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CENTRAL AND PERIPHERAL MECHANISMS REGULATING ENERGY AND GLUCOSE HOMEOSTASIS

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Matthew 7:7 “Ask, and it will be given to you; seek, and you will find; knock, and it will be opened to you.”

ABSTRACT

Obesity is a major health concern across the globe and is often associated with insulin resistance and type 2 diabetes. The coupling of energy intake and expenditure is tightly regulated by central and peripheral mechanisms and is dysregulated in obesity. This thesis focuses on the role of central and peripheral mechanisms regulated by signaling via the leptin receptor and AMP-activated protein kinase (AMPK) in regulating energy and glucose homeostasis.

Leptin signaling through the long form of the leptin receptor (LepRb) plays a role in the regulation of glucose and energy homeostasis. Leptin action is mediated by phosphorylation of several tyrosine residues on LepRb, of which Tyr985 plays a major role. The aim of Study I was to elucidate the role of LepRb-Tyr985 in glucose metabolism. LepRb-Tyr985 mutant mice (*l/l* mice) that lack feedback inhibition of LepRb signaling had improved glucose tolerance and insulin sensitivity. Euglycemic-hyperinsulinemic clamp studies performed in *l/l* mice revealed enhanced hepatic and peripheral insulin sensitivity. Thus, LepRb-Tyr985-mediated signals regulate whole-body glucose metabolism and insulin sensitivity.

Tissue-specific alterations in mitochondrial respiration have been implicated in obesity and type 2 diabetes. The aim of Study II was to determine the role of leptin in regulating tissue-specific mitochondrial respiration in obese leptin-deficient *ob/ob* mice. Hepatic mitochondrial respiration was reduced in *ob/ob* mice and unaltered by short-term leptin treatment. Mitochondrial electron transport capacity was enhanced in glycolytic extensor digitorum longus (EDL) muscle, whereas mitochondrial function in oxidative soleus muscle was unaltered by obesity or leptin treatment in *ob/ob* mice. The present study highlights the tissue-specific mitochondrial adaptations imposed by obesity and its modulation by short-term leptin treatment.

AMPK is activated in response to cellular energy demand and turns on cellular processes that restore energy balance. Skeletal muscle overexpression of an activating form of AMPK (AMPK$\gamma 3^{R225Q}$) provides protection from high-fat diet induced insulin resistance. Study III was designed to test the hypothesis that skeletal muscle-specific expression of the AMPK$\gamma 3^{R225Q}$ isoform rescues the metabolic abnormalities associated with leptin deficiency in *ob/ob* mice (*ob/ob*-$\gamma 3^{R225Q}$). The AMPK$\gamma 3^{R225Q}$ mutation confers favorable metabolic adaptations including increased skeletal muscle glycogen content and decreased intramuscular triglyceride content, but glucose tolerance and skeletal muscle insulin-stimulated glucose uptake was unaltered. This implies that central defects arising from leptin deficiency overrides many of the positive benefits brought about by peripheral AMPK signaling.

Collectively, the results presented in this thesis highlight the role of leptin receptor and AMPK signaling in the regulation of glucose and lipid metabolism from a whole-body perspective. Strategies targeted at improving leptin sensitivity and skeletal muscle-specific AMPK activation open up new venues for the treatment of metabolic complications associated with diabetes and obesity.

LIST OF PUBLICATIONS

I. **Tom RZ**, Sjögren RJ, Vieira E, Glund S, Iglesias-Gutiérrez E, Garcia-Roves PM, Myers MG Jr., Björnholm M.
Increased hepatic insulin sensitivity in mice lacking inhibitory leptin receptor signals. *Endocrinology* 152(6):2237-46, 2011.

II. Holmström MH, **Tom RZ**, Björnholm M, Garcia-Roves PM, Zierath JR.
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III. **Tom RZ***, Garcia-Roves PM*, Sjögren RJ, Jiang LQ, Holmström MH, Deshmukh AS, Vieira E, Björnholm M, Zierath JR.
Effects of AMPK activation on insulin sensitivity and metabolism in leptin-deficient *ob/ob* mice. Under Revision, *Diabetes* *These authors have contributed equally to the work

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The Rab-GTPase-activating protein TBC1D1 regulates skeletal muscle glucose metabolism. *Am J Physiol Endocrinol Metab* 303(4):E524-33, 2012.

II. Deshmukh AS, Glund S, **Tom RZ,** Zierath JR.
Role of the AMPKgamma3 isoform in hypoxia-stimulated glucose transport in glycolytic skeletal muscle. *Am J Physiol Endocrinol Metab* 297(6):E1388-94, 2009.

CONTENTS

1 Introduction ... 1
  1.1 Obesity and insulin resistance ... 1
  1.2 Role of the hypothalamus in energy metabolism ... 4
    1.2.1 Hypothalamus and feedback control system ... 4
    1.2.2 Lipostat theory and afferent signals to the hypothalamus ... 4
    1.2.3 Neuropeptides and hypothalamic nuclei in energy balance ... 6
    1.2.4 Leptin receptor signaling ... 7
    1.2.5 Regulation of glucose homeostasis by leptin ... 8
  1.3 Mitochondria and metabolic disorders ... 10
    1.3.1 Mitochondrial structure ... 10
    1.3.2 Mitochondrial DNA transcription ... 10
    1.3.3 Mitochondrial respiration ... 10
    1.3.4 Mitochondria and metabolic disorders ... 11
  1.4 AMP-activated protein kinase (AMPK) ... 12
    1.4.1 Regulation of AMPK and upstream kinases ... 12
    1.4.2 AMPK and metabolism ... 13
    1.4.3 Drugs and mutations that modulates AMPK activity ... 13
2 Aims ... 15
3 Experimental Section ... 16
  3.1 Animals ... 16
  3.2 Experimental techniques to measure glucose and insulin sensitivity ... 16
    3.2.1 *In vivo* measurements ... 16
    3.2.2 *In vitro* measurement of insulin sensitivity ... 18
  3.3 *In vitro* measurement of lipid oxidation ... 19
  3.4 Analytical methods ... 19
    3.4.1 Immunoblot analysis ... 19
    3.4.2 Determination of triglyceride content ... 20
    3.4.3 Determination of glycogen content ... 20
  3.5 RNA purification and quantitative real time RT-PCR ... 20
  3.6 Mitochondrial respirometry ... 21
    3.6.1 General methodology ... 21
    3.6.2 Respirometry measurements ... 21
  3.7 *In vivo* leptin treatment ... 22
  3.8 Statistical analyses ... 22
4 Results and Discussion ... 23
  4.1 Leptin signaling and glucose homeostasis ... 23
    4.1.1 LepRb-Tyr985 regulates whole-body glucose homeostasis ... 23
    4.1.2 LepRb-Tyr985 mediates hepatic insulin sensitivity ... 25
    4.1.3 Limitations of Study I ... 26
  4.2 Mitochondrial function in *ob/ob* mice: Role of leptin ... 26
    4.2.1 Mitochondrial respiration in glycolytic skeletal muscle ... 27
    4.2.2 Mitochondrial respiration in soleus muscle from *ob/ob* mice is unaltered ... 29
    4.2.3 Hepatic mitochondrial respiration is impaired in *ob/ob* mice ... 30
    4.2.4 Effect of leptin treatment on whole-body energy metabolism ... 32

4.2.5 Limitations of Study II 33
4.3 AMPK activation in the context of insulin resistance associated with leptin deficiency 33
4.3.1 AMPK activation and insulin sensitivity 33
4.3.2 AMPK $\gamma3^{R225Q}$ increases skeletal muscle glycogen content 34
4.3.3 AMPK$\gamma3^{R225Q}$ and lipid oxidation and mitochondrial respiration . 34
4.3.4 AMPK activation and obesity 35
4.4 Summary 38
5 Conclusions and Future Perspectives 39
6 Acknowledgements 41
7 References 43

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
AgRP	Agouti related peptide
AICAR	5-aminoimadazole-4-carboximide-1-β-4-ribofuranoside
PKB	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
CAMKKβ	Calmodulin-dependent protein kinase kinase beta
CART	Cocaine and amphetamine related transcript
CBS	Cystathione-β-synthase
CNS	Central nervous system
CPT 1	Carnitine palmitoyl transferase 1
CRF	Corticotropin releasing factor
DAG	Diacylglycerol
DNMIL	Dynamin-1-like protein
EDL	Extensor digitorium longus
ERK	Extracellular signal-regulated kinase
ETS	Electron transport system
FADH2	Flavin adenine dinucleotide
FCCP	Carbonylcyanide 4-(trifluoromethoxy)pheny hydrazone
FCR	Flux control ratio
FFA	Free fatty acid
G6Pc	Glucose-6-phosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter 4
GS	Glycogen synthase
GSK	Glycogen synthase kinase
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ICV	Intracerebroventricular
IKKB	Inhibitor of nuclear factor-kappa B kinase-B
IL-6	Interleukin-6
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin receptor
IRS	Insulin receptor substrate
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
KHB	Krebs-Henseleit buffer
LepR	Leptin receptor
LHA	Lateral hypothalamic area
LKB1	Liver kinase B1
MC3/MC4	Melanocortin receptors 3/4

MFN2	Mitofusin 2
MSH	Melanocyte-stimulating hormone
MTCO1	Mitochondrially encoded cytochrome c oxidase 1
MTERF3	Mitochondrial transcription termination factor 3
NADH	Nicotinamide adenine dinucleotide
NDUFA9	NADH dehydrogenase 1 alpha subcomplex subunit 9
NPY	Neuropeptide Y
OPA1	Mitochondrial dynamin-like 120 kDa protein
OXPHOS	Oxidative phosphorylation
PAI-1	Plasminogen-activator inhibitor 1
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
POMC	Proopiomelanocortin
PPARα	Peroxisome proliferator-activated receptor α
PVDF	Polyvinylidene difluoride
PVN	Paraventricular nucleus
RBP-4	Retinol-binding protein 4
SCD1	Stearoyl-CoA desaturase 1
SDHA	Succinate dehydrogenase complex subunit A
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFKs	Src family kinases
SH2	Src-homology-2
SHP2	SH2 domain-containing tyrosine phosphatase
SIRT1	Silent mating type information regulation 2 homolog 1
SOCS3	Suppressor of cytokine signaling 3
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline containing 0.02% Tween 20
Tfam	Mitochondrial transcription factor A
TNF-α	Tumor necrosis factor alpha
UQCRC1	Ubiquinol-cytochrome c reductase core protein 1
VMH	Ventromedial hypothalamus

1 INTRODUCTION

Obesity is derived from the Latin word “Obesus” meaning fat. There is a rapid increase of obesity worldwide. The World Health Organization defines overweight and obesity as a condition in which abnormal or excessive fat accumulation poses a health risk (WHO-report 2000; Ofei 2005). Body mass index (BMI) which is the ratio of body weight in kilograms to the height in meter square defines the border that determines whether a person is underweight (BMI <18.5 kg/m^2), normal (BMI 18.5-24.9 kg/m^2), overweight (BMI 25-29.9 kg/m^2) or obese (BMI >30 kg/m^2 (WHO-report 2000). The caveat with BMI is that it neither takes into consideration whether the weight is predominantly attributed to fat or lean mass nor the distribution of the fat mass. Another more reliable and accurate way to measure adiposity is by calculating the waist to hip ratio (Kissebah et al. 1994). As the prevalence of obesity is increasing dramatically, efficient treatment strategies for the prevention and treatment need to be developed.

Obesity is often associated with metabolic abnormalities. The excess accrual of visceral fat acts as an endocrine tissue, secreting a variety of hormones and cytokines that can trigger metabolic abnormalities across various organ systems, which conglomerate and bring about the metabolic syndrome. The metabolic syndrome is a cluster of metabolic complications (dyslipidemia, hypertension, dysregulated glucose homeostasis, visceral adiposity and insulin resistance) that increases the risk of coronary heart disease and type 2 diabetes (Alberti et al. 1998; Zhu et al. 2002; Kassi et al. 2011). Obesity also predisposes a person to develop stroke, osteoarthritis and certain types of carcinomas (uterus, ovaries, breast, colon, rectum and prostate) (Aleksandrova et al. 2013; Esposito et al. 2013; Olsen et al. 2013; Rundle et al. 2013). Understanding the etiology and development of obesity can shed light into the development of effective strategies that can bring down the rate and rise of obesity and its associated comorbidities.

Obesity mainly results from an imbalance of energy intake and expenditure. Excess energy that is not immediately required is stored in the form of glycogen and triglyceride for later use (Owen et al. 1979). The coupling of energy expenditure and energy intake is a tightly regulated process that is brought about by interaction between different organ and hormonal systems. The central nervous system (CNS) plays a crucial role by acting as a master regulator, integrating signals from the periphery and regulating metabolic processes to ensure optimum energy balance of the organism. When there is a persistent mismatch between energy intake and energy expenditure, there will be weight gain in the form of fat accumulation if the organism is in positive energy balance and weight loss if there is a negative energy balance. Persistent positive energy balance leads to obesity (Stanhope et al. 2008; Kelly et al. 2009).

1.1 OBESITY AND INSULIN RESISTANCE

Obesity and insulin resistance are closely connected and the constellation predisposes for type 2 diabetes and associated metabolic complications. Obesity is often associated with a dysregulation of endocrine, neural and inflammatory pathways that contribute to the development of insulin resistance. Insulin resistance is defined as a condition in which normal insulin levels elicit a subnormal biological response in insulin-sensitive

tissues such as skeletal muscle, adipose tissue and liver (Lima et al. 2002). In insulin resistant conditions, insulin-stimulated glucose uptake is compromised in skeletal muscle and adipose tissue with concomitant defects in the suppression of hepatic glucose production. Understanding the molecular pathways of insulin signaling is important to bring about appropriate therapeutic interventions to treat metabolic disease.

Metabolic perturbations associated with obesity affect the insulin signaling cascade in insulin-sensitive tissues. Binding of insulin to the insulin receptor (IR) leads to auto-phosphorylation of the tyrosine residues and a conformational change of the receptor (White et al. 1994). This allows the insulin receptor substrate (IRS) proteins to interact and associate with the IR, and in turn undergo phosphorylation (Sun et al. 1991). Phosphorylated IRS allows Src-homology-2 domain (SH2 domain) containing molecules to bind to phosphorylated tyrosine residues on IRS (White 1998; Virkamaki et al. 1999). The SH2 domain containing enzyme phosphoinositide (PI) 3-kinase interacts with phosphorylated IRS and in turn activates Akt/PKB (protein kinase B), which is a key molecule regulating insulin-mediated metabolic actions (Cross et al. 1995; Kohn et al. 1996; Harris et al. 2003). Insulin-stimulated glucose uptake in skeletal muscle accounts for 75% of glucose metabolism in the postprandial state (DeFronzo 1988; Zierath et al. 1998; DeFronzo et al. 2009). Under insulin-stimulated conditions, Akt is phosphorylated in skeletal muscle. Akt phosphorylates and inactivates the Akt substrate of 160 kDa (AS160), which in turn leads to translocation of glucose transporter (GLUT) 4 to the plasma membrane, and facilitates glucose entry to the cell (Karlsson et al. 2005; Larance et al. 2005; Miinea et al. 2005) (Fig 1). In parallel, Akt also stimulates glycogen synthesis in skeletal muscle. The ability of skeletal muscle to take up glucose in response to insulin is critical for the maintenance of glucose homeostasis and is blunted in conditions of insulin resistance (DeFronzo et al. 1982; DeFronzo et al. 1985; Zierath et al. 1998).

In liver, insulin signaling has a crucial role in the regulation of hepatic glucose production and lipogenesis. In response to insulin stimulation, phosphorylated Akt inactivates forkhead transcription factor 3, which results in decreased expression and activity of gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pc), which suppress hepatic glucose production (Matsuura et al. 1975; Haeusler et al. 2010; Zhang et al. 2012). At the same time, hepatic insulin signaling stimulates lipogenesis via Akt, and regulates the expression of a key lipogenic gene namely sterol regulatory element-binding protein-1C (Foretz et al. 1999; Fleischmann et al. 2000; Ono et al. 2003; Leavens et al. 2009). In insulin resistant states, the ability of insulin to suppress hepatic glucose production is blunted (DeFronzo et al. 1982).

In adipocytes insulin stimulates glucose uptake via GLUT4 and favors lipogenesis with concomitant suppression of lipolysis. Insulin suppresses the activity of the rate limiting lipolytic enzyme, hormone sensitive lipase, thereby inhibiting lipolysis (Anthonsen et al. 1998; Kitamura et al. 1999). Insulin resistance in adipose tissue is accompanied by defective suppression of lipolysis resulting in increased circulating free fatty acid levels further leading to insulin resistance in other tissues (Landin et al. 1990; Magkos et al.

2012). Defects in the insulin signaling cascade affect multiple tissues and contribute to the metabolic derangements associated with obesity.

In obesity, visceral adipose tissue hypersecretes proinflammatory adipokines and free fatty acids, which affect hepatic and peripheral insulin sensitivity and ultimately lead to hyperglycemia. Increased levels of circulating fatty acids can compete with glucose for oxidation and consequently reduce insulin-stimulated glucose uptake in the peripheral tissues (Randle et al. 1963; Shulman 2000; Hue et al. 2009). Fatty acid metabolites such as diacylglycerol (DAG), ceramides and acyl-CoAs induce insulin resistance by activating protein kinases such as protein kinase C (PKC), c-Jun N-terminal kinase (JNK) and IKKB (inhibitor of nuclear factor-kappa B kinase-B) (Griffin et al. 1999; Samuel et al. 2004). These kinases increase serine phosphorylation of IRS and consequently reduce insulin signal transduction (Li et al. 2004). In addition to fatty acids, several lines of evidences suggests that elevated levels of adipokines including resistin, plasminogen-activator inhibitor-1 (PAI-1), interleukin (IL)-6, tumor necrosis factor (TNF)-α and retinol-binding protein (RBP) -4 can induce insulin resistance (Hotamisligil et al. 1993; Hotamisligil et al. 1996; Mitrou et al. 2011). Furthermore, adiponectin, which confers insulin sensitivity, is decreased in obese conditions (Stefan et al. 2002). These changes affect the ability of insulin to inhibit hepatic glucose production and stimulate peripheral glucose uptake in skeletal muscle and adipose tissue. Pancreatic β-cells hypersecrete insulin to compensate for the insulin resistance (Clark et al. 2001). When the β-cells of the pancreas fail to secrete enough insulin to overcome the insulin resistance, clinical manifestation of diabetes in the form of hyperglycemia ensues (Guillausseau et al. 2008).

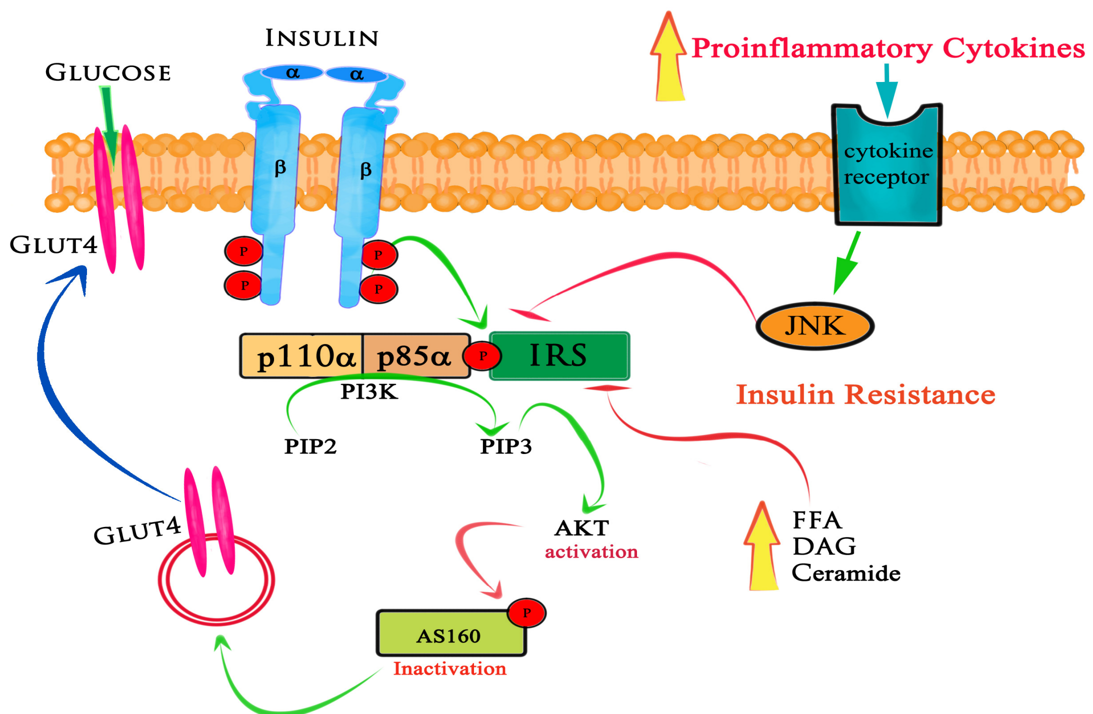

Figure 1: Glucose uptake in insulin-sensitive peripheral tissues. In insulin-resistant conditions, increased levels of FFA and intracellular lipid metabolites, along with proinflammatory cytokines, negatively affect the insulin signaling cascade.

1.2 ROLE OF THE HYPOTHALAMUS IN ENERGY METABOLISM

Early studies of hypothalamic lesions in rats stress the role of the hypothalamus in regulating adipose tissue mass (Hetherington 1940). Bilateral symmetrical lesions in the ventromedial hypothalamic nuclei (VMH) resulted in substantial hyperphagia and consequently adiposity. The same lesions in the lateral hypothalamic area (LHA) led to complete absence of spontaneous eating (Anand et al. 1951). These hypothalamic lesions led to the conclusion that the LHA is a feeding center and the medial hypothalamic region, a satiety center (Anand et al. 1955).

1.2.1 Hypothalamus and feedback control system

Hypothalamic lesion studies in parabiotic rats suggested that body weight changes are sensed by the hypothalamus (Hervey 1959). Parabiosis is the surgical technique of anastomosing blood vessels of two animals of the same genetic background in such a way that exchange of blood occurs between the animals through the peritoneal capillaries. Hypothalamic lesions in one of the parabiotic pair led to hyperphagia and subsequent weight gain due to increased adiposity. This in-turn led to aphagia in the other animal in the pair and was thought to be mediated by changes in the levels of blood metabolites from the lesioned rat (Kennedy 1953). Parabiotic experiments carried out on genetically diabetic and obese *db/db* mice with normal mice recapitulated the results from parabiotic studies of rats with hypothalamic lesions. Parabiosis between *db/db* mice and lean mice resulted in decreased food intake in the lean mice (Coleman et al. 1969). These results underscore the concept of a circulating factor released in *db/db* mice that in turn signals the satiety center to regulate food intake in normal mice. Parabiotic studies on another genetically obese and diabetic mouse model (*ob/ob* mice) with lean mice did not affect food intake in both parabionts. In addition, when *ob/ob* mice were parabiosed with *db/db* mice, the *ob/ob* mice had reduced food intake. These experiments led to the conclusion that *ob/ob* mice have normal hypothalamic satiety centers that are responsive to a circulating factor secreted by the *db/db* mice (Coleman 1973). These hypothalamic lesion studies in rats and parabiotic experiments in mice established the concept of a feedback system regulating food intake at the level of the hypothalamus (Fig 2).

1.2.2 Lipostat theory and afferent signals to the hypothalamus

The lipostat theory proposes that the CNS regulates body fat mass, and that the metabolized fat products circulating in plasma affect energy balance through hypothalamic interactions (Kennedy 1953). Later, a positional cloning approach was used to identify the product of the obese (*ob*) gene secreted from the adipose tissue. The *ob* gene encodes an adipose tissue messenger RNA of 4.5 kb with 84% amino acid sequence similarity between human and mouse and has the characteristics of a secreted protein. The *ob/ob* mouse strain has a nonsense mutation in codon 105 resulting in the expression of twenty-fold higher *ob* RNA levels which was associated with a non-functional gene product resulting in increased mRNA levels as part of a possible feedback loop (Zhang et al. 1994). This data provides evidence to suggest that the adipose tissue mediated *ob* gene product is a signal to regulate the size of the body fat depot (Zhang et al. 1994). The *ob* gene product of 16 kDa was cloned, and protein was produced and administered to lean wild-type, *ob/ob* and *db/db* mice. Administration of

the isolated protein resulted in a reduction of both food intake and body weight in wild-type and *ob/ob* mice, whereas *db/db* mice did not respond. This not only proved the endocrine hypothesis of the *ob* gene product, but was the first evidence for the biological action of the adipocyte-secreted hormone "Leptin", rooted from the Greek word "Leptós" meaning thin (Halaas et al. 1995). Several other candidate molecules were discovered and proposed to act as afferent signals to hypothalamus and mediate food intake and body weight. Among them glucose, free fatty acids and peptides like cholecystokinin, neuropeptide Y (NPY), corticotropin releasing factor (CRF) were evaluated, but none were not found to be involved in the long-term regulation of food intake and body weight (Arase et al. 1988; Kulkosky et al. 1988; Peikin 1989). Thus, adipocyte-derived leptin signals to the hypothalamus to regulate food intake.

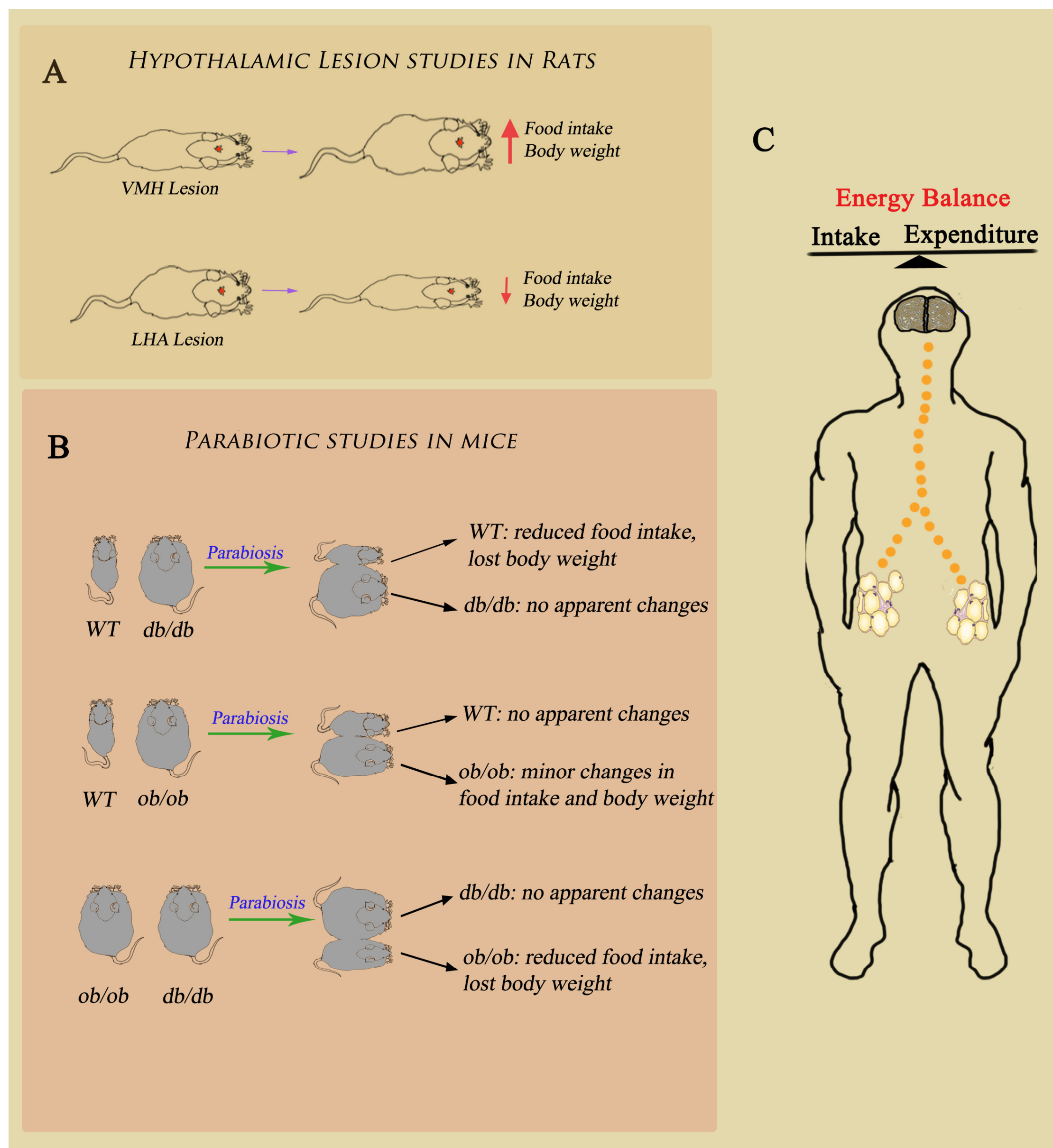

Figure 2: A: Hypothalamic lesion studies in rats highlight the importance of the hypothalamus in the regulation of food intake. B: Parabiotic experiments in mice underscoring the concept of integration of peripheral signals in the hypothalamus, thereby regulating food intake and energy expenditure. C: Integrated view of the regulation of energy homeostasis.

1.2.3 Neuropeptides and hypothalamic nuclei in energy balance

Various neuropeptides in the hypothalamic circuitry are involved in the regulation of energy homeostasis. The reduction in body weight and food intake in response to intracerebroventricular (ICV) injection of leptin into the third ventricle of wild-type and *ob/ob* mice implies that one or more hypothalamic nuclei are target sites for leptin action (Campfield et al. 1995). Leptin acts in various hypothalamic nuclei (arcuate nucleus, paraventricular nucleus (PVN), VMH and LHA) to modulate the levels of neuropeptides in the hypothalamus to regulate energy homeostasis.

1.2.3.1 Arcuate nucleus

The arcuate nucleus contains both orexigenic (appetite-stimulating) and anorexigenic (appetite-suppressing) neurons that are responsive to leptin. Orexigenic neurons include the agouti related peptide (AgRP) and NPY expressing neurons. The most abundant and potent orexigenic neuropeptide NPY (Adrian et al. 1983) is co-expressed with the leptin receptor in the arcuate neuronal population, which suggests a role for leptin in the regulation of NPY (Hakansson et al. 1996; Mercer et al. 1996). NPY mediates orexigenic actions by signaling via G-protein coupled receptors (Y1 to Y5). The regulation of NPY expression by leptin was underscored through studies of *ob/ob* mice, where hormone supplementation reduced high expression levels of NPY, independent of changes in body weight (Wilding et al. 1993; Schwartz et al. 1996). High levels of AgRP mRNA in the arcuate nucleus of *ob/ob* and *db/db* mice suggest a possible role for leptin in its regulation (Shutter et al. 1997). Immunohistochemistry studies on mediobasal arcuate nucleus identified co-localization of AgRP and the leptin receptor neurons. Leptin treatment of *ob/ob* mice resulted in a substantial reduction of AgRP mRNA levels (Wilson et al. 1999). Furthermore, leptin blunted the fasting induced up-regulation of AgRP in arcuate nucleus. This suggests that part of the anorexigenic response to leptin is mediated by reducing the orexigenic neuropeptides in the hypothalamus.

Anorexigenic neurons in the arcuate nucleus constitute proopiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART) expressing neurons. The hypothalamic POMC system is important for the regulation of body weight and energy homeostasis and mutations in the POMC gene lead to obesity (Comuzzie et al. 1997). Proopiomelanocortin neurons express POMC, which is proteolytically cleaved into α-melanocyte-stimulating hormone (α-MSH) and binds to melanocortin receptors (MC3/MC4) and mediates anorexigenic responses (Mountjoy et al. 1994; Harrold et al. 1999). POMC gene expression is reduced with short-term food restriction and is restored by leptin treatment (Cheung et al. 1997; Cowley et al. 2001). Another anorexigenic neuropeptide that regulates feeding behavior is CART. CART is predominantly found in the dorsomedial part of the arcuate nucleus and is responsive to leptin. Food deprivation reduced CART mRNA levels in the arcuate nucleus and leptin treatment of *ob/ob* mice, which lacks CART expression, restored CART mRNA levels (Kristensen et al. 1998). These experiments in the hypothalamic arcuate nucleus suggest a role of leptin in the regulation of food intake by modulating anorexigenic neuropeptides.

1.2.3.2 VMH, LHA and PVN

Leptin responsive neurons implicated in energy homeostasis are also present in the VMH, LHA and PVN. Conditional knock-out of leptin receptors in VMH neurons leads to the development of obesity (Bingham et al. 2008). Leptin action in LHA modulates the incentive to feed and decreases food intake and body weight (Leinninger et al. 2009). PVN has dense fenestration of NPY/AgRP neurons along with POMC expressing neurons. In response to leptin, α-MSH binds to MC-3/MC-4 receptors and antagonizes the action of NPY on food intake (Cowley et al. 1999). Thus, the action of leptin in VMH, LHA and PVN, along with the neurons in the arcuate nucleus plays a prominent role in the regulation of energy homeostasis and food intake.

1.2.4 Leptin receptor signaling

1.2.4.1 Leptin receptor

The leptin receptor (LepR) belongs to class I cytokine family and is encoded by the *db* locus. Several splice forms of the receptor (LepRa, b, c, d and e) exists. LepRb is the long form of the receptor, which has a functional cytoplasmic domain (Tartaglia et al. 1995; Chen et al. 1996; Lee et al. 1996). Among the different spliced forms, LepRe does not have cytoplasmic domain and forms a soluble receptor that has been implicated as a transport protein (Lee et al. 1996). The long LepRb form has conserved intracellular domains with sequence motifs important for binding of Janus kinase (Jak) and signal transducer and activator of transcription (STAT) that are involved in mediating intracellular signal transduction (Chen et al. 1996).

1.2.4.2 Signaling through the long form of the leptin receptor

Leptin receptor signaling is primarily mediated through LepRb. LepRb exists as a homodimer in the inactive state and has constitutively associated Jak2 and Src family kinases (SFKs). Binding of leptin to the LepRb induces a conformational change of the receptor that leads to transphosphorylation and transactivation of the Jak2 and SFKs (Ghilardi et al. 1997). Phosphorylated Jak2 and SFKs in turn phosphorylate critical tyrosine residues of LepRb namely Tyr985, Tyr1077 and Tyr1138 that mediates downstream signaling events. Phosphorylated Tyr1138 acts as a docking site for STAT3, which is phosphorylated by Jak2 (Baumann et al. 1996; Banks et al. 2000). This allows STAT3 to dimerize and translocate to the nucleus where it is involved in the transcription of a variety of genes, including suppressor of cytokine signaling (SOCS) 3. Leptin-mediated signaling through Tyr1077 is implicated in downstream signals originating from STAT5 (Gong et al. 2007). Tyr985 undergoes phosphorylation by Jak2, which allows for binding and activation of the tyrosine phosphatase SHP2. Phosphorylated Tyr985 does not only mediate SHP2/Grb2/ERK signaling, but also facilitates SOCS3-mediated feedback inhibition of LepRb signaling (Bjorbaek et al. 2000) These signaling pathways emerging from LepRb regulate multiple physiological functions that are important for energy homeostasis (Fig 3).

1.2.4.3 Physiological functions mediated by leptin receptor signaling

Besides long-term energy storage and regulation of food intake, leptin receptor signaling is involved in many physiological functions. Lack of functional leptin action in *ob/ob* mice results in sterility, whereas repeated leptin administration rescues

reproductive function (Chehab et al. 1996). Leptin signaling through melanocortin receptors regulate thyroid hormonal levels, which plays a role in setting the basal metabolic rate (Kim et al. 2000). In addition, the pleiotropic actions of leptin are also involved in the regulation of growth, bone remodeling, thermogenesis and immunity (Lord et al. 1998; Elefteriou et al. 2005; Rahmouni et al. 2009). Furthermore, the hormone plays an important role in the regulation of energy metabolism by mediating glucose and lipid metabolism. Thus multiple physiological functions are regulated by leptin receptor signaling.

1.2.5 Regulation of glucose homeostasis by leptin

The physiological role of functional leptin action in the regulation of glucose metabolism is highlighted from studies on *ob/ob* and *db/db* mice. The hormone exerts multiple effects on glucose homeostasis. The CNS is the main target for leptin action. Intracerebroventricular leptin infusion suppresses hepatic glucose production and stimulates peripheral glucose uptake (Kamohara et al. 1997; Liu et al. 1998). In addition to central action of leptin on glucose metabolism, *in vitro* studies provide evidence for a direct effect of leptin on glucose metabolism in isolated skeletal muscle and hepatocyte (Harris 1998; Aiston et al. 1999). Furthermore, leptin acutely inhibits insulin secretion from the β-cells (Khan et al. 2001). In contrast, chronic leptin treatment enhances glucose-induced insulin secretion (Khan et al. 2001). These studies point to the importance of leptin signaling in the regulation of glucose homeostasis, but the critical nodes on the leptin receptor mediating these actions are poorly understood. Evidence from studies of mice with disrupted LepRb-STAT3 signaling suggests that LepRb-STAT3 independent signals regulate glucose homeostasis (Bates et al. 2005). Leptin induces phosphorylation on LepRb at several tyrosine residues and among them Tyr985 has been implicated in leptin resistance and attenuation of LepRb signaling (Bjorbaek et al. 2000). Fed and fasted leptin and insulin levels are reduced in mice expressing a mutant form of LepRb-Tyr985 (Björnholm et al. 2007), which indicates that Tyr985 might play a role in the regulation of whole-body glucose metabolism.

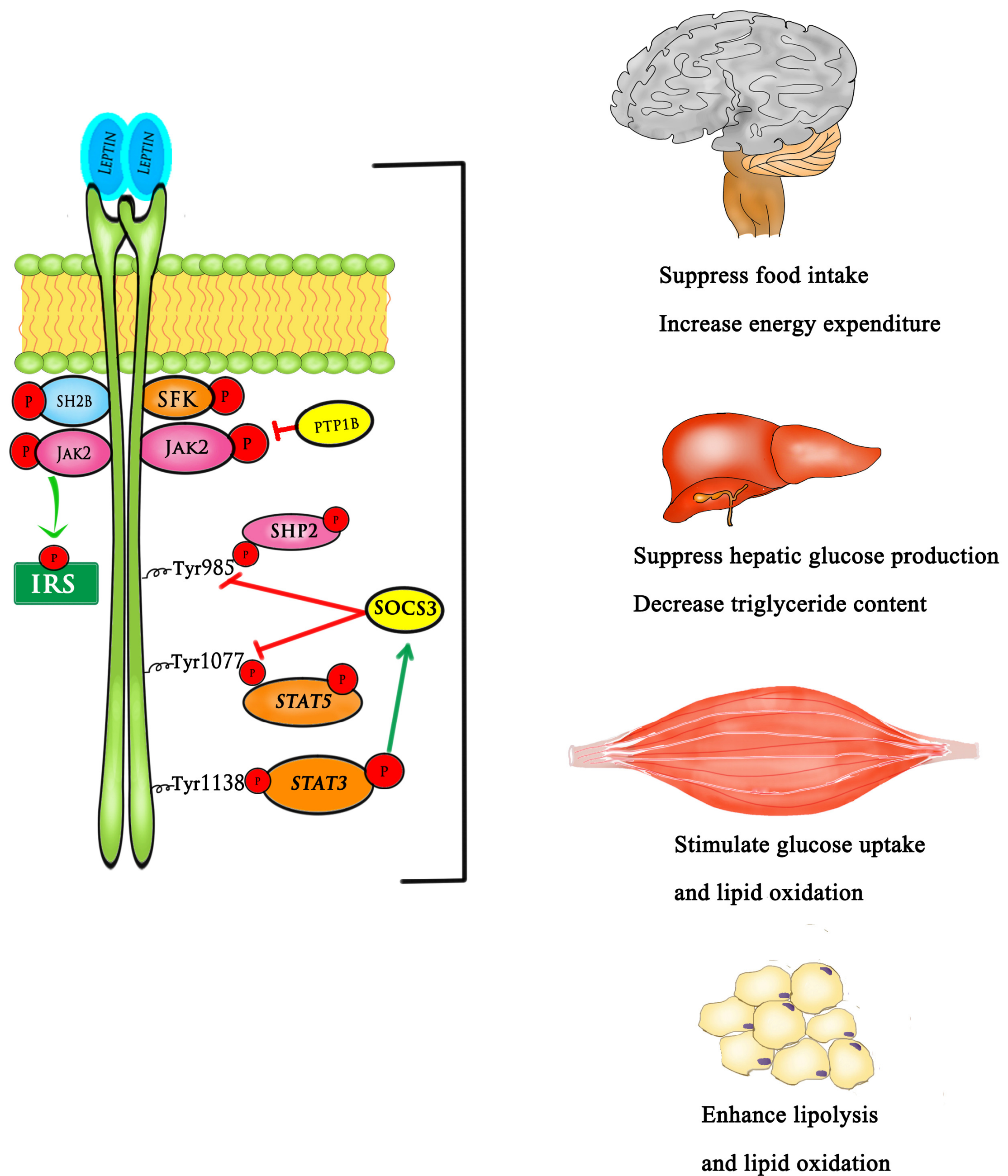

Figure 3: Leptin receptor signaling. Leptin binding to LepRb initiates a signaling cascade that leads to various leptin-mediated physiological functions. The central action of leptin is involved in long-term regulation of food intake and energy expenditure. Leptin also suppresses hepatic glucose production, stimulates peripheral glucose uptake and lipid oxidation by direct and indirect mechanisms. Leptin regulates adiposity by increasing lipolysis and lipid oxidation which are mediated by central and peripheral mechanisms.

1.3 MITOCHONDRIA AND METABOLIC DISORDERS

1.3.1 Mitochondrial structure

Mitochondria are DNA containing organelles that serve as the power house of the cell. Mitochondria generate ATP from different sources (glucose, fatty acids and amino acids), which fuel the citric acid cycle. The shape, size and number of mitochondria within a cell vary and are influenced by the type of tissue (Fernandez-Vizarra et al. 2011). Oxidative tissues like soleus muscle have more mitochondria compared to glycolytic muscle like extensor digitorum longus (EDL). Cardiac myocytes contain mitochondria that are larger in size and fewer in number compared to other tissues (Veltri et al. 1990). These mitochondrial adaptations are compatible with the different metabolic demands imposed on the tissue. Besides ATP and substrate production via the citric acid cycle, mitochondria have other functions including regulation of apoptosis, Ca^{2+} signaling and reactive oxygen species generation. These organelles are capable of morphological changes and redistribute within the cell upon demand by a process of fission and fusion, resulting in a dynamic network instead of an isolated mitochondrion. These functions of the mitochondria, along with the ATP generating capacity, emphasize its role in the regulation of energy homeostasis.

1.3.2 Mitochondrial DNA transcription

The mitochondrial genome (mtDNA) encodes approximately 3% of mitochondrial proteins and the remaining is transcriptionally regulated by nuclear DNA. Out of the 90 proteins of the respiratory chain complexes, 13 are encoded by mitochondrial DNA, which implies that the remaining proteins of the complexes are regulated by nuclear DNA (Peralta et al. 2012). Nuclear encoded regulatory proteins and transcription factors regulate mtDNA transcription and are targeted to the mitochondria by special transport proteins for assembly. Among the various transcription factors, mitochondrial transcription factor A (Tfam), the first described mitochondrial transcription factor, regulates mtDNA by binding and changing the structure of mtDNA by unwinding the promoter region (Fisher et al. 1988; Fisher et al. 1992). In response to cellular energy demand, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) or PGC-1β are activated and contribute to a coordinated expression of specific genes that regulate mitochondrial biogenesis (Bergeron et al. 2001; Amat et al. 2009). Lipid oxidation and membrane uncoupling are regulated at the gene level by the peroxisome proliferator-activated receptor (PPAR) family of transcription factors. The mitochondrial transcription termination factor 3 (MTERF3) is a negative regulator of mitochondrial transcription that interacts with the promoter region and suppresses transcription initiation (Linder et al. 2005; Park et al. 2007). Silent mating type information regulation 2 homolog 1 (SIRT1) has been implicated in the direct regulation of mitochondrial biogenesis (Aquilano et al. 2010). Thus, mitochondrial biogenesis and activity are integrated to meet cellular energy demands and are transcriptionally regulated by nuclear and mitochondrial encoded genome products.

1.3.3 Mitochondrial respiration

Energy production within the mitochondria is driven through the electron transport chain and is coupled to oxidative phosphorylation (OXPHOS). Complex I to complex V comprises the oxidative phosphorylation system and is located inside the inner

mitochondrial membrane. Among these complexes, complex II is exclusively encoded by mtDNA whereas the remaining complexes are derived from proteins encoded by both nuclear DNA and mtDNA (Falkenberg et al. 2007). Ultimate breakdown of carbohydrates and fatty acids generate reducing equivalents (NADH and FADH2) which will be oxidized in OXPHOS to NAD^+ and FAD. The oxidation generates electrons that are transferred to complex I and complex II and move along to complex IV and finally to molecular oxygen yielding water. The electrons flowing through the complexes drive proton translocation through the inner mitochondrial membrane, thereby generating a proton gradient across the inner mitochondrial membrane (Saraste 1999). This proton movement is mediated through complex I, III and IV (Saraste 1999) whereas complex II only serve as an entry point for electrons (Hagerhall 1997). The proton motive force that builds up finally drives the ATP synthase leading to the formation of ATP from ADP and inorganic phosphate (Boyer 1997). The synthesis of ATP is coupled to the energy requirement and, depending on cellular energy demand, oxidative phosphorylation and energy production are tightly regulated.

1.3.4 Mitochondria and metabolic disorders

1.3.4.1 Mitochondrial regulation of lipid metabolism

The regulation of lipid metabolism is a complex process in which mitochondria play a prominent role. Mitochondrial carnitine palmitoyl transferase (CPT)-1 residing on the outer mitochondrial membrane converts the intracellular fatty acyl-CoAs into fatty acyl carnitine that is translocated into the inner mitochondrial membrane by carnitine acyl carnitine translocase (Cohen et al. 1998). The inner mitochondrial membrane harbors CPT2, which converts fatty acyl carnitine back to fatty acyl-CoA and undergoes β-oxidation generating acetyl-CoA moieties that fuels the citric acid cycle. Mitochondrial CPT1 and acetyl-CoA carboxylase (ACC) play a role in the regulation of mitochondrial lipid oxidation. ACC regulates malonyl-CoA levels (Wakil et al. 1983). An increase in malonyl-CoA leads to inhibition of CPT1 thereby reducing mitochondrial entry of long chain fatty acyl-CoA, which in turn leads to fatty acid synthesis and triglyceride formation (McGarry et al. 1983; Rasmussen et al. 2002). Given the fact that ectopic lipid accumulation has detrimental effects on insulin sensitivity (Morino et al. 2006), understanding the defects in β-oxidation might be helpful in correcting this metabolic abnormality.

1.3.4.2 Mitochondria and type 2 diabetes

The primary role of mitochondria in the pathogenesis of insulin resistance and type 2 diabetes is controversial. Mitochondrial protein abundance and mitochondrial respiratory capacity is decreased in skeletal muscle biopsies obtained from type 2 diabetic patients and obese individuals (Kelley et al. 2002; Patti et al. 2003; Ritov et al. 2010). Mitochondrial ATP production has also been reported to be reduced in oxidative skeletal muscle from insulin resistant subjects. Studies performed using diabetic animal models provide evidence for defects in mitochondrial bioenergetics (Rogers et al. 1986). Other studies in skeletal muscle from type 2 diabetic patients provide evidence that mitochondrial performance is unaltered or even improved (De Feyter et al. 2008; Nair et al. 2008). Diabetic Goto-Kakizaki rats display enhanced hepatic mitochondrial bioenergetics (Ferreira et al. 1999). Glucose homeostasis and insulin sensitivity in

skeletal muscle are unaltered in a mouse model of respiratory chain dysfunction (Wredenberg et al. 2006). Animal models with impairments in mitochondrial oxidative phosphorylation displayed enhanced glucose tolerance and insulin sensitivity (Pospisilik et al. 2007). Furthermore, mitochondrial capacity in skeletal muscle is enhanced in animal models of obesity induced by high fat diet (Stephenson et al. 2012). Studies in *db/db* mice addressing the perturbations of mitochondrial function in different tissues points to tissue-specific adaptability of mitochondria in the facet of whole-body metabolic derangements (Holmström et al. 2012). These studies in obese *db/db* and high fat fed mice indicate leptin plays a role in mediating tissue-specific adaptation and/or alterations in mitochondrial function. Even though, a wealth of information regarding mitochondrial function and metabolic dysregulation exists, the exact role of leptin in mediating these effects is incompletely resolved.

1.4 AMP-ACTIVATED PROTEIN KINASE (AMPK)

The evolutionary conserved serine threonine kinase, initially identified as a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG)-CoA reductase kinase regulating cholesterol metabolism, was later identified as a master regulator of cellular energy metabolism (Ingebritsen et al. 1978; Clarke et al. 1990). AMPK, is a heterotrimeric complex comprised of an anchoring catalytic α-subunit coded by two different genes (α1 and α2), a regulatory β-subunit, encoded by β1 and β2, and a regulatory γ-subunit encoded by three different genes (γ1, γ2 and γ3), is highly responsive to stress signals that affect cellular energy status, thereby acting as a cellular fuel gauge. The catalytic activity of the α-subunit requires both the β- and γ-subunits and the triad can exist in 12 tissue-specific heterotrimeric combinations (Chen et al. 1999; Mahlapuu et al. 2004). Being highly sensitive to intracellular changes in AMP to ATP and ADP to ATP ratio (Oakhill et al. 2011), AMPK regulates a plethora of metabolic reactions with the final outcome of restoring the energy status of the cell. Stress signals like exercise, hypoxia, glucose deprivation and osmotic changes activate AMPK, which in turn shuts down energy demanding anabolic processes and simultaneously activates catabolic processes that yield energy, thereby restoring the intracellular energy balance (Fig 4). Therefore, AMPK regulates cellular energy homeostasis by monitoring and integrating the nutritional status of a cell.

1.4.1 Regulation of AMPK and upstream kinases

AMP activates AMPK by allosteric modulation and inhibits dephosphorylation of AMPK by protein phosphatase. This regulation requires an intact cystathione-β-synthase (CBS) domain in the γ-subunit (Davies et al. 1995; Sanders et al. 2007). In addition to AMP, ADP activates AMPK by binding to the γ-subunit (Oakhill et al. 2011). Activation of AMPK by AMP requires phosphorylation of the α-subunit on Thr172 on the activation loop by upstream kinase LKB1 (liver kinase B) and the extent of Thr172 phosphorylation is regulated by AMP levels (Hong et al. 2003; Woods et al. 2003; Sanders et al. 2007). The Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CAMKKβ) is another upstream kinase that activates AMPK in response to a rise in Ca^{2+} concentration, independent of AMP levels (Hawley et al. 1995; Hawley et al. 2005; Gormand et al. 2011). AMPK regulates energy homeostasis and the activation and inhibition of the enzyme are tightly regulated by AMP/ADP and upstream kinases.

1.4.2 AMPK and metabolism

AMPK has a potential role in regulating multiple metabolic pathways in relation to energy homeostasis. AMPK activation in the hypothalamus increases food intake (Minokoshi et al. 2004), while peripheral activation increases glucose and lipid metabolism (Merrill et al. 1997; Carling 2005). At the same time, activation of AMPK in response to cellular energy demand turns off energy consuming anabolic processes (Li et al. 2003; Chan et al. 2004). The final outcome of AMPK activation is the restoration of the energy balance of the organism.

1.4.2.1 AMPK and glucose metabolism

Activation of AMPK by exercise, muscle contraction, hypoxia or AMPK activators like 5-aminoimadazole-4-carboximide-1-β-4-ribofuranoside (AICAR) increases skeletal muscle glucose uptake (Mu et al. 2001; Jessen et al. 2003; Yu et al. 2003) via translocation of GLUT4 to plasma membrane (Kurth-Kraczek et al. 1999). The major fate of glucose entering the cell is either oxidation to meet immediate energy demands or storage in the form of glycogen. AMPK regulates the fate of glucose in both directions. *In vitro* studies have provided evidence that AMPK phosphorylates critical serine residues on glycogen synthase, which consequently inhibits glycogen synthesis and enhances glucose oxidation (Miyamoto et al. 2007). Conversely, chronic AMPK activation favors glycogen synthesis (Hunter et al. 2011; Vitzel et al. 2013). Increased glucose transport associated with AMPK activation results in glucose-6-phosphate accumulation that allosterically activates the glycogen synthase (GS) and overrides the inhibition imposed by AMPK (Hunter et al. 2011). These pathways can be potentially targeted to increase glucose transport and glycogen synthesis in the context of insulin resistance to provide a metabolic benefit for people with type 2 diabetes and obesity.

1.4.2.2 AMPK and lipid metabolism

Skeletal muscle relies on fatty acids as the major fuel source at rest and during sustained exercise. This process is regulated at the level of AMPK. AMPK activation inactivates ACC by phosphorylating critical serine residues on both ACCα and ACCβ (Kudo et al. 1996; Hutber et al. 1997). This in turn decreases malonyl-CoA levels that negatively regulates CPT1, allowing transport of long chain fatty acids into the mitochondria for β-oxidation. In addition to exercise, AICAR, leptin and adiponectin also activate AMPK and increase lipid oxidation (Hutber et al. 1997; Kaushik et al. 2001; Minokoshi et al. 2002; Yamauchi et al. 2002). AMPK-induced lipid oxidation can decrease the elevated intramuscular triglyceride levels associated with insulin resistance and obesity and this may confer an insulin sensitizing action on glucose metabolism.

1.4.3 Drugs and mutations that modulates AMPK activity

Anti-diabetic drugs such as metformin and rosiglitazone and hormones such as leptin and adiponectin activate AMPK by distinct mechanisms and have therapeutic benefits to ameliorate insulin resistance (Fryer et al. 2002; Minokoshi et al. 2002; Yamauchi et al. 2002). In addition, a single naturally occurring point mutation on AMPK ($\gamma 3^{R225Q}$) plays a positive role in glucose and lipid metabolism. The mutation initially identified in RN^- Hampshire pigs was associated with increased glycogen content in skeletal

muscle and increased citrate synthase activity, indicative of an increased oxidative capacity (Milan et al. 2000). Overexpression of this gain-of-function mutation in glycolytic skeletal muscle of mice fed a high-fat diet improves insulin sensitivity, increases lipid oxidation and reduces intramuscular triglycerides. *In vitro* experiments in COS7 cells show this mutation increases basal AMPK activity independent of changes in the AMP:ATP ratio (Barnes et al. 2004). Furthermore, transgenic mice bearing the AMPKγ3^{R225Q} mutation in skeletal muscle have increased mitochondrial biogenesis (Garcia-Roves et al. 2008). A similar mutation in the AMPK γ3-subunit (R200W) in skeletal muscle of humans showed increased glycogen content and reduced intramuscular triglyceride content. Although these studies have highlighted the importance of AMPK activation in improving insulin sensitivity, whether expression of the activating AMPKγ3^{R225Q} mutation in glycolytic skeletal muscle can ameliorate the metabolic disturbances observed in the leptin-deficient *ob/ob* mice is unknown.

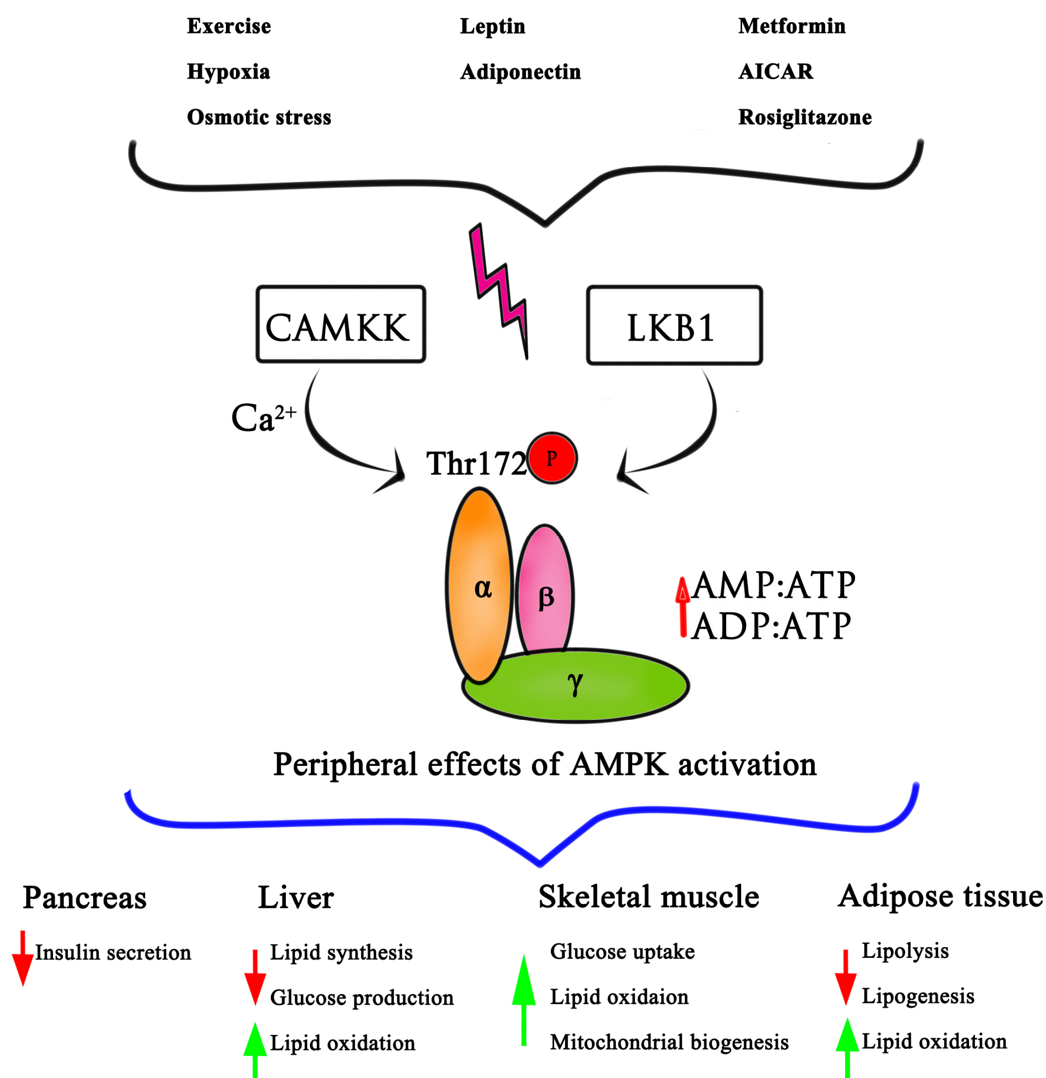

Figure 4: AMPK signaling. Various physiological stimuli, hormones and drugs activate AMPK. AMPK regulates cellular energy homeostasis by activating pathways that yield energy (catabolism) and turning off energy demanding processes (anabolism).

2 AIMS

Leptin and AMPK play prominent roles in the regulation of energy homeostasis. Leptin signaling through the long form of the leptin receptor regulates whole-body energy metabolism. Leptin action in the brain regulates energy intake and expenditure. Furthermore, leptin suppresses hepatic glucose production and stimulates peripheral glucose uptake and fatty acid oxidation and may play a role in mitochondrial function. AMPK regulates cellular energy homeostasis and its activation leads to increase in glucose uptake and lipid oxidation, which further improves metabolic abnormalities associated with obesity and insulin resistance. Therefore, the overall aim of this thesis was to study the role of leptin and AMPK in the regulation of whole-body glucose and energy homeostasis.

The specific sub-aims of this thesis are to elucidate the role of:

- the leptin receptor Tyr985 in mediating glucose metabolism
- leptin in the regulation of tissue-specific mitochondrial function in a mouse model of leptin deficiency
- the AMPKγ3 isoform in ameliorating metabolic disturbances arising from deficiencies in leptin signaling

3 EXPERIMENTAL SECTION

3.1 ANIMALS

All the animals used in Study I, Study II and Study III were on a C57Bl/6J background, had free access to food and water and were kept in a temperature controlled environment with 12 hour dark and light cycle. For Study I, male and female mice in the age range of three to four months were used. Wild type *(+/+)* and $Lepr^{tm2Mgmi/tm2Mgmi}$ *(l/l)* mice were generated as previously described (Björnholm et al. 2007). The exon at 18b of leptin receptor gene was homologously replaced in such a way that Tyr985 of LepR was replaced to Leu985, thereby encoding $LepRb^{Leu985}$. Heterozygous $Lepr^{Leu985}$/+ *(l/+)* animals were intercrossed to generate +/+, *l*/+ and homozygous *l/l* littermates. In Study II, *ob/ob* mice and lean littermates were studied at 14-20 weeks of age. In Study III, four genotypes namely WT, AMPKγ3^{R225Q} (γ3^{R225Q}), *ob/ob*-WT (*ob/ob*) and *ob/ob*-γ3^{R225Q} mice were studied. The generation of γ3^{R225Q} has been described (Barnes et al. 2004). Heterozygous *ob/*+-γ3^{R225Q} mice were generated by intercrossing *ob/*+ mice with γ3^{R225Q} mice. These mice were further intercrossed again with *ob/*+ mice to generate WT, γ3^{R225Q}, *ob/ob* and *ob/ob*-γ3^{R225Q} mice.

Study	Animal model	Pathway studied
Study I	$Lepr^{tm2Mgmi/tm2Mgmi}$ *(l/l)*	LepRb-Tyr985 regulates the SHP2/ERK pathway and SOCS3-mediated feedback inhibition. Role of LepRb-Tyr985-mediated signals for whole-body glucose homeostasis.
Study II	*ob/ob*	Leptin receptor signaling in the context of tissue-specific regulation of mitochondrial metabolism.
Study III	γ3^{R225Q} *ob/ob*-γ3^{R225Q}	AMPKγ3-mediated signaling pathways in the regulation of glucose homeostasis in the absence of a functional leptin action.

All animal experiments were approved by the Regional Animal Ethical Committee, Stockholm North, and the mice were treated in accordance with the regulation for protection of laboratory animals.

3.2 EXPERIMENTAL TECHNIQUES TO MEASURE GLUCOSE AND INSULIN SENSITIVITY

3.2.1 *In vivo* measurements

3.2.1.1 Intraperitoneal glucose tolerance test (IPGTT)

In Study I, four hour fasted +/+ and *l/l* mice were used and they were individually housed at the time of experiment. An intraperitoneal glucose load of 2 g/kg was given after measuring basal glucose values (0 min) and subsequently glucose values were measured at 15, 30, 60 and 120 minutes using a glucose meter (One Touch Ultra Lifescan, Milpitas, CA, USA). Blood samples were collected from the tail at 0 and 15 minutes for the determination of insulin levels using an ultrasensitive insulin ELISA kit

(Crystal Chem Inc, Downers Grove, IL). In Study III, all the study procedures were similar except that the intraperitoneal glucose load was 1 g/kg in order to prevent the overshoot in glucose values in *ob/ob* and *ob/ob*-$\gamma 3^{R225Q}$ mice.

3.2.1.2 Whole-body glucose turnover rate

Euglycemic-hyperinsulinemic clamps were performed in four hour fasted conscious mice for determining whole-body insulin sensitivity. Five days prior to the experiment, mice underwent jugular vein cannulation under isoflurane anesthesia and the catheter was exteriorized and placed under the skin on the back of the mouse and secured with suture. Caprofen (5 mg/kg) was given as an analgesic on the day of surgery and one day after the surgery. Body weight and overall health of the mice were monitored daily. Any animal that lost body weight more than 10% of their pre-surgery weight was not included in the study. Glucose turnover rate and hepatic glucose production were determined as described previously (Chibalin et al. 2008). A constant infusion of [3-^{3}H] glucose (2.5 µCi bolus and infusion flow rate of 0.09 µCi/min) was used to measure glucose turnover rate in the basal state and during euglycemic-hyperinsulinemic condition. Basal glucose turnover rate was measured after 60 to 70 minutes of constant tracer infusion, which was followed by a bolus of insulin (12.5 mU/kg for female mice and 25 mU/kg for male mice). Thereafter, a continuous insulin infusion (1.25 mU/kg/min for female mice and 2.5 mU/kg/min for male mice) was maintained. During the insulin clamp, a variable infusion of glucose (30%) was maintained to ensure euglycemia. A steady state clamped condition was achieved by 60 to 70 minutes after the start of insulin infusion and glucose turnover rate at the clamped condition was measured similar to that of basal condition in deproteinated blood. Hepatic glucose production was estimated by subtracting the average glucose infusion rate during the clamped condition from that of glucose utilization during the clamp. Blood samples were collected at basal and clamped condition to measure insulin levels using an ultrasensitive insulin ELISA kit (Crystal Chem Inc, Downers Grove, IL, USA). At the end of the experiment, animals were euthanized by an overdose of sodium pentobarbital and liver and skeletal muscles were rapidly dissected, frozen in liquid nitrogen, and stored at -80°C for subsequent signaling experiments.

3.2.1.3 Tissue-specific glucose uptake

Animals underwent surgery and recovery as described for the whole-body glucose turnover rate clamp studies. Tissue specific glucose uptake clamp was performed as described (Chibalin et al. 2008). On the day of experiment, after a four hour fast, baseline glucose measurements were obtained. A bolus dose of insulin was given through the exteriorized jugular vein catheter followed by a continuous constant rate insulin infusion. During this period, a variable rate of glucose (30%) was infused to ensure euglycemia and a steady state glucose levels (clamped at the basal level) was achieved ~60 to 70 minutes from the start of insulin infusion. A bolus of 2-deoxy-D-[1-^{14}C] glucose (3 µCi) was injected and blood glucose was subsequently measured at 3, 6, 10, 15, 20, 30, 40 and 60 minutes. Blood samples (20 µl) were deproteinized to measure glucose specific activity. Mice were euthanized by overdose of sodium pentobarbital and tissues (detailed in Study III) were quickly dissected and digested in 1 N NaOH. An aliquot of each sample was processed in per-chloric acid and $ZnSO_4$-$Ba(OH)_2$ mixture to measure tissue specific glucose uptake. In Study III, insulin

infusion rates were 10 mU/kg/min for lean mice (WT and $\gamma3^{R225Q}$) and 75 mU/kg/min in obese mice (*ob/ob* and *ob/ob* $\gamma3^{R225Q}$). Obese mice were clamped at higher insulin levels due to severe insulin resistance associated with this model.

3.2.2 *In vitro* measurement of insulin sensitivity

3.2.2.1 Insulin-stimulated glucose transport

Insulin-stimulated glucose transport was estimated in isolated skeletal muscle from male and female +/+ and *l/l* mice. Mice were fasted for four hours and under avertin anesthesia (2,2,2-tribromoethanol 99% and tertiary amyl alcohol (1:1 w/v), 500 mg/kg), EDL and soleus muscles were isolated with intact tendons and allowed to recover in a Krebs-Henseleit buffer (KHB) supplemented with 0.1% radioimmunoassay grade bovine serum albumin (BSA) and 5 mM HEPES. Muscles were then incubated in absence (basal) or presence of insulin (0.36 or 60 nM corresponding to a submaximal or maximal dose, respectively) in a shaking waterbath at 30ºC under a constant gas phase of 95% O_2 and 5% CO_2. Muscles were subjected to the following experimental protocol.

Experimental protocol		
Condition	**Constituents**	**Stimulus**
Recovery – 30 min	5 mM glucose 15 mM mannitol	no insulin
Preincubation – 30 min	5 mM glucose 15 mM mannitol	basal/submax/max insulin
Rinse – 10 min	20 mM mannitol	basal/submax/max insulin
Hot incubation – 20 min	19 mM mannitol 1 mM 2-deoxyglucose (^{3}H) 2-deoxyglucose (2.5 mCi/ml) (^{14}C)-mannitol (0.7 mCi/ml)	basal/submax/max insulin

At the end of the experiment, muscles were blotted on a pre-wet filter paper, trimmed of tendons, and freeze clamped with tongs pre-cooled to liquid nitrogen temperature. Muscles were stored in -80°C until further analysis. Glucose transport was estimated by measuring the accumulation of intracellular 2-^{3}H deoxyglucose-6 phosphate (Hansen et al. 1994).

3.3 *IN VITRO* MEASUREMENT OF LIPID OXIDATION

Isolated EDL muscle was used to measure palmitate oxidation as described previously (Chadt et al. 2008). EDL muscles were carefully isolated from anesthetized mice. After a 30 minute recovery in KHB buffer containing 0.1% BSA and 5 mM HEPES, muscles were transferred to a vial containing KHB buffer with tritium labeled palmitate (Palmitic Acid, [9,10-^{3}H(N)]) and incubated for 120 minutes. Palmitate oxidation was measured by determining the amount of tritium labeled water in the incubation medium, which was the byproduct of palmitate oxidation. To separate the non-metabolized palmitate from the tritium labeled water, 200 µl of the incubation medium was mixed with 800 µl of activated charcoal slurry (0.1 g activated charcoal powder in 1ml 0.02 M Tris-HCl buffer, pH 7.5), shaken for 30 minutes to facilitate adsorption of non-metabolized palmitate to charcoal. Samples were subjected to centrifugation at 12000 g for 15 minutes. An aliquot of the supernatant was used to measure tritium labeled water using a liquid scintillation beta counter.

3.4 ANALYTICAL METHODS

3.4.1 Immunoblot analysis

Homogenates were prepared by placing ~10 to 15 mg of tissue in Eppendorf Safe Lock Tubes™ containing ice cold homogenization buffer (300 to 500 µl per tube). A steel bead was added to each tube and samples were loaded onto a tissue lyzer (Qiagen TissueLyser II) set at 20 Hz (two times for 60 seconds with a 15 second gap). The tubes were subjected to end over end rotation at 4ºC for 60 minutes. Samples were subjected to centrifugation at 10000 g for 15 minutes, and the supernatant was collected for protein measurements using a Pierce BCA protein assay kit (Nordic Biolabs, Täby, Sweden). The homogenization buffer contained the following constituents: NaCl 137 mM, KCl 2.7 mM, $MgCl_2$ 1 mM, $Na_4P_2O_7$ 5 mM, NaF 10 mM, Triton X-100 1%, Glycerol 10%, Tris pH 7.8 20 mM, EDTA 1 mM, PMSF 0.2 mM, Na_3VO_4 0.5 mM, Protease inhibitory cocktail 1X (Merck Millipore, Nottingham, UK). Homogenates were normalized to an equal protein concentration using Laemmli buffer and homogenization buffer and heated at 55°C for 20 minutes. For Study III, tissue homogenates were subjected to three freeze/thaw cycles to disrupt mitochondria (freezing at -80°C overnight, defrost for 1-2 hours at 4°C, vortex followed by -80°C for 30 min, defrost and vortex). In Study I and III, proteins were separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA) that were pre-activated in methanol and blocked in 7.5% (w/v) nonfat dry milk. For Study II, proteins were transferred onto nitrocellulose membranes. Membranes were incubated with following primary antibodies overnight at 4ºC for the determination of phosphorylation and expression of various proteins: phospho-Akt-Ser473, phospho-glycogen synthase kinase (GSK)-3-α/β-Ser21/Ser9, Akt, GSK-3-α/β, IRS-1, ACC, ACCα^{Ser79}, phospho-AMPKα^{Thr172} and AMPKα were from Cell Signaling Technology, Danvers, MA, USA. Dynamin like 120 kDa protein (OPA1) and TFAM were from Abnova, Taipei, Taiwan. NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9 (NDUFA9), complex I; Succinate dehydrogenase complex, subunit A (SDHA), complex II; ubiquinol-cytochrome c reductase core protein I (UQCRC1), complex III; Mitochondrial encoded cytochrome c oxidase I (MTCO1), complex IV and ATP5B, ATP synthase were from Invitrogen, Carlsbad, CA, USA.

Phospho-ACCβ-Ser219/221; MFN2, GAPDH, DNM1L, PPARα and PEPCK were from Santa Cruz Biotechnology, CA, USA. Tubulin and IRS-2 was from Millipore, Billerica, MA, USA.

The AMPKγ3 antibody was a kind gift from Prof. Grahame Hardie, University of Dundee, Dundee, UK and the GLUT4 antibody was a kind gift from Dr Geoffrey Holman, University of Bath, Bath, UK. Membranes were incubated overnight in primary antibodies, washed in Tris-buffered saline containing 0.02% Tween 20 (TBST), further incubated with appropriate secondary antibodies for one hour, and finally washed again in TBST. Immunoreactive proteins were visualized by enhanced chemiluminescence (GE Healthcare, Little Chalfront, UK) and quantified by GS-800 calibrated densitometer using Quantity One analytical software.

3.4.2 Determination of triglyceride content

A frozen piece of tissue (~10 to 15 mg) was cut under liquid nitrogen and placed in Eppendorf Safe Lock Tubes™. A steel bead and triglyceride extraction buffer (3:2 heptane-isopropanol solution containing 1% v/v Tween-20) was added to each tube. Homogenization of the tissues in the extraction buffer was achieved as described above. The tubes were subjected to centrifugation at 1500 g for 15 minutes at 4ºC for phase separation. The upper phase was collected and an aliquot was dried in a glass tube under vacuum centrifugation. Samples were then used for the determination of triglyceride content using a standard enzymatic colorimetric method that estimates the glycerol liberated by the hydrolysis of triglyceride present in the sample. A triglycerides/glycerol blanked kit was used along with Precinorm L standard (Roche Diagnostics Scandinavia, Sweden).

3.4.3 Determination of glycogen content

A portion of frozen tissue was cut under liquid nitrogen and placed in tight seal micro tubes. To each tube, 500 µl of 1N HCl was added and subjected to heating at 100ºC on a heated shaking block for 2 hours. The tubes were subjected to centrifugation at 2000 g for 10 minutes at 4ºC and an aliquot of the supernatant was used for the determination of glycogen content (Passonneau et al. 1967). Briefly, 10 µl of the extract was mixed with 2 ml of assay buffer (50 mM Tris buffer (pH 8.1), 300 µmol/l ATP, 2 mM $MgCl_2$, 0.02% BSA, 40 µmol/l NADP, 1 µg/ml glucose-6-phosphate dehydrogensase and 2 µl Hexokinase (HK). HK (50-100 µl) was subjected to centrifugation at 4000 g for 5 min and the pellet was resuspended in an equal volume of enzyme diluting buffer (20 mM imidazole-HCl, pH 7.1; 0.02% BSA). The above reaction mix was thoroughly mixed with a vortex and incubated at room temperature for 30 minutes. Glycogen content was estimated using fluorometry (TD-700, Tuner Design, Sunnyvale, CA, USA). When liver samples were used, a 1:4 dilution with 1 N HCl was required for the detection in the normal standard curve range.

3.5 RNA PURIFICATION AND QUANTITATIVE REAL TIME RT-PCR

Real time polymerase chain reaction using TaqMan technique was used for the determination of mRNA expression in tissue samples. Total RNA was isolated from tissues using Trizol reagent (Invitrogen) according to manufactures protocol. Isolated

RNA was further treated with deoxyribonuclease using a DNA-free kit (Ambion, Huntington, UK). The mRNA concentration and purity was measured spectrophotometrically using a Nanodrop 1000 (Thermo Scientific, Wilmington, MA, USA). Samples were diluted to equal concentrations with sterile and RNase-free water and cDNA was synthesized using a Super-Script First-Strand Synthesis system (Invitrogen) for Study I and Super-Script III First-Strand Synthesis (Invitrogen) for Study II. The mRNA expression of *Pck-1* (Mm00440636_m1), *G6pc* (Mm00839363_m1), *Scd-1*(Mm00772290_m1) [TaqMan gene assay from Applied Biosystems] were calculated using ΔCt method and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Study I) and β-actin (Study II) were used as reference gene.

3.6 MITOCHONDRIAL RESPIROMETRY

3.6.1 General methodology

A high resolution closed two chamber mitochondrial respirometer was used to measure mitochondrial function (Oroboros Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Several tissues were isolated and used for the estimation of mitochondrial function in this thesis. In Study I, the right liver lobe was dissected and analyzed. In Study II, the EDL muscle, soleus muscle and right liver lobe were dissected and analyzed. In Study III, the EDL muscle was dissected and analyzed.

Skeletal muscle samples used in Study II and III were placed in ice cold BIOPS relaxing solution (2.8 mM Ca_2K_2EGTA, 7.2 mM K_2EGTA, 5.8 mM ATP, 6.6 mM $MgCl_2$, 20 mM taurine, 15 mM sodium phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol and 50 mM MES, pH 7.1). Muscle samples were freed of fat and tendon using a fine forceps under a dissection microscope. Fibers were individually combed to maximize surface area. Sarcolemmal permeabilization of muscle membrane was achieved by transferring finely combed muscle samples into ice-cold BIOPS, supplemented with 0.005% (w/v) saponin. Samples were subjected to gentle shaking on ice for 10 minutes. Tissue samples were equilibrated in ice cold MiR05 (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) for 30 minutes before transferring them into mitochondrial respirometry chamber. Muscle was blotted on a filter paper for 30 seconds and samples weighing 1 to 2 mg were transferred into the chamber and mitochondrial respirometry was determined. Mild mechanical permeabilization in amino acid-depleted MiR05 was used for liver samples and for mitochondrial respirometry, an equivalent of 1 mg of tissue per chamber was used along with MiR05 as respirometry medium.

3.6.2 Respirometry measurements

The "LEAK" respiration, owing to endogenous uncoupling, was measured in the absence of ADP by adding malate (final concentration 2 mM) and pyruvate (10 mM). Addition of ADP (5 mM) allowed the quantification of oxidative phosphorylation capacity or OXPHOS. Complex I mediated oxidation (*C I*) was evaluated by addition of 20 mM glutamate, followed by 10 mM succinate for the convergent electron flow through both complex I and II (*C I+II*). An exogenous protonophore carbonylcyanide-4-(trifluoromethoxy)-phenyl-hydrazone (FCCP) (titrated to a final concentration of 0.7

μM for liver and 0.3 μM for skeletal muscle) was added to measure maximum flux through the electron transfer system (*ETS I +II*). Rotenone (0.1 μM) and antimycin A (2.4 μM) were added in subsequent steps to inhibit electron transport through complex I *(ETS II)* and complex III inhibition (Zero respiration) respectively. Zero oxygen flux, which is not directly related to electron transfer system, was deducted from the values of each previous steps. Absolute oxygen flux (J_{o2}, [pmol O_2/mg/s]) was expressed relative to tissue wet weight per second. The relative contribution of each respiratory state to maximum oxygen flux described as flux control ratio (FCR) was expressed as ratio over *ETS I+II*.

3.7 *IN VIVO* LEPTIN TREATMENT

In Study II and III, a five day leptin treatment protocol was used to assess the effect of the hormone on various parameters. Mice (14 to 20 weeks old) were studied. Mice were weight-matched within genotypes and between saline or leptin treatment groups. Animals were individually housed and acclimatized for a period of 2 to 3 days, during which all the experimental animals were handled and injected with saline prior to the start of saline/leptin treatment. Recombinant leptin (Peprotech, Rocky Hill, NJ, USA) was reconstituted in sterile saline and administered intraperitoneally (1 mg/kg) once daily between 15:00 and 16:00 hrs for a period of five days, during which body weight and food intake were recorded. Body composition analysis (fat mass and lean mass) was performed using MRI scans (Echo MRI, Houston, TX, USA) before and after saline/leptin treatment. On the sixth day morning, mice were anesthetized with avertin (2,2,2-tribromoethanol 99% and tertiary amyl alcohol (1:1 w/v), 500 mg/kg) and tissues were collected for various experimental protocols. In Study III, mice were fasted for four hours prior to tissue collection.

3.8 STATISTICAL ANALYSES

Data are presented as mean ± SEM. Unpaired student's *t*-test, one-way ANOVA or two-way ANOVA was used to identify significance between the different groups as further detailed in each study. Results that were identified as statistically significant using a one-way ANOVA or two-way ANOVA were further evaluated by an appropriate *post hoc* analysis as further detailed in each study. $p<0.05$ was considered to be statistically significant.

4 RESULTS AND DISCUSSION

4.1 LEPTIN SIGNALING AND GLUCOSE HOMEOSTASIS

4.1.1 LepRb-Tyr985 regulates whole-body glucose homeostasis

Signaling through the long form of the leptin receptor activates the Jak2-mediated phosphorylation of Tyr985, Tyr1077 and Tyr1138 of the leptin receptor. Among these residues, phosphorylated Tyr985 mediates the SHP2-Grb2-ERK signaling pathway and also acts as a docking site for SOCS3 (Banks et al. 2000). Attenuation of leptin signaling by ligand activation, as well as mediation of leptin resistance, is facilitated by SOCS3 binding to Tyr985 (Bjorbaek et al. 2000), which indicates that this pathway mediates leptin sensitivity. The role of LepRb-Tyr985 in mediating glucose homeostasis is poorly understood. Study I focuses on the role of LepRb-Tyr985 in regulating whole-body glucose homeostasis.

4.1.1.1 Glucose tolerance is improved in LepRb-Tyr985 mutant (l/l) mice

Initial characterization of *l/l* mice showed that glucose levels in the fed state were normal in male and female mice with reduced insulin levels in female mice (Björnholm et al. 2007). Intraperitoneal glucose tolerance revealed improved insulin sensitivity and glucose tolerance. Female *l/l* mice had lower insulin levels under basal conditions and in response to a glucose challenge, along with normal glucose tolerance. In contrast, male mice showed improved glucose tolerance with normal insulin levels compared to that of +/+ mice (Fig 5)

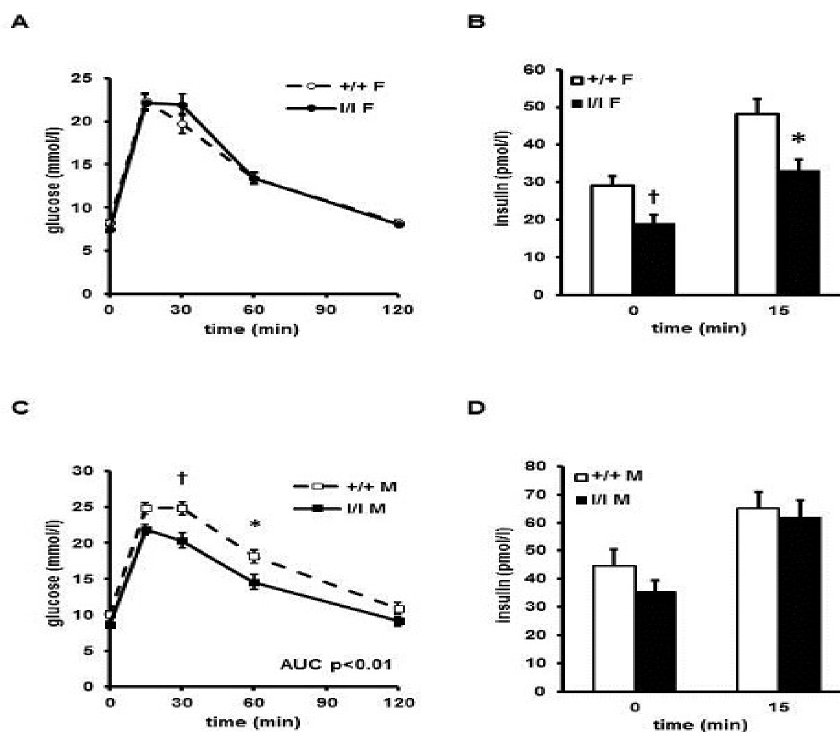

Figure 5: LepRb-Tyr985 mutation confers improved glucose homeostasis. Intraperitoneal glucose tolerance tests were performed in 4 hour fasted +/+ (white bars) and *l/l* (black bars) mice. Plasma glucose (mM) and insulin (pM) values during the glucose tolerance test in female (F; A and B) and male (M; C and D) +/+ and *l/l* mice. Results are mean ± SEM. *, $p<0.05$ vs. +/+; †, $p<0.01$ vs. +/+ mice (n = 9-12). (Tom et al. 2011).

Previous studies provide evidence that *l/l* mice have slightly lower body weight, reduced adipose tissue mass, and enhanced leptin sensitivity (Björnholm et al. 2007). These moderate alterations in the body composition, combined with increased leptin sensitivity, can confer improvements in glucose homeostasis. Experimental approaches leading to reduction in adipose tissue mass improve glucose tolerance (Wetter et al. 1999; Ntambi et al. 2002; Yan et al. 2013). Leptin signaling influences insulin sensitivity (Carvalheira et al. 2005). The improved glucose tolerance and insulin sensitivity in *l/l* mice could stem from enhanced hepatic insulin sensitivity and/or an enhanced peripheral glucose disposal. To differentiate hepatic versus peripheral insulin sensitivity, a euglycemic-hyperinsulinemic clamp in conscious mice was performed.

4.1.1.2 LepRb-Tyr985-mediated signals are important for hepatic and peripheral insulin sensitivity

The euglycemic-hyperinsulinemic clamp is considered as a golden standard to asses insulin sensitivity (DeFronzo et al. 1979). This method, when combined with a radio isotope technique, allows for the determination of hepatic vs. peripheral glucose fluxes during the clamp (DeFronzo et al. 1979). Female mice were clamped at an insulin infusion rate of 1.25 mU/kg/min, whereas male mice were clamped at 2.5 mU/kg/min insulin. The glucose turnover rate in the basal state, which is indicative of endogenous glucose production, was similar between female and male +/+ and *l/l* mice (Fig 6 A and C). The above results are in agreement with the glucose tolerance results, which show basal glucose values were similar between +/+ and *l/l* mice. In female mice, insulin-stimulated peripheral glucose utilization was similar between +/+ and *l/l* mice and increased ~1.8 fold over basal values (Fig 6 A and C). During the clamped condition, hepatic glucose production was completely suppressed in female *l/l* mice ($p<0.001$ vs. +/+) in response to insulin, whereas this suppression was only 46% in the +/+ mice. This indicates that LepRb-Tyr985 influences hepatic insulin sensitivity (Fig 5 B). The LepRb-Tyr985 mutation in the male mice not only improved suppression of hepatic glucose production ($p<0.05$ vs. +/+), but it also enhanced peripheral glucose utilization during the clamp ($p<0.01$ vs. +/+) (Fig 6 C and D).

Female *l/l* mice displayed enhanced hepatic insulin sensitivity, whereas the male *l/l* displayed both hepatic and peripheral insulin sensitivity. This difference in insulin sensitivity between males and females could be partly attributed to the influence of testosterone levels (Sato et al. 2008). Castration of male *l/l* mice significantly reduces fat pad mass, whereas testosterone treatment restored fat mass to levels in wild-type mice (Johnson et al. 2012).Experiments evaluating glucose tolerance in castrated *l/l* mice may be performed in the future to directly address the influence of the sex on peripheral insulin sensitivity.

Body weight is one factor that can influence insulin sensitivity. Body weight of female and male *l/l* mice was significantly lower than the corresponding +/+ mice (Study I, Table 1). Clamp data was analyzed in a subset of body weight-matched +/+ and *l/l* mice to exclude the possibility that enhanced insulin sensitivity in *l/l* mice is due to reduced body weight or adiposity. Hepatic insulin sensitivity was increased in body weight-matched female *l/l* mice as evidenced by an enhancement in the suppressive effect of insulin on of hepatic glucose production ($p<0.05$ vs. +/+). Glucose utilization during

the basal, as well as insulin-stimulated conditions was similar in +/+ and *l/l* mice (body weight: +/+, 19.7 ± 0.8 g vs. *l/l,* 19.3 ± 0.5 g). Male +/+ and *l/l* mice matched body weight (body weight: +/+, 28.0 ± 0.2 g vs. *l/l,* 27.3 ± 0.3 g) had similar rates of basal glucose utilization. Insulin-stimulated glucose utilization and suppression of hepatic glucose production tended to be enhanced in male *l/l* mice, but this difference was not statistically significant compared to +/+ mice. These results highlight a primary role for LepRb-Tyr985 in mediating insulin sensitivity. Moreover, they point to only a secondary role for reduced body weight.

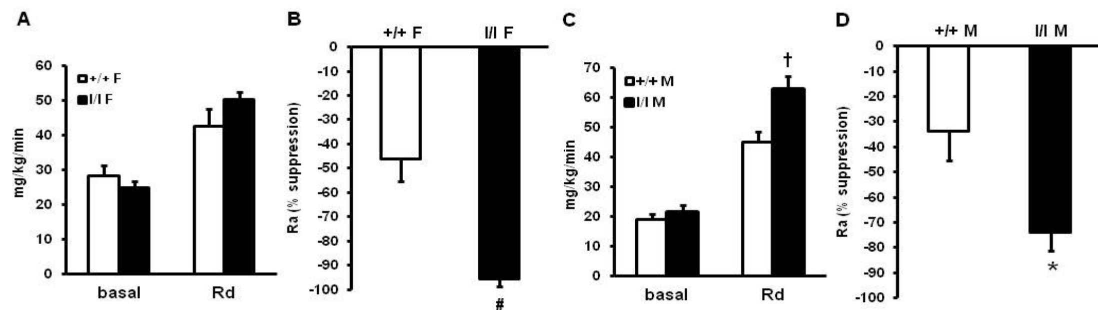

Figure 6: LepRb-Tyr985 mutation enhances whole-body insulin sensitivity. Basal and insulin-stimulated peripheral glucose utilization (Rd) and hepatic glucose production (Ra) during a euglycemic-hyperinsulinemic clamp in 4-hour fasted +/+ (white bars) and *l/l* (black bars) mice. Glucose utilization and insulin-mediated suppression of hepatic glucose production are presented for female (A and B) and male (C and D) +/+ and l/l mice. n=7-9. Results are mean±SEM, *$p<0.05$, †$p<0.01$ and #$p<0.001$ versus +/+ mice. (Tom et al. 2011).

4.1.2 LepRb-Tyr985 mediates hepatic insulin sensitivity

Leptin signaling interacts with canonical insulin signaling cascades by central and peripheral mechanisms (Liu et al. 1998; Carvalheira et al. 2003). In the present study, canonical insulin signaling pathways involved in the regulation of hepatic insulin sensitivity were assessed. Liver was frozen at the end of a 75 minute insulin infusion and phosphorylation and abundance of insulin signaling proteins (tyrosine phosphorylation of the insulin receptor, IRS-1 and IRS-2 and phosphorylation of Akt-Ser473) was evaluated by Western blot. Interestingly, insulin signaling was similar between +/+ and *l/l* mice. During the clamp experiment, *l/l* mice responded to the insulin infusion very rapidly and blood glucose dropped faster compared to the +/+ mice. This prompted an evaluation of hepatic insulin signaling cascades after a 10 minute insulin infusion. Tyrosine phosphorylation of the insulin receptor, IRS-1 and IRS-2 and phosphorylation of Akt-Ser473 was similar between +/+ and *l/l* mice. We also determined whether alterations in gluconeogenic genes could account for the enhanced hepatic insulin sensitivity in *l/l* mice. Hepatic gene expression of PEPCK and G6Pc were unaltered between +/+ and *l/l* mice. These results provide evidence to suggest that increased hepatic insulin sensitivity conferred by LepRb-Tyr985 may not be a direct consequence of enhanced LepRb signaling in the liver.

The role of hypothalamus in the regulation of hepatic glucose production is well established (Obici et al. 2002; Pocai et al. 2005). Acute intracerebroventricular administration of leptin or insulin regulates hepatic glucose production (Liu et al. 1998;

Obici et al. 2002). The precise mechanism by which the LepRb-Tyr985 mutation influences hepatic insulin sensitivity is unclear. An enhanced hypothalamic leptin signaling may possibly play a role in mediating hepatic insulin sensitivity in *l/l* mice. pSTAT3 is enhanced in hypothalamic arcuate nucleus of *l/l* mice in the presence of low leptin levels as compared to +/+ mice, indicative of enhanced leptin signaling in the hypothalamus (Björnholm et al. 2007). Given that leptin signaling influences insulin sensitivity (German et al. 2009) and central insulin signaling regulates hepatic glucose production (Liu et al. 1998), any enhancement in leptin signaling in the hypothalamus might amplify the central insulin mediated inhibition of hepatic glucose production. SOCS3 is another molecule that is connected to leptin sensitivity. SOCS3 is a negative regulator of leptin signaling and is implicated in leptin resistance (Bjorbaek et al. 2000). SOCS3 haploinsufficient mice showed enhanced leptin sensitivity and improved glucose homeostasis (Howard et al. 2004; Kievit et al. 2006). Moreover, overexpression of SOCS3 in hypothalamus leads to leptin resistance, obesity and disturbances in glucose homeostasis (Reed et al. 2010). SOCS3 has also been implicated in mediating insulin resistance (Rui et al. 2002). In *l/l* mice, SOCS3-mediated feedback inhibition of leptin signaling was blunted since phosphorylated Tyr-985 of the leptin receptor is needed for this feedback loop to function (Bjorbaek et al. 2000). This suggests that a lack of SOCS3 signaling in *l/l* mice could enhance hepatic insulin sensitivity by central mechanisms. Furthermore, AgRP levels in the hypothalamus are reduced in *l/l* mice (Björnholm et al. 2007). Given that insulin-mediated suppression of AgRP levels in the AgRP neurons plays a role in the regulation of suppression of hepatic glucose production (Konner et al. 2007; Lin et al. 2010), reduced AgRP levels in the *l/l* mice might potentiate insulin ability to suppress hepatic glucose production. These results suggest that enhanced central leptin signaling might enhance hepatic insulin sensitivity in *l/l* mice.

4.1.3 Limitations of Study I

We have not been able to differentiate as to whether the LepRb-Tyr985-mediated changes in glucose metabolism are due to primary effects brought about by central regulation or secondary effects via adaptive responses in peripheral tissues. Another limitation is that the present study cannot determine whether the enhanced suppression of hepatic glucose production is due to decreased glycogenolysis and/or decreased gluconeogenesis during the euglycemic-hyperinsulinemic clamp. Thus further studies are required to identify the molecular signaling mechanisms connecting the enhancement in hepatic insulin sensitivity with the insulin signaling cascade.

4.2 MITOCHONDRIAL FUNCTION IN *OB/OB* MICE: ROLE OF LEPTIN

The *ob/ob* mice are hyperphagic, hyperglycemic, obese and exhibit perturbations in whole-body energy metabolism owing to the lack of the hormone leptin. Leptin supplementation to *ob/ob* mice improves insulin sensitivity and other complications associated with obesity (Halaas et al. 1995). Mitochondrial function has a major role in regulating whole-body metabolism. The role of mitochondrial dysfunction in the development of insulin resistance and type 2 diabetes is well documented (Patti et al. 2003; Petersen et al. 2004; Lowell et al. 2005), but nevertheless some controversy exists. Tissue-specific differences in mitochondrial properties such as mitochondrial mass, cytochrome *c* oxidase activity, mitochondrial DNA copy number are adapted to

integrate varying metabolic demands unique to each specific tissue (Weibel et al. 1969; Eisenberg et al. 1975; Eisenberg et al. 1976; Gagnon et al. 1991; Wiesner et al. 1992). Tissue-specific differences in mitochondrial function might be altered in the context of obesity and type 2 diabetes (Brady et al. 1985; Ferreira et al. 2003; Hancock et al. 2008; Raffaella et al. 2008; Holmström et al. 2012). Study II was designed to address the role of leptin in modulating tissue-specific mitochondrial function in the setting of obesity and associated insulin resistance.

4.2.1 Mitochondrial respiration in glycolytic skeletal muscle

Glycolytic skeletal muscle adapts to obesity in *ob/ob* mice through increases in mitochondrial respiration. Maximum electron transfer capacity measured at ETS *I +II* was increased in EDL muscle ($p<0.05$) compared to lean littermates, along with a trend for enhanced electron transport capacity at ETS *II* ($p=0.069$) (Fig 7 A). These mitochondrial adaptations in glycolytic skeletal muscle were also observed in obese diabetic *db/db* mouse (Holmström et al. 2012). Consistent with the functional data, protein abundance of the SDHA subunit of complex II ($p<0.01$), MTCO1 subunit of complex IV ($p<0.05$) and ATP5B subunit of complex V ($p<0.05$) are increased (Fig 7 C-E). The reason for these adaptations can be multifactorial. EDL muscle from *ob/ob* mice has fiber type differences, with increased type II A fibers having larger surface area and proportionately lower type II B fibers (Warmington et al. 2000). The fiber type alteration, coupled with increased lipid availability and elevated circulating free fatty acids, increases mitochondrial biogenesis in glycolytic skeletal muscle (Garcia-Roves et al. 2007). This metabolic milieu might lead to an increase in mitochondrial respiration in EDL muscle from *ob/ob* mice. We also confirmed our findings in Study III, where an increase in mitochondrial respiration was seen in glycolytic EDL muscle from *ob/ob* mice. Collectively, these studies provide evidence that glycolytic skeletal muscle can adapt to increase mitochondrial respiration in the context of obesity.

With an increase in mitochondrial respiration in EDL muscle from *ob/ob* mice, an increase in markers of mitochondrial biogenesis and stability was expected. However, TFAM protein abundance was significantly lower in *ob/ob* mice, the OPA1 ratio of the short to long form was increased along with increased DNM1L protein level. These points suggest that mitochondria are undergoing fission or fragmentation, since MFN2, which is responsible for fusion, is unaltered. The results of the present study are compatible with the previous finding of increased mitochondrial fission or fragmentation in EDL muscle from *db/db* mice. The long-term or age-dependent consequences of this "obesity-associated" mitochondrial profile might decrease mitochondrial respiration over time.

Analysis of flux control ratio in EDL muscle provided evidence for reduced *C I* and *CI + II* contribution in *ob/ob* mice compared to lean mice. Since the protein constituents that comprise the complexes of the respiratory chain were unaltered (*C II*, *C IV* and *C V* were increased), the decrease in FCR could indicate mitochondrial inefficiency. This might have arisen from the relatively increased *ETS I + II* value that is used for normalizing oxygen flux.

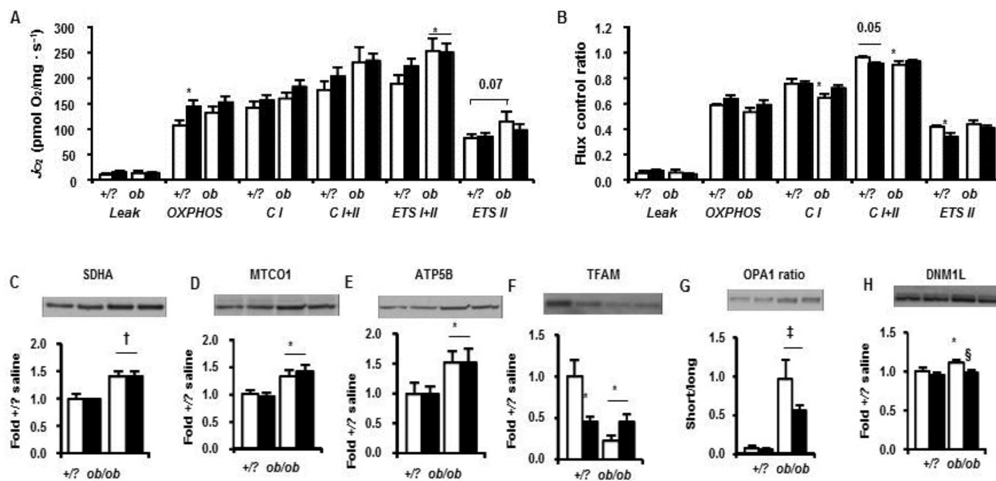

Figure 7: Mitochondrial respiration in EDL muscle from *ob/ob* mice. (A) Oxidative phosphorylation and electron transport system, n=4-9. (B) Flux control ratio highlighting the relative contribution of each given respiratory state to *ETS I +II,* n=4-9. (C-E) Protein markers of ETS complexes II, IV and V; (F-H) protein markers of mitochondrial biogenesis and dynamics, n=6-8. Results are mean ± SEM. Open bar - saline-treated; closed bar - leptin-treated. *p<0.05, †p<0.01, ‡p<0.001 vs. lean (+/?) saline-treated mice. §p<0.05 vs. *ob/ob* saline-treated mice.

Mitochondrial respiration in glycolytic EDL muscle was unaltered after five days of leptin treatment. Leptin stimulates lipid oxidation in under *in vivo* and *in vitro* conditions through activation of AMPK (Muoio et al. 1997; Minokoshi et al. 2002). Activation of AMPK is associated to mitochondrial biogenesis (Garcia-Roves et al. 2008). In the present study, leptin action through AMPK was expected to increase mitochondrial biogenesis and mitochondrial respiration. Initial studies addressing the effect of leptin on lipid oxidation indicate that the EDL muscle is unresponsive to leptin (Minokoshi et al. 2002). The effect of leptin on mitochondrial biogenesis is mediated by multiple mechanisms. Leptin treatment increases PGC-1α expression and induces endothelial nitric oxide synthase, highlighting a role in mitochondrial biogenesis. Concomitantly, proinflammatory cytokines like TNF-α negatively regulate mitochondrial biogenesis. Indeed, TNF-α levels are markedly higher in *ob/ob* mice. Future studies testing higher doses of leptin or more frequent administration may enhance mitochondrial biogenesis.

4.2.2 Mitochondrial respiration in soleus muscle from ob/ob mice is unaltered

Mitochondrial respiration in soleus muscle was similar between lean and *ob/ob* mice. Moreover, leptin treatment did not alter the respiratory state or the flux control ratios (Fig 8 A). Protein abundance of several markers of the respiratory chain complex was unaltered between saline- and leptin-treated lean and *ob/ob* mice. Saline-treated lean mice had higher TFAM protein abundance compared with saline- and leptin-treated *ob/ob* mice (p<0.001), whereas leptin treatment reduced TFAM levels in lean mice (p<0.01) (Fig 8 B). The mitochondrial fusion-mediating protein MFN2 tended to increase with leptin treatment (Fig 8 C). Oxidative tissues, such as the soleus muscle, display defective glucose and abnormal lipid metabolism in the context of obesity and type 2 diabetes (Cuendet et al. 1976). Obesity arising from a high fat diet increases mitochondrial mass and hence respiratory capacity in rat soleus muscle (Stephenson et al. 2012). Thus, mitochondrial adaptations might be occurring as a consequence of metabolic perturbations associated with obesity and type 2 diabetes. However, these adaptations are insufficient to correct the metabolic defects in glucose and lipid metabolism in oxidative tissues in the obese mice. Conversely, mitochondrial respiration is decreased in soleus muscle from diabetic and obese *db/db* mice (Holmström et al. 2012). The difference in mitochondrial respiration in soleus muscle from *db/db* and *ob/ob* mice may be attributed to strain differences. The data reported from soleus muscle in *ob/ob* mice indicates that metabolic disturbances in glucose and lipid metabolism in oxidative skeletal muscle are dissociated from mitochondrial function. Furthermore, short-term leptin treatment does not alter the mitochondrial respiratory capacity.

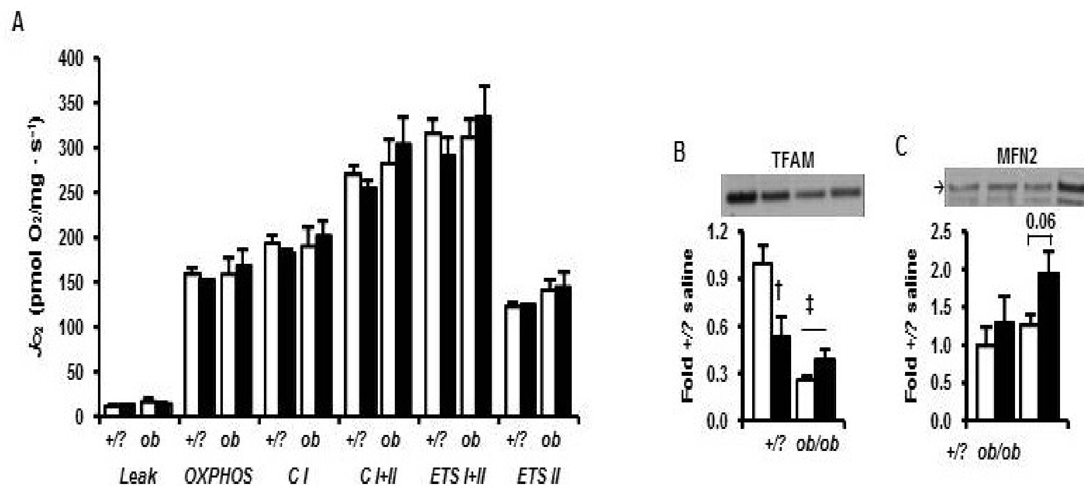

Figure 8: Mitochondrial respiration and protein markers of mitochondrial biogenesis and dynamics in soleus skeletal muscle from *ob/ob* mice. (A) Oxidative phosphorylation and electron transport system (ETS), (B, C) TFAM and MFN2 protein levels. Open bar - saline-treated; closed bar - leptin-treated. Results are mean ± SEM. (n=6-8). †p<0.01, ‡p<0.001 vs. lean (+/?) saline-treated mice.

Previous studies in *ob/ob* mice provide evidence to suggest that leptin treatment alters fiber type composition in oxidative muscle, with a net increase in type II B fibers and a concomitant decrease in type II A fibers (Warmington et al. 2000). This shift to a

greater proportion of fast twitch glycolytic fibers may mitigate any effect of slight increase in mitochondrial biogenesis markers after leptin treatment. The similarities in mitochondrial respiration between lean *+/?* and *ob/ob* saline-treated mice suggest that obesity, due to a lack of functional leptin action, does not affect mitochondrial respiration in oxidative skeletal muscle. The effect of leptin on lipid oxidation is biphasic involving a direct activation of AMPK by shifting ADP to ATP ratio and indirect activation via the sympathetic nervous system. Experimentally, these effects of leptin on lipid oxidation are diminished after six hours (Minokoshi et al. 2002). Thus, the peak effect of leptin on mitochondrial respiration may have been missed, since tissues were isolated 16 hours after the last leptin injection.

4.2.3 Hepatic mitochondrial respiration is impaired in *ob/ob* mice

The liver influences energy metabolism by regulating glucose fluxes under fed and fasting conditions. In addition, the liver also plays an integral role in lipid metabolism. Ectopic lipid accumulation in hepatic tissues leads to insulin resistance. Moreover, an increase in hepatic glucose production is a characteristic feature of obese insulin resistant or type 2 diabetic patients, animal models of obesity and diabetes. The role of mitochondria in regulating hepatic energy metabolism is incompletely resolved. Leptin receptor deficient *db/db* mice are hyperglycemic, exhibit hepatic triglyceride accumulation and have decreased hepatic mitochondrial respiration (Holmström et al. 2012). Treating *ob/ob mice* with leptin and studying hepatic mitochondrial respiration will highlight the role of leptin in regulating hepatic oxidative capacity.

Mitochondrial respiratory capacity at *ETS I + II* and *ETS II* were reduced in saline-treated *ob/ob* mice compared to saline-treated lean (*+/?*) mice ($p<0.01$ and $p<0.001$ respectively). A similar trend in *C I+II* was also observed in saline-treated *ob/ob* mice ($p=0.062$). In the present study, leptin treatment did not alter mitochondrial respiration in liver (Fig 9 A). Flux control ratio for *OXPHOS* and *C I+II* was increased in saline-treated *ob/ob* mice ($p<0.001$; $p<0.05$ vs. saline-treated lean (*+/?*) mice respectively (Fig 9 B). Leptin treatment increased *Leak* and *OXPHOS* respiration in lean mice ($p<0.05$ vs. saline-treated lean mice). Protein levels of SDHA, which is the catalytic subunit of *C II*, was decreased in *ob/ob* mice and was unaltered by leptin treatment ($p<0.05$ vs. saline-treated lean (*+/?*) mice (Fig 9 D). Abundance of the *C I* subunit protein, NDUFA9, tend to be increased in saline treated *ob/ob* mice ($p=0.058$) (Fig 9 C). TFAM levels were decreased in saline-treated *ob/ob* mice ($p<0.05$ vs. saline-treated lean mice) and increased by leptin treatment ($p<0.05$ vs. *ob/ob* saline-treated mice), (Fig 9 E). The increased OPA1 ratio (short to long isoform) in saline-treated *ob/ob* mice was reduced by leptin treatment ($p<0.05$ vs. saline-treated *ob/ob* mice) (Fig 9 F). PPARα levels were lower in *ob/ob* mice ($p<0.001$ vs. saline-treated lean mice) (Fig 9 G). With respect to the hepatic mitochondrial respiratory capacity, the results from the present study are compatible with previous results (Garcia-Ruiz et al. 2007; Perfield et al. 2013). Thus, the reduction in hepatic mitochondrial oxidative capacity in *ob/ob* mice could be due to multiple defects in respiration. Furthermore, short-term leptin treatment failed to fully restore hepatic mitochondrial oxidative capacity.

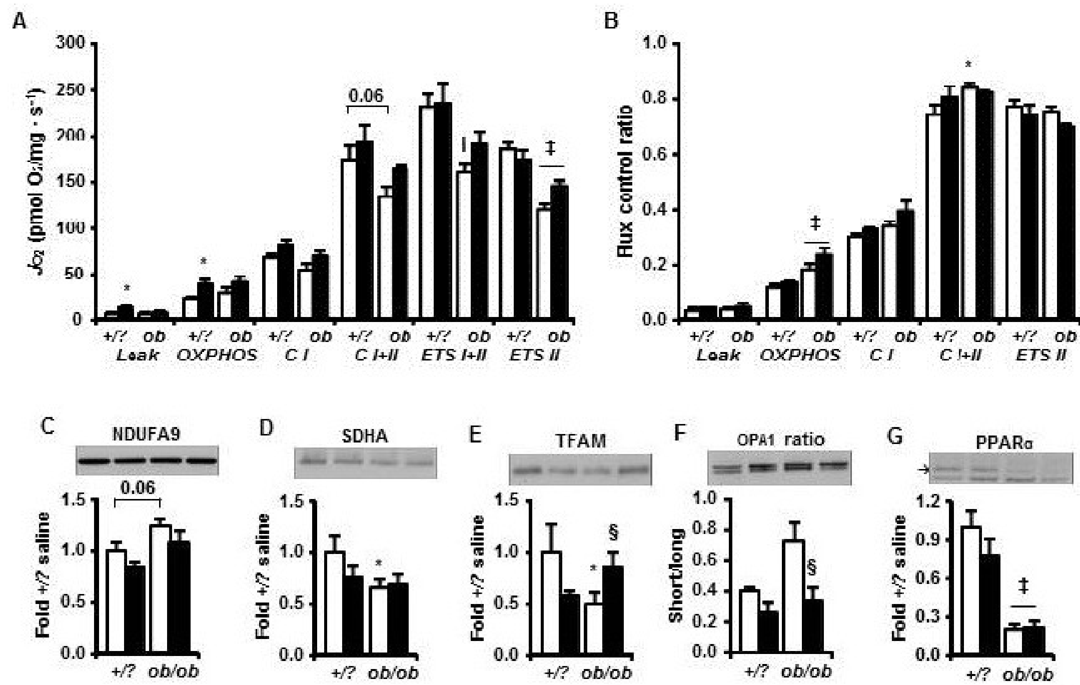

Figure 9: Hepatic mitochondrial respiration is reduced in *ob/ob* mice. (A) Oxidative phosphorylation and electron transport system (n=5-9). (B) Flux control ratios (n=4-9). (C-D) Protein markers of complex I and II. (E) TFAM protein levels, (F) OPA1 ratio. (G) PPARα protein abundance, (n=6-8, for immunoblot analysis). Results are mean ± SEM. *p<0.05, †p<0.01, ‡p<0.001 vs. lean (*+/?*), saline-treated mice. §p<0.05 vs. *ob/ob* saline-treated mice.

Results from mitochondrial respiration in *ob/ob* mice suggest that the primary defect in the electron transport capacity lies at the level of *C II*. The reduction in electron transfer at the level of *C II* could be attributed to the relatively low abundance of the catalytic subunit of *C II*, SDHA. Succinate dehydrogenase connects the citric acid cycle to the electron transport chain and catalyzes the oxidation of succinate to fumarate. The *C II* activity is decreased in patients with non-alcoholic hepatic steatosis (Perez-Carreras et al. 2003). The present data in *ob/ob* mice is compatible with previous results (Perez-Carreras et al. 2003) showing a massive accumulation of intrahepatic triglyceride content (Study II, Fig 1E) in obesity. In the present study, leptin treatment decreased liver weight and intrahepatic triglyceride content, but mitochondrial respiration was unaffected. Leptin-mediated depletion of hepatic triglyceride content is independent of mitochondrial respiration (β-oxidation) and is mediated by increased hepatic lipid export (Singh et al. 2009). Conversely, leptin treatment represses the elevated stearoyl-CoA desaturase-1 (SCD1) activity in the liver from *ob/ob* mice and is thought to enhance β-oxidation with concomitant triglyceride depletion (Cohen et al. 2002). Other studies have provided evidence that hepatic mitochondrial respiration is enhanced in *ob/ob* mice (Brady et al. 1985; Singh et al. 2009). The discrepancy between previous studies and the present results could stem from different reasons. One major difference is the experimental methodology used between studies. Previous studies were performed in isolated mitochondrial fractions, whereas the present results were obtained using mechanically permeabilized liver tissues. Isolation techniques might unknowingly lead to a bias in mitochondrial selection. Mitochondria in most pathological states have altered volume and density. Differential centrifugation, which

is essential for mitochondrial isolation procedures, may lead to a selection of a specific pool of mitochondria that might yield very different results. Mitochondria usually exist as a network of structures and isolation procedures disrupts this network, which might influence the final experimental readout (Kuznetsov et al. 2008). Decreased TFAM protein abundance along with increased OPA1 (short to long form) protein abundance in the saline-treated *ob/ob* mice indicates increased mitochondrial stress. Even though, leptin treatment did not increase mitochondrial respiration, improved mtDNA stability and decreased mitochondrial stress, as evidenced by increased levels of TFAM and reduced OPA1 levels, was observed.

4.2.4 Effect of leptin treatment on whole-body energy metabolism

Leptin treatment decreased food intake and concomitantly reduced body weight in both *ob/ob* mice and lean mice. Moreover, the amplitude of these effects was more pronounced in obese mice. In Study II, lean and fat mass were decreased by leptin treatment, whereas in Study III, the effect of leptin treatment was more pronounced on fat mass. The effect of leptin on food intake is well-established and is under central control (Zhang et al. 1994; Halaas et al. 1995; Pelleymounter et al. 1995). Leptin reduces fat mass by direct and indirect mechanisms, even at doses that do not reduce food intake (Levin et al. 1996). These effects of leptin on food intake and body composition are consistent with the physiological role of leptin in energy homeostasis.

Leptin treatment partially corrected the hepatic steatosis in *ob/ob* mice (Study II). Liver weight was higher in saline-treated *ob/ob* mice ($p<0.001$ vs. lean, saline-treated mice). Leptin treatment for five days reduced liver weight in *ob/ob* mice ($p<0.001$ vs. *ob/ob*, saline treated mice) and lowered the hepatic triglyceride level ($p<0.01$ vs. *ob/ob* saline-treated mice). These effects of leptin in the absence of any increase in mitochondrial respiration suggest that the reduction in hepatic triglyceride content might have resulted from an increase in hepatic lipid export (Singh et al. 2009) or a decrease in triglyceride synthesis. Acute infusion studies have provided evidence that leptin-mediated hepatic triglyceride depletion is a result of increased hepatic β-oxidation, with no change in lipid export. This increase in β-oxidation was associated with increased ACC phosphorylation (Huang et al. 2006). In the present study, both lipid export and increased β-oxidation might have occurred. We cannot exclude the possibility that an increase in β-oxidation may have occurred, since the mitochondrial respiration in isolated liver was performed approximately 16 hours after the last leptin injection. Leptin also has direct inhibitory effect on insulin secretion, which might indirectly decrease triglyceride synthesis (Milstein et al. 1956; Emilsson et al. 1997; Kieffer et al. 1997).

The intramuscular triglyceride content was reduced in leptin-treated *ob/ob* mice (Study II, Fig 1 E and Study III, Fig 6 B). Leptin treatment also depletes intramuscular triglyceride content (Shimabukuro et al. 1997) by stimulating lipid oxidation by central and peripheral mechanisms (Minokoshi et al. 2002). Leptin also opposes the lipogenic effect of insulin on lipid partitioning and favors lipid oxidation (Muoio et al. 1999) and is regulated by AMPK-mediated inhibition of ACC activity (Minokoshi et al. 2002). The inhibitory effect of leptin on insulin secretion and gene expression also normalizes

insulin levels in *ob/ob* mice (Seufert et al. 1999), which when combined with an inhibition on ACC activity, leads to triglyceride depletion in skeletal muscle.

Skeletal muscle glycogen content in *ob/ob* mice was reduced by leptin treatment (Study II, Fig 1F and Study III, Fig 6 A). Leptin also inhibits glycogen synthesis under basal, as well as insulin-stimulated condition in skeletal muscle (Liu et al. 1997). This effect of leptin on glycogen synthesis could partly be mediated by AMPK-induced inhibition of GS (Bultot et al. 2012). Even though our results are consistent with this finding, others have reported that leptin increases basal and insulin-stimulated glycogen synthesis (Harris 1998). Indeed, leptin may regulate skeletal muscle glycogen content at multiple levels. For instance, leptin may inhibit insulin secretion and insulin-mediated gene expression (Seufert et al. 1999). Alternatively, leptin may activate AMPK and inhibit GS (Bultot et al. 2012). In addition, the reduction in food intake may favor glycogenolysis. Indeed, all of these possibilities may account for the reduced glycogen content after leptin treatment.

4.2.5 Limitations of Study II

Leptin has a potent effect on food intake. Therefore, to exclude the possibility that leptin-mediated reduction in food intake may influence the results, a group of pair fed animals could have been included. However, one strength of the work was the inclusion of lean wild-type (leptin-positive) mice treated with saline or leptin. The use of the present mitochondrial respiration protocol (glutamate and succinate) to assess respiration may not completely reflect β-oxidation. Therefore a modified protocol using palmitoyl-carnitine might give a more precise tool to assess leptin-mediated responses on mitochondrial respiration (β-oxidation). The acute effect leptin on β-oxidation was not assessed in the present study and therefore future studies to study mitochondrial respiration over a time course (i.e. 15 minutes to 6 hours) may address this issue.

4.3 AMPK ACTIVATION IN THE CONTEXT OF INSULIN RESISTANCE ASSOCIATED WITH LEPTIN DEFICIENCY

4.3.1 AMPK activation and insulin sensitivity

AMPK is comprised of a heterotrimeric complex with α, β and γ subunits that act collectively as a metabolic sensor to regulate energy metabolism (Davies et al. 1994; Hardie et al. 1997). AMPK is highly sensitive to changes in intracellular AMP/ATP and ADP/ATP ratio in response to cellular stress (Corton et al. 1994; Oakhill et al. 2011), AMPK activation restores cellular energy status by stimulating glucose uptake and lipid oxidation (Merrill et al. 1997; Bergeron et al. 1999; Hayashi et al. 2000; Kaushik et al. 2001). This effect of AMPK on glucose uptake is insulin-independent (Hayashi et al. 2000). Physiological or experimental approaches that activate AMPK have been shown to ameliorate insulin resistance and improve energy metabolism in type 2 diabetes and obesity (Musi et al. 2001; Halseth et al. 2002; Song et al. 2002). A point mutation (R225Q) on the AMPKγ3 subunit has been shown to activate the kinase and confers metabolic improvements in a high-fat diet model of insulin resistance (Barnes et al. 2004). Study III addressed whether skeletal muscle expression of

AMPKγ3^{R225Q} can confer metabolic improvements in a leptin deficient *ob/ob* mouse model of insulin resistance.

4.3.2 AMPK γ3^{R225Q} increases skeletal muscle glycogen content

Skeletal muscle glycogen content in gastrocnemius was increased in lean transgenic γ3^{R225Q} mice ($p<0.05$ vs. WT mice). This result is consistent with reported effects of this (R225Q) and similar mutation (RN^{-} pig, R200Q), (humans, R225W) on skeletal muscle glycogen content (Milan et al. 2000; Barnes et al. 2004; Costford et al. 2007). Several mechanisms can be attributed to the increased glycogen content associated with AMPK activation. Acute activation of AMPK phosphorylates and thereby inhibits GS activity (Miyamoto et al. 2007) and this could lead to reduced glycogen content. AMPK activation also increases glucose uptake (Krook et al. 2004), with a concomitant increase in glucose-6-phosphate level, which allosterically overrides the inhibition imposed by AMPK on GS (Luptak et al. 2007; Hunter et al. 2011) thereby increasing glycogen synthesis (Study III, Fig 1). Defects in skeletal muscle glucose uptake and glycogen synthesis contribute to impairments in whole-body glucose metabolism in type 2 diabetic patients (Shulman et al. 1990; Zierath et al. 1998). Therefore, targeting skeletal muscle specific activation of the AMPKγ3 subunit can potentially improve glucose metabolism in metabolically dysregulated condition as in diabetes and obesity.

4.3.3 Effect of AMPKγ3^{R225Q} mutation on lipid oxidation and mitochondrial respiration

Palmitate oxidation in EDL muscle from γ3^{R225Q} mice tended to be increased ($p=0.06$ vs. WT). Previously, oleate oxidation was shown to be increased in EDL muscle from γ3^{R225Q} mice fed high fat diet and unaltered in muscle from mice fed a normal chow diet (Barnes et al. 2004). Activation of AMPK is associated with increase in lipid oxidation. This is a net result of ACC phosphorylation and inhibition by AMPK, leading to decreased malonyl-CoA levels, that negatively regulates CPT1 activity. Therefore, by decreasing malonyl-CoA levels, AMPK increases mitochondrial β-oxidation (Winder et al. 1997). Glycogen content negatively regulates AMPK activity and hence lipid oxidation in skeletal muscle (Wojtaszewski et al. 2003; Steinberg et al. 2006). Therefore, the γ3^{R225Q} mutation conferring increased glycogen content might prevent an increase in basal lipid oxidation during the palmitate oxidation experiments. The increased lipid oxidation in EDL muscle from γ3^{R225Q} mice fed a high fat diet (Barnes et al. 2004) could imply that hyperleptinemia associated with high fat feeding (Ahren et al. 1997) augments the effect of the γ3^{R225Q} mutation on lipid oxidation.

Mitochondrial respiration was analyzed in permeabilized EDL muscles. Consistent with palmitate oxidation, mitochondrial respiration in EDL muscle fibers was similar between WT and γ3^{R225Q} mice (Study III, Fig 4). The AMPKγ3^{R225Q} mutation was reported to increase mitochondrial biogenesis, but not mitochondrial respiration (Garcia-Roves et al. 2008). Increased mitochondrial biogenesis is expected to increase mitochondrial respiration. The discordance between mitochondrial biogenesis and mitochondrial respiration could be due to the normalization method used in the respiration experiment. As mentioned earlier, glycogen content was higher in EDL muscle from γ3^{R225Q} mice and therefore water content of the skeletal muscle was likely

to be increased (MacKay et al. 1932). Consequently, when normalizing respiration data with respect to muscle weight, the $\gamma 3^{R225Q}$ mutant EDL muscle might have effectively less proteins and mitochondrial complexes. This could influence the final readout of the mitochondrial respiration data.

4.3.4 AMPK activation and obesity

Obesity and type 2 diabetes affect energy metabolism in insulin-sensitive tissues (liver, adipose tissue and skeletal muscle). Changes in energy metabolism may subsequently lead to defects in glucose and lipid metabolism. In the liver, metabolic dysregulation increases triglyceride content and glucose production (Murthy et al. 1979; DeFronzo et al. 1982; DeFronzo et al. 1985). The ability of insulin to suppress lipolysis from adipocytes is diminished in obesity (Hickner et al. 1999). Defective glucose uptake and glycogen synthesis in skeletal muscle is a hallmark of type 2 diabetes (Shulman et al. 1990; Zierath et al. 1998). AMPK activation can overcome several of the metabolic disturbances in insulin sensitive tissues. AMPK activation in hepatic tissues improved lipid and glucose metabolism (Brusq et al. 2006; Cool et al. 2006) where as in adipocytes, lipolysis is reduced and lipogenesis is inhibited (Sullivan et al. 1994). AMPK activation in skeletal muscle increases glucose uptake and glycogen synthesis, with a concomitant increase in fatty acid oxidation (Merrill et al. 1997; Hunter et al. 2011). Thus, targeting AMPK sensitive pathways may offer a potential therapeutic strategy to ameliorate metabolic abnormalities associated with type 2 diabetes and obesity.

Skeletal muscle accounts for the majority of insulin-stimulated glucose uptake under fed conditions (DeFronzo et al. 1982; DeFronzo et al. 1985). Whole body insulin-mediated glucose uptake is decreased in insulin resistant and type 2 diabetic patients. Defective insulin-stimulated GLUT4 translocation can account for reduced glucose uptake in type 2 diabetes (Kahn et al. 1991; Kahn et al. 1992). Signaling defects at multiple levels in the insulin signaling cascade have been implicated in defective GLUT4 translocation to cell surface (Björnholm et al. 1997; Vind et al. 2011). Pharmacological and physiological approaches that activate AMPK improve glucose uptake through increased GLUT4 levels and increased insulin-stimulated glucose uptake (Kurth-Kraczek et al. 1999; Musi et al. 2001; Koistinen et al. 2003; O'Gorman et al. 2006; Vind et al. 2011). Besides the above mentioned approaches, certain mutations associated with AMPKγ3 subunit results in increased glucose uptake and skeletal muscle glycogen content (Milan et al. 2000; Barnes et al. 2004; Costford et al. 2007). Furthermore, humans bearing the AMPK$\gamma 3^{R225W}$ mutation in skeletal muscle had higher mitochondrial content, oxidative capacity, glucose uptake, and glycogen synthesis rates and reduced intramuscular triglyceride levels (Costford et al. 2007; Crawford et al. 2010). These studies highlight the important role of AMPK in regulating glucose homeostasis even in situations of metabolic perturbations as in type 2 diabetes and obesity.

Increased intramuscular triglyceride content is negatively correlated with skeletal muscle insulin sensitivity in sedentary and obese individuals (Sinha et al. 2002). AMPK activation enhances lipid metabolism in skeletal muscle (Merrill et al. 1997). From a molecular view point, AMPK activation leads to the phosphorylation and

subsequent inactivation of ACC (Kudo et al. 1996; Hutber et al. 1997), which leads to a decrease in malonyl-CoA synthesis. Malonyl-CoA, by negatively regulating mitochondrial CPT1 activity, results in reduced β-oxidation (McGarry et al. 1983; Rasmussen et al. 2002), with a subsequent increase in long chain acyl-CoA and triglyceride synthesis. Thus by inhibiting ACC, AMPK activation increases β-oxidation. AMPK activation also increases mitochondrial biogenesis (Garcia-Roves et al. 2008; Crawford et al. 2010; Koltai et al. 2012; Price et al. 2012) which, when combined with increased β-oxidation augments the depletion of intramuscular triglyceride stores. Thus activation of AMPK by various means offers a metabolically favorable effect on glucose and lipid metabolism. Pathways mediated by AMPK can be targeted to improve insulin sensitivity in metabolically dysregulated conditions as in obesity and type 2 diabetes.

Leptin activates AMPK in skeletal muscle by direct and indirect mechanisms (Minokoshi et al. 2002). AMPK activation by leptin subsequently leads to increase in lipid oxidation. Leptin also has a peripheral effect to increase skeletal muscle glucose uptake (Kamohara et al. 1997). This has potential therapeutic implications for common forms of obesity, which is associated with leptin resistance rather than leptin deficiency (Frederich et al. 1995). Leptin resistance is associated with lower basal metabolic rate and reduced glucose oxidation (Niskanen et al. 1997), which in turn leads to further weight gain. Further studies are required to address whether AMPK signaling is affected by leptin resistance. Whether activating AMPK in the presence of leptin resistance can confer leptin sensitivity and thereby improving insulin sensitivity also remains to be elucidated. Studies using a combination of peripheral leptin sensitizers and peripheral AMPK activators might open up new therapeutic strategies in the context of obesity and leptin resistance.

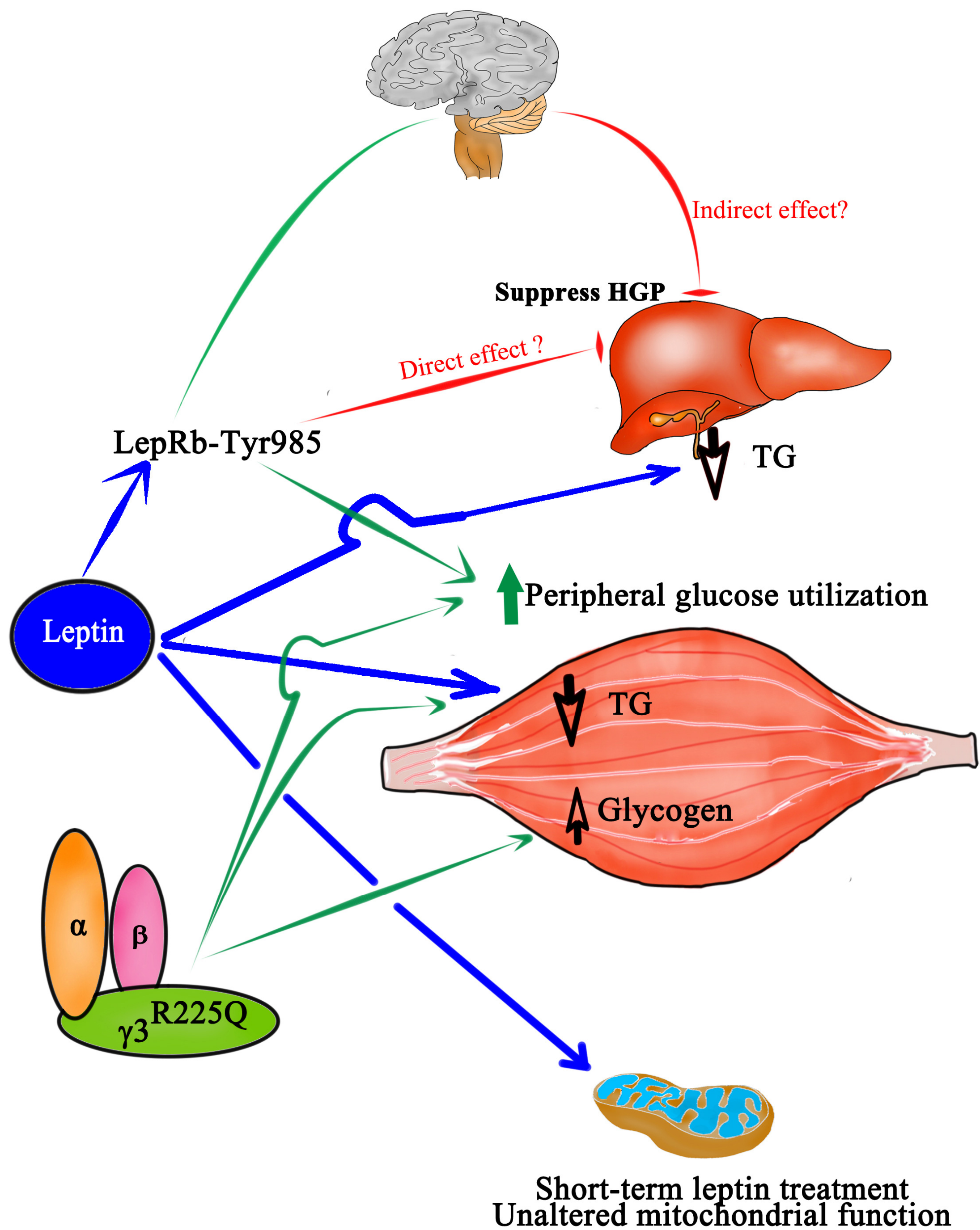

Figure 10: Regulation of whole-body energy metabolism by LepRb and AMPK signaling. Mutation of LepRb-Tyr985 enhances whole-body insulin sensitivity. The AMPKγ3^{R225Q} mutation increases skeletal muscle glycogen content and decreases triglyceride content. Short-term leptin treatment reduced hepatic and skeletal muscle triglyceride content in leptin deficient mice.

4.4 SUMMARY

- The leptin receptor plays a prominent role in the regulation of hepatic and peripheral glucose metabolism. Attenuation of SOCS3-mediated feedback on LepRb-Tyr985 improves insulin-mediated suppression of hepatic glucose production. Enhanced leptin sensitivity conferred by this mechanism also improves whole body insulin sensitivity. Strategies that can reduce SOCS3 binding at LepRb-Tyr985 may offer a potential approach to improve leptin resistance and thereby enhance hepatic and peripheral insulin sensitivity in the context of obesity and insulin resistance.

- Metabolic perturbations as in the leptin-deficient obese condition bring about tissue-specific programming of mitochondrial function and dynamics. Hepatic mitochondrial function is impaired in the absence of functional leptin action and is not improved by short-term leptin treatment. Obesity-associated changes in glycolytic skeletal muscle increase mitochondrial electron transport capacity. Oxidative soleus muscle exhibits robust mitochondrial respiration, which is unaltered by either obesity or short-term leptin treatment. Understanding the molecular mechanisms behind these tissue-specific mitochondrial adaptations may bridge the gap between mitochondrial function and insulin resistance, and may pave the way for new treatments to improve insulin sensitivity.

- AMPK activation by the $\gamma3^{R225Q}$ mutation increases skeletal muscle glycogen content and increases skeletal muscle glucose uptake in lean insulin-sensitive mice. The same AMPK mutation imposes a metabolically favorable milieu (increased skeletal muscle glycogen content and reduced intramuscular triglyceride) in insulin resistant and obese *ob/ob* mice. Nevertheless, these metabolic adaptations were insufficient to ameliorate the whole-body metabolic disturbances arising from the life-long leptin-deficiency.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The overall aim of this thesis was to investigate the role of central and peripheral mechanisms regulating energy and glucose homeostasis. To address this issue, two pathways were studied: 1) leptin receptor-mediated signaling, 2) AMPK-mediated signaling. These signaling pathways were put in the context of insulin sensitivity and energy homeostasis.

Studies in this thesis highlight the role of leptin receptor and AMPK signaling in the regulation of whole-body insulin sensitivity. Furthermore, the effect of leptin treatment on tissue-specific mitochondrial function is evaluated in the context of metabolic disturbances associated with obesity. Results from Study I provide evidence for a role of LepRb-Tyr985 in the regulation of hepatic glucose production and peripheral insulin sensitivity. Binding of the negative regulator, SOCS3 to the LepRb is blocked when Tyr985 on LepRb is mutated to Leu985 (Bjorbaek et al. 2000). Reduced binding of SOCS3 on LepRb increases leptin sensitivity (Björnholm et al. 2007) and thereby enhances hepatic and peripheral insulin sensitivity, implying strategies to improve leptin sensitivity may also increase whole-body insulin sensitivity. This has therapeutic potential that can be utilized to address the leptin resistance observed in the common forms of obesity. Study I, while highlighting an important role for LepRb-Tyr985 in mediating whole-body insulin sensitivity, has not identified the insulin-sensitive tissues responsible for mediating these actions. Future research should therefore be aimed at identifying the tissue-specific effects of LepRb-Tyr985 mutation in mediating whole-body insulin sensitivity. Furthermore, additional studies to explore the beneficial effects of the LepRb-Tyr985 mutation in preventing leptin resistance and improving whole-body insulin sensitivity in a mouse model of leptin resistance and insulin resistance (high fat diet studies) should be performed.

Mitochondrial respiration studies of isolated tissues from *ob/ob* mice indicate that tissue-specific alterations in mitochondrial function occur in the context of obesity. In glycolytic skeletal muscle mitochondrial respiration is increased in obesity, whereas in oxidative soleus muscle mitochondrial respiratory capacity is unaltered and unresponsive to leptin treatment. Hepatic mitochondrial capacity is negatively affected by obesity, whereas short-term leptin treatment partially improves hepatic steatosis in the absence of further improvements in mitochondrial capacity. The present work evaluating tissue-specific mitochondrial respiration in *ob/ob* mice highlights a neutral role for short-term leptin treatment in correcting altered mitochondrial function in obesity. These results indicate that the improvement in whole body insulin sensitivity after short-term leptin treatment (Yaspelkis et al. 2001; Nagao et al. 2008; Denroche et al. 2011) is dissociated from changes in mitochondrial respiratory capacity. Diabetes and obesity are chronic metabolic disorders that require long-term treatment interventions. Therefore, further studies are warranted to address whether long-term leptin treatment affects tissue-specific mitochondrial function and whether leptin has an effect on mitochondrial function in leptin-sensitive tissues like adipocyte or brain.

The effect of chronic AMPK activation (AMPK$\gamma 3^{R225Q}$) to improve whole-body insulin sensitivity in leptin-deficiency remains incompletely resolved. Study III provides

evidence to suggest that skeletal muscle-specific AMPK activation improves insulin sensitivity in a tissue-specific manner and functional leptin action is required for this effect. The increased glycogen content in the gastrocnemius muscle from the leptin-deficient *ob/ob*-$\gamma3^{R225Q}$ mice indicates that leptin is not required for AMPK-mediated glycogen synthesis. Moreover, the effect of the AMPK$\gamma3^{R225Q}$ mutation on glycogen content is independent of adiposity. Intramuscular triglyceride content is also reduced in *ob/ob*-$\gamma3^{R225Q}$ transgenic mice, presumably due to decreased triglyceride synthesis, since basal lipid oxidation was unaltered. However, the improvements in muscle biochemistry arising from the AMPK$\gamma3^{R225Q}$ mutation are insufficient to ameliorate whole-body insulin resistance in the leptin-deficient mice. Consequently, a permissive amount of leptin may be required to fully confer the AMPK$\gamma3^{R225Q}$-dependent improvements in skeletal muscle insulin-sensitivity. Thus, lack of central leptin signaling in *ob/ob* mice may override the favorable metabolic milieu conferred by peripheral expression of the AMPK$\gamma3^{R225Q}$ mutation to improve glucose and energy homeostasis. The present study underscores the role of leptin in mediating whole-body insulin sensitivity in a metabolically challenged situation as in *ob/ob* mice.

Collectively, the studies presented in this thesis highlight the role of two important signaling pathways in the regulation of various aspects of glucose and lipid metabolism from a whole body perspective. The leptin and AMPK signaling pathways were explored to determine their beneficial effects in the context of insulin resistance. Treatment strategies targeted at improving leptin sensitivity and skeletal muscle insulin sensitivity via AMPK signaling may provide new opportunities for the treatment of metabolic complications associated with diabetes and obesity.

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