From THE DEPARTMENT OF MOLECULAR MEDICINE
AND SURGERY, SECTION OF INTEGRATIVE
PHSYIOLOGY
Karolinska Institutet, Stockholm, Sweden

NOVEL PATHWAYS REGULATING GLUCOSE AND LIPID METABOLISM IN HUMAN SKELETAL MUSCLE

Reginald L. Austin

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ABSTRACT

The utilization of glucose and lipids as energy substrates in skeletal muscle is strictly regulated. As skeletal muscle is the body's chief consumer of glucose and lipids, it plays a critical role in the maintenance of whole-body homeostasis. Under normal physiological conditions, skeletal muscle displays a certain metabolic flexibility, allowing the tissue to switch between the utilization of glucose and lipids. Generally, skeletal muscle manifests a preference for lipids as the primary energy substrate, in the fasting state, or during such metabolic challenges as starvation or exercise. In these situations, glucose availability is low. However, in the post-prandial state, glucose availability is high and energy substrate utilization is shifted toward glucose. A compromised or reduced plasticity in this ability to shift substrate utilization results in metabolic inflexibility. Conditions associated with metabolic inflexibility include the metabolic syndrome, insulin resistance and type 2 diabetes mellitus. To address the etiology and pathogenesis of these disorders, investigation of the molecular events governing these processes is warranted. In this thesis, novel pathways regulating glucose and lipid metabolism in human skeletal muscle were investigated with the specific aim of discovering and validating new potential therapeutic targets to treat type 2 diabetes.

Cultured primary human skeletal muscle cells were used to determine the consequence of targeted malonyl CoA decarboxylase (MCD) (*Study I*), and inhibitor of nuclear factor κB kinase (IKKβ) (*Study II*), reduction on metabolic and signaling parameters. The effect of exogenous fibroblast growth factor 21 (FGF-21) (*Study III*) and clenbuterol treatment (*Study IV*) on metabolic and signaling parameters were also determined. Targeted genetic reduction of either MCD or IKKβ increased glucose uptake and improved insulin signaling in cultured muscle cells. MCD reduction was sufficient to shift substrate utilization from lipid to glucose oxidation. IKKβ gene silencing prevented TNF-α-mediated insulin resistance in cultured human skeletal muscle. FGF-21 is a novel member of the fibroblast growth factor family of proteins, distinguished by its "hormone-like" action on metabolism. FGF-21 treatment positively impacted glucose metabolism in human skeletal muscle cells and in incubated intact mouse skeletal muscle. Finally, chronic clenbuterol exposure was sufficient to increase glycogen synthesis, and reduce lipid oxidation in human skeletal muscle myotubes and in cultured rat L6 muscle cells.

In conclusion, the studies presented in this thesis provide insight into molecular pathways governing glucose and lipid metabolism in skeletal muscle. Several new targets have been identified and validated for potential therapeutic intervention to treat insulin resistance. Moreover, these investigations provide insight not only into the signaling paradigm of a particular pathway but rather into the interconnectivity of complex metabolic regulation.

LIST OF PUBLICATIONS

I. Bouzakri K*, **Austin R***, Rune A, Lassman ME, Garcia-Roves PM, Berger JP, Krook A, Chibalin AV, Zhang BB, Zierath JR. (2008) Malonyl Coenzyme A Decarboxylase Regulates Lipid and Glucose Metabolism in Human Skeletal Muscle. *Diabetes* 57(6): 1508-1516. * Both authors contributed equally to this work.

II. **Austin RL**, Rune A, Bouzakri K, Zierath JR, Krook A. (2008) siRNA-Mediated Reduction of Inhibitor of Nuclear Factor-kappaB Kinase Prevents Tumor Necrosis Factor-α-Induced Insulin Resistance in Human Skeletal Muscle. *Diabetes* 57(8): 2066-2073.

III. **Austin RL**, Deshmuk AS, Zierath JR, Krook, A. Direct Effect of FGF-21 on Skeletal Muscle Glucose Uptake.

IV. **Austin RL**, Fahlman, R, Long YC, Krook A, Al-Khalili L**.** Effects of Clenbuterol on Glucose and Lipid Metabolism in Skeletal Muscle.

CONTENTS

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ANOVA	Analysis of variance
AS160	Akt substrate of 160 kD
ATP	Adenosine-5'-triphosphate
BMI	Body mass index
CD36	Cluster of differentiation 36
CPT	Carnitine palmitoyl transportation
CRP	C-reactive protein
DAG	Diacylglycerol
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular signal regulated kinases
FBS	Fetal bovine serum
FFA	Free fatty acid
GLUT	Glucose transporters
GSK3	Glycogen synthase kinase 3
HDL	High-density lipoproteins
HSMC	Human skeletal muscle
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
IκBα	Inhibitor of nuclear factor κβ
IKKβ	Inhibitor of nuclear factor κβ kinase
IR	Insulin receptor
IRS	Insulin receptor substrate proteins
JNK	c-jun N-terminal kinase
LCFA	long chain fatty acid
LDL	Low-density lipoproteins
MAPK	Mitogen activated protein kinases
MCD	Malonyl CoA decarboxylase
MHC	Myosin heavy chain
mRNA	messenger Ribonucleic Acid
NFκB	Nuclear factor κβ kinase
OGTT	Oral glucose tolerance test
PDK	Phosphatidyl inositol dependent kinase
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3 Kinase
PIP_2	PI 4, 5-biphosphate
PIP_3	PI 3, 4, 5-triphosphate
PKC	Protein Kinase C
PMSF	Phenylmethylsulphonyl Fluoride

PPAR	Peroxisome proliferator-activated receptor
PTB	Phosphotyrosine binding domain
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SH2	Src homology 2
siRNA	Small interfering ribonucleic acid
TGF-β	Transforming growth factor beta
Thr	Threonine
TNF-α	Tumor necrosis factor alpha
TZD	Thiazolidinediones
VLDL	Very-low density lipoprotein
WHO	World Health Organization

1 INTRODUCTION

Energy metabolism and homeostasis are among the most imperative biological processes to the survival of a living organism. Energy homeostasis is governed by the consumption, storage and utilization of substrates to drive the reactions necessary for normal physiological function. Glucose and lipids are the key substrates in energy balance and their metabolism is closely regulated in human physiology. Normal metabolic function is distinguished by an organism's ability to switch between glucose and lipids as the principal energy substrate. The ability to select between glucose and lipids is based on energetic demand, as well as the availability of the substrate thus establishing metabolic flexibility (1; 2). Generally, when glucose availability is high, it is the preferred substrate. The level of blood glucose is typically increased after a meal. Conversely, when glucose availability is low, lipid metabolism is increased. Metabolic inflexibility manifests as a result of dysregulated substrate utilization. Several factors including excessive caloric intake and physical inactivity correlate with the occurrence of metabolic inflexibility.

Lifestyles, marked by sustained over-nutrition and lack of exercise, frequently result in obesity and imbalances in substrate utilization. Dysregulated substrate utilization and metabolic inflexibility have been implicated (1; 3) in the development of metabolic disorders including type 2 diabetes mellitus, which is a disease characterized by failed insulin action and secretion, also with associated co-morbidities. According to the most recent estimates by the World Health Organization (WHO) [http://www.who.int/diabetes/facts/world_figures/en/], the worldwide prevalence of type 2 diabetes will rise to 366 million in the year 2030. This is a dramatic increase from the estimated prevalence of 171 million in the year 2000. The predicted rise in the number of new cases of type 2 diabetes warrants the development of new treatment strategies.

Skeletal muscle is quantitatively the most important tissue for glucose (4) and lipid (5) metabolism. Under the euglycemic hyperinsulinemic clamp, skeletal muscle accounts for 80-90% of glucose disposal (4; 6), and although adipose tissue is important in the storage of lipids, skeletal muscle is the largest consumer of lipids as an energy substrate. Insulin plays a critical role in skeletal muscle metabolism (7). It is a hormone secreted from the pancreas with multiple metabolic and mitogenic roles. One of the key roles of insulin in skeletal muscle metabolism is to stimulate glucose uptake and concomitantly suppress lipid oxidation. These effects in skeletal muscle make insulin vital to energy homeostasis. Insulin resistance is an emblematic feature of type 2 diabetes. Insulin resistance is characterized by a reduced ability of insulin-sensitive tissues to respond to the effects of insulin on glucose and lipid handling.

Insulin resistance and metabolic inflexibility potentiate the development of type 2 diabetes (2; 8). Therefore, an investigation of the essential molecular mechanisms and signaling pathways that regulate glucose/lipid metabolism and improve insulin action in skeletal muscle is important for the development of novel therapeutics to treat type 2 diabetes.

1.1 THE METABOLIC SYNDROME

The metabolic syndrome is a composite of medical conditions and risk factors that potentiate the development of cardiovascular disease and type 2 diabetes (9-12). Hallmarks of the metabolic syndrome include central obesity, hyperinsulinemia, hypertension, dyslipidemia and debilitated glucose metabolism (9). The precise clinical definition of the metabolic syndrome is a subject of much debate, which challenges its

practicality as a tool for identifying and treating its intrinsic risk factors. Although there are several extant definitions of the metabolic syndrome, the definitions provided by the National Cholesterol Education Program – Adult Treatment Panel III (Expert Panel on Detection, Evaluation and Treatment of High Blood Pressure in Adults, 2001, (13) and the International Diabetes Federation (14) seem to be the most widely used. Despite the slight departures in defining the metabolic syndrome, there is a fundamental and collective agreement on the cluster of risk factors that make up the metabolic syndrome phenotype.

Other key features of the metabolic syndrome include; increased very-low density lipoprotein (VLDL) triglyceride and decreased high-density lipoprotein (HDL) - cholesterol (9) (**Figure 1**). Increased VLDL cholesterol levels are associated with obesity (15) Both the National Cholesterol Education Program – Adult Treatment Panel III and the International Diabetes Federation give particular importance to obesity as a risk factor for the development of the metabolic syndrome and type 2 diabetes. In fact, the International Diabetes Federation's definition of the metabolic syndrome mandates the presence of central abdominal obesity [http://www.idf.org/webdata/docs/MetS_def_update2006.pdf]. Two commonly used measurements to diagnose obesity are body mass index (BMI) and waist circumference. BMI is closely associated with total body fat content and it compares a patient's weight (kg) in relation to height (m^2). Patients with a body mass index (BMI) ≥ 30 are considered to be obese (16). A key disadvantage of using BMI as a diagnostic tool for obesity is that BMI does not account for the proportional contribution of such factors as bone, muscularity and others to a patient's weight. A further clinical consideration in the use of BMI is that it may not correspond to the same degree of body fat content within different populations, which is largely due to varying body proportions. For example, the WHO Expert Consultation determined that the percentage of Asian people with a high risk of developing type 2 diabetes and co-morbidities is substantial at BMI measurements lower than the existing WHO cut-off point for overweight individuals (= 25 kg/m^2). However, due to a high degree of variance observed in cut-off points within different Asian populations, the WHO Expert Consultation recommended retention of current BMI cut-off points. Parameters governing the clinical use of BMI and its relation and interaction with other diagnostic tools such as weight circumference are under review.

The rationale for giving obesity such particular consequence as a risk factor in the etiology of the metabolic syndrome is based on evidence, which shows that other risk factors including elevated serum triglycerides, LDL-cholesterol, blood glucose and hypertension are attenuated with a reduction in body weight through diet or exercise (17-20). The features of the metabolic syndrome are inherent to the development and pathogenesis of type 2 diabetes. This establishes a clear link between these two multifactorial disorders and indicates that shared molecular mechanism may contribute to the underlying pathogenesis of insulin resistance in multiple diseases.

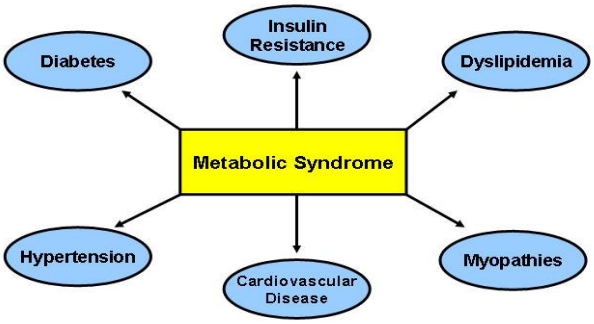

Figure 1: Features of the metabolic syndrome. The physiological abnormalities that comprise the metabolic syndrome.

1.1.1 Cellular glucose metabolism

The monosaccharide glucose serves as a critical energy substrate and metabolic intermediate in living organisms. The uptake, storage and utilization of glucose are important to the continuity of cell and tissue metabolic function. In vivo blood glucose levels are under tight hormonal control (8; 21; 22). In response to a meal, pancreatic β-cells release a pulsatile secretion of insulin into circulation (23; 24). The presence of insulin facilitates glucose uptake into insulin-sensitive tissues and decreases hepatic glucose output and lipid oxidation. In the starved-state, glucose availability is low. The limited access to glucose as an energy substrate stimulates the pancreatic α-cells to release glucagon, which promotes the increase of hepatic glucose production and fatty acid oxidation (23). The ability to differentially utilize alternate substrates based on availability, is termed metabolic flexibility, and allow for efficient use of metabolic resources (1; 8).

1.1.2 Type 2 Diabetes Mellitus

Failure of insulin-sensitive peripheral tissues to respond to insulin-mediated glucose uptake results in an increase in serum glucose levels, which leads to a compromised homeostatic state (25; 26). Chronically elevated levels of glucose in the circulation i.e. hyperglycemia, which if left unmanaged, potentiates the development of cardiovascular disease and renal failure, as well as severe microvascular, nerve and retinal damage (27-30). Peripheral insulin-resistance, sustained hyperglycemia concomitant with pancreatic β-cell failure, and increased hepatic glucose output are intrinsic to manifest type 2 diabetes mellitus (31-33). The attenuation of hyperglycemia and maintenance of euglycemia is vital to impede the manifestation of type 2 diabetes mellitus and its co-morbidities.

1.1.3 Medical diagnosis of type 2 diabetes mellitus

The most current World Health Organization (WHO, 2006) diagnostic definition of type 2 diabetes is a fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or plasma glucose ≥ 11.1 mmol/l (200 mg/dl) 2 h post an oral glucose tolerance test (OGTT; 1.75 g glucose/kg of body weight, maximum 75 g glucose). The WHO retains and recommends OGTT as a medical diagnostic tool for type 2 diabetes because measurements of fasting glucose alone fail to properly identify roughly 30% of previously undiagnosed diabetes. OGTT is also the only current metric for determining impaired glucose tolerance and it is often required to establish glucose tolerance abnormalities in asymptomatic cases.

1.1.4 Type 2 diabetes development and pathogenesis

Peripheral insulin resistance, i.e. a diminished response to insulin-mediated glucose uptake in peripheral insulin sensitive tissues, is the putative initiation of type 2 diabetes development and pathogenesis. The causation of insulin resistance is variable, but the fundamental end result is failed insulin action. In the pre-diabetic state, key tissues including adipose, liver and skeletal muscle are recalcitrant to the glucose lowering effects of insulin (34). As a result, serum glucose levels are elevated, which stimulates the pancreatic β-cells to increase insulin secretion beyond normal physiological levels. Insulin resistance in the liver further exacerbates the elevation in serum glucose levels, as insulin fails to exact its normal suppression of hepatic glucose production (35). The protracted over-stimulation of the pancreatic β-cells ends with β-cell failure, which together with hyperglycemia establishes type 2 diabetes (36-38).

The development and progression of type 2 diabetes has a number of distinct but often-related contributors. Indeed, the roles of such dysyregulated factors as hormones (39; 40), immunity (41; 42) , free fatty acids (8; 43) and others, have been studied extensively and contribution to the development and pathogenesis of the disease. A more informed knowledge of the basic molecular events and pathways that drive insulin resistance is required. Additionally, studies investigating the interactions between these factors are needed. A more comprehensive understanding of type 2 diabetes may potentiate improved diagnosis and treatment.

1.2 SKELETAL MUSCLE METABOLISM

The metabolic properties of mammalian skeletal muscle are heavily influenced by their respective fiber type composition (44; 45). Proportionate numbers of the fiber types present in a given muscle determines its contractile and metabolic disposition. There are four myosin heavy chain (MHC) gene isoforms (*I, IIA, IIX/IID and IIB*) and the relative expression of these genes makeup the muscle's phenotype (44). Type I or slow-twitch fibers are enriched with mitochondria and have an increased oxidative capacity, whereas type II or fast-twitch fibers have a high expression of glycolytic enzymes (44; 45). Muscle fiber type composition may play a role in the overall metabolism of glucose, as type I fibers are more insulin sensitive than type II fibers (46; 47). This is evidenced the finding of reduced relative number of type I fibers in diabetics (48; 49). The relative proportion of type I fibers is positively correlated with insulin sensitivity and glucose transport is increased in type I fibers when compared to type II fibers (50; 51).

Glucose undergoes oxidative or non-oxidative metabolism in skeletal muscle. The oxidative or glycolytic metabolism of glucose involves its immediate degradation in a series of steps to produce pyruvic acid. This alpha-keto acid is then converted to acetyl-CoA, which enters the citric acid cycle to produce adenosine-5'-triphosphate

(ATP) (52; 53). In non-oxidative glucose metabolism, glycogen is formed. The glycogen utilization is deferred until muscle needs to produce more energy.

Skeletal muscle can also metabolize lipids to satisfy its energy needs (54; 55). The tissue depends upon the catabolism of lipids during periods of low glucose availability. Lipids enter skeletal muscle as fatty acids through passive and facilitated transport. The albumin-bound long-chain fatty acids (LCFA) form of lipid exists in the blood stream (54). Upon the facilitated transport of a LCFA into skeletal muscle through the membrane integral membrane protein cluster of differentiation 36 (CD36), the metabolic fate of LCFA as a fuel depends on its subsequent facilitated transport into the mitochondria where it can be oxidized (54). The transfer of LCFA into the mitochondria is under the control of the energy sensor 5' AMP-activated protein kinase or AMPK (56; 57).

The control of AMPK over LCFA mitochondrial entry is actuated via two mechanisms. In the first mechanism, AMPK can phosphorylate and activate malonyl coenzyme A decarboxylase (MCD), which subsequently inactivates malonyl CoA. This action promotes LCFA entry into the mitochondria. However, in the absence of MCD, malonyl CoA acts downstream to inhibit LCFA mitochondrial entry via binding to carnitine palmitoyltransferase 1 (CPT1) (56; 58). CPT1 is responsible for facilitating LCFA transfer into the mitochondria and the allosteric binding of malonyl CoA to its regulatory domain blunts the capacity of CPT1 to carry out this action (**Figure 2**). The second mechanism by which AMPK can control LCFA mitochondrial entry is via phosphorylation of acetyl-CoA carboxylase (ACC) (56; 58; 59). The muscle specific isoform, ACCβ is the rate-limiting enzyme in malonyl-CoA synthesis as it catalyzes the carboxylation of acetyl CoA to form malonyl CoA (59). Thus, AMPK appears to have a duel role in controlling fatty acid oxidation. Importantly, an increase in malonyl CoA can potential promote a switch in substrate utilization toward glucose instead of lipid thus leading to a reduce serum glucose profile. As such, the genetic and pharmacological inhibition of MCD is currently being investigated as a possible therapeutic to correct substrate inflexibility.

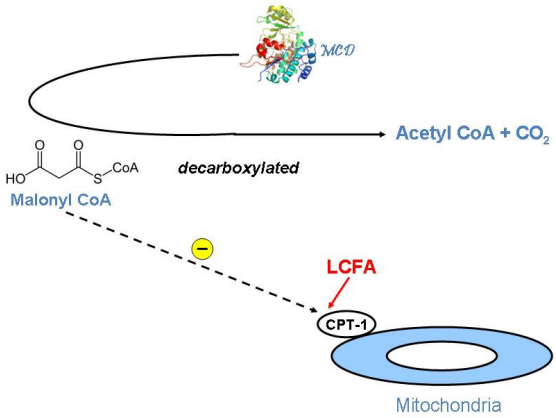

Figure 2: The action of malonyl CoA on long chain fatty acid (LCFA) metabolism. Malonyl CoA is produced from the carboxylation of acetyl CoA. Malonyl CoA then goes on regulates the metabolism of LCFA by allosterically binding to the regulatory domain of CPT1. This action blocks CPT1-facilitated transport of LCFA across the mitochondria outer membrane for β-oxidation.

1.2.1 Glucose metabolism and insulin resistance in skeletal muscle

Insulin facilitates the entry of glucose into skeletal muscle through glucose transporter 4 (GLUT4) (60; 61). Insulin increases glucose transport in sensitive tissues by facilitating the translocation of GLUT4 from intracellular vesicles to the plasma membrane. When GLUT4 is in the plasma membrane, glucose enters the cell via facilitated diffusion. Skeletal muscle accounts for the majority of postprandial glucose utilization making it the chief site of glucose transport (4; 6). Type 2 diabetics have a dampened response to insulin-stimulated glucose uptake in skeletal muscle following a meal (4; 6). As skeletal muscle is the principal tissue for glucose disposal in the body, it has become one of the main target tissues for therapeutics focused on the normalization of glucose-homeostasis and the treatment of type 2 diabetes.

1.2.2 Insulin signaling network

Insulin sensitive tissues express insulin receptors at the cell surface, which allows them to regulate metabolism through insulin signaling cascades (62). The events of insulin signaling are initiated upon insulin binding to the insulin receptor (IR). The IR consists of two extracellular α-subunits and two transmembrane β-subunits (62; 63). The tyrosine kinase activity of the IR is responsible for autophosphorylation upon binding insulin (62; 64). The activated receptor's phosphorylated tyrosine residues provide docking sites for several downstream molecules including the family of insulin receptor substrate (IRS) proteins which are subsequently phosphorylated by the insulin receptor on tyrosine residues (Krook, Whitehead et al. 1997; White 1997; Virkamäki, Ueki et al. 1999) (**Figure 3**). There are four members in the IRS family of proteins (65). However, IRS-1 and IRS-2 are the two family members most prominently involved in skeletal muscle metabolic regulation. IRS-1 and IRS-2 have distinct signaling roles despite their high degree of sequence homology (66). Investigations into their specialized downstream signaling roles show that IRS-1 is important for glucose metabolism while IRS-2 is essential for lipid metabolism (66). The IRS family of proteins, particularly IRS-1 and IRS-2, is indispensable to insulin signaling as they orchestrate insulin-mediated metabolic and mitogenic actions (66-68).

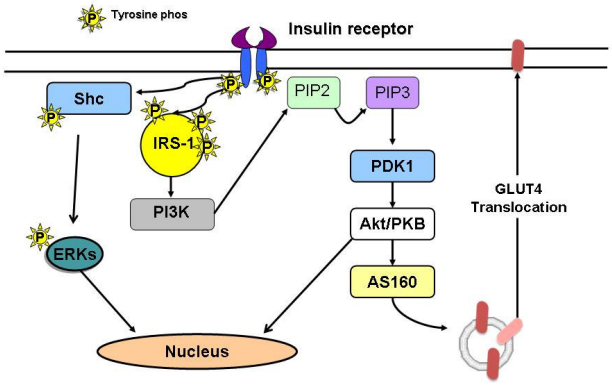

Figure 3: Insulin signaling to glucose transport and gene regulation. Upon insulin binding to the insulin receptor, insulin receptor substrate 1 (IRS1) is recruited to the cell membrane. IRS1 then recruits phosphatidolinositol 3 kinase (PI3), which converts PI 4,5-biphospahet (PIP_2) to PI 3, 4, 5 triphosphate (PIP_3). This leads to the allosteric activation of phosphatidyl inositol dependent kinase 1 (PDK1), which in turn activates Akt. The activation of Akt results in the regulation of various metabolic and mitogenic processes including the phosphorylation of its downstream target Akt substrate 160 (AS160). Phosphorylation of AS160 leads to GLUT4 translocation to the cell membrane and the facilitation of glucose uptake.

1.2.2.1 Insulin-mediated metabolic action

Activated IRS's function as scaffolding proteins and tyrosine phosphorylation on chief IRS residues enhance the binding of proteins containing *src* homology (SH) 2 domains including phosphatidylinositol 3 kinase (PI3K) (69-71). The association of PI3K with IRS affords PI3K a closer proximity to the plasma membrane. This magnifies its lipid substrate availability for the production of second messengers in which PI3K phosphorylates PI 4,5-biphosphate (PIP_2) to PI 3,4,5-triphosphate (PIP_3) (72). Phosphoinositide dependent kinase 1 (PDK1) is allosterically activated downstream of PIP_3 (73). PDK1 activation results in the subsequent phosphorylation of the atypical Protein Kinase C (PKC) family member PKCζ as well as Akt (74; 75). PKCζ activation may play a role in GLUT4-mediated skeletal muscle glucose transport (76). Akt is also believed to be essential to skeletal muscle GLUT4-mediated glucose transport (77). There are currently three known isoforms of Akt (Akt 1-3) but only Akt1 and Akt2 are expressed in skeletal muscle (78). The phosphorylation of Akt leads to the subsequent phosphorylation and deactivation of its downstream targets Akt-Substrate 160-kD (AS160) (also known as TBC1D4) and TBC1D1 (79). These proteins are postulated to be the proximal steps to GLUT4 vesicle translocation to the plasma membrane (80). Interestingly, Akt1 and Akt2 have been determined to have differential downstream metabolic regulation (66). Akt1, together with IRS-2, appears be important for lipid metabolism, while Akt2 and IRS-1 was shown to be essential for glucose metabolism (66).

1.2.2.2 Insulin-mediated gene regulation and signaling

Insulin mediates alterations in gene expression through IRS phosphorylation and subsequent downstream targeting of Mitogen Activated Protein Kinases (MAPK) (71). Extracellular signal Regulated Kinases (ERK) 1 and 2, c-Jun N-terminal Kinase (JNK) and p38 are MAPK family members responsible for interposing the mitogenic effects of insulin. ERK 1/2 MAPK is dispensable for the acute effects of insulin on glucose transport in skeletal muscle (81). This is evidenced through the inhibition of ERK 1/2 with the selective, cell-permeable pharmaceutical PD 98059, which impedes MAPKK phosphorylation (82). However, JNK phosphorylation and activation negatively impacts insulin signaling to glucose uptake via serine phosphorylation on IRS1 (83; 84). The full phosphorylation of crucial tyrosine residues on IRS1 is disrupted by serine phosphorylation (84). This interferes with the interaction between IRS1 and IR and perturbed downstream signaling to GLUT4 translocation (85). The consequence of p38 MAPK on insulin-mediated glucose transport is ambiguous and is in need of further investigation. External factors such as cellular stress and cytokines activate p38 MAPK. Cytokines are involved in JNK activation (84);(86; 87) .

1.2.3 Defective insulin signaling and insulin resistance

Phosphorylation of the IR promotes interaction of its catalytic domain with the phosphotyrosine binding (PTB) domian of IRS1 (85). Serine phosphorylation of IRS1 on various residues attenuates its tyrosine phosphorylation and disrupts its association with the IR (72; 77). Incomplete activation of IRS1 results in perturbations in its signaling to downstream targets such as PI3K. Diminshed insulin-mediated PI3K activity in the skeletal muscle of pre-diabeteic and diabetic subjects has been described under *in vivo* and *in vitro* conditions (71; 88; 89). GLUT4 translocation to the cell surface is an event that occurs downstream of PI3K activation. Impairments in PI3K activity correlate with reduced cell surface GLUT4 translocation and abated glucose uptake (89; 90).

Although reduced IRS1 tyrosine phosphorylation in the skeletal muscle of type 2 diabetics has been reported, its total protein expression is unaltered (71; 88; 91) Similarly, protein expression of targets downstream of IRS1 including PI3K, Akt and GLUT4 are unchanged (92-94). These observations suggest that phosphorylation of these proteins is the principal mechanism by which they are regulated, as protein expression seems to play little or no discernable role in the pathology of type 2 diabetes.

In addition to its role in metabolic regulation, insulin mediates changes in gene expression (95)Defective insulin action on JNK has been well described in skeletal muscle. JNK activation and expression is abnormally elevated in skeletal muscle from obese and type 2 diabetic patients (87). Escalated JNK activity has been shown to be associated with increased IRS1 phosphorylation and downstream signaling disturbances (84; 87). The isoform of the JNK gene, JNK1 is posited to have specific role as the deletion of JNK1 led to decreased adiposity, enhanced insulin sensitivity and improved IR signaling capacity in obese mouse models (96). P38 MAPK has also been suggested to play a possible role in disturbed insulin signaling as an earlier study in our lab showed basal levels of p38 to be elevated in type 2 diabetes patients (97). Conversely, insulin action ERK 1/2 MAPK is unchanged in the skeletal muscle of patients with type 2 diabetes (71).

The insulin signaling network is circuitous and this obscures efforts to clearly define the progression of type 2 diabetes from a mechanistic standpoint. The putative defects in insulin signaling may not simply be attributed to alterations in the phosphorylation or gene expression of a single target, but rather to a complex set of

alterations within the signaling cascades. In this view, consideration must be given to the full composite of contributing signaling defects. This may allow further insight into the mechanisms charting the course to type 2 diabetes development and pathogenesis.

1.2.4 Lipid metabolism and insulin resistance in skeletal muscle

Central obesity has an intrinsic association with increased serum free fatty acid (FFA) levels (11). Obesity in the central region of the body is a result of immoderate visceral adipose deposition (98). Visceral adipose is the fat tissue that is connected to the internal organs. This tissue appears to be metabolically different from subcutaneous adipose, which is the fat tissue that lies just beneath the skin. In contrast to subcutaneous fat, visceral fat is believed to make a greater contribution to circulating FFA levels possibly due to its more ready availability (99; 100). The increase in circulating FFA levels is linked to the activation of JNK, which may lead to IRS1 serine phosphorylation and perturbed signaling to glucose uptake (101); (87; 102). Lipid metabolism may also negatively impact the transduction of insulin signaling in skeletal muscle via increased intramuscular triglyceride deposits. The ectopic deposition of triglycerides into skeletal muscle impairs insulin signaling principally via inflexibility in substrate switching (103; 104). With increases in intramuscular triglyceride deposits the muscle fails to block lipid oxidation in response to insulin stimulation (105-108). Conversely, lipid oxidation is not suitably increased in response to energetically demanding metabolic challenges.

1.3 SUBSTRATE UTILIZATION FLEXIBILITY AND ITS ROLE IN THE DEVELOPMENT OF TYPE 2 DIABETES

The metabolic demands of fasting and exercise are met chiefly through increased lipid oxidation in skeletal muscle (109; 110). Skeletal muscle is the predominate site for lipid utilization in the body. In fasting conditions, skeletal muscle utilization of lipids as an energy substrate is about 50% (111; 112). Circulating FFAs account for approximately half of the lipids being oxidized during a fast, while intramuscular triglyceride stores account for the remaining proportion (113; 114). Substrate utilization in response to exercise is weighted toward the oxidation of lipids during low intensity challenges (~ 30 % VO_{2max}) and changes proportionately along a continuum with increasing intensity. During high intensity exercise (~ 75-85 % VO_{2max}), substrate utilization switches to glucose as main energy substrate (55). Thus, skeletal muscle has the capacity to switch between glucose and lipid utilization based on energetic demands and substrate availability.

Insulin-mediated glucose transport and oxidation is increased in the fed state (109). The ability of skeletal muscle to switch from lipid to glucose oxidation is particularly important postprandially, as failure to do so reflect metabolic inflexibility, which can lead to hyperglycemia and the development of type 2 diabetes. The possibility that increased and dysregulated lipid metabolism could hamper insulin-mediated glucose metabolism in skeletal muscle was posited by Randle and colleagues (115). Since then, a number of studies have provided evidence in support of their argument (100; 116; 117). Central to these investigations is the finding that obese and type 2 diabetic patients manifest an increased rate of lipid oxidation under insulin-stimulated conditions compared to control subjects. These reported observations implicate aberrant substrate utilization in the etiology of insulin resistance and subsequent metabolic disorders. The key molecular events involved in the development of errant substrate utilization flexibility require further study. A better understanding

these mechanisms could lead to improved intervention and treatment of resulting diseases including type 2 diabetes.

1.4 THE ROLE OF CIRCULATORY FACTORS IN TYPE 2 DIABETES

The number of scientific reports describing roles for endogenous circulatory factors in type 2 diabetes is vast and rapidly expanding. The impact of circulatory factors that contribute to the development of the disease, as well as circulatory factors that act to resist it, has become an intriguing area of research in recent years. Of the circulatory factors shown to have a positive correlation with the manifestation of type 2 diabetes, pro-inflammatory cytokines have perhaps the best appreciation. An extensive body of evidence has linked such inflammatory markers as C-reactive protein (CRP) (118), TNF-α (87) and interleukin-6 (IL-6) ((118; 119) to insulin resistance through various mechanisms. Studies using pharmacological and/or genetic interventions to prevent pro-inflammatory cytokine-mediate insulin resistance have been performed in skeletal muscle (87); (120; 121), indicating attenuation of these pro-inflammatory targets improve insulin signaling. Further investigation into the molecular pathways governing pro-inflammatory-mediated insulin resistance is warranted.

1.4.1 Pro-inflammatory cytokines

Cytokines are small, secreted intercellular signaling proteins with molecular weights ranging from approximately 6 to 70 kD. The differential classification of cytokines is challenging, as they do not appear to have any distinctively conserved amino acid sequence motif or tertiary protein structure. Cytokines are chiefly involved in host response to disease or infection. Thus they play an intuitive and essential role in innate and adaptive immunity (122; 123). Despite current difficulties in classifying cytokines, the major physiological actions of different cytokine populations are either anti-inflammatory or pro-inflammatory. This is a central principle to cytokine biology in clinical medicine.

Anti-inflammatory cytokines include interleukin 10 (IL-10), IL-13, transforming growth factor β (TGF-β) (124) and others (125). These cytokines prevent or suppress the inflammatory process. The action of anti-inflammatory cytokines is important in maintaining a balance in the immune response. Pro-inflammatory cytokines include such proteins as IL-6 (118), TNF-α (87) and others, which induce inflammation in response to immune stimulus. The role of IL6 in the development and pathogenesis of type 2 diabetes is controversial (119), as there is no consensus on whether IL6 is enhances glucose upake metabolism or not (126-128). Studies have shown muscle derived IL-6 to stimulate fat oxidation and mediate anti-inflammatory actions in humans. These described IL-6 properties could lead to improved whole body homeostasis (129; 130). In contrast, other studies have shown to IL-6 to promote inflammation sub-clinical the sub-clinical state manifest in type 2 diabetics (131; 132) Pro-inflammatory cytokines typically mediate their inflammatory promoting effects via binding and signaling through their respective receptors.

The role of pro-inflammatory cytokines in the development of metabolic disease has been intensely investigated in recent years (42; 87; 133). Dysregulated pro-inflammatory cytokine production and activity is strongly associated with type 2 diabetes pathogenesis. The role of the pro-inflammatory cytokine TNF-α is of particular interest, as its protein levels are elevated in adipose tissue (134), blood plasma (135; 136) and skeletal muscle (137) of patients with type 2 diabetes. More importantly, *in vivo* studies provide evidence that an acute infusion of TNF-α into healthy human subjects precipitates skeletal muscle insulin resistance (87). The

signaling mechanisms through which TNF-α mediates insulin resistance are not entirely clear. However, current evidence links TNF-α to the upregulation of several different downstream targets including JNK and inhibitor of nuclear factor κβ kinase (IKKβ) (42; 87). These targets then contribute to insulin resistance through various mechanisms resulting in IRS1 serine phosphorylation (83; 84; 87).

1.4.1.1 The TNF-α/IKKβ/NFκB nexus

TNF-α mediates its pleiotropic biological effects through two distinct membrane receptor subtypes (138). Signaling through the TNFR2 subtype results in the activation of the caspase cascade. This unalterably commits the cell to the apoptotic program. Signaling through TNFR1, however leads to an upregulation in the transcription of pro-inflammatory cytokines (138). Upon binding to TNFR1, TNF-α acts downstream to phosphorylate and activate all three MAPK cascades, as well as serine/threonine kinase IKKβ (139). TNF-α-mediated activation of JNK can lead to its subsequent direct serine phosphorylation of IRS1 (87). Activation of IKKβ results in the downstream phosphorylation of inhibitor of nuclear factor κB α (IκBα) and this specifies its degradation in the proteosome (140). Once IκBα is phosphorylated, it releases necrosis factor κB (NFκB), which translocates to the nucleus and drives the expression of more pro-inflammatory cytokines including TNF-α (141) (**Figure 4**). This establishes a vicious cycle of pro-inflammatory cytokine production and contributes to the sub-clinical inflammatory state of type 2 diabetic patients.

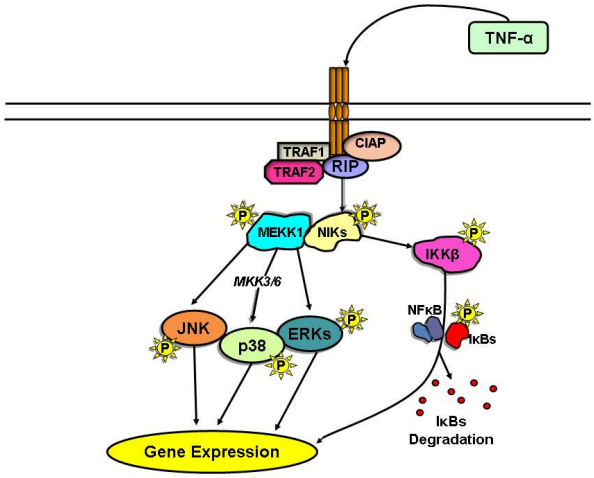

Figure 4: The TNF-α/IKKβ/NFκB nexus. TNF-α acts through its receptor subtype TNFR1 to activate mitogen activated protein kinases (MAPKs), as well as inhibitor of nuclear factor κB kinase (IKKβ). Activation of IKKβ leads to the subsequent phosphorylation of inhibitor of nuclear factor κB α (IκBα) and this action results in the release and nuclear translocation of necrosis factor κB (NFκB). Once in the nucleus, NFκB drives the expression of pro-inflammatory cytokines including TNF-α, which can go on to act on its receptor. This sets up a vicious cycle of sub-clinical inflammation.

1.4.2 Novel endogenous circulatory anti-diabetic factors

A number of endogenous circulatory factors have been found to oppose the development and progression of type 2 diabetes. Among these factors are the adipokine

adiponectin (142), which is involved in glucose regulation and lipid catabolism. Other endogenous circulatory anti-diabetic factors include hormones and polypeptides associated with immune response as previously mentioned.

The emergence of a novel endogenic anti-diabetic circulatory factor is commanding attention due to its ability to improve obesity-consorted hyperglycemia and hyperlipidemia in insulin-sensitive peripheral tissues. These effects are mediated by the atypical fibroblast growth factor family member fibroblast growth factor-21 (FGF-21). The discovery of FGF-21's anti-diabetic actions is of great interest and could have potential therapeutic applications in the treatment of type 2 diabetes (143-146).

1.4.2.1 Fibroblast growth factor (FGF-21)

FGF-21 is a unique member of the FGF family of proteins (147; 148). In contrast to classical FGFs, FGF-21 is not mitogenic and it does not require heparin as a co-factor for full activity (148). FGF-21 is further differentiated from classical FGF family members by its hormone-like actions (148; 149). The hormone-like properties of FGF-21 confer the protein membership to a novel subfamily of "hormone-like" FGFs, which include FGF19 and FGF23 (148; 149). This subfamily is distinguished by their ability to regulate metabolic processes. Current evidence demonstrates the capacity of FGF-21 to effectively regulate glucose and lipid homeostasis (143; 150). These studies were performed in nonhuman primates and different rodent models and they show that FGF-21 treatment leads to attenuated insulin resistance, enhancemented pancreatic β-cell function and mass, normalized glucose and lipid balance, as well as improvements in cardiovascular and lipoprotein risk factors. The effects of FGF-21 are thought to be realized through its binding, together with its required co-receptor β-klotho to subtypes 1 or 2 of the FGF receptor (151). FGF-21 is then postulated to promote glucose uptake directly via an increased GLUT1 expression (152). The mechanisms controlling the metabolic actions of FGF-21 are currently being resolved. Further investigation of this protein is needed as new reports supporting and contrasting the current body of evidence is rapidly growing. FGF-21 may have the potential to positively impact obesity and type 2 diabetes. Thus, an investigation of FGF-21 is one of the central studies in this thesis.

1.5 NOVEL BIOPHARMACEUTICALS

The current biopharmaceutical approach to type 2 diabetes treatment is centered on four major drug classes. These include α-glucosidase inhibitors, sulfonylureas, metformin and thiazolidinediones (TZDs). Exogenous insulin is also frequently used as a therapeutic in concert with these agents. However, current therapeutics are not entirely sufficient to control glucose homeostasis. This fact necessitates the discovery of new biopharmaceuticals to treat the increasing prevalence of the disease. Many of the current therapeutic approaches are focused on the catabolic processes of glucose and lipid metabolism (153-156). The investigation of clenbuterol departs from this model by virtue of the fact that its actions are anabolic (157; 158). Study of this drug expands the scope of our search for novel biopharmaceuticals. A therapeutic approach which includes both catabolic and anabolic treatment options is more comprehensive and may lead to overall metabolic improvements for type 2 diabetic patients. Clenbuterol affects glucose and lipid metabolism (159; 160) and may offer potential use in the development of type 2 diabetes treatments.

1.5.1 Clenbuterol

Clenbuterol is a β-2-adrenergic agonist with a defined role in cardiac and skeletal muscle hypertrophy (161). The increase in clenbuterol-stimulated protein synthesis in skeletal muscle, which leads to enhanced growth of the tissue, appears to be part of its repartitioning effect (160). Clenbuterol mediates concomitant reductions in body fat, which account for the other portion of its repartitioning effect (160). Clenbuterol plays a potential role in skeletal muscle fiber type transformation in rats by increasing the ratio of fast- to slow-twitch fiber in extensor digitorum longus (EDL) and soleus muscles (157). This histochemical investigation and others suggest a role for clenbuterol in the regulation of fiber type composition in addition to its role in muscle growth. The historical clinical use of clenbuterol has been in the treatment of chronic obstructive pulmonary disease (COPD) and acute asthma exacerbations in humans (162). However, clenbuterol positively impacts glucose handling in skeletal muscle (160). Long-term clenbuterol treatment improved insulin-mediated glucose transport in the adipose and skeletal muscle tissue of diabetic rodent models (160). Clenbuterol treatment also leads to mitogenic changes in skeletal muscle. Genes regulating transcription and translation were altered in response to clenbuterol exposure in a genetic screen (163). Although data supports a role for clenbuterol in metabolic regulatory processes such as glucose and lipid handling, the mechanisms by which clenbuterol manipulates these processes are unclear and are in great need of further study. Clenbuterol reportedly upregulates GLUT4 expression and this may be how it imposes its effects on glucose uptake. Clenbuterol conversely downregulated the expression of peroxisome proliferators-activated receptor δ (PPARδ) (164).

2 AIMS

Skeletal muscle plays an essential role in glucose and lipid homeostasis. The utilization of glucose and lipids are under stringent regulation in this tissue and impairments or dysreglation of the utilization of these energy substrates is closely associated with the development of type 2 diabetes. As skeletal muscle is the principal site for glucose and lipid handling, investigation of its metabolism would inform our understanding of the molecular events which lead to metabolic diseases effecting this tissue. This enhanced knowledge could potentially result in the discovery of novel therapeutic approaches. Therefore, the central objective of this thesis is to identify and validate the molecular events that govern metabolism in skeletal muscle. Studies were conducted to explore the roles of substrate utilization, inflammation and the effects of novel biopharmaceuticals on metabolic function in skeletal muscle.

The following specific questions were addressed:

- Does malonyl CoA decarboxylase play a direct role in metabolic flexibility in skeletal muscle?
- Is the genetic silencing of the downstream TNF-α target, IKKβ sufficient to prevent TNF-α-mediated insulin resistance in skeletal muscle?
- Does FGF-21 directly impact glucose metabolism in skeletal muscle?
- What are the acute and chronic effects of clenbuterol on glucose and lipid metabolism in primary human skeletal muscle cells? What are the signaling mechanisms that direct its effects?

3 EXPERIMENTAL PROCEDURES

3.1 MATERIALS

Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, fetal bovine serum (FBS), penicillin, streptomycin, and fungizone were obtained from GibcoBRL (Invitrogen, Stockholm, Sweden). Radioactive reagents, 2-[G – ^{3}H] deoxy – D – glucose, D - [U – ^{14}C] glucose, [9, 10 (n) - ^{3}H] palmitic acid and 1 - [^{14}C] palmitate were purchased from Amersham (GE Healthcare) (Uppsala, Sweden). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (GE Healthcare) (Uppsala, Sweden). For *Study I*, small interfering RNA (siRNA) oligos directed against MCD were from Dharmacon (Chicago, IL, USA). siRNA oligos directed against IKKβ in *Study II* were from Ambion (Austin, TX, USA). In *Studies I* and *II*, the transfection agent Lipofectamine 2000 was obtained from Invittrogen (Stockholm, Sweden). For *Study III*, FGF-21 was kindly provided by Dr. Alexi Kharitonenkov (Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, USA). Oligonucleotide primers and TaqMan probes used in all studies were purchased from Applied Biosystems (Stockholm, Sweden). General phospho-specific and total protein antibodies were from Cell Signaling Technology (Danvers, MA, USA) or Upstate/Millipore (Billerica, MA, USA). For *Study II*, mitogen-activated protein kinase kinase (MEK) isoform 4 (MAP2K4) and MAP4K4 antibodies were obtained from Abgent (San Diego, CA, USA), glyceraldehyde-3-phosphate dehydrogenase from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Desmin from Abcam (Cambridge, UK). TNF-α and clenbuterol used in *Studies II* and *IV* respectively as well as general laboratory reagents were purchased from Sigma (St. Louis, MO, USA).

3.2 SUBJECTS

The subjects in *Studies I*, *II*, *III* and *IV* were scheduled for abdominal surgery at Huddinge or Karolinska University Hospital (Stockholm, Sweden). The subjects had no manifest metabolic disorders and they presented with normal fasting glucose values. The clinical parameters of the healthy subjects are presented in each of the respective papers. Informed consent was obtained from the subjects prior to their participation in the studies performed in papers I-IV and the ethical committee at Karolinska Institutet approved all study protocols.

3.3 CELL CULTURE

3.3.1 Muscle biopsies and primary skeletal muscle cell culture preparation

Abdominis rectus muscle biopsies (~1 – 3 g) of normally active, non-diabetic subjects were obtained during general surgery. The tissue was collected in cold phosphate-buffered saline (PBS) supplemented with 1% PeSt (100-units/ml-penicillin/100 µg/ml streptomycin). Discernable connective and fat tissues were removed from the muscle via dissection before the muscle was finely diced and transferred to a trypsin digestion solution [0.015 g collagenase IV, 8% 10 x trypsin, 0.015 g bovine serum albumin (BSA), 1% PeSt, in Ham's F-10 medium]. The skeletal muscle was incubated in the trypsin digestion solution at 37°C for 15 – 20 min with gentle agitation. Undigested muscle tissue was allowed to collect at the bottom of the tube and the supernatant containing free satellite cells was collected and mixed with

growth medium (Ham's F-10 with 20% FBS, 1% PeSt) in a 1:1 ratio. The remaining undigested muscle tissue was transferred to fresh trypsin digestion solution and incubated further (37°C for 20 min). The resultant supernatant was pooled with the previously collected supernatant and centrifuged for 10 min at 350 g. The pelleted cells were re-suspended in 5 ml Ham's F-10/20% FBS and incubated in a non-coated (bacteriological) petri dish for 1 h to selectively promote adherence of non-myogenic cells. The supernatant was then transferred and cells were seeded in 150-cm^2 Costar culture flasks. The cells were exposed to fresh growth media every 2 -3 days. Cells were trypsinised and sub-cultured upon reaching confluence (> 80%). The flask containing cells after the first trypsinisation was designated 'passage 1'. Myoblasts were allowed to reach > 80% confluence prior to being submitted to the differentiation program. This protocol was used prior to all subsequent experimentation. The initiation of skeletal muscle myocytes into differentiated myotubes began with the replacement of growth media with differentiation media (DMEM with 4% FBS, 1% PeSt, 1% fungizone). Cells were incubated in this media for 48 h before being switched to DMEM with 2% FBS, 1% PeSt, 1% fungizone. Fused and multinucleated cells were observed on the 3rd day of the of differentiation program. All experimentation was performed on cultures from the second to fifth passages (165).

3.3.2 Human skeletal muscle cell (HSMC) culture

Cells were seeded at a density 2- 3 x 10^4 cells/cm^2 and cultivated in (Ham's F-10 with 20% FBS, 1% PeSt, 1% fungizone) or [DMEM (1 g/L glucose) with 20% FBS, 1% PeSt, 1% fungizone) in uncoated Costar culture dishes. Cells were grown to confluence (> 80%] and differentiated as described in the previous section. The cells were optically controlled for myotube formation prior for to > 18 h serum starvation (Ham's F-10 or DMEM 1g/L glucose supplemented with 1% PeSt, 1% fungizone).

3.3.3 Rat L6 skeletal muscle cell culture

Rat L6 skeletal muscle cells were cultivated in alpha-MEM growth media (alpha-MEM with 10% FBS, 1% PeSt and 1% fungizone). Upon reaching confluence (> 80%), cells were switched to alpha-MEM differentiation media (alpha-MEM with 2% FBS, 1% PeSt and 1% fungizone). Cells were changed to fresh alpha-MEM differentiation media every 2 – 3 days for 8 days. The cells were optically controlled for myotube formation prior to overnight serum starvation (alpha-MEM media with 1% PeSt, 1% fungizone).

3.3.4 Mouse fibroblast 3T3-L1 cell culture

3T3-L1 cells were cultured in DMEM (4.5 g/L glucose) supplemented with 10% FBS, 1% PeSt and 1% fungizone. 2 days after reaching confluence (> 80%), cells were switched to differentiation media [DMEM supplemented with 10% FBS, insulin (1 mg/ml), dexamethasone (0.39 mg/ml in 100% ethanol), isobutylmethylxanthine (11.5 mg/ml in dH_2O)]. Following a 48 h incubation, the cells were switched back to DMEM (4.5 g/L glucose) supplemented with 10% FBS, 1% PeSt and 1% fungizone. Cells were allowed to differentiate for 6 days after the induction of differentiation. Cells were optically controlled for expression of the adipocyte phenotype i.e. when >90% of the cells contained lipid droplets before experimental use.

3.4 CELL CULTURE MANIPULATION/METABOLIC METRICS IN CELLS

3.4.1 Glycogen synthesis

Skeletal muscle myotubes grown on six-well plates and serum starved for 16-18 h following 5 days of differentiation. Myotubes were randomized into control conditions (received no insulin stimulation) or experimental conditions (stimulated with 120 nM insulin) and incubated at 37°C for 30 min. Myotubes were then incubated with 5 mmol/l glucose containing D - [U – ^{14}C] glucose (1 mCi/ml; final specific activity, 0.18 mCi/μmol) for 90 min. Placing the six-well plates on ice terminated the reaction. Myotubes were washed with ice-cold PBS and lysed in 1 ml of 0.03% sodium dodecyl sulfate (SDS). Lysate (0.85 ml) was transferred to 10 ml tubes and 100 ml (2 mg/sample) carrier glycogen was added. Samples were heated at 95°C for 30 min and 95% ethanol was added. The samples were incubated at -20°C overnight to precipitate glycogen. The samples were subjected to centrifugation at 1700g for 35 min. The resultant glycogen pellets were washed once with 70% ethanol and re-suspended in 200 ml of dH_2O. Liquid scintillation counting was used to measure radioactivity (WinSpectral 1414 Liquid Scintillation Counter; Wallac/PerkinElmer, Waltham, MA, USA). Each experiment was performed in triplicate wells. Protein concentration was measured using the remaining lysate from the experiment.

3.4.2 Glucose uptake

3.4.2.1 HMSC and Rat L6 muscle cells

Differentiated skeletal myotubes were grown in six-well plates and serum starved in DMEM (*HMSC*) or alpha-MEM (*Rat L6 muscle cells*) for > 18 h. Myotubes were then incubated in glucose free DMEM in the absence or presence of 120 nM insulin at 37°C for 30 min. Thereafter, 5 mmol/l 2-[G – ^{3}H] deoxy – D – glucose (0.33 mCi/well) was added and myotubes were incubated for 10 min. Media was aspirated following this incubation and the myotubes were washed three times with ice-cold PBS. The cells were lysed in 1 ml 0.5 M NaOH. The lysate (500 μl) was transferred to 4 ml scintillation tubes and 3.5 ml of scintillation fluid was added. Radioactivity was measured by liquid scintillation counting (WinSpectral 1414 Liquid Scintillation Counter; Wallac/PerkinElmer, Waltham, MA, USA). Each experiment was performed in triplicate wells. Protein concentration was measured using the remaining lysate from the experiment.

3.4.2.2 3T3-L1 adipocytes

Upon completion of their differentiation program, adipocytes were serum starved in DMEM for 16 h. Adipocytes were then incubated with or without insulin plus stimuli at 37°C for 6 h. Glucose uptake was initiated with the addition of 50 μmol/l 2-[G – ^{3}H] deoxy – D – glucose and 1 μCi in 1 ml of Krebs-Ringer phosphate buffer (pH 7.4) for 5 min at 37°C. Placing the six-well plates on ice terminated the reaction. The cells were washed three times with ice-cold PBS and cells were lysed in 1 ml of lysis buffer containing 0.1% Triton X-100 for 45 min. Radioactivity was measured by liquid scintillation counting (WinSpectral 1414 Liquid Scintillation Counter; Wallac/PerkinElmer, Waltham, MA, USA). Each experiment was performed in triplicate wells. Protein concentration was measured using the remaining lysate from the experiment.

3.4.3 Palmitate oxidation

For *Study I*, myoblasts were cultured in a 25-cm^2 cell culture flask and differentiated into myotubes at > 80% confluence. Prior to the initiation of the experiment, a 2-mm hole was made in the lid of each flask and two sheets of 24 mm Whatman filter (VWR International, Stockholm, Sweden) were encircled with a gauze bandage compass. The filter compass was then pressed into the inside of the culture flask lid. Following 8 days of differentiation, myotubes were serum starved > 16 h and treated for 3 h with 0.4 µCi 1-[14C] palmitate in 2 ml serum-free DMEM (containing 5 mmol/l glucose) with or without 120 nmol/l insulin at 37°C. Following treatment, 200 µl Solvable reagent (benzethonium hydroxide; Packard Bioscience Co. Meridian, CT, USA) was added dropwise through the hole of the flask lid to soak the filter. Thereafter, 300 µl of 70% perchloric acid was injected through the hole and filter. The lids were sealed with Parafilm; (Nordic EM Supplies, Espoo, Finland). Flasks were then laid down with slight agitation for 1 h at room temperature. Thereafter, the filter compass was transferred to a scintillation tube with 10 ml scintillation liquid and 200 µl ice-cold methanol was added. [$^{14}CO_2$] captured in the filter was then counted in a liquid scintillation counter. Protein content of each sample was determined by the BioRad method.

For *Study IV*, myotubes were exposed to clenbuterol 100 µM (for acute experiments) or 100 nmol/l (for long-term experiments). Differentiated myotubes were serum starved > 16 h and incubated with radioactive media (1 ml of MEM-alpha media or DMEM (1 g glucose/L), supplemented with 0.2 % fatty acid free albumin bovine serum and 0.5 µCi palmitic acid [9, 10 (n) - ^{3}H]) in the presence of either 100 µmol/l (short-term treatment) or 100 nmol/l (chronic treatment) clenbuterol, with or without insulin (120 nmol/l for short-term and 6 or 60 nmol/l for chronic clenbuterol treatment) for 6 hours. Ethanol (0.1%) was used as vehicle. The six- well plates were placed on ice to terminate the reaction and 0.2 ml of media from each well was collected in an Eppendorf tube. A 0.8 ml aliquot of charcoal slurry (1 g charcoal per 10 ml 0.02M Tris-HCl pH 7.5) was added to each tube and the samples were incubated for 30 min with agitation. Samples subjected to centrifugation at 13,000 rpm for 15 min and 0.2 ml of the supernatant was withdrawn and placed in a 4 ml vial with 2.8 ml of scintillation liquid. Radioactivity was counted in the total protein concentration was measured using the Pierce method.

3.4.4 Intracellular accumulation of radioactive palmitate

The 25-cm^2 culture flasks from the described palmitate oxidation experiment were washed five times with Tris - buffered saline plus Tween (TBST). Cells were then lysed with 2 ml 0.03% SDS for 2 h at room temperature with slight agitation. 400 µl of the cell lysates were then transferred to 4 ml of scintillation liquid. Palmitate uptake was determined by 1-[^{14}C] accumulation in the lysate. This measurement was obtained from liquid scintillation counting. The total lipid accumulation was calculated by adding the amount oxidized to the amount accumulated.

3.4.5 Lactate production

Myotubes were randomized into control conditions (received no insulin stimulation) or experimental conditions (stimulated with 120 nmol/l insulin). The insulin stimulation was performed in serum-free DMEM and the myotubes were incubated for times specified in each study. The media (100 µl) was collected following the incubations and lactate concentration was measured using a lactate kit (catalog no. A-108; Biochemical Research Service Center, University at Buffalo, New York, USA).

3.4.6 Glucose oxidation

Skeletal muscle myotubes were grown in 25-cm^2 culture flasks. Before the start of the experiment, a 2-mm hole was made in the lid of each flask and two sheets of 24 mm Whatman filter (VWR International, Stockholm, Sweden) were encircled with a gauze bandage compass. The filter compass was then pressed into the inside of the culture flask lid. Myotubes were incubated at 37°C for 1 h in 2 ml serum-free DMEM containing 5 mmol/l glucose and 2 mCi/ml D- [U-^{14}C] glucose with or without insulin (120 nmol/l). Trapped $^{14}CO_2$ was measured to determine the amount of labeled glucose that was metabolized by the myotubes. Scintillation counting of the filter compasses' radioactivity was as described for palmitate oxidation. Protein content of each sample was determined by the BioRad method.

3.4.7 Malonyl CoA, acetyl –CoA, CoASH, diacylglycerol (DAG) and ceramide measurements

For *Study I*, treated myotubes were lysed in 10% sulfosalicylic acid with 10 mmol/l dithiothreitol (DTT). The lysates were then centrifuged at 15, 000g the supernate analyzed with liquid chromatography–mass spectrometry without dilution or sample treatment. An Agilent 1100 series capillary pump (Wilmington, DE, USA) was interfaced with a Linear Ion Trap Quadrapole ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA; USA). The concentration of the analytes was determined through a comparison of the signal from triplicate injections to that of a standard curve. The standard curve was obtained prior to and after the sample queue. The content of ceramide and diacylglycerol in the human skeletal muscle samples was determined by the conversion to phosphorylation products by the addition of exogenous diacylglycerol kinase from E. coli in the presence of [γ - 32P] ATP (166; 167).

3.4.8 siRNA transfection of myotubes

Myotubes were grown in serum-free DMEM on the 2nd day of differentiation. On day 3 of the differentiation program, myotubes were transfected with specific siRNA oligos against MCD in *Study I*, and IKKβ in *Study II*, (1μg/ml) using Lipofectamine 2000 in serum-free DMEM (1 g/L glucose). The myotubes were incubated with respective siRNA oligos for > 16 h. Pools of siRNA against MCD in *Study I*, or IKKβ in *Study II*, or scrambled sequence were used. Following incubation with respective siRNA sequences, myotubes were washed with PBS and 2 ml of DMEM with 2% FBS was added to each well of the six –well plates. Myotubes were serum starved for 16 h on day six of the differentiation program. Subsequent experiments were then performed on the genetically altered myotubes.

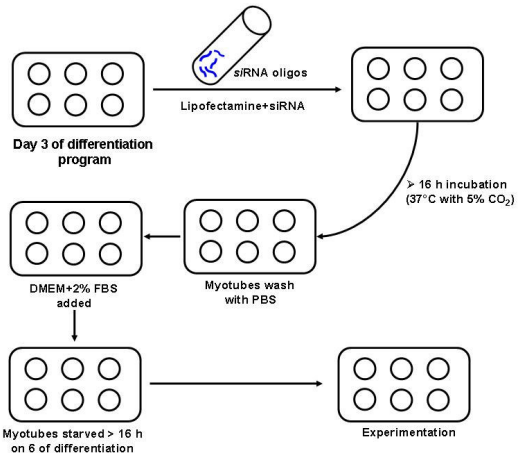

Figure 5: siRNA experimental scheme. On day 3 of the differentiation program, cells are transfected with sequence specific siRNA oligos using the transfection agent Lipofectamine. Cells are then incubated at 37°C with 5% CO_2 for > 16 h. Myotubes are then wash with PBS and switched to DMEM (1 g/L glucose) / 2% FBS. On day 6 of the differentiation, myotubes are serum starved for > 16 h and experimentation is performed.

3.4.8.1 siRNA constructs

siRNA controls (scrambled) and against MCD or IKKβ were purchased from Dharmacon (Perbio Science, Belgium).

Order numbers:

siGENOME Non-Targeting siRNA, D-001210-01-05

siGenome duplex D-009626-02-0010, Human MLYCD, NM_012213

ON-TARGETplus SMARTpool L-003503-00-0020, Human IKBKB, NM_001556

3.4.9 Cell surface GLUT1 and GLUT4 measurements

For *Study I*, cell surface GLUT1 and GLUT4 content was determined in myotubes transfected with siRNA sequences against MCD or scrambled sequences. Skeletal muscle myotubes were incubated as described for glucose uptake. This incubation was followed by incubation at 18°C for 5 min. Myotubes were then washed and incubated with Krebs - Henseleit bicarbonate buffer (KHB) supplemented with 5 mmol/l HEPES and 0.1% BSA, with 100 µmol/l Bio - LC - ATB - BGPA {4, 4 - O - [2 - [2 - [2 - [2 - [2 - [6 - (biotinylamino) hexanoyl] amino] ethoxy] ethoxy] ethoxy] - 4 – (1 - azi - 2, 2, 2, rifluoro-ethyl) benzoyl] amino-1, 3-propanediyl bis-D-glucose} for 8 min. Dishes were irradiated for 3 min and myotubes were washed with PBS. Cells were solubilized and scraped into 1 ml PBS with 2% thesit ($C_{12}E_9$) and protease inhibitors [10 µg/ml aprotinin, 10 µg/ml antipain, 10 µg/ml leupetin, and 200 µmol/l phenylmethylsulfonyl fluoride (PMSF)]. Cell extracts were transferred to microtubes (Sarstedt, Nümbrecht, Germany) and rotated for 60 min at 4°C. Lysates were subjected to centrifugation at 20,000 g for 10 min and the supernatant was collected. Protein

concentration was determined and equal amounts of protein were mixed with 50 µl PBS-washed streptavidin agarose beads (50% slurry; Pierce, Inc., Rockford, IL, USA). The streptavidin-biotin complex was incubated > 16 h at 4°C with end-to-end rotation. The streptavidin agarose beads were washed three times with PBS-1% thesit, three times with PBS-0.1% thesit, and twice with PBS. Photolabeled glucose transporters were eluted from the streptavidin agarose beads by heating in 50 µl Laemmli (pre-heated with a lamp for 40 min) buffer at 56°C for 20 min. Protein samples were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for separation and immunoblot analysis was used to determine the cell surface content of GLUT1 and GLUT4 (168).

3.4.10 Animal models

Male wild-type C57BL/6 mice were maintained on a 12 h light-dark cycle and allowed free access to standard rodent chow. Mice were anesthetized via intraperitoneal injection of 2.5% avertin /0.02ml/g of body weight and extensor digitorum longus (EDL) and soleus muscles were removed for *in vitro* incubation. The mice were humanely killed by cervical dislocation immediately after muscle dissection. The Ethics Committee on Animal Research in Northern Stockholm approved all experimental procedures.

3.4.11 Muscle tissue collection and incubations

Pre-gased (95% O_2, 5% CO_2) KHB containing 5 mmol/l HEPES and 0.1% bovine serum albumin (RIA grade) was used to prepared incubation media solutions. Surgically removed EDL and soleus muscles were incubated at 30°C in a shaking water bath. The incubated muscles were exposed to continuous gas phase of 95% O_2 and 5% CO_2.

3.4.12 *In vitro* glucose transport

Pre-incubated muscles were collected into KHB buffer supplemented with 1 mM 2-[G – ^{3}H] deoxy – D – glucose (2.5 mCi/ml) and 19 mmol/l [^{14}C] mannitol (0.7 mCi/ml). The addition of insulin or FGF-21 was at concentrations identical to the pre-incubation conditions. 2-[G – ^{3}H] deoxy – D – glucose transport into the muscle was assessed for 20 min at 30°C. At the end of incubations, connective and fat tissues were dissected from the muscle. The muscle was then snap frozen in liquid nitrogen and stored at -80°C for later analysis.

Frozen muscles were crushed and homogenized in 0.5 M NaOH. Protein concentration was determined using Coomassie Plus (Pierce, Inc., Rockford, IL, USA). Radio-labeled glucose transport activity was determined by liquid scintillation counting and expressed as nmol 2-[G – ^{3}H] deoxy – D – glucose per mg protein per min in *Study III*.

3.5 ASSAYS AND ANALYSIS

3.5.1 Protein concentration measurements

Protein concentrations for SDS-PAGE/Western blot analysis were measured in skeletal muscle cell or muscle biopsy lysates by Braford (Bio-Rad, Richmond CA, USA) or Pierce (Rockford, IL, USA) methods. Measurements were performed as recommended by the respective manufacturer.

3.5.2 Western blot analysis

Protein expression in skeletal muscle cell or muscle biopsy lysates was determined using Western blot analysis. Equal concentrations of proteins were diluted in 4 x Laemmli buffer (final concentration 1 x) and heated at 56°C for 20 min. Protein samples were then submitted to SDS – PAGE. Separated proteins were then transferred to polyvinylidenediflouride (Immobilon – P, Millipore, Bedford, MA, USA). Following protein transfer, membranes were incubated in 7.5% low fat milk in TBST at room temperature for 1 – 2 h. Membranes were washed to remove the milk solution and incubated > 16 h with primary antibodies in TBST and 0.01% sodium azide. Membranes were washed 4 times 10 min with TBST and incubated with the appropriate secondary antibody (horse-radish peroxidase-conjugated) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry. Results were expressed as arbitrary units or fold over respective controls.

3.5.3 Real Time PCR/primers and probes

Real Time (RT) polymerase chain reaction (PCR) was used in used in all four studies. Gene expression analysis was performed using Syber green gene expression assays to quantify specific mRNA expression (ABI-PRISMA 7000 Sequence Detector, Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Reactions were carried out in 96 – well MicroAmp Optical plates. Total volume of reactions was 25 µl and contained diluted complementary DNA (cDNA), 2 x Syber green PCR Master Mix as well as forward and reverse primers for the targeted gene. Resulting data was analyzed by ABI Prism 7000 SDS Software version 1.1.

3.5.4 PI3 kinase activity

For *Study I*, differentiated myotubes were stimulated with insulin and homogenized in ice – cold PI3 kinase buffer (50 mmol/l HEPES pH 7.6, 150 mmol/l NaCl, 1% Triton X 100, 1 mmol/ Na_3VO_4, 10 mmol/l NaF, 30 mmol/l $Na_4P_2O_7$, 10% (v/v) glycerol, 1 mmol/l benzamidine, 1 mmol/l DTT, 10 µg/ml leupeptin, 1 mmol/l PMSF and 1 µmol/l microcystin). An aliquot of the protein lysate was immunoprecipitated with anti-IRS1 antibody coupled to protein A-sepharose (Sigma) at 4°C for > 16 h. The immunoprecipitates were washed 3 times with buffer A (homogenization buffer, twice with buffer B (500 mmol/l LiCl, 100 mmol/l Tris-HCL, pH 8.0) and once in buffer C (150 mmol/l NaCl, 10 mmol/l Tris-HCL, 1 mmol/l ethylenediaminetetraacetic Acid (EDTA), pH 7.6) and once with buffer D [(20 mmol/l HEPES, 1 mmol/l (DTT), 5 mmol/l $MgCl_2$, pH 7.6]. Beads were re-suspended in 40 µl of buffer (10 mmol/l β-glycerophosphate, 5 mmol/l $Na_4P_2O_7$, 30 mmol/l NaCl, 1 mmol/l DTT, pH 7.2), 20 µl of phosphatidylinositol/cholate solution (3 mg/ml in 1% (w/v) sodium cholate) was added to each tube and the reaction was initiated with the addition of 5 µCi of [γ - ^{32}P] ATP in 40 µl of reaction mix (3 µmol/l Na_2ATP, 7.5 mmol/l $MgCl_2$) and incubated at 37°C for 15 min. The addition of 450 µl $CHCl_3$: CH_3OH (1:2 v/v) terminated the reaction. Product was extracted by the addition of 150 µl of $CHCl_3$ and 150 µl of 0.1 mmol/l HCL and then again 300 µl of $CHCl_3$ and 300 µl of 0.1 mmol/l HCL. Extracted lipid was dried down under vacuum prior to being re-dissolved in 25 µl of $CHCl_3$, CH_3OH, 0.1 mol/l HCL (200:100:1). Thin layer chromatography was used to separate the reaction products (run in a pre-equilibrated tank containing methanol:chloroform:ammonia:water, 300:210:45:75). A PhosphoImager (Bio-Rad) was used to quantify reaction products.

3.5.5 IRS1 tyrosine phosphorylation

For *Study I*, the phosphorylation of tyrosine was measured. Aliquots of cell lysates were immunoprecipitated with anti-IRS1 antibody for > 16 h. Protein A sepharose beads were then added to the lysates and incubated at 4°C for 2 h. Immunoprecipitated samples were washed three times with homogenization buffer, two times with buffer B (0.1 mol/l Tris, pH 8 and 0.5 mol/l LiCl) and once with buffer C (10 mmol/l Tris, pH 7.6, 0.15 mmol/l NaCl and 1 mM EDTA). Laemmli buffer supplemented with β-mercaptoethanol was used to re-suspend the pellets. Samples were heated at 95°C for 4 min and submitted to SDS-PAGE. Separated proteins were then transferred to polyvinylidenediflouride (Immobilon – P, Millipore) and blocked with 7.5% low fat milk in TBST at room temperature for 1 – 2 h. Membranes were washed to remove the milk solution and incubated > 16 h with HRP conjugated anti-phosphotyrosine antibody in TBST and 0.01% sodium azide. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

3.6 STATISTICS

Data are expressed as means ± S.E.M. Details of statistical analysis is provided in each of the separate papers. Significance was accepted at $p < 0.05$.

4 RESULTS AND DISCUSSION

Studies designed to investigate the molecular mechanisms and pathways governing whole body glucose and lipid metabolism are of critical importance. The urgency of such inquests is driven by the dramatic rise in global type 2 diabetes incidence. Another contributing factor to the need for these investigations is the fact that treatment of type 2 diabetes with the current schedule of pharmacological therapies is not entirely sufficient to achieve sustained control of glucose homeostasis. Evidence from studies presented here and elsewhere, convincingly link disturbances in glucose and lipid metabolism to the development of type 2 diabetes and co-morbidities. As skeletal muscle is the largest consumer of the body's metabolic budget (4; 6), it is one of the most suitable tissues in which to conduct metabolic and molecular signaling investigations. The studies presented here were performed in human skeletal muscle cells, thus conferring a unique opportunity to gain insight into the metabolic and molecular signaling events that control glucose and lipid signaling in human tissue.

4.1 SUBSTRATE UTILIZATION AND ENERGY BALANCE IN SKELETAL MUSCLE

The utilization of carbohydrates and lipids as energy substrates is crucial to whole body energy balance (21). More importantly, the maintenance of energy balance requires that skeletal muscle have metabolic flexibility or "a clear capacity to utilize lipid and carbohydrate fuels and to transition between them" (169). This capacity is characteristic of a metabolically healthy individual. During fasting conditions, the skeletal muscle of healthy lean subjects has increased rates of lipid oxidation, concomitant with high lipid uptake (3). Upon the consumption of a meal, their skeletal muscles rapidly shift to glucose metabolism. Skeletal muscles from insulin resistant subjects have an impairment in the postprandial switch to glucose metabolism (3). Moreover, their muscles fail to increase lipid utilization in response to exercise or fasting and they have a failed secretion of cephalic-phase insulin (1; 3).

A genetic or pharmacological approached focused on the targeted suppression of lipid metabolism in the fed state is intriguing, as a suppression of postprandial lipid oxidation could lead to an increased dependency on glucose as an energetic substrate in patients with impaired metabolic flexibility. Successful targeting of the perturbations leading to disturbed substrate utilization and metabolic inflexibility could improve whole body glucose utilization and restore homeostasis.

4.2 INCREASED MALONYL CoA IMPROVES THE METABOLIC PHENOTYPE

Within lipogenic tissues, lipogenesis begins with acetyl – CoA being carboxylated by ACC to form the coenzyme A derivative malonyl CoA (56; 57). This biomolecule is the first intermediate regulator in the synthesis of LCFAs and an inhibitor of CPT1 (170). The focus of *Study I* was to explore the effects of increased malonyl CoA content on substrate utilization through the genetic silencing of the upstream enzyme MCD (Figure 6). Malonyl CoA acts downstream to allosterically bind to CPT1 (171). This binding leads to an inhibition in the transfer of LCFAs into the mitochondria as CPT1 is responsible for the facilitated transfer of LCFAs across the outer membrane of the mitochondria. In *Study I*, genetic silencing of MCD was achieved with sequence specific siRNA oligos. The results from *Study I* show that *MCD* gene silencing improves the metabolic phenotype by shifting bioenergetics toward glucose utilization.

The siRNA-mediated silencing of MCD in cultured human skeletal muscle cells resulted in a 75% reduction *MCD* gene expression and a twofold increase in the levels of malonyl CoA. Although a possible increase in levels of intracellular metabolites was expected, no changes in were detected for coenzyme A (CoASH), acetyl-CoA, DAG or ceramide in response to *MCD* gene silencing. Genetic or pharmacological inhibition of MCD leads to a corresponding decreased lipid oxidation in mice with an ischemic heart episode.(172). Therefore, we determined the consequence of *MCD* gene silencing in human skeletal muscle cells. Fatty acid oxidation of radio – labeled palmitate was decreased 2.8-fold. This finding was accompanied by a 1.5-fold increase in the intracellular accumulation of palmitate. The decrease in lipid oxidation as a consequence of *MCD* gene silencing was the first confirmation of our hypothesis and encouraged the measurement of other metabolic parameters. Intriguingly, *MCD* gene silencing-mediated suppression of palmitate in the basal state was similar to insulin-mediated suppression of palmitate in control cells. Moreover, *MCD* gene silencing had an additive effect on lipid oxidation when combined with insulin. We also measured the effect of *MCD* gene silencing on the mRNA expression of fatty acid transport protein member (FATP)-1 and pyruvate dehydrogenase kinase (PDK)-4. FATP-1 is important for the transport of fatty acids and PDK-4 inactivates the pyruvate dehydrogenase complex, which controls the entry of carbohydrates into the tricarboxylic cycle. *MCD* gene silencing increased FATP-1 mRNA expression, but was without effect on (PDK)-4.

We next determined the effect of *MCD* gene silencing on glucose metabolism to assess whether there was a shift in bioenergetics toward the utilization of glucose as the principal energy substrate. The effect of *MCD* gene silencing on glucose metabolism in human skeletal muscle is of particular interest as animal data shows that long-term inhibition of the *MCD* gene using whole-body knockout mice conferred protection against the development of glucose intolerance and hyperglycemia associated with high-fat diet (173). Moreover, $MCD^{-/-}$ animals had a higher respiratory exchange ratio than their wild-type littermates, which suggest that they have increased rates of glucose, rather than lipid, oxidation at the whole-body level. Thus we determined the direct effect of *MCD* gene silencing on glucose uptake, glucose oxidation and glycogen synthesis in cultured human muscle cells. *MCD* gene silencing increased basal glucose transport compared to control cells. We observed an increased insulin-stimulated glucose transport in response to *MCD* gene silencing. However the fold effect of this result was similar to that observed in control cells. Thus, we attributed this enhancement in glucose uptake to the increases observed under basal conditions. Glucose oxidation was increased in the basal 1.4- and insulin-stimulated 2.6-fold conditions in response to *MCD* gene silencing. Glycogen synthesis was increased 1.3-fold. *MCD* gene silencing was also sufficient to reduce lactate production in the basal and insulin-stimulated conditions. Together, theses results suggest that *MCD* gene silencing may lead to improvements in the skeletal muscle metabolic profile in type 2 diabetes by shifting metabolism away from lipid and increasing glucose metabolism.

In an effort to elucidate a possible mechanism for the observed effects of *MCD* gene silencing on the metabolic parameters in skeletal muscle, we measured the cell signaling parameters that are important to glucose and lipid metabolism. GLUT1 and GLUT4 are members of the glucose transport family that are respectively responsible for basal and insulin-mediated glucose transport (174). *MCD* gene silencing increased GLUT4 content at the cell-surface and this increased was comparable to the changes that we reported for glucose transport and oxidation. Conversely, GLUT1 appearance at the cell surface was unchanged. This is consistent with the evidence that GLUT1 is associated with basal glucose uptake in skeletal muscle. Phosphorylation and activation

of signaling targets upstream of GLUT4 translocation including Akt, PI3 kinase, GSK3 α/β and IRS1 were unaltered in response to *MCD* gene silencing. These data suggest that observed changes in the metabolic parameters are dissociated from alterations in signaling. A possible explanation for the observed metabolic changes may be mediated by spare insulin receptor signaling capacity as maximally activated glucose transport corresponds to <15% of maximal receptor kinase activity (71). Lastly, *MCD* gene silencing had no discernable impact on AMPK phosphorylation. The activation of AMPK was of interest due to its regulation of malonyl CoA via MCD and ACC. In summary, *Study I* provides evidence to suggest that *MCD* gene silencing mediates an insulin independent bioenergetic shift from lipid to glucose oxidation in human skeletal (Figure 6). *MCD* gene silencing may be a potential therapeutic strategy to treat metabolic inflexibility and normalize whole body glucose levels.

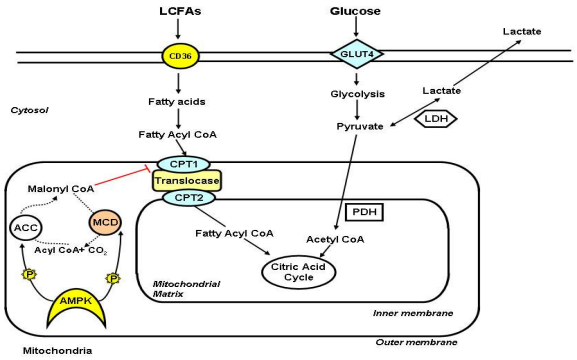

Figure 6: Malonyl CoA regulation of fatty acid oxidation in skeletal muscle. Malonyl CoA regulates LCFA via allosterically binding to CPT1. This binding blocks CPT1 from facilitating the transfer of LCFAs into the mitochondria for oxidation. Malonyl CoA is regulated by AMPK in through two mechanisms (i.e. the phosphorylation of ACC, which block malonyl CoA production or through phosphorylation of MCD, which breaks it down.

4.3 THE ACTION OF ELEVATED PRO-INFLAMMATORY CYTOKINES IN SKELETAL MUSCLE

Correlative studies link elevated levels of the pro-inflammatory cytokine TNF-α with whole-body insulin resistance (175; 176). In an earlier study, our lab demonstrated that an *in vivo* infusion of TNF-α precipitated insulin resistance in healthy subjects (177). The data from this study, together with evidence provided by similar investigations, inspired a further study with the specific aim of identifying and characterizing TNF-α-sensitive targets in human skeletal muscle (Figure 7). Data from such a study could provide insight into the molecular mechanisms governing TNF-α-mediated insulin resistance. A better understanding of TNF-α action on the insulin cascade could provide for the development of novel therapeutics to treat insulin resistance and type 2 diabetes. In *Study II*, siRNA oligos were used to selectively mediate the silencing of the *IKKβ* gene in cultured human skeletal muscle. Transfection of the muscles cells with specific siRNA sequences against IKKβ led to a 73% reduction in its protein expression. The targeted deletion of the *IKKβ* gene also led to a 55% reduction in its mRNA expression. The gene silencing of IKKβ was further confirmed through an observed increased in IκBα protein expression. IκBα is the downstream target of IKKβ.

Upon confirming the suppression of *IKKβ* gene expression, metabolic parameters were assessed to determine the direct effect of *IKKβ* gene silencing on glucose handling. Pre-treatment of the skeletal muscles cells with TNF-α resulted in a marked reduction of insulin-mediated glucose uptake. Gene silencing of IKKβ had no effect on either basal or insulin-stimulated glucose uptake. However, silencing of the *IKKβ* gene prevented the inhibitory effect of TNF-α on insulin-stimulated glucose uptake. Similarly, *IKKβ* gene silencing had no effect on insulin-stimulated glucose incorporation into glycogen. Pre-treatment of muscle cells with TNF-α, lead to an impaired insulin response. *IKKβ* gene silencing was sufficient to rescue the negative effect of TNF-α.

As outlined in section 1.2.1, insulin mediates several effects through Akt. Under normal conditions, insulin activates Akt via phosphorylation on residues Thr^{308} and Ser^{473}. Activated Akt then phosphorylates and deactivates AS160, leading to GLUT4 translocation to the plasma membrane to facilitate glucose uptake (178; 179). TNF-α treatment impaired insulin action of Akt phosphorylation, but *IKKβ* gene silencing protected against the TNF-α-mediated impairments. To further evaluate the effect of *IKKβ* gene silencing on insulin signaling, we assessed phosphorylation of downstream Akt substrates, AS160 and GSK3β. TNF-α pre-treatment prevented insulin-mediated phosphorylation of AS160. However, *IKKβ* gene silencing prevented the TNF-α-mediated inhibition of insulin signaling to AS160. TNF-α treatment increased basal GSK3β phosphorylation. Although this result is intriguing, its functional consequence is not immediately clear. *IKKβ* gene silencing restored normal insulin-mediated signaling to GSK3β. ERK 1/2 MAPK is a downstream insulin signaling target, which is important in mediating the mitogenic effects of insulin. An evaluation of its insulin-mediated phosphorylation showed that TNF-α exposure did not alter the action of insulin on this target. Further the effect of the pharmacological inhibition of ERK 1/2 MAPK with the MEK inhibitor PD 98050 showed that ERK 1/2 MAPK had no effect on glycogen synthesis in response to insulin or TNF-α exposure. The effect of *IKKβ* gene silencing on JNK was also measured. As expected TNF-α exposure increased JNK phosphorylation, but *IKKβ* gene silencing was without effect on the TNF-α-mediated increase of JNK.

Elevated TNF-α is associated with IRS1 serine phosphorylation (Ser^{312}). Therefore, we determined whether IKKβ plays a role in TNF-α-mediated IRS-1 Ser^{312} in phosphorylation in primary human skeletal muscle. TNF-α exposure increased the phosphorylation of IRS1 on Ser^{312} , however, surprisingly this effect was not rescued through *IKKβ* gene silencing. Next, we performed a gel mobility shift assay to assess the total phosphorylation of IRS1. Both insulin and TNF-α caused an upward shift indicating an increase in phosphorylation. Lastly we assessed the mRNA expression of glucose GLUT1 and GLUT4 transporters. Our results showed that *IKKβ* gene silencing was insufficient to mediate detectable changes in mRNA levels of these transporters. In conclusion, *IKKβ* gene silencing using siRNA provides protection against TNF-α-mediated insulin resistance at the level of Akt and AS160 (Figure 7). *IKKβ* gene silencing also mediated favorable changes in glucose and glycogen synthesis. These data highlight IKKβ as a potential therapeutic to treat peripheral insulin resistance. Further experiments are warranted to determine the precise molecular targets of *IKKβ* gene silencing to improve insulin action.

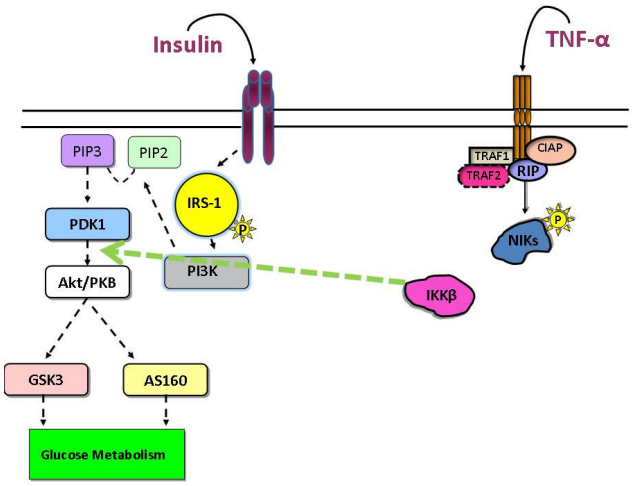

Figure 7: **siRNA mediated reduction of IKKβ ameliorates TNF-α-induced insulin resistance at the level of Akt**. Arrow indicates possible level of action. Whether siRNA mediated reduction of IKKβ has effects on PDK1 or PI3 kinase remains to be determined.

4.4 FGF-21 AS A POTENTIAL THERAPEUTIC IN HUMANS

Studies performed in animal models, 3T3-L1 adipocytes, and human adipocytes have shown the atypical fibroblast growth factor FGF-21 to be a regulator of metabolism (143; 146; 180). The observed effect of FGF-21 on glucose metabolism shows that FGF-21 stimulates non-insulin dependent glucose uptake (180). This effect is believed to be mediated through the binding of FGF-21 to receptor subtypes FGFR1 or FGFR2 and β-klotho (151; 180; 181). Resultant downstream signaling to GLUT1 cell surface protein expression is thought to then facilitate glucose uptake. To date, very little experimental data has been published on the effects of FGF-21 in skeletal muscle. As this tissue has been shown to be one of the most consequential organs in whole-body metabolism, we decided to investigate the direct role of FGF-21 on skeletal muscle metabolism and molecular signaling.

In *Study III*, animal models and HSMC were used to determine whether FGF-21 treatment could impact skeletal muscle metabolism. One of the first experiments in this study was simply to validate the bioactivity of our stock of FGF-21. Previous studies that described the effects of FGF-21 on glucose metabolism were performed in adipocytes. Thus, these cells could serve as an experimental model in which to test our FGF-21. 3T3-L1 adipocytes were cultured and differentiated. We then treated the cells with FGF-21 and measured pERK 1/2 MAPK as marker of FGF-21 activity. The phosphorylation of pERK 1/2 MAPK was determined to be a suitable marker, as it has been validated as an FGF-21 target in other experimental studies. Upon confirmation that our FGF-21 stock was biologically active, we continued our investigation in HSMC.

To determine the optimal time point at which the effects of FGF-21 could be realized on glucose uptake, we performed a time course study, which included time points 0, 2 h, 6 h, and 24 h. Upon data analysis, we determined the 6 and 24 h time points to be optimal for FGF-21 effects on glucose uptake. Indeed, after 6 h of FGF-21 treatment, glucose uptake showed a trend toward an increase in HSMC. This increased reached significance after 24 h of treatment. The FGF-21-mediated increase in glucose uptake in 3T3-L1 cells is reported to be insulin-independent (180). Thus, we validated

this finding in the HSMC model. Our results showed insulin-stimulated glucose to be enhanced by FGF-21 treatment at both the 6 h and 24 h time points. Following the results obtained for the effects of FGF-21 treatment on glucose uptake, we next determined the impact of the growth factor on glucose incorporation into glycogen and lactate production. FGF-21 had no effect on glucose incorporation into glycogen. Similarly, lactate concentration in the media was unaltered after FGF-21 exposure.

As we had observed an increase in glucose uptake in HSMC response to 6 or 24 h treatment with FGF-21, we endeavoured to validate these results *in vivo*. Mouse EDL muscles were isolated and pre-exposed to FGF-21 for 6 h and thereafter incubated for 20 min with insulin. Unlike the effects observed in HSMC, FGF-21 did not independently mediate an increase in glucose uptake. However, pre-exposure of isolated EDL muscle to FGF-21 significantly increased insulin-stimulated glucose. This result is consistent with data collected in HSMC. Further, FGF-21 had a similar effect to enhance insulin-stimulated glucose uptake in incubated mouse soleus muscle.

To elucidate the mechanism responsible for the observed *in vivo* effects of FGF-21 on glucose metabolism, we measured the phosphorylation of molecular signaling proteins Akt, ERK 1/2 MAPK, AS160, AMPK and GSK3β following 6 hours exposure to FGF-21. At this time point FGF-21 treatment was without effect on either basal or insulin-stimulated phosphorylation of AKT Ser^{473}, AS160, ERK1/2 or GSK3β. Neither insulin nor FGF-21 altered phosphorylation of AMPK. Similar results were obtained in mouse soleus muscle. These results do not exclude the possibility that FGF-21 may alter phosphorylation of these molecules at other time points, and this remains to be investigated.

In summary, the results from *Study III*, provide evidence that the range of tissues targeted by FGF-21 is broader than initially believed (Figure 8). The targeting of FGF-21 by skeletal muscle provides and additional point of regulation that may positively contribute to establishment and maintenance of glucose homeostasis and whole-body energy balance. Therefore, FGF-21 may be a novel therapeutic to treat metabolic disorders associated with insulin resistant conditions, including type 2 diabetes.

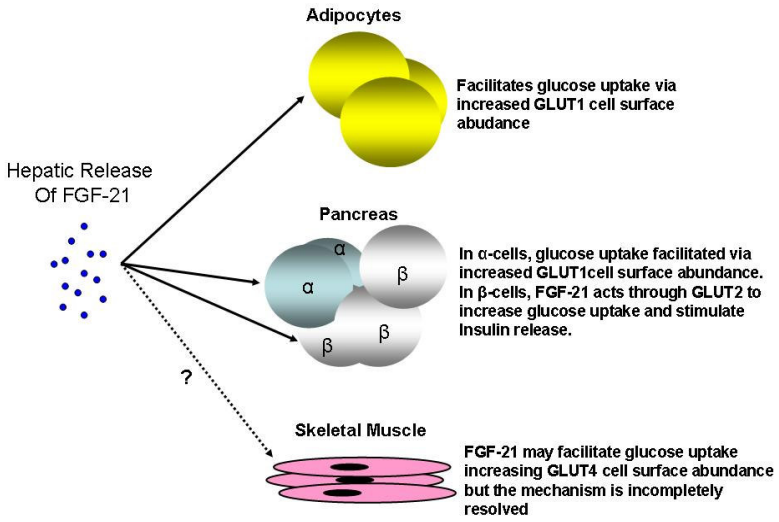

Figure 8: Summary of FGF-21 signaling in adipose, pancreas and muscle.

4.5 A POSSIBLE ROLE FOR ANABOLIC PATHWAYS IN THE TREATMENT OF TYPE 2 DIABETES

Studies I, II and III were focused on catabolic signaling pathways and metabolic mechanisms important to the control of glucose homeostasis. The aim of *Study IV* represents a departure from this paradigm, as the action of clenbuterol *in vivo* is anabolic. Investigations in this arena are suitable, as they afford a different approach to the same problem. The pathways in this of field diabetes research are incompletely understood and further study is required to elucidate the mechanisms responsible for observed beneficial effects of clenbuterol on glucose handling. Clenbuterol is a β-2-adrenergic agonist with a wide range of effects. In *Study IV*, the effects of short term and long effects of clenbuterol exposure was examined in primary human and rat L6 skeletal muscle cells.

Short-term clenbuterol treatment suppressed glycogen synthesis in cultured rat L6 muscle cells (*Study IV*). Following short-term treatment, glycogen synthesis was decreased under basal and insulin-stimulated conditions. Curiously, the insulin fold-effect of glycogen synthesis was conserved following acute clenbuterol (2.9 over basal) exposure. This observation was remarkably close to the control insulin effect of 2.4-fold over basal. Conversely, short-term clenbuterol exposure was without effect on glycogen synthesis in primary human myotubes.

Next, we measured fatty acid oxidation in response to acute clenbuterol treatment and found that clenbuterol decreases fatty acid oxidation. This was a surprising result, as the effects of clenbuterol as popularly known for it lipolytic effects (182). This would intuitively suggest an increased rate of beta oxidation. However, our results showed the opposite to be true.

The effect of clenbuterol treatment on signal transduction was determined. In culture rat skeletal muscle, acute clenbuterol exposure, decreased basal and insulin-stimulated ERK 1/2 MAPK phosphorylation. Similar results were obtained in primary human skeletal muscle cells. Insulin-stimulated Akt Ser^{473} phosphorylation was also decreased following acute exposure to clenbuterol, although modestly. The clenbuterol-mediated decreases were restored after 2 h. Acute clenbuterol treatment did not mediate any change in AMPK phosphorylation in cultured rat skeletal muscle. p38 MAPK phosphorylation was increased in both cultured rat and human skeletal. Chronic clenbuterol exposure had the opposite effect of on glycogen synthesis in human muscle, as this metabolic parameter was increased after an 8 day clenbuterol exposure. Similar to the acute data results, chronic clenbuterol exposure decreased fatty acid oxidation. Finally, we performed a expression analysis of key genes involved in glucose transport, fatty acid uptake and metabolism, differentiation, transcription and energy metabolism. Chronic clenbuterol exposure increased mRNA of glucose transporter 4 (GLUT4), fatty acid transporter 4 (FATP4), myocte enhancing factor 2d (MEF2d), peroxisome proliferator-activated receptor γ co-activator 1 (PGC1), peroxisome proliferator-activated receptor isoforms α δ and γ (PPARα, PPARδ and PPARγ) in human skeletal muscle cells.

Dysregulated lipid utilization is a paramount feature of metabolic inflexibility (2). One of the most popularly proposed mechanisms for this dysregulation is increased ectopic skeletal muscle triglyceride deposition or intramuscular triglyceride (IMTG) accumulation (108; 183). Studies in skeletal muscle show that elevated levels of IMTGs have the capacity to impair insulin signal transduction via the release of FFAs into the circulation (2; 107; 108; 183). The FFAs from IMTG stores often act in concert with FFAs liberated from excessive visceral adipose tissue. Irrespective of the source of the elevated circulatory FFAs species, the collective impact is the same i.e. negative

signaling inputs on insulin signal transduction which ultimately lead to insulin resistance and hyperglycemia (2; 184). FFAs can exact their negative effects on insulin transduction through the activation of MAPKs, particularly JNK and the novel protein kinase C isoform PKCθ (184). Elevated phosphorylation of JNK leads to subsequent serine phosphorylation of IRS1 on residue 307 in rodents and 312 in humans (83; 101). IRS1 serine phosphorylation disrupts IR/IRS1 association and perturbs downstream signaling to glucose uptake. FFAs action on PKCθ results in a subsequent activation of JNK and IKK (184). The action of FFAs on these targets provide a clear link between FFAs and inflammation as both JNK and IKK play a role in mediating the inflammatory response (87; 184). FFAs are further linked to inflammation by virtue of the fact that they are PPAR ligands (185). The focus of *Study I* was to determine whether a targeted genetic deletion of the *MCD* gene could correct dysregulated lipid oxidation. In *Study II* we focused on improving pro-inflammatory cytokine-mediated insulin resistance through the targeted deletion of a downstream target. Thus, the two studies are linked by the mechanisms described here for dysregulated lipid metabolism and inflammation.

4.6 REDUCTION OF MCD AND CHRONIC EXPOSURE TO CLENBUTEROL HAS SIMILAR EFFECTS ON GLUCOSE AND LIPID METABOLISM.

In *Study I* we investigated MCD as a potential therapeutic in the treatment of insulin resistance and type 2 diabetes through targeting its deletion. In *Study IV* the β-2-adrenergic agonist clenbuterol was investigated with the same objective, which was to assess its potential as a therapeutic in the treatment of metabolic disorders. In both of these studies, we employed the use of glucose and lipid metabolism metrics to determine the respective efficacies of each approach on these important physiological parameters. Thus, based on the data compiled from each study, a comparison of MCD *gene* silencing and clenbuterol exposure could be performed. Our data shows that the targeted deletion of MCD was sufficient to increase glucose metabolism. Indeed, every measured parameter in glucose handling including; glucose oxidation, glucose incorporation into glycogen and glucose uptake were increased in response to *MCD gene* silencing. These findings almost independently highlight *MCD gene* silencing as an attractive potential therapy, as its deletion satisfies one of the most essential aims in the treatment of insulin resistance and type 2 diabetes, which is to normalize glucose metabolism and establish glucose homeostasis. Clenbuterol treatment yielded model-specific effects with respect to glycogen synthesis. We show that while chronic clenbuterol exposure increased glycogen synthesis in cultured human skeletal muscle, it had the opposite effect on cultured rat L6 skeletal muscle after acute exposure. The rationale for these contrasting results is not entirely clear. It is tempting to attribute the contrasting response, at least in part to exposure time. Perhaps the effect of clenbuterol is to initially reduced glycogen synthesis. Alternatively, the contrasting results may be ascribed to differences in the experimental model.

Lipid oxidation was also measured in both studies. In *Study I*, MCD gene silencing resulted in a decrease in lipid oxidation and an increase in lipid uptake. Similarly, clenbuterol was sufficient to mediate a potent reduction in lipid oxidation in both the culture skeletal muscle cell models. This was true after acute or chronic clenbuterol exposure. Lipid uptake was not measured in *Study IV*. Given the similar effects on lipid and glucose handling, it would be interesting to determine whether clenbuterol treatment alters cellular expression of MCD and/or levels of Malonyl CoA. Based on our results for glucose metabolism and lipid oxidation, as well as results

reported by others (172; 173), MCD inhibition currently appears to be an attractive therapeutic target for the treatment of insulin resistance and type 2 diabetes. It must be noted that this is suggested based on the current results. In summary, the data from both *Study I* and *Study IV* highlight potential targets. Further investigation of MCD and clenbuterol as potential therapeutics is warranted.

5 SUMMARY

The central objective of this thesis has been to identify and validate the molecular events that govern metabolism in skeletal muscle. The results presented may be summarized as follows:

- Suppression of malonyl CoA decarboxylase plays a direct role in metabolic flexibility in skeletal muscle. MCD silencing suppresses lipid uptake and enhances glucose uptake in primary human myotubes.

- Suppression of IKKβ is sufficient to prevent TNF-α-mediated insulin resistance with respect to glucose metabolism in primary human myotubes.

- FGF-21 directly increases glucose metabolism in skeletal muscle.

- Chronic exposure of cultured skeletal muscle cells to clenbuterol suppresses lipid oxidation and enhances glycogen synthesis.

The results presented in *Studies I-IV* highlight different approaches to alter skeletal muscle metabolism, with the aim to enhance glucose metabolism. A summary of metabolic effects noted are presented in **Table 1**.

Table 1. Summary of metabolic responses after different treatment approaches.

	Targeted deletion of MCD	Targeted deletion of IKKβ*	FGF-21	Clenbuterol
Glucose Oxidation	↑			
Glucose Uptake	↑	↔	↑	
Glycogen Synthesis	↑	(↑)	↔	↑
Lactate Production	↓		↔	
Lipid Uptake	↓			
Lipid Oxidation	↓			↓

* Effects of IKKβ silencing were only observed in presence of TNF-α

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of this thesis was to investigate novel molecular signaling pathways governing glucose and lipid metabolism. Studies from this thesis provide evidence that the deletion of molecular targets from two distinct signaling pathways was sufficient to positively impact the glucose and lipid metabolism. Moreover, studies performed here provide further evidence that many of the signaling pathways under current investigation may be linked. This was particularly true for work performed in *Studies I* and *II*. The consequence of abnormal and excessive lipid oxidation was central to *Studies I.* Here, the concept of increased FFAs as a negative regulator of insulin action came into play. Indeed, the goal of targeted deletion of the *MCD* gene was to suppress the transfer of LCFA into the mitochondria. We demonstrated the benefit of this action was to shift the bioenergetics from fatty acid oxidation to glucose oxidation. Increased FFAs in the circulation is widely accepted to have the capacity to impact and activate an immune response. This serves as a link between the investigations performed in *Study I* and the investigations performed in *Study II*. As delineated in **Figure 9**, elevated FFAs can directly activate JNK, which can serine phosphorylate IRS1 and drive the expression of other pro-inflammatory cytokines. More importantly, FFAs are linked to the immune response by virtue of its ability to activate PKCθ, which is a potent activator of IKKβ, the very target that we silenced in *Study II.*

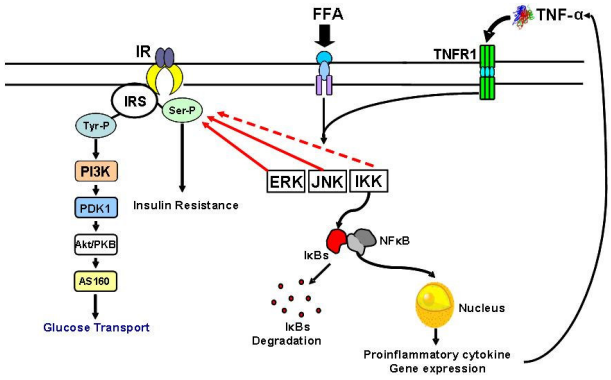

Figure 9: The link between free fatty acid and inflammatory signaling pathways. Elevated free fatty acids levels are intimately connected to inflammation through the action of mitogen activated protein kinases (MAPKs).

The nexus between these signaling pathways may provide an opportunity to develop therapeutics that target(s) each pathway at their point(s) of conversion. The development of such therapeutics could provide a more comprehensive approach in the treatment of type 2 diabetes, where one agent could potentially address perturbations in more than one relevant signaling pathway simultaneously.

The discovery of the beneficial effects of FGF-21 on whole body glucose homeostasis (144) served as the basis for the investigations performed in *Study III*. The majority of the data compiled on the glucose handling and insulin sensitizing effects of FGF-21 were performed in adipocytes (146; 180; 186). Until recently, the glucose uptake effects of FGF-21 were believed to limited to adipose tissue. The novelty of the work presented in this thesis is that we were able to show that FGF-21 had similar

effects in cultured human skeletal muscle. This study is important, as it may offer direct relevance to this potential treatment in humans. The novelty of using primary human skeletal muscle cells was carried over into the investigations performed in *Study IV* where we assess the effects of clenbuterol on metabolic and signaling parameters. Perhaps the most interesting facet of this study, in terms of its scientific register, is the fact that this it focused on a pharmacological agent that is classically anabolic. As stated previously this could provide a broader perspective and understanding of the potential therapeutics.

The work presented in this thesis emphasizes the need and relevance of continued research to discover novel molecular targets. The elucidation and characterization of such targets could prove to be beneficial in the fight against insulin resistance and diabetes. The substance of this thesis endeavors to contribute to the tremendous global effort to combat the ever increases prevalence of type 2 diabetes.

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