyHC type I (D-5) antibodies, kindly donated by Professor Stefano Schiaffino, together with the Vector M.O.M Immunodetection Kit (Vector Laboratories Inc.) AMI. Briefly, after allowing the sections to air dry, they were blocked with an Avidin/Biotin Blocking reagent (Vector Laboratories Inc.) as instructed. The sections were then incubated with mouse immunoglobulin (Ig) blocking reagent for 1 hour, briefly pre-incubated with antibody diluent and probed with primary antibody diluted 1:500-1:1000 for 30 min. Subsequently, slides were incubated in biotinylated rabbit anti-mouse IgG reagent for 10 min, followed by fluorescein Avidin DCS for 5 min. Each step was followed by washing in PBS (10 mM Na_3PO_4, 0.15 M NaCl, pH 7.4-7.8). Finally, slides were mounted using VECTASHIELD® mounting medium (Vector Laboratories Inc., Burlingame, CA) for image capture.

4.3.2 Succinate Dehydrogenase Staining

Succinate dehydrogenase (SDH), ETC complex II, is an indicator of oxidative capacity. Histochemical staining intensity of SDH differs between skeletal muscle fiber types IIb, IIa, and I; the latter staining the darkest. Slides were treated with incubation solution for 8 min at RT (6.5 mM NaH_2PO_4, 43.5 mM Na_2HPO_4, 0.6 mM nitro blue tetrazolium, 50 mM sodium succinate, pH 7.6), washed in 0.9% saline and fixed in 15% ethanol. Coverslips were then mounted using aqueous mounting medium (Dako, Glostrup, Denmark).

4.3.3 Myosin ATPase Staining

Myosin II ATPase staining exploits the differential sensitivity of ATPase I, IIa, IIx and IIb eznymes to inhibition by acidic or alkaline conditions. Acidic preincubation inhibits type IIa, IIx and IIb enzymatic activity, resulting in dark staining (cobalt sulfide) of only type I fibers. Skeletal muscle fiber sections were preincubated under acidic conditions for 4 min (0.2 M sodium acetate, 0.1 M KCl, pH 4.43), washed (0.1 M NaOH, pH 9.4) and incubated with ATP

(90 mM glycine, 65 mM $CaCl_2$, 90 mM NaCl, 3 mM ATP, pH 9.4) for 20 min at 37°C. Differential fiber type staining was then resolved by sequential incubation with 1% (w/v) $CaCl_2$ (aqeous solution) for 3 min and 2% (w/v) $CoCl_2$ (aq) for 3 min. Following a wash in dH_2O and 1% (v/v) $(NH_4)_2S$ for 1 min, the slides were again rinsed thoroughly with dH_2O and air dried. Coverslips were mounted using aqueous mounting medium (Dako, Glostrup, Denmark).

4.3.4 Image Capture and Analysis

Image capture in study I was performed using an Olympus DP70 camera (Olympus Corporation, Japan) and Cast software (Visiopharm, Horsholm, Denmark), unless stated otherwise. Quantification of fiber type composition was performed using Image J software (National Institutes of Health). Fiber types were assigned based on threshold values of particle density: type I fibers <40, type IIa 40 - 210, and type IIb >210, as determined by Image J. Approximately 100 fibers counted per muscle section and the percentage of each fiber type was calculated.

4.4 ELECTRON MICROSCOPY

In study I, mitochondrial ultrastructure in white gastrocnemius skeletal muscle was evaluated by transmission electron microscopy (TEM; Morgagni 268, FEI Co., Hillsboro, OR). The muscle was perfused-fixed (2.5% [v/v] glutaraldehyde, 300 mM sucrose, 0.1 M cacodylate, pH7.2–7.4), dissected, cut into approximately 1x1x2-mm pieces, incubated in the same fixative overnight and post-fixed for 1 hour in 1% (v/v) osmium tetroxide. Samples were then dehydrated through a graded series of ethanol and acetone (30% ethanol for 10 min, 50% ethanol for 20 min, 70% ethanol for 30 min, 70% ethanol for 20 min, 85% ethanol for 20 min, 95% ethanol for 20 min, 99% ethanol 3 x 20 min, 99% ethanol:acetone 1:1 for 20 min, and 100% acetone x3). Samples were pre-embedded in acetone/Durcupan resin (Fluka, Sigma-Aldrich) 3:1 for 1 hour with rotation, followed by acetone:Durcupan 1:1 with rotation overnight. Samples were then embedded in Durcupan for 4 hours with rotation, placed in Durcupan-filled moulds, de-gassed for 10-20 min at 50°C and left to harden at 50°C for 24 hours. Ultrathin 70 nm sections were cut with a Leica Ultracut UCT (Leica Microsystems, Wetzlar, Germany) and placed on copper mesh grids (Electron Microscopy Sciences, Hatfield, PA). Sections were then post-fixed with 2% (w/v) uranyl acetate for 6 min, and counterstained with lead citrate (80 mM $Pb[NO_3]_2$, 160 mM sodium citrate, 160 mM NaOH) in the presence of NaOH (s) for 3 min in a closed container. Post-fix and counterstaining were both followed by gentle wash in dH_2O. Grids were air dried and stored until viewing by TEM. TEM image capture was done using an AMT camera system (Advance Microscopy Techniques Corp., Danvers, MA) at x 20,400 magnification. Quantification of mitochondrial volume, area percentage and number was performed using Image J software (National Institutes of Health), version 1.37.

4.5 MITOCHONDRIAL RESPIROMETRY

Mitochondrial function was assessed using a closed, two chamber, high-resolution respirometry system (Oroboros Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) (355; 356; 380; 381). In study I, mitochondrial respirometry was measured in white gastrocnemius. In study II and III, respiration was measured in glycolytic EDL and oxidative soleus skeletal muscle, as well as in liver.

Skeletal muscle samples in all three studies were processed the same manner. Following dissection, sample were placed in ice-cold BIOPS relaxing solution (2.8 mM Ca_2K_2EGTA, 7.2 mM K_2EGTA, 5.8 mM ATP, 6.6mM $MgCl_2$, 20mM taurine, 15mM sodium phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, and 50 mM MES, pH 7.1). Using fine forceps and a microscope, the muscle samples were cleaned of fat, blood and tendons; and fibers were separated to expose maximum surface area. The muscle samples were then transferred to ice-cold BIOPS supplemented with 0.005% (w/v) saponin for permeabilization of the sarcolemma, and incubated on ice with agitation for 10 min. This was followed by equilibration in ice-cold MiR05 respirometry medium (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 0.1% [w/v] bovine serum albumin, pH 7.1) with agitation for 30 min. Muscle samples were blotted for 30 sec before 1-2 mg of tissue was added to the experimental chamber. Liver (biopsy from the right lobe) samples were subjected to mild mechanical permeabilization (355) in amino-acid depleted MiR05 (20 µl/mg body weight), to allow for later protein concentration measurement. The equivalent of 1 mg of tissue was added to the experimental chamber. All respirometry experiments were performed using MiR05.

Oxygen flux caused by endogenous uncoupling, denoted "*LEAK*" in figures (see Table 1 and Fig. 4), was measured by adding malate (final concentration 2 mM) and pyruvate (10 mM), in the absence of ADP. Oxidative phosphorylation capacity, an indication of coupling efficiency, was quantified by adding ADP (5 mM, *OXPHOS*), followed by glutamate (20 mM) for additional complex I-supported oxidation (*C I*), and succinate (10 mM) for convergent electron flow through both complex I and II (*C I+II*). For maximum flux through the electron transfer system (*ETS I+II*), the exogenous protonophore carbonylcyanide-4-(trifluoromethoxy)-phenyl-hydrazone (FCCP) was titrated, to a final concentration of 0.7 µM for liver and 0.3 µM for skeletal muscle. Finally, electron transport through complex I was inhibited (*ETS II*) by adding rotenone (0.1 µM), followed by complex III inhibition (*ZERO*, data not shown in figures) by antimycin A (2.4 µM). *ZERO* oxygen flux, which is independent of the electron transfer system, was subtracted from the values of each of the previous steps. Absolute oxygen flux values are expressed relative to tissue wet weight per second (J_{O2}, [pmol O_2/mg/s]). The flux control ratio (FCR) describes the relative contribution of each state to maximum oxygen flux, and is expressed as a ratio over *ETS I+II*.

The implications of comparing data from studies using isolated mitochondria versus permeabilized tissue mainly concern aspects of processes directly dependent on membrane intactness. The first is complex I function; all citric acid cycle enzymes, except for succinate dehydrogenase (complex II), are soluble and located in the mitochondrial matrix. In the process of mitochondrial isolation, it is unavoidable that some of the matrix contents will leak out, while components of the cytosol and the experimental buffer leak in, as shearing forces disrupt parts of the mitochondrial networks and membranes (382-384). A second concern related to membrane damage is that ADP availability, one key controller of oxidative phosphorylation, is regulated by transporters in the outer mitochondrial membrane (385). Studies on isolated mitochondria (385) and permeabilized skeletal muscle fibers (386) provide evidence that mitochondrial isolation reduced the ADP Km (concentration at half maximum reaction speed) approximately by a factor of ten (387). Finally, there is a risk that the mitochondrial isolation process results in differential recovery of mitochondria, which would lead to a selection bias in terms of actual versus measured respiratory capacity.

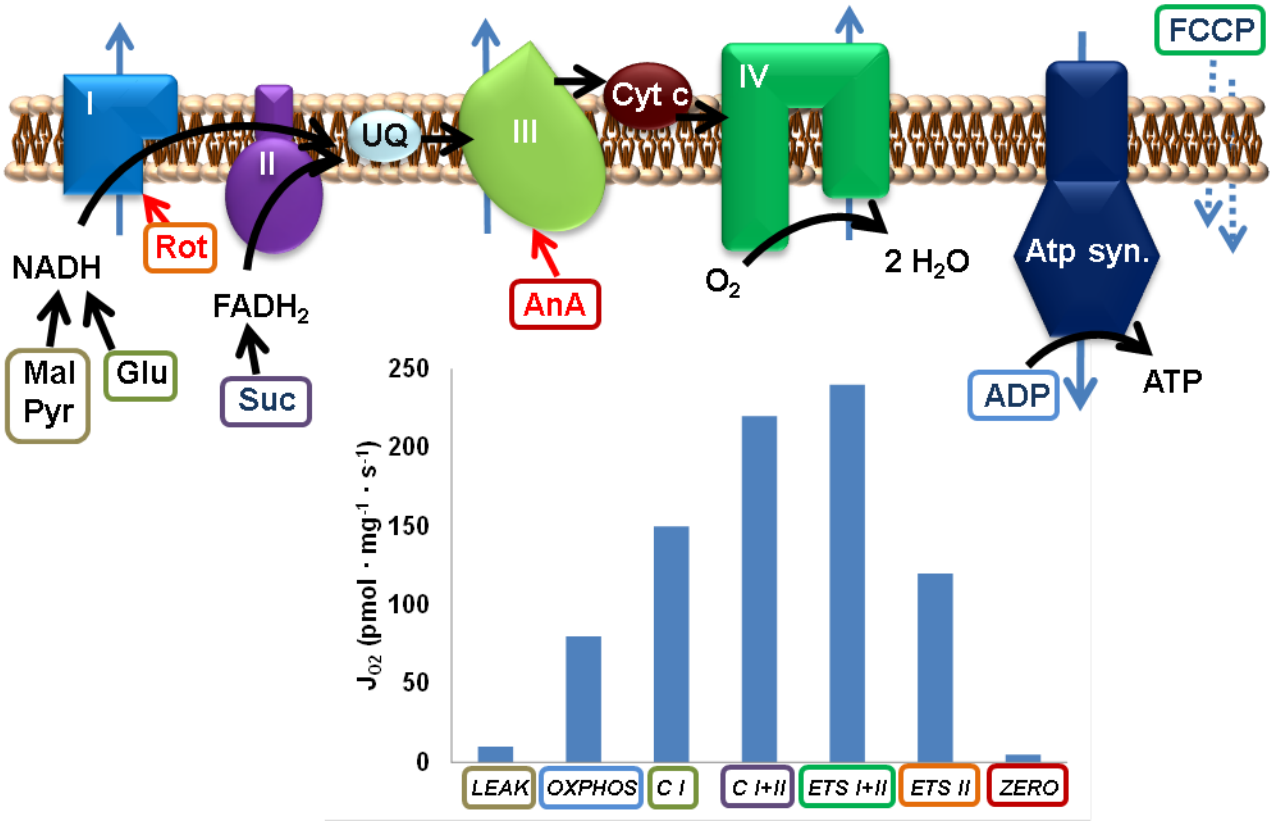

Figure 4. Schematic depiction of compounds added during the respirometry experiment, their targets and the corresponding respiratory flux as presented in figures. Mal – malate, Pyr – pyruvate, Suc – succinate, AnA – antimycin A, FCCP - carbonylcyanide-4-(trifluoromethoxy)-phenyl-hydrazone.

4.5.1.2 Sample Handling

In study I, all measurements were made in duplicate. The duplicate measurements were highly reproducible. However, interexperimental variation was identified as an important issue to address. Therefore tissues, rather than sample duplicates, were run in parallel in study II and III. If an experiment had to be terminated prematurely, or results diverged greatly from the expected, the experiment was repeated whenever possible. Examples of such circumstances are extremely low respiratory rates or response to substrate, unexplained "spikes" in the respiration measurements, or human error.

If the skeletal muscle samples could not be used for respirometry right away, they were kept in MiR05 at 4°C for up to 6 hours (381), following permeabilization. Previous studies in muscle have demonstrated that appropriate storage for up to 24 hrs does not affect mitochondrial performance. Analysis of our own data sets did not reveal any differences in mitochondrial function between muscle samples that had been used for respirometry immediately following equilibration and those that had been properly stored in this manner. Although one would normally strive to use all samples immediately, there were several reasons for this compromise in study II and III. First, we planned to measure mitochondrial respiration in several tissues from

the same mouse, and preferably analyze tissues representing more than one genotype or treatment condition per day to minimize experimental variation. Second, the time required for experiments in different tissues vary; experiments on liver and brain samples normally take half the time (45-60 min) of a typical muscle experiment (1.5-3 hrs). Third, the viability of liver samples is poor with respiratory responses to substrates being visibly lower after less than 1 hour (unpublished observations). A typical experimental design would then be to run storage-sensitive tissues from the first preparation while collecting tissues from the second. Following appropriate between-experiment cleaning, the second round of storage-sensitive tissues is performed while keeping processed skeletal muscle from both donors in MiR05 on ice. Finally, the muscle experiments are performed, starting with the first set in order to equalize storage times.

4.5.1.3 Data Presentation – Flux Control Ratio

The FCR is a means of obtaining a theoretical minimum and maximum limit of oxygen consumption capacity, by normalizing the oxygen flux for each state by that of the maximum (in our case, *ETS I+II*). Importantly, the FCR is independent of mitochondrial mass. However, changes in *ETS I+II* will be propagated to all FCR values. An alternative means of normalization is substrate control ratio, in which the measured states of oxygen flux are normalized by a common *coupled* reference state. This method presents the same problem with calculation artifacts as the FCR, with the additional limitation that substrate control ratio only provides relative values without an absolute maximum.

4.6 BIOCHEMICAL ANALYSES OF GLYCOGEN AND TRIGLYCERIDE CONTENT

In study II and III, glycogen and triglyceride content was measured in liver and skeletal muscle (soleus and gastrocnemius in study II, gastrocnemius in study III).

4.6.1 Glycogen Content Measurement

Glycogen content was measured using a standardized fluorometry-based biochemical method. In this method, intracellular glucose is phosphorylated *in vitro* by hexokinase, and subsequently converted to gluconic acid by glucose-6-phosphate dehydrogenase. This reaction produces nicotinamide adenine dinucleotide phosphate (NADPH), which is measured fluorometrically. To determine glycogen, tissue samples were lysed in 1 N HCl at 100°C for 2 hours and centrifuged at 2,000 x g. Liver samples required a further dilution of 1:4 with 1 N HCl. Samples were then mixed 1:200 with fluorometry assay buffer (50 mM Tris-HCl [pH 8.1], 300 µM ATP, 2 mM $MgCl_2$, 0.02% (w/v) fatty acid-free bovine serum albumin [BSA], 40 µM NADP, and 1 µg/ml glucose-6-phosphate dehydrogenase, supplemented with 1 µl/ml hexokinase in dilution buffer [20 mM imidazole, 0.02% (w/v) fatty-acid free BSA]), followed by fluorometry (TD-700, Turner Design, Sunnyvale, CA). Glycogen content was calculated using a standard curve.

4.6.2 Triglyceride Measurement

Triglyceride content was measured by a standard enzymatic colorimetric method. In this procedure, free glycerol is first eliminated enzymatically, followed by enzymatic hydrolysis of triglyceride in the presence of lipase and 4-aminophenazone. Liberated glycerol is determined spectrophotometrically (Libra S22, Biochrom Ltd., Cambridge, UK). Samples were homogenized in a 3:2 heptane-isopropanol solution with 1% (v/v) Tween-20. Thereafter, a

Trig/GB kit (triglycerides/glycerol blanked kit; Roche Diagnostics Scandinavia, Bromma, Sweden) was used AMI along with a Precinorm L standard (Roche Diagnostics Scandinavia).

4.7 ANALYSIS OF PROTEIN ABUNDANCE

Reagents for denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and precast gels were purchased from Bio-Rad, Hercules, CA.

4.7.1 Western Blot

Tissue samples used for protein analysis were homogenized in ice-cold lysis buffer (study I - 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5], 10% [v/v] glycerol, 1% [v/v] Triton X-100, 2 mM dithiotreitol, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM benzamidine, 1 µg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µM microcystin, 5 mM $Na_4P_2O_7$, 10 mM NaF, 0.5 mM Na_2VO_4; study II and III – 20 mM Tris-HCl [pH 7.8], 1 mM $MgCl_2$, 2.7 mM KCl, 135 mM NaCl, 10 mM NaF, 0.5 mM Na_2VO_4, 1% [v/v] protease inhibitor cocktail [VWR]). Following homogenization, samples were rotated end-over-end for 1 h at 4°C and subjected to three freeze/thaw cycles to disrupt the mitochondria; freezing at -80°C overnight, defrost for 1-2 hours at +4°C, vortex → two cycles of -80°C for 30 min, defrost and vortex). Samples were then centrifuged at 10,000 x g for 15 min at 4°C. Supernatants were removed for protein determination using Pierce BCA protein assay kit AMI (Nordic Biolabs, Täby, Sweden). Finally, samples were diluted to equal concentrations using lysis buffer and Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] sodium dodecyl sulphate, 10% [w/v] glycerol, ~0.002% [w/v] bromophenol blue and 5% [v/v] β-mercaptoethanol).

To examine protein abundance, Western blot analysis and chemiluminescent detection were performed. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA) (transfer buffer: 0.03 M Tris base, 0.24 M glycine, 20% methanol) and blocked in 5% (w/v) nonfat dry milk in Tris-buffered saline with Tween (TBST; 20 mM Tris base, 137 mM NaCl, pH 7.6, containing 0.1% (v/v) Tween-20). SDS-PAGE in study I was performed using gels prepared as follows: stacking gel: 0.12 M Tris-HCl [pH 6.8], 3.8% [v/v] 37.5:1 acrylamide/bis, 0.1% [v/v] sodium dodecyl sulphate [SDS], 0.1% [v/v] ammonium persulphate [APS], 1‰ tetramethylethylendiamine [TEMED]; resolving gel: 0.375 M Tris-HCl [pH 8.8], 7.5% [v/v] 37.5:1 acrylamide/bis, 0.1% [v/v] SDS, 0.045% [v/v] APS, 0.5‰ TEMED. Proteins were separated overnight at 14-20 mA with water cooling, with running buffer (0.025 M Tris-base, 0.19 M glycine, 0.1% [w/v] SDS, pH 8.3). Membrane transfer was conducted on ice at 500 mA for 3 hours with transfer buffer (0.03 M Tris base, 0.24 M glycine, 20% [v/v] methanol). In study II and III, precast Criterion gradient gels were used for protein separation (4-12%, Tris-based; XT-MES running buffer; approximately 500 mA at room temperature for 45 min) and transfer was performed on ice for 90 min at approximately 600 mA. Additionally, in study III the membranes were stained with Ponceau S solution (2 min staining and 1 min + 2 min wash in distilled water) and scanned prior to blocking.

The PVDF membranes were treated with methanol pre- and post-transfer AMI. After a quick wash with TBST, the membranes were probed with primary antibody (overnight at 4°C or 1 hour at room temperature), washed thoroughly in TBST, and incubated with the appropriate

horseradish peroxidase (HRP)-conjugated anti-IgG antibody for 1 hour at room temperature. Antibody-bound protein was detected using enhanced chemiluminescence AMI (GE Healthcare, Little Chalfront, UK), and quantified by densitometry (Quantity One analytical software in conjunction with GS-710 calibrated densitometer in study I, and a GS-800 Calibrated Densitometer in study II and III; both densitometer and software were from Bio-Rad). For repeated probing, the membrane was stripped of bound antibodies in stripping buffer (0.06 M Tris-HCl [pH 6.8], 2% [w/v] SDS, 0.7% [v/v] β-merkaptoethanol) for 45 min at 56°C, followed by thorough washing in TBST and blocking at 4°C overnight. Each membrane was probed a maximum of three times.

Target	Alternative name	Function/ Pathway	Manufacturer	Specificity
ATP5A1		ATP synthesis	Molecular Probes/ Invitrogen	M
Cyt *c*		ETC	PharMingen International	M
DNM1L	DRP1	Mitochondrial fission	Santa Cruz Biotech., Inc.	P
GAPDH		Glycolysis	Santa Cruz Biotech., Inc.	P
MFN-2		Mitochondrial fusion	Santa Cruz Biotech., Inc.	P
MT-CO1	COX1	ETC complex IV	Molecular Probes/ Invitrogen	M
NDUFA9		ETC complex I	Molecular Probes/ Invitrogen	M
NRF-1		TF, nuclear	Santa Cruz Biotech., Inc.	P
OPA1		Mitochondrial fusion	Abnova	M
PEPCK		Gluconeogenesis	Santa Cruz Biotech., Inc.	P
PGC-1		Transcription	Chemicon International/ Millipore	P
PPARα		TF, nuclear	Santa Cruz Biotech., Inc.	P
SDHA		ETC complex II	Molecular Probes/ Invitrogen	M
SOD2		ROS scavenging	Abcam	M
TFAM		TF, mito.	Santa Cruz Biotech., Inc. (study I) Abnova (study II)	P M
Tubulin		Structural	Millipore	P
UCP3		Transport	Chemicon International/ Millipore	P
UQCRC1		ETC complex III	Molecular Probes/ Invitrogen	M
UQCRC2		ETC complex III	Molecular Probes/ Invitrogen	M

Table 2. Antibodies used for Western blot protein detection. ETC – electron transport chain, TF – transcription factor. ROS – reactive oxygen species, M – monoclonal, P – polyclonal.

The primary antibodies used are listed in Table 2. In study I, commercially available secondary HRP-conjugated goat anti-mouse, and donkey anti-rabbit/goat/mouse IgG antibodies were obtained from the Jackson ImmunoResearch Laboratories (West Grove, PA). In study II and III, HRP-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Bio-Rad.

In all three studies, total protein abundance is expressed as fold change compared to the appropriate control group. Data for OPA1 represents a ratio of long (*a*-*b*) to short (*c*-*e*) isoforms. The appearance of OPA1 proteolytic cleavage isoforms is an indicator of mitochondrial stress (388; 389). Values presented are mean ± standard error of the mean (S.E.).

4.7.2 Myosin Heavy Chain Isoform Abundance

The separation of MyHC isoforms was performed to examine muscle fiber type composition of mouse models. The white skeletal muscle portion of the gastrocnemius skeletal muscle was homogenized in ice-cold lysis buffer (20 mM Tris-HCl [pH 7.8], 10% [v/v] glycerol, 1 mM $MgCl_2$, 2.7 mM KCl, 137 mM NaCl, 1% [v/v] Triton X-100, 1 mM EDTA, 1 µg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin, 1 µM microcystin, 5 mM $Na_4P_2O_7$, 10 mM NaF, 0.5 mM Na_3VO_4) and 50 µl was removed for electrophoretic separation. Total protein content was assessed using a Pierce BCA protein assay kit AMI (Nordic Biolabs) and the sample aliquots were diluted to equal concentrations using lysis buffer and Laemmli sample buffer (62.5 mM Tris base, 2% [w/v] sodium dodecyl sulphate, 10% [w/v] glycerol, ~0.002% [w/v] bromophenol blue and 5% [v/v] β-merkaptoethanol; pH 6.8). Separation of MyHC isoforms by glycerol SDS-PAGE (stacking gel: 4% [v/v] 40:1 acrylamide/bis, 0.8% [v/v] 2:1 bis, 37% [v/v] glycerol; resolving gel: 8% [v/v] 40:1 acrylamide/bis, 0.2% [v/v] 2:1 bis, 37% glycerol) was performed with upper (100 mM Tris base, 150 mM glycine, 0.1% [w/v] SDS; pH 8.6) and lower running buffer (50 mM Tris base, 75 mM glycine, 0.05% [w/v] SDS) at equal levels (390). One µg of total protein was loaded and separated at 4°C for 28 hrs at 5-10 mA. Gels were then fixed overnight (10% (v/v) acetic acid, 40% (v/v) ethanol), and stained using the SilverQuest™ silver staining kit (Invitrogen) AMI. Finally, the gels were scanned with a GS-710 calibrated imaging densitometer (Bio-Rad), the different MyHC isoforms identified according to migration characteristics as described (390) and their relative proportion determined using Quantity One software (Bio-Rad).

4.8 GENE EXPRESSION ANALYSES

4.8.1 Quantitative PCR

Skeletal muscle total mRNA was extracted using TRIzol (Invitrogen). The mRNA was purified further using the RNeasy Mini Kit AMI (Qiagen, Hilden, Germany). Liver total mRNA was extracted using QIAshredder (Qiagen) AMI, followed by purification with the Rneasy Mini Kit. The mRNA concentration and purity was measured spectrophotometrically (study I: Eppendorf BioPhotomer, Eppendorf AG, Hamburg, Germany; study II and III: Nanodrop 1000, Thermo Scientific, Wilmington, MA). Samples were diluted to equal concentrations with sterile and RNase-free water prior to cDNA synthesis. Converson of mRNA to cDNA was performed AMI using the SuperScript First Strand Synthesis System (oligo[dT] primers; Invitrogen) in study I, and SuperScript III First-Strand Synthesis Supermix (oligo[dT_2] primers; Invitrogen) in study II and III, according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was then performed in duplicate in a 96-well format (SYBR Green: 1X SYBR green mix, 0.7 µM each of forward and reverse primer; TaqMan assay [Applied Biosystems, Foster City, CA]: 1X TaqMan PCR Master Mix, 1X primer/probe mix; 10-25 ng cDNA) using an ABI Prism 7000 Sequence Detector (Applied Biosystems).

In study I, quantitative PCR was performed with SYBR Green primer/probe sets selected using the Primer Express software (Applied Biosystems). Relative expression was normalized against acidic ribosomal phosphoprotein PO (36B4). In study II and III, mRNA expression quantitative PCR was performed in multiplex with predesigned TaqMan assays (Applied Biosystems, Foster City, CA) and normalized against β-actin. Primer sequences are shown in Table 3. Quantification

of mRNA expression was normalized to a designated housekeeping gene and calculated using the standard curve method (391).

SYBR green primers		
Target	Forward Primer	Reverse Primer
36B4	CCCTGAAGTGCTCGACATCA	TGCGGACACCCTCCAGAA
ALAS	CGCCGATGCCCATTCTT	ACAACAGAGACTTTCCTGCCTTCT
β-actin	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC
Catalase	TGAGAAGCCTAAGAACGCAATTC	CCCTTCGCAGCCATGTG
COX4	GCAGCCTTTCCAGGGATGA	TCTCGGCGAAGCTCTCGTTA
CS	TGTCCTGCCCCTCCTCATC	GTGCTGGAGTTGGGTTCCA
Cyt *c*	GGAAAAGGGAGGCAAGCATAA	GCAGCCTGGCCTGTCTTC
Drp-1/Dnm1l	GCCCAGCCTACGGTGTGT	GCTGTAATTGCTACAATGCTGAATG
ERRα	GGGAAGCTAGTGCTCAGCTCTCT	CAGGATGCCACACCGTAGTG
LonP	GTGGGCAAGACCAGCATTG	CACCAACACTGAAACGGAAGTATT
LPL	CTGGGCTATGAGATCAACAAGGT	AGGGCATCTGAGAGCGAGTCT?
MCAD	TTCCCAAGGAAATGAGATCAAAG	TCCTCCGCCATGGGAAT
MFN-2	TGAAAGTCACTGTGCATTTGATAAAGT	GGCGCCCATCAGTCATTC
MT-CO1	TCAGTATCGTATGCTCAACAAATTTAGA	TGGTTCCTCGAATGTGTGATATG
MyHC I	TTGTGCTACCCAGCTCTAAGGG	CTGCTTCCACCTAAAGGGCTG
MyHC IIa	AAGCGAAGAGTAAGGCTGTC	CTTGCAAAGGAACTTGGGCTC
MyHC IIb	GAAGAGCCGAGAGGTTCACAC	AGGACAGTGACAAAGAACGTC
MyHC IIx	GAAGAGTGATTGATCCAAGTG	ATCTCCCAAAGTTATGAGTACA
NRF-1	CCACGTCCTTCACCAAAGCT	CACGCTGTGTCCTGGATCTTC
NRF-2	CAAGAGCAACAGATGAATGAG	ACTTTAATCGTAGTCGGTGTAG
OPA1	GGCATTTTATAGATTCTGAGCTGGAA	GCGAGCATGCGCTGTATTC
PDK4	GAAAGTCGAGTTCAAAAGGGAGAT	TTTCCCAACATGCACAATCCT
PGC-1α	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA?
PGC-1β	TGCTCTGCCCTCGATGTGCC	GACTACTGTCTGTGAGGCTGCCC
PPARα	CGATGCTGTCCTCCTTGATGA	CTCGCGTGTGATAAAGCCATT
PPARβ/δ	TCTCCGCAAGCCCTTCAGT	TCCAGCGCATTGAACTTGAC
SOD2	CACTCACGGCCACATTGAGT	CAGTCATAGTGCTGCAATGCTCTA
TFAM	AGGCTTGGAAA AATCTGTCTC	TGCTCTTCCCAAGACTTCATT
TaqMan assays		
Target	Accession number/Custom primer set	
ATP5A1	Mm00431960_m1	
β-actin	Mm00607939_s1	
Cyt *b*	FWD: AAAGCCACCTTGACCCGATT TAMRA probe: CGCTTTCCACTTCATCTTACCATT	REV: GATTCGTAGGGCCGCGATA
Drp-1/Dnm1l	Mm01342903_m1	
MFN-2	Mm00500120_m1	
MT-CO1	Mm04225243_g1	
NDUFA9	Mm00481216_m1	
OPA1	Mm00453879_m1	
SDHA	Mm01352366_m1	
SOD2	Mm01313000_m1	
TFAM	Mm00447485_m1	
UQCRC1	Mm00445911_m1	

Table 3. Primers used for quantification of mRNA expression.

5. RESULTS AND DISCUSSION

The aim of this thesis is to dissect out pathways which regulate mitochondrial biogenesis and to identify molecular targets that can modulate mitochondrial function, with an emphasis on ameliorating insulin resistance.

5.1 TISSUE-SPECIFIC MITOCHONDRIAL FUNCTION

Tissue-specific mitochondrial respiration in glycolytic and oxidative skeletal muscle, and liver was characterized in wild-type mice. This question has been addressed previously by others (18; 141; 392-397), but investigations of permeabilized biopsies from various tissue collected from the same animal have not been performed.

Comparing mitochondrial respiration in glycolytic EDL and oxidative soleus skeletal muscle, and liver, these tissues are easily distinguished by their respiratory phenotypes. The oxygen consumption in oxidative soleus muscle is consistently 80-90% higher than in glycolytic EDL muscle (Fig. 1A), which is in line with previous findings of oxidative muscle having more mitochondria with a higher oxidative phosphorylation activity (394; 396; 398; 399). In both muscle types, *C I+II* (maximal coupled respiration) is nearly equal to *ETS I+II* (maximal uncoupled respiration); indicating that in the resting state, membrane uncoupling may not be a significant feature of skeletal muscle mitochondrial function.

Liver respiration is low in the *LEAK*, *OXPHOS* and *C I* states. The comparatively low response to ADP appears to be consequent of low *C I* activity, demonstrating that the liver preferentially uses glucose for lipogenesis and other biosynthesis processes (400), while only a small proportion is used for oxidation. Maximal coupled and uncoupled electron transport capacity, in contrast, is comparable to that of EDL muscle; proving the liver to be a highly metabolically active tissue (395).

Apart from the difference in scale between EDL and soleus respiratory capacity, they can also be distinguished by FCR, the contribution of each respiratory state to maximal electron transport capacity (Fig. 1B). Complex I dominates respiration in both EDL and soleus. The complex I contribution is also somewhat higher in EDL, which corresponds to its glycolytic properties. Liver, in contrast, is more reliant on complex II; *C I+II* contribution to maximal electron transport capacity is three times higher than that of *C I* alone (Fig. 1A). Consequently, the response to complex I inhibition in liver (*ETS II*) is low. The low degree of endogenous uncoupling (*LEAK*) in liver is further emphasized by the marked increase in respiration by uncoupling (Fig. 1A and B).

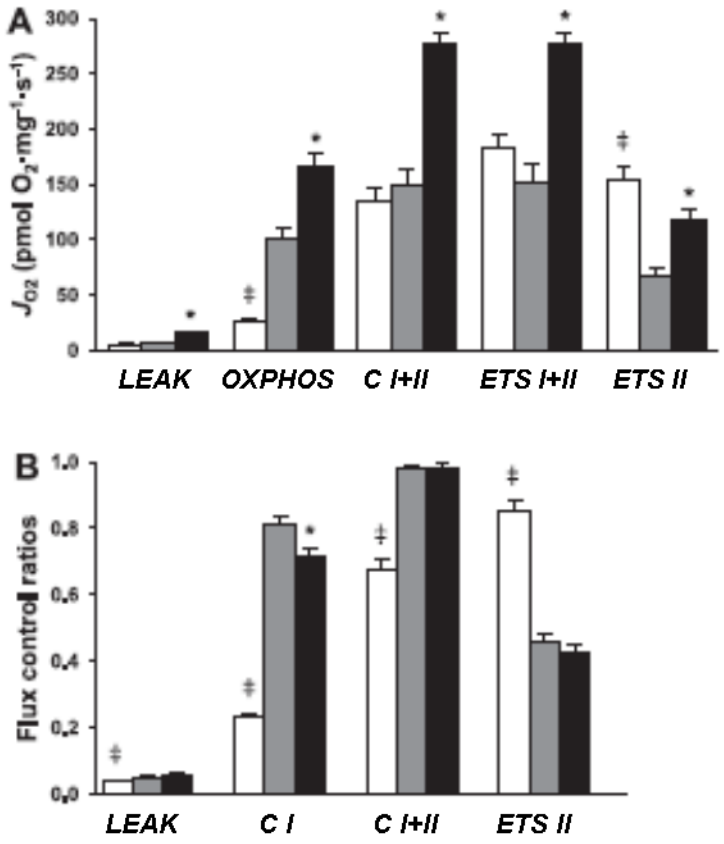

Figure. 4. Tissue-specific mitochondrial respiratory capacity in wild-type mice studied in Holmström *et al*, 2012 (401). A. Mitochondrial respiratory capacity expressed as mass-specific oxygen flux (J_{O2}, pmol·mg^{-1}·s^{-1}). B: Flux control ratio (FCR). Open bar - liver; gray bar - EDL, closed bar - soleus. Data are presented as mean±S.E. (N = 6–8). *p<0.05 vs. liver and EDL muscle; ‡p<0.05 vs. EDL and soleus muscle.

The functional implication of the *C I+II* FCR value is that a value below 1.0 means that ATP synthesis, the main site for proton re-entry, is rate-limiting for electron transport. In both glycolytic and oxidative skeletal muscle, the FCR for *C I+II* is close to 1.0, suggesting that the electron transport efficiency of the oxidative phosphorylation system, from complex I to ATP synthase, under these conditions, is close to 100%. Thus, electron transport cannot be further enhanced by membrane uncoupling. This is reasonable for skeletal muscle, an organ where both frequency and amplitude of energy output may be intermittent. Conversely, in liver, the *C I+II* FCR is closer to 0.7, leaving almost 30% of "spare capacity". The biological rationale for this phenomenon is incompletely resolved, but there are clues. One study investigated the hepatic transcriptional response to high-fat diet in two mouse strains; one sensitive (C57Bl/6J) and the other resistant (A/J) to high fat diet-induced non-alcoholic liver disease (402). A key difference between the two strains appeared to be the ability of the A/J mouse to induce expression of genes associated with mitochondrial uncoupling (402; 403). This uncoupling flexibility could be a mechanism of dissipating surplus charge elicited by increased fuel supply in the absence of increased need for ATP synthesis. Also, increased coupling efficiency (i.e. low proton leak) has implications for production of ROS, which act as signaling molecules in several cell types. In liver, ROS are important signaling molecules for induction and activation of detoxifying pathways (404-406); e.g. mediated by NRF-2 (407).

5.2 AMPK ACTIVITY INCREASES MITOCHONDRIAL BIOGENESIS, BUT NOT FUNCTION IN GLYCOLYTIC SKELETAL MUSCLE

AMPK is a metabolic switch which enables the cell to favor energy production in times when the energy charge falls, i.e. the ATP/AMP ratio decreases (408-410). The AMPKγ3^{R225Q} mutation transgenically expressed in the mouse introduces a conformational change that surpasses the allosteric regulation by AMP and ATP. Hence, the degree of phosphorylation and activation of the catalytic AMPKα subunit is increased (213). The aim of this study was to determine if the γ3 subunit of the AMPK enzyme has a role in signaling pathways mediating mitochondrial biogenesis in skeletal muscle and if the AMPKγ3 subunit is necessary for mitochondrial function Three mouse strains were used; AMPKγ3$^{-/-}$, expressing no γ3 subunit, and Tg-AMPKγ3^{WT} and Tg-AMPKγ3^{R225Q} expressing a transgene containing the wild-type γ3 subunit and the mutated γ3^{R225Q} subunit (213), respectively.

5.2.1 AMPKγ3^{R225Q} Induces Robust Changes in Gene Expression

The mRNA expression of several markers of mitochondrial function and biogenesis, intracellular metabolism and transcriptional control was investigated. Removal of the AMPKγ3 subunit did not result in altered transcription of any of the targets measured, compared to wild-type littermates. Overexpression of AMPKγ3^{WT} reduced transcription of PGC-1β only. Expression of AMPKγ3^{R225Q}, in contrast, was associated with marked changes in transcriptional profile, increasing expression of the transcriptional regulators PGC-1α, NRF-1, NRF-2 and TFAM; the metabolic markers medium-chain acyl-CoA dehydrogenase (MCAD), citrate synthase and ALAS; the ROS scavengers SOD2 and catalase, and the mediators of mitochondrial dynamics MFN-2, OPA1, DNM1L and Lon protease. Furthermore, the mitochondrial-to-nuclear DNA ratio was unchanged in AMPKγ3$^{-/-}$ and Tg-AMPKγ3^{WT} muscle compared to wild-type littermates, but increased in Tg-AMPKγ3^{R225Q}. Although the link between mRNA transcription and protein expression is tenuous and subject to translational regulation that varies greatly between targets, the general increase in transcription of genes related to mitochondrial dynamics and function, as well as the mitochondrial to nuclear DNA ratio, could indicate increased mitochondrial mass.

Protein abundance analysis of key markers of transcriptional regulation and metabolic function, and the mitochondrial oxidative phosphorylation system revealed no alterations in skeletal muscle from AMPKγ3$^{-/-}$ mice. A marker of ETC complex II was reduced in Tg-AMPKγ3^{WT} mice, but the remaining targets were similar compared to wild-type littermates. In Tg-AMPKγ3^{R225Q} mice, protein abundance of PGC-1α, uncoupling protein 3 (UCP3) and pyruvate dehydrogenase kinase 4 (PDK4), as well as markers of ETC complex I-IV was increased; further underscoring the possibility of increased mitochondrial density and function. The marker of ATP synthase, PPARα and PPARδ, were unaltered.

5.2.2 Increased AMPKγ3 Signaling is not associated with Altered Fiber Type Profile

AMPK signaling partly overlaps with that of the protein phosphatase calcineurin; activation of these enzymes in response to reduced ATP/AMP ratio and increased intracellular Ca^{2+} release, respectively, results in changes in gene expression mediated by PGC-1α (265) and PPARs (411).

They both stimulate mitochondrial biogenesis and lipid oxidation, and enhance insulin sensitivity. Overexpression of calcineurin in mouse skeletal muscle reduces AICAR-induced glucose uptake and is specifically associated with decreased AMPKγ3 expression (412); while inducing fiber type reprogramming to a more slow-twitch profile (413). Furthermore, although protein abundance of PPARδ was unaltered, enhanced activity is implicated in a switch to type I fibers (414). The changes in mRNA and protein abundance in Tg-AMPKγ3^{R225Q} mouse skeletal muscle could therefore be associated with a change in fiber type profile towards a more slow-twitch, oxidative phenotype. This was also indicated by SDH staining, which was unaltered in AMPKγ3$^{-/-}$ and Tg-AMPKγ3^{WT} muscle, but increased in Tg-AMPKγ3^{R225Q} compared to wild-type littermates.

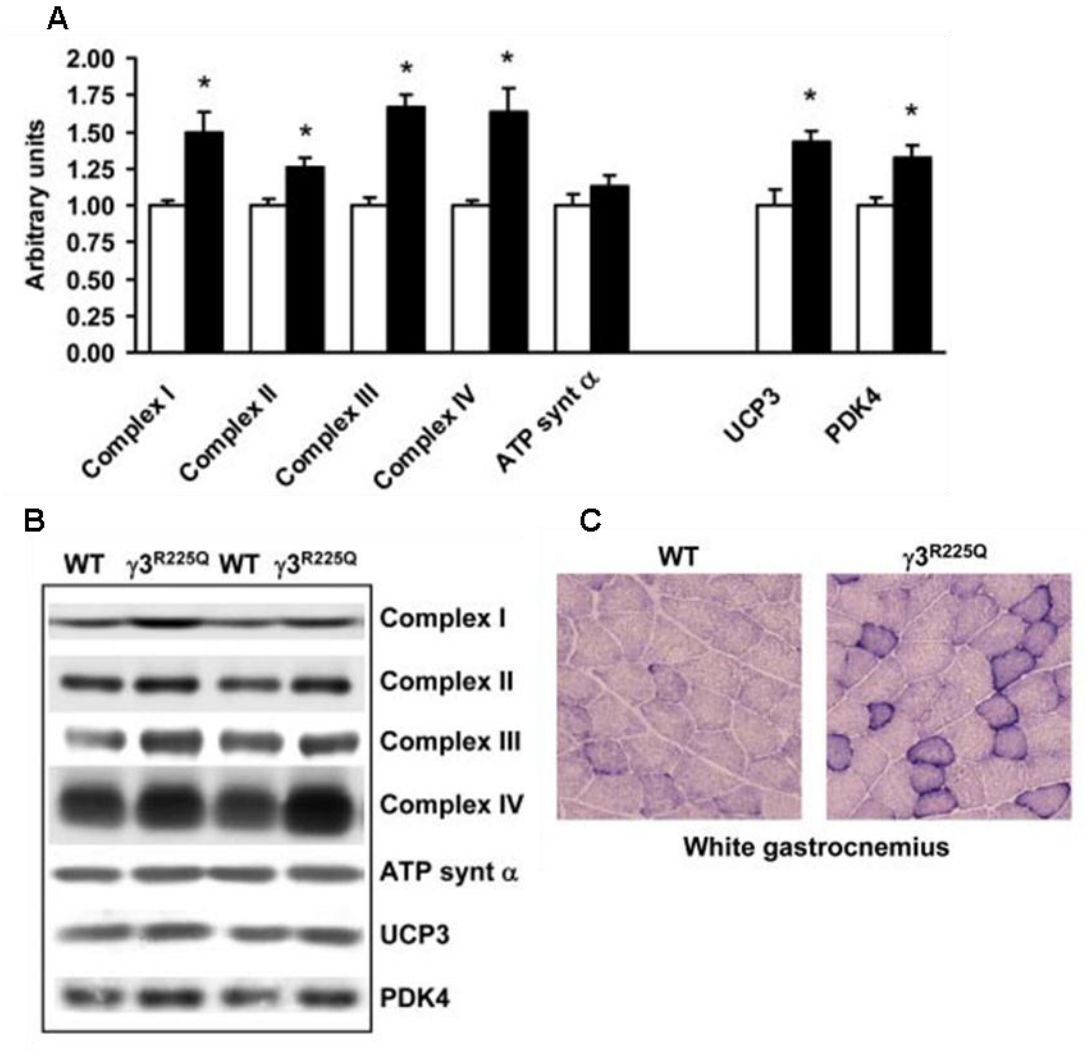

Figure 5. Mitochondrial protein expression studied in Garcia-Roves *et al*, 2008 (415). A. Protein content of markers of ETS complexes and mediators of oxidative metabolism (N=8). B. Representative blots. C. SDH staining in white gastrocnemius. Open bar – wild-type, closed bar – Tg-AMPKγ3R225Q. Data are presented as mean±S.E.. *p<0.05 vs. wild-type mice.

Analysis of MyHC isoform mRNA transcription (Fig. 3A) and protein abundance in white gastrocnemius revealed similar expression patterns in Tg-AMPKγ3^{R225Q} mice compared to wild-type littermates. Furthermore, quantification of immunocytochemical staining of MyHC isoforms and myosin II ATPase staining in EDL (fast-twitch, mainly glycolytic) and soleus (slow-twitch, mainly oxidative) (Fig. 3B and C), as well as plantaris (fast-twitch, mainly glycolytic) and gastrocnemius (mixed, but primarily fast-twitch, glycolytic) (416), demonstrated no change in fiber type percentages in wild-type and Tg-AMPKγ3^{R225Q} mice.

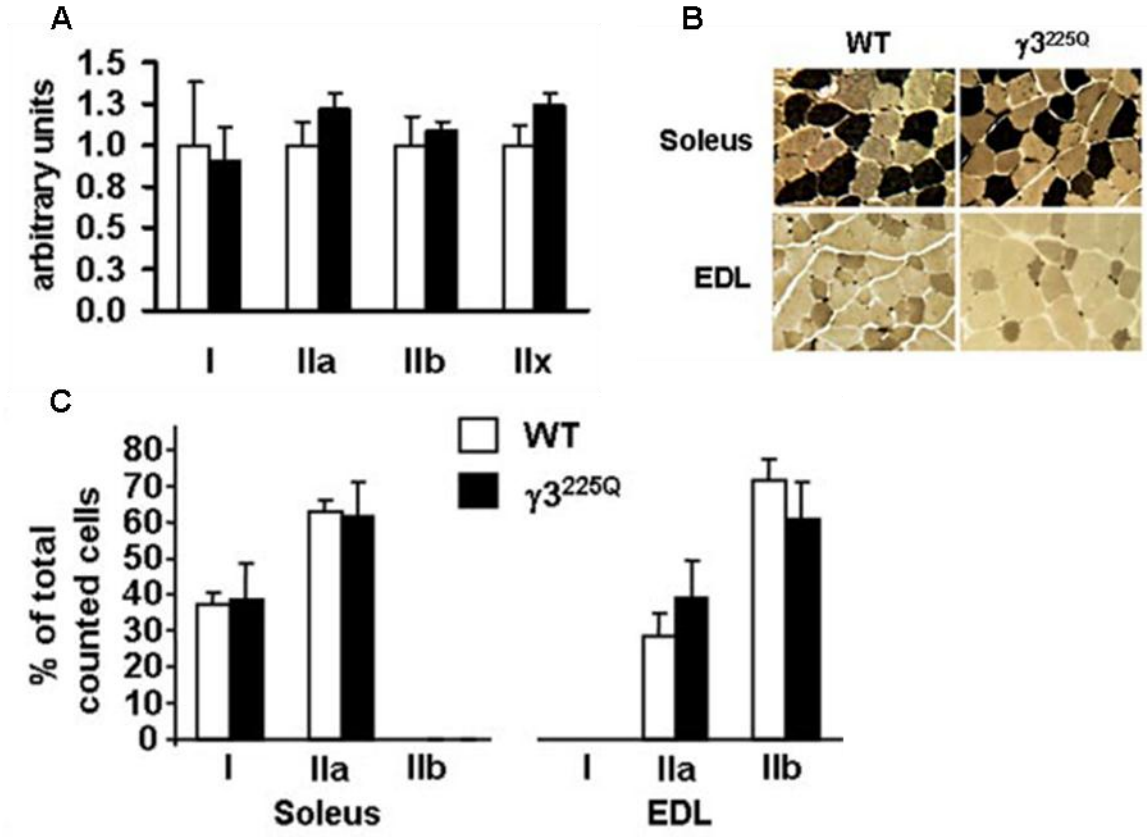

Figure 6. Fiber type composition in skeletal muscle from Tg-AMPKγ3R225Q mice studied in Garcia-Roves *et al*, 2008 (415). A. MyHC isoform mRNA expression in white gastrocnemius muscle (N=7-9). B. Myosin ATPase staining in soleus and EDL. C. Quantification of skeletal muscle fiber types in soleus and EDL based on myosin ATPase staining (N=4). Open bar – wild-type, closed bar - Tg-AMPKγ3R225Q. Data are presented as mean± S.E.

5.2.3 The γ3^{R225Q} Mutation Increases Mitochondrial Dynamics, but not Respiratory Function

AMPK enhances mitochondrial biogenesis (201; 269), and substrate oxidation induced by exercise (204) and energy deprivation (32). The changes in gene expression and SDH staining in Tg-AMPKγ3^{R225Q} mice suggested enhanced mitochondrial oxidative capacity or structural adaptations in sedentary mice. Mitochondrial area density was increased in Tg-AMPKγ3^{R225Q} mice compared to wild-type littermates (Fig. 4A) and, consistent with increased mRNA expression of markers of mitochondrial dynamics, the degree of mitochondrial clustering was increased. However, mitochondrial respiration and the relative contribution of each respiratory

flux state were unchanged between genotypes (Fig. 4B). This is in contrast to results from studies using chronic AICAR injections in rats (201). Chronic treatment with this AMP analogue increased abundance of cytochrome *c* and ALAS, and activity of enzymes of the citric acid cycle. This discrepancy may be explained by the fact that AICAR provides an artificial signal of increased energy demand for all AMP-regulated processes (eg. phosphofructokinase) (417). In contrast, the signaling through the AMPKγ3^{R225Q} heterotrimer is subject to posttranslational modifications and downstream regulation.

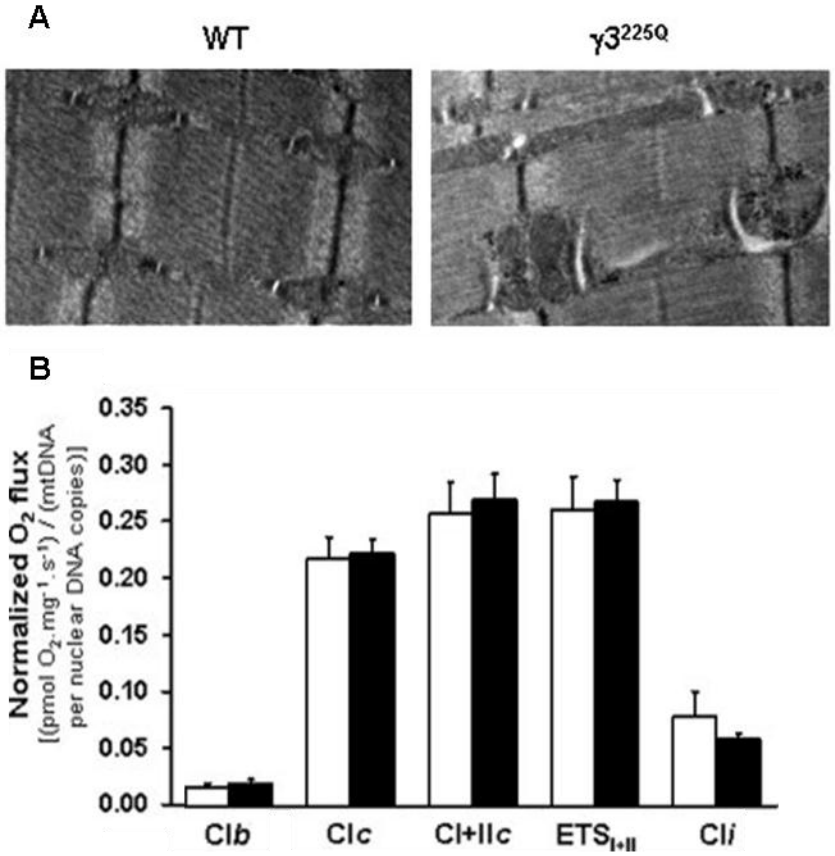

Figure 7. Mitochondrial biogenesis and respiratory capacity in white gastrocnemius muscle studied in Garcia-Roves *et al*, 2008 (415). A. Transmission electron microscopy of mitochondrial ultrastructure. B. Mitochondrial respiration (N=8-10). Open bar – wild-type, closed bar - Tg-AMPKγ3R225Q. Data are pre-sented as mean± S.E. CI*b* = malate + pyruvate, CI*c* = ADP + glutamate, CI+II*c* = succinate. ETS_{I+II} = FCCP, CI*i* = rotenone.

5.2.4 Summary of Mitochondrial Biogenesis and AMPKγ3^{R225Q}

The single-point mutation R225Q in the AMPKγ3 subunit stimulates mitochondrial biogenesis and mass in glycolytic skeletal muscle from sedentary mice, along with increased abundance of enzymes of lipid oxidation. These adaptations were not caused by increased subunit availability, as no major changes were evident after overexpressing the wild-type γ3 subunit. However, the AMPKγ3 subunit is also not strictly necessary for normal mitochondrial function, since whole-body ablation did not result in altered expression of mitochondrial proteins or factors regulating mitochondrial function.

The primary importance of skeletal muscle AMPK activation is to favor energy production under conditions of metabolic stress, as well as glucose and NEFA salvage in the recovery phase. Taken together with our results, this indicates that the functional enhancements caused by the γ3^{R225Q} mutation are superfluous in the absence of metabolic challenge (as evidenced by the lack

of enhanced protein abundance of ATP synthase). However, increased signaling by AMPK may prime the cell for such a challenge. A previous study demonstrated increased lipid oxidation capacity *in vitro*, reduced intramuscular lipid accumulation and protection from insulin resistance in high-fat fed Tg-AMPKγ3^{R225Q} mice compared to wild-type littermates. Conversely, high-fat feeding in AMPKγ3$^{-/-}$ mice resulted in enhanced lipid accumulation and reduced lipid oxidation in skeletal muscle, while insulin resistance was not significantly affected (213). In the present study, abundance of PPARδ, an AMPK signaling target (418), was unaltered. In contrast, PPARδ abundance is increased following endurance exercise (234) and after long-term low-intensity exercise (419) in obese humans. This suggests that the elevated basal activity of the AMPKγ3^{R225Q} holoenzyme does not result in downstream increases in lipid oxidation in the absence of metabolic challenge.

The AMPK β subunit contains a carbohydrate-binding module (420; 421). This subunit binds to glycogen *in vitro* (422) and in cells (420), in addition to mediating glycogen-dependent inhibition of AMPK activity (423). As part of the α2β2γ3 heterotrimer, the β2 subunit predominates in human skeletal muscle (208) and has increased affinity for glycogen, compared to the β1 isoform (424). The activating γ3^{R225Q} mutation is associated with increased intramuscular glycogen and glycogen resynthesis after strenuous exercise (425), as well as glycogen content-sensitive AMPK activity *in vitro*. AMPKγ3 ablation was associated with reduced glycogen resynthesis, but did not affect resting state glycogen levels (425). In both strains, muscle ergogenics were positively correlated with glycogen content (425). A whole-body knockout mouse model exploring the role of the AMPKα2 subunit displayed normal cardiac function, in spite of aberrations in mitochondrial ultrastructure, cardiolipin content and complex I function (426). Hence, the α2 subunit, and corresponding heterotrimers, modulate insulin action in tissues other than muscle, but plays an integral role in tissue-specific capacity to adapt to energy deprivation (427). In conclusion, in the sedentary state the activating R225Q mutation in the AMPKγ3 subunit stimulates mitochondrial biogenesis, but does not enhance oxidative capacity. These findings provide the molecular basis for one key pathway mediating the improvements in skeletal muscle metabolism in response to decreased energy charge, such as fasting or exercise.

5.3 TISSUE-SPECIFIC MITOCHONDRIAL FUNCTION IS NOT UNIFORMLY ALTERED IN LEPTIN RECEPTOR-DEFICIENT *db/db* MICE

5.3.1 Leptin-Receptor Deficiency Increases Mitochondrial Function in Glycolytic Skeletal Muscle

Mitochondrial respiration in *db/db* mouse EDL muscle was increased in all flux states investigated (Fig, 5A), while FCRs were similar between genotypes. This indicates a quantitative increase rather than a change in function directly. Elevated circulating NEFA is associated with enhanced mitochondrial respiration and density in skeletal muscle from high-fat fed animal models (124; 226; 227; 231) and in leptin receptor-deficient rats (230). EDL protein abundance of ETC complex I-IV markers was increased in *db/db* mice, as was TFAM and MFN-2 abundance and OPA1 proteolytic cleavage (Fig. 5B and C). TFAM plays a role in maintenance of the mitochondrial genome (428; 429), in addition to interacting directly with the mitochondrial transcription machinery (430). Together with unaltered DNM1L abundance and mitochondrial-to-nuclear DNA ratio, this could be interpreted as an adaptation to emerging

mitochondrial stress. Interestingly, even in light of the limitations of our protocol in terms of analyzing complex II-mediated function, there does not appear to be any additional functional or translational adaptations affecting it. Therefore, the EDL muscle in *db/db* mice does not appear to increase lipid oxidation, but rather oxidative capacity as a whole. Furthermore, since cell permeabilization (or mitochondria isolates) precludes glycolytic activity, an assessment of adaptations in the *s,n*-glycerophosphate shuttle, which is an important contributor of glycolysis-derived electrons to $FADH_2$ (431) in glycolytic skeletal muscle, is not possible.

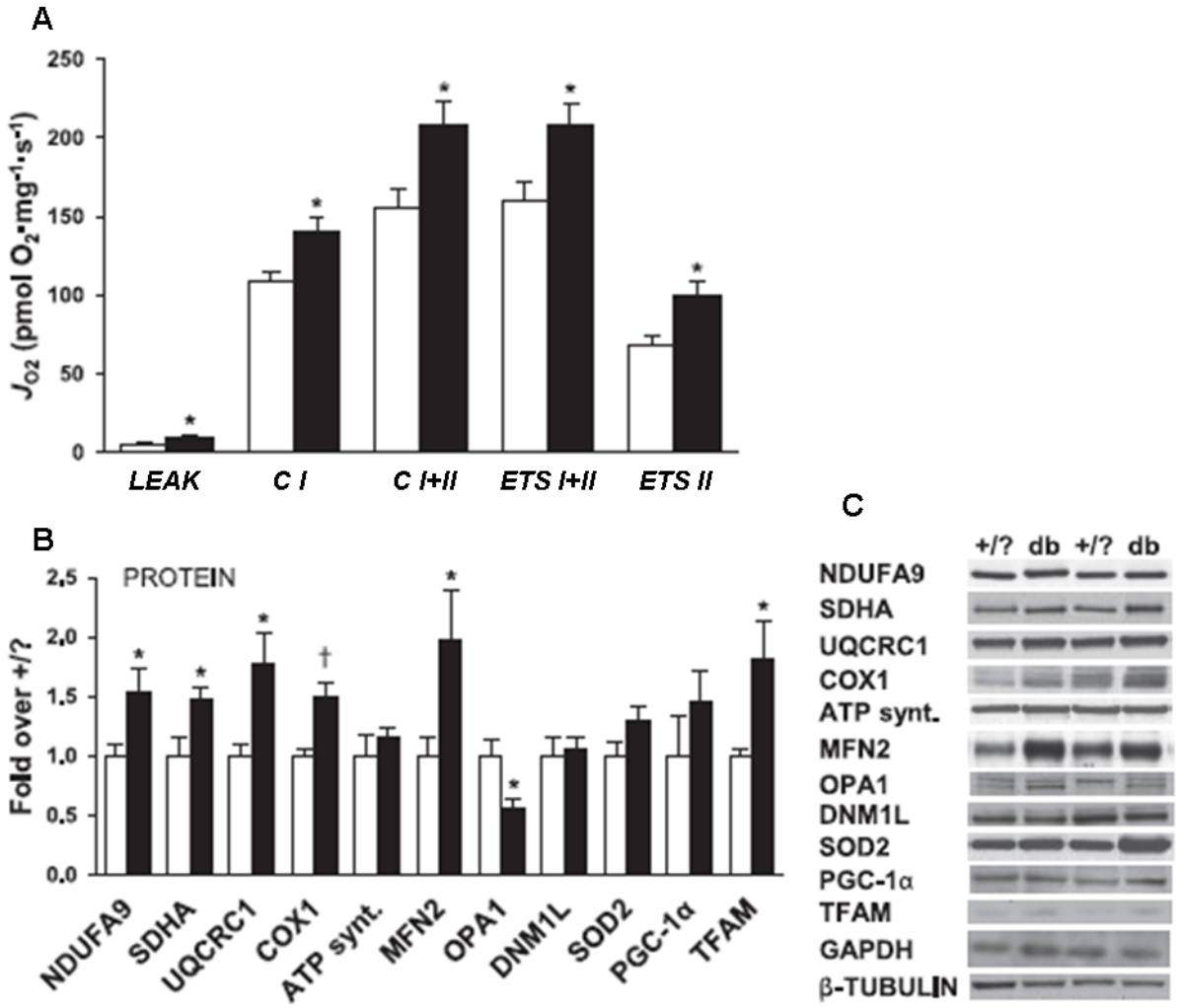

Figure 8. Mitochondrial respiratory capacity and protein abundance in glycolytic EDL muscle studied in Holmström *et al*, 2012 (401). A. Mitochondrial respiration (N=9–13). B. Protein abundance of markers of the ETS and mediators of biogenesis (N=6–9). C. Representative blots. Open bar – lean mice, closed bar - *db/db* mice. Data are presented as mean±S.E. *p<0.05 vs. lean mice.

5.3.2 Mitochondrial Dynamics are enhanced in Oxidative Skeletal Muscle from db/db Mice

The *db/db* mice have decreased *OXPHOS* respiration in comparison to lean littermates in soleus muscle. The other respiratory states and FCRs were unaltered. *OXPHOS* respiration is only

partly coupled with limited NADH production. Although there was a tendency for decreased *C I* respiration, this is not reflected in the FCR or in protein abundance of the complex I marker. The regulation of ETC complexes subunits is incompletely resolved, but mitochondrial NADH dehydrogenase 1α subcomplex 9 (NDUFA9) and subunits of complex III and IV are subject to inhibitory posttranslational modification in the form of added *N*-acetylglucosamine units (O-GlcNAcylation) in cultured cardiomyocytes under conditions of hyperglycemia (432). Furthermore, complex I activity and complex III structure is dependent on interaction with the inner mitochondrial membrane phospholipid cardiolipin (433; 434).

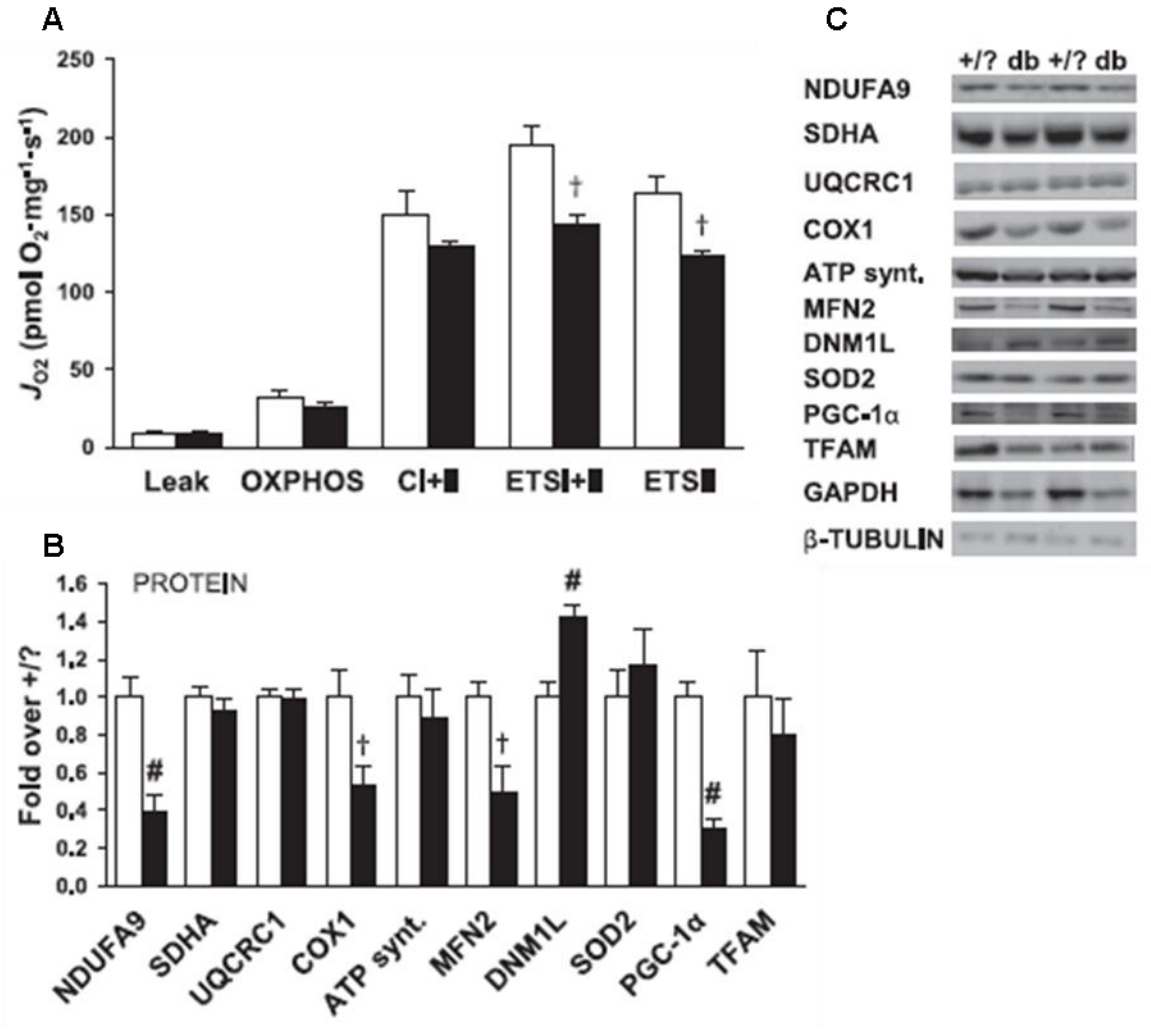

Figure 9. Mitochondrial respiratory capacity and protein abundance in liver, studied in Holmström *et al*, 2012 (401). A. Mitochondrial respiration (N=9–13). B. Protein abundance of markers of the ETS and mediators of biogenesis (N=6–9). C. Representative blots. Open bar – lean mice, closed bar - *db/db* mice. Data are presented as mean±S.E.. *p<0.05 vs. lean (+/?) mice.

Abundance of markers of the other ETS complexes was similar between genotypes. MFN-2 and DNM1L abundance, as well as OPA1 long-to-short ratio were enhanced, while PGC-1α was decreased. The net result of this would appear to be increased mitochondrial dynamics, in the absence of direct stimulation of biogenesis. Also, both TFAM abundance and mitochondrial-to-

nuclear DNA ratio were reduced; demonstrating a concerted reduction in mitochondrial DNA content. However, the maintained mRNA expression of the mitochondria-encoded gene MT-CO1 would indicate that mtDNA transcriptional activity persists. The *db/db* mouse skeletal muscle is insulin resistant (435; 436), resulting in reduced glucose uptake under conditions of high lipid availability. Maintaining ETS complex abundance with active mitochondrial turnover, could be an adaptation to these conditions. In that context, decreased *OXPHOS* respiration is in line with a notion of enhanced efflux of citric acid cycle intermediates for purposes other than ATP production and, in accordance with that hypothesis; soleus triglyceride content is markedly increased.

5.3.3 Reduced Mitochondrial Function in *db/db* Mouse Liver

Mitochondrial respiration was similar in *db/db* mice and lean littermates, with the exception of maximal electron transport capacity, *ETS I+II*, which was reduced (Fig. 6A). The *C I+II* FCR in *db/db* mice, in contrast, was increased. In the absence of altered FCR for states *C I* or *ETS II*, or succinate dehydrogenase complex subunit A flavoprotein (SDHA) protein abundance, this may reflect other means of regulation of complex II activity that is not sensitive to membrane uncoupling. The protein abundance of ETC complex I and III was decreased in *db/db* mice, while markers of the remaining ETS were unaltered. Reduced complex III abundance may be correlated to the blunted response to exogenous membrane uncoupling, whereas the decrease in complex I does not appear to translate into a functional impairment. However, the liver relies primarily on complex II function in the healthy state, so it is possible that in the steatotic liver of the *db/db* mouse, insulin resistance and other pathological processes combine to maintain complex I function in spite of protein downregulation. A study comparing mitochondrial gene expression and mitochondrial function in two mouse strains resistant (A/J) or sensitive (C57Bl/6) to high-fat diet-induced liver non-alcoholic liver disease revealed that protection against steatosis appeared associated with the ability to induce mitochondrial uncoupling (402). Furthermore, functional analysis confirmed this hypothesis since the A/J strain has increased state 2, 3 and 4 respiration after high-fat feeding, without increased ATP synthesis. Liver from C57Bl/6 mice, however, had unaltered respiratory capacity, but enhanced P/O ratio; an indication of increased coupling efficiency (403).

PGC-1α and MFN-2 protein abundance was decreased in the *db/db* mice, with a concurrent increase in DNM1L (Fig. 6B and C); suggesting enhanced mitochondrial fission and reduced signaling for biogenesis. OPA1 proteolytic isoforms were also altered, but with no clear long-to-short isoform shift. SOD2 and TFAM protein levels were unaltered, while the mitochondrial to nuclear DNA ratio was increased. Hence, the apparent stimulation of mitochondrial fission is likely a controlled adaptive process in the *db/db* mouse although mitochondrial stress cannot be excluded. Previous analyses on hepatocytes and isolated liver mitochondria from *fa/fa* rats, with an equivalent genetic defect in the leptin receptor, showed no difference in mitochondrial performance (437). However, in difference to the *db/db* mouse, the *fa/fa* rats in that particular study also had no sign of increased liver weight, lipid accumulation or steatosis. In contrast, rats fed a high-fat diet had decreased respiratory function in isolated mitochondria, hepatic steatosis, insulin resistance and increased oxidative stress (103). Complex I- and II-mediated respiratory capacity, as well as complex IV enzymatic activity in conjunction with oxidative damage to mitochondrial proteins and hypoxic stress were also reduced in high-fat fed mice (104).

5.4 LEPTIN REPLETION ALTERS MITOCHONDRIAL DYNAMICS, BUT NOT FUNCTION IN *ob/ob* MICE

The aim study III was to characterize tissue-specific mitochondrial function in leptin-deficient *ob/ob* mice and lean littermates, and to determine if it can be modulated by exogenous leptin-repletion.

5.4.1 Increased Respiratory Capacity in *ob/ob* Mouse Glycolytic Skeletal Muscle

Glycolytic EDL muscle is shorter and 26% lighter in *ob/ob* mice bred on an Aston background (438), compared to lean littermates. Furthermore, *ob/ob* mice have a higher oxidative type IIa fiber percentage and fiber cross-sectional area, with a corresponding decrease in glycolytic type IIb fibers (438). Chronic leptin treatment did not modify these parameters. Nevertheless, acute leptin treatment stimulates lipid oxidation through AMPKα2 activation in skeletal muscle (174). Therefore, leptin treatment may also induce mitochondrial functional adaptations in EDL and soleus muscle.

In EDL muscle from saline-treated *ob/ob* mice, *ETS I+II* was increased, along with a similar trend in *ETS II* (Fig. 7A). The FCR for *C I* and *C I+II* was reduced, which could be an artifact of the increase in the factor used for normalization; especially considering the changes in ETS protein abundance (Fig. 7B-D). Markers of complex II and IV, and ATP synthase were increased – explaining the enhanced maximal electron transport capacity – while complex I and III were unaltered compared to saline-treated lean mice. Leptin treatment in *ob/ob* mice revealed a trend for enhanced *C I* FCR (Fig. 7A), which could be an effect of leptin-modulation of insulin signaling. Lean leptin-treated mice had increased *OXPHOS* respiration, while *ETS II* FCR decreased, along with a similar trend in *C I+II* FCR. In the absence of altered complex II abundance, this indicates decreased complex II contribution to maximal electron transport capacity. Leptin treatment also reduced body weight and fat mass, which indicates enhanced white adipose tissue lipid mobilization. However, there were no changes in intracellular glycogen or triglyceride stores. Taken together, this implies that the leptin treatment-mediated stimulation of lipid oxidation in lean mice is superseded by other pathways signaling sufficient intracellular energy charge.

DNM1L (Fig. 7F) and total OPA1 abundance was increased in saline-treated *ob/ob* mice, along with an OPA1 long-to-short isoform shift and reduced TFAM (Fig, 7E). This strongly suggests mitochondrial fission or fragmentation, since there is no corresponding response in MFN-2 compared to saline-treated lean littermates. In leptin-treated *ob/ob* mice DNM1L abundance was normalized.

5.4.2 Minor Mitochondrial Adaptations in Oxidative Skeletal Muscle from *ob/ob* Mice

Oxidative soleus muscle from *ob/ob* mice on the Aston genetic background is smaller in the *ob/ob* mouse and has a lower glycolytic type IIb fiber percentage (438). Type IIb fibers also have increased cross-sectional area in the *ob/ob* mouse soleus compared to lean littermates. Chronic leptin treatment increased type IIa and IIb fiber percentage in both *ob/ob* and lean mice (438).

On the B101 background, *ob/ob* mice have smaller slow, oxidative fibers, with no difference in fast, glycolytic fibers (439).

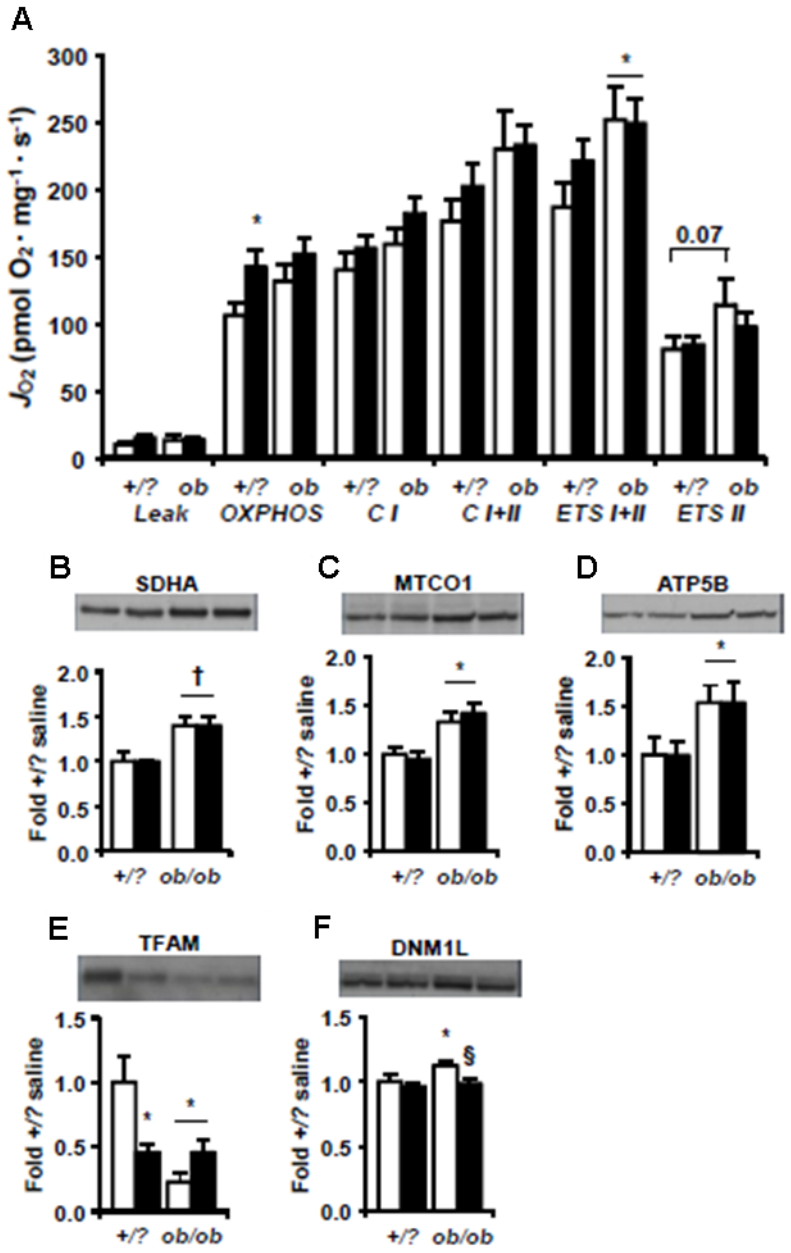

Figure 10. Mitochondrial respiration and abundance of mitochondrial proteins in glycolytic EDL muscle, studied in Holmström *et al*. A. Mitochondrial respiratory capacity. B-D. Protein abundance of markers of ETS complexes I, IV and ATP synthase. E-F. Protein abundance of mediators of mitochondrial biogenesis. Open bar - saline-treated; closed bar - leptin-treated. Data are presented as mean±S.E (N=6-8). *p<0.05, †p<0.01 vs lean (+/?), saline-treated mice; §p<0.05 vs *ob/ob*, saline-treated mice.

Mitochondrial respiration in soleus (Fig. 8A) and FCRs were unchanged between saline-treated *ob/ob* and lean mice. The similarities persisted in both *ob/ob* and lean mice following leptin treatment. This would suggest pathways regulating muscle substrate oxidation are intact even in obesity. Thus, further leptin stimulation through peripheral and neuronal pathways seems to be superfluous. Accordingly, protein abundance of markers of the ETS complexes and mitochondrial dynamics was unaltered after leptin treatment. TFAM was reduced in both groups of *ob/ob* mice. Leptin-treatment did not alter protein abundance of markers of the ETS in neither lean nor *ob/ob* mice. Leptin-treatment was insufficient to rescue TFAM abundance in *ob/ob*

mice. In lean mice, leptin treatment itself reduced TFAM abundance. Hence, these results provide evidence to suggest that severe obesity does not induce mitochondrial respiratory dysfunction in oxidative soleus muscle, and that short-term leptin treatment is insufficient to further improve oxidative phosphorylation.

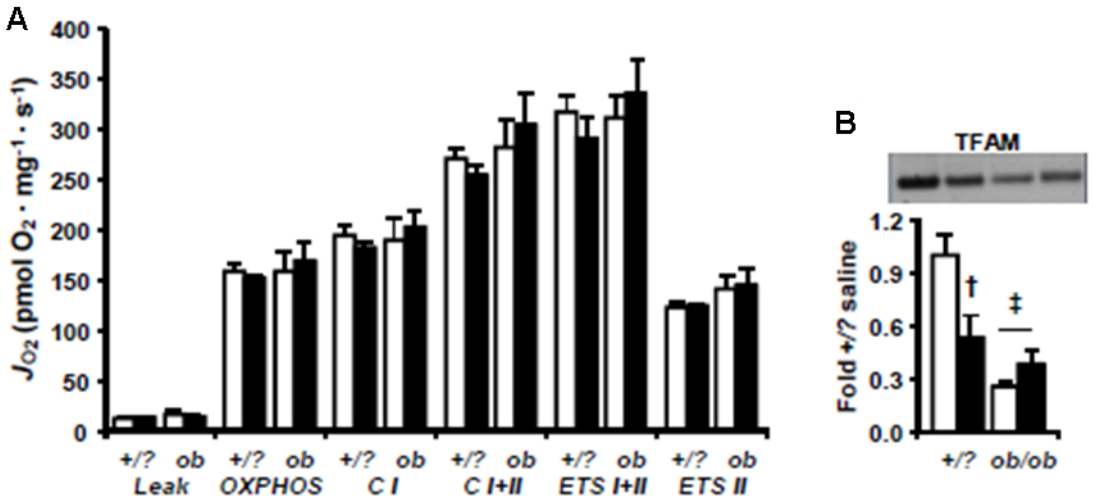

Figure 11. Mitochondrial function (A) and TFAM protein abundance (B) in oxidative soleus muscle, studied in Holmström *et al*. Open bar - saline-treated; closed bar – leptin-treated. Data are presented as mean±S.E. (N=6-8). †p<0.01, ‡p<0.001 vs lean (+/?), saline-treated mice

5.4.3 Reduced Complex II Function in Liver from *ob/ob* Mice, and Improved Biogenesis with Leptin Treatment

In the saline-treated *ob/ob* mice (Fig. 9A), both *ETS I+II* and *ETS II* were depressed compared to saline-treated lean littermates, with a similar trend in *C I+II*. Leptin treatment in *ob/ob* mice was insufficient to alter the reduced respiratory capacity, although there was a strong trend for increased *ETS II*, compared to saline-treated *ob/ob* mice. Thus, these results provide evidence to suggest that ATP synthesis may be limiting to electron transfer capacity. This was compatable with our finding in *db/db* liver. In addition, the respirometry data suggests that the reduced electron transfer capacity is dependent on complex II function and that leptin-repletion in *ob/ob* mice enhances $FADH_2$ generation (e.g. lipid or amino acid oxidation).

Protein abundance of markers of complex III and IV, and ATP synthase were unaltered; so the cause of reduced maximal electron transport capacity is not immediately clear. However, the marker of succinate dehydrogenase (SDHA) (Fig. 9C) was reduced in saline-treated *ob/ob* mice and there was a trend towards increased levels of complex I NDUFA9; which is consistent with the functional data. SDHA is the catalytic subunit of complex II, mediating the conversion of succinate to fumarate and a decrease would affect $FADH_2$ supply and citric acid cycle efficiency. Hence, the source of reduced electron transport capacity in the uncoupled state appears to be different in *ob/ob* mice, compared to *db/db* – i.e. reduced $FADH_2$ electron supply through complex II, rather than turnover capacity at complex IV. Furthermore, the abundance of PPARα was reduced in saline-treated *ob/ob* mice and unaltered by leptin treatment in both genotypes (Fig. 9F). This transcription factor is central to hepatic β-oxidation gene expression (241; 440)

and the lack of enhanced expression further supports the hypothesis that the hepatic response to leptin treatment is focused on lipid mobilization over oxidation. Accordingly, increased lipid export (180) and reduced lipogenesis (176; 180) has been reported with leptin treatment. Hence, reduced electron transport capacity in liver from *ob/ob* mice is mediated by reduced complex II activity, which may be partly restored with leptin repletion in *ob/ob* mice.

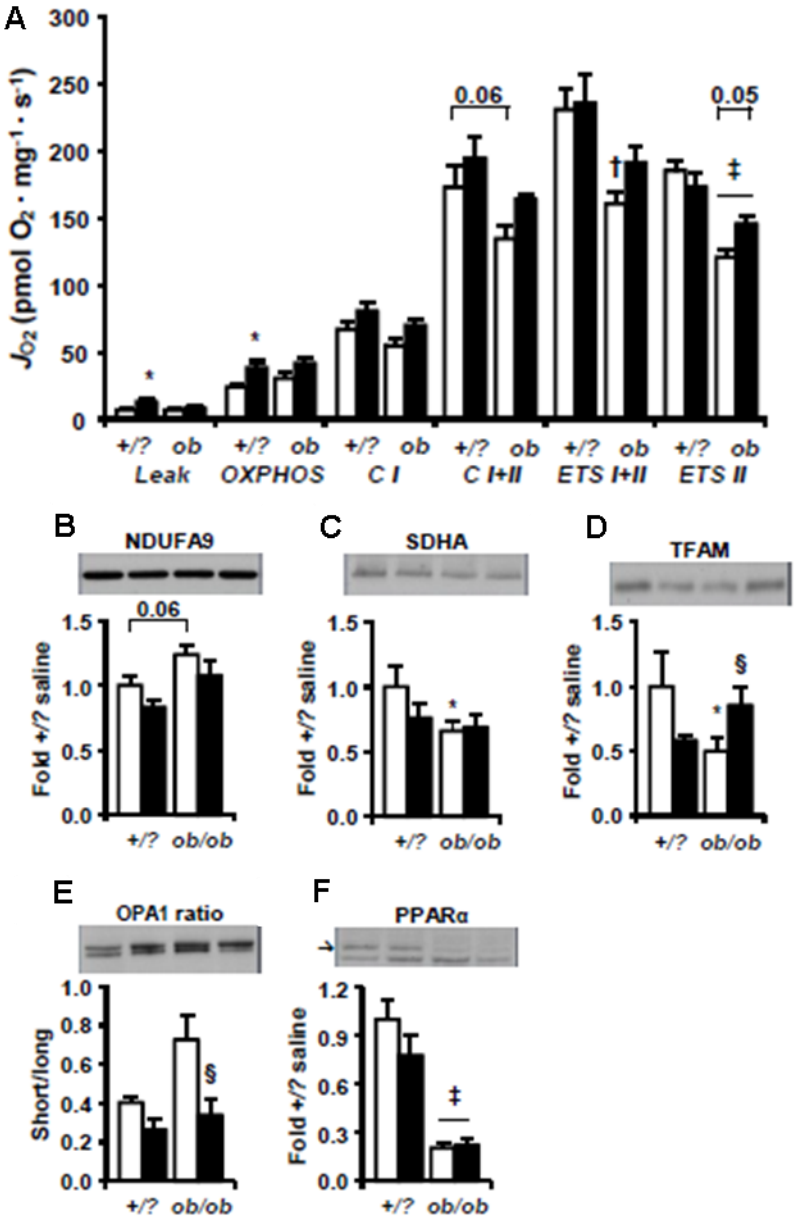

Figure 12. Mitochondrial respiration and abundance of mediators of mitochondrial function in liver, studied in Holmström *et al*. A. Mitochondrial respiratory capacity. B-C. Protein abundance of markers of ETC complexes I and II. D-F. Protein abundance of mediators of mitochondrial biogenesis and lipid oxidation. Open bar - saline-treated; closed bar - leptin-treated. Data are presented as mean±S.E. (N=6-8). *p<0.05, †p<0.01, ‡p<0.001 vs. lean (+/?), saline-treated mice; §p<0.05 vs. *ob/ob*, saline-treated mice.

The FCR for *OXPHOS* and *C I+II* was increased in saline-treated *ob/ob* mice; whereas only *OXPHOS* was increased in *ob/ob* mice treated with leptin. The increased *C I+II* FCR value is likely a mathematical artifact, caused by the fact that the tendency for *C I+II* respiration (Fig. 9A) to decrease was smaller than the significant decrease in *ETS I+II* respiration. The rise in *OXPHOS* FCR cannot be as easily explained. *OXPHOS* respiration is measured under conditions of partial citric acid cycle NADH production. Hence, its contribution to maximal electron transport capacity could be subject to alterations caused by endogenous uncoupling or

components affecting NADH supply. The latter seems more likely, because *LEAK* oxygen flux (Fig. 9A) and *LEAK* FCR remain unaltered. Also, once glutamate is added the difference disappears. These data imply that *OXPHOS* NADH production may be affected by reduced citrate and/or α-ketoglutarate exchange. Indeed, the tricarboxylate carrier, which provides citrate for lipogenesis, is downregulated in rats fed a high-fat diet inducing insulin resistance (441) and its activity is reduced in starved rats (442); both conditions in which lipogenesis is reduced in favor of gluconeogenesis and hepatic glucose production. Furthermore, citrate is an allosteric activator or ACC (443) and an allosteric inhibitor of phosphofructokinase (444), suggesting that reduced efflux is a means of reducing lipogenesis in favor of gluconeogenesis. Thus, mitochondrial respiratory function under non-saturating conditions in the *ob/ob* mouse may be subject to additional regulation at the level of mitochondrial substrate transport, which is not affected by leptin treatment.

MFN-2 and DNM1L were unaltered in saline-treated *ob/ob* compared to saline-treated lean littermates, while TFAM (Fig. 9D) was reduced with obesity. Leptin treatment in *ob/ob* mice normalized TFAM abundance and improved the OPA1 ratio (Fig. 9E). Hence, the degree of mitochondrial stress appears to be reduced by leptin treatment in *ob/ob* mice, along with improved mtDNA stabilization.

5.4.4 Summary of Mitochondrial Function and Leptin Signaling Deficiency

In studies II (*db/db*) and III (*ob/ob*), mitochondrial function and markers of biogenesis in *db/db* and *ob/ob* mice were investigated. Interestingly, even though the overall physiology is similar in the *db/db* and the saline-treated *ob/ob* mice – blunted ETS capacity in liver, enhancements in ETS capacity in EDL and modest to no alterations in soleus – the specific molecular adaptations are different. The first obvious source of difference is the fact that the molecular background to the *db* and *ob* pathologies is different; lack of the long isoform (isoform b) of the leptin receptor, and lack of the leptin hormone, respectively. The precise configuration of some of the leptin receptor isoforms with leptin, as well as possible interactions with other cytokine receptors, remain to be determined (445). Thus, the *ob/ob* mouse should perhaps be considered to be the more profound model, as absence of the hormone would imply a complete ablation of leptin receptor signaling, regardless of receptor isoform. However, even in the *ob/ob* mouse, the receptors are still expressed (446). The second source of difference lies in the experimental design. Study I and II were performed using mice taken straight from the animal facilities, whereas the mice in study III were subject to comparatively intensive handling and once-daily treatment, as well as separate housing in smaller cages. These are all factors which could influence stress and activity levels, and, by extension, whole-body metabolism.

5.5 WHOLE-BODY EFFECTS OF LEPTIN TREATMENT IN *ob/ob* MICE

Leptin treatment had profound effects on food intake, body weight and body composition in *ob/ob* mice. Lean mass, fat mass and body weight were reduced in the lean mice as well, but to a smaller degree than in *ob/ob* mice. The effect of leptin treatment on body weight and composition in *ob/ob* mice is well established (144; 145; 438; 447); body weight is decreased as a result of reduced food intake and increased energy expenditure. Importantly, these effects are functionally separate as doses too low to elicit depressed food intake still decrease body weight (146). Conversely, pair-feeding to the levels of lean littermates does not affect thermogenesis

(374), nor does it improve cold adaptation or skeletal muscle growth (448). The effect of leptin treatment in lean mice, in contrast, is more varied. Using a similar design as the present study, reduced food intake, body weight and fat mass after five days of leptin treatment of lean *ob/ob* mouse littermates has been reported (144). The same was found in young (5 wks) mice (145). However, reduced body weight was demonstrated in lean mice in the absence of food intake reduction after intracerebroventricular leptin-administration (449). Hence, the reduction in lean mass is an unusual finding, but not unique; in the *ob/ob* mice reduced lean mass could be caused by reduced food intake and enhanced thermogenesis, whereas the lean littermates may be further affected by reduced ambulatory behavior (146), and single-housing in smaller cages.

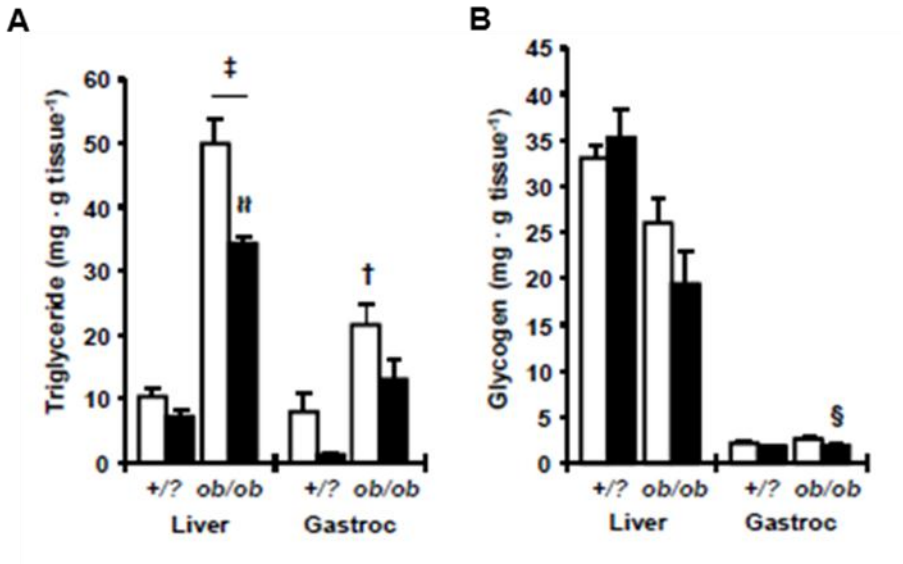

Figure 13. Effect of leptin treatment on fuel storage, studied in Holmström *et al*, 2012 (401). A. Triglyceride content in liver and gastrocnemius muscle. B. Glycogen content in liver and gastrocnemius. Open bars - saline-treated; closed bars - leptin-treated. Data are presented as mean± S.E. N=6-8. †p<0.01, ‡p<0.001 vs. lean (+/?), saline-treated mice; §p<0.05, ǂp<0.01 vs. *ob/ob*, saline-treated mice.

The degree of leptin action in hypothalamus and periphery is in part dose-dependent *in vivo*; plasma and cerebrospinal concentrations are correlated up to circulating levels of 15 ng/ml, but this association is lost with increasing levels (450; 451). While the hypothalamic feeding regulation plateaus after further increases in leptin concentrations, peripheral action on lipid metabolism continues (450). Although circulating leptin was not measured in the present study, the lean mice may transiently experience a higher dose of hormone than the *ob/ob* mice, because the exogenous leptin is added to endogenous production.

Measurement of tissue-specific fuel storage showed that while there was no leptin-treatment effect in the lean mice, liver weight, liver triglyceride (Fig. 10A), and muscle glycogen (Fig. 10B) were reduced in leptin-treated *ob/ob* mice, in the absence of reduced muscle triglyceride. This suggests that reduced fat mass and lean mass in *ob/ob* mice could then be caused by increased lipid mobilization from white adipose tissue and, possibly, increased protein breakdown in skeletal muscle, respectively. This is also consistent with the mitochondrial respirometry data showing enhanced maximal electron transport capacity in EDL from *ob/ob* mice (Fig. 7A), with no further potentiation in oxidative capacity after leptin treatment, and no

change with neither obesity nor leptin in soleus (Fig. 8A). Peripheral leptin action is evident in rats in which the ventromedial hypothalamus has been ablated, indicating that the hypothalamic leptin target site is not necessary for peripheral effects (452). Leptin action is also evident in *fa/fa* leptin receptor-deficient rats expressing the receptor only in liver, demonstrating functional signaling pathways and that hypothalamic unresponsiveness is inconsequential to local leptin signaling (453). Hepatic leptin-mediated stimulation of lipid disposal is mediated partly by inhibition of stearoyl-CoA desaturase 1, causing increased lipid export (176; 180) and reduced hypertriglyceridemia (162). This was associated with increased hepatic glucose production in conjunction with increased uptake in brain, brown adipose tissue and heart (454). In lean mice, however, acute leptin infusion caused increased glucose turnover and uptake, dominated by EDL muscle and brown adipose tissue, without altering circulating insulin or glucose (455). Likely, these differences can be traced to both the profound insulin resistance in *ob/ob* skeletal muscle and white adipose tissue, and differential regulation of lipid mobilization (180).

6. PERSPECTIVES AND CONCLUDING REMARKS

The aim of this thesis was to investigate the pathways regulating mitochondrial biogenesis and function, as well as to identify potential molecules that can be targeted in order to modulate mitochondrial function in insulin resistance.

Study I provides evidence that the AMPKγ3 subunit can influence mitochondrial biogenesis in sedentary mice. Although, the γ3 subunit does not appear to be required for fully functional mitochondria in glycolytic skeletal muscle, elevating the basal activity in the heterotrimer by introducing the $\gamma3^{R225Q}$ mutation markedly increases markers of biogenesis and regulators of lipid oxidation. However, in the sedentary state, these adaptations do not translate into alterations in mitochondrial respiratory function. In study II, data are presented that show that respiratory function is markedly different in oxidative and glycolytic skeletal muscle, and liver, from sedentary wild-type mice. These results from intact permeabilized cell, using high-resolution respirometry, are in line with previous studies on mitochondrial protein expression, enzyme activity, and respiration measured in isolated mitochondria. Furthermore, tissue-specific adaptations in obese and insulin resistant *db/db* mice were demonstrated. The mitochondrial functional adaptations vary in accordance with metabolic characteristics; glycolytic EDL skeletal muscle has increased mitochondrial biogenesis and oxidative capacity, while mitochondrial function in oxidative soleus muscle is similar in lean and *db/db* mice. Finally, the *db/db* liver has blunted maximal electron transport capacity, possible due to an ETC complex IV bottleneck and decreased abundance of complex I. In study III, evidence is provided supporting tissue-specific differences in mitochondrial adaptations in *ob/ob* mice versus *db/db* mice; especially in liver. Liver mitochondrial respiration was depressed to a similar degree as found in *db/db* mice, but protein abundance of specific targets differed; the complex II marker was decreased, with a trend for increased abundance of the complex I marker. EDL muscle from *ob/ob* mice has enhanced maximal electron transport capacity, with corresponding increases in markers of complex II, IV and ATP synthase. In oxidative soleus muscle, mitochondrial respiratory capacity was unaltered in response to both obesity and leptin-treatment.

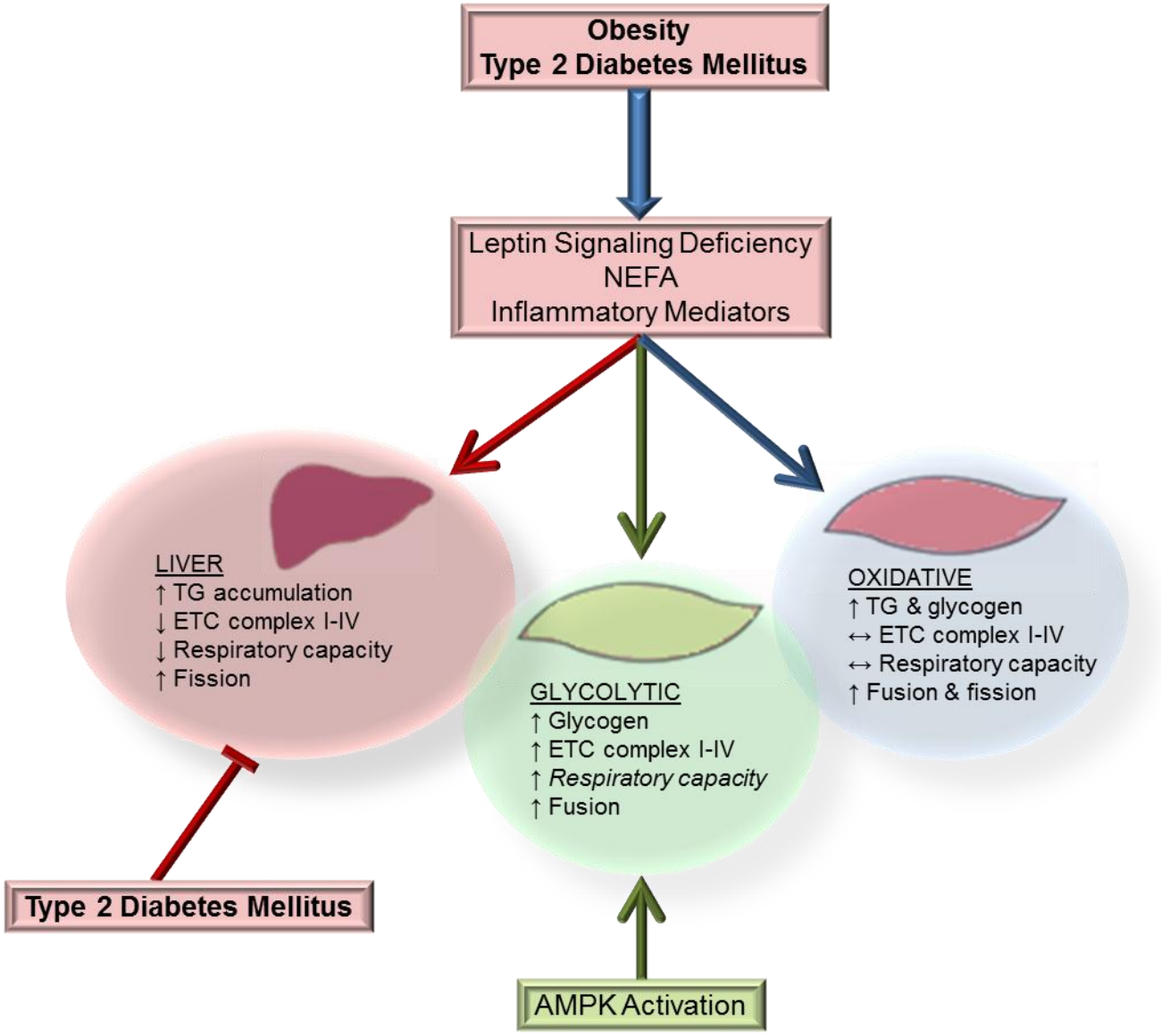

Figure 14. Summary of the findings presented in this thesis. Obesity caused by deficient leptin signaling results in tissue-specific adaptations in mitochondrial function and biogenesis. Hepatic mitochondrial function is blunted, which is reflected in specific electron transport chain complexes, in addition to extopic lipid accumulation and increased mitochondrial fission. Glycolytic skeletal muscle adapts to this condition by enhancing glycogen storage, mitochondrial respiration, electron transport chain proteins and mitochondrial fusion. Oxidative muscle mitochondrial function remains unaltered, while biogenesis and fuel storage is enhanced. AMPK activation in glycolytic muscle also stimulates glycogen storage, electron transport chain protein abundance and mitochondrial biogenesis, but does not alter respiratory function. ETC – electron transport chain, NEFA – non-esterified fatty acids, TG – triacylglyceride.

In conclusion (Fig. 14), these three studies provide evidence that mitochondrial biogenesis, in terms of dynamics and regulators of substrate oxidation, is sensitive to both chronic and more acute perturbations in metabolic status. Mitochondrial respiratory function appears more stable in the tissues investigated and does not readily change in response to short-term leptin treatment, in spite of profound whole-body effects on fuel mobilization. Monogenetic obesity, in contrast,

results in notable and tissue-specific adaptations. Leptin and AMPK signaling are interconnected in the central nervous system and peripheral tissues. These studies show that tissue-specific AMPK activation and systemic stimulation of the leptin/AMPK signaling axes are insufficient to induce improvements in mitochondrial respiratory function in the absence of increased energy demand.

There are several aspects of mitochondrial function terms of substrate oxidation that remain to be explored in light of metabolic disease – especially in peripheral tissues. First, the apparent disconnect between white adipose mass and centrally stimulated food intake may constitute a central deficiency in obesity. Hence, the combined effect of restored leptin signaling and exercise warrants further study, as this would alter both signals of systemic energy storage and tissue-specific energy demand. Although leptin treatment has proven inefficient in people (likely due to saturation of transporters and cellular desensitization), there may be promising pharmaceutical targets in terms in leptin sensitizers. Second, the integration of the complex network of mitochondrial substrate transporters, mtDNA maintenance and membrane dynamics remains to be elucidated in both human and experimental obesity. Of the targets investigated in this thesis, the ones most responsive to obesity induced by leptin resistance/deficiency, short-term leptin treatment and tissue-specific AMPK activation were proteins mediating membrane dynamics and mitochondrial DNA maintenance. Furthermore, mitochondrial ultrastructural alterations have been identified in various tissues in association with human obesity and T2DM. Since the function of the electron transfer system complexes is affected by mitochondrial membrane structure and composition, it would be of great interest to determine the temporal relationship between alterations in mitochondrial biogenesis and function, and how they can be modulated by exercise, diet and pharmacological treatment. Third, there is much to be discovered in the field of posttranslational modification of ETS complex activity – especially in terms of ROS signaling – and how it is affected by metabolic deregulation or improvements. Experimental obesity is correlated with mitochondrial protein hyperacetylation, and ROS-mediated oxidation exerts functional regulation of specific mitochondrial enzymes; including ETC complex I. Hence, regulation of enzymatic activity by substrate oxidation intermediates may play an important role in mitochondrial metabolism. Finally, tissue-specific mitochondrial function may be an important issue to address in light of the multi-organ nature of insulin resistance; investigating oxidative phosphorylation in white adipose tissue, gut, specific regions of the brain, and vascular tissue may expand our understanding of the integration and timing of pathological processes in different tissues throughout the body,

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